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PLATELET THROMBOPLASTIC FUNCTION

A technique for its measurement and its uses.

By

K. Cheney M.B.,B.S., Adelaide, 1956.

A thesis for the degree of Doctor of Medicine of the University of Adelaide, based on work conducted at the Institute of Medical and Veterinary Science, Adelaide.

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PREFACE

I. Indications for the Study.

The gravity of diseases characterized by a tendency to abnormal haemorrhage based upon a defect of blood coagulation has never been minimized. Recent years have seen spectacular and rewarding progress in the understanding of blood coagulation and its disorders. Many of the advances have had their beginning in the publication of qualitative techniques which have made it possible to study effectively various phases of normal blood coagulation and thereafter to distinguish if not isolate individual factors and to implicate specific deficiencies as the causative defects in various haemorrhagic diatheses. Side by side with progress in diagnosis, advances in treatment have demanded the development of appropriate quantitative techniques whereby not only the character of a bleeding tendency might be uncovered but its initial severity and subsequent response to therapy gauged.

The long delayed recognition of blood platelets as an integral part of normal intrinsic blood coagulation had provided a background for the interest in haemorrhagic diseases dependent on platelet abnormalities, (especially of their thromboplastic component) existing in the laboratory wherein the work contained in this thesis was undertaken. Leading this work, Dr. J.A. Bonnin had modified the thromboplastin generation test described by Biggs and Douglas in 1953, to quantitate platelet thromboplastic factor. In a series of papers, published or in preparation when this present work was undertaken, Bonnin was able to provide an essential clue whereby discrepancies between platelet numbers and the severity of haemorrhage in diseases affecting platelets began to be resolved.

Briefly his work suggested that haemorrhage was related to the degree of impairment of platelet thromboplastic function and, equally, quite unrelated to platelet numbers. This suggestion was more than a possible solution to an academic problem for it found an important clinical application in the assessment and management of the haemorrhagic state in diseases affecting blood platelets. It was realized, however, that, despite its practical importance, this work would probably find limited routine adoption for the technical difficulties inherent in the thromboplastin generation test and the attendant need for skilled and experienced staff mitigated against its use in laboratories other than those able to undertake the more complex coagulation investigations. So, too, with increasing local experience it was accepted that the thromboplastin generation test, modified to quantitate platelet thromboplastic function, presented certain disadvantages which rendered it less than ideal for this purpose. Accordingly it was considered worthwhile to seek an alternative method, the aim being, if possible, to develop a less complicated procedure than the modified thromboplastin generation test and, thus, one likely to find wider acceptance and greater clinical application along lines suggested by earlier studies published from the Institute of Medical and Veterinary Science.

For the most part this thesis describes the development of a new method to quantitate platelet thromboplastic function together with a thorough investigation of the factors participating in the reaction. A further section illustrates the uses of the established technique as applied in a series of appropriate cases in which haemorrhage is a common if not the dominant feature. Moreover while this work was in progress it was realized that, while the technique to be described was primarily a reliable means of detecting and assessing platelet thromboplastic dysfunction, it would almost certainly be possible to adapt the basic reaction to serve other uses. Further sections describe preliminary experiments modifying the reaction to assay factor X, and others in which the reaction is displayed as a means to study the behaviour of plasma

and serum anti-thromboplastic activity. In accordance with the regulations, the original content of this thesis is introduced with an historical review of relevant antecedent work.

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2. Acknowledgements.

The work described in this thesis was conducted at the Institute of Medical and Veterinary Science, Adelaide. I am grateful to the Council of the Institute upon whom, ultimately, the opportunity and facilities to undertake this work depended. I am especially indebted to Dr. James A. Bonnin, now Director of the Institute of Medical and Veterinary Science, for it was in discussions with him that the principle elaborated herein arose, while his help, interest and criticism throughout have been invaluable. Although the author personally conducted the majority of the experiments in their entirety, and participated and supervised in all, some required additional technical help. It is my pleasure to record in this instance the capable and valued assistance of Mr. Bruce Duncan. My thanks are due to Dr. Rosemary Biggs (of Churchill Hospital, Oxford) and Dr. J.B. Graham (of the University of North Carolina), both of whom generously supplied scarce and valuable reagents, thus enabling outstanding problems to be investigated more completely. So, too, the Honorary Staff of the Royal Adelaide Hospital who generously permitted investigations to be performed on their patients, and, no less, the patients themselves, and the innumerable normal volunteers who willingly and often repeatedly donated blood, have contributed in no small way to the successful completion of this work. Finally, my sincere appreciation is due to Miss Helen Gosling whose patience and unsurpassed capabilities in typing both the text and tables deserve the highest recognition.

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3. Declaration of Originality.

The author declares that the composition of this thesis is entirely his own and that it is a true record of original work pursued by him in the development of a technique to assay platelet thromboplastic function. This material has not been submitted by him to any other University for the award of any other degree or diploma, nor, to the best of his knowledge has the same material been presented or published by any other person.

TO WHOM IT MAY CONCERN

This is to certify that Dr. Cheney worked with me in my Department of Haematology at the Institute of Medical and Veterinary Science where he personally undertook the technical work concerned in the development of a new technique for the estimation of platelet thromboplastic function. Following this he applied this test to a series of uraemic patients and at the same time studied the status of their other coagulation factors. He also initiated some studies on the role of Factor X in the particular blood coagulation system with which he worked.

I wish to verify that Dr. Cheney personally undertook the majority of the technical work involved and I hereby give my consent for him to use the results of our joint publications in any way in which he desires.

J. A. BONNIN,
DIRECTOR,
INSTITUTE OF MEDICAL AND VETERINARY SCIENCE.

6th December, 1963.

SECTION I

An Historical Review

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INTRODUCTION

Since they were first described with conviction and accepted as distinct morphological elements by one man, Alexander Donné in I842, blood platelets have provided a topic for widespread speculation and controversy. When in I906, Wright was able to produce irrefutable evidence that platelets were derived from megakaryocytes in bone marrow, most workers were prepared to accept them as a constant feature in blood, but few were prepared to grant them any significant physiological function. In one sense this is perhaps strange, for there had been many reports associating reduced platelet numbers with various haemorrhagic phenomena. On the other hand, it is not so difficult to understand, for the recurring and well-remembered dissociation between platelet numbers and the severity of haemorrhage seemed the more significant point to many workers and, on this, they based their conclusions. It was not until I953, when Biggs and Douglas introduced the thromboplastin generation test, that it was possible to show that relatively few platelets could participate in the formation of a powerful thromboplastin. Following this, the attention of many workers formerly pre-occupied with tissue thromboplastin, was re-directed to study the formation of intrinsic thromboplastin in general, and, inevitably, to platelets in particular. The recent magnificent volume devoted entirely to blood platelets following a symposium at the Henry Ford Hospital in New York, gives some indication of the current interest in these tiny particles, but it is but a meagre portion of the sum total that has been written about them and closely related subjects. Indeed, if one accepts that any study of blood platelets cannot be entirely dissociated from a consideration of "purpuric" diseases, the relevant literature becomes immense, and one must delve as far back as the writings of Hippocrates to find the first recorded descriptions of illnesses accompanied by what may well have been purpura.

As an introduction to this thesis, therefore, an historical

survey is presented to cover briefly some of the more significant milestones in the fascinating story of blood platelets and the broader field of "purpuric" diseases in general. So too, early work directed towards the production of purpura and haemorrhage experimentally is described, and this progresses to a consideration of some more recent concepts of the function of blood platelets in relation to haemostasis and coagulation. Finally, some of the techniques used to detect qualitative platelet abnormalities are discussed.

For clarity, although somewhat artificially, and, with some overlap, this historical section is divided into four parts following the topics outlined above. These are:-

Part I. A review of the work preceding the general acceptance of platelets as constant formed elements in blood, and of megakaryocytes as their parent cells.

Part II. A review of the history of "purpuric" diseases and the data forming the basis for a present day classification of diseases affecting platelets.

Part III. A review of the clinical and experimental work contributing to an understanding of the normal haemostatic processes and the mechanism underlying the production of thrombocytopaenia and haemorrhage in diseases affecting platelets.

Part IV. A consideration of the techniques that may be used to detect or assess abnormalities of platelet function.

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HISTORICAL REVIEW

Part I

A review of the work preceding the general acceptance of platelets as constant formed elements in blood and of megakaryocytes as their parent cells.

Signs of awareness that forms other than red or white cells existed in blood were evident in some medical writings in the early years of the nineteenth century. In particular, in Hewson's and Andral's works, references had been made to certain white globules and bodies, distinct from white cells or red corpuscles but of uncertain nature, occurrence and identity.

The Cyclopaedia of Anatomy and Physiology, I835-I836, edited by Robert B. Todd, devoted one chapter, twelve pages in all, to a consideration of the knowledge of normal blood accumulated throughout Europe and England since the introduction of the microscope. It is evident from this, that, although interest in blood was both keen and widespread, most workers were concerned primarily with comparative studies of the "globules" of blood in different animal species. A few, however, were more interested in determinations of the actual size and true shape of mammalian red cells, while a minority was attempting to define the chemical composition of "serum".

The author of this section, H. Milne Edwards indicated that independent workers had proposed three principal shapes for red cells. Della Torre and Styles believed that they were shaped like rings, having perforated centres. Lister and Hodgkin postulated biconcave discs, while Hewson and Prevost, and Dumas, were convinced that they were flattened vesicles and possessed a central nucleus. Edwards himself was convinced that red cells had the latter shape and claimed to have separated the central nucleus by treating red cells with acetic acid. Although he made no mention whatever of white cells in his article, it is obvious that Edwards had seen but failed to recognize platelets, for he said, "It frequently happens that other smaller corpuscles than the globules of which we have treated hitherto, are observed swimming in the serum. These are of whitish colour and similar to the molecules that occur in almost all the fluids of the animal economy. The resemblance that exists between these corpuscles and the central nuclei of the proper globules of the blood might lead to the belief that they were nothing more than the central nuclei divested of their coloured envelope; but in several of the inferior tribes, as the river crabs and

certain mollusca, in the blood of which they occur in very considerable numbers, the central nuclei of the globules are much larger and it is impossible to confound the two together. These then are to be regarded, not as globules of the blood, properly so-called, altered in any way, but as globules of albumen or fibrine. These substances in fact have always the appearance of being made up of circular corpuscles of extreme minuteness when by any means they are brought into the solid state; and we are led to believe that even when suspended in water they still preserve this peculiar disposition, and only escape detection by their dissemination and transparency". And so, once again the platelet was mentioned only to be forgotten.

A few years later, on March 7th, I842, Alexander Donné presented a review of his studies on the microscopy of blood to a meeting of the "Academie des Sciences", in Paris. In this, he stated quite clearly, that he believed that there existed in blood red and white globules and little globules, "globulins". Two years later, in his book "Cours de Microscopie", Donné repeated and so amplified his original description, to leave no doubt of his own belief in the existence of a third distinct morphological element in blood. There seems to be no earlier record of the platelet as a separate and constant feature in blood, and Donné must be given full credit for this recognition.

Four years after Donné's original description, Zimmerman described what were, undoubtedly, platelets. He found certain structures, "Elementar Blarschen", in horse blood rendered incoagulable with magnesium sulphate. These, he noted, had a tendency to gather in clumps, and he thought that they were precursors of red cells.

Schultz, in I865, observed small elements in normal blood which he thought were of a protoplasmic nature. These, too, had an affinity to gather in clumps and form granular masses but he denied that they were the same as Zimmerman's "Elementar Blarschen". He believed that they resulted from the destruction of white cells. This hypothesis was corroborated in I872 and

subsequent years by Riess, who thought that white cells underwent fragmentation in anaemic and cachectic states.

It is not unreasonable that variable platelet morphology, exaggerated perhaps by differing conditions of collection and observation, caused other workers to regard them as extrinsic matter peculiar to certain disease states. Thus in the third quarter of the last century they were occasionally described as bacteria and accepted as such.

Vulpian, in I873,found small colourless corpuscles in blood and again the tendency for these structures to adhere to glass or to accumulate in clumps was recorded.

In the same year Ranvier observed granules, distinct from white and red cells, in the centre of the fibrinous network that appeared during clotting. He advanced an ingenious hypothesis to explain this observation. As a crystal of salt causes crystallization in a saturated solution of that salt, in like manner, he postulated, that these masses had a function to determine coagulation in blood.

It was not until I874, however, that platelets really began to gain acceptance more as normal constituents of circulating blood and less as artefacts or byproducts of changes in shed blood. This era began when Osler pointed out that the "granular masses" of Schultz resulted from the agglutination of small bodies which existed as single units in the circulation. His statements were based on experiments performed in the venules of recently killed young rats, when he was able to show that the "single units" agglutinated when blood was shed.

A few years later, in I878, Hayem revived interest in platelets. He believed that they had the basic and most important function of red cell regeneration, and that they were in fact red cell precursors. He suggested however, that in addition to this they had a second function, the property of accelerating blood coagulation. His conclusions were founded on changes he observed in platelets after haemorrhage and in acute diseases.

Bizzozero confirmed Osler's work in I882 and gave impetus to

the concept of the platelet as a distinct blood element. This idea had been gaining more general acceptance since Osler's original publications, but it was not until Bizzozero, too, had stressed that the granular masses" of Schultz were neither residues of fragmented white cells nor fibrin gramulations, as maintained by Ranvier, that wide interest was aroused. Bizzozero was the first to observe platelets actually circulating in the blood of living animals, and he was certainly the first to show with any conviction that they played a role in thrombosis. If Donne has the credit for the earliest description of the platelet as a distinct entity, Bizzozero must be recognized for establishing a secure foundation for the present day concept of the platelet as a distinct functional element of great importance.

Before Bizzozero published his work it was generally believed that white thrombi consisted almost exclusively of white cells. Bizzozero's classic experiments in living animals demonstrated that the white portion of a thrombus consisted almost entirely of platelets, gradually accumulated at a point in a vessel where its wall had been damaged or the circulation obstructed. Furthermore, he showed that, once aggregated, either in vivo or in vitro, platelets changed in appearance and became unusually sticky. These changes he described as "viscous metamorphosis".

Bizzozero's ideas were similar in many respects to those put forward some years earlier by Osler, but, without doubt were of greater merit. Bizzozero's experiments had the distinction that they were performed on the intact circulation of living animals, whereas Osler's observations had been based on the vessels of dead animals. Osler himself emphasized that he was unsure in his own mind whether the bodies he described might not be bacteria.

It is interesting to note that it was Osler who introduced the English word "platelet". One of the first to translate Bizzozero's publications into English, he disliked the literal translation "blood plate" for Bizzozero's "Blut Platchen", and introduced "platelet" in preference to other names in common use for these bodies, "blood plaques", "disklets", "third corpuscles", "fugitive

corpuscles", "fugitive discs", and even "invisible colourless discs".

The eminent physiologist, William H. Howell, supported Bizzozero's claims in full and in I884 published a paper corroborating and extending his ideas. Even so, these new concepts did not pass unchallenged. In particular the followers of A. Schmidt and Lowit adhered firmly to the theory that "Plattchen" resulted from leucocyte fragmentation or fibrin deposition.

Without doubt much of the criticism and contradiction that followed Bizzozero's papers stemmed from technical difficulties inherent in handling platelets. Weigert, one of Bizzozero's most trenchant critics was convinced that Bizzozero's findings were nothing more than artefacts produced by vessel compression, circulatory disturbances or the anaesthesia employed. Bizzozero quickly and cleverly countered these objections when he repeated and confirmed his original experiments using, this second time, the intact vessels in the wing of a living, unanaesthetized bat.

Another worker, Wooldridge, in I887 experimented with blood rendered incoagulable, especially after the injection of peptone. He concluded that platelets did not exist as distinct entities, but were simply precipitates of the globulin fraction of plasma. In this belief he was supported by Lowit, who, in I885, had produced much evidence denying the existence of platelets.

Although Bizzozero had formidable opponents he had equally eminent supporters. As had Howell, so, too, Eberth and Schimmelbusch, (I885) and later, Laker, (I889) confirmed and extended his original concepts, while studying circulating blood in the vessels of living dogs and other mammals.

Meanwhile some of the early workers who had accepted the platelet as a distinct element, attempted to assess actual platelet numbers. The first recorded counts were made by Riess in I872. Remembering the technical difficulties involved, it is not surprising that his figures were quite inaccurate, for he recorded increased platelet numbers in diseases which we now know are accompanied by thrombocytopaenia. Notwithstanding, it was only six years later in I878 that the first accurate counts were published by Hayem.

His figures for the average number of platelets per cubic millimetre of normal blood did not differ significantly from those accepted today.

Hayem maintained his interest in platelets for some years, and, in I882, after careful consideration of Bizzozero's work, he concluded that the platelet thrombus did play an important role in arresting haemorrhage. He showed that when experimental wounds were made in dogs' jugular veins haemostasis was effected by platelet agglutination. Following these observations he postulated that decrease or absence of platelets would disrupt the haemostatic mechanism. S. Lubnitzky, in I885, reported haemostatic platelet thrombosis in similar wounds of rabbits' crural arteries, while nearly forty years later K. Apitz and M.B. Zucker confirmed and extended these findings.

In I883, Krauss reported reduced platelet numbers in one form of haemorrhagic disease, purpura haemorrhagica. This was followed by increasing numbers as haemorrhage ceased. Denys, I887, confirmed this finding and in I89I, Hayem cited a platelet count of 62,000 per cubic millimetre of blood in a patient with purpura. In this instance and again in I896, Hayem commented upon the large size of the platelets, and the soft quality and poor retractility of blood clots in these patients. He attributed the latter defects to the decrease or absence of platelets.

While the main interest centred on attempts to prove or disprove the existence of the platelet as a distinct entity, some workers had begun to seek the source of these provocative particles. Among those who had accepted the platelet, many believed that they were derived from nucleated or more mature red cells. Engel (I893) thought that they originated from the nuclei of normoblasts, while Wlassov (I894) and Bremer (I894), still opposing the true nature of platelets, believed they were derived from the remnants of disintegrated erythrocytes. The latter view was corroborated by Arnold in I897, and later in succession by Muller, Determan, Maximow and Schwalbe. The views held by the latter workers, were based on experiments performed in the years I898 - I902, and can hardly be considered acceptable for they had all followed from

observations made on clotted or aged specimens of blood.

There can be no doubt that much of the confusion surrounding platelets in early years stemmed from two causes. In the first instance widely different methods were used by different workers to prepare their material, and this cannot be considered as a fair basis for comparison. Indeed, few experiments were made on living vessels or fresh blood, while most were made on dried, aged or clotted specimens. Antagonists of the theory that platelets were degradation products of erythrocytes were quick to realize the fallacies inherent in some methods. Petrone (I897), Sacerdotti (I900) and Dominici (I900) severely critical of the views held by Arnold and his followers, pointed out that their conclusions were, in fact, based on artefacts produced in dried smears of blood. The fact that differentiating stains were not generally available, was the second cause of confusion. It was not until the introduction and dissemination of the polychrome Romanowsky stains and azure dyes that it became possible, for the first time, to separate the red-violet azurophilic platelet granules from the mass of confusing intra and extra-cellular granular material. With this advance, renewed interest in platelets and their origin was stimulated.

In I900, Dominici introduced a new and partly correct concept of platelets. He held that they were organites, formed elements liberated by cells but lacking a nucleus. He went so far as to describe parent cells. These, he said, were large mononuclear cells with protoplasm distributed in long pedicles, the tips of which became broken off to form platelets. Twenty years earlier, however, two Italian workers Foa and Salvioli, had come very close to the correct solution of the origin of platelets. While studying red cell formation, they had seen the marrow giant cell, (first described by Bizzozero in I869) fragmented into numerous hyaline bodies. Their initial project foremost in their minds, they erroneously described these structures as the red cell precursors they were seeking. Still other workers suggested other marrow giant cells, osteoclasts, as likely parent cells.

With the ever increasing variety and use of staining

techniques, Bizzozero's concept of platelets as independent functional units, was extended further by some European investigators who claimed for them cellular characteristics common to most cells. Deetjen (I90I) claimed to have demonstrated both a nucleus and protoplasm by the use of osmic acid fixation. By the use of more specialized techniques he claimed further, to have seen platelets extend protoplasmic processes akin to leucocyte pseudopodia. Both Deckhuysen and Koppsch corroborated the latter view in the same year, and it was confirmed later in I9I0 by Wright. Some years earlier, in I885, Eberth and Schimmelbusch, had described in the blood of oviparous animals certain nucleated bodies that underwent changes, similar to those of platelets, during haemorrhage and coagulation. Deckhuysen, after full consideration of the known facts, came to regard the mammalian platelet as homologous to these nucleated spindle cells or thrombocytes of lower species.

In the years I889-I90I, numerous workers had found structures resembling platelets in organs, other than the circulation, principally in the spleen and lymph nodes. Among those who had made such observations were Foa and Carbone, and Vassale. Foa and Carbone differed from Vassale by regarding these structures as fragments of mononuclear cells and not true platelets.

Almost a century following the first vague references to platelets, opinion was still divided on two major issues. Despite the wealth of information already gathered there were still those who disputed the true nature of platelets. Secondly, their site of origin was still unknown. The final answer to both questions appeared in I906, when J. Homer Wright published his first classic paper on the origin of platelets.

When Wright's original article appeared there were still six current theories regarding the nature of platelets. These were:-

I. That platelets were fragments of leucocytes.

2. That platelets were derived from extruded nuclei of red blood cells.

3. That platelets were derived from certain parts or constituents of red blood cells other than the nucleus.
4. That platelets were a definite and independent type of blood cell.
5. That platelets were of the nature of albuminous precipitates.
6. That platelets were cells; the precursors of red cells.

With full consideration of all the known facts Wright was able to disprove each of these concepts, and for the first time described the origin of platelets from a marrow giant cell, named the megakaryocyte by Howell in 1890, to distinguish it from other multinucleated giant cells, more closely related to bone and called osteoclasts. Significant though it was, Wright's first article, published in the Boston Medical and Surgical Journal, received unfavourable comment in some quarters, possibly due to poor reproduction of some of his illustrations. Four years later Wright published his experiments in greater detail and this time he included numerous beautifully coloured plates to illustrate his conclusions. Wright's success followed the use of his special eosin - methylene blue stain (Wright's stain) which enabled him to clearly distinguish platelets in stained fixed tissue, and differentiate them from other histologic elements. In this second paper he described his studies of the bone marrow of the cat, rabbit, guinea pig, white mouse, possum and man with special reference to megakaryocytes and platelets. He had found that the giant cells of the marrow often contained granules which were most numerous in the pseudopodial process of cytoplasm projecting into the sinusoids of the marrow. In some megakaryocytes or their pseudopods, one or more small groups of granules were separated from the rest of the cell by a zone of hyaline cytoplasm. These masses of granules with their intervening cytoplasm had an identical staining reaction with platelets. Furthermore he found that bodies identical with platelets were often found near pseudopods and that, at times, detached pseudopods were seen in blood channels. He concluded therefore that megakaryocytes produced platelets by direct budding from

from pseudopods which had entered the circulation of the marrow. In further support of his theory, Wright emphasized two important facts, the absence of platelets in embryonic life until the appearance of megakaryocytes, and the absence of platelets in species lacking cells comparable to megakaryocytes.

Bunting confirmed Wright's findings in 1909, when he demonstrated that in rabbits increased megakaryocyte activity could be induced by bleeding and that the granules of megakaryocytes and platelets gave identical staining reactions with supravital dyes, brilliant cresyl blue and neutral red. Additional confirmation was supplied in the human by Downey in 1913 and more evidence based on staining techniques was supplied by Smith, Robinson and Tyson in 1938. The origin of platelets from megakaryocytes and the fragments issuing from their pseudopods was correlated with the number of blood platelets as modified by repeated bleeding (Bunting 1920), the action of Benzol (Weiskotten, Wyatt and Gibbs, 1924), the use of anti-platelet serum (Bedson and Johnston 1925), the use of saponin (Firket and Campos 1922) and by blocking of the reticulo-endothelial system (Volterra 1928).

Wright's work and its confirmation by other workers seemed to have proved the origin of platelets beyond doubt. However, while European histologists generally accepted megakaryocytes as the parent cells, during the next thirty years occasional publications appeared still disputing the nature of platelets or their origin.

Brown in 1913 claimed that under conditions of excessive demand, platelets could develop from monocytes, while in 1915, Martelli claimed that they developed by cytoplasmic fragmentation of leucocytes. In 1920 Bunting, despite his other work, claimed that in epidemic influenza accompanied by thrombocytopaenia, platelets or platelet-like bodies developed in the blood stream from lymphocytes, and in the same year Pianese discussed the possibility that they might be a precipitate of shed blood. Bedson published an article in 1922 in which he first produced evidence supporting the existence of platelets as independent elements and then proceeded to demonstrate that not infrequently they entered

the circulation as nucleated elements, losing their nuclei as maturation proceeded. He stressed the analogy between this process and development of red cells from their nucleated precursors. He concluded by directing attention to the frequent occurrence of structures, in reality "nuclear rests" that could be found in platelets, comparing these to Howell-Jolly bodies in erythrocytes. In 1930, Matthews revived an idea first proposed by Wooldridge in 1887, suggesting that platelets were a precipitate of plasma proteins and did not exist as distinct formed elements in blood. Finally, one of the last relevant articles to appear disputed neither the nature nor the parent cells of platelets but rather their site of production. The author was William R. Howell, whose first contributions to the platelet controversy had appeared almost fifty years before. In this paper published in 1937, Howell and Donahue on the basis of arterial and venous counts, histologic studies and perfusion experiments, suggested that new platelets were added to the circulation in the lungs and not marrow, a view hardly tenable in the light of present knowledge.

Thus originally based on purely morphologic grounds, the evidence from both histologic and experimental studies that platelets are formed from megakaryocytes of the bone marrow can hardly be more complete.

HISTORICAL REVIEW

Part II

A review of the history of purpuric diseases and the data forming the basis for a present day classification of diseases affecting platelets.

Purpura, the Latin derivative of the Greek word "Porphyra" did not appear in medical records until the sixteenth century. In its original sense "purpura" referred to a certain purple fish whose gills were a source of purple dye valued so highly in early Greece and Rome. Later and for some centuries, it designated the colour purple. On the other hand, descriptions of "purpuric" diseases in the loosest sense, appeared in the earliest medical writings.

Hippocrates was aware of the association of petechial haemorrhages and fever, describing four cases, one of which was particularly suggestive of purpura, displaying foetid breath, gum swelling and large haemorrhagic ulcers on the legs. Centuries later Celsus recorded similar cases, but these displayed haemorrhages from the nose and elsewhere in addition, aspects he attributed to "morbid enlargement of the spleen". Galen cited epistaxis, glandular swelling, and the appearance of red spots as complications of epidemic fevers, while the medical monk, Francastorius, describing fevers he had encountered in Italy in the years I505-I506, distinguished one form characterized by the appearance of red spots. None-the-less, prior to the sixteenth century there had been no clear cut description of a "purpuric" state independent of fever.

At the beginning of the sixteenth century epidemics of "spotted" fevers (plague, typhus and cerebro-spinal fever) were common. It was at this time that these diseases became known as purpuric fevers, and subsequently any eruption of purple colour was called purpura. Before long, however, it became apparent that purple spots occurred, not infrequently, independent of any fever and the term soon acquired a narrower meaning, and with the introduction of the terms "purpura sine fibre" and "petechiae sine fibre", came the earliest classifications of purpuric diseases.

Perhaps the first to describe purpura without fever was the Portuguese physician Amatus Lusitanus. In I557 he recorded the case of a young boy whose entire body was covered with dark macules resembling flea bites, and who had extensive haemorrhages from various sites for some days, but no fever. Lusitanus called

the condition "morbus pulicaris absque fibre". Later in I658, Eugalenus gave a clear description of purpura under the title of "scurvy". He portrayed four cases each of which had a purple eruption and haemorrhages from the nose, lung and bowel.

A much more precise separation of purpuric states was published in the mid-seventeenth century when La Riviere's Praxis Medica appeared. Culpeper translated this book into English in I678. From the section on pestilential fevers he translated, "But there is one Symptome proper and peculiar to a pestilential feaver which doth not happen in other Feavers; viz. Purple Specks or Spots on the whole body --- which the Italian Physicians name Peticulae or Petechiae; and these Feavers which have these symptoms are commonly named Purpurate or Petechiales --- and sometimes they are very large or possess whole members --- and then the parts appear tainted with redness which in a few hours oftentimes vanisheth away and then returns again --- and are commonly called ebullitions of the blood ---. There do appear in other Diseases, spots very like unto the aforesaid, but springing from a far different course; viz. from the over thinness of the blood, which being exagitated by the heat of the expulsive faculty doth sprout forth of the capillary veins into the skin. These spots want for the most part to appear in such as have some Flux of the blood because the blood in such is more thin and watery; and also in Splenetick persons and in such as have the Jaundice and old obstructions of the Bowels and in all such --- who are apt to fall into Cachexy".

It is obvious then that La Riviere had suggested infections, blood dyscrasias, malignancy, diseases associated with splenomegaly, jaundice and Cachexia, were all of aetiologic importance in the production of purpura, while his mention of the occasional transient redness might have represented the earliest reference to an allergic basis for some types of purpura.

A tendency to subdivide purpuric states into more exact clinical entities appeared early in the eighteenth century. In I734, Hornung proposed a classification of purpura into three groups, simplex, febrile and scorbutic, thus recognizing in his third group what we

now known to be deficiency states as important causative factors in some cases.

The following year, Paul Gottlieb Werlhof described what was, in retrospect, a case of idiopathic purpura, under the name "morbus maculosus haemorrhagicus". It was from this description, but not until one hundred years later, that the term "morbus maculosus Werlhofii" arose. Werlhof described the classical case of an "adult girl, robust, without manifest cause, attacked towards the period of her menses, with sudden severe haemorrhage from the nose — and about the neck and on her arms, spots, partly black, partly violaceous or purple". It is well to recall that no knowledge of thrombocytopaenia or other coagulation defect existed in the eighteenth century, but regardless of the presence or absence of such knowledge this seems to be the first recognition of endocrine factors in the causation or aetiology of purpura. However, due credit must be given to the little known German physician Behrens who, some months earlier, had set forth this same condition in a letter to Werlhof, giving it a similar name. The combined observations of both these men were not published until I775, when Wichmann collected and edited Werlhof's writings.

In I808, Willan published in London a Treatise "On Cutaneous Diseases". This contained descriptions of many varieties of purpura even as we know them today, but Willan failed to separate many of these into distinct entities, a task accomplished by later workers who consequently gained the renown. This is typified by a case described of a woman presenting acute gastro-intestinal symptoms, bloody stools and purpura, later to be known as Henoch's purpura. Willan classified purpura under the headings, simplex, haemorrhagica, urticans, contageosa and senilis.

Schonlein, I837, submitted a classification of purpura, recognizing three broad groups, namely, purpura haemorrhagica, (or peliosis Werlhofii), peliosis senilis and peliosis rheumatica. The latter he included because he had collected several cases with a characteristic symptom complex in which purpura was associated

with multiple joint involvement, a condition later to bear his name.

Thirty years later (in I868), Henoch criticized Schonlein's third group, in the belief that the haemorrhagic and joint manifestations had the same aetiology. Further, he said, the element of exposure was not invariably present to explain joint symptoms. However, he described five cases in children which he believed merited separate consideration, while for all other types of purpura he reserved the term purpura haemorrhagica. The former type, since known as Henoch's purpura, had a common symptomatology of purpura, vomiting, abdominal pain, intestinal haemorrhage and joint symptoms but no fever. They differed from purpura haemorrhagica by virtue of pain, intestinal symptoms and joint swelling. Moreover, under separate heading he described purpura-fulminans, the rapidly progressive, fatal type of purpura, now known to be associated with overwhelming infections. Many years later Glanzmann (I9I8) reviewing the particular symptom complexes described by Henoch and Schonlein, suggested that the basic mechanism of both types was of an allergic nature and proposed that they be termed "anaphylactoid purpura", a view almost universally accepted.

From the beginning of the nineteenth century with its widespread interest in blood microscopy it was inevitable that numerous observations both clinical and experimental, were made which left no doubt of an association between blood platelets and purpuric diseases. From this time forward any consideration of the historical background of these diseases cannot be separated from various aspects of platelet dysfunction.

The first suggestion that purpura might be a disease of blood coagulation was made by Andrew Duncan in I822. Duncan, Professor of Materia Medica at Edinburgh and editor of the Edinburgh Medical and Surgical Journal published the case of a fifteen year old boy who had died of a purpuric disease. When a post-mortem revealed widespread petechiae throughout the body he questioned the

propriety of considering purpura a cutaneous disease, suggesting it might be due,
"to increased tenuity of the blood allowing it to escape from the superficial extremities of the minute arteries". Furthermore he reported, "--- the blood coagulated very slowly and the coagulum was not firm, the serum did not separate and the coagulum had the appearance of shaking jelly". Duncan had apparently been stimulated to publish his case report with its significant observations by an earlier communication detailing a case of purpura with no clot retraction sent to him for publication by one George Johnston. Describing this case Johnston (I822) said,
"the blood drawn in the morning had not separated into serum and crassamentum. It possessed little consistence or tenacity but there are traces of coagulable lymph diffused through it".

The bleeding time of the skin, introduced by Duke as a clinical test in I9I0, had been performed almost ninety years earlier by Stoker. In I823 Stoker, who was Professor of Medicine in the Eccles Street School in Dublin, published a book of "pathological observations on dropsy, purpura --- and particularly on the morbid changes of the blood and their influence on the production and course of these diseases". For this publication he had performed experiments to determine the coagulation time and "flowing" time of the blood in various states. The former, he found, varied between nine and nineteen minutes and the latter between three and seven minutes. Stoker stressed the importance of making successive punctures as uniform as possible in assessing the "flowing" time but neglected to indicate the site of the puncture. During this work he found a prolonged "flowing" time in purpuric diseases.

Following Stoker's observations other writers of the era made isolated reports on bleeding times in purpura. Henoch reported prolonged bleeding times in purpuric states, while others noted difficulty in checking bleeding after venipuncture, but the matter was not critically studied until Duke emphasized the distinction between bleeding and clotting times in I9I0.

Meanwhile both Hayem and Bizzozero had attributed a haemostatic

function to platelets and in the following years various workers had begun to perform platelet counts and some had recorded, on occasions, reduced platelet numbers in purpuric states.

Many years before this, however, in 1810, a chance observation saw the beginning of the capillary resistance test. In that year Sir John Pringle's volume "Observations on Diseases of the Army" appeared. In this Pringle described how petechiae appeared below the applied tourniquet during or after venesection in hospital fevers. Later numerous workers noted this association with purpuric diseases and it acquired several synonyms, becoming the focal point for various investigations, particularly in the first years of the twentieth century. The year 1911 saw the publication of many of these, including that of Frugoni from Italy, (Grocco-Frugoni's sign) Weill, from France ('le signe du lacet') Leede from Germany (Rumpel Leede phenomenon) and later from America came the work of Hess (the capillary resistance test of Hess). Of all these papers Frugoni's was the most comprehensive, for he had investigated capillary resistance in various forms of purpura while Leede was concerned primarily with changes in scarlet-fever and Hess with the changes in scurvy.

As early as 1897 Delezenne and later Sourd and Pagniez (1906) Gratia (1914) and Hess (1917), showed that partial or complete removal of platelets by centrifugation, caused marked lengthening of the clotting time of plasma even when in contact with glass and in the presence of ionized calcium. However, Delezenne and some later workers worked with avian and amphibian blood where the less fragile nucleated "platelets" are more readily removed and only with difficulty can these results be reproduced using mammalian blood.

Cramer and Pringle in the early years of the present century thought that the incoagulability of mammalian blood after filtration through Berkfield filters was due to the removal of platelets. It is now realized that such filtration causes complex and variable adsorption of factors other than platelets, but even so, there remained a mass of information both clinical and

experimental to suggest that platelets did play an important role in blood coagulation.

Despite the evidence, however, the platelet was largely ignored and relegated to a place of secondary importance in haemostasis. Most workers, adhering to the classical scheme of blood coagulation, believed that thromboplastin, the initiator of the whole process of clotting, was derived from damaged tissues. The fact that blood collected with the greatest care to avoid the least contamination with tissue products clotted rapidly and firmly, and therefore, must have contained its own intrinsic source of thromboplastin was ignored. Together with this neglect, realization that an intrinsic source of thromboplastin may be of greater physiological importance than the tissue thromboplastin, studied so extensively, went almost unheeded.

The concept that plasma might contain its own intrinsic source of thromboplastin had been clearly and definitely proposed by Collingwood and McMahon in I9I2. They considered that platelets provided a precursor of thromboplastin. Bordet and Delange too, in I9I2, had concluded that platelets provided a precursor of thromboplastin. Their experiments, similar to those of Delezenne (I897) showed that both bird and rabbit plasma freed from platelets became incoagulable and that serum obtained after the coagulation of platelet poor plasma contained a large amount of residual prothrombin.

Wright and Minot in I9I7, described platelet changes during coagulation (the "viscous metamorphosis" of Bizzozero). In I926 Tait and Burke published a detailed study of these changes, describing the extrusion from platelets of clear blebs or spherules which travelled through the plasma. Significantly, perhaps, they stated that the first filaments of fibrin seemed to appear behind these spherules as they moved.

Notwithstanding, the concept of an intrinsic thromboplastic mechanism contained within blood gained little serious attention until comparatively recent times even though Nolf had always

maintained the existence of such a system. It was generally accepted that platelets had little thromboplastic activity and as recently as 1948 this view was upheld by Ware, Fahy and Seegers, following their experiments based on the addition of platelets to recalcified oxalated plasma.

The question was conclusively resolved in favour of the platelet in 1953, when McFarlane and his colleagues showed that a powerful thromboplastin was developed in blood during coagulation. The introduction of the thromboplastin generation test by Biggs and Douglas in 1953, provided a relatively easy method of demonstrating intrinsic thromboplastin generation, and added further to the appreciation of platelets as a powerful component of intrinsic thromboplastin generation.

Long before this latter work had been published, however, indeed by the middle of the nineteenth century, a multiplicity of aetiologic factors in the production of purpura was well recognized. It was known that purpura might occur as a result of, or in association with, a varied group of clinical states including infections, blood diseases, malignancies, cachexia, endocrine disturbances and deficiency states in addition to gastro-intestinal, skin and rheumatic syndromes that are now regarded as of an allergic nature.

Notwithstanding, until the latter part of the nineteenth century purpura was considered largely as a vascular disease because of the paucity of knowledge of blood coagulation although an occasional worker, for example, Duncan, had suggested that purpura might be a disease of blood coagulation. Subsequently, in the decade or two that followed the discovery of the relation of platelet reduction to purpura, profound changes occurred in the interpretation of the disease and it became possible to separate cases into two broad groups, thrombocytopaenic and non-thrombocytopaenic.

Wright's demonstration of platelet production by marrow megakaryocytes provided a valuable clue to the mechanism by which platelet reduction might occur as a result of decreased production

and it was soon established by Duke (I9I5), Minot (I9I7), and others that platelet deficiency did occur in bone marrow diseases, such as leukaemia and aplastic anaemia. Duke also showed that platelet reduction might occur in severe infections, after massive x-ray irridation, following the use of chemical toxins of the benzol type, and, perhaps most significantly, he produced thrombocytopaenia experimentally in an hypersensitivity mechanism in rabbits sensitized to horse serum.

At this stage Frank, in I9I5, and Kaznelson, in I9I6, each proposed an hypothesis in respect of the condition purpura haemorrhagica. Thrombocytopaenia being a constant feature, Frank attributed this to reduced thrombocytopoetic marrow activity, while Kaznelson believed it followed abnormal peripheral destruction of platelets, primarily in the spleen.

As a consequence of Kaznelson's idea, Professor Schloffer performed the first splenectomy as treatment for purpura. This first operation was a spectacular success but the great hopes aroused were soon dampened when it became apparent that uniformly good results were not obtained. The next twenty five years saw few advances although many workers had sought evidence both clinical and experimental, in support of one theory or the other. The position in I94I was well summarized by Nygaard in his book "Haemorrhagic Diseases". When discussing purpura haemorrhagica, he said, " —— the impression remains that the rationale of surgical treatment in this disease is not as clear cut as was assumed in the I920's" and later, when considering the hypotheses of Frank and Kaznelson, "The question has not been resolved in favour of either viewpoint".

At this time patients presenting with purpura could be readily subdivided into two groups, those in which platelet numbers were reduced below accepted normal values and those in which platelet numbers remained normal. Moreover, in the thrombocytopaenic group, lessened platelet numbers could often be explained by some obvious

feature, either a primary disease or a history of contact or ingestion of a physical or chemical agent with known noxious properties to marrow generally, or specifically to megakaryocytes and platelets. This knowledge led naturally to an initial further subdivision of the thrombocytopaenic group into cases symptomatic of some primary disease and true idiopathic thrombocytopaenic purpuras.

During the last twelve years a mass of information has been forthcoming and has led to a fuller understanding of the causation of at least some of the idiopathic cases. This work will be considered in detail later and it suffices to say here that the approach has been from widely differing lines of research. Briefly, the major sources of enlightening data have been:-

I. Elucidation of the mechanism of Sedormid, Quinidine and allied purpuras.
2. Detailed studies of the similarities between haemolytic anaemias and idiopathic thrombocytopaenic purpuras.
3. Investigations into thrombocytopaenic activity of plasma from individuals with idiopathic thrombocytopaenic purpura when injected into normal individuals.
4. Experiments involving the transfusion of normal platelets into subjects with thrombocytopaenic purpura.
5. Detailed studies of the effects of steroids when used in this group of diseases.

As yet, however, the picture is still far from complete, but with current widespread interest in platelets, a clearer picture must eventually emerge.

The second group, the non-thrombocytopaenic purpuras were, in general, not so readily classified. In some instances a simple or obvious cause was present to explain the presence of purpura and appropriate descriptive names were applied. Thus one is confronted with purpura simplex, senile and cachectic purpura, mechanical purpura, the presence of infectious diseases to name but a few.

In other cases it was obvious that, in addition to purpura and haemorrhage, patients manifested symptoms usually associated with allergy, erythema, urticaria and effusions of serum into submucous and subcutaneous tissues and viscera. If accompanied by joint involvement this type was already known as Henoch-Schonlein purpura. It was found too that some cases may have an obvious allergic basis yet remain distinct from classical Henoch-Schonlein purpura. While the aetiology remains obscure, many symptoms and signs may be due to increased capillary permeability from toxic or unknown causes.

A second group overlapped the symptomatic thrombocytopaenic purpuras. The causative agent in these cases may produce purpura without thrombocytopaenia or obvious signs of allergy in some people. These cases may be explained on the basis of a primary and more severe effect on capillary permeability alone or together with a qualitative platelet defect.

A suggestion that haemorrhagic disorders might result from the latter cause, qualitative platelet defects, was made early in the present century, but, even so, these have remained the most obscure and confused group of disorders. The confusion has resulted in large part because different names have been applied to the same clinical condition, and partly because the investigations carried out were sometimes inadequate to justify conclusions as to the cause of the abnormality.

A beginning in the study of qualitative platelet defects was made in 1918 by Glanzmann. Under the name thrombocyto-asthenia, he described cases in which purpura and excessive bleeding were accompanied by a normal bleeding time, normal clotting time and normal platelet numbers but bizarre platelet morphology and defective clot retraction. Later the picture became confused when other workers described examples of quite different defects, usually with normal platelet morphology and normal clot retraction, under the same name and Glanzmann's work and concept became discredited.

Further impetus to the concept of functional platelet defects was given by Von Willebrand in I93I when he described a familial disease on the Aland island close to Finland. This disease, named thrombocytopathia, by Von Willebrand was inherited as a Mendelian dominant, affected both sexes, and was characterized by a prolonged bleeding time, normal clotting time and normal platelet count. Two years later Von Willebrand and Jurgens sought to prove that the basic defect in this condition was defective platelet agglutination but their results were inconclusive.

In I94I McFarlane described 5 cases resembling those of Von Willebrand. He was unable to demonstrate any platelet abnormality and clot retraction was normal in all. The nail fold capillaries, however, were abnormal in shape and failed to contract after puncture. He suggested that bleeding in these patients was due to an inherited capillary defect and not due to functional platelet deficiency.

The number of similar cases reported multiplied quickly and by I946 Estren, Medal and Dameshek were able to review 62 cases from the literature, adding eleven of their own. Similar surveys on the ever-growing list were made by Revol, Favre-Gilly and Olagnier (I950), Soulier and Larrieu (I954) while McFarlane and Simpkiss studied one family of 2I affected members in I954. All of these workers stressed the apparent normality of platelets; the only consistent defect was a prolonged bleeding time. Approximately half the patients studied had a positive tourniquet test, while platelet counts, platelet morphology, prothrombin times, prothrombin consumption and clot retraction were normal in all. McFarlane's findings of bizarre capillary morphology and defective function were confirmed by Levy in I947, Perkins in I946, Casal and Izarn I950 and O'Brien in I950; and it became generally accepted that bleeding was secondary to capillary dysfunction. This simple explanation was complicated in I953 when Larrieu and Soulier, and Alexander and Goldstein described low levels of anti-haemophilic globulin in two patients falling into this category. This

finding was confirmed in eight more cases by Biggs and McFarlane (I957), but, to date, its significance is still unknown.

A further complication had been introduced by Bernard and Soulier in I948 when they described a case fulfilling the criteria of Von Willebrand's thrombocytopathia but associated with giant-sized platelets and defective prothrombin consumption. Later they proved that these platelets were defective in thromboplastin generation. Similar findings were reported by Favre-Gilly et al in I950.

In I953 Braunsteiner et al, studying platelets by electron microscopy defined a disease characterized by an inability of platelets to form pseudopods and especially to spread in contact with a foreign surface. These cases had either manifest or latent defects of clot retraction as revealed by thrombo-elastography, and the authors found it logical to call them thrombocyto-asthenia.

Later, in I956, Braunsteiner and Pakesch reported the most detailed investigation yet of qualitative platelet abnormalities. They studied 23 cases presenting a uniform clinical picture of a severe bleeding tendency from all mucous membranes, a greatly prolonged bleeding time, petechiae and ecchymoses, normal platelet numbers, normal plasma coagulation factors and no detectable anticoagulant. Their detailed investigations included electron microscopy of platelets, examination of platelets on the smear, coagulation time, bleeding time, tourniquet test, clot retraction, thrombo-elastography, prothrombin time, prothrombin consumption test, heparin tolerance test, thromboplastin generation and, in some instances, quantitative determinations of Factors V, VII and anti-haemophilic globulin, and capillary microscopy. Based on their results Braunsteiner and Pakesch subdivided these cases into two broad categories.

I. Five patients had a well-defined disease caused by a constant reproducible platelet defect, manifest as an inability to form pseudopodia and an absolute lack of platelet spreading. Clot retraction was obviously disturbed or a latent defect was

evident by thrombo-elastography. Prothrombin consumption, platelet thromboplastin generation and all plasma coagulation factors were normal. In deference Glanzmann's original article instancing defective clot retraction, the authors chose to classify this group as thrombocyto-asthenias.

2. The remaining cases were not so readily classified, the results being much less uniform. All cases had normal pseudopod formation, platelet spreading, clot retraction and thrombo-elastography. On the basis of coagulation tests, however, they could be classified in 3 groups.

   a. Five patients had constantly and severely impaired prothrombin consumption and thromboplastin generation. All known coagulation factors and capillary microscopy were normal in each case. Three had abnormal platelet morphology; thus resembling cases reported by Bernard and Soulier (1948) and Hirsch, Favre-Gilly and Dameshek (1950).

   The remaining two had normal platelet morphology. One patient had a sister who had a severe bleeding tendency and in whom a probable platelet defect existed as well as a temporary diminution of anti-haemophilic globulin. In this she resembled cases reported by Alexander and Goldstein and Larrieu and Soulier, characterized by a prolonged bleeding time and reduced anti-haemophilic globulin levels. A further patient had a brother with a moderate bleeding tendency but normal prothrombin consumption and thrombo-plastin generation. The disease in these five patients they called thrombocytopathia.

   b. Nine patients had prothrombin consumption and thrombo-plastin generation which were either temporarily abnormal or at the limit of normal values. This group included 3 cases of the original thrombocytopathia of Von Willebrand-Jurgens from the Aland islands. Capillary microscopy as performed by the authors showed no abnormality. Braunsteiner and Pakesch chose to call these cases

probable thrombocytopathia in the expectation in the future, more sensitive, evaluation of platelet function would reveal hitherto undisclosed defects.

c. In the remaining four cases despite the undoubted severe bleeding tendency no defect could be demonstrated at any time. These cases they designated possible thrombocyto-pathia, stressing that in some laboratories these and possibly some of their cases of "probable thrombocyto-pathia" would be classified as "vascular pseudohaemo-philia" (Von Willebrand's disease). In the absence of positive identification of any structural or functional capillary defect, however, they preferred to leave classification indefinite.

Knowledge of qualitative platelet defects has advanced little since this study, and the broad general groups of thrombocyto-pathia, Von Willebrand's disease, and thrombocyto-asthenia re-main. The interesting fact, however, brought out by Braunsteiner and Pakesch that closely related members of the one family, each with severe bleeding disease, might have different laboratory findings, suggested that such a division might be purely arbitrary. Further work with new and more sensitive techniques may show that thrombocyto-asthenia, Von Willebrand's disease and thrombocytopathia, in reality represent different phases of the same condition or that such a subdivision represents artificial selections in a naturally heterogenous group in which many variants of hereditary or acquired abnormalities of platelets and blood vessels may be combined to find expression in different ways.

HISTORICAL REVIEW

Part III

A review of the clinical and experimental work contributing to an understanding of the normal haemostatic processes and the mechanism underlying the production of thrombocytopaenia and haemorrhage in diseases affecting platelets.

Interest in purpuric diseases has not been restricted to purely aetiological aspects and for many years attempts have been made to induce thrombocytopaenia and haemorrhagic manifestations experimentally. The only positive fact that has emerged, however, has been the complexity of the problem and a singular lack of correlation between platelet numbers and the severity of haemorrhage.

The earliest recorded attempts to induce haemorrhage were those of Magendi, who, in I833, produced extravasations of blood in experimental animals by the intravenous injection of putrefactive material. He concluded that purpura resulted from poisoning of the blood by an unknown morbid principle.

Hayem during his period of active interest in platelets, produced thrombocytopaenia by peptone injection, thus precipitating an anaphylactoid reaction, and secondly by injecting heterologous serum. The latter provided the background for tremendous interest in the production of thrombocytopaenia by the use of anti-platelet sera, evident in the early part of the twentieth century.

Marino produced anti-platelet sera in I905 and subsequently its effects were investigated by Chevral and Roger (I907), Le Sourd and Pagniez (I908), Cole (I908), Sacerdotti (I908), Stchastnyi (I909) and Aynaud (I9II), all of whom demonstrated the species specifity of such sera.

Early in I9I4 Ledingham demonstrated that anti-guinea pig platelet serum was toxic for guinea pigs, producing a state closely resembling human purpura haemorrhagica. Subsequently in I9I5, Ledingham and Bedson re-affirmed the species specifity of this serum, proved that anti-red and white cell sera had no such effects and showed that while a characteristic feature in animals injected with anti-platelet serum was an early extensive fall in platelets, other sera had little or no such effect on platelets. This work was confirmed in America in I9I6 by several workers (Musser and Krumbhaar, Lee and Robertson) in Japan in I9I7-I9I8 by Watabiki, and again in America by Gottlieb in I9I9, but no new

facts emerged.

The sera used by the various workers, however, were found to agglutinate and lyse red cells in vitro to a higher titre than they would alter platelets. Haemagglutination was also apparent to a large extent in vivo and was thought to play a prominent part, in a purely mechanical way, in the production of haemorrhage.

Bedson set out to determine whether anti-platelet sera contained an anti-body specific for platelets, and, if so what part it played in the production of purpura. In a series of classic experiments Bedson prepared anti-sera against each of the formed blood elements, titrated these against their own antigens and adsorbed each with different antigens, and was thus able to show that anti-platelet sera contained an antibody specific for platelets after it had been adsorbed with red cells whereas, prior to this, it agglutinated both red cells and platelets. After adsorption with red cells platelet specific anti-sera remained. Moreover only the latter could induce purpura, both anti-red and anti-white cell anti-sera being quite without effect.

The ability of anti-platelet sera, unabsorbed with red cells, to induce intense haemagglutination in vivo and in vitro, raised the possibility that this might lead to haemorrhage by rupture of capillaries secondary to mechanical plugging. This was investigated although it was quite obvious that this was not the sole factor since red cell anti-sera while inducing intense haemagglutination did not produce purpura. Since it was possible to adsorb the red cell agglutinin from anti-platelet sera, it was possible to test this theory experimentally.

Anti-platelet sera, adsorbed and not adsorbed with red cells were injected into separate guinea pigs. In both, almost complete depletion of platelets and extensive haemorrhages resulted. In one, however, there was widespread haemagglutination, in the other, none. Bedson concluded, therefore, that haemagglutination played no part in the production of haemorrhage.

Was thrombocytopaenia alone sufficient to cause haemorrhage?

This question was easily tested for both Aynaud (I9II) and Roskam (I92I) had shown that intravenous injection of substances like peptone induced marked but transient thrombocytopaenia. Bedson was unable to produce haemorrhage in rabbits in which pure thrombocytopaenia was induced by injecting agar serum.

Thus while thrombocytopaenia was insufficient to produce haemorrhage and haemagglutination played no part, no other obvious change in blood had been demonstrated to explain the findings. Attention was then directed towards capillaries. It had long been thought that capillary damage was essential to the production of haemorrhage and this idea was corroborated by microscopic examination of capillaries in guinea pigs after the injection of anti-platelet serum. The endothelium was swollen and oedematous, individual cells standing off the vessel wall.

Bedson then postulated further that should endothelial damage be the other factor, it should be produced by anti-sera prepared against any of the blood cells since they were genetically related to endothelial cells. Two facts are pertinent to this consideration. The first of these is the common origin of vascular endothelium and the different varieties of blood cells from primitive embryonic mesenchyme. The second is Wright's belief that some of the fore-runners of megakaryocytes seemed to be formed by a transformation of endothelial cells of blood vessels, for he had seen megakaryocytes forming part of the endothelium of a vessel in the yolk sac of a guinea-pig embryo. The fact that neither anti-red nor anti-white cell sera produced haemorrhage, presumably through lack of thrombocytopaenic factor, made it possible to subject the dual hypothesis of endothelial damage and thrombocytopaenia to experimental confirmation.

Bedson injected guinea pigs with anti-red cell serum, and after allowing sufficient time for capillary damage to ensue, induced thrombocytopaenia in the same animals by injecting agar serum. This combination of factors resulted in a haemorrhagic state similar to that following injection of anti-platelet serum.

At about the same time Roskam (I92I) reported that the bleeding time was only slightly prolonged in animals made thrombocytopaenic by gelatin injection, and suggested that another factor must be involved, probably endothelial damage. Similar ideas were held by Nelf and Ledingham (I9I4), and were supported by histological evidence of swollen, oedematous capillary endothelial cells in sections taken from purpuric animals.

During the next twenty years Japanese workers had conducted stimulating work which was largely forgotten until Clarke and Jacobs reviewed the relevant literature in I950. Katsura (I923-4), OhKubo (I928), Okana abd Kawakani (I938) and Hiramatsu (I94I), had published papers on experimental non-thrombocytopaenic purpura, induced by anti-endothelial serum. In each case anti-serum was produced by the intravenous injection into rabbits of antigen obtained from guinea pig aorta. It was able to agglutinate guinea pig platelets, red and white cells and endothelial cells. The latter were obviously damaged as evidenced by swelling and lysis. Katsura's serum produced generalized purpura when injected into homologous animals, but Hiramatsu thought that this was less severe than purpura induced by anti-platelet serum.

In further work Katsura found that when subpurpuric amounts of both anti-endothelial and anti-platelet sera injected into the same animal, synergism resulted, and marked haemorrhagic purpura followed. The anti-platelet serum agglutinated endothelial cells in vitro and in vivo, causing them to swell and slough, and a positive complement fixation test was obtained whenever endothelial cells were used as antigen in a reaction containing anti-platelet serum as antibody. Moreover, adsorption of anti-platelet serum with platelets caused marked reduction of its ability to agglutinate platelets in vitro, but adsorption with endothelial cells had no such effect and its injection induced thrombocytopaenic purpura in vivo.

The Japanese workers in general concluded that purpura secondary to the use of anti-vascular endothelium sera resulted

from specific lesions of vascular endothelium, while purpura induced by anti-platelet serum followed from the dual effects of almost any type of cyto-toxin on vascular endothelium together with thrombocytopaenia. These views agreed with those expressed by Bedson and later by Quick, Shanberge and Stefanini, (I948) and Tocantins and Stewart, (I939).

Clarke and Jacobs (I950), succeeded in producing anti-sera against the vascular endothelium of dogs and produced severe haemorrhagic purpura without associated thrombocytopaenia by its injection.

The picture was confused, however, for many years before Duke, (I9I0) had been impressed by the striking improvement in the haemorrhagic tendency in some cases of thrombocytopaenic purpura when the platelet count was raised by transfusion. He thought that thrombocytopaenia was probably an important cause of bleeding, haemorrhages resulting from an insufficiency of platelets to repair capillary defects. Tidy, (I926) on the other hand held an opposite view believing that thrombocytopaenia was largely secondary to capillary damage, platelets being utilized to repair the damage. In respect of these two opinions, however, there were numerous reports of the occurrence of purpura without thrombocytopaenia (Mackay, I93I: Morrison, Lederer and Fredkin, I928: Roskam, I929) presumably due to pure capillary damage, while other workers had shown that platelets might be reduced to a very low level without producing purpura. (Brill and Rosenthal, I923: Thompson et al, I934). Indeed it was this very lack of correlation recognized long ago, that caused Bedson and others to investigate the problem of purpura and thrombocytopaenia, and while many workers believed that purpura, or more particularly haemorrhage was the result of combined capillary and platelet defects, it was widely held that the main lesion was increased capillary fragility, and that thrombocytopaenia was of secondary importance.

Basic understanding of the problem advanced slowly and as recently as I946 Dameshek and Miller remarked "The reasons for the development of sudden generalized bleeding from all the mucous membranes and into the skin are almost as obscure today as they

were in Werlhof's time".

In the Western world and for twenty years following Bedson's experiments with anti-platelet sera very little was contributed towards the mechanism of haemorrhage in purpuric diseases. Interest was centred principally on the mechanism by which thrombocytopaenia might be produced. As mentioned earlier, in 1915, Frank made accurate studies of essential thrombocytopaenia and postulated a marked diminution in platelet production by megakaryocytes. In the following year Kaznelson suggested splenectomy as a therapeutic measure in a chronic relapsing case of idiopathic thrombocytopaenic purpura, assuming by analogy with haemolytic anaemia, that the spleen might have an unusual thrombolytic action. The first operation was a brilliant success and although this was not maintained in all succeeding cases, the spectacular recovery of not a few desperately ill patients, led many workers to implicate the spleen as a prime pathogenetic importance in the disease. Whether the spleen participated by fostering excessive thrombocytolysis or whether the prime defect was decreased platelet production by megakaryocytes was still unknown in the early nineteen-forties although numerous investigations had reviewed marrow megakaryocytes in idiopathic thrombocytopaenic purpura since Frank and Kaznelson made their respective suggestions. Indeed Frank himself demonstrated a definite disorder of platelet production by marrow megakaryocytes, but the significance of his observations was not appreciated and most observers simply concluded that the spleen destroyed platelets excessively. Minot, (1917) with the help of J.H. Wright studied the marrow in a fatal case of the disease and reported that megakaryocytes were increased, but they were unable to say if there was any altered histology of these cells. Similar studies were made by Seeliger (1924), Gaspar (1926), Wiener and Kaznelson (1926), Schmincke (1930), Gerlach (1930), Nickerson and Sunderland (1935), Krjukof (1935), Rosenthal (1938) and Scott (1939) all of whom agreed that megakaryocytes were increased in idiopathic thrombocytopaenic purpura. Besides noting increased numbers of these

cells Jedlicka and Altschuler (1926) and Limarzi and Schleicher (1940), reported histological changes including vacuolization, lack of granularity, degenerative nuclei and cytoplasmic hyalinization.

In 1946 Dameshek and Miller attempted to reconcile the two theories of Frank and Kaznelson, and endeavoured to develop a concept of the pathogenesis of idiopathic thrombocytopaenic purpura centering about the failure of platelet growth from megakaryocytes dependent upon an abnormal inhibitory factor in the spleen. They studied the megakaryocytes obtained at sternal marrow biopsy in eleven cases of acute idiopathic thrombocytopaenic purpura and compared the numbers and morphology of these with marrows from normal control subjects, cases of thrombocytopaenic purpura associated with various types of splenomegaly, and a large group of miscellaneous haematologic conditions, including leukaemia, accompanied by thrombocytopaenia. They found that in both acute and chronic idiopathic thrombocytopaenic purpura, while megakaryocytes were greatly increased, most were immature and very few showed platelet formation; of the platelets that were formed most were abnormal in appearance. These findings were the reverse of those in all the other conditions studied (aplastic anaemia, leukaemia and other diseases invading or destroying bone marrow) in which thrombocytopaenia was readily explained on the basis of reduced numbers of megakaryocytes, which, none-the-less, always had normal appearance and showed normal platelet formation. In serial bone marrow examinations before and after splenectomy in some of their acute and chronic cases, Dameshek and Miller demonstrated a dramatic change in the appearance of megakaryocytes shortly after operation when extreme degrees of platelet production were evident. This finding strongly suggested a pathogenetic relationship of the spleen to the disease and Dameshek and Miller concluded that idiopathic thrombocytopaenic purpura was probably a form of hypersplenism in which, possibly through a hormonal mechanism, the bone marrow

megakaryocytes were inhibited from normal platelet production and delivery.

A big advance in the understanding of this disease came in I949, when Ackroyd published his work on the production of thrombocytopaenic purpura secondary to sedormid ingestion. Besides its contributions to pathogenesis this work gave further credence to the concept that haemorrhage in this disease was the result of dual capillary and platelet defects. Prior to Ackroyd's publication there had been numerous reports of thrombocytopaenia due, apparently, to an hypersensitivity reaction to a wide range of drugs. Many explanations for the occurrence of thrombocytopaenia had been forthcoming. While some workers on the basis of megakaryocyte morphology postulated maturation inhibition (Lieberherr I937, Lietner I945), others had found no or but slight megakaryocyte changes, and suggested the existence of further factors, probably a peripheral action on platelets, (Lieberherr). Moeschlin (I942) and Falconer and Epstein (I940), on the basis of rapid disappearance of platelets following test doses of the offending drug, invoked this latter theory and Moeschlin endeavoured to show the presence of a thrombocytolysin by transfusing blood from a patient with sedormid purpura to a normal recipient but failed. Quick, Ota and Baronofski (I946) proposed another hypothesis to explain the rapid thrombocytopaenia invoking a mechanism similar to that ensuing in anaphylactic shock in which, prior to the onset of thrombocytopaenia, platelets became clumped and later lodged in the capillary bed.

Skin testing in drug purpura had usually given negative results. Scarborough however, (I94I) recorded generalized lowering of capillary resistance throughout the whole skin area but particularly over the area of application when patch testing was carried out on a boy recovered from thrombocytopaenic purpura due to sulpha-thiazole. There was no accompanying fall in platelet count, a significant finding, suggesting as it did a specific effect on capillaries independent of any effect on

platelets. In his work Ackroyd demonstrated that local application of Sedormid to the skin of some patients recovered from thrombocytopaenic purpura due to this drug, caused petechial haemorrhages in the area of application. The absence of any associated thrombocytopaenia seemed to indicate, at least in so far as sedormid was concerned, that capillary lesions might be produced independent of any fall in the platelet count. Ackroyd was able to show, however, that sedormid did have an effect on platelets in these subjects by demonstrating in vitro agglutination and lysis of their platelets in the presence of sedormid. Because of this in vitro effect it was reasonable to infer that thrombocytopaenia in sedormid purpura occurred as a phenomenon distinct from capillary lesions. Ackroyd believed that these findings confirmed the view originally propounded by Nolf and supported later by Bedson (I922), Brill and Rosenthal (I923). Elliott and Whipple (I940), Poncher (I935) and Quick (I942), that two separate conditions existed in thrombocytopaenic purpura, a capillary defect and reduced platelet numbers. He felt that the latter aggravated the haemorrhagic tendency that existed because of capillary defects.

Since I942, occasional reports had appeared commenting on similarities between acquired haemolytic anaemia and idiopathic thrombocytopaenic purpura. Wiseman and Doan (I942) recorded the occurrence of leucopenia and thrombocytopaenia in a case of primary splenic neutropenia. A similar case was recorded by Rogers and Hall in I945. In I947 Fisher commented on the presence of leucopenia and thrombocytopaenia in one of a series of cases of acquired haemolytic anaemia, and Dameshek and Estren discussed similar findings in I948, but described their cases as hypersplenic haemolytic anaemia attributing the features of leucopenia and thrombocytopaenia to an unusual degree of splenic inhibition of bone marrow.

It remained for Evans and Duane (I949) after their studies on a series of cases of acquired haemolytic anaemia to suggest that neutropenia and thrombocytopaenia in these patients was

due to an immune body with a broader range of activity than erythrocytes or to a separate substance or substances more specific for platelets and leucocyte tissue. The latter seemed the more likely hypothesis for there was no correlation between the severity of the haemolytic process and the depression of either leucocytes or platelets. Subsequently in 1951 they adduced evidence for the common aetiology of primary thrombocytopaenic purpura and acquired haemolytic anaemia. They stressed that in many cases either haemolysis or thrombocytopaenia so predominated the picture that other features went unrecognized. They presented a series of cases representing the two extremes with intermediate examples to support their hypothesis. They demonstrated further that an antibody similar to those developed in acquired haemolytic anaemia could be detected in the plasma of patients with idiopathic thrombocytopaenic purpura.

Closely following this work Harrington et al (1951) demonstrated the presence of a thrombocytopaenic factor in the blood of eight of ten patients with idiopathic thrombocytopaenic purpura. Transfusion of 250-500 ccs. of blood from these patients to normal donors caused a profound drop in the platelet count of the recipients while two developed haemorrhagic manifestations typical of purpura haemorrhagica. In two patients of three with secondary thrombocytopaenic purpura no platelet reducing factor could be demonstrated.

At this time current concepts of the genesis of idiopathic thrombocytopaenic purpura were still those of reduced platelet formation by megakaryocyte suppression or increased platelet lysis or phagocytosis peripherally. The rapid disappearance of platelets after transfusion in this study strongly supported peripheral destruction as the major factor. However, marrow examination in one patient when thrombocytopaenia was most severe, showed typical immature, non-functioning megakaryocytes, and indicated that, in fact, thrombocytopaenia resulted from the

dual mechanism of impaired production and peripheral destruction. Overall, the study tended to confirm the concept of a circulating thrombocytopaenic factor as an important element in the production of thrombocytopaenia in purpura haemorrhagica and indicated similarities between the production of this disease and acquired haemolytic anaemia.

Meanwhile several authors had reported the occurrence of thrombocytopaenic purpura secondary to the drug quinidine (Nudelman et al I948, Norcross I950, Hirsch and Dameshek I950). In I953 Larson conducted a detailed study of such a case. Prior to this the rare complication of quinidine ingestion, thrombocytopaenic purpura, had been considered as a manifestation of specific sensitivity or allergy rather than drug toxicity. The then recent publications of Ackroyd and Harrington caused Larson to assess his patient in the light of this new knowledge.

When his patient had recovered and her platelet count had returned to normal, Larson attempted to demonstrate the aetiology of her condition by administering a test dose of 0.2g. of quinidine. Within 30 mins. her platelet count had fallen from 325,000 to 25,000/c.mm. and she began to bleed from the gums. At the same time her tourniquet test formerly very slightly positive became markedly so. Larson showed further that the effect of quinidine could only be produced in the presence of a factor in the patient's serum. Complete inhibition of clot retraction in normal blood could be produced by the addition of quinidine and the patient's serum together but neither quinidine nor serum was effective alone.

These experiments confirmed and extended the importance of Ackroyd's work, but in this case quinidine rather than sedormid was the antigenic agent. The rapid onset of thrombocytopaenia following the test dose precluded the immediate effect as one of megakaryocyte inhibition, and the effect could only be explained on the laws of a peripheral action on circulating platelets. In this, his work supported the concept of Harrington

et al, but Larson stressed that platelets being fragments of megakaryocytes, any agent likely to damage them could quite easily affect the latter. Indeed the first obvious effect, thrombocytopaenia would be due to the peripheral action of the causative factor, but later effects associated with the typical marrow appearances of inactive megakaryocytes would result from the dual effects of reduced platelet formation and their peripheral destruction.

At the same time Stefanini et al conducted a detailed investigation of the properties and mechanism of action of a potent platelet agglutinin in the serum of a patient with chronic idiopathic thrombocytopaenic purpura. This factor in high dilution was able to clump platelets in suspension and was able to interfere with the activity of normal platelets. The patient's platelets were found to be "coated" with an agent capable of reacting with anti-globulin rabbit serum. All indications pointed to the characterization of the agglutinin as a platelet iso- and auto-antibody. Normal platelets injected into the patient disappeared promptly from the circulation. The patient's plasma injected into normal recipients was followed by a series of events:- I. striking degenerative changes in bone marrow megakaryocytes accompanied by lack of platelet formation. 2. an extreme degree of platelet reduction with the development of haemorrhagic phenomena. 3. the presence of detectable platelet agglutinins in the patient's serum persisting for I2-I4 days, while the recipient's platelets were found to be coated with a substance capable of reacting with anti-human globulin serum. These workers postulated that in this case thrombocytopaenia was probably due to direct injury of circulating platelets and marrow megakaryocytes by a circulating agglutinin, leading to increased destruction and reduced formation of platelets.

Meanwhile interest in the survival times and effects of transfused platelets was becoming evident. Duke (I9I0) was apparently the first to investigate the survival time of plate-

lets in the peripheral circulation of man. From serial platelet counts in two patients with idiopathic thrombocytopaenia transfused with fresh blood, he concluded that the life span of transfused platelets was very short and in the order of two days. Lawrence, Valentine and Adams (1948) failed to gain any response in two patients, with aplastic anaemia and chronic thrombocytopaenic purpura respectively. Using siliconized apparatus, Hirsch, Favre-Gilly and Dameshek (1950) transfused blood from a polycythaemic donor to a patient with thrombopathic thrombocytopaenia. The patient's platelets were very large and amorphous and could be readily distinguished from those of the donor in serial platelet counts. By simple enumeration and morphological differentiation it was possible to determine that the donor platelets survived for five to six days after transfusion. In 1952 Hirsch and Gardner described the applications of the technique of transfusing polycythaemic blood with siliconized apparatus, to various primary and secondary thrombocytopaenic states.

In their series platelet transfusions were successful in raising the platelet counts of twenty two of the twenty three patients studied. Of these the highest counts and longest platelet survivals were found in patients with aplastic anaemia, acute leukaemia and pan-cytopaenia with normal bone marrow. The lowest counts and shortest survivals were found in cases of acute thrombocytopaenic purpura, while in the chronic forms of the disease, while initial elevations of the platelet count were obtained, survival was reduced but never to the extent of that seen in acute cases.

Clinically the most striking effects were the rapid cessation of haemorrhagic symptoms whenever these were present and their absence throughout the period during which transfused platelets survived. Coincident with this improvement bleeding times and tourniquet tests showed a marked return towards normality. Following the dramatic cessation or amelioration of bleeding in their patients following transfusion of normal platelets, Hirsch and Gardner concluded that haemorrhage could not have been due

primarily to vascular defects. Excellent correlation between platelet survival times and the duration of improvement strongly suggested that improvement was due to transfused platelets. Normal survival times of 4-5 days of transfused platelets in aplastic states indicated that lack of formation was the only cause of thrombocytopaenia in these cases. On the other hand, rapid platelet destruction and their apparent survival for a few hours only after transfusion in acute post infectious thrombocytopaenic purpura, was comparable to the rapid development of thrombocytopaenia in sedormid and quinidine purpura, and suggested the presence in blood of these patients, of a factor causing rapid peripheral elimination of platelets distinct from defective production. The sequence of events in chronic idiopathic thrombocytopaenic purpura suggested that peripheral platelet destruction did occur but at a slower rate than in acute cases. When considered in relation to the work of Harrington et al, Larson and Stefanini et al, outlined earlier, Hirsch and Gardner concluded that their work favoured rapid peripheral destruction of platelets in all types of acute thrombocytopaenia whether post infectious, idiopathic or due to drug sensitivity, for while their cases had been post infectious, those of Harrington et al. were quite idiopathic while, Larson and Stefanini et al. had described cases secondary to drug sensitivity.

However, despite conclusive evidence in support of a thrombocytopaenic factor acting peripherally, the authors reviewed their work in the light of that of Dameshek and Miller. They stressed that platelet destruction in thrombocytopaenic states might be an incidental finding, and that lack of platelet formation, demonstrated by Dameshek and Miller, might be the basic defect, for under these circumstances no platelets would be released to be destroyed by factors in the plasma or elsewhere. In support of the latter hypothesis, the normal survival of transfused platelets in aplastic states indicated lack of formation by megakaryocytes as the cause of thrombocytopaenia.

In the same year 1952, Stefanini et al, conducted an almost

identical investigation in twenty two patients including twelve cases of acute idiopathic thrombocytopaenic purpura, two cases of the chronic variety and eight cases of thrombocytopaenia due to primary bone marrow disease (acute or subacute leukaemia, hypoplastic and aplastic anaemia). Their results were remarkably in agreement with those of Hirsch and Gardner.

They found that platelets disappeared rapidly from the circulation (one half to twelve hours) in the twelve patients with acute idiopathic thrombocytopaenia. Disappearance was slower in chronic idiopathic thrombocytopaenic purpura (24 hours) while in cases of secondary thrombocytopaenia, survival of platelets in the recipient's circulation was much longer averaging forty eight to ninety six hours.

Again improvement in haemorrhagic symptoms was noted in every case after transfusion, and was maintained throughout the time in which transfused platelets survived and for many hours after the platelet count had returned to its original level. One of the most interesting features of their work, however, was the dramatic improvement in bleeding times and capillary fragility evident in every case within a few minutes of transfusion. Again this was usually maintained for twenty four to forty eight hours after platelet counts had regained their pre-transfusion levels. These features were far more obvious in this work than in that of Hirsch and Gardner.

From the prompt disappearance of transfused platelets in acute idiopathic thrombocytopaenic purpura Stefanini et al, concluded that a humoral destructive mechanism was active in these which was not present in "secondary" or amegakaryocytic cases. They indicated, however, that the existance of such a factor did not exclude an inhibitory effect upon platelet production from megakaryocytes in the bone marrow. They stressed too, that despite the rapid disappearance of transfused platelets in acute cases, haemorrhagic manifestations were greatly improved, but they were unable to offer an explan-

ation for this.

Since 1949, then, many authors had contributed to clarify the mechanisms underlying the production of thrombocytopaenia, but little information had been forthcoming to clarify the mechanism of haemorrhage. The relative importance of capillary defects and thrombocytopaenia was still unknown, and the factors governing the severity of haemorrhage in each case remained a mystery. Thus, it was only natural that the long recognized association of thrombocytopaenia and a prolonged bleeding time should have claimed the attention of several workers.

One of the earliest was Duke who, in 1912, published his investigations conducted on cases of primary and secondary thrombocytopaenia and on the experimental production of purpura in laboratory animals. One reads not infrequently that Duke found strict correlation between the bleeding time and the degree of thrombocytopaenia. Careful consideration of his original article makes it evident that this was not so. In many instances he found that thrombocytopaenia was accompanied by a very prolonged bleeding time, but he also recorded in detail the many occasions on which thrombocytopaenia and a normal bleeding time existed together. Further, he remarked on the frequent disocciation between a greatly prolonged bleeding time, severe thrombocytopaenia and lack of progressive clinical haemorrhage, if indeed such might be expected should the bleeding time and thrombocytopaenia be considered directly related to severity of haemorrhage.

None-the-less, Duke was convinced of the usefulness of serial bleeding time estimations in assessing progress in thrombocytopaenic states, and as a test of the general tendency to bleed. He deplored reliance solely on clinical judgement as an index of the progression of haemorrhagic phenomena, believing they were influenced too much by local conditions including voluntary activity of the patient and passive handling by staff to be of any substantial reliability. His faith in the

reliability of the bleeding time was based to a large extent on his belief that the major factor in the production of the bleeding time was the formation of a platelet plug in severed capillaries. The capillaries themselves were of secondary importance. A prolonged bleeding time depended principally on the absence of or defective platelets. Extensive investigations since Duke's original publication have left no doubt that platelets do play a role in determining the bleeding time, but it is now apparent that the process is not simple plugging of severed capillaries by a platelet mass, but is the resultant of extra-vascular, vascular and intra-vascular factors.

To some extent assessment of haemorrhage and its control in thrombocytopaenic states has been confused by failure to differentiate between haemostasis and coagulation, two vastly different processes. Indeed few observations on the mechanism of haemostasis have been published since I882 when Hayem and Bizzozero first reported on the form and function of mammalian blood platelets. This void is a striking contrast to the mass of literature dealing with haemorrhagic diseases, blood coagulation and isolated blood elements.

The most obvious function of coagulation is the control of bleeding, and the popular conception of its working was extremely simple; blood issuing from damaged tissues solidified into an impervious and adherent mass, effectively preventing further haemorrhage. Closer examination of the problem, however, quickly invalidates any such simple explanation of haemostasis, for clinically normal haemostasis often arrests haemorrhage from arterioles, venules and capillaries in a few seconds, although coagulation may take 5-I0 minutes. Further the mechanism of securing haemostasis from small stab wounds differs from that operating in the case of open wounds. In those conditions where clotting is defective, the bleeding time is usually normal but haemorrhage from open wounds is protracted. When platelets are numerically or functionally defective both the bleeding time and bleeding from open wounds may be prolonged. In still other

states when both platelets and clotting mechanism are apparently normal, prolonged bleeding may ensue in both small stab wounds and large open wounds. On the other hand thrombocytopaenia may exist without any abnormal bleeding whatsoever.

There are few reported investigations of the way in which vascular factors, platelet agglutination and blood clotting react together to arrest bleeding. So much attention has been paid to the details of each of these factors, that the larger and more basic problem of haemostasis has been neglected.

Haemostasis effected by platelet agglutination was first demonstrated by Hayem in I882, in an experimental wound of a dog's jugular vein. In I885 Lubnitzsky reported haemostatic platelet thrombosis in similar wounds of rabbit's crural arteries. Lubnitzsky studied the rate of platelet agglutination in vascular wounds, and showed that in rabbits an incomplete thrombus filled the arterial gap within fifteen seconds, but that open pathways in this were not closed until the end of the first minute. She also demonstrated that unsupported platelets were unable to resist the blood pressure in large arteries since platelet thrombi could only be produced in rabbit's arteries if moderate proximal pressure was applied.

Magnus (I923) and Stegemann (I924) noted that arteries contracted after injury. Magnus (I924) and later McFarlane (I94I) found that human capillaries disappeared from view after injury, even when the venous pressure was raised, reappearing after some 20 minutes to 2 hours. In thrombocytopaenic purpura and Von Willebrand's disease, however, severed capillaries did not disappear and sustained haemorrhage occurred from visible, damaged vessels.

After a lapse of nearly 60 years Apitz (I942) and M.B. Zucker (I947) confirmed the early work of Hayem and Lubnitzsky and extended it in studies involving experimental interference with coagulation and haemostasis by the use of heparin, dicumarol and anti-platelet serum. They watched the formation of platelet thrombi in vascular wounds of small

animals, and found that these appeared within 10-30 seconds of injury and became haemostatically effective within four minutes despite gentle irrigation. Zucker found that neither dicumarol nor heparin in therapeutic doses affected platelet agglutination after vascular injury in vivo, and haemostasis was unaffected. She also studied reaction to injury of the small vessels of the meso-appendix of the rat, and found that non-muscular venules did not contract after injury but that a plug of refractile material, presumably platelets formed at the tip of each severed vessel. Small arterioles and veins with muscle coats did contract in response to injury, however, as did nearby uninjured vessels. This latter remote contraction only occurred if platelet plugs formed at injured sites. Similar marked contraction of uninjured vessels followed the local application of platelet extracts.

Chen and Tsai (1948) confirmed these findings in frogs and rabbits but found that in certain sites, capillaries were capable of active contraction, but not in others. In the latter sites pressure or cutting caused adhesion of endothelial cells with consequent obliteration of the lumen. Haemorrhage induced by section of venules in the ear, brain and mesenteric vessels was stopped by the formation of platelet plugs which became haemostatically effective within 3 minutes, while arteries and arterioles contracted actively and remained contracted for an hour or more. During this period of contraction, blood which had escaped clotted in situ, while removal of the clot from the vessel wall shortened the period of contraction.

Apitz (1942) had studied the histology of bleeding time puncture wounds obtained at autopsy. In a similar study H.D. Zucker (1949) used serial sections to study the histology of small puncture wounds in human skin, removed by biopsy after the wounds had ceased to bleed. The material examined included wounds in both normal individuals and four with idiopathic thrombocytopaenic purpura.

Zucker was impressed with the great variation in structure

of wounds in successive cases despite care to unify the technique used in their production, for while some penetrated but half the skin thickness, others extended into the subcutaneous fat. The most striking feature, however, was the extreme variability in the size and number of the vessels cut and the lack of difference in appearance between the endothelium of control and purpuric subjects. Only rarely could severed capillaries be identified, and Zucker concluded that their recognition was obscured by traumatic distortion of their endothelium, collapse or endothelial agglutination. In the control series, puncture wounds could be visualized as tubular tracts into which a variable number of small vessels (precapillary arterioles and post-capillary venules) opened, but capillaries were few. In each case the actual wound was filled by a mass of erythrocytes and fibrin. The open end of each vessel larger than a capillary was sealed by a platelet plug most of which projected into the lumen of the track while an occasional platelet mass was incorporated in the red-cell-fibrin mass. The bulk of the platelets in these masses had undergone viscous metamorphosis. Any true capillaries that could be recognized were conspicuous by the absence of platelet plugs, their open ends being sealed by fibrin in the wound tracks. The fibrin lay across the ends of the capillaries but never entered the lumen.

In the thrombocytopaenic subjects the structure of the wound track and the number and size of the vessels cut, was essentially the same as in the normal controls. The wound tracts were filled with a fibrin-red cell coagulum. Fibrin strands or masses closed some capillaries, but in all instances the outstanding feature was the complete absence of platelets or platelet plugs in any vessel or the wound tract itself. In one instance the wound was filled with uncoagulated blood, while, in another, capillaries and larger vessels were prominently dilated.

Each of these workers McFarlane, M.B. Zucker, H.D. Zucker

and Chen and Tsai, used their respective findings as a basis for speculation on the mechanism of haemostasis.

McFarlane proposed a two-stage mechanism. He supposed that normally loss of blood from damaged small vessels was arrested by their temporary contraction and that, during this period of haemostasis, blood lying in the wound had time to clot firmly. This adherent clot prevented a recurrence of bleeding on vascular re-dilatation. Based on this theory, abnormal bleeding might occur for two main reasons.

I. In the absence of vascular contraction, bleeding would be continuous from any wound, since normal coagulation itself was incapable of arresting an actual flow of blood.

2. If coagulation itself was deficient, bleeding would ensue when vascular contraction relaxed, for no firm clot would have formed in the interval.

McFarlane's theory, then, was based on the ability of human capillaries to contract, a property of capillaries that has been denied by some workers (Clark and Clark I943), (Chambers and Zweifach I944). Other workers, however, proved that capillaries in certain tissues do contract for Chen and Tsai (I948) and Lewis (I923, 24, 27) showed that human skin capillaries do contract under suitable stimulation, while Sanders, Ebert, Florey (I940) showed that capillaries in the rabbit's ear were capable of active contraction due to rapid swelling of endothelial cells.

McFarlane's hypothesis explained most of the observed phenomena in coagulation and haemostasis, but was criticized for its failure to recognize a role for the blood platelet. The associations of a prolonged bleeding time with normal platelet numbers and a normal bleeding time with thrombocytopaenia were recalled in answer to this criticism and as evidence that platelets were not essential for haemostasis. The commoner occurrence of a prolonged bleeding time and thrombocytopaenia was attributed to antigenic similarities between platelets

and vascular endothelium. For this reason agents toxic for platelets were likely to be toxic for capillary endothelium with consequent alteration of the bleeding time.

Although she had noted the vaso-constrictor effect of platelet extracts, M.B. Zucker (I947) regarded both this and inherent vascular contraction unimportant, believing that platelet plugs played the dominant role in haemostasis.

Chen and Tsai (I948) developed a theory which was the reverse of Zucker's in some respects and in others similar to McFarlane's two-stage mechanism. They believed vascular contraction was most important, but, in addition, platelet agglutination, endothelial adhesions and clot formation, all played a part, the relative importance of each mechanism varying with the type of vessel involved.

In preface to his theory of haemostasis H.D. Zucker stated that any such theory must account for the arrest of bleeding within a stated time interval (I-3 minutes for small skin wounds) and must explain the prolonged absence of renewed bleeding. While fibrin formation had often been suggested as the mechanism whereby open vessels were sealed, Zucker stressed that fresh blood would be continually passing through the cut ends of vessels and that such blood would not clot in vitro in many minutes. In the laboratory, in the presence of excess thromboplastin, human blood would not clot in less than several seconds. Accordingly, even if fibrin was a sufficient seal, a given drop of blood would have to be delayed for several seconds at the tips of a severed vessel if haemostasis was to be accomplished. He was unable to accept unaided vascular contraction, as proposed by McFarlane, as sufficient to account for haemostasis by allowing sufficient time for extravasated blood to clot. He did not believe the evidence in favour of vascular contraction sufficient to be accepted without reservation. On the other hand, both he and Apitz had shown that platelet thrombi normally form in most skin vessels larger than capillaries,

while he had demonstrated that such thrombi developed within the expected time interval, and that they were able to resist considerable pressure. The importance of platelets in the haemostatic mechanism preceding clotting was emphasized further by failure of fibrin formation in briskly bleeding thrombocytopaenics who had normal clotting and prothrombin times, and by delayed fibrin formation in other thrombocytopaenics.

After consideration of the foregoing, Zucker postulated that in haemostasis, platelet thrombi act as cofferdams, which, aided by local vaso-constrictor mechanisms, stop or slow blood flow to the extent that extravasated blood within the wound is allowed sufficient time to clot. Not all vessels need to be plugged, however, for once blood flow had been sufficiently slowed, coagulation would follow and seal small vessels still lacking a platelet plug. Fibrin formation in the wound track and its later retraction represented the formation of a permanent concrete dam which reinforced and anchored the earlier platelet plug. Once fibrin formation had reinforced platelet thrombi, resumption of normal pressure relationships as vaso-constriction relaxed, could be accomplished without renewed bleeding. By contrast, coagulation alone, secondary to tight vaso-spasm or obliteration of a vessel lumen by pressure would fail to maintain haemostasis on the resumption of normal blood pressure, because without blood flow, platelet thrombi could not form. On the other hand he considered it unlikely that platelets played any part in capillary haemostasis for neither he nor Apitz had been able to demonstrate platelet plugs in capillaries. The small difference in intra-capillary and tissue pressures, assisted by reflex contraction at the capillary mouths would permit only a slight ooze of blood from opened capillaries, and coagulation of extravasated blood would proceed unimpeded. Fibrin formed in the latter process would be a sufficient seal for capillaries.

The absence of platelet plugs in Zucker's sections cannot

be accepted as proof that platelets play no part in capillary haemostasis, however. It is only reasonable to assume that in the production of bleeding time tracks, at least as many capillaries as arterioles and venules have been cut. Their lack of recognition in subsequent sections may have been hindered by contraction while platelet plugs might not have formed because bleeding had not persisted for any length of time.

Zucker reviewed the bleeding time in relation to his findings. He stressed that because of the variation in depth of punctures, in some instances only capillary bleeding time was tested while others tested the entire mechanism of haemostasis. This, he thought was an adequate explanation for the tendency of bleeding in thrombocytopaenic purpura to vary in duration at different skin sites. He concluded that the prolonged bleeding time in acute idiopathic thrombocytopaenic purpura was explained by loss of the primary haemostatic mechanism, platelet plugs, in severed muscular vessels. This was due to either quantitative or qualitative platelet deficiencies and was sufficient explanation for the abnormal bleeding in thrombocytopaenic conditions; the implication of other factors was not necessary.

This explanation of the bleeding time, however, is not totally acceptable, for such estimations must test capillary bleeding time which is usually normal in those conditions in which fibrin formation is greatly delayed or absent. In Zucker's interpretation the reverse would be the case, should fibrin be the means of securing capillary haemostasis.

From consideration of the work of these four authors it was apparent that the mechanism of haemostasis was dependent on a summation of factors which included:-

1. Contraction of blood vessels - arterioles, venules and probably capillaries.
2. Formation of platelet masses in damaged vessels.
3. Fibrin formation.

Further, it was possible to conceive a sequence of events as adequate explanation for the securing and maintenance of haemo-

stasis. Following injury to a small vessel, platelets would start to adhere to each other and to the injured site forming a platelet plug covering the lesion. At first this plug would be permeable but, shortly, and probably secondary to viscous metamorphosis, it would become impermeable and bleeding would stop. This process, aided by contraction of vessels would be sufficient to secure early haemostasis. Viscous metamorphosis of platelets probably initiated or accelerated coagulation by liberating platelet thromboplastic factor. New platelets adhering to the fibrin network would disintegrate under the influence of thrombin releasing more lipid, enlarging the thrombus and establishing a cycle, conducive to the establishment of permanent haemostasis.

This explanation emphasizes two phases of "total" haemostasis, namely immediate haemostasis of freshly injured vessels and the maintenance of normal haemostasis. There is ample evidence to suggest that "total" haemostasis is dependent on normal functioning of these two processes and that separation into two phases is quite valid. Further, the relative importance of platelets and coagulation in the two stages would appear to be quite distinct; coagulation itself would seem to be unnecessary to attain effective immediate cessation of haemorrhage. In support of this assertion one can say,

I. Fibrin is not detected in platelet plugs which arrest bleeding from freshly injured small vessels (Zucker I947) and patients with congenital afibrinogenaemia usually have a normal bleeding time (Penniger and Prunty I946), (Alexander et al I954).

2. Patients with congenital defects in coagulation factors usually have a normal bleeding time (Borchgrevink and Waaler I958).

3. Dicoumarol treatment of rats does not prevent the formation of platelet plugs following injury of small vessels (Zucker I947).

4. Heparin in therapeutic doses did not prevent the form-

ation of white thrombi in rats in vivo nor did it prevent the formation of thrombi in arterio venous glass shunts (Zucker, I947).

5. In certain arthropods no fibrin is detectable in blood and in these "haemostasis" is due solely to a plug formed by amoebocytes. In comparative phylogenetic studies it was found that haemostasis in lower species was based on cellular aggregation only. In higher species an additional mechanism, coagulation, has been developed. The cellular mechanism, however, always precedes coagulation.

Thus coagulation is probably not necessary to achieve immediate haemostasis, but it is essential for the maintenance of haemostasis. Patients who have deficiencies of coagulation factors usually have a normal bleeding time but an haemorrhagic diathesis characterized by "late" or "after" bleedings. This observation indicates a difference between the process which arrests bleeding in freshly injured vessels and the process which secures permanent haemostasis. Such differences in the mechanism of "primary" and permanent haemostasis have recently been demonstrated by Borchgrevink and Waaler (I958). Their work strongly supported the concept that platelets were, in large part, responsible for "primary" haemostasis while both platelets and coagulation factors belonging to the intrinsic system were responsible for permanent haemostasis in man.

Thus while ample evidence was forthcoming to establish the importance of platelets in normal haemostasis, the factors underlying their deposition, cohesion and adhesion were poorly understood until very recently. Despite the obvious importance of platelet adhesiveness to both haemostasis and thrombus formation, the subject had received scant attention, and of the few investigations made, most were at best semi-quantitative, while in others the results obtained could in no way be accepted as dependent solely on platelet adhesiveness. In I960 Hellem published details of a new and satisfactory technique, in which, by means of an electrically driven mechanical device blood was

pushed from a graduated syringe through a standardized glass bead column at a constant rate. The reduction in platelet count after passage through the glass bead column was taken as a measure of platelet adhesiveness. It was assumed that the reduction in platelet numbers by the procedure was caused by their adhesion to glass beads.

Hellem applied this technique in a comprehensive survey of platelet adhesiveness in specimens of citrated whole blood from 104 normal donors. In these the total platelet count ranged from 138,000 per c.mm. to 421,000 per c.mm. with a mean of 215,000 per c.mm. The number of adhesive platelets ranged from 52,000 to 150,000 with a mean of 90,000. The percentage of adhesive platelets ranged from 26-68 percent with a mean of 42 percent. He then reassessed platelet adhesiveness in citrated platelet rich plasma from sixty of the same donors and found that in every case platelet adhesiveness was virtually zero.

Hellem proceeded to show that there was an almost linear relationship between the number of red cells in the plasma and platelet adhesiveness and that their presence was essential for immediate adhesion to glass. He proved that the alteration of platelets in blood was secondary to changes in red cells which had been in contact with a foreign surface. In his apparatus contact with glass beads caused red cells to liberate a water soluble, heat stable substance (Factor R) which increased the adhesiveness of platelets in citrated plasma. In further studies he found an almost linear relationship between platelet adhesiveness in citrated plasma and the logarithm of the concentration of added Factor R.

The presence of Factor R in erythrocytes raised the exciting possibility that this, or a similar substance might be present in other body cells. Of special interest in this respect was the vascular wall, for its presence here would explain the immediate adhesion of platelets to injured intima in thrombosis and haemostasis. Although Hellem was unable to demonstrate any factor R in either human brain tissue or the aorta of a freshly killed

cow, this does not preclude its existence, perhaps in smaller blood vessels. Future work may clarify this important possibility.

Another significant finding was the effect of factor R on platelet clumping. Increasing concentrations of factor R produced increasing platelet clumping as well as increased adhesion of platelets to glass. The higher the concentration of factor R added to a drop of platelet rich plasma on a slide, the greater was the number of platelets that remained firmly adherent to the glass after gentle irrigation with saline.

Hellem applied his technique to an investigation of the relationship between the number of adhesive platelets and the bleeding time in patients with decreased or variable platelet adhesiveness. He was able to establish a fairly close inverse relationship between the number of adhesive platelets and the bleeding time, whereas there was no correlation between the bleeding time and the total number of platelets. In absolute numbers, adhesive platelets had to be reduced below an approximate level of 40,000 per c.mm. before any prolongation of the bleeding time was evident. Of nine patients with acute idiopathic thrombocytopaenic purpura, all with greatly prolonged bleeding times, eight had percentages of adhesive platelets within normal limits, but in all cases the absolute numbers were below the critical level. The platelets of three people with thrombo-asthenia had zero platelet adhesiveness, while factor R prepared from their erythrocytes was of normal concentration and activity. Further, since their platelet's adhesiveness was not improved by the addition of factor R prepared from normal erythrocytes, Hellem concluded that in this disease the defect was resistance to, but not lack of, factor R. In haemophilia, Christmas disease and anti-coagulant therapy platelet adhesiveness was normal, as was the case in fifteen cases of Von Willebrand's disease. All of the latter cases had prolonged bleeding times, and Hellem was unable to decide whether the defect in this disease was a vascular, platelet or a plasma defect.

This work added even further support to the concept of the major role played by platelets and platelet aggregates in primary haemostasis, and the non-essentiality of coagulation at this stage. The relationship between the bleeding time and platelets was clarified. It is interesting to consider Hellem's findings in relation to those of Humble on the mechanism of petechial haemorrhage formation.

Humble (1949) used capillary microscopy to study the mechanism of petechial haemorrhage formation during the performance of tourniquet tests in 17 cases of haemorrhagic disease of widely differing aetiologies. These included 5 cases of idiopathic thrombocytopaenic purpura, 7 of secondary thrombocytopaenia (drug induced, leukaemia, aplastic anaemia) and 5 of non-thrombocytopaenic purpura (including anaphylactoid purpura, scurvy and essential hypoprothrombinaemia).

In all cases the purpuric lesions arose from the same position in the capillary loop, at the arteriolar-capillary junction. Humble emphasized some important features peculiar to this site. It was the area from which fluid normally left for the tissues, and where the tightly constricted pre-capillary arteriole suddenly dilated to form a capillary loop. The intra-capillary pressure at this point was higher than anywhere else in the capillary loop and this very area was of great importance in the maintenance of blood flow and tissue nutrition generally. In Humble's opinion these features made the arteriolar end of the capillary loop peculiarly vulnerable to selective poisoning or damage by toxic agents, and more conducive to haemorrhage under pressure.

The purpuric lesions themselves were of two types, small, produced by erythrocytes leaking from a single capillary, and large, produced by confluence of haemorrhages from adjacent loops. In all cases, save one, the behaviour of the lesions was the same, in that a shower of erythrocytes spread cone-wise from the capillary defect for a longer or shorter distance but soon stopped. The one case in which a defect of the clotting

mechanism was assured, was characterized by the curious way effused blood tracked superficially around the capillary loop. One could, perhaps, attribute this to lack of total haemostasis.

From Hellem's work, therefore, one can visualize escaped red cells, in contact with a foreign surface, releasing factor R. The latter would immediately effect platelet adhesion and clumping to seal the leak and establish primary haemostasis. Coagulation would follow promptly and permanent haemostasis would be established. In the case of essential hypoprothrombinaemia the second phase would be wanting and escaped fluid blood would be enabled to track around capillaries to a greater extent.

Two major questions remained, however, for the mechanisms governing the severity of haemorrhage and the action of ACTH and cortico-steroids in thrombocytopaenic purpura were still obscure. Although Duke had warned of the dangers inherent in clinical appraisal alone, nearly half a century later the clinical state of the patient remained the best guide to the severity of the disease and its progress. As for laboratory techniques the platelet count was useful in diagnosis but the complete dissociation between platelet numbers and haemorrhagic manifestations was all too evident, and cited as indicative of the major role of vascular damage in these diseases.

In 1957, Bonnin suggested that the severity of haemorrhagic manifestations might be related to levels of platelet thromboplastic function and quite independent of platelet numbers. He modified the thromboplastin generation test of Biggs and Douglas (1953) to quantitate platelet thromboplastic function. In this the thromboplastic function of a standard number of patients' platelets was compared with the thromboplastic activity of the same number of normal platelets by means of a calibration graph.

Bonnin applied this technique originally to 121 estimations of platelet thromboplastic function in 30 individuals with thrombocytopaenic purpura of various types and correlated these results with the haemorrhagic state of the patient and the platelet count at the time of the estimations. He found that

when platelet thromboplastic function was below I2% of normal, haemorrhage occurred in every patient. Between I2% and 25% function, while some patients had frank bleeding, others had purpura only, and he considered that this was a range of function in which haemorrhage was likely to occur. Patients whose platelet thromboplastic function ranged between 25% and 53% often had purpura but no frank haemorrhage, but others in this range had no haemorrhagic signs whatsoever. This range was considered that in which purpura was likely to occur. Above these levels no patients had either purpura or haemorrhage, save one, whose bleeding was explained by other complicating factors. Thus, Bonnin demonstrated reasonable correlation between platelet thromboplastic function and haemorrhagic manifestations and emphasized the complete dissociation between these and platelet numbers.

In the same paper Bonnin proposed a possible mechanism whereby purpura and haemorrhage might be produced. These two, he considered, were related but independent phenomena. He suggested that haemostasis was dependent on 3 principal factors: I). The efficiency of the vascular mechanism including the ability of damaged vessels to contract. 2). The degree of damage to vascular endothelium and 3). The effectiveness of the haemostatic mechanism. If vascular damage was severe enough, the occurrence of frank bleeding would be related to the degree of platelet thromboplastic dysfunction, or, when other coagulation factors were wanting, to the total thromboplastic efficiency. If the same factor produced both vascular and platelet functional defects, both would be affected together and the degree of each would be roughly proportional. Under these circumstances, estimations of platelet function would give an approximate guide of both the degree of vascular damage and the state of the coagulation mechanism.

Considering the results of thromboplastic function estimations in relation to this mechanism Bonnin suggested that when vascular damage was sufficient to produce a purpuric leak obvious

platelet dysfunction could be detected by the thromboplastin generation test. However, because one was not dependent upon the other there was a relatively wide range over which purpura might occur. It was assumed therefore that purpura was a manifestation of capillary damage alone. In these circumstances, when the coagulation mechanism was not greatly impaired, the lesion was repaired and blood flow was re-established through an intact vessel leaving a small purpuric spot or larger ecchymotic area according to the degree of vascular damage. When platelet thromboplastic function (or total thromboplastic efficiency) was so reduced that the coagulation mechanism was severely impaired, repair was no longer possible and frank haemorrhage would ensue. Frank haemorrhage, therefore, depended not only upon vascular damage but directly upon platelet thromboplastic function, thus explaining the narrower range of platelet function over which it occurred.

In his initial series Bonnin recorded dramatic improvement in platelet thromboplastic function, coincident with amelioration or cessation of haemorrhagic manifestations in patients receiving ACTH or cortico-steroids. This improvement often preceded rising platelet counts by days. Bonnin suggested that the beneficial action of these drugs lay in their ability to protect vascular endothelium and platelets against the damaging action of serum or plasma factors present in thrombocytopaenic purpura.

In two subsequent papers, Bonnin (I96I) has described the application of his platelet thromboplastic function test to the management of thrombocytopaenic states. The first of these dealt with idiopathic and secondary thrombocytopaenic states in which normal or increased numbers of megakaryocytes were present in marrow. Again reasonable correlation was found between levels of platelet thromboplastic function and haemorrhage, and often platelet function had returned to normal and haemorrhagic manifestations ceased after steroid hormone therapy was instituted, long before any change in platelet numbers was evident. The relationship between platelet thromboplastic function and purpura remained obscure, however,

and Bonnin suggested that it might indicate the necessity of platelets for the maintenance of the integrity of vascular endothelium or that vascular endothelium and platelets were damaged in parallel by a common antibody. He further indicated that the prime effect of ACTH and steroids might be to protect megakaryocytes from such an antibody and thus assist in the production of qualitatively normal platelets.

His second article devoted to leukaemic and aplastic thrombocytopaenia demonstrated the usefulness of serial estimations of platelet thromboplastic function in assessing the initial severity and progress of haemorrhage and the unimportant role played by platelet numbers. In this group, however, the response to ACTH or cortico-steroids was much less satisfactory than in the first series.

Bonnin's work would seem to have established the importance and reliability of estimations of platelet thromboplastic function in the control of thrombocytopaenic states. It provided a means whereby patients could be assessed and their therapy controlled without reliance on purely clinical impressions. However, his original hypothesis suggesting parallel damage to capillary endothelium and platelets was not wholly acceptable, for one factor, proportionate capillary damage was beyond the bounds of experimental proof. He has however, modified his ideas in the light of recent knowledge.

It is highly likely that the basic defect in thrombocytopaenic states is one of megakaryocytes which, once damaged from whatever cause, are either unable to produce platelets or produce abnormal platelets. Peripheral destruction of platelets is relatively unimportant in the production of thrombocytopaenia, as suggested earlier by Hirsch and Gardner. The circulating platelet, however, is of major importance for the maintenance of normal haemostasis. While it is likely that megakaryocytes, platelets and vascular endothelium will all be damaged by agents toxic to one because of their antigenic similarities, recent but, as yet, unconfirmed, data has contributed to an understanding

of a mechanism whereby capillary damage might ensue distinct from direct toxic effects.

Luscher and Asper (I960) claim to have isolated a protein factor from platelets which they believe is essential for the maintenance of normal capillary integrity. Should this be so, it is possible that defective circulating platelets will lack this factor or that it will be functionally imperfect or limited and that ultimately capillary damage will follow from its lack. The existence of such a factor is supported by the findings of Hirsch and Gardner (I952) and Stefanini et al (I952) in their work on transfused platelets. Both groups of workers found dramatic improvement in the bleeding time and Hess test immediately following transfusion of normal platelets in thrombocytopaenic states. The speed of this improvement was such that Hirsch and Gardner used it as evidence contravening capillary damage as the prime cause of haemorrhage. Equally well it can now be explained on the assumption that the protein essential for capillary integrity was transfused in normal platelets and quickly utilized to repair capillary damage, with consequent rapid improvement in the bleeding time and Hess test.

Thus in less severe states of platelet dysfunction capillary damage, supposedly, would be less. A small shower of erythrocytes leaking from damaged capillaries would precipitate a release of factor R, with immediate alterations in platelet adhesiveness and clumpings and "primary" haemostasis would follow. In this state, the coagulation mechanism, although impaired, would be sufficient to ensure permanent haemostasis. With progressive platelet damage, capillary damage would progress until a stage was reached when widespread leaking from many capillaries would come. At this stage it is conceivable that platelet adhesiveness may be reduced to critical levels when insufficient adhesive platelets would be available to effect primary haemostasis. Insufficient adhesive platelets may be apparent for two reasons; firstly if damage is sufficiently widespread to require more platelets than are available to

repair the damage, or if damage is sufficiently severe in a more limited area. This failure of the primary haemostatic mechanism may occur despite apparent adequate total numbers of platelets. Once failure of "primary" haemostasis has ensued, haemorrhage would follow, the severity depending on the degree of disruption of the coagulation mechanism reflected in estimations of platelet thromboplastic function. Indeed in the severest states both primary haemostasis and permanent haemostasis would become ineffective.

HISTORICAL REVIEW

Part IV

A consideration of the techniques that may be used to detect or assess abnormalities of platelet function.

I. Bleeding time.

The mechanism of the bleeding time and some factors influencing it have already been discussed. It has been shown that the cessation of blood flow from a small puncture wound cannot be explained simply on a mechanical plugging of capillaries by a mass of platelets in a gross sense, but is a delicate process in which both platelets and vascular factors are important. A possible third factor, an extravascular component is often forgotten. If one had to define the bleeding time therefore, one could say that it was "a complex process in which extravascular, vascular and intravascular factors combined to stop blood flow from a small puncture wound within certain time limits". Even so, with increasing recognition that the bleeding time may be within normal limits and yet be abnormal, one should add "and with a blood loss not in excess of a certain normal volume within this time".

At present it is difficult to describe the underlying mechanism of each component other than in the most general terms, although some of the processes are becoming clearer. Thus it has been shown that not only platelet numbers, but their function, is an important consideration. In particular, platelet adhesiveness is a significant feature and this is influenced by the presence or absence of an "R" factor liberated from erythrocytes on contact with a foreign surface. From this it follows that platelet adhesiveness and thereby, the bleeding time, may be influenced by the haematocrit value. Whether Serotonin liberated from platelets helps induce capillary constriction is still debatable, and what part another platelet constituent may play in maintaining normal capillary integrity, is still subject to experiment, but it is quite clear that the role of platelets in the control of bleeding time and their activation is complex.

Even less well defined is the vascular component of the process. Morphological abnormalities of capillaries may be associated with a prolonged bleeding time but sometimes apparently morphologically normal capillaries and the absence of

any demonstrable platelet abnormality, may be accompanied by an abnormal bleeding time. This may reflect a functional capillary defect, but such a conclusion is mere presumption, for capillary physiology and the response of normal capillaries to trauma are still largely unknown quantities.

In aged or otherwise debilitated persons, prolongation of the bleeding time may be secondary to poor tissue tone and atrophy of subcutaneous tissue whereby the hydrostatic pressure in surrounding tissues may not rise to effective levels when bleeding occurs, and such tissue is not conducive to the proper mechanical plugging of the open end of a vessel when it retracts.

Prolongation of the bleeding time, then, may follow from 3 basic causes, and it is useful in that it will demonstrate a defect of haemostasis. However, it does not permit a specific diagnosis to be made, and in any particular case, an abnormal bleeding time may result from a defect in one component or a more complex lesion involving 2 or all 3 components. In the absence of further confirmatory tests, it is impossible to differentiate the relative importance of each. Furthermore, while a prolongation of the bleeding time may be of significance, the degree of prolongation can only be roughly quantitative. It is well known that the bleeding time may vary in duration at different sites in the body, and while this may follow from differences in the production of a puncture at various sites, equally well, local differences in conditions of capillaries, tissue tone etc. may be active, and influence results. That estimations can at best be roughly quantitative is all the more obvious if one recognizes that blood flow from a bleeding time wound may be quite abnormal while the actual duration of flow remains within normal limits.

It is possible therefore, for platelet abnormalities to be reflected in an abnormal bleeding time, but this is not a process specific for such defects and its interpretation is thereby limited.

2. The tourniquet test.

Among other things, the bleeding time reflects the ability of capillaries to react to trauma from without. The tourniquet test may be considered primarily a test of their ability to resist stress from within, namely, a raised intra-vascular pressure. First and foremost it is a test of the vascular component of haemostasis, but the evidence suggests that the fundamental mechanism differs from that in the bleeding time. Thus, the latter may be normal while the tourniquet test is markedly positive and vice versa. This difference possibly stems from the fact that in the bleeding time, the mechanism involves several distinct processes acting together to stop blood flow from a definite incised wound, while the tourniquet would seem to act first at an earlier stage, testing initially, the ability of capillaries to resist trauma and second, if leaks occur, the processes by which damage is repaired. Therefore, although capillaries may be able to withstand a raised intravascular pressure and the tourniquet test be negative, the bleeding time may yet be prolonged if other capillary functions in relation to haemostasis, for example, retraction are abnormal. Similarly, if they are abnormally fragile, capillaries may rupture under stress resulting in a positive tourniquet test, yet the bleeding time may be normal. In simplest form then, the tourniquet test and the bleeding time reflect different aspects of capillary physiology and because one is abnormal, the other is not necessarily so. In an uncomplicated case where a mild increase in capillary fragility is the only finding, it may be said that the tourniquet test illustrates this one feature, and that every purpuric spot indicates the escape of erythrocytes from one or possibly two adjacent capillaries that have ruptured under the force of the raised intravascular pressure. Considering the work of Humble and later Hellem, one can visualize the process active in these cases. Once a capillary has ruptured escaped erythrocytes in contact with a foreign surface liberate factor R and thus

effect platelet adhesiveness, and in the presence of fundamentally normal haemostasis and coagulation the leak is soon repaired, and a small purpuric spot results. Under the condition where capillary fragility only is involved, the Hess tourniquet test is a direct measure of this defect and may be made roughly quantitative by counting the number of purpuric spots produced in a given area.

However, such an assessment of the tourniquet test must be limited, for it makes little or no allowance for the character or extent of individual lesions. In those cases where a positive tourniquet test is indicative of an increased capillary fragility, only, the lesions are usually small and circumscribed. In these, the number of lesions is, for practical purposes, a sufficient guide to the severity of the defect. In more complicated conditions for example, thrombocytopaenic purpura, the character of the lesions may vary widely and in the performance of the tourniquet test small and large purpuric spots and even ecchymoses may be produced. In some of the more complex cases, fundamentally the production of any haemorrhagic lesion must depend on the escape of erythrocytes from capillaries and thus on capillary fragility, but once such leaks occur, the extent or character of the lesions may be affected by other factors. Thus, in acute thrombocytopaenic purpura, because of their antigenic similarities, capillaries and platelets are likely to be damaged by the same factor. This primary capillary damage may or may not be exaggerated, by quantitative or qualitative defects of platelet components essential to maintain their normal integrity. Be this as it may, the result is an increase in capillary fragility, the essential defect underlying the production of any positive tourniquet test. In contradistinction to other cases in which increased capillary fragility is the sole defect, concomitant defects of other capillary functions or platelet defects affecting both haemostasis and coagulation, may not permit prompt normal repair of lesions, this resulting in a greater escape of blood from each

site and the production of more varied lesions.

It is probably better then to use a more arbitrary system to roughly quantitate the tourniquet test giving due recognition to the character of the lesions as well as their number. Interpretation of the tourniquet test thus may provide valuable information to an experienced observer, indicating not only definite capillary damage in the form of increased fragility, but it may also provide non-specific information to suggest a concomitant more severe haemostatic defect. It is a useful test in the investigation of coagulation defects, especially thrombocytopaenic states, but it has definite limitations which must be clearly recognized. These are:-

I. It is first and foremost a test of capillary fragility and an increased fragility is the only definite conclusion that can be drawn from a positive result.

2. That any information obtained from a tourniquet test is but roughly quantitative.

3. In those cases in which the character and extent of the tourniquet test suggest a concomitant defect of other components of the haemostatic mechanism, or where other defects are obvious, for example, thrombocytopaenia, a return to normal of the tourniquet test cannot be interpreted as indicative of a general improvement in the complete defect. In primary thrombocytopaenia for instance, primary capillary lesions are usually accompanied by other defects of haemostasis and coagulation secondary to platelet damage. During hormone therapy there may be a rapid improvement in capillary fragility with amelioration or cessation of spontaneous haemorrhagic phenomena and a return of the tourniquet test to negative. This improvement may be isolated and restricted to capillaries while other defects of haemostasis remain unchanged. In like manner, if the tourniquet test is negative at the outset, such a finding can only be interpreted as indicating that one aspect of capillary physiology, their fragility, is normal, and it cannot be used to judge the integrity of other important capillary functions or to

exclude a defect of haemostasis or coagulation.

Thus, the tourniquet test is a useful procedure and in thrombocytopaenia, helps to provide a "complete" picture of a total haemostatic defect if interpreted with care and in the knowledge of the limitations. However, it provides definite information of but one part of a complex haemostatic mechanism and cannot be used to gauge response of the whole defect to appropriate therapy.

3. Clot Retraction

While the earliest observations pertinent to the bleeding time and capillary fragility were made nearly I50 years ago, so too, the fact that newly formed clots contract squeezing out serum and a few red cells is an old observation. As early as I772 Howson noted its occurrence and in I8I9 Thackrah observed that it tended to vary in different conditions and he subsequently attempted to measure its extent. The term "retraction" was introduced by Schklarewsky in I868, and in I878 and later in I895 Hayem described a number of factors concerned in the process. Most important, he showed that platelets were probably involved. His was not the first such suggestion linking reduced platelet numbers with deficient clot retraction, however, for in I822, twenty years before platelets themselves had been described, Duncan had recorded deficient clot retraction in a case of purpura haemorrhagica. Delesenne confirmed Hayem's work in I897 when he showed that blood deprived of platelets produced non-retractile clots, and later Le Sourd and Pagniez (I906,I908,I9I3) showed that even moderate reduction in the platelet count decreased the amount of retraction so that the two values were directly proportional to each other. This was confirmed by McFarlane in I938, who found a similar relationship once the platelet count had been reduced below I00,000 per c.mm. although deficient retraction might occur in the absence of reduced platelet numbers.

How platelets exert their function in relation to clot

retraction is not known. There is no doubt that during this process the fibrin fibres shorten and so reduce the volume of the clot. Various theories have been proposed.

Following the observation that platelets adhere to fibrin and that small agglutinated masses form especially at the intersection of fibres, Duke (I9I2) suggested that their action was to bind fibres together, and that without this retraction would be ineffective in reducing clot volume, the individual fibres tending to slip over one another. Presumably he believed that shortening of the fibres was inherent in the fibrin strands themselves. With some modifications Tocantins (I936) held a similar view, but it has since been shown by Budz-Olsen (I95I) that this theory is inadequate to explain all the observed facts.

Glanzmann (I9I8) and later Fonio (I95I) favoured the idea that a specific retraction enzyme (retracto-enzyme) was present in platelets and that this acted on fibrin to induce its shortening. Fonio claimed to have separated this factor from platelets by ultrasonic disintegration, but this work awaits confirmation.

A remaining and very plausible theory was proposed by Budz-Olsen in I95I. This was based on vital activity of platelets as living structures and the analogy between them and the primitive thrombocyte upon which coagulation in lower animals depends. In some of the latter haemostasis is secured not by fibrin but by a network formed by extrusions from special cells in blood. Observations of morphological changes in platelets during coagulation, the sending out and pulling in of long processes have been made for almost a century, being reported by Vulpian and Ranvier in I873, Bizzozero in I882, Eberth and Schimmelbusch in I885, Frank in I9I5 and Tait and Green in I926. In I950 Bessis and later Braunsteiner et al produced convincing photographs obtained during electron microscopy of long pseudopodia extruded by platelets. Based on these and his own observations during which he watched long pseudopodial processes sent out from platelets link up with those sent out

from adjacent platelet masses and to fibrin threads, Budz-Olsen suggested that clot retraction was not dependent on any inherent activity of fibrin itself but on the contraction of the interlacing and interconnected filamentous pseudopodia sent out by platelets. He suggested this process was analagous to and possibly may have been a sort of vestigial function, dating from a remote primitive coagulation mechanism. This theory has gained support from observations which have shown that anything which might be expected to kill living cells will inhibit clot retraction, and that platelets have certain features common to living cells in so far as they consume oxygen and glucose and release lactic acid.

Not all theories of clot retraction have involved platelets, however, and some authorities have attributed it to an inherent property of fibrin threads which, like some other colloids, had the property of undergoing a process of shrinking or syneresis. Others had suggested that it was the first phase in the process of fibrinolysis, fibrin being shortened before being dissolved. Whatever the full and final explanation may be, there can be no doubt that platelets do play a part in the process and that reduced clot retraction may be present in thrombocytopaenia and even in some cases when platelet numbers are normal but these are presumably functionally defective.

Nevertheless it has been adequately shown that platelets are not the only factor concerned in the production of normal clot retraction and its degree is influenced to a greater or lesser extent by physical factors such as the size, shape and surface of the vessels used for its determination, pH and temperature, the volume of packed red cells and the fibrinogen concentration. Thus impaired clot retraction is not specific for platelet abnormalities. Furthermore its usefulness must be limited for it would seem to revolve around one apparently isolated platelet function whose importance is not known. Although impaired clot retraction may be attributed to a platelet defect, such a finding cannot be assumed to reflect a general disturbance of

other platelet functions. Indeed the reverse would seem to be the case and it is the author's impression that in many instances when platelet functions are disturbed, there is a greater or lesser degree of selective impairment, their thromboplastic function, for example, being markedly reduced while clot retraction is apparently unaffected.

Furthermore, despite many observations of phenomena concerned in the process and numerous techniques devised to measure its degree, the function of clot retraction remains unknown. It has been said that in conditions where clot retraction is deficient such as idiopathic thrombocytopaenic purpura or liver disease, an haemorrhagic state is often present. None-the-less, the latter finds more ready explanation in coincident impairment of thromboplastin formation or reduced prothrombin levels than in any significant function of clot retraction. More commonly haemorrhage is present when clot retraction is normal. It has been suggested that by adhering to wound margins clots act as ligatures and when retraction occurs the edges of the wound are drawn together. A further possibility suggests that by retraction the recanalization of thrombosed vessels is assisted. Both of these theories seem unlikely.

Clot retraction then, is an interesting process whose observations may demonstrate a platelet abnormality. In the light of present knowledge, however, it can be said to reflect but one function of a complex particle, and that it has not been possible to link such a defect convincingly with the onset, presence or degree of haemorrhagic phenomena. Further, by comparison with newer techniques available to assess platelet functions more directly associated with haemorrhage, either its onset or degree, observations of clot retraction are of limited use, while actual measurements of its degree which may be influenced by many factors other than platelets, have little clinical application.

4. Thromboelastography.

Mention must be made of an instrument, the thromboelastograph devised by Hartert and intended to study the rigidity of blood clots. Determinations using this instrument are based on the following principles. A small amount of whole blood or recalcified plasma is introduced into a steel cuvette and covered with a layer of paraffin oil. A cylindrical steel pin suspended from a torsion steel wire is inserted into the cuvette. A small mirror is mounted on the wire. By means of a motor, the cuvette is rythmically rotated back and forth on its axis through a small arc of $4^{\circ}\ 5I'$, every 9 seconds. As soon as the first fibrin threads appear and adhere to the walls of the cuvette and the pin itself, the pin is rotated, increasing in speed and amplitude as clot formation proceeds. These movements are transmitted to the mirror by means of the steel wire and recorded on photographic paper. After reaching a maximum, the movements decrease in amplitude following the decreased adhesion of the fibrin clot to the walls of the cuvette and the pin. Fibrinolysis is then in progress.

Several distinct phases can be distinguished in thromboelastographic determinations. Initially the tracing forms a straight line known as the r value or reaction phase. Measurement of this allows one to calculate the time taken until the first detectable evidence of clotting occurs. This is followed by a coagulation phase in which two lines separate, these corresponding to the two directions of the movements. The rate at which the two lines separate is proportional to the speed of clot formation. A measure of this is obtained from the distance of the point where clotting begins to the point at which the lines are 20mm. apart. This is known as the K value. In a third phase the two lines become parallel, and the distance between the two at this stage, the maximum amplitude (ma) is an indication of clot firmness. In normal tracings the ma value decreases slightly in I-2 hours following a small amount of fibrinolysis, but under some experimental conditions,

fibrinolysis is slow and might last for several days. Finally when fibrinolysis is complete a single line is obtained.

Consideration of a tracing therefore can provide information on several aspects of coagulation. Indeed variations in the coagulogram have been found in several coagulation defects such as haemophilia, thrombocytopaenia, thrombocytopathies, hypoprothrombinaemia, anticoagulant treatment and so on. For example in haemophilia, both the r and k values are greatly prolonged but the ma value is normal, while in thrombocytopaenia and thrombocytopathia typically the r value is normal, while the k value is prolonged and the ma value markedly decreased.

De Nicola and Mazzetti (1956) stated that ma values varied in direct proportion to platelet numbers and function. Von Kaula and Weiner in 1955 had made a similar observation, but they also recorded that the ma value was reduced not only when platelet numbers were reduced but when the fibrinogen content of a specimen was lowered or fibrinolytic activity was increased. Their findings were extended by Weisborg in 1957.

The reports mentioned above demonstrated that thromboelastography could be used to demonstrate defects in platelet function. However, it is not possible with the work published to date to assess its sensitivity in this respect, for these features of the coagulogram altered in platelet functional disorders are also altered by other specific deficiencies and possibly by a variety of technical factors. Nevertheless, the technique is undoubtedly interesting and might well prove useful after more detailed evaluation.

5. Tests for prothrombin consumption.

A simple and logical sequence of events and predictions has provided the basis for many tests designed to detect coagulation defects by determining the amount of prothrombin remaining in serum after coagulation is complete. The background to these may be explained concisely. If the formation of thromboplastin is impaired the maximum conversion

of prothrombin to thrombin does not take place and an excess of residual prothrombin is found in serum after clotting is complete. It follows from this that if there is a deficiency of any factor participating in thromboplastin formation an excess of residual prothrombin should be found in serum. To this should be added the rider that even under normal circumstances when thromboplastin formation is optimum not all the original prothrombin is used and some 10-20 percent of the original amount remains in serum. Indeed values as high as 40 percent have been found by some workers.

The first relevant observations were made by Bordet and Delange in 1912. They found that when platelet poor plasma was allowed to clot thrombin was formed slowly and in small amounts, and the resulting serum would react with platelets to form more thrombin. Serum derived from the clotting of platelet rich plasma did not react in this way. Thus Bordet and Delange had established a role for platelets in the early stages of coagulation but they did not enlarge their findings nor did they apply their experiments to clinical thrombocytopaenia or other haemorrhagic conditions.

It was not until 24 years later in 1936, that the subject of prothrombin conversion was taken up by Warner, Brinkhouse and Smith. They described a method for measuring prothrombin in serum or plasma and applied it in determinations of the rate of utilization of prothrombin during clotting. Later Brinkhouse (1939) using this method, demonstrated that prothrombin conversion was greatly slowed in haemophilia.

This finding was confirmed by Quick in 1947 using a technique based on a modification of his one stage test. He also reported similar findings in plasma made thrombocytopaenic by centrifugation. In Quick's technique, one of the simplest, prothrombin in plasma or serum was measured by adding 0.1 ml. of thromboplastin derived from acetone-treated brain, and 0.1 ml. of fibrinogen to 0.1 ml. of serum or plasma. The clotting time of the mixture was converted into a percentage prothrombin

concentration by means of a calibration graph. He found that 80-85 percent of prothrombin was normally converted to thrombin within one hour, but prothrombin consumption was slight in blood from 3 haemophiliacs even after 24 hours. So too, in blood collected into a siliconized syringe and rendered platelet-free by centrifugation in siliconized tubes, prothrombin consumption was markedly delayed and even after 24 hours most was unchanged.

Quick, Shanberge and Stefanini in I949 showed that prothrombin consumption was markedly delayed in platelet poor plasma but was gradually restored to normal as the platelet count was increased by adding platelet rich plasma. They found poor prothrombin consumption in 2 cases of thrombocytopaenic purpura, and, significantly, in these there was no correlation between platelet numbers and consumption, the patient with the higher count showing the least utilization of prothrombin. This finding they attributed to a greater functional defect in the patient with the higher count. In a further paper later in the same year the authors reported similar findings in 3 more patients with thrombocytopaenic purpura, while Alexander and De Vries (I949) recorded defective prothrombin consumption in both haemophilia and thrombocytopaenic purpura. The latter workers used a method similar to Quick's.

In I948, Soulier used a simplified technique to determine the residual prothrombin in both venous and capillary blood. In his technique venous and capillary blood which had been allowed to clot was allowed to stand at 37°C. for 4 hours. Prothrombin was determined using a simplified two-stage technique in which 0.I ml. of serum was mixed with 0.I ml. of a tissue thromboplastin preparation and after incubation for 60 seconds 0.2 ml. of fibrinogen was added. The clotting time of fibrinogen gave a measure of thrombin generated during the 60 seconds incubation. In I7 normal subjects the clotting time was always greater than 54 seconds and frequently greater than 3 minutes when serum obtained from venous blood was examined.

When capillary blood was tested the clotting times were always in excess of 3 minutes.

In I9 haemophilic subjects, the clotting times ranged from 4-8 seconds with serum from both venous and capillary blood. In I8 subjects with thrombocytopaenia the clotting times ranged from 6-II seconds in II and in all but one of the remainder were less than 2I seconds when venous blood was tested. When capillary blood was tested an abnormality was found in only 4, and in these the clotting times ranged between I0 and 24 seconds.

One of the most recent and widely used techniques was described by Merskey in I950. He described a carefully controlled test in which the prothrombin present in serum one hour after coagulation was measured and expressed as a percentage of that present in plasma. This figure was known as the prothrombin consumption index. The technique of this test and the rigid standardization of the procedure and equipment warrants a more detailed description, for, as will be discussed later, they have an important bearing on the reliability and reproducibility of results. Serum was obtained under standard conditions by carrying out the whole blood clotting time as described by Merskey (I950). The prothrombin in serum was measured in specimens which had been kept for exactly one hour at 37°C. after coagulation was complete. A two-stage technique was employed in which 0.2 ml. of serum or plasma to be tested was placed in 2.5" x $\frac{3}{8}$" test tube in a water bath at 37°C. and 0.2 ml. of 0.85 percent sodium chloride solution and 0.2 ml. of brain thromboplastin were added. 0.2 ml. of 0.255 M calcium chloride solution was then added and subsamples of 0.I ml. were removed after I0,20,60,I20 and I80 seconds and blown into tubes containing 0.4 ml. of fibrinogen preparation. The clotting time of the fibrinogen in each tube was measured, the shortest time being inversely proportional to the prothrombin content of the plasma or serum, these values then being used to calculate the index.

When 37 normal persons were tested the prothrombin consumption index was less than 40 percent in each and less than 25

percent in most. The index was 40 percent or more in 19 patients with haemophilia save one. In this patient the index was less than 40 but difficulty had been experienced in obtaining the blood by venepuncture. In 7 of 8 cases of thrombocytopaenia of differing aetiologies, the index was greater than 40 percent.

The techniques described and others with a similar purpose have had wide application in the diagnosis of defect of thromboplastin formation. Their interpretation has been limited however, for they are not specific, and a deficiency of any factor participating in thromboplastin formation could be responsible for an abnormal result. In the years in which these tests received most attention, knowledge of the factors participating in thromboplastin formation was very incomplete and reports of their use were concerned largely with haemophilia and thrombocytopaenia. As knowledge of the factors concerned in thromboplastin formation increased and new factors were described, better techniques to detect deficiencies have been evolved and these have largely replaced prothrombin consumption tests.

Apart from considerations such as these, with experience of various techniques, it became obvious that results were influenced to a considerable extent by a variety of technical factors. Soulier found quite different results in many of his cases of thrombocytopaenia when parallel tests were conducted on venous and capillary blood, while Merskey reported an unexpected result in a case of haemophilia when difficulty with the venepuncture had been experienced. These findings can be explained by the contamination of specimens by tissue products, but quite distinct from "contaminant" cases it has been shown that technical variations such as the size of the needles and syringes used to collect specimens, the size of the clotting tubes, and the amount of blood added to them, may produce surprisingly large variations in results. Even when adequate precautions are taken, as in the technique described by Merskey, the results for normal series have not been constant, and while the variation in normals has usually been 10-20 percent residual prothrombin, values as high

as 40 percent have been recorded.

Factors such as these have contributed to limit the interpretation of prothrombin consumption tests and have made it difficult to utilize the technique quantitatively with confidence. Thus, it is that they and similar procedures have been replaced by the thromboplastin generation test, but before considering this, it is necessary to consider briefly, tests of thrombin generation.

6. Thrombin generation tests.

In the broadest concept tests based on the generation of thrombin during coagulation and prothrombin consumption tests are closely related, for in the former, the techniques used provide a direct measure of the amount of thrombin generated during clotting, and in the latter the aim is to determine the amount of unused prothrombin in serum after clotting is complete.

Several techniques have been evolved all stemming from a view held by Warner, Brinkhouse and Smith (I936), that prothrombin is converted stoichiometrically to thrombin. Should such be the case the rate and amount of thrombin formed during clotting should give a measure of the rate and amount of prothrombin conversion, and thus indirectly of the amount of thromboplastin generated. In all the techniques the amount of thrombin is measured at intervals during clotting, and by plotting on graph paper the amount of thrombin generated against the time of estimation, thrombin formation and removal can be studied from the resulting curve. The rate of thrombin formation can be judged from the steepness of the curve and should reflect the speed of thromboplastin generation. The area of the curve is proportional to the amount of prothrombin converted. If prothrombin conversion is complete this area could be expected to be proportional to the amount of prothrombin present in the blood before clotting.

In I953 McFarlane and Biggs described a technique for the estimation of thrombin formation during the clotting of whole blood. It was applied to 6 normal subjects of each sex and

when the results were plotted graphically, they were all found to fall within a well-defined area. Seven patients with thrombocytopaenia, all with platelet counts of 25 x 10^3 per c.mm. or less showed reduced thrombin formation but no delay in the first appearance of thrombin. These results were confirmed on plasma made thrombocytopaenic by centrifugation.

In the same year, Pitney and Dacie described the measurement of thrombin during the clotting of recalcified citrated plasma. They too, found reduced thrombin formation in plasma made thrombocytopaenic by centrifugation and in cases of pathological thrombocytopaenia.

Both of these methods had disadvantages, however. That described by McFarlane and Biggs necessitated immediate performance of the test as soon as blood was collected, and therefore it had to be carried out at the bedside. The technique described by Pitney and Dacie was possibly open to error in that centrifugation of the blood, especially thrombocytopaenic blood, might lead to a reduction in platelet numbers and thus erroneous results. Accordingly in 1955, Hicks described a technique in which he attempted to utilize the advantages of the former two methods and yet overcome the disadvantages. In basic principle, his technique was essentially the same as the others, save that his estimations were performed on anticoagulated whole blood. He did not apply his technique to cases of clinical thrombocytopaenia.

Meanwhile in 1954 Fantl evolved a technique employing a different principle. He studied thrombin generation in mixtures of diluted blood to which an inhibitor of anti-thrombin had been added. He found that mixtures containing this substance, pyrocatechol, yielded higher concentrations of thrombin than those without. Furthermore, he produced evidence to show that to obtain accurate results blood must be tested immediately after collection in order to reduce errors due to the activation of platelets after blood was shed.

As had been the case with tests to measure prothrombin

consumption, then, it had been possible to demonstrate defects of platelet function in thrombin generation tests. However, it is doubtful whether the latter techniques were more sensitive than prothrombin consumption techniques in the detection of defects and, indeed, mild cases of haemophilia and thrombocytopaenia with platelet functional defects have sometimes given normal results. Thrombin generation tests too, are subject to much the same criticism as pertain to prothrombin consumption tests. Thus, they are not specific, and are subject to poorly defined technical errors. Fantl showed that unless scrupulous care was exercised in the collection and performance of tests quite erroneous results could result. Added to these disadvantages was the fact that all of these techniques required considerable experience and technical skill for their performance. Like the prothrombin consumption tests, however, thrombin generation tests have largely been replaced by thromboplastin generation tests in the diagnosis of defects of the first stage of coagulation.

7. The thromboplastin generation test.

In I9I2, Bordet and Delange suggested that during clotting a plasma substance serozyme, reacted with a platelet factor which they called cytozyme. In I947 Quick and Brinkhouse independently postulated that thromboplastin was formed in blood by an interaction between platelets and a plasma substance. This limited concept remained as such until I953 when Biggs, Douglas, and McFarlane discovered that aluminium hydroxide-treated plasma, serum, platelets and calcium reacted together to form thromboplastin. Since all reagents were necessary before the reaction could proceed it was apparent that platelets reacted with more than one factor to form thromboplastin. This observation led to the development of the thromboplastin generation test.

In this, the various factors required are prepared under set conditions and brought together in optimum concentrations and allowed to react and form thromboplastin. No prothrombin

is present in the mixture, being removed from plasma by aluminium hydroxide treatment, and the thromboplastin formed does not react further but is allowed to accumulate and is only removed by naturally occurring anti-thromboplastins. The formation and removal of thromboplastin can therefore be studied without the generation of thrombin complicating the picture. Thus, this test provides a degree of specificity not present in those described earlier. Any of the reagents, platelets, serum or aluminium hydroxide-treated plasma, can be tested separately by completing the mixture with normal reagents.

By the use of this technique it has been possible not only to distinguish certain disorders from others, but to detect entirely new coagulation factors and clarify the sequence of events in thromboplastin formation. It has been widely used to specify platelet thromboplastic defects. Furthermore it has proved more sensitive than other methods in demonstrating mild defects of thromboplastin formation. This is exemplified by 65 cases of haemophilia studied by Pitney (1957). In these the prothrombin consumption test was normal in 13, and the thrombin generation test normal in 10, yet in all, the thromboplastin generation test showed a defect in the patient's aluminium hydroxide-treated plasma. Bianco and Crolle (1957) produced similar findings in 8 cases of haemophilia and one of Christmas disease.

With increasing use, and as experience was gained with the thromboplastin generation test, it became obvious that although it was very sensitive in the detection of abnormalities affecting the early stages of coagulation, it did not provide quantitative information. Furthermore, together with the growing application of the technique and the description of new factors, new therapeutic measures came to hand, and, with these, an increasing demand for assay procedures to quantitate various factors. In this latter respect, as has happened so often before, interest was focussed principally on the assay of anti-haemophilic globulin, and several authorities devised modifications of the thromboplastin generation test applicable to this.

It has been mentioned already that in studies of thrombocytopaenia, using the prothrombin consumption test, Quick, Shanberge and Stefanini found that in at least one such patient the defect seemed to be relatively unrelated to platelet numbers, a discrepancy they attributed to differences in platelet functional defects. This was in keeping with the common occurrence that platelet numbers were quite dissociated from the severity of haemorrhage. Subsequently Bonnin (1956,1957) conceived the idea that the purpuric and other bleeding manifestations in thrombocytopaenic purpura might be closely related to platelet thromboplastic function. The thromboplastin generation test offered an excellent opportunity to investigate this problem.

In the original technique as described by Biggs and Douglas, platelets to be used in the thromboplastin generation test were prepared from blood collected by careful venepuncture using a silicone coated syringe and mixed in a silicone coated centrifuge tube with one-ninth part of 3.8 percent sodium citrate. Platelet rich plasma was separated by centrifugation at 1500 r.p.m. for 15 minutes, and the platelets were removed by further centrifugation at 3000 r.p.m. for 15 minutes. The platelets deposited were resuspended in saline and washed twice by further centrifugation at 3000 r.p.m. They were finally reconstituted in a volume of normal saline equal to one-third of the original plasma volume.

Bonnin (1956-1957) modified the thromboplastin generation test as originally described and made it more accurately quantitative in respect of platelet thromboplastic function, by carrying out platelet counts on the low spun plasma. The platelets were then separated, washed and finally reconstituted to give a concentration of 1,600,000 platelets per c.mm. The thromboplastin generation test was carried out using a volume of this suspension and equivalent volumes of the other reagents prepared from normal donors. Pathological platelets were treated in an identical fashion and the maximum percentage of thromboplastin generated was determined from a calibration graph. The

calibration curve was constructed by testing dilutions of a potent thromboplastin mixture prepared as above (and using normal platelets) and kept at 0°C. to retain its activity.

The results of Bonnin's studies have been described earlier and will be re-iterated only briefly here. He found abnormal thromboplastin generation in patients suffering from thrombocytopaenic purpura. The defect was qualitative as in all cases platelets had been reconstituted to the level mentioned above. Bonnin found this test to be a useful guide to the expected severity of purpuric and bleeding manifestations in thrombocytopaenic patients and stated that purpura was likely to occur when the thromboplastic function falls below 53 percent, and spontaneous haemorrhages were likely to occur in addition to purpura when the platelet thromboplastic function falls below 25 percent, while below I2 percent function all patients were invariably, actively haemorrhagic. In further studies Bonnin found a similar but less strict relationship in secondary thrombocytopaenia.

The test has been found very useful in the control of the haemorrhagic state in thrombocytopaenic purpura by steroid therapy, as it permits the dose to be adjusted to keep the patient free from the risk of dangerous haemorrhage. The success or failure of therapy can be predicted and the dose increased or decreased depending on the level of platelet thromboplastic function.

This test, then, provided much valuable information but it had certain practical objections which rendered it not ideal as a routine procedure. It was even more time consuming than the thromboplastin generation test, and, being subject to the same technical details as the latter was tedious and technically difficult. From day to day in any one test there were three variable factors, platelets, aluminium hydroxide-treated plasma and serum, all of which conceivably might influence the results. Furthermore, in cases of severe thrombocytopaenia, a large amount of blood was needed to produce the necessary platelet

numbers, and this limited its application. Considering this test and its uses and limitations Hicks (1959) stated:-
"If platelet function could be assessed by a more simple method than that of Bonnin, it would be extremely useful for diagnostic and therapeutic purposes. As the test stands, it will probably only be used for research purposes or in specialized laboratories".

Bonnin himself, was aware of the limitations of his test, and accepted the need for an alternative and simplified technique which might receive a more general application. In consideration of this, he suggested to the author that to seek such a technique would be a worthwhile research project. In subsequent discussions, the idea arose that if a phospho-lipid extracted from brain thromboplastin could be used as an effective substitute for platelets in thromboplastin generation tests, might it not be possible to reverse the process and use platelets to reactivate the tissue residue remaining after the preparation of phospholipid from acetone-treated brain? From this simple beginning, the original work now to be described was initiated.

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SECTION II

Original Experimental Work

Reagents and Techniques

Throughout the whole of the experimental work to be described and, subsequently, in the application of that work, certain reagents and established techniques have been used repeatedly. It seemed unnecessarily cumbersome to supply complete descriptive details of these on each occasion they were used and, accordingly, many have been described in an abbreviated form. The following is a list of the reagents and techniques commonly used together with the appropriate descriptive detail. Where applicable an abbreviated term, if used in the text, is given in brackets. Unless specified in the text the use of a particular reagent or procedure refers to that as detailed hereunder.

I. Reagents.

(I) Acetone. Analytic and laboratory grade chemical (B.D.H.)

(2) Aluminium hydroxide for adsorption. Prepared as described by Biggs and McFarlane (I957).

(3) Aluminium hydroxide treated plasma. Blood collected with a siliconized syringe was anticoagulated with one-tenth volume of 3.8 percent trisodium citrate in a siliconized tube. The blood was centrifuged for I5 minutes at 3,000r.p.m. at I5°C in a M.S.E. medium refrigerator centrifuge. 0.2 ml. of aluminium hydroxide gel was added to I.8 ml. of the platelet poor plasma so obtained and the two were mixed with continuous agitation in a water bath at 37°C for 5 minutes. The plasma was again centrifuged and the supernatant removed. The treated plasma was used if the clotting time exceeded 3 minutes on the addition of brain extract and calcium chloride.

(4) Asbestos: Pulverized refined asbestos (Gooch).

(5) Brain thromboplastin for use in the one stage prothrombin time test.

(Acetone treated brain): Prepared as described by Biggs

and McFarlane (I957).

(6) Chloroform. Laboratory grade reagent (May and Baker Ltd.)

(7) Chloroform extract of brain for the thromboplastin generation test (Lipoid). Prepared as described by Bell and Alton (I954).

(8) M/40 calcium chloride (calcium chloride). Prepared as described by Biggs and McFarlane (I957).

(9) Owren's buffer. Prepared as described by Biggs and McFarlane (I957).

(I0) Sodium citrate. 3.8 percent tri-sodium citrate was used as anti-coagulant throughout and was prepared by dissolving the appropriate weight of tri-sodium citrate in distilled water.

2. Technical Methods.

I. Bleeding time; Duke's method.
An attempt was made to standardize the procedure by using only uniform lancets, appropriately guarded, to unify the size and depth of punctures in the ear lobes. If any doubt surrounded the validity of any estimation it was repeated at once.

2. Clotting time; Lee and White method at 37°C as described by Biggs and McFarlane (I957).

3. Tourniquet test.
A sphygmomanometer cuff was held for 5 minutes at a pressure of I00mm. of mercury. After releasing the pressure the number, extent and character of the purpuric spots down the arm were estimated in an arbitrary + to + + + + classification.

4. One stage 'prothrombin' test of Quick; as described by Biggs and McFarlane (I957).

5. Whole blood platelet count; as described by Dacie (I956). Platelet counts on platelet 'rich' plasma were performed in the same way.

6. Clot retraction and fibrinolysis.
   The clots in Lee and White clotting tubes were examined macroscopically for retraction after one hour at 37°C, and subsequently at 2, 6 and 24 hours for both retraction and evidence of fibrinolytic activity.
7. Screening test for thromboplastin generation (screening test): as described by Hicks and Pitney (1957).
8. Thromboplastin generation test: as described by Biggs and Douglas (1953) except that a chloroform extract of acetone-dried human brain (Bell and Alton, 1954) was used as a substitute for platelets.

PART I. The development of a technique to assay platelet thromboplastic function.

In the laboratory in which this work was undertaken, acetone-treated human brain has always been used as a source of thromboplastin for use in Quick's "prothrombin" time estimation. Lipid platelet substitute has always been prepared from the same product using the technique described by Bell and Alton. In common with other laboratories the particulate residue remaining after the preparation of lipid, has always been discarded, and the possibility that it might retain active factors capable of adaption to a useful purpose had never been explored. This thesis deals with the investigation of this residue and its uses; as a reagent in a system to assay platelet thromboplastic function, and in a modification of this system to quantitate factor X (Stuart-Prower factor).

Initial theoretical considerations made it clear that any "brain residue" to be investigated should retain negligible inherent thromboplastic activity. Accordingly preliminary experiments were undertaken to gauge the extent to which such activity was reduced by chloroform treatment of acetone-treated brain (as in the standard Bell and Alton procedure) and to develop a technique for the preparation of a product sufficiently inert to be suitable for further investigations.

Experiment I. To assess residual thromboplastic activity in acetone-treated human brain extracted with chloroform by the Bell and Alton technique.

Reagents: I. Acetone-treated human brain prepared as for use in Quick's "prothrombin" time estimation.

2. Chloroform.
3. Normal platelet-poor plasma as substrate.
4. Normal saline.

TABLE 1. The substrate clotting times obtained on the addition of brain suspensions and calcium chloride to normal plasma.

Raw Material Number	Treatment of Raw Material	Substrate Clotting Times In Seconds
1	Acetone	10
	Chloroform	60
2	Acetone	10
	Chloroform	48
3	Acetone	10.5
	Chloroform	37
4	Acetone	12
	Chloroform	72

5. Calcium chloride.

Technique: Using suitable screw-capped containers, and with the aid of an electrically driven Matburn rotor, 0.5 gramme amounts of acetone-treated human brain suspended in 20 ml. volumes of chloroform were mixed continuously for 2 hours. After this, the supernatant lipid-chloroform suspension was discarded, and the separate samples of brain residue were pooled, dried in air at room temperature, ground to a coarse powder in a mortar and pestle, and stored in air-tight containers at -20°C. until used. Subsequently five percent suspensions (weight/volume) in normal saline, of both the brain residue and its parent acetone-treated brain, were prepared, and incubated in a water bath at 37°C. for 20 minutes. After incubation 0.I ml. volumes of each suspension in turn were transferred to 0.I ml. volumes of normal plasma in each of five 3½" x ½" test tubes, and the ten tubes thus prepared were placed in a water bath and warmed to 37°C. Finally each tube in turn was recalcified with 0.I ml. calcium chloride, previously warmed to 37°C. Coincident with recalcification a stop-watch was started and the substrate clotting times recorded. The five substrate clotting times obtained with each suspension were averaged to give a final result. This experiment was repeated on four occasions using acetone-treated brain prepared from different batches of raw material in each case. The results are shown in Table I.

Results: The substrate clotting times obtained with the suspensions of chloroform-treated brain residue were 60,48,37 and 72 seconds, while the corresponding times obtained with the suspensions of parent acetone-treated brain were I0,I0,I0.5 and I2 seconds respectively.

Discussion: The longer substrate clotting times obtained when the suspension of chloroform-treated brain was used must have reflected loss of inherent thromboplastic activity secondary to such treatment. Those preparations associated with substrate clotting times in excess of 60 seconds could be considered to retain little thromboplastic activity, but others in which

substrate clotting times were faster than this could not be regarded as sufficiently "inert" to warrant further investigation. Furthermore, the procedure was time-consuming, and with the limited capacity of the rotor, at best, only 4-5 grammes of residue could be prepared at the one time. Because of these considerations, attention was directed towards the development of an alternative technique for the preparation of larger yields of a constantly satisfactory residue in a minimum of time.

It was thought that these early difficulties might be overcome, wholly or in part, if a constant weight of acetone-treated brain was extracted, not once, but several times, with an excess of chloroform, each extraction being of shorter duration than the two hours required in the Bell and Alton procedure. Through experiments based on this idea, it was possible to develop a technique which resolved the early problems of small yield for prolonged extraction, but invariably produced a preparation with minimal residual activity. Several experiments were conducted, these differing only in details of the relative proportions of brain and chloroform, the time of individual treatments, and the number of treatments necessary to produce a uniformly satisfactory product. Eventually these three variables were standardized to formulate a fixed procedure which has been used throughout the remainder of this work to prepare successive batches of "brain residue". The relevant experiments in this work differing only in details it seems unnecessary to describe each at length. Therefore, only the final, standardized method will be detailed, together with data obtained during a typical preparation to show progressive loss of thromboplastic activity in acetone-treated brain with successive chloroform extractions. Here-after the term "brain residue" will refer to acetone-treated brain further treated with chloroform in this way.

Experiment 2. Standardized technique for the preparation of "brain residue".

Reagents: I. Acetone-treated human brain.

TABLE 2. Substrate clotting times obtained with suspensions of acetone-treated brain and chloroform-treated, acetone-treated brain.

Brain Preparation Number	Acetone or Acetone and Chloroform Treatment	Substrate Clotting Times In Seconds
1	Acetone	10
	Acetone and Chloroform	112
2	Acetone	10
	Acetone and Chloroform	162
3	Acetone	11
	Acetone and Chloroform	153
4	Acetone	10
	Acetone and Chloroform	187
5	Acetone	10.5
	Acetone and Chloroform	152

2. Chloroform.

Technique: I5 grammes of acetone-treated brain are placed in a suitable screw-capped container. 400 ml. of chloroform are added and the two reagents are vigorously shaken for 4 minutes. After this the chloroform-brain suspension is strained through several thicknesses of gauze, thereby allowing the chloroform and extracted lipid to drain away, while the particulate residue is easily recovered. The whole process is repeated to a total of six chloroform extractions, after which the final residue is dried in air at room temperature, ground to a powder in a mortar and pestle, and stored at -20°C. After preparation, each new batch of residue is tested for residual thromboplastic activity as outlined in experiment I. Table 2 shows the substrate clotting times obtained when 5 successive preparations were tested in this way.

Results: The substrate clotting times obtained with each preparation were well in excess of I00 seconds. The range of substrate clotting times obtained with the corresponding parent acetone-treated brain was I0-II seconds.

Discussion: The residual thromboplastic activity in each of these preparations was negligible, and all batches of "residue" subsequently prepared in this way have been equally satisfactory. The whole procedure takes far less than half the time required by the original method, while the final yield is some 3-4 times greater. The residual thromboplastic activity in such residue being so small, the interpretation of subsequent experiments could not be complicated by consideration of possible fallacies introduced by the use of an insufficiently inert residue.

Experiment 3. To demonstrate progressive loss of thromboplastic activity in acetone-treated human brain with succeeding chloroform extractions.

Reagents: I. Acetone-treated human brain.
2. Chloroform.

TABLE 3. Progressive loss of thromboplastic activity with successive chloroform extractions of acetone-treated brain.

	BRAIN PREPARATION AND EXTRACTIONS						
Substrate Number	Acetone Treated	1	2	3	4	5	6
1	11.5	19.5	33.5	>60	>60	>60	>60
2	11.5	19.5	32.5	>60	>60	>60	>60
3	12	19	35	>60	>60	>60	>60
4	11.5	18.5	33.5	>60	>60	>60	>60
5	11.5	19.5	33.5	>60	>60	>60	>60
6	11.5	19.5	37	>60	>60	>60	>60
7	11.5	19.5	34.5	>60	>60	>60	>60
8	14	22	37	>60	>60	>60	>60
9	11.5	19.5	32	>60	>60	>60	>60
10	11.5	19.5	33	>60	>60	>60	>60

3. Normal saline.
4. Plasma, as substrate, collected from 10 normal donors.
5. Calcium chloride.

Technique: Brain residue was prepared as described in experiment 2, save that, after each extraction, a small aliquot of residue was set aside, dried in air at room temperature, and ground to a powder in a mortar and pestle. Each sample was kept apart from the others and when the final extraction was completed, a 5 percent suspension of each in saline was prepared. These-together with a control suspension of the original acetone-treated brain were incubated at 37°C. for 20 minutes. After incubation, 0.1 ml. volumes of each suspension in turn, were subsampled into 0.1 ml. volumes of each of the ten normal plasma substrates, with coincident recalcification with 0.1 ml. calcium chloride, and the substrate clotting times recorded. The whole procedure was carried out at 37°C. The results are shown in Table 3.

Results: The suspension of acetone-treated brain caused the plasma substrates to clot in 11.5-12 seconds, with one exception. In this the clotting time was 14 seconds. After a single extraction with chloroform, a suspension of the same brain clotted the same substrates in 18.5-22 seconds, after two extractions in 32-37 seconds, and, after the third and all subsequent extractions, always in excess of 60 seconds.

Discussion: The rapid lengthening of substrate clotting times with serial chloroform extractions of acetone-treated brain, clearly demonstrated progressive loss of inherent thromboplastic activity. Indeed, after 3 extractions the substrate clotting times were in excess of 60 seconds, and retained thromboplastic activity was slight. In practice, however, acetone-treated brain has always been extracted at least 6 times, before use, in attempts to standardize experiments on a preparation with a minimum of retained thromboplastic activity.

It was possible therefore to produce sufficient brain

residue at the one time to permit a sequence of experiments to be conducted with a constant preparation. However, while these preparations were quite "inert" in prothrombin time estimations, there was no indication of the exact mechanism responsible for this inactivation. Doubtless it was due, in part at least, to removal of tissue lipids, but whether other factors, known to be present in tissues and active in "prothrombin" time estimations had been altered or destroyed, remained unknown. Some preliminary experiments were designed to ascertain whether the introduction of suspensions of brain residue had any influence on thromboplastin generation in systems containing plasma, platelet suspensions, saline and calcium chloride, in varying combinations.

Experiment 4. To ascertain the effects of adding suspensions of brain residue to incubation mixtures containing plasma, platelet suspensions, saline and calcium chloride in varying combinations.

Reagents: I. A 5 percent suspension (W/V) of brain residue in normal saline, incubated at 37°C. for 20 minutes.

2. Suspensions of normal platelets prepared as follows:- Using siliconized glassware throughout, a normal donor was bled of I8 ml. of blood. This was immediately anti-coagulated with 2 ml. of 3.8 percent sodium citrate, and centrifuged at I000r.p.m. for I0 minutes at I5°C. in a M.S.E. Medium Refrigerator centrifuge. The supernatant "platelet rich" plasma was removed and divided into two equal volumes, one of which was centrifuged for a further I5 minutes at 3000r.p.m. The supernatant "platelet poor" plasma was removed from the platelet concentrate so obtained and set aside for use as substrate. The platelet concentrate itself was washed by resuspending with agitation in an excess

TABLE 4. The substrate clotting times obtained with incubation mixtures containing a suspension of brain residue, plasma, suspensions of normal platelets and calcium chloride in varying combinations.

Incubation Mixture*	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS					
	1	2	3	4	5	6
Brain suspension Saline Calcium chloride	> 60	>60	>60	>60	>60	55
Brain suspension Platelet suspension Calcium chloride	41	40	42	40	41	42
Brain suspension Platelet susp. 1:4 Calcium chloride	49	47	50	44	48	41
Brain suspension Platelet susp. 1:8 Calcium chloride	>60	>60	55	>60	>60	>60
Saline Platelet suspension Calcium chloride	>60	>60	>60	>60	>60	>60
Brain suspension Low spun plasma Calcium chloride	31	15	11	12	11½	15
Brain suspension Low spun plasma 1:2 Calcium chloride	23	12	11	10	9½	11
Brain suspension Low spun plasma 1:4 Calcium chloride	22	14	14	12	10	11
Brain suspension Low spun plasma 1:8 Calcium chloride	35	16	14	13	12	12½

TABLE 4. (cont'd)

Incubation Mixture*	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS					
	1	2	3	4	5	6
Brain suspension Low spun plasma 1:10 Calcium chloride	46	23	20	15	16	14½
Saline Low spun plasma Calcium chloride	>60	45	29	22	16	15
Saline Low spun plasma 1:2 Calcium chloride	>60	>60	38	30	25	21
Saline Low spun plasma 1:4 Calcium chloride	46	43	30	27	21	17
Saline Low spun plasma 1:6 Calcium chloride	>60	40	31	28	24	22
Saline Low spun plasma 1:8 Calcium chloride	>60	>60	50	46	45	39
Saline Low spun plasma 1:10 Calcium chloride	>60	45	36	32	30	36

* Incubation mixtures were prepared by adding 0.3ml. quantities of each of the reagents.

of normal saline, and again centrifuged for I5 minutes at 3000r.p.m., after which the supernatant saline was removed and the platelets were resuspended in a volume of saline equivalent to the original plasma volume from which they had been obtained. Finally aliquots of this suspension were further diluted I in 4 and I in 8 with normal saline.

3. Aliquots of the "platelet rich" plasma described above were diluted I in 2, I in 4, I in 6, I in 8 and I in I0 with normal saline.
4. Platelet "poor" plasma as substrate.
5. Normal saline.
6. Calcium chloride.

Technique: Incubation mixtures were prepared in the following combinations, 0.3 ml. of each reagent being added in each instance.

(I) Brain residue suspension, saline and calcium chloride.
(2) Brain residue suspension, platelet suspensions and calcium chloride.
(3) Brain residue suspension, platelet "rich" plasma and calcium chloride.
(4) Saline, platelet suspensions and calcium chloride.
(5) Saline, platelet "rich" plasma and calcium chloride.

Details of each mixture are shown in Table 4. In every case calcium chloride was the last reagent added, and, coincident with this, a master stop-watch was started, and the mixture incubated at 37°C. in a water bath. At minute intervals for 6 minutes 0.I ml. volumes of incubation mixture were subsampled into 0.I ml. volumes of the normal, platelet poor, plasma substrate, with coincident recalcification with 0.I ml. calcium chloride, and the substrate clotting times recorded. The results are shown in Table 4.

Results: By far the shortest substrate clotting times (and thereby maximum thromboplastin generation) were obtained in mixtures containing brain residue suspension, platelet rich plasma (and

its dilutions) and calcium chloride. By comparison, in corresponding mixtures in which saline had been substituted for brain residue suspension, thromboplastin generation was greatly reduced. Indeed, in that mixture containing brain residue and a I in IO dilution of platelet rich plasma, the minimum substrate clotting time, I4.5 seconds, was much the same as that obtained with undiluted plasma and saline, namely I5 seconds. The minimum substrate clotting time obtained in the combination of saline, a I in IO dilution of platelet rich plasma and calcium was 30 seconds. Thromboplastin formation was negligible in the combinations of brain residue, saline and calcium: saline, suspensions of normal platelets and calcium; and was slight when suspensions of brain residue were incubated with platelet suspensions and calcium.

Discussion: The results of this experiment were interesting. The greater thromboplastin generation in mixtures containing brain residue suspensions, platelet rich plasma and its dilutions and calcium chloride suggested that brain residue did influence thromboplastin formation. It was tempting to postulate a reaction between brain residue and platelets. However, it was equally evident that such improvement was not solely dependant on a simple brain residue-platelet reaction, for a system containing brain residue, suspensions of washed platelets and calcium, was productive of negligible thromboplastin formation. Should improved thromboplastin generation be due to a reaction between brain residue and platelets therefore, it seemed likely that this reaction was dependant upon the presence of additional factors or a factor supplied by normal plasma. The similarity between this hypothesis and the activation of tissue thromboplastin in the presence of factors V, VII and X, supplied by normal plasma was immediately apparent. It was equally possible, however, that improved thromboplastin generation might not be dependant on a reaction between brain residue and platelets at all, for one could postulate that, in some way, plasma might determine direct activation of previously inactive brain residue

without the intervention of platelets. This theory was unlikely for an essential component of tissue thromboplastin, the lipid fraction, had certainly been removed by chloroform extraction. It was subject to simple confirmation or denial, however, for all plasma coagulation factors except prothrombin could be tested in combination with brain residue, in the absence of platelets, by the use of serum and aluminium hydroxide treated plasma, either alone or in combination. This was done in experiment 5.

Experiment 5. To determine the effects of incubating a suspension of brain residue with serum, aluminium hydroxide treated plasma and calcium chloride.

Reagents: I. A suspension of brain residue prepared and incubated as before.

2. A I in I0 dilution of normal serum in sterile normal saline. I0 ml. of blood, collected from a normal donor was allowed to clot. After incubation at 37°C. for 2 hours the serum was separated and diluted.

3. Aluminium hydroxide treated normal plasma diluted in I in 5 with normal saline and prepared as follows. At the same time as blood was collected for serum, 9 ml. of blood from the same donor was anti-coagulated with I ml. of 3.8% sodium citrate. Platelet poor plasma was obtained by centrifugation at 3000r.p.m. for I5 minutes. Subsequently I.8 ml. of the plasma was treated for 5 minutes at 37°C. with 0.2 ml. of aluminium hydroxide, centrifuged and the supernatant diluted.

4. Normal saline.

5. Platelet poor normal plasma as substrate.

6. Calcium chloride.

TABLE 5. The effects of incubating suspensions of brain residue, serum, aluminium hydroxide treated plasma and calcium chloride in varying combinations.

Example Number	Incubation Mixture	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS					
		5	10	15	20	25	30
1	Brain residue Serum Saline Calcium chloride	>60	>60	>60	>60	>60	>60
	Brain residue Saline Treated plasma Calcium chloride	>60	>60	>60	>60	>60	>60
	Brain residue Serum Treated plasma Calcium chloride	>60	>60	52	49½	54	55

TABLE 5. (cont'd)

Example Number	Incubation Mixture	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS					
		5	10	15	20	25	30
2	Brain residue Serum Saline Calcium chloride	>60	>60	>60	>60	>60	>60
	Brain residue Saline Treated plasma Calcium chloride	>60	>60	>60	>60	>60	>60
	Brain residue Serum Treated plasma Calcium chloride	>60	50	48½	47½	49½	50
3	Brain residue Serum Saline Calcium chloride	>60	>60	>60	>60	>60	>60
	Brain residue Saline Treated plasma Calcium chloride	>60	>60	>60	>60	>60	>60
	Brain residue Serum Treated plasma Calcium chloride	>60	>60	46	48	52½	56

TABLE 5. (cont'd)

Example Number	Incubation Mixture	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS					
		5	10	15	20	25	30
4	Brain residue Serum Saline Calcium chloride	>60	>60	>60	>60	>60	>60
	Brain residue Saline Treated plasma Calcium chloride	>60	>60	>60	>60	>60	>60
	Brain residue Serum Treated plasma Calcium chloride	>60	56	44	46	45	45
5	Brain residue Serum Saline Calcium chloride	>60	>60	>60	>60	>60	>60
	Brain residue Saline Treated plasma Calcium chloride	>60	>60	>60	>60	>60	>60
	Brain residue Serum Treated plasma Calcium chloride	>60	50½	45½	53	49	53½

Technique: Using 0.5 ml. of each reagent, the following incubation mixtures were prepared.

(I) Brain residue suspension, serum, saline, calcium chloride.
(2) Brain residue suspension, saline, aluminium hydroxide treated plasma, calcium chloride.
(3) Brain residue suspension, serum, aluminium hydroxide treated plasma, calcium chloride.

In each case calcium chloride was the last reagent added. Coincident with this a master stop-watch was started and at intervals of 5,I0,I5,20,25 and 30 minutes, 0.I ml. volumes of incubation mixture were transferred to 0.I ml. volumes of normal plasma substrate with immediate recalcification with 0.I ml. of calcium chloride, and the substrate clotting times recorded. The whole procedure was performed at 37°C. This experiment was repeated on 5 days using serum and plasma obtained from a different donor on each day. The results are shown in Table 5.

Results: Thromboplastin formation was not evident in mixtures containing either serum or adsorbed plasma alone, and was but slight in the presence of both reagents.

Discussion: This experiment proved beyond doubt that improved thromboplastin generation in mixtures containing a suspension of brain residue, platelet rich plasma and calcium, was not due to reactivation of residue by a factor or factors in plasma other than platelets. The added serum supplied Hageman factor, plasma thromboplastin antecedent, factor VII, factor IX and factor X, and the treated plasma the first two of these factors, factor V and factor VIII. The presence of both serum and adsorbed plasma would have supplied all known intrinsic coagulation factors other than platelets and prothrombin. The virtual absence of thromboplastin formation in the presence of any or all of these factors lent strong support to the original concept that the greater generation of thromboplastin in the presence of brain residue, platelets and plasma, followed a reaction between the residue and platelets, plus a factor or factors supplied by plasma. The identity of the factors

concerned, and indeed, of the whole mechanism was obscure, although the possible similarity between it, and the activation of tissue thromboplastin, as stressed earlier, was obvious. With this in mind, experiments were begun to clarify the basis of any such mechanism. The first of these, experiment 6, compared the relative effects of dilutions of platelet poor plasma and serum, on thromboplastin formation, in mixtures containing suspensions of brain residue, suspensions of normal platelets and calcium chloride.

Experiment 6. To determine the effects of platelet poor plasma and serum in combination with brain residue, platelets and calcium chloride.

Reagents: I. A suspension of brain residue prepared as before.
2. A suspension of normal platelets in saline, prepared as before.
3. A I in 5 dilution of platelet poor plasma in normal saline.
4. A I in IO dilution of normal serum in normal saline prepared as in experiment 5.
5. Normal saline.
6. Normal platelet poor plasma as substrate.
7. Calcium chloride.

Technique: Several incubation mixtures were prepared, details of which are given in Table 6. 0.4 ml. volumes of each reagent were used in every case. Again calcium chloride was the last reagent added. Coincident with this a master stop-watch was started and at minute intervals for up to 9 minutes 0.I ml. volumes of the incubation mixture were subsampled into 0.I ml. volumes of a normal plasma substrate with coincident recalcification with 0.I ml. calcium chloride, and the substrate clotting times recorded. The whole procedure was carried out at 37°C. The experiment was repeated 3 times using platelet poor plasma serum and platelet suspensions prepared from 3 different donors.

TABLE 6. The relative effects of dilutions of plasma and serum in combination with brain residue and platelet suspensions.

Incubation Mixture	Donor No.	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS								
		1	2	3	4	5	6	7	8	9
Brain susp.	1	>60	23	13	13½	13½	14	14	-	-
Platelet susp	2	>60	27	18	15	14½	12	12	-	-
Plasma 1 in 5	3	52	21	16	13	12	11	13	-	-
Calcium chloride										
Brain susp.	1	>60	47	44	36	31	29	-	-	-
Saline	2	>60	51	32	28	19	23	-	-	-
Plasma 1 in 5	3	57	39	30	31	27	29	-	-	-
Calcium chloride										
Saline	1	37	20	13½	14½	15	15	-	-	-
Platelet susp.	2	43	21	17	13	13	18	-	-	-
Plasma 1 in 5	3	35	19	13	14	13	15	-	-	-
Calcium chloride										

TABLE 6. (cont'd)

Incubation Mixture	Donor No.	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS								
		1	2	3	4	5	6	7	8	9
Brain susp. Platelet susp. Serum 1 in 10 Calcium chloride	1	41	30	23	15	14	12	12	10½	10½
	2	>60	40	30	24	20	-	17	15	14
	3	-	-	-	-	25	22	21	19	13
Brain susp. Saline Serum 1 in 10 Calcium chloride	1	>60	>60	>60	>60	>60	>60	57	59	60
	2	>60	>60	>60	>60	>60	58	>60	>60	>60
	3	>60	>60	>60	>60	>60	>60	>60	>60	>60

TABLE 6. (cont'd)

Incubation Mixture	Donor No.	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS								
		1	2	3	4	5	6	7	8	9
Saline	1	>60	>60	46	40	33	28½	25	25	26
Platelet susp.	2	>60	49	39	32	26	-	23	23	23
Serum 1 in 10 Calcium chloride	3	-	-	-	-	37	37	36	32	32
Brain susp.	1	49	53	52	50	50	50	53	50	51
Platelet susp.	2	>60	>60	58	57	52	52	57	53	57
Saline Calcium chloride	3	>60	>60	>60	>60	57	53	49	51	51

The substrate clotting times shown against donor numbers indicate the times obtained with platelets and serum or plasma prepared from the same donor.

The results are shown in Table 6.

Results: In the control test containing a suspension of brain residue, platelet suspension, saline and calcium chloride, thromboplastin generation was negligible. In mixtures containing brain residue suspension, platelet suspensions, plasma and calcium chloride, there was rapid formation of a powerful thromboplastin, minimum substrate clotting times of 11-13 seconds being reached after 3-6 minutes incubation. When saline was substituted for platelets thromboplastin generation was markedly reduced, the minimum substrate clotting times attained being 19-31 seconds. Substitution of saline for brain residue in this mixture had little effect on thromboplastin formation, substrate clotting times of 13 - 13.5 seconds being reached in each case after 4-5 minutes incubation. There was marked thromboplastin formation in mixtures containing brain residue suspension, platelet suspension, serum and calcium chloride. In these the minimum substrate clotting times were much the same as those reached in the corresponding mixture containing plasma, namely 10.5-14 seconds. However, in the presence of serum, slightly more prolonged incubation was necessary before these times were attained, usually in 8-9 minutes. When saline was substituted for platelet suspensions, there was negligible thromboplastin formation, substrate clotting times being in excess of 60 seconds; in the corresponding mixture containing plasma, they varied between 19 and 31 seconds. In contra-distinction to the corresponding mixture containing plasma, thromboplastin formation was markedly reduced in the presence of serum, when saline was substituted for the suspension of brain residue, minimum substrate clotting times of 23-32 seconds being reached after 7-8 minutes incubation.

Discussion: Three significant features were evident from this experiment.

The first of these was the confirmation of the suggestion

that brain residue and platelets might react with "serum" factors to generate thromboplastin. True, tissue thromboplastin requires factor V for its complete activation, and this factor is absent from serum, but it was conceivable that sufficient of this factor might be introduced adsorbed onto platelets to complete the reaction under discussion.

The second important feature was the slight interference with thromboplastin generation when saline was substituted for suspensions of brain residue in mixtures containing plasma, and the opposite effect in those containing serum. Apparently, therefore, brain residue supplied factors essential for thromboplastin generation in mixtures containing serum, but could be removed from mixtures containing plasma probably because the latter was able to supply the same factors or their equivalent. The obvious difference between serum and plasma in this respect was the presence of factors V and VIII in the latter. It was concluded that after it had been subjected to chloroform treatment, acetone-treated brain retained activity comparable to anti-haemophilic globulin or factor V, and was thus able to replace certain fractions of plasma.

The third point was the slower generation of thromboplastin in "serum" mixtures. This was not marked but was consistent. In the presence of plasma maximum thromboplastin generation was evident after 3-4 minutes incubation, but in the presence of serum this was not reached before 7-8 minutes, and even then seemed "incomplete" or still proceeding when the experiment was stopped after 9 minutes. Further investigations were undertaken to clarify this feature of "serum" mixtures and to investigate a point neglected until this juncture. While earlier experiments had demonstrated the necessity for brain residue, platelets and serum before thromboplastin generation could proceed, a similar necessity for calcium chloride had not been shown. Experiment 7 was directed towards this end.

Experiment 7. To determine the effect of calcium chloride on

thromboplastin generation in mixtures containing brain residue, platelets and serum, and the effects of prolonged incubation of such mixtures.

Reagents: I. A suspension of brain residue in saline prepared as before.

2. A suspension of normal platelets prepared as before.
3. Normal serum, prepared as before and diluted I in 20 and I in IO with normal saline.
4. Normal platelet poor plasma as substrate.
5. Calcium chloride.

Technique: In addition to the suspension of brain residue in saline, further suspensions were prepared using the two serum dilutions as the suspending media. All suspensions were incubated at 37°C. for 20 minutes prior to use. "Primary" incubation mixtures were prepared using the suspension of brain residue in saline and the I in IO dilution of serum. The details of these are shown in Table 7 (part Ia). These mixtures, lacking calcium chloride, were incubated at 37°C., and at the start of incubation and thereafter at intervals of I5,30 and 60 minutes, 0.I ml. volumes were subsampled into 0.I ml. volumes of a normal plasma substrate with coincident recalcification, and the substrate clotting times recorded. After I5 minutes incubation, "secondary" incubation mixtures were prepared from the "primary" mixtures by transferring 0.2 ml. volumes of each in turn, to a second series of incubation tubes containing 0.2 ml. volumes of calcium chloride. Subsamples were taken from each of the "secondary" mixtures after IO and 30 minutes incubation, transferred to a normal plasma substrate with coincident recalcification, and the substrate clotting times recorded. The results are shown in Table 7 (part Ib). The whole procedure was repeated using the suspensions of brain residue in saline and the I in 20 dilution of serum. (Table 7 part 2a and b).

TABLE 7. The effect of calcium chloride on thromboplastin generation in mixtures containing brain residue, platelets and serum, and the effect of prolonged incubation on such mixtures.

Part 1 (a)

Time of Sub-sampling	INCUBATION MIXTURE					
	1 0.5ml.brain in serum1:10 0.5ml. plat. suspension	2 0.5ml.brain in saline 0.5ml.plat. suspension	3 0.5ml.brain in serum1:10 0.5ml.saline	4 0.5ml.brain in saline 0.5ml.saline	5 0.5ml.serum 1:10 0.5ml.plat. suspension	6 0.5ml.serum 1:10 0.5ml.saline
	Substrate Clotting Time In Seconds					
Statim	38	41	94	73	28	98
15min.	34	38	90	71	29	83
30min.	38	41	100	70	33	95
60min.	38	44	102	82	36	121

Part 1 (b)

Time of Sub-sampling	0.2ml. of 1 0.2ml.calc. chloride	0.2ml. of 2 0.2ml.calc. chloride	0.2ml. of 3 0.2ml.calc. chloride	0.2ml. of 4 0.2ml.calc. chloride	0.2ml. of 5 0.2ml.calc. chloride	0.2ml. of 6 0.2ml.calc. chloride
	Substrate Clotting Time In Seconds					
10min.	10	36	65	71	22	74
30min.	$8\frac{1}{2}$	33	63	65	37	81

TABLE 7.

Part 2 (a)

Time of Sub-sampling	INCUBATION MIXTURE					
	1 0.5ml.brain in serum1:20 0.5ml. plat. suspension	2 0.5ml.brain in saline 0.5ml.plat. suspension	3 0.5ml.brain in serum1:20 0.5ml.saline	4 0.5ml.brain in saline 0.5ml.saline	5 0.5ml.serum 1:20 0.5ml.plat. suspension	6 0.5ml.serum 1:20 0.5ml.saline
	Substrate Clotting Time In Seconds					
Statim	37	38	50	52	60	88
15min.	32	39	54	46	42	90
30min.	35	37	46	48	53	90
60min.	36	38	42	49	50	90

Part 2 (b)

Time of Sub-sampling	0.2ml. of 1 0.2ml.calc. chloride	0.2ml. of 2 0.2ml.calc. chloride	0.2ml. of 3 0.2ml.calc. chloride	0.2ml. of 4 0.2ml.calc. chloride	0.2ml. of 5 0.2ml.calc. chloride	0.2ml. of 6 0.2ml.calc. chloride
	Substrate Clotting Time In Seconds					
10min.	15	43	67	80	49	90
30min.	5	35	45	60	42	90
60min.	13	32	36	60	47	90

Results: Thromboplastin formation was poor in all "primary" mixtures. The presence of calcium chloride in "secondary" mixtures was accompanied by marked thromboplastin formation only in those mixtures containing brain residue, serum and platelets. In the absence of the latter, thromboplastin generation was much reduced. In "secondary" mixtures, moreover, substrate clotting times were faster after 30 minutes incubation, than they were after 10 minutes, but, in the mixtures containing the I in 20 dilution of serum, after 60 minutes they showed some lengthening.

Discussion: This experiment provided ample proof that calcium chloride was essential for thromboplastin generation in a combination of brain residue, serum and platelets. Furthermore the indication in experiment 6, that the whole reaction was slower in the presence of serum was confirmed. In view of this it seemed that the incubation mixtures containing serum would need to be more prolonged to gain a complete picture of the whole reaction.

In summary to date, then, it had been established that a suspension of acetone and chloroform treated brain, platelets, serum and calcium chloride reacted to form a powerful thromboplastin. The mechanism of this reaction appeared to be similar to the activation of tissue thromboplastin by plasma coagulation factors, but had not been defined. Direct activation of brain residue by plasma factors other than platelets had been excluded. All the reagents mentioned above were essential to the process, and although preliminary evidence suggested that one of the factors in brain residue behaved like anti-haemophilic globulin or factor V, the precise nature of the active components in each reagent had not been clarified. It seemed certain, however, that platelet thromboplastic factor participated in the reaction. In support of this concept was the fact that chloroform treatment of acetone-treated brain, certainly removed the lipoid platelet substitute. The addition of platelets to a suspension of brain residue was tantamount to

replacing this factor. Further, in the intrinsic thromboplastin system, although all other factors may be present, thromboplastin cannot be formed in the absence of platelet thromboplastic factor or its equivalent. So too, in the system under discussion, brain residue was unable to react with all other intrinsic factors supplied in the form of serum and treated plasma to form any appreciable thromboplastin. In further support of the participation of platelet thromboplastic factor, it was considered that the only other platelet factor likely to affect the reaction was adsorbed factor V. Factor V, introduced other than in platelets, in the form of aluminium hydroxide treated plasma did not influence thromboplastin formation in mixtures containing brain residue, serum and calcium. It was reasonable to suppose that platelet thromboplastic factor and possibly factor V absorbed onto platelets, reacted with brain residue and unknown serum factors to generate thromboplastin. Should this be so it was thought that it might be possible to modify this reaction to quantitate platelet thromboplastic function. With this in mind, experiment 8 was undertaken to assess the effects of incubating progressively reducing numbers of platelets with constant volumes of all other reagents.

Experiment 8. To assess the effects of adding serial dilutions of a platelet suspension to constant volumes of all other reagents.

Reagents: I. Brain residue.

2. Normal serum diluted I in IO with normal saline.

3. A suspension of normal platelets. Platelet "rich" plasma was prepared from a normal donor as outlined in experiment 4. Platelet counts were performed on this, after which the platelets were concentrated and washed as in the same experiment. In this case, however, the volume of saline added to the final concentrate was adjusted to give a

TABLE 8. The effect of adding serial dilutions of platelets to standard volumes of brain residue, serum and calcium chloride.

Case No.	Platelet Concentration (per c.mm.)	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS					
		4	10	15	20	25	30
1	400×10^3	18	15	16	16	18	34
	200×10^3	19	18	17	17	18	36
	100×10^3	22	20	20	20	22	39
2	400×10^3	18	12	13	12	14	15
	200×10^3	19	15	15	15	18	17
	100×10^3	22	18	19	19	21	19
3	400×10^3	17	12	13	13	11	15
	200×10^3	15	13	13	13	13	14
	100×10^3	20	18	18	18	26	34

final suspension of $400xIO^3$ platelets per c.mm. Aliquots of this suspension were diluted further to give final concentrations of $200xIO^3$ and $IOOxIO^3$ platelets per c.mm. respectively.

4. Normal plasma as substrate.
5. Calcium chloride.

Technique: A five percent (W/V) suspension of brain residue in the I in IO dilution of serum was prepared and incubated at 37°C. for 20 minutes. Subsequently 3 incubation mixtures were prepared each containing 0.3 ml. of one of the platelet dilutions, 0.3 ml. of brain residue suspension and 0.3 ml. of calcium chloride. Coincident with the addition of calcium chloride, a stop-watch was started and all mixtures were incubated at 37°C. in a water bath. In consideration of the findings of experiment 7, incubation was continued for 30 minutes during which time, at 5 minute intervals, 0.I ml. of each incubation mixture in turn was subsampled into 0.I ml. of normal plasma with coincident recalcification with 0.I ml. of calcium chloride, and the substrate clotting times recorded. The experiment was repeated 3 times using different samples of brain residue and platelets and serum prepared from different donors each time. The results are shown in Table 8.

Results: In each of the 3 tests, reduction of the platelet concentrations produced lengthening of the corresponding minimum substrate clotting times.

Discussion: In this reaction, the substrate clotting times obtained with any mixtures must have been dependent on the strength of thromboplastin generated in that mixture. In this experiment the only variable in the mixtures was the number of platelets, and this therefore, must have been the limiting factor in determining the strength of thromboplastin formed, and thereby the minimum substrate clotting times attained. This experiment then, gave the first positive support for the concept that the reaction under study might be modified to quantitate platelet thromboplastic function.

TABLE 9. The effects of using serial dilutions of normal platelets in combination with brain residue, serum and calcium chloride.

Clotting times using $400x10^3$ platelets.

Case No.	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
	4	10	15	20	25	30
1	14.5	10.5	10.5	9.5	9	9.5
2	16	11	10	10.5	10.5	11
3	20	14.5	12	11	12.5	12.5
4	19.5	12.5	10.5	10.5	10.5	12.5
5	20	13	10	9.5	9	9
6	26	15	12	10	11	10.5
7	24.5	17	14	12	12	12
8	32	19	15.5	13.5	13	14.5
9	28	14.5	11	10.5	10.5	10
10	33	27	15	12	13	12
11	42	15	12.5	11.5	11.5	11
12	23	16	14.5	13	13	13
13	21	14.5	12	12	12.5	12
14	23	16	14	13	13	13
15	20	15	14	12.5	13.5	15
16	25	16.5	15	15	16	15
17	21	15	13.5	12	12	-
18	20.5	13.5	11.5	11.5	12	11.5
19	-	15	13.5	13	13	13.5
20	18.5	13	12	11.5	10	10.5
21	15	13	12	12	13	13.5
22	13	12.5	12	11.5	11.5	11.5
23	15	12.5	12.5	12.5	14	16

TABLE 9. Cont'd.

Case No.	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
	4	10	15	20	25	30
24	16	11.5	11.5	11.5	12	12.5
25	20	14	12	11.5	11	11
26	22.5	15.5	14.5	13	13	13.5
27	22.5	19	16	15	14.5	14
28	19	15	13.5	12	12	11
29	28.5	16.5	14.5	11.5	12	11.5
30	21.5	15.5	13	12	12.5	13
31	17.5	14.5	12	12.5	13.5	12.5
32	18	13	11.5	11.5	11.5	12.5
33	31	17.5	16	15.5	16.5	16

TABLE 9. Cont'd.

Clotting times using $200x10^3$ platelets.

Case No.	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
	4	10	15	20	25	30
1	17.5	14	12	12	12	12
2	22.5	15	12	12	12	12
3	16.5	14	12	14	15	15.5
4	24.5	16.5	13	13	13	14
5	30	16.5	12	11.5	11	11
6	25	16	13	11.5	11.5	11.5
7	32	21	18	16	17	16
8	33	24	18	17	15.5	17.5
9	37	24	17	15	14	14
10	35	23	17	14	15	15
11	48	21	17	14.5	14	14.5
12	25	18	17.5	17	17	17
13	26	20	18	18	19	21
14	27	20	17	17	18	17
15	19.5	16.5	15	15	14.5	16
16	29	21	19.5	19	21.5	26
17	25	18.5	15	15	15	-
18	21.5	16.5	14.5	13.5	14	14.5
19	-	19.5	18.5	16	17	16
20	18	14	11	10	10	11
21	16.5	13.5	12.5	12.5	12.5	13
22	28	19	17	16.5	17	17.5

TABLE 9. Cont'd.

Clotting times using $100x10^3$ platelets.

Case No.	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
	4	10	15	20	25	30
1	23	16	15.5	15.5	16	16
2	22	15.5	15.5	15.5	16	16
3	20.5	17.5	17.5	17	-	19
4	23	17.5	17	18.5	19	19
5	26	20	16	16	15.5	15.5
6	32	24	17	17	17.5	18
7	33	25	23	21	21	21.5
8	40	26	18.5	17	16	18
9	42	28	18.5	19	15.5	15
10	48	35	28	28	25.5	23
11	46	28	24	21	18.5	18.5
12	33.5	26.5	24	24	22.5	24
13	40	30	26	25	26.5	27
14	37	27	25	25	25	25
15	26	20.5	20.5	20.5	21	21
16	40	28	25	25	-	34
17	33	20	18.5	18	18.5	-
18	25	18	16	16	16.5	20
19	27	24	22.5	23	23	23
20	20	16	15	15.5	16	16.5
21	18	16.5	15	15	16.5	18
22	-	26	22.5	23	26	26.5

Following this experiment, and over a period of some weeks, platelet thromboplastic function estimations were made on dilutions of platelets prepared from a series of normal donors. This project is described as experiment 9.

Experiment 9. To assess the effects of serial dilutions of platelets from a series of normal donors.

Reagents and Technique: These were the same as for experiment 8 save that in the tests themselves, 0.5 ml. of each reagent was used instead of 0.3 ml. Platelet suspensions were prepared from 33 normal donors. In 22 of these platelet suspensions containing $400xI0^3$, $200xI0^3$ and $I00xI0^3$ platelets per c.mm. respectively were tested. In the remaining II concentrations of $400xI0^3$ platelets per c.mm. only were tested. Throughout this series three different batches of brain residue were prepared and used in turn. No attempt was made to standardize the serum used, and on each day, this was taken from sources of pooled human serum that had been stored at -20° C. for periods varying from 3-6 months. The results of the tests in the order in which they were performed are shown in Table 9.

Results: In each of the 22 cases in which 3 platelet concentrations were tested, successive dilutions were accompanied by progressive lengthening of the minimum substrate clotting times. With any platelet concentrate, a wide range of minimum substrate clotting times was obtained over the whole series. For example, when concentrations of $400xI0^3$ platelets were used the range of minimum substrate clotting times in 33 tests was 9.5 - I6 seconds. Although no record was kept of the batch of brain used in any particular test, consideration of the minimum substrate clotting times obtained with concentrations of $400xI0^3$ platelets per c.mm. revealed an interesting feature. The 33 tests could be divided into 3 groups, these including cases I-I6, I7-27, 28-33 respectively. In each of these groups, the specimens tested early in the series appeared to be accompanied

by faster minimum substrate clotting times than the later tests. Overall, the impression gained was one of progressive lengthening of substrate clotting times as the number of specimens tested in any particular group increased.

Discussion: The wide range of minimum substrate clotting times in these tests directed attention to the problem of minimising this variation. It was not possible to say with certainty that a different batch of brain residue had been used exclusively for each of the 3 groups of tests separated above, but, in retrospect, this seemed likely. Assuming this to be so, the later in any group a test was performed, it would be associated with a progressive ageing of the brain residue used. Under this circumstance the range of variation in substrate clotting times might well have been exaggerated by a factor remote from platelets themselves, namely, deterioration of brain residue under the existing conditions of storage, namely, in bulk in air-tight, screw-capped containers at -20°C. It was conceivable, therefore, that the range of variation might be reduced if progressive deterioration of residue could be demonstrated and overcome.
Two other considerations were related to platelets themselves. Firstly, it was thought that the vigorous washing of platelets per SE might be associated with variable loss of thromboplastic factor. Secondly, it was difficult to obtain absolutely uniform platelet suspensions, and many suspensions, of necessity, contained large clumps of unknown numbers of platelets. Depending on the uniformity of platelet suspensions, in practice, unknown numbers of platelets were being used in successive tests, and successive estimations could not be accepted as strictly comparable. Experiments IO and II were used to clarify some of these considerations.

Experiment IO. To determine the stability of brain residue under different conditions of storage.

Reagents and Technique: A new batch of brain residue was

TABLE 10. The effects of differing conditions of storage on the stability of brain residue.

Brain Preparation	Age of Residue	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
1*	24 hrs.	13	10	10	10	10	10.5
2*	24 hrs.	19	10.5	10.5	10.5	10.5	11.5
3*	24 hrs.	12	10	10	10	10.5	10
1	4 wks.	22.5	15	13	13.5	13	13
2	4 wks.	18	14.5	11.5	11	11.5	12
3	4 wks.	17	10.5	10.5	10.5	10.5	11.5

1* = Brain residue stored in bulk.

2* = Residue stored in small aliquots in screw-capped containers.

3* = Residue stored in ampoules.

prepared. Immediately after preparation 4 amounts each of 0.5 gramme were weighed and set aside. Two of these were transferred to suitable small ampoules and the latter were evacuated over P_2O_5 to 0.04 mm. mercury on a freeze-dry apparatus and sealed. The other two amounts were placed in individual small air-tight, screw-capped containers. The rest of the residue was stored in bulk in the usual way. All samples were stored at -20°C. until used. On the day following the preparation of the residue, a platelet suspension containing 400×10^3 platelets per c.mm. was prepared from a normal donor in the usual way. A I in IO dilution of normal serum in normal saline was prepared, the serum having been incubated on the clot for 4 hours prior to use. Using this serum dilution, three brain suspensions were prepared using a sample of brain residue stored under the different conditions described above, for one of each of the suspensions. Incubation mixtures were prepared using, in turn, 0.5 ml. of one of the suspensions of brain residue, 0.5 ml. of the platelet suspension and 0.5 ml. of calcium chloride. In each case calcium chloride was the last reagent added and coincident with this a stop-watch was started. At intervals of 5 minutes for 30 minutes, sub-samples were taken in the usual way and the substrate clotting times recorded. The whole procedure was carried out at 37°C. The remaining samples of brain residue were stored in their respective containers for 4 weeks, after which the whole experiment was re-peated using platelets and serum prepared in an identical way, from the original donors, and these samples of residue. The results are shown in Table IO.

Results: After 24 hours storage, the minimum substrate clotting times obtained with the 3 samples of brain residue were almost identical, despite the different methods of storage. After 4 weeks, the minimum substrate clotting time obtained with the suspension of residue prepared from the ampouled sample, was virtually unchanged, but the substrate clotting times obtained with suspensions of residue prepared from that stored in bulk or individual small amounts in screw-capped containers were some

seconds slower than the earlier values obtained with the corresponding preparations.

Discussion: These results confirmed the suggestion that brain residue stored in screw-capped containers might deteriorate and so contribute to the wide range of variation in substrate clotting times found earlier. There seemed to be no other adequate explanation for the marked differences in substrate clotting times found after a period of storage of brain residue in screw-capped containers, and the slight variation when residue had been stored for the same period of time in properly evacuated ampoules. It was decided therefore, that, in future, all residue would be stored in 0.5 gramme amounts in sealed ampoules, and that the latter would be opened only immediately prior to use, and then only in sufficient quantities to accomodate the anticipated work for any one day.

Meanwhile attention was focussed on the second possible source of error in the technique hitherto used, namely, the uncertainty that comparable platelet numbers were being used in successive tests. In experiment 9, when platelet suspensions of 0.5 ml. volumes were used, and when the suspension contained 400×10^3 platelets per c.mm., the total number of platelets in the final incubation mixture was 200×10^6. This number of platelets gave convenient substrate clotting times, and it was decided to continue using this concentration but to modify their preparation whereby errors due to the uneven distribution of platelets in suspensions might be overcome. It was thought that this might be accomplished in the following way.

Specimens of blood would be collected from normal donors in the usual way, using siliconized glassware throughout and 3.8 percent sodium citrate as anti-coagulant. The specimens of whole blood would be centrifuged at 1000r.p.m. for 10 minutes at 15°C. in an M.S.E. Medium Refrigerator Centrifuge and the platelet "rich" plasma, pipetted into a siliconized centrifuge tube. The contained platelets would be distributed as evenly as possible in the plasma by repeated inversion of the centrifuge

tube. After this duplicate platelet counts would be performed on the platelet rich plasma. If the difference between the two counts was less than 5 percent of the average of the two counts, the average count would be used as the "true" value. If the difference between the two counts was greater than 5 percent, the specimen would be mixed again and further counts prepared until agreement was reached. The "true" value would be used to calculate that volume of plasma containing $200xI0^6$ or any desired number of platelets. This exact volume of plasma would be transferred to another siliconized centrifuge tube and centrifuged at 3000r.p.m. for I5 minutes. The supernatant plasma could be carefully pipetted from the resulting platelet concentrate, which would then be carefully washed with 4-5 ml. of normal saline, and centrifuged for a further I5 minutes at 3000r.p.m. If care was observed the resulting platelet concentrate, after removal of the supernatant saline, should contain $200xI0^6$ platelets or the desired number.

At this juncture, while radical changes in the preparation of reagents were in progress, it was thought opportune to standardize the preparation of the serum to be used. Until this stage, at various times, serum had been obtained from sources of pooled serum stored in the frozen state for 3-6 months, or more frequently from fresh specimens incubated on the clot for 4-6 hours prior to use. Both sources had proved equally effective. In consideration of the fact that some residual prothrombin may be active in serum for a short time after coagulation, it was decided to use only that serum which had been freshly collected and incubated on the clot at $37^{\circ}C$ for I8 hours, or samples from a source of serum that had been prepared in this way, separated from the clot and stored frozen in small aliquots, these being thawed immediately before use.

These changes in the preparation and standardization of basic reagents, necessitated re-appraisal of the whole technique used until this stage. The new method for obtaining platelet concentrates in particular, necessitated radical changes in

technique. After due consideration, the new technique, outlined as experiment II, was introduced.

Experiment II. The revised technique for the estimation of platelet thromboplastic function.

Reagents: I. A five percent suspension (W/V) of brain residue in normal saline, incubated at 37°C. for 20 minutes.

2. Platelet "rich" normal plasma.
3. Serum incubated on the clot for I8 hours at 37°C.
4. Platelet poor plasma as substrate.
5. Calcium chloride.

Technique: Using the revised technique for their preparation, platelet concentrates containing $200xI0^6$, $I00xI0^6$ and $50xI0^6$ platelets were prepared. After washing, the supernatant saline was removed from the platelet concentrates, care being taken to disturb them as little as possible. 0.I ml. volumes of serum were added directly to the platelets, and with the aid of a wooden probe, the platelet masses were broken up and roughly suspended in the serum. I.0 ml. volumes of the brain residue suspension were added to each platelet-serum suspension, and finally each mixture was recalcified with I.0 ml. of calcium chloride. A stop-watch was started, coincident with recalcification, and at intervals during 30 minutes incubation at 37°C., 0.I ml. volumes of each incubation mixture were subsampled into 0.I ml. volumes of a normal plasma substrate with coincident recalcification with 0.I ml. of calcium chloride and the substrate clotting times recorded. In conjunction with these tests it was decided to re-affirm the need for all four basic reagents in an incubation mixture before thromboplastin generation could proceed. Accordingly in turn, additional incubation mixtures were prepared in which, in turn, an equivalent volume of saline was substituted for each reagent. This whole experiment was repeated on each of ten successive days, using suspensions of brain residue prepared

TABLE 11. Assays of platelet thromboplastic function using a revised technique.

Case No.	Incubation Mixture*	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
1	A	22.5	15	13	13.5	13	13
	B	22	16.5	14.5	15	15.5	15
	C	22	19	19	20	20.5	19
	D	> 60	> 60	> 60	> 60	> 60	> 60
	E	> 60	33.5	28	26	24	19
	F	> 60	56	41	37	38.5	38
	G	40	21	17	17.5	20	37
2	A	18	14.5	13	13	13	14.5
	B	19	16.5	16	15.5	15.5	16
	C	22.5	19	19	20	22	19
	D	> 60	53	> 60	> 60	> 60	> 60
	E	56.5	35.5	28.5	28.5	28	27.5
	F	49	37	31	31	29	32
	G	18.5	22.5	32	39	44.5	47

TABLE 11. cont'd.

Case No.	Incubation Mixture*	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
3	A	12	12.5	12.5	13.5	13.5	13
	B	14	14	14.5	16	16	16.5
	C	18	16.5	16	16.5	17.5	20
	D	> 60	> 60	58.5	53.5	53	> 60
	E	50	33	28.5	27	26	29
	F	60	54	50	49	43	43
	G	> 60	45.5	48	59	> 60	> 60
4	A	17.5	13	13	13.5	14.5	16
	B	16	14	14.5	15.5	16	16
	C	48	22.5	18	18.5	23	23
	D	> 60	> 60	> 60	53	50	48
	E	> 60	49	46	43	37	38.5
	F	61	55	44	39	27	24
	G	16	19	24	29	34	39

TABLE 11. cont'd.

Case No.	Incubation Mixture*	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
5	A	14.5	11	11	11	11	15
	B	16.5	15	14	14.5	15.5	16
	C	40	20	19.5	19.5	22	23
	D	> 60	> 60	> 60	> 60	> 60	> 60
	E	> 60	49	46	43	37	38.5
	F	> 60	38	36.5	36.5	31	31
	G	27.5	17.5	22	29	35	36
6	A	15.5	12	12.5	12.5	13	14
	B	21.5	16	15	14.5	15.5	16
	C	21	16.5	16.5	17.5	19	21
	D	> 60	> 60	58	> 60	> 60	> 60
	E	> 60	> 60	42.5	35	36	37.5
	F	58	57.5	51	49	38	41
	G	27	19	23	31	37	43

TABLE 11. cont'd.

Case No.	Incubation Mixture*	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
7	A	17	14	12.5	12.5	12	12.5
	B	23.5	16.5	15	14.5	15	15
	C	43.5	22	18	18	18.5	18
	D	> 60	> 60	> 60	> 60	> 60	> 60
	E	> 60	> 60	45	37	35	36
	F	> 60	> 60	53	47.5	45	39.5
	G	23	17	19	24	32	41
8	A	18	13.5	13.5	12	12	12
	B	15	15.5	15	15.5	15.5	15.5
	C	28	16	16.5	16.5	17	18
	D	> 60	50	50	45	45	51
	E	50	35.5	27	28	28	27.5
	F	> 60	> 60	47.5	38	37.5	34
	G	> 60	49	21	23	29	40

TABLE 11. cont'd.

Case No.	Incubation Mixture*	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
9	A	19.5	14.5	12	12	12	12
	B	24.5	17	15.5	15	15	15
	C	39	21	18.5	19	19	19.5
	D	> 60	> 60	> 60	> 60	> 60	> 60
	E	> 60	57.5	46	35	36	35.5
	F	49	29.5	26	25.5	25	27
	G	> 60	37	42	33	49	> 60
10	A	16	12.5	12.5	12.5	13	13
	B	29	17	16	15.5	15.5	15.5
	C	40	21.5	19	18	18.5	19
	D	53	> 60	> 60	> 60	> 60	> 60
	E	55	35	25.5	25	23	22
	F	54	48	44	43.5	41	43
	G	> 60	18	23	33	41	45

TABLE 11. cont'd.

INCUBATION MIXTURES

A = 1ml. brain residue suspension, $200x10^6$ platelets, 0.1ml. serum, 1ml. calcium chloride.

B = 1ml. brain residue suspension, $100x10^6$ platelets, 0.1ml. serum, 1ml. calcium chloride.

C = 1ml. brain residue suspension, $50x10^6$ platelets, 0.1ml. serum, 1ml. calcium chloride.

D = 1ml. brain suspension, 0.1ml. serum, 1ml. calcium chloride. (No platelets).

E = 1ml. brain suspension, $200x10^6$ platelets, 1ml. calcium chloride. (No serum).

F = 1ml. brain suspension, $200x10^6$ platelets, 1ml. serum, 1ml. saline. (No calcium).

G = 1ml. saline, $200x10^6$ platelets, 0.1ml. serum, 1ml. calcium chloride.

from the same batch of residue on each day, but platelets and serum prepared from ten different donors. The results are shown in Table II.

Results: In every case thromboplastin generation was negligible or slight in any mixture lacking any one of the four basic reagents. When all reagents were present thromboplastin generation was rapid and powerful. As in experiment 9, when succeeding dilutions of platelets were used progressively slower, minimum substrate clotting times were obtained. The range of minimum substrate clotting times obtained with any concentration of platelets was much narrower than that found earlier. In this series of ten cases the range of minimum substrate clotting times obtained with 200×10^6 platelets was 11-13 seconds, with 100×10^6 platelets 14-15.5 seconds, and when 50×10^6 platelets were used 16.5-19.5 seconds.

Discussion: The preliminary tests with the revised technique were most encouraging. The necessity for all four reagents had been reaffirmed. Under the new conditions of preparation and storage the reagents reacted well to produce a uniform pattern of thromboplastin generation, and, despite the relatively small number of tests performed, it seemed likely that the range of variation in minimum substrate clotting times would be considerably narrowed by the modifications and new technique introduced. It was decided to standardize all future work on the exact procedure outlined in experiment 11, and to conduct a similar experiment with platelet concentrates prepared from a large number of normal individuals. Before doing so, however, it was thought essential to investigate the effects of using washed and unwashed platelets in the system, lest considerable amounts of platelet thromboplastic factor should be lost during the washing process.

Experiment 12. To investigate the relative effects of washed and unwashed platelets in combination with brain residue, serum and calcium chloride.

TABLE 12. The effects of using washed and unwashed platelets in the standard procedure.

Test No.	Platelet Preparation	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Unwashed	40	27.5	17	14.5	13.5	13	12	12	12.5
	Washed	46	31	23	18	17	14.5	13.5	12.5	12.5
2	Unwashed	23	22	19.5	17.5	15.5	13	12.5	12.5	12.5
	Washed	40	27.5	21	19	18	16	14	14	13
3	Unwashed	44	30	17.5	15.5	14	12	11.5	12	12.5
	Washed	50	30	23	19	17	14	13	12	12.5
4	Unwashed	34.5	22.5	17.5	16.5	15.5	13.5	13	13	13.5
	Washed	45	26	20	17.5	16	15.5	14.5	13.5	13.5
5	Unwashed	22	11.5	11	11	11.5	12	12	11.5	12
	Washed	36	22	17.5	14.5	12.5	12.5	12	12	12.5

TABLE 12. Cont'd.

Test No.	Platelet Preparation	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
6	Unwashed	43	-	16	14.5	13.5	13	12.5	12.5	12.5
	Washed	42	32.5	28	24	20.5	16.5	16.5	15	13.5
7	Unwashed	46	24	18	16.5	14.5	13	12.5	12.5	12.5
	Washed	54	45.5	36.5	29	24	22.5	20	17.5	16.5
8	Unwashed	42	26	20.5	18	17	15	13.5	13	13
	Washed	52.5	44	37.5	28	23	18.5	17.5	16	16
9	Unwashed	43	21	19	16	15.5	12.5	12.5	12.5	12
	Washed	>60	57	48	42	41	28	23.5	21	20
10	Unwashed	37.5	24	19.5	18.5	17	15	13	13.5	13
	Washed	46	41	39	34	33.5	29.5	28.5	26.5	24

Reagents: I. A suspension of brain residue prepared as before.
2. Platelet "rich" plasma.
3. Serum prepared as before.
4. Platelet poor plasma as substrate.
5. Calcium chloride.

Technique: Ten preparations of platelets collected from different normal donors were tested on successive days. On each day concentrates containing $200xI0^6$ platelets were prepared in duplicate. On each occasion the supernatant plasma was pipetted carefully from both concentrates, one of which was set aside for use as the "unwashed" preparation. The remaining concentrate of each pair was treated as follows. In the first 5 tests, 7-8 ml. of normal saline was carefully added to the platelet concentrate, by allowing the saline to run gently down the side of the centrifuge tube, and with as little agitation of the platelets as possible. In the remaining 5 cases the platelet concentrate was washed much more vigorously with the same amount of saline. In the latter tests each concentrate was separated from the bottom of the centrifuge tube and largely broken up by vigorous shaking, and distributed throughout the saline. Subsequently, the washed platelets were re-centrifuged at 3000r.p.m. for I5 minutes to re-concentrate the total number; and the "washed" and "unwashed" preparations of each pair were tested by the technique outlined in experiment II. The results are shown in Table I2.

Results: In the first 5 tests, when platelets were gently washed, the minimum substrate clotting times of either the washed or unwashed platelets did not differ by more than 0.5 seconds. When washed platelets were used, however, the reaction seemed somewhat slowed, and slightly longer incubation times were needed before maximum thromboplastin generation was reached. In the remaining 5 tests, again the reaction was obviously slower when washed platelets were used, but in these instances the substrate clotting times after 30 minutes incubation were some seconds slower by

comparison with the unwashed controls. In retrospect, it seemed that in the latter cases, the minimum substrate clotting times had not been reached when the experiments were stopped.

Discussion: These results were interesting but difficult to interpret. In all cases a common feature was the more prolonged incubation necessary to attain minimum substrate clotting times whenever washed platelets were used. This tendency was more marked when vigorously washed platelets were tested. Despite this, however, there was no significant difference between the minimum substrate clotting times attained with gently "washed" platelets and the corresponding unwashed preparation, while marked differences were apparent in most tests when vigorously washed platelets had been used. In the latter examples however, the differences might well have been reduced by longer incubation, for it seemed that in most cases the substrate clotting times were still reducing when incubation and subsampling were discontinued. Two principal factors had to be considered in relation to these findings. The first of these was the possible loss of platelet thromboplastic factor during washing. Should such happen, one would have expected greater loss with increasing vigour of washing, and the differences in substrate clotting times between gently and vigorously washed platelets, would find ready explanation. This explanation, however, could hardly be regarded as adequate to resolve all of the observed facts, for pure loss of thromboplastic factor would be unlikely to cause a general slowing of the whole reaction as was evident in all ten cases. Furthermore, this occurred in the first 5 cases despite the insignificant difference between minimum substrate clotting times obtained with washed and unwashed platelets. Thus, the second feature to be considered was the distinct possibility that factor V might be eluted from platelets during washing, and, here, too, the degree of loss would certainly vary with the vigour of the washing process. Such loss would certainly be a sufficient explanation for the slowed reaction, in all cases. Indeed, it may have been

unnecessary to postulate loss of thromboplastic factor, for, as suggested earlier, continued incubation in the last five cases, may have permitted the establishment of the same minimum substrate clotting times in the case of vigorously washed platelets as in the unwashed controls. The less marked changes when gently washed platelets were used, would be dependant on the lesser loss of factor V, and the marked changes in the examples using more vigorously washed platelets, on more exaggerated loss of factor V with or without the loss of thromboplastic factor. These possible explanations were open to experimental confirmation as detailed in experiment I3.

Experiment I3. To determine the relative effects of elution of factor V and loss of thromboplastic factor incurred in the washing of platelets.

Reagents: The same as for experiment I2.
Technique: Platelet concentrates containing $200xI0^6$ platelets were prepared in triplicate from a specimen of platelet "rich" plasma. The supernatant plasma was removed from the concentrates, and the respective volumes of plasma removed from each tube were kept separate from the others. One of the platelet concentrates was set aside for use as the unwashed specimen. The remaining two concentrates were vigorously washed as described in experiment I2, and the platelets re-concentrated by centrifugation at 3000 r.p.m. for I5 minutes. The supernatant saline was removed from both platelet concentrates, one of which was set aside as the "washed" platelet specimen. The second of this pair was treated in the following way. A volume of the platelet poor plasma, equivalent to that from which $200xI0^6$ platelets had been derived, was added to the third concentrate, and the platelets were suspended in this by shaking. The suspended platelets were incubated in this plasma for 30 minutes at room temperature, after which they were concentrated by a third centrifugation, and

TABLE 13. The effects of washing platelets: Comparison of loss of factor V and elution of platelet thromboplastic factor.

Test Number	Platelet Preparation	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Unwashed	28.5	15.5	11.5	11	11	11	11	11	11
	Washed	29.5	20	14.5	13	11.5	11.5	11.5	11.5	11.5
	Incubated	35	16.5	12	11.5	11	11	10.5	11	11.5
2	Unwashed	16	12	10.5	10	10	9.5	10	10	11
	Washed	> 60	54	33	26	20.5	15.5	13.5	13	13
	Incubated	28.5	14.5	12	11	10	10	10	10.5	11.5
3	Unwashed	35.5	20.5	15	13	12	12	12	12	12
	Washed	> 60	48	36	31	25	18.5	16	16	14.5
	Incubated	45	22.5	16.5	14	12.5	12	12	13	13
4	Unwashed	30	17.5	14	12.5	12.5	11.5	11.5	11.5	12.5
	Washed	> 60	51	28	24.5	21.5	16	15	13.5	13.5
	Incubated	-	54	-	-	13	12.5	13	13	14.5
5	Unwashed	35.5	19.5	14.5	13	11.5	11.5	11.5	11	11.5
	Washed	45	36.5	30	25	21.5	18	15	12	11.5
	Incubated	31	17	13	11.5	11.5	11	11	11	11.5

finally the supernatant plasma was removed. The three platelet concentrates, thus prepared, were incubated with brain residue suspension, serum and calcium, in the standard way, and the appropriate substrate clotting times recorded. The experiment was repeated on 5 occasions. The results are shown in Table I3.

Results: In the first example the minimum substrate clotting times obtained with the unwashed, washed and the washed and incubated platelet concentrates were II, II.5 and II seconds respectively. In examples 2 and 3, the reaction was slowed by washing the platelets and the minimum substrate clotting times obtained with these preparations were slower than those of the corresponding unwashed preparation. Both these features were corrected by incubation of the third platelet concentrate in platelet poor plasma, in each example. After this procedure, both the incubation time required to reach minimum substrate clotting times and the latter themselves were much the same as in the unwashed controls. In case 4, again the reaction was slower and the minimum substrate clotting times slower than in the unwashed controls. Incubation with plasma in this case, however, corrected the overall speed of the reaction but did not affect the longer substrate clotting time. In the fifth example, again the reaction was slowed by washing, but the minimum substrate clotting time attained by this preparation was finally the same as the unwashed controls. Incubation in plasma, in this case restored the speed of the reaction while the minimum substrate clotting times obtained with this third preparation were slightly faster than either of the corresponding unwashed or washed preparations.

Discussion: The results of this experiment were difficult to interpret for it was impossible to be sure that the two platelet concentrates subjected to washing in each example, had been treated in a sufficiently identical way during washing to be considered strictly comparable. Assuming this to be approximately so, it was apparent that whenever the reaction was slowed by washing, the speed of the reaction was restored by incubation in platelet poor plasma. In cases 2 and 3, the slower substrate

clotting times evident after washing were also returned to normal by incubation. It is difficult to accept that this latter correction in these two examples was due to the addition of that exact amount of thromboplastic factor, that had been lost by washing by additional incubation and centrifugation of platelets in platelet poor plasma. Even if additional thromboplastic factor was supplied by this procedure it would hardly be sufficient, and indeed, too great a coincidence for this to be the exact amount to correct these tests to normal. A more likely explanation would seem to be that the correction of the substrate clotting times and the restored incubation times had a common basis and this, the re-adsorption onto platelets of factor V eluted during washing. Test 5, perhaps supplies confirmation for this concept, for in this, after more prolonged incubation the minimum substrate clotting times reached when washed platelets were used were the same as in the case of the corresponding unwashed preparation. In this instance incubation in plasma restored the speed of the reaction but the substrate clotting times were not significantly altered. However, it seemed likely that in isolated cases actual loss of thromboplastic factor might contribute to the lengthened substrate clotting times evident after platelets had been vigorously washed, and that under these circumstances, incubation in plasma could not restore this loss. Case 4 is an example of this category, for while incubation restored the speed of the reaction to normal, the minimum substrate clotting times remained prolonged. Overall, experiments I2 and I3 indicated that the gentle washing of platelets as usually practised, had no significant effect on their activity in the system under consideration. Vigorous washing, however, caused loss of factor V and this produced slowing of the reaction and possibly reduced thromboplastin formation. In most instances these defects could be corrected by re-adsorption of factor V onto platelets during incubation in platelet poor plasma, this suggesting that platelet thromboplastic factor was not usually lost during washing, and that such an occurrence was

TABLE 14. The action of thrombin in the assessment of platelet thromboplastic function.

Test Number	Substrate	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS								
		2	4	6	8	10	15	20	25	30
1	Normal plasma	51.5	27	22	16.5	15.5	11.5	11.5	11.5	11.5
	$Al(OH_3)$ treated plasma	55	54	53.5	54	53	50	54	> 60	> 60
2	Normal plasma	47	23	16.5	14.5	14	13	13	13.5	13.5
	$Al(OH_3)$ treated	> 60	> 60	> 60	> 60	58.5	> 60	> 60	> 60	> 60
3	Normal plasma	25	13	10.5	9	9.5	9.5	9.5	10	12
	$Al(OH_3)$ treated plasma	> 60	> 60	57	55	50	50	57	> 60	> 60

TABLE 14. (cont'd)

Test Number	Substrate	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS								
		2	4	6	8	10	15	20	25	30
4	Normal plasma	21	15	11.5	11	10.5	10.5	11	10.5	12
	$Al(OH_3)$ treated	> 60	> 60	> 60	>60	>60	> 60	> 60	>60	>60
5	Normal plasma	37	18.5	14	12.5	12	12	12	12.5	12
	$Al(OH_3)$ treated plasma	> 60	>60	58	55	49.5	52	53	58	> 60

unusual. If loss of thromboplastic factor was incurred during washing, the speed of the reaction was still restored by incubation in plasma but the lost thromboplastic factor was not recoverable.

No doubt the loss of factor V when platelets were vigorously washed, followed the disruption of a large platelet mass, with the consequent exposure of a larger area of platelet surface from which factor V could be eluted. It was decided, therefore, that in all future investigations the platelet concentrates would be gently washed, as described, for in this way, platelet thromboplastic component would be unaffected, while any plasma surrounding the platelet concentrate would be greatly diluted. However, a minute volume of plasma trapped in the depths of the platelet mass would not be affected by dilution with saline. Experiment I4 was undertaken to see if this small amount of plasma yielded sufficient thrombin to influence the substrate clotting times obtained in the standard procedure.

Experiment I4. To assess the effect of any thrombin generated in the incubation mixture.

Reagents: I. All normal reagents prepared as for use in the standard procedure.

2. Aluminium hydroxide treated plasma for substrate.

Technique: Incubation mixtures, prepared in the usual way, were incubated at 37°C. At intervals of 2,4,6,8,I0,I5,20,25 and 30 minutes, 0.I ml. volumes were subsampled into 0.I ml. volumes of normal plasma as substrate, and into a second substrate of aluminium hydroxide treated plasma. Both substrates were recalcified on the addition of incubation mixture and the substrate clotting times recorded. The results of 5 experiments using different preparations of brain residue, serum and platelets on each occasion are shown in Table I4.

Results: The minimum clotting times of the normal plasma sub-

strates ranged between 9.5 and I3 seconds. None of the aluminium hydroxide treated plasma substrates clotted in less than 49.5 seconds. The minimum substrate clotting times of the plasma substrates, once reached, were maintained at a constant level for some time. On the other hand in cases I,2,3 and 5, the minimum clotting times of the adsorbed plasma substrates were less stable and showed rapid decline.

Discussion: Potency of any thrombin preparation is usually assessed by the ability of 0.I ml. volumes of that preparation to clot a substrate composed of 0.4 ml. of pure fibrinogen or the same volume of aluminium hydroxide treated plasma containing added pyrocatechol to neutralize any contained anti-thrombins. No calcium is added. For the sake of this work the concern was to determine the possible effect on substrate clotting times of any thrombin generated in incubation mixtures, when and if added to usual substrate volumes. Normal proportions were maintained therefore, by using 0.I ml. volumes of adsorbed plasma, and adding the usual amount of calcium chloride on the addition of incubation mixture. It was evident therefore that thrombin formation was slight in the usual incubation mixture and had no effect to determine the substrate clotting times of the usual plasma substrate. The reasons for the conclusion were two. The first of these, and the most obvious, was the marked difference between the minimum substrate clotting times of normal and adsorbed plasma on the addition of incubation mixture. The second feature precluding a role for formed thrombin in determining the substrate clotting times either directly or as a result of the combined effects of formed thrombin and thromboplastin, was the maintenance of constant minimum substrate clotting times of whole plasma, while those of adsorbed plasma were declining. Should minimum substrate clotting times have been determined in whole plasma as a result of the combined action of thrombin and thromboplastin, one would have expected the substrate clotting times of whole plasma to decline in parallel with the decline of thrombin. Since this was not the case, it could only

TABLE 15. A range of variation in substrate clotting times obtained with normal platelets in combination with a constant preparation of brain residue and individual samples of serum.

Part (a) Clotting times using 200 x 10^6 platelets.

Case No.	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
	2	4	6	8	10	15	20	25	30
1	45	22.5	17	16	15	13	13.5	13	13
2	35.5	18	16.5	15	14.5	13	13	13	14.5
3	15	12	12	12	12.5	12.5	13.5	13.5	13
4	40	17.5	14.5	13.5	13	13	13.5	14.5	16
5	39	14.5	12.5	11	11	11	11	11	15
6	23	15.5	12.5	12.5	12	12.5	12.5	13	14
7	25	17	16.5	15.5	14	12.5	12.5	12	12.5
8	43.5	18	14.5	15	13.5	13.5	12	12	12
9	32	19	15.5	14.5	14.5	12	12	12	12
10	38	16	15	13	12.5	12.5	12.5	13	13
11	35	17	15.5	14.5	13	12.5	12.5	12.5	13
12	27.5	18	14.5	14.5	13.5	12.5	12.5	13	12.5

TABLE 15. Part (a) cont'd.

Case No.	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
	2	4	6	8	10	15	20	25	30
13	33	18.5	16.5	14.5	14	13	12.5	12.5	12.5
14	28	13.5	12.5	12	12.5	12	12.5	12.5	12
15	50	17.5	15.5	13.5	13	12.5	12	12.5	12
16	40	22	17	14	13	12	12	11.5	12
17	28	17	14.5	14	12.5	12	12	12	12
18	34	14	12.5	12	12	11.5	11.5	11.5	11.5
19	19	13	12	11	12	12	12	11.5	11.5
20	40	19.5	18	16	13	12.5	12	12	12.5
21	> 60	32	16.5	14.5	15	13	13	13	12
22	25	17	15.5	14.5	14	12.5	12.5	13	12.5
23	37	21	17	15.5	15	13.5	13.5	13	13
24	37.5	16.5	14	12.5	12	12.5	11.5	12	12
25	41	25.5	16	16	14.5	12.5	12.5	12	11.5
26	50	24.5	18	15	14	13	13	12.5	12.5
27	42	26.5	17	14.5	13.5	12.5	11.5	12	12

TABLE 15. Part (a) cont'd.

Case No.	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
	2	4	6	8	10	15	20	25	30
28	41.5	24.5	19.5	17	14.5	13	11.5	12	12
29	46.5	26	18.5	15.5	15	12.5	12	12	12
30	36	23	18.5	18	15.5	13.5	13	12.5	12.5
31	41.5	28.5	24	18.5	16.5	13.5	13	12.5	12
32	30	26	21	20.5	20	14.5	13.5	13.5	13.5
33	35	22	21	18	16	13.5	13	13	13
34	40	22.5	16.5	13.5	13	12	12	12	12
35	40	27.5	17	14.5	13.5	13	12	12	12.5
36	23	22	19.5	17.5	15.5	13	12.5	12.5	12.5
37	44	30	17.5	15.5	14	12	11.5	12	12.5
38	34.5	22.5	17.5	16.5	15.5	13.5	13	13	13.5
39	22	11.5	11	11	11.5	12	12	11.5	12
40	28	22.5	19	16	14	13	12	12	12
41	43	-	16	14.5	13.5	13	12.5	12.5	12.5
42	46	24	18	16.5	14.5	13	12.5	12.5	12.5

TABLE 15. Part (a) cont'd.

Case No.	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
	2	4	6	8	10	15	20	25	30
43	32	21	18	15.5	15	13.5	12.5	12	12.5
44	42	26	20.5	18	17	15	13.5	13	13
45	47.5	24	19	17.5	15.5	13	12.5	12.5	12.5
46	43	21	19	16	15.5	12.5	12.5	12.5	12
47	37.5	24	19.5	18.5	17	15	13	13.5	13
48	25	20	17	16	14	13.5	12.5	12	12
49	42.5	18.5	15	14	12.5	12.5	12	12.5	12
50	35	21.5	18	15.5	14.5	13.5	13	13	13
51	36	18.5	15.5	14.5	14.5	11	11	11	11
52	21	15.5	14	14	13.5	12.5	11.5	11.5	11.5
53	42	25.5	21.5	18	16	14	13.5	13.5	13.5
54	53	23.5	19.5	17.5	14.5	12.5	12.5	12.5	14
55	> 60	39	25.5	19	16.5	13.5	13	13	13.5

TABLE 15. Part (b)

Clotting times using 100×10^6 platelets.

Case No.	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
	2	4	6	8	10	15	20	25	30
1	36	22	18.5	18	16.5	14.5	15	15.5	15.5
2	38	19	17	16	16.5	16	15.5	15.5	16
3	23	14	13	13.5	14	14.5	16	16	16.5
4	29	16	14.5	14.5	14	14.5	15	16	16
5	30	16.5	15	15	15	14	14.5	15.5	16
6	43.5	21.5	17	14.5	16	15.5	16	15.5	16.5
7	43.5	23.5	18.5	18	16.5	15	14.5	15	15
8	28.5	17	15	15	15.5	15	15.5	15.5	15.5
9	32	24.5	20	18	17	15.5	15	15	15
10	46	29	21	18.5	17	16	15.5	15.5	15.5
11	46	22	17	16	15.5	15.5	16	17	16

TABLE 15. Part (b) cont'd.

Case No.	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
	2	4	6	8	10	15	20	25	30
12	32	16.5	16.5	16	15.5	15	15.5	14.5	14.5
13	30	17	16	16	14.5	15.5	15.5	15.5	15.5
14	55	24	18	15.5	16	14.5	15	14.5	14.5
15	50.5	20	17	15	15	14.5	15	15	15
16	35	16	14	13.5	13	14	13.5	14	15
17	29.5	16	16	15	15	15.5	15	14.5	14.5
18	53	17	15.5	15	13	13	13	13.5	13.5
19	60	28	18	17	15.5	15	15	15.5	15.5
20	38.5	19	16.5	15	14.5	14.5	14.5	14.5	14.5
21	51	19.5	18	15.5	16	15	15.5	16	15
22	35	21	18	17	16	15	14.5	14.5	14.5

TABLE 15. Part (c)

Clotting times using 50×10^6 platelets.

Case No.	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
	2	4	6	8	10	15	20	25	30
1	-	22	-	-	19	19	20.5	20	19
2	-	22.5	-	-	19	19	20	22	19
3	44	18	15.5	16	16.5	16	16.5	17.5	20
4	> 60	48	37.5	27	22.5	18	18.5	23	23
5	> 60	40	30	23	20	19.5	19.5	22	23
6	47.5	21	16.5	16	16.5	16.5	17.5	19	21
7	> 60	43.5	29	24.5	22	18	18	18.5	18.5
8	57	28	24	20.5	16	16.5	16.5	17	18
9	59	39	27	23	21	18.5	19	19	19.5
10	58	40	30.5	22	21.5	19	18.5	18.5	19
11	55	30	21	18	17	17	18	17	17.5

TABLE 15. Part (c) cont'd.

Case No.	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
	2	4	6	8	10	15	20	25	30
12	53.5	25	19	18.5	18	18	18	18	19
13	59	44	25.5	22	21	19	19.5	18.5	19
14	39.5	18.5	17	16	16	16.5	15.5	16	16
15	47	28	20	20	17.5	17.5	17.5	17.5	19
16	58	39	24.5	18.5	17.5	17	16.5	16.5	17
17	61	41	30	21	19.5	17.5	17.5	17.5	16.5
18	46	19	17	15	15	15.5	15	15.5	16
19	43	34	29	24.5	21	20	17.5	17.5	16.5
20	50	22	17	16	16.5	16	16.5	17	17
21	54	52	40	28	23.5	21	19	19.5	19
22	46	34	24	21	20	18.5	18.5	18	18.5

be concluded that the substrate clotting times in the present system must have been dependent solely on the formation of thromboplastin.

Following the results of the last 3 experiments then, attention was re-directed to the establishment of a range of variation in the estimation of thromboplastic function in normal platelets. The revised technique, outlined in experiment II was used, and all platelet concentrates were gently washed with saline, the aim being to dilute any entrapped plasma, rather than actively wash platelets.

Experiment I5. To establish a range of normal variation in platelet thromboplastic function, using a constant preparation of brain residue and separate samples of serum for each estimation.

Reagents and Technique: The one preparation of acetone-treated human brain was used to prepare several batches of residue at the one session. The individual preparations were pooled and thoroughly mixed to provide a large quantity of a constant residue to be used throughout this experiment. Serum collected from a different normal donor and prepared in the standard way, was used with each estimation. Platelets from 55 normal donors were tested in the way formerly outlined. All 55 subjects had estimations performed on concentrates of $200xI0^6$ platelets, while in 22, additional estimates were made using $I00xI0^6$ and $50xI0^6$ platelets. The results are shown in Table I5.

Results: When $200xI0^6$ platelets were used the minimum substrate clotting times ranged between II and I3.5 seconds with a mode of I2 seconds and a mean of I2.3 seconds and a standard deviation of 0.53 seconds. In the 22 subjects where further estimations were made the range of substrate clotting times obtained with $I00xI0^6$ platelets was I3-I5.5 seconds and when $50xI0^6$ platelets were used I6-I9.5 seconds.

Discussion: A similar series had been tested earlier using a

somewhat different technique, and before the stability of brain residue had been critically studied. In the earlier group, when platelet concentrations of $200xI0^6$ platelets from 33 normal donors were examined, the range of minimum substrate clotting times was 9.5-I6 seconds. This range in the second survey, using comparable platelet numbers was considerably reduced, an improvement attributed to 3 factors: improved methods of storage of brain residue with attendant stability; an improved technical procedure; and increasing technical ability of the author. Using these figures obtained during 55 tests using $200xI0^6$ platelets each time the standard deviation was 0.53 seconds. One could expect therefore, that in most normal subjects, using this particular preparation of brain residue and $200xI0^6$ platelets most minimum substrate clotting times would fall in the range I2.3±0.53 seconds, and that 95 percent would be within I2.3±I.06 seconds. It was apparent however, that independent of any range, the degree of substrate clotting times was dependent on factors other than platelets, and in particular, on the activity of brain residue in current use. To demonstrate this point a further survey was undertaken using an entirely new preparation of brain residue. This will be described as experiment I6.

Experiment I6. To establish a range of normal variation in platelet thromboplastic function in a further series of normal subjects using a constant preparation of brain residue and separate samples of serum for each estimation.

Reagents and Technique: As in the previous experiment a large batch of brain residue was prepared and maintained constant throughout the series. The basic acetone-treated brain used initially was different from that used in experiment I5. Again separate specimens of serum, prepared from different normal donors were used for each estimation. The technique was the

TABLE 16. A range of variation in substrate clotting times obtained with normal platelets in combination with a constant preparation of brain residue and individual samples of serum.

Test Number	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS					
	4	10	15	20	25	30
1	16	9	9	9	9	9
2	15	10	9.5	9.5	9.5	9.5
3	19	12	11.5	11.5	11.5	11.5
4	15.5	9	9.5	9.5	9.5	9.5
5	17	10.5	10	10	10	10
6	12.5	9	9	9	9	9
7	18	10.5	10	10	10	10
8	13	9	9	9	9	9
9	12.5	9.5	9.5	9.5	9.5	9.5
10	13	9.5	9.5	9.5	9.5	9.5
11	16.5	10	10	10	10	10
12	13	9	9	9	9	9
13	15.5	10.5	10.5	10.5	10.5	11
14	12	9.5	10	10	10	10
15	18	11	10.5	10.5	10.5	11
16	12	10	10	10	10	10
17	13	9	9	9	9	9
18	14	10	10	10	10	11
19	12	9.5	9.5	9.5	9.5	9.5
20	11	9.5	9	9	9	9.5
21	17	10.5	9.5	9.5	9.5	9.5
22	16	10	10	10	10	10
23	17	10.5	10.5	10.5	10.5	10.5
24	15.5	10.5	10	10	10	10
25	20.5	11	10	10	10	10

TABLE 16. (cont'd)

Test Number	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS					
	4	10	15	20	25	30
26	17.5	11.5	10	10	10	10
27	15.5	10.5	9.5	9.5	9.5	9.5
28	15.5	10.5	9.5	9.5	9.5	9.5
29	15	11	9.5	9.5	9.5	9.5
30	16	11.5	10.5	10	10.5	10
31	14.5	10	10	10	10	10.5
32	15	10.5	10	10	10.5	11
33	20	14	11	11	11	11
34	15	11	11	11	11	11
35	14.5	10	10	10	10	10
36	13	9.5	10	10	10	10
37	16	11	11	11	11	12
38	13	10	10	10	10.5	10.5
39	14.5	10.5	10	10	10	10
40	15	11	11	11	11	11
41	15	9.5	9.5	9.5	9.5	9.5
42	17.5	11.5	10.5	10.5	10.5	10.5
43	21	13	11	11	11	11
44	17.5	10.5	10.5	10.5	10.5	10.5
45	20.5	10.5	10.5	10.5	10.5	10.5
46	13	9.5	9.5	9.5	9.5	9.5
47	19	10.5	10.5	10.5	10.5	10.5
48	18	13.5	11.5	11.5	11.5	11.5
49	15.5	10.5	9.5	9.5	9.5	9.5
50	20.5	11	11	11	11	11

same as for experiment I5, save that concentrations of $200xI0^6$ platelets only were tested, and these from 50 normal donors. The results in the order in which the tests were performed are shown in Table I6.

Results: The range of minimum substrate clotting times in this series was 9-II.5 seconds with a mode of I0 seconds and a mean of I0.0I seconds. The standard deviation was 0.65 seconds.

Discussion: Although the minimum substrate clotting times in this series were much faster than those found in experiment I5, the range of variation was almost identical in both experiments, when comparable numbers of cases had been examined. The two experiments differed only in the batch of residue used, and one could not but conclude that the degree of substrate clotting times, was dependant on the activity of individual preparations of brain residue. In this second group of tests the standard deviation was 0.65 seconds, and the minimum substrate clotting times of most normal individuals tested with this brain preparation should fall within the range $I0.0I \pm 0.65$ seconds, while 95 percent of cases would have minimum substrate clotting times in the range $I0.0I \pm I.3$ seconds. These figures, then, agreed very well with those of experiment I5.

In similar vein, two further experiments were undertaken to determine whether the range of normal variation was influenced to any great extent by the serum used. The first of these was experiment I7.

Experiment I7. To establish a range of variation of platelet thromboplastic function in a series of normal individuals using a constant preparation of brain residue and samples of pooled human serum for each estimation.

Reagents and Technique: The brain residue used, was kept constant throughout and was the same as that used in experiment I6. The technique was the same as that used in experiment I6, save that

TABLE 17. A range of variation in substrate clotting times obtained with normal platelets in combination with a constant preparation of brain residue and samples of pooled serum.

Test Number	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS					
	4	10	15	20	25	30
1	18	10	9.5	9	9	9
2	19.5	12	10	10	10	10
3	20.5	12.5	11	11.5	11.5	11.5
4	12.5	9.5	9.5	9.5	9.5	10
5	15	10	9	9	9	9
6	14	10.5	10.5	10.5	10.5	10.5
7	18	9.5	9	9	9	9
8	12.5	9	8.5	8.5	8.5	8.5
9	12.5	9.5	9.5	9.5	9.5	9.5
10	13	9.5	9.5	9.5	9.5	9.5
11	16	10	10	10	10	10
12	13	9	9	9	9	9
13	15.5	10.5	10.5	10.5	10.5	11
14	12	9.5	10	10	10	10
15	18	11	10.5	10.5	10.5	11
16	12	10	10	10	10	10
17	13	9	9	9	9	9
18	14	10	10	10	10	11
19	12	9.5	9.5	9.5	9.5	9.5
20	11	9.5	9	9	9	9.5
21	14.5	11	10.5	10.5	10.5	11
22	11.5	9	9.5	9.5	9.5	10
23	16	12	11	11	11	11
24	14.5	10.5	10.5	10	10.5	10.5
25	14	10	10	10	10	10.5

TABLE 17. (cont'd)

Test Number	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS					
	4	10	15	20	25	30
26	12	9	9	9	9	9
27	15	10.5	10	10	10	11
28	11	9	9	9	9	10
29	15	10.5	10.5	10.5	10.5	10.5
30	19	11	11	11	11	11.5
31	13	9	9	9	9	10
32	14.5	10	9	9	10	10
33	17.5	11	10.5	10	10	10
34	13	10	10	10	10	10
35	17.5	10	10	10	10	10
36	13	9	9	9	9	9
37	17.5	11.5	11	11	11	12
38	18	12	10.5	10	10	10
39	13.5	9.5	9.5	9.5	9.5	9.5
40	12	10	9	9	9	9
41	15.5	11	10.5	10.5	10.5	10.5
42	12.5	10	10.5	10.5	10.5	10.5
43	13	11	10.5	10.5	10.5	10.5
44	15	11.5	11	11	11	11
45	18	11.5	10.5	10.5	10.5	10.5
46	12.5	11	10.5	10.5	10.5	10.5
47	11.5	10	10	10	10	10.5
48	12	9.5	9.5	9.5	9.5	9.5
49	19.5	10.5	10.5	10.5	10.5	10.5
50	14.5	10	10	10	10	10

for each estimation, samples of serum prepared in the standard way from 2 or 3 normal donors were pooled prior to use. Platelet concentrations of 200×10^6 platelets collected from 50 normal donors were tested. The results are shown in Table I7.

Results: In this series the range of minimum substrate clotting times was 8.5-II.5 seconds with a mean of 9.9 and a mode of I0 seconds. The standard deviation was 0.69 seconds.

Discussion: In this group although the range of minimum substrate clotting times was slightly greater than the other series, the increased range was not more than 0.5 seconds and the standard deviation, 0.69 seconds, was not vastly different from that found in experiments I5 and I6. It could be expected therefore, that under the conditions of this experiment, 95 percent of normal platelets would give minimum substrate clotting times in the range $9.9 \pm$ I.38 seconds. It was possible to say therefore, that the range of minimum substrate clotting times obtained with a constant preparation of brain residue and constant numbers of platelets, was not affected significantly whether pooled human serum or separate, individual samples of serum were used for the estimations.

In a final experiment in this group the range of variation in substrate clotting times obtained with normal platelets was determined using a constant preparation of brain residue and the one preparation of serum collected from a single normal donor.

Experiment I8. To establish a range of normal variation of platelet thromboplastic function using a constant preparation of brain residue and a constant serum preparation.

Reagents and Technique: As for the last 3 experiments a batch of brain residue was prepared and maintained constant throughout this experiment. Ideally fresh samples of serum should have been collected from the same donor prior to each estimation, but, as it was intended to survey a large population of normal individuals

TABLE 18. A range of variation in substrate clotting times obtained with normal platelets, a constant preparation of brain residue and a constant serum preparation.

Case Number	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS								
	2	4	6	8	10	15	20	25	30
1	20	13	11.5	10.5	9.5	9.5	10	9.5	9.5
2	18	12.5	10.5	10	9.5	9.5	9.5	9.5	9.5
3	18	12.5	10.5	10	9.5	9.5	9.5	9.5	9.5
4	22	16	13	12	10	9.5	9.5	9.5	9.5
5		12			9.5	9.5	9.5	9.5	9.5
6	27.5	12.5	11.5	10.5	10	9.5	9.5	9.5	9.5
7	26	14.5	11	10	10	9.5	9.5	9.5	9.5
8	31.5	15.5	12.5	11.5	11	10	9.5	9.5	9.5
9	24.5	13.5	11	11	10	10	10.5	10.5	10.5
10	33	19.5	14.5	12	11	10.5	10.5	10.5	10.5
11	19.5	13.5	11.5	10.5	9.5	10	9.5	9.5	9.5
12	25.5	15.5	12.5	11	10.5	10.5	10.5	10.5	10.5
13	43	21	14	12.5	11	11	11	11.5	11
14	33.5	16.5	12.5	10.5	10	10	10	10	11

TABLE 18. (cont'd)

Case Number	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS								
	2	4	6	8	10	15	20	25	30
15	16	12.5	12	9.5	9.5	10	9.5	10	11
16	25	15.5	12.5	11	11	10.5	10.5	11	11.5
17	28.5	15.5	11.5	11	11	11	11	11	11
18	16	12	10.5	10	10	9.5	10	10	11
19	27	16	13	11	9.5	9.5	9.5	9.5	9.5
20	37	17	13	11.5	10.5	9.5	9.5	9.5	9.5
21	51.5	27	22	16.5	15.5	11.5	11.5	11.5	11.5
22	25	13	10.5	9	9.5	9.5	9.5	10	12
23	21	15	11.5	11	10.5	10.5	11	10.5	12
24	21.5	15.5	11.5	10.5	10.5	10.5	12	13	14
25	18.5	13	11.5	10.5	10	10	10	10	10
26	31	16	13	11.5	10	10	10	10	10
27	19	13.5	11.5	10.5	10.5	10.5	10.5	11.5	11.5
28	28	15.5	12	11.5	10.5	10.5	10.5	10.5	11.5
29	> 60	30	18.5	15	14	11.5	11.5	11.5	11.5

TABLE 18. (cont'd)

Case Number	Time of Subsampling In MINUTES Substrate Clotting Time In SECONDS								
	2	4	6	8	10	15	20	25	30
30	> 60	39	19	15	12	10.5	10.5	10.5	10.5
31	53	29	18.5	14.5	12.5	10.5	10.5	10.5	10.5
32	16	12	10.5	10	10	9.5	10	10	11
33	22	10.5	10	9.5	9.5	10.5	9.5	9.5	9.5
34	18.5	13	12	11	11	9.5	10	10	10
35	19	16	12	11	10	10	10	10.5	10

this ideal was manifestly difficult to attain. Instead of this procedure therefore, and since in experiment 9, it had been shown that frozen and thawed, stored serum was active in this system, a normal donor was bled of 50 ml. of blood, from which serum was prepared in the usual way, and stored frozen in small aliquots each of approximately 0.5 ml. On each day one of these stored preparations was thawed and warmed to 37°C. prior to use. Platelets prepared from 35 normal donors, chosen at random were tested in the usual way. The results are shown in Table I8.

Results: In this group the minimum substrate clotting times ranged from 9.5-II.5 seconds with a mean of I0.I seconds and a mode of 9.5 seconds. The standard deviation was 0.59 seconds.

Discussion: The range of minimum substrate clotting times was essentially the same as in the 3 earlier groups. The standard deviation was 0.59 seconds, and one could say that 95 percent of normal platelets, tested with this particular batch of brain residue and this one sample of serum, would have minimum substrate clotting times in the range of I0.I$\pm$I.I8 seconds.

Considering the last four experiments as a group, the standard deviation in each group was 0.53, 0.65, 0.69 and 0.59 seconds respectively. The degree of the minimum substrate clotting times in each group was determined to a major extent by the activity of the batch of brain residue being used, but, quite independant of the mean, the range of normal variation appeared to be unaffected by this activity and to be a function of platelets. These four experiments were conducted with great care, and the range of normal variation was remarkably constant in the four surveys, despite the fact that the samples of serum used in each experiment differed in their preparation. With normal platelets and a uniform preparation of brain residue the range of substrate clotting times did not vary to any marked extent whether separate individual samples of serum, samples of pooled serum, or the one specimen of serum was used for each estimation in a series. It was concluded, therefore, that serum played no significant part to determine the range of

TABLE 19. The effect of different preparations of serum in combination with a constant suspension of brain residue and a constant platelet preparation.

Sub-sampling time in minutes	Serum Preparation and Substrate Clotting Times In Seconds				
	1	2	3	4	5
4	23	24	23	16.5	15
10	12.5	12	13	10.5	10.5
15	10.5	10.5	11	10.5	10.5
20	10.5	10.5	11	10.5	10.5
25	11.5	10.5	11	10.5	10.5
30	10.5	10.5	11	10.5	10.5

normal platelet thromboplastic function as assessed by this technique, and that the range found in each of these experiments was a true reflection of normal variation in platelet thromboplastic function in a group of normal individuals assessed over a period of some weeks, together with variation that must be inherent in any technical procedure. In confirmation of the impression that serum as used, played no part in determining the degree of minimum substrate clotting times experiment I9 was performed.

Experiment I9. To determine the effect of different preparations of serum in combination with a constant preparation of brain residue and a single sample of normal platelets.

Reagents: I. A suspension of brain residue prepared and incubated as before.
2. Platelet "rich" plasma.
3. Five specimens of normal serum, collected from five normal donors chosen at random, and prepared in the standard way.
4. Normal platelet poor plasma as substrate.
5. Calcium chloride.

Technique: The usual procedure was repeated 5 times using 200×10^6 platelets prepared from the one sample of platelet "rich" plasma, and the one suspension of brain residue, but serum from a different donor in each instance. The results are shown in Table I9.

Results: In four tests the minimum substrate clotting time was 10.5 seconds and in the fifth 11 seconds.

Discussion: The results supported the concept that minimum substrate clotting times were not affected or determined to any extent by the activity of the particular preparation of serum used, for in these five tests the minimum substrate clotting times were identical in 4 and only 0.5 second longer in the

TABLE 20. The reproducibility of results.

Sub-sampling time in minutes	Test Number and Substrate Clotting Time In Seconds									
	1	2	3	4	5	6	7	8	9	10
4	15	13	14.5	13.5	12	12.5	14.5	10.5	13.5	12.5
10	10.5	10	10.5	10	10	10	10	9.5	10.5	10
15	10	10.5	10	10	10	9.5	10	9.5	10	10
20	10	10	10	10	10	9.5	10	9.5	10	10
25	10	10	10	10	10	9.5	10.5	9.5	10	10
30	10	10	10	10	10	9.5	10	9.5	10	10

fifth, despite the fact that 5 different samples of serum were used. It was possible that in this last instance the longer substrate clotting time might have been dependent on technical error. Further experiments to be described later in sections devoted to the isolation of the serum components of the reaction, and anti-thromboplastins confirmed the results of this experiment. Meanwhile experiment 20 demonstrated the reproducibility of the results obtained with this technique.

Experiment 20. To demonstrate the reproducibility of results by the present technique.

Reagents: All normal reagents prepared as for use in the standard technique.

Technique: The standard procedure was repeated 10 times using 200×10^6 platelets each time, all reagents being kept constant. The results are shown in Table 20.

Results: Minimum substrate clotting times of 10 seconds were obtained in 8 estimations, and 9.5 seconds in the remaining 2.

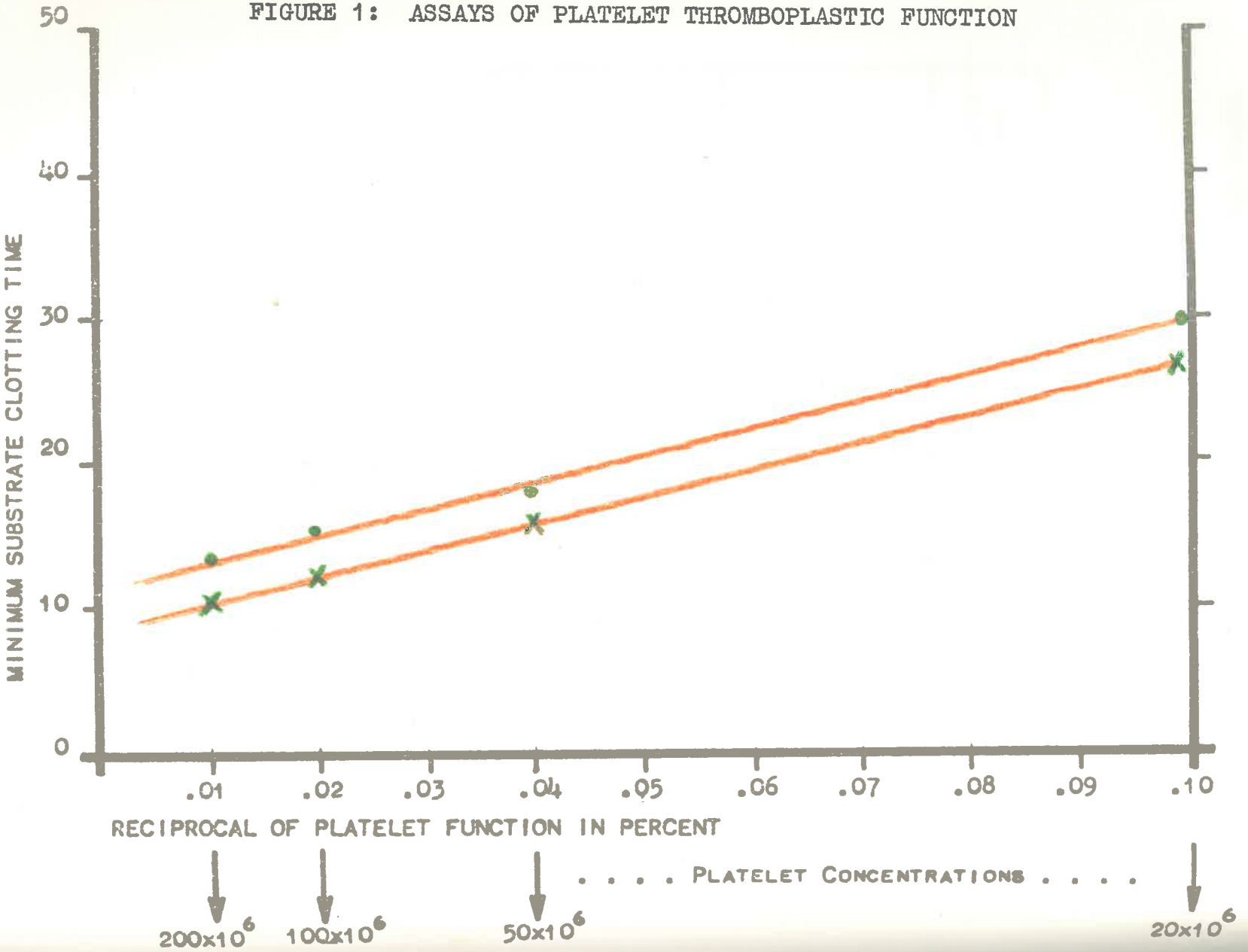
Discussion: The results show that estimations of platelet thromboplastic function by this method are eminently reproducible. Since all reagents were maintained constant throughout, the two estimations in which faster substrate clotting times were obtained must have reflected technical error. It is probable, however, that the difference of 0.5 second was an over-estimate for throughout this work, substrate clotting times have been given to the nearest 0.5 second only, and truer estimates may have been in the region of 9.75 seconds. Albeit small, on the basis of this series, one would expect that no matter how many times the estimates were repeated, ninety five percent of the substrate clotting times should fall in the range of 9.5-10.5 seconds, and most should be in the range of 9.75-10.25 seconds.

Meanwhile attention had been centred on the use of the technique described to assay platelet thromboplastic function using reducing numbers of normal platelets and constant volumes

TABLE 21a. First assays of platelet thromboplastic function.

Test Number	Platelet Count	Platelet Function (%)	Min. Substrate Clotting Time
1	200×10^6	100	12.5
	100×10^6	50	14.5
	50×10^6	25	17.0
	20×10^6	10	29.0
2	200×10^6	100	10.0
	100×10^6	50	12.0
	50×10^6	25	15.0
	20×10^6	10	26.0

FIGURE 1: ASSAYS OF PLATELET THROMBOPLASTIC FUNCTION



of all other reagents. The first of these experiments was experiment 2I.

Experiment 2I. Technique for the assay of platelet thromboplastic function.

Reagents: I. A suspension of brain residue prepared as before.
2. Platelet "rich" plasma obtained from 5 normal donors.
3. Normal serum prepared in the usual way.
4. Platelet "poor" plasma as substrate.
5. Calcium chloride.

Technique: Using each of the preparations of platelet "rich" plasma in turn, concentrates containing $200xI0^6$, $I00xI0^6$, $50xI0^6$ and $20xI0^6$ platelets respectively were prepared. Standard volumes of all the other reagents were added to each of these concentrates and the usual technique followed. After each of the five specimens of platelets had been tested, the minimum substrate clotting times obtained with each corresponding concentration were averaged to give a final result. On the following day the same procedure was followed using platelets obtained from another 5 normal donors, a different preparation of serum, and a preparation of brain residue known to be associated with faster substrate clotting times than that used on the previous day. Accepting, quite arbitrarily, the thromboplastic function of $200xI0^6$ platelets as equivalent to I00 percent, $I00xI0^6$ platelets equivalent to 50 percent function etc. graphs were drawn on arithmetic graph paper, wherein the reciprocal of the platelet thromboplastic function in percent, was recorded along the abscissa and the corresponding minimum substrate clotting time along the ordinate. The results of these two assays are shown in Table 2I(a) and graphically in Figure I.

Results: On the first day the average minimum substrate clotting

times obtained with concentrations of platelets representing 100%, 50%, 25% and 10% thromboplastic function were 12.5, 14.5, 17.0 and 29.0 seconds respectively, and on the second day 10, 12, 15 and 26 seconds. The two graphs drawn from these figures were both straight lines and very nearly parallel.

Discussion: These results were exciting and of fundamental importance for the production of two parallel calibration graphs when two preparations of brain residue of differing activities were used suggested that, quite independant of the degree of substrate clotting times obtained with normal platelets (a reflection of the inherent activity of the residue used) a constant relationship existed between the substrate clotting times obtained with any particular concentration of platelets and any subsequent concentration. That is to say, if two different preparations of brain residue were incubated with $200x10^6$ normal platelets, serum and calcium chloride, and when maximum thromboplastin generation was attained, the two mixtures caused a normal plasma substrate to clot in the presence of calcium chloride in x and y seconds respectively, the substrate clotting times obtained with $100x10^6$ and $50x10^6$ platelets in the same system would be $(x+2)$ and $(y+2)$ and $(x+5)$ and $(y+5)$ seconds respectively. Should this be so all calibration graphs obtained by the use of platelets and preparations of brain residue, no matter how the different preparations of the latter should vary in inherent activity, should be parallel. In like manner, different preparations of residue, of comparable activity should be associated with identical calibration graphs. For the reasons stated, and with the aid of the two graphs established in experiment 21, it was possible to predict on purely theoretical grounds, the clotting time to be expected with any lesser concentration of platelets once the clotting time obtained with $200x10^6$ platelets had been established by incubating such platelets with any unknown brain residue, serum and calcium chloride. For example, if a concentration of $200x10^6$ normal platelets in a given incubation mixture was accompanied by the generation of

TABLE 21 b. Theoretical and actual minimum substrate clotting times obtained in assays of platelet thromboplastic function.

Test Number	Theoretical or Actual Values	Platelet Concentration and Substrate Clotting Time In Seconds		
		200 x 10^6	100 x 10^6	50 x 10^6
1	Theoretical	9.5	11.25	14.75
	Experimental	9.5	12.0	14.5
2	Theoretical	10	11.75	15.25
	Experimental	10	12.0	15.0
3	Theoretical	10.5	12.25	15.75
	Experimental	10.5	12.5	15.0
4	Theoretical	11.0	12.75	16.25
	Experimental	11.0	12.5	16.0
5	Theoretical	11.5	13.25	16.75
	Experimental	-	-	-
6	Theoretical	12.0	13.75	17.25
	Experimental	12.0	14.0	17.0

TABLE 21 b. (cont'd)

Test Number	Theoretical or Actual Values	Platelet Concentration and Substrate Clotting Time In Seconds		
		200 x 10^6	100 x 10^6	50 x 10^6
7	Theoretical	12.5	14.25	17.75
	Experimental	12.5	14.5	18.25
8	Theoretical	13.0	14.75	18.25
	Experimental	13.0	15.0	18.0
9	Theoretical	13.5	15.25	18.75
	Experimental	13.5	15.0	18.5

thromboplastin capable of clotting a normal plasma substrate in II seconds at its maximum, then the substrate clotting times obtained in the same mixture with any lesser concentration of platelets should fall on a straight line drawn through the points I3 seconds (100×10^6 platelets) I6 seconds (50×10^6 platelets) and 26.5 seconds (20×10^6 platelets). Following these considerations a table was prepared in which the calculated substrate clotting times expected with concentrations of 200×10^6, 100×10^6, 50×10^6 (and 20×10^6) platelets were set down over the range of theoretical minimum substrate clotting times obtained with 200×10^6 platelets, from 9.5 - I3 seconds. Subsequently and over a period of months frequent assays were performed in the way outlined, using many different preparations of brain residue. Ultimately the figures obtained for successive assays having a common minimum substrate clotting time when 200×10^6 platelets were used were averaged to produce the "true" figures shown in table 2Ib. In this table the theoretical "expected" minimum substrate clotting times are given together with the average values obtained in practice. The latter figures in every case represent the average of at least 5 assays.

The most superficial consideration of these figures makes it plain that in every case the calculated and true minimum substrate clotting times were very close, and they would seem to prove beyond any doubt that the probable constant relationship between substrate clotting times and platelet numbers (or function) discussed earlier does in fact, hold true. Indeed, in the majority of single assays the minimum substrate clotting times obtained with 100×10^6 and 50×10^6 platelets were either identical with or close to the theoretical values expected from the minimum substrate clotting times obtained with the concentration 200×10^6, of the same platelets. The results obtained with 20×10^6 platelets or less in isolated assays have been less reliable. This is readily understood, however, if one appreciates that at these levels of platelet function, slight differences in function are

reflected in relatively great differences in minimum substrate clotting times. For example, the difference between 12.5 and 10 percent platelet function is accompanied by a difference of 4.5 seconds in minimum substrate clotting times. By contrast, at the other end of the scale a difference in minimum substrate clotting times of this magnitude would reflect differences in thromboplastic function of more than 50%, and in the case of normal platelets, this could only result from gross technical error.

On the basis of this work it was considered unnecessary to construct a new calibration graph every time an estimation of the thromboplastic function of possibly defective platelets was undertaken. The technique was proving so reliable and reproducible that one could compare any unknown estimation with a predetermined calibration graph. It seemed adequate to perform one or two assays with any new batch of residue as soon as it had been prepared and again during the period of its use to ensure that its behaviour was comparable to that of other batches of residue. The important question arose, however, as to what calibration graph should be used to assess the thromboplastic function of unknown platelets when a particular batch of residue was in use. It had been shown that with any batch of residue, a series of normal platelets tested over a period of time, produced a range of minimum substrate clotting times, and it was accepted that this range reflected variation in the thromboplastic function of normal platelets. This being so, it was reasonable to suppose that the thromboplastic function of any unknown preparation of platelets should be assessed from the calibration graph related to the mean substrate clotting time of the range obtained with any particular batch of residue. Closer consideration of the problem, however, revealed that this interpretation might be fallacious. In those experiments in which the range of variation was established, it was usually the case that one or two estimations were performed on any one day, in conjunction with other experiments, and the overall limits were determined over a period

of weeks. Although the same batch of residue was used throughout any one series, the suspension of residue used on any one day was prepared from individual small aliquots taken from the bulk residue immediately after its preparation. It was quite possible that while the gross deterioration of residue stored in bulk had been overcome by the revised method of storage, slight variations might be present in individual samples used for daily estimations, and that such variations might contribute to a range of normal variation found over a period of time. So too, external conditions, remote from the reagents themselves, might be active and exaggerate variation, independant of variation in the thromboplastic function of normal platelets. It is well established that a range of daily variation may be quite different from the range of variation found in a series of identical experiments conducted over a period of time. Accordingly experiment 22 was undertaken to determine whether daily variation in the present technique was different from the variation found over a more prolonged period of time. Ideally to clarify this issue, comparable numbers of platelet samples to those examined in the earlier series, should have been tested daily, but this was quite impossible because of the difficulties inherent in obtaining sufficient daily controls and because of the time required to prepare individual platelet concentrates and to perform each test. Alternatively, the procedure outlined below was substituted.

Experiment 22. To determine a range of daily variation in the estimation of platelet thromboplastic function of normal platelets.

Reagents: I. A suspension of brain residue prepared as before.
2. Platelet "rich" plasma prepared from 5 normal donors.
3. Serum prepared as before.
4. Normal platelet "poor" plasma as substrate.

TABLE 22. Daily variation in the thromboplastic function of normal platelets.

Group Number	Sub-sampling time in minutes	Test Number and Clotting Time In Seconds				
		1	2	3	4	5
1	4	23	21	23	24.5	23
	10	12.5	12.5	12.5	14	14
	15	12	12.5	12	12.5	12.5
	20	12	12.5	12	12.5	12.5
	25	12	12.5	12	14.5	12.5
	30	12	14	12	16	12.5
2	4	22.5	25	20.5	19	16
	10	12.5	13	12	12	11.5
	15	12	12.5	12	12	11.5
	20	12	12.5	12	12	11.5
	25	12	12.5	12.5	12	11.5
	30	12	12.5	12	12.5	11.5
3	4	16	17	24	20	25
	10	12	12	14	12.5	14
	15	12	12	12	12	12.5
	20	12	12	12	12	12.5
	25	12	12	12	12	12.5
	30	12	12.5	12	12	13
4	4	19	25	16	18.5	19.5
	10	12	15	12	13	12.5
	15	12	12.5	12	12	12
	20	12	12.5	12	12.5	12
	25	12	12.5	12	12.5	12.5
	30	12	12.5	12	12.5	12

TABLE 22. (cont'd)

Group Number	Sub-sampling time in minutes	Test Number and Clotting Time In Seconds				
		1	2	3	4	5
5	4	42	54	29	32	24.5
	10	17	20	15.5	12.5	12.5
	15	12	12.5	12	12	12.5
	20	12	12.5	12	12.5	12.5
	25	12	12.5	12	12.5	12.5
	30	12.5	12.5	12	12.5	13.5
6	4	32	28	33	29	35
	10	15	15.5	15.5	15	14.5
	15	11.5	12	12	12	12.5
	20	11.5	12	12	12.5	12
	25	11.5	12.5	12	12	12
	30	11.5	12	12	12	12
7	4	20.5	22.5	28.5	15.5	19
	10	12.5	13.5	12.5	12.5	12
	15	12	12	11.5	12	11.5
	20	12	12	11.5	12	11.5
	25	12	12	11.5	12.5	11.5
	30	12	12	11.5	13	11.5
8	4	35	17	41	30	25
	10	15	12	17	12.5	14
	15	12.5	12	12.5	12	12.5
	20	12.5	12	12.5	12	12.5
	25	12.5	12	12.5	12	12.5
	30	13	12.5	12.5	13	13

TABLE 22. (cont'd)

Group Number	Sub-sampling time in minutes	Test Number and Clotting Time In Seconds				
		1	2	3	4	5
9	4	33.5	34	19	24.5	20
	10	17.5	16.5	13.5	13.5	13.5
	15	13.5	16	13	13.5	13.5
	20	13.5	13.5	13	13.5	13.5
	25	13.5	13.5	13	13.5	13.5
	30	13.5	13.5	13.5	13.5	14
10	4	19	20	19	17.5	17.5
	10	13	13.5	13	13	13.5
	15	13	13.5	13	13	13.5
	20	13	13.5	13	13	13.5
	25	13	13.5	14	13	14
	30	13	14.5	14	13	14.5
11	4	36	25.5	30	25	32
	10	16.5	15	16.5	15.5	17
	15	14	14	14	14	15.5
	20	13.5	14	13.5	13.5	14
	25	13.5	14	13.5	13.5	14
	30	13.5	15	13.5	13.5	14
12	4	17.5	25	21	17	36
	10	13	15	13	13	15
	15	13	13	13	13	13
	20	13	13	13	13	13
	25	13	13	13	13	13
	30	13	13	14.5	13	13

5. Calcium chloride.

Technique: Using a constant suspension of brain residue and the same serum preparation on any one day, platelet concentrations of 200×10^6 normal platelets prepared from five normal donors were tested in turn using the standard technique. In all, 70 platelet preparations prepared from the same number of normal donors, chosen at random, were tested in groups of 5, different preparations of residue and serum being used for different groups. The results are shown in table 22.

Results: The standard deviation was calculated for each of these groups and in the order of the group as shown in table 22 was found to be 0.27, 0.35, 0.24, 0.27, 0.27, 0.22, 0.27, 0.27, 0.24, 0.27, 0.27, 0.0, 0.14 and 0.0 seconds respectively.

Discussion: While the number of tests in each of these groups was small, the results suggested that for practical purposes the daily range of variation in platelet thromboplastic function of normal platelets was considerably smaller than the variation found over a period of weeks. Indeed, in the whole series the standard deviation was approximately one half of that found in the earlier but larger groups. One could anticipate therefore, that on any one day using constant preparations of residue and serum, that of the minimum substrate clotting times obtained with a series of preparations of normal platelets, most would fall in the range of the mean $\pm$ (approximately) 0.25 seconds, and that 95 percent would be in the range of the mean $\pm$ 0.5 second. Since minimum substrate clotting times were recorded to the nearest 0.5 second only, for practical purposes, the range of normal daily variation could be accepted as the actual recorded time $\pm$ 0.5 second. The range of daily variation thus derived was somewhat less than half the range of variation found in a more protracted period of time.

It was decided then, that in determinations of the relative function of pathological platelets, it would be more accurate to obtain one's assessment from the calibration graph pertinent to

TABLE 22a. Accuracy of the technique described as applied in the investigation of possible coagulation defects.

Test No.	Clinical Notes	Platelet Count	Test or Control	Platelet Function Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
				4	10	15	20	25	30
1 E.G.	Acute hyperthyroidism. Hepatosplenomegaly. Positive Hess test.	220×10^3	Cont. 1	31	15	13	11	11	11
			Test	34	14	12.5	11	11	11
2 D.G.	Scurvy - extensive purpura.	280×10^3	Control	24	12.5	11	11	11	11
			Patient	24	12.5	11	11	11	11
3 P.T.	Alcoholic cirrhosis. Haematemesis.	660×10^3	Control	31	15	13	11	11	11
			Patient	30	15	13	11	11	11
4 H.J.	Asthmatic - haematuria Slight bruising tendency.	140×10^3	Control	16.5	11.5	9.5	9	9.5	9.5
			Patient	16.5	12	9.5	9.5	9.5	9.5
5 H.R.	Severe asthmatic. Spontaneous bruising.	160×10^3	Control	31	12	11	11	11	11
			Patient	30	13	11	11	11	11

TABLE 22a. Cont'd.

Test No.	Clinical Notes	Platelet Count	Test or Control	Platelet Function Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
				4	10	15	20	25	30
6 H.W.	C.V.A. - bruises easily.	$104x10^3$	Control	17	10.5	10	10	10	10
			Patient	19	11	10.5	10	10	10
7 M.L.	Recurrent thromboses.	$260x10^3$	Control	25	15	13	12	12	12
			Patient	23	14	13	12	12	12
8 H.P.	Epistaxis. Slightly +ve Hess test.	$190x10^3$	Control	15.5	10.5	10.5	10.5	10.5	11
			Patient	17	10.5	10.5	10.5	10.5	10.5
9 G.F.	Recovering from acute maturation arrest.	$100x10^3$	Cont. 1	20	12	10.5	10.5	10.5	10.5
			Cont. 2	22	11	10.5	10.5	10.5	10.5
			Patient	26	11	10.5	10.5	10.5	11
10 E.McC.	Pancytopaenia - hepatosplenomegaly.	$124x10^3$	Control	18.5	10.5	10	10	10	10.5
			Patient	24	12	10	10	10	11

TABLE 22a. Cont'd.

Test No.	Clinical Notes	Platelet Count	Test or Control	Platelet Function Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
				4	10	15	20	25	30
11 A.C.	Cirrhosis, C.C.F., diabetes, thrombocytopaenia - no haemorrhagic signs.	$26x10^3$	Control	29	12	11	11	11	11
			Patient	27	12	11	11	11	11
12 B.M.	Family history of bleeding. For tonsillectomy.	$372x10^3$	Control	30	14.5	12	11	11	11
			Patient	27	14	11.5	11	11	11
13 L.P.C.	Bruising tendency throughout life.	$200x10^3$	Control	24	13	12	11	11	11
			Patient	21	12	11.5	11	11	11
14 R.G.	Prolonged bleeding after dental extraction.	$260x10^3$	Control	19	12	10	10	10	10
			Patient	21	12	10.5	10	10	10
15 B.W.	Bruises readily on slight trauma.	$282x10^3$	Control	31	13	10.5	10.5	10.5	10.5
			Patient	22	12.5	10.5	10.5	10.5	10.5

TABLE 22a. Cont'd.

Test No.	Clinical Notes	Platelet Count	Test or Control	Platelet Function Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
				4	10	15	20	25	30
16 B.H.	Recurrent epistaxes.	$400x10^3$	Control	20	12	10.5	10.5	10.5	10.5
			Patient	22	11	10.5	10.5	10.5	10.5
17 V.Z.	Family history of congenital thrombo-cytopaenia.	$260x10^3$	Control	30	17	14.5	13	13	13
			Patient	34	17	14.5	14.5	13	13
18 C.A.	3 years after acute I.T.P. No haemorrhagic signs.	$210x10^3$	Control	14	10	10	10	10	10
			Patient	11	10	10	10	10	11
19 D.C.	9 months after acute I.T.P. No haemorrhagic signs.	$140x10^3$	Control	12.5	10	9.5	9.5	9.5	9.5
			Patient	14.5	10	9.5	9.5	9.5	9.5
20 E.L.	Chronic lymphatic leukaemia. No haemorrhagic signs.	$110x10^3$	Control	12.5	10	9.5	9.5	9.5	9.5
			Patient	15.5	11	10	9.5	9.5	9.5

TABLE 22a. Cont'd.

Test No.	Clinical Notes	Platelet Count	Test or Control	Platelet Function Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
				4	10	15	20	25	30
21 W.	Family history of bleeding.	$268x10^3$	Control	15	9.5	9.5	9.5	9.5	9.5
			Patient	12.5	9.5	9.5	9.5	9.5	9.5
22 B.H.	Thrombocytopaenia in course. Infectious mononucleosis. No haemorrhagic signs.	$50x10^3$	Control	22	13	12	12	11.5	12
			Patient	16.5	12	12	12	12	12
23 R.F.	Multiple myelomatosis, thrombocytopaenia. No haemorrhagic signs.	$60x10^3$	Control	17	12.5	12	12	11.5	12
			Patient	14.5	13	12	12.5	12	12
24 L.E.	Chronic lymphatic leukaemia. No haemorrhagic signs.	$234x10^3$	Control	17.5	12.5	12	12	11.5	12
			Patient	22	14	11.5	12	12	12.5
25 V.R.	Reticulosis. No haemorrhagic signs.	$30x10^3$	Control	30	14	12	12	13	12.5
			Patient	16	12.5	12	12	11.5	12.5

TABLE 22a. Cont'd.

Test No.	Clinical Notes	Platelet Count	Test or Control	Platelet Function Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
				4	10	15	20	25	30
26 C.O.	6 months after acute I.T.P. No haemorrhagic signs.	$116x10^3$	Control Patient	37 20	21 16.5	15 14.5	13.5 13.5	13.5 13.5	13.5 13.5
27 D.A.	C.C.F. Spontaneous bruising.	$210x10^3$	Control Patient	16.5 20.5	12.5 15.5	12 13	12 13	12.5 12	12 12
28 V.W.	Chronic myeloid leukaemia in remission.	$300x10^3$	Cont. 1 Cont. 2 Cont. 3 Patient	12.5 14.5 15.5 12.5	10 10 10 9.5	9.5 9.5 10 10	9.5 9.5 9.5 9.5	9.5 9.5 9.5 9.5	9.5 9.5 9.5 9.5
29	Chronic thrombocyto-paenia. No haemorrhagic signs.	$60x10^3$	Control Patient	22 32	11.5 13	11 11.5	11 11	11 11	11 11
30 H.S.	Family history of bleeding following dental extraction.	$300x10^3$	Control Test	16.5 16.5	12 12	10.5 10.5	10.5 10.5	10.5 10.5	10.5 10.5

the minimum substrate clotting time obtained with a control experiment with normal platelets on each day rather than the calibration graph of the mean substrate clotting time of the wider range of variation found in a series of tests conducted over a period of time. In this way potential errors due to variation in activity in small individual samples of residue taken from a bulk preparation and errors due to external and perhaps unknown factors would be minimised. Thus, if as discussed earlier, one had ascertained that the activity of the residue being used was the same as prior preparations, all that seemed essential in daily estimations was to test concentrations of 200×10^6 platelets from 2 to 3 normal donors to ensure that the range of daily variation was within accepted limits and to gauge the relative function of the unknown from the appropriate calibration graph pertinent to the mean substrate clotting time of the control tests. In actual practice it has been found unnecessary to test more than one concentration of normal platelets on any one day, for the technique has proved so constant that, the results obtained with a single daily control have proved eminently satisfactory. Table 22a is included to support this contention. In this table are shown the actual figures obtained in 30 estimates of platelet thromboplastic function in 30 patients referred to the laboratory for investigation of a possible bleeding tendency. Routinely the investigation of these subjects included estimations of the bleeding time, clotting time, Hess test, one-stage prothrombin time, the screening test for thromboplastin generation, whole blood platelet count, observations of clot retraction and for any evidence of excess fibrinolytic activity. Irrespective of the findings in each of the subjects in this table, estimations of platelet thromboplastic function were performed. The results are virtually self-explanatory and leave no doubt either of the accuracy of the method when performed with all due care and of the narrow limits of variation of thromboplastic function in samples of normal platelets. Further,

since this technique has been adopted elsewhere, other workers have confirmed the institution of assessing relative platelet function from the daily normal as eminently satisfactory.

Part II. Identification of the factors in serum and brain residue participating in the reaction.

Section I. Serum factors.

This problem was approached through the use of sera lacking specific factors as substitutes for normal serum in the standard technique. The results of the earliest relevant experiments suggested that it might be possible to modify the basic reaction to assay factor X. Later experiments directed towards this aim showed that some of the earlier conclusions were incomplete and to fully clarify the function of the "serum" factors it has been necessary to include some of these later experiments in this section rather than in their original place in that section describing attempts to assay factor X.

At the outset a serum specifically lacking factor VII as a mild congenital deficiency, and a second deficient solely in factor IX were available for testing, while there was an unlimited supply of sera to be obtained from patients having routine anti-coagulant (Dindevan) therapy, and presumed to be deficient in factors VII and X, and, possibly, in some cases, factor IX. Tests using each of these sera are considered collectively as experiment 23, together with similar tests conducted at a later stage using aluminium hydroxide treated normal serum (which should retain P.T.A. and Hageman factor only) and aluminium hydroxide treated normal plasma (which should retain factors V and VIII, P.T.A. and Hageman factor). The individual sera lacking either factor VII or factor IX were tested 5 times on successive days, but in the case of the other reagents listed above, specimens from 5 different individuals were studied in each case.

Experiment 23. To determine the effects of substituting serum lacking specific coagulation factors for normal

TABLE 23. To show the effects of substituting reagents lacking specific coagulation factors for normal serum in the reaction.

Part (a). Factor IX deficient serum.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
1	Control	16.5	14.5	12	12	12	12
	Test	18	14.5	12	12	12	12
2	Control	16	12.5	12.5	12.5	13	13
	Test	18.5	14	12.5	12.5	13.5	13.5
3	Control	16.5	14.5	13	13	13	12
	Test	13.5	14.5	14	14	15	16
4	Control	17	14.5	12.5	12.5	13	12.5
	Test	16	13	13	12	12	12
5	Control	17	15.5	13.5	13.5	13	13
	Test	16	14	13.5	13	13.5	14

TABLE 23. Cont'd.

Part (b). Factor VII deficient serum.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
1	Control	16.5	14.5	12	12	12	12
	Test	13.5	11	11.5	12	12.5	12
2	Control	16	12.5	12.5	12.5	13	13
	Test	26	11.5	11.5	12.5	12.5	12.5
3	Control	16.5	14.5	13	13	13	12
	Test	14.5	11.5	11.5	11.5	11.5	12
4	Control	17	14.5	12.5	12.5	12.5	12.5
	Test	16	13	12.5	13	13	13.5
5	Control	17	15.5	13.5	13.5	13	13
	Test	14.5	10.5	11	11	12	11.5

TABLE 23. Cont'd.

Part (c). Dindevan serum.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
1	Control	24.5	14	13	13	12.5	12.5
	Test	34.5	32	31	30.5	27.5	26
2	Control	26.5	13.5	12.5	11.5	12	12
	Test	42	34	30.5	27.5	26	27
3	Control	24.5	14.5	13	11.5	12	12
	Test	45	31	29.5	27	27.5	25
4	Control	26	15	12.5	12	12	12
	Test	37.5	27.5	25.5	22.5	21	20.5
5	Control	33	15.5	13.5	13	12.5	12.5
	Test	49	35	34	28	31.5	29.5

TABLE 23. Cont'd.

Part (d). Aluminium hydroxide treated serum.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
1	Control	24.5	14.5	13	11.5	12	12
	Test	34	32	31.5	28	28	26
2	Control	26	15	12.5	12	12	12
	Test	41	31	30	31	28	26.5
3	Control	23	15.5	13.5	13	12.5	12.5
	Test	35	22	20	21	20	21
4	Control	28.5	16.5	13.5	13	12.5	12.5
	Test	36	34	35.5	33	31.5	29.5
5	Control	26	20.5	14.5	13.5	13.5	13.5
	Test	47	30	28.5	27.5	26	24

TABLE 23. Cont'd.

Part (e). Aluminium hydroxide treated plasma.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
1	Control	28	14	12	12	12	12
	Test	55.5	42	37	37.5	38.5	36
2	Control	19	14	12	12	12	12
	Test	52	44	40.5	39.5	36	33
3	Control	20	12.5	11.5	11.5	11.5	12.5
	Test	43	36	31	29	26.5	24.5
4	Control	16.5	12	10.5	10.5	10.5	10.5
	Test	44	39	38	32	32	31.5
5	Control	16.5	12	10.5	10.5	10.5	10.5
	Test	52	44	38.5	34.5	37	28

serum in the standard technique.

Reagents: I. All normal reagents prepared as for use in the standard technique.
2. Factor VII deficient serum.
3. Factor IX deficient serum.
4. Serum from patients having "Dindevan" therapy.
5. Aluminium hydroxide treated normal serum.
6. Aluminium hydroxide treated normal plasma.

Technique: In every instance a control experiment using all normal reagents was conducted parallel with the reagent to be tested. Subsequently the serum or reagent to be examined was substituted for the normal serum in the usual incubation mixture and the standard procedure followed. The results are shown in Table 23.

Results: When the sera lacking either factor VII or factor IX alone were tested, the minimum substrate clotting times were much the same as those obtained in the corresponding control tests. Thromboplastin generation was reduced in the presence of "Dindevan" serum, aluminium hydroxide treated normal serum and aluminium hydroxide treated normal plasma, however.

Discussion: Normal thromboplastin generation despite reduced concentrations of either factor VII or factor IX seemed to preclude both as essential reagents in the system being considered. In similar vein, while aluminium hydroxide treated normal serum and plasma both supplied P.T.A. and Hageman factor, thromboplastin formation was markedly impaired in the presence of these reagents. This suggested that these reagents were not essential or that if they were, other factors too must be concerned. The essential factor or factors therefore must have been removed by treating serum with aluminium hydroxide and it followed that either factor VII, IX or X must participate in the reaction. Other tests in this experiment tended to exclude the former two factors and it was concluded that only factor X was likely to be involved. This assumption received support from those tests

using "Dindevan" sera, for these certainly lacked both factors VII and X and their use was accompanied by impaired thromboplastin formation. Serum specifically lacking factor X was not available at this stage to confirm this concept but additional evidence was obtained shortly when 2 patients presented with bleeding manifestations, due in one case to a pure, albeit mild, deficiency of factor VII and in the other to a combined deficiency of factors VII and X. The relevant data concerning these patients is as follows.

M.F. a 23 year old female presented with protracted bleeding following tonsillectomy. T.C. a 40 year old male progressed to acute hepatic failure during infectious hepatitis. In both, the bleeding time, clotting time, tourniquet test, platelet count and clot retraction were normal. Neither showed abnormal fibrinolytic activity.

M.F. had a "prothrombin" time of 20 secs. (N - II seconds), a normal "Stypven" time and the screening test for thromboplastin generation was normal. The factor VII deficiency suggested by these results was confirmed by the inability of plasma specifically lacking factor VII to correct this patient's prolonged "prothrombin" time.

T.C. had a prolonged "prothrombin" time of 32 secs. (N - II seconds), a prolonged "Stypven" time of I5 secs. (N - 8.5 seconds) and an abnormal screening test. The full thromboplastin generation test was abnormal only when the patient's serum was used. A deficiency of factor IX was excluded when it was shown that the serum could correct the defect in serum specifically lacking this factor. A deficiency of factor X was thereby proved but an additional deficiency of factor VII was evident by the inability of the patient's plasma to correct the prolonged "prothrombin" time of a plasma specifically lacking factor VII or that of M.F. above.

Sera obtained from these 2 patients were used as outlined in

TABLE 24. To show the effects of serum deficient in factor VII and serum deficient in both factor VII and factor X when substituted for normal serum.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds										
		2	4	6	8	10	15	20	25	30	35	40
1	Control	53	23.5	19.5	17	14.5	12.5	12.5	12.5	14	15	17
	VII deficient.	> 60	26.5	16.5	13.5	12.5	12	12.5	12.5	12.5	13	14.5
2	Control	28	20	18	11	10.5	10.5	10.5	10.5	10.5	10.5	10.5
	VII & X deficient	44	42	39	33	27.5	26.5	24	23.5	22	22	20.5

Test No. 2 Cont'd.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						
		45	50	55	60	65	70	75
2	Control	12	13	-	-	-	-	-
	VII & X deficient	19.5	19	18.5	16.5	16.5	16.5	16.5

experiment 24.

Experiment 24. To determine the effect of substituting serum specifically lacking factor VII and a second lacking both factor VII and factor X for normal serum.

Reagents: I. All normal reagents prepared as before.

2. Serum obtained from M.F. above, specifically lacking factor VII and prepared in the standard way.

3. Serum lacking factors VII and X obtained from T.C. above. (Only a small volume of serum remained after the routine investigations described above had been completed, and since the patient died a few hours later, another specimen could not be obtained. The serum used in this case was not prepared in the usual way).

Technique: The technique used was the same as for experiment 23. The results are shown in Table 24.

Results: When factor VII deficient serum was used the minimum substrate clotting times obtained were the same as those in the control test and were reached after much the same period of incubation as in the control. With the doubly deficient serum, however, thromboplastin formation was both slowed and incomplete, the minimum substrate clotting time of 16.5 seconds being reached after 60 minutes incubation.

Discussion: These results supported the findings of the previous experiment which had indicated that factor X was likely to be the only serum factor participating in the reaction. In this experiment incubation of that mixture containing the doubly deficient serum was prolonged beyond the usual 30 minutes, further subsamples being taken at 5 minute intervals for a total of 75 minutes. The substrate clotting times continued to

shorten until, after 60 minutes incubation, the minimum of 16.5 seconds was reached and maintained. The slowed rate of thromboplastin formation in this instance was accepted as being in keeping with a function of factor X as an accelerator of thromboplastin formation, but the reason why thromboplastin formation should be "incomplete" even after prolonged incubation was less readily explained.

Meanwhile Dr. J.B. Graham of the University of North Carolina had generously forwarded a specimen of lypophilised serum from his patient Mr. Stuart in whom a deficiency of factor X had first been described. The conditions of collection and preparation of this specimen were not known, nor could one be certain that deterioration of other coagulation factors had not ensued in transit. Thus, while this serum could not be considered strictly comparable to the standardized serum normally used, it was indeed a useful and most essential reagent to be tested. Before any tests directly related to this work were undertaken, preliminary experiments were performed to confirm that the specimen was still deficient in factor X alone. It seems unnecessary to record these in detail and, suffice to say here, that once reconstituted in normal saline, the specimen forwarded by Dr. Graham was able to correct the defects in plasma or serum specifically lacking in factors VII and IX, but gave an abnormal result when substituted for normal serum in a full thromboplastin generation test. Furthermore, it was unable to correct the prolonged "Stypven" time (29 seconds, normal 10 seconds) of a plasma obtained from a patient having routine Dindevan therapy. These tests confirmed that the serum on arrival was still deficient in factor X only, and it was subsequently used as described in experiment 25.

Experiment 25. To determine the activity of serum specifically lacking factor X in the standard technique.

TABLE 25. To show the effect of serum specifically lacking factor X.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds										
		2	4	6	8	10	15	20	25	30	35	40
1	Control	24.5	13.5	11	11	10	10	10	10.5	10	-	-
	X deficient.	-	-	-	-	21.5	18.5	17.5	17	17	16.5	16.5

Test No. 1 Cont'd.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						
		50	60	75	90	105	120	
1	Control	-	-	-	-	-	-	
	X deficient.	16.5	16.5	16.5	16.5	17	18	

TABLE 25. Cont'd.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds										
		2	4	6	8	10	15	20	25	30	35	40
2	Control	24.5	13.5	11	11	10	10	10	10.5	10	-	-
	X deficient.	-	-	-	-	20.5	17.5	17	17.5	16	16.5	16

Test No. 2 Cont'd.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						
		50	60	75	90	105	120	
2	Control	-	-	-	-	-	-	
	X deficient.	16.5	16.5	16.5	16.5	17.5	19	

TABLE 25. Cont'd.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds										
		2	4	6	8	10	15	20	25	30	35	40
3	Control	33	19.5	14.5	12	11	10.5	10.5	10.5	10.5	10.5	-
	X deficient	-	29	-	-	23	21	19	18.5	18.5	18	18

Test No. 3 Cont'd.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		50	60	75	90	105	
3	Control	-	-	-	-	-	
	X deficient.	17.5	18	17.5	19	20	

TABLE 25. Cont'd.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds										
		2	4	6	8	10	15	20	25	30	35	40
4	Control	33	19.5	14.5	12	11	10.5	10.5	10.5	10.5	10.5	
	X deficient.	-	32.5	-	-	21	20	19.5	19	18	18	17

Test No. 4 Cont'd.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		50	60	75	90	105	
4	Control	-	-	-	-	-	
	X deficient.	17	17	17	18.5	20	

TABLE 25. Cont'd.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds										
		2	4	6	8	10	15	20	25	30	35	40
5	Control	19.5	13.5	11.5	10.5	9.5	9.5	10	9.5	9.5	10	-
	X deficient.	-	30	-	20.5	18	18	17	16.5	16.5	16.5	16.5

Test No. 5 Cont'd.

Test No.	Control or Test Serum	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						
		50	60	75	90	105	120	
5	Control	-	-	-	-	-	-	
	X deficient.	16.5	16.5	17	17	17.5	18.5	

Reagents: I. All normal reagents prepared as before.
2. Factor X deficient serum.

Technique: Using the standard procedure parallel experiments using, firstly, all normal reagents and, secondly, the test serum as a substitute for normal serum were conducted and repeated 5 times on three successive days. The results are shown in Table 25.

Results: In each of the 5 tests using the factor X deficient serum, thromboplastin generation was slowed and the minimum substrate clotting times once reached were always some seconds slower than those obtained with the corresponding control serum.

Discussion: These results were virtually identical with those obtained in experiment 24 when serum deficient in both factor VII and factor X was studied, and confirmed an essential role for factor X in promoting thromboplastin generation in a system containing a suspension of brain residue, platelets, serum and calcium chloride. Again, however, and as had been the case in the previous experiment, a disconcerting feature was the failure of the minimum substrate clotting times obtained with the test serum to approximate to those of the control mixtures even after more prolonged incubation. While it was possible that both the slowing of the reaction and this latter feature were both dependent on reduced factor X levels, other possibilities had to be considered. Among these, although unlikely, it was possible that another factor not detected by the present techniques was involved. A second explanation stemmed from the possibility that the thromboplastin formed might be unstable or that the substrate clotting times might reflect a state of equilibrium between thromboplastin formation and neutralization. In these circumstances a slower rate of thromboplastin formation consequent upon a reduced factor X concentration, might permit relatively greater breakdown, destruction or neutralization of thromboplastin before equilibrium was reached, with consequently slower minimum substrate clotting times. A third consideration was based on the fact that in both experiments 24 and 25 neither of the serum

TABLE 26. The effects of serially diluting normal serum.

Test No.	Serum Dilution	Time of Subsampling In Minutes Substrate Clotting Time In Seconds												
		4	10	15	20	25	30	35	40	50	60	70	80	90
1	Full strength	11.5	10	10	10	10	10.5	11	11.5	11.5	12	12	12	13
	1 in 2	12	9.5	9.5	9.5	9.5	9.5	10	9.5	9.5	11	10.5	10.5	11
	1 in 4	15	11	10	10	9.5	9.5	9.5	9.5	9.5	10	9.5	9.5	9.5
2	Full strength	14.5	10	10	10	10	10	10	10	10	10	10	9.5	10
	1 in 2	18	11.5	11	10	10	9	9	9	9.5	9.5	9	9	9
	1 in 4	26	15.5	12.5	11.5	11	10	10	10	9.5	9.5	9.5	9.5	9.5
3	Full strength	20	12	12	12	12	12.5	12	13	-	-	-	-	-
	1 in 2	29	15	12.5	12	11.5	11.5	11	11	11.5	11	11.5	11.5	11.5
	1 in 4	34.5	18.5	14.5	14	12.5	12.5	12	12	12.5	12	12	12	12

TABLE 26. Cont'd.

Test No.	Serum Dilution	Time of Subsampling In Minutes Substrate Clotting Time In Seconds												
		4	10	15	20	25	30	35	40	50	60	70	80	90
4	Full strength	17	11	11	11	11	11	12	-	-	-	-	-	-
	1 in 2	21.5	13	12	11	10.5	10	10	10	10	10.5	11	10.5	12
	1 in 4	29.5	21.5	18.5	16	14.5	13.5	12.5	12	11.5	11.5	11.5	11.5	11.5
5	Full strength	15	10.5	10.5	10.5	11	12	-	-	-	-	-	-	-
	1 in 2	21.5	15	13.5	12	11	11	11	11.5	11	11	11	11	12
	1 in 4	32.5	19	15.5	13	12	11.5	11.5	11.5	11	10.5	10.5	10.5	10.5

preparations associated with slower minimum substrate clotting times had been prepared under accepted standard conditions. It was not unreasonable to suppose that these 2 preparations retained factors capable of "neutralizing" relatively greater amounts of thromboplastin and thus cause slower minimum substrate clotting times. In an attempt to clarify some of these points experiment 26 was undertaken.

Experiment 26. To determine the effect of serial dilutions of normal serum on thromboplastin formation in the standard procedure.

Reagents: All normal reagents prepared as for use in the standard procedure.

Technique: Aliquots of normal serum prepared under standard conditions were diluted I in 2 and I in 4 with sterile normal saline. The standard procedure was followed using in turn 0.I ml. volumes of undiluted serum and the two dilutions. In each instance incubation and subsampling were prolonged for a total of 90 minutes. The results of 5 experiments are shown in Table 26.

Results: With each preparation of serum tested, the minimum substrate clotting times obtained did not differ significantly with dilution. On the other hand with increasing dilutions of serum the incubation times required before minimum substrate clotting times were reached increased progressively.

Discussion: These results suggested that the only function of serum in this reaction was to accelerate thromboplastin formation and that dilution of serum did not influence the ultimate strength of thromboplastin formed. Such an interpretation had to be reconciled with the results of experiments 24 and 25, wherein reduced thromboplastin formation followed the use of sera lacking factor X. Subsequently it was shown that serum incubated on the clot for periods of less than 6 hours retains an activity capable of neutralizing thromboplastin, but that this activity is reduced to negligible proportions after I8 hours incubation. This work

will be described later, but, at this stage suffice to say that it was thought that the anomalous results obtained in experiments 24 and 25 depended on such activity present in those sera which had not been prepared and incubated as prescribed.

Following these experiments it was assumed that only the factor X content of serum was necessary for the reaction to proceed and this phase of the work was discontinued in favour of other problems. After some months, and based on this conclusion, work on the "serum factor" was resumed in an attempt to modify the basic reaction to quantitate factor X.

From a consideration of the results of experiment 26 it was evident that since it was the rate rather than the degree of thromboplastin formation that was influenced by the concentration of factor X, any such technique would need to be based on the speed of maximum thromboplastin formation in otherwise comparable incubation mixtures containing appropriate dilutions of normal serum. It was thought that in such a way a calibration graph could be constructed by plotting the concentration of serum (i.e. the concentration of factor X) against the corresponding time interval required to reach maximum thromboplastin generation. The concentration of factor X in any unknown serum could be determined by referring the incubation time required to reach maximum thromboplastin generation in an identical system containing the unknown serum to such a graph. It was clear that the construction of calibration graphs in the way outlined would be a time consuming process, and in an attempt to minimise this feature, experiment 27 was undertaken. In this an attempt was made to define the optimum concentration of serum that might be used by assessing the effects of increasing the concentration of serum relative to the amounts of the other reagents in the standard procedure.

Experiment 27. To determine the effect of increasing the concentration of serum relative to the normal volumes of all other reagents.

TABLE 27. The effect of increased serum concentrations on thromboplastin formation.

Volumes Used.	Serum Concentration	Minimum Clotting Time	Incubation Time (Minutes)
0.8 ml.	400 percent	12 seconds	< 2 minutes
	200 percent	10 seconds	8 minutes
	100 percent	9.5 seconds	12 minutes
	80 percent	9.5 seconds	18 minutes
	40 percent	9.5 seconds	40 minutes
0.6 ml.	300 percent	8.5 seconds	< 2 minutes
	225 percent	8.5 seconds	8 minutes
	150 percent	9 seconds	8 minutes
	75 percent	9.5 seconds	28 minutes
	30 percent	10 seconds	46 minutes

TABLE 27. Cont'd.

Volumes Used.	Serum Concentration	Minimum Clotting Time	Incubation Time (Minutes)
0.4 ml.	200 percent	9.5 seconds	8 minutes
	100 percent	9.5 seconds	16 minutes
	40 percent	9.5 seconds	22 minutes
	20 percent	19 seconds	60 minutes
	10 percent	25 seconds	102 minutes

Reagents: All normal reagents prepared as before.
Technique: Normal serum was incubated at 37°C for 18 hours, separated from the clot and stored frozen in several 3cc. aliquots until used. In this and related experiments the aim was to determine not the degree of thromboplastin formation but the incubation time required to reach maximum thromboplastin formation. To do this accurately it seemed that more frequent subsamples would need to be taken from each incubation mixture and in anticipation of any prolonged test each mixture in this experiment contained twice the usual quantities of brain residue suspension, platelets and calcium chloride. On 3 successive days an aliquot of the same serum preparation was thawed and in turn 0.8 ml., 0.6 ml. and 0.4 ml. volumes of undiluted serum and appropriate dilutions contained in the same volume of saline were tested in combination with the other reagents. Subsamples were taken from each incubation mixture at 2 minute intervals until a minimum substrate clotting time had been reached and maintained. These times and the incubation times required to attain them were recorded. The results are shown in Table 27.
Results: For purposes of explanation, that volume of serum normally used in the standard procedure, 0.1 ml. (in combination with 1.0 ml. of a suspension of brain residue, 200×10^6 platelets and 1.0 ml. of calcium chloride) has been chosen as equivalent to 100 percent "serum" concentration. In Table 27 the concentrations of serum used in every test in this experiment are shown as percentages relative to this concentration. Thus when 0.8 ml. volumes of serum were used the concentrations of serum ranged from 400 to 40 percent, and with 0.6 ml. and 0.4 ml. volumes from 300 to 30, and 200 to 10 percent respectively. In addition to the relative concentration of serum the table shows the minimum substrate clotting times obtained with each concentration and the corresponding incubation times. When 0.8 ml. volumes of serum and its dilutions were tested the minimum substrate clotting times ranged from 12 to 10 seconds, the slowest time being associated with the highest serum concentration. Maximum thromboplastin

formation was so rapid when 0.8 ml. of undiluted serum was used that the minimum substrate clotting times were attained before the first subsample was taken at 2 minutes. Thereafter progressive dilution of serum necessitated increasingly longer incubation before maximum thromboplastin formation was reached. Similar results were obtained with 0.6 ml. volumes of serum. Again, with the most concentrated serum maximum thromboplastin formation was almost immediate and thereafter with succeeding dilutions more prolonged incubation was necessary to attain maximum thromboplastin formation. The range of minimum substrate clotting times was 8.5 - 10 seconds, but in these tests the minimum time obtained with the most concentrated serum was not the slowest. More dramatic changes were apparent when 0.4 ml. volumes of serum were used. Here, too, increasing dilutions of serum necessitated increasing periods of incubation to achieve maximum thromboplastin formation. Over a range of serum concentrations of 200 to 40 percent the minimum substrate clotting times did not vary, all being 9.5 seconds. At concentrations of 20 and 10 percent, however, there was a marked change, for minimum substrate clotting times of only 19 and 25 seconds respectively, were reached and maintained after prolonged incubation.

Discussion: In some essentials these results conformed to the pattern to be anticipated from earlier work, and supported the concept that an essential function of serum in this reaction was to accelerate thromboplastin formation. Indeed, this very function was a factor limiting the volume of serum that might be used in any modification of the basic reaction to assay factor X, for when the proportion of serum was increased 3 to 4 times that normally used, maximum thromboplastin formation was so rapid that normal subsampling, and, thereby, accurate timing, was precluded. The slower minimum substrate clotting time obtained with the most concentrated serum used in this experiment could be explained on the basis of other work contained in this thesis. In the latter work it has been shown that the usual volume of standardized

TABLE 27 (b). The effect of concentrations of serum in relation to minimum substrate clotting times.

Serum No.	Serum Concentration (Percent) Minimum Substrate Clotting Times In Seconds												
	300	200	190	180	170	160	150	140	120	110	100	90	80
1	-	10	-	-	-	-	-	-	-	-	10		
2	-	9.5	-	-	-	9.5	-	-	-	-	-	-	8.5
3	11	11	-	-	-	-	10.5	-	-	-	-	-	-
4	-	-	-	8.5	-	-	9.5	-	10.5	-	-	10	-
5	-	8.5	-	-	-	-	-	-	8.5	-	8.5	-	9
6	9.5	-	-	9.5	-	-	-	-	9.5	-	-	-	-
7	10.5	10.5	-	11.5	-	-	11.5	-	-	-	-	-	-
8	-	10.5	-	-	-	9.5	-	-	10.5	-	-	-	9
9	-	12	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	-	9	-	-	-	9	-	-
11	-	-	-	-	-	-	9.5	-	-	-	-	-	-

TABLE 27 (b). Cont'd.

Serum No.	Serum Concentration (Percent) Minimum Substrate Clotting Times In Seconds						
	70	60	50	40	30	20	10
1	-	-	9	-	12.5	18	-
2	-	-	-	9	-	15	-
3	-	10.5	-	-	11	-	-
4	-	9.5	-	-	10.5	-	-
5	-	9.5	-	9.5	-	12	-
6	-	9.5	-	-	10	-	-
7	11.5	-	-	12.5	-	-	20
8	-	-	-	10.5	-	13	20
9	-	-	-	12.5	-	-	20
10	-	10	-	-	-	15	-
11	9.5	9.5	-	9.5	-	11.5	-

serum (0.I ml.) contains negligible "inhibitory" activity. However, it can be appreciated that a four-fold increase in serum concentration could easily result in a detectable neutralization of thromboplastin and cause slower minimum substrate clotting times. Taking the experiment as a whole but excluding this one test, the range of minimum substrate clotting times evident with serum concentrations corresponding to 300 - 30 percent of that normally used, was quite small being 8.5 to I0 seconds. It could be said that in this range of serum concentrations the chief function of serum was to accelerate thromboplastin formation, and to influence the ultimate degree of its formation little. At concentrations below 30 percent both the rate and degree were affected however, and the minimum substrate clotting times thereafter showed marked changes.

Additional evidence in support of this suggestion is shown in Table 27b. This table shows the results of experiments similar to that just described in which on separate days varying concentrations of normal sera obtained from II normal donors were studied to clarify their activity in relation to minimum substrate clotting times. The concentrations of serum are shown relative to a 0.I ml. volume of normal serum as equivalent to I00 percent. It can be seen that at concentrations of 300 - 30 percent dilution of any individual serum did not have much effect on the minimum substrate clotting times, but below 30 percent the substrate clotting times lengthened markedly.

In view of this finding it was thought desirable to review any possible activity of factor VII in relation to this reaction, for in experiments 23 and 24, the level of factor VII in the sera used was not markedly reduced. With the passage of time it was possible to study two further sera, one specifically lacking factor X and the other markedly deficient in factor VII alone. The relevant details concerning these specimens are as follows.

I. <u>Factor X deficient serum</u>.

A 23 year old female presented with an acute self-limited

haemorrhagic episode whose aetiology has not been explained although it may have had an allergic basis. During the acute phase her bleeding time was $\frac{3}{4}$ minute, clotting time 10 minutes, tourniquet test negative, platelet count 520×10^3 per c.mm., and her clot retraction was normal. There was no excess fibrinolytic activity. Her "prothrombin" activity was 21 percent and the screening test for thromboplastin generation was abnormal. The full thromboplastin generation test demonstrated a defect in the patient's serum only. The "Stypven" time was prolonged (15 seconds) (Normal - 8 seconds). Plasma and serum deficient in factors VII and IX as isolated defects and this patient's serum were mutually corrective.

2. Factor VII deficient serum.

The patient was the same woman whose serum had been tested in experiment 23. During pregnancy her deficiency became more marked and on this occasion her "prothrombin" activity was less than 5 percent. Her screening and full thromboplastin generation tests and her "Stypven" time were normal.

The study of these two sera is considered as experiment 28.

Experiment 28. To reaffirm the effects of sera deficient in either factor VII or factor X.

Reagents: 1. All normal reagents prepared as before.
2. Factor X deficient serum.
3. Factor VII deficient serum.

Technique: Normal serum and the two test sera were all incubated on the clot at 37°C. for 18 hours prior to use. It was convenient to test dilutions of normal serum at the same time as the factor X deficient serum was tested, and accordingly incubation mixtures were prepared in the usual way to contain concentrations corresponding to 200, 160, 120, 80, 40, 20 and 10 percent normal serum in parallel with the test serum. In these mixtures

TABLE 28 (a). The effects of factor X deficient serum.

Time of Sub-sampling in minutes.	Concentration of Control Serum Substrate Clotting Time In Seconds							Test Serum
	200	160	120	80	40	20	10	
2	19	40	-	-	-	-	-	-
4	-	-	-	-	-	-	-	68
6	11	13	-	-	-	-	-	-
8	10.5	-	13	-	-	-	-	-
10	10.5	9.5	11.5	11	-	-	-	50
12	10.5	-	11	10.5	17	-	-	-
14	11	9.5	10.5	10.5	-	-	-	-
16	-	9.5	10.5	10	14	22	38	31
18	11.5	10	10.5	10	-	-	-	-
20	-	-	10.5	9.5	13	19	31	-
22	-	-	10.5	9.5	-	-	-	24
24	-	-	10.5	9.5	12	-	-	-
26	-	-	10.5	9.5	11.5	16	28	20
28	-	-	10.5	9.5	11	-	-	-
30	-	-	10.5	9.5	10.5	-	-	18

TABLE 28 (a). The effects of factor X deficient serum. Cont'd.

Time of Sub-sampling in minutes.	Concentration of Control Serum Substrate Clotting Time In Seconds							Test Serum
	200	160	120	80	40	20	10	
32	-	-	-	-	10.5	15	26	-
34	-	-	-	-	10.5	14	-	17
36	-	-	-	-	10.5	14	-	-
38	-	-	-	-	10.5	13.5	24	16
40	-	-	-	-	10.5	13	23	-
42	-	-	-	-	10.5	13	-	16
44	-	-	-	-	-	13	22	-
46	-	-	-	-	-	13	21	16
48	-	-	-	-	-	13	20.5	-
50	-	-	-	-	-	13	20	16
52	-	-	-	-	-	-	20	-
54	-	-	-	-	-	-	20	16
56	-	-	-	-	-	-	20	-
58	-	-	-	-	-	-	20	-
60	-	-	-	-	-	-	20	-

TABLE 28 (b). The use of factor VII deficient serum.

Time of Sub-sampling in minutes.	Substrate Clotting Time In Seconds	
	Control Serum	Test Serum
2	60	>60
4	41	>60
6	27	>60
8	19	75
10	14	60
15	11	46
20	9.5	37
25	10	32.5
30	9.5	28
35	9.5	25
55	10	22
60	9.5	9.5
75	10	17.5
90	10	15
105	10	14.5
120	11	14.5
130	11.5	14.5

subsampling was practised at appropriate 2 minute intervals, each mixture containing finally I.0 ml. of brain residue suspension, $200xI0^6$ platelets, I.0 ml. of calcium chloride and 0.2 ml. of serum or an equivalent volume of a saline dilution of serum. The test mixture contained the same volumes of brain residue suspension, platelets and calcium chloride and 0.2 ml. undiluted factor X deficient serum. The factor VII deficient serum did not become available until some time later and it and a normal control serum were tested in the usual way in an incubation mixture containing reagents in the proportions normally used for an estimation of platelet thromboplastic function. In this case subsamples were taken at 5 minute or other appropriate intervals for a total of 90 minutes. The results are shown in Table 28, parts (a) and (b).

Results: I. Factor X deficient serum.

With concentrations of normal serum from 200 to 40 percent the range of minimum substrate clotting times was narrow being 9.5 - I0.5 seconds. Below 40 percent serum, however, there was a marked alteration of minimum substrate clotting times and with 20 and I0 percent concentrations the minimum times were I3 and 20 seconds respectively. Despite the slight difference in substrate clotting times through the range 200 to 40 percent, however, the rate of the reaction was more obviously affected and with every increase in dilution more prolonged incubation was needed to attain maximum thromboplastin generation. When the factor X deficient serum was used both the rate and degree of thromboplastin formation were affected, a minimum substrate clotting time of I6 seconds being reached after 38 minutes incubation.

2. Factor VII deficient serum.

In the control test a minimum substrate clotting time of 9.5 - I0 seconds was reached after 20 minutes incubation, but with the factor VII deficient serum the minimum of only I4.5 - I5 seconds was reached after 90 minutes incubation.

Discussion: The "pattern" of the reaction with dilutions of normal serum as used in the first part of this experiment confirmed the

indications of experiment 27, that there was a considerable range of serum concentrations in which the only function of serum was to influence the rate of thromboplastin formation. In the region of 30 percent concentration, however, the degree as well as the rate of thromboplastin formation was affected, and it appeared that once this critical concentration was passed, further small reductions in concentration produced a disproportionately great effect on the amount of thromboplastin generated. That such must be the case was evident from the results of this experiment for the range of minimum substrate clotting times was only 9.5 - I0.5 seconds with serum concentrations of 200 to 40 percent, yet I3 to 20 seconds in the range of 20 to I0 percent serum. That factor X played an essential role in the reaction was reaffirmed by the slow rate of thromboplastin generation in the mixture containing factor X deficient serum. Maximum thromboplastin generation was attained in this mixture after some 38 minutes incubation, this time corresponding to the incubation time required to achieve maximum thromboplastin generation in the mixture containing 20 percent normal serum. It could be inferred therefore, that the content of factor X in the test serum was in the region of 20 percent of normal, that is in the range where one might expect reduced as well as slowed thromboplastin formation. That such was the case is evident from the results for the minimum substrate clotting time obtained with the factor X deficient serum was I6 seconds.

The presence of both reduced and slowed thromboplastin formation in association with a reduced factor VII concentration was a disconcerting feature and the opposite of the findings in earlier experiments. The only significant feature seemed to be the fact that the deficiency at this time was much more severe than when her serum was first tested (experiment 23) and it was certainly more marked than the defect in the serum tested in experiment 24. It was thought that it was perhaps only at very low concentrations did factor VII influence the reaction, and to assess this possibility experiment 29 was proposed.

TABLE 29. The effect of diluting normal serum with factor VII deficient serum.

Time of Sub-sampling in minutes.	DILUTIONS OF NORMAL SERUM					Factor VII* deficient serum
	FS+	1:2	1:3	1:4	1:10	
4	41	35	38.5	45	49	63
10	22	17	25	18.5	27.5	47
15	15	13	19	13.5	19.5	42
20	13.5	11.5	16	12	15.5	36
25	12	10	13	11	12.5	31.5
30	11	10	12	10.5	12.5	30.5
35	10.5	10	11	10	12	28
40	10.5	10.5	11	10	11	26
45	10.5	10	10.5	10	11	-
50	10.5	9.5	11	10	10.5	24
55	10.5	10	11	10.5	10.5	23.5

TABLE 29. Cont'd.

Time of Sub-sampling in minutes.	DILUTIONS OF NORMAL SERUM					Factor VII* deficient serum
	FS+	1:2	1:3	1:4	1:10	
60	10.5	10	11	-	10	24
65	11	10.5	11.5	10.5	10	22.5
70	-	10	11	11	9.5	-
75	10.5	9.5	11	-	10	22
80	10.5	10	11	-	-	21
85	10.5	10	11	10	10	21
90	10.5	10	11	10	10	21

+FS: Control undiluted normal serum.

The dilutions 1 in 2, 1 in 3 etc. refer to normal serum diluted with factor VII deficient serum.

* Undiluted Factor VII deficient serum.

Experiment 29. To assess the effects of diluting normal serum with serum specifically lacking factor VII.

Reagents: I. All normal reagents prepared as before.
2. Serum severely deficient in factor VII and incubated on the clot at 37°C. for I8 hours prior to use.

Technique: Normal serum was diluted I in 2, I in 3, I in 4 and I in I0 with factor VII deficient serum. Incubation mixtures were prepared to contain I.0 ml. of a suspension of brain residue, 200×10^6 platelets, I.0 ml. of calcium chloride and 0.I ml. of normal serum or one of its dilutions, or 0.I ml. of factor VII deficient serum. Each mixture was incubated at 37°C., and at 5 minute intervals subsamples were taken and thromboplastin formation assessed in the usual way. The results are shown in Table 29.

Results: With undiluted normal serum a minimum substrate clotting time of I0.5 seconds was reached after 30-35 minutes incubation. With the dilutions I in 2, I in 3, I in 4 and I in I0, minimum substrate clotting times of I0, II, I0 and I0 seconds were reached after periods of 20-25, 30-35, 30-35 and 55-60 minutes incubation, respectively. When factor VII deficient serum was used a minimum substrate clotting time of 2I seconds was reached after 75-80 minutes incubation.

Discussion: These results were interesting and explained the anomalous results mentioned above. By diluting normal serum with serum specifically lacking factor VII, the content of factor X would be kept constant while factor VII would be progressively diluted. Assuming arbitrarily, the factor VII content of the normal serum to be I00 percent, and that in the deficient serum to be less than 5 percent (prothrombin activity less than 5 percent) the factor VII concentration in the various mixtures tested would have been approximately I00 percent, 50, 33, 25, I0 and less than 5 percent respectively. This experiment showed that the factor

VII content in an incubation mixture had to be reduced to levels less than 25 and approaching 10 percent of normal before it had any effect on thromboplastin formation. Furthermore, in this range it was only the rate of the reaction that was slowed while the degree of thromboplastin formation remained unaffected. At concentrations higher than this neither the speed or degree of thromboplastin formation differed from normal. When the concentration of factor VII was reduced to very low levels, however, (that is concentrations approaching or less than 5 percent of normal) both the rate and degree of thromboplastin formation were reduced.

It has been shown in earlier experiments that when serial dilutions of normal serum are used in this system the first and most pronounced effect is to cause progressive slowing of thromboplastin generation with succeeding dilutions, and this is apparent with relatively small dilutions. In these experiments both factor X and factor VII were diluted. In experiment 29, when factor X was kept relatively constant and factor VII was progressively reduced, no such slowing of the reaction was apparent until a concentration of factor VII approaching 10 percent of normal was reached. It was obvious then that factor X played the major role in determining the rate of the reaction.

These last experiments had proved that the two "serum factors" influenced the reaction, factor X and factor VII. Of these, factor VII was relatively unimportant and it was necessary to reduce its concentration markedly before either the rate or degree of thromboplastin formation was affected. On the other hand factor X played a major role, and the system was more sensitive to its reduction. Progressive dilution of factor X was immediately reflected in progressive lengthening of the incubation times necessary to attain maximum thromboplastin formation. When the concentration of factor X was reduced to concentrations approaching 20 percent of those present in the usual incubation mixtures, the degree of thromboplastin formation

as well as the rate was affected. In the case of both factors, however, once the concentration of either was reduced to levels sufficient to cause reduced thromboplastin formation, further dilutions caused a disproportionate increase in minimum substrate clotting times.

It was possible therefore to explain discrepancies in experiments 23, 24 and 28. Obviously in the earlier 2 experiments the factor VII concentration in the serum used was outside the critical level of IO percent below which the reaction might be affected.

Section 2. The properties and function of acetone-treated brain, further treated with chloroform.

Acetone-treated brain, as used in Quick's one-stage 'prothrombin' time estimation is insensitive to deficiencies of P.T.A., Hageman factor and factors VIII and IX. It seems likely that tissue thromboplastin contains these factors or an effective functional equivalent. Apart from the removal of lipid platelet substitute there was no indication as to how or to what extent any other active components of tissue thromboplastin might be destroyed or altered by the action of chloroform. A further consideration in relation to the function of brain residue in this system, stemmed from the fact that a suspension of brain residue supplied an immense foreign surface to facilitate platelet disintegration. This section of the thesis deals with investigations designed to assess the relative effects of a suspension of brain residue as a source of a large surface area or as a possible source of active factors essential for thromboplastin formation.

All known coagulation factors likely to be present in brain residue are inactivated at temperatures in excess of 60°C. It was thought that if a suspension of residue was treated in excess of this level, any active coagulation factors would be destroyed while the purely passive aspects of residue as a large foreign surface would be retained. This consideration formed the basis of experiment 30.

Experiment 30. To determine the effects of heat on a suspension of brain residue.

Reagents: I. All normal reagents prepared as for use in the standard technique.

Technique: After incubation at 37°C. for 20 minutes, a suspension of brain residue was divided into equal portions, both of which were placed in suitable screw-capped containers. One of these was set aside for use as a control specimen, while the other was

TABLE 30. To demonstrate the effects of heat on a suspension of brain residue.

Test No.	Brain Preparation	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Normal	46	24	18	16.5	14.5	13	12.5	12.5	12.5
	Heated	32	19.5	18	16.5	16.5	16	15.5	16	16.5
2	Normal	40	27.5	17	14.5	13.5	13	12	12	12.5
	Heated	41	32	23.5	22	20	19	20	21.5	21
3	Normal	23	22	19.5	17.5	15.5	13	12.5	12.5	12.5
	Heated	43	27.5	21.5	19	.8.5	18.5	22	22	21.5
4	Normal	44	30	19.5	17.5	14	12	11.5	12	12.5
	Heated	> 60	55	39	27.5	22.5	22	21.5	22	21
5	Normal	22	11.5	11	11	11.5	12	12	11.5	12
	Heated	23	18	17	17	18	18.5	18	17	18.5

stood in a beaker of water. The water was heated to 100°C. and maintained at this temperature for 10 minutes, thereby raising the temperature of the brain residue suspension to levels where any active coagulation factors would be inactivated. Subsequently the heated residue was allowed to cool, and both it and the control suspension were used in the usual way in the standard procedure. The results of 5 such experiments are shown in Table 30.

Results: Whenever heated brain residue was used, the substrate clotting times were much longer than in the corresponding control series. Despite the longer times, however, a plateau of maximum generation was evident in each case, but while the minimum times tended to be reached slightly faster than in the control series, the plateau was less well maintained and in some cases more rapid lengthening of substrate clotting times was evident in the test than in the control series.

Discussion: Despite heating, suspensions of brain residue still supplied a large foreign surface to facilitate platelet disintegration. The marked interference with thromboplastin generation in these tests, therefore, indicated that the provision of such a surface by brain residue had a small part to play in the production of minimum substrate clotting times. The effects of heated residue must have been mediated through the inactivation of essential components in residue itself. The importance of a purely passive role for residue was excluded and the presence of essential active components confirmed. Furthermore, the fact that the incubation times required to reach maximum thromboplastin formation (albeit reduced) in the presence of heated residue, were not prolonged, suggested that the active factors in residue were "thromboplastic" factors rather than "accelerators". The tendency for maximum generation to be less well maintained in the presence of heated residue suggested that residue itself exercised a stabilizing effect on the whole reaction, and in ways unknown helped to maintain maximum thromboplastin formation at a constant level once this was reached. Alternatively one could postulate that

TABLE 31. To demonstrate the effects of centrifugation of a suspension of brain residue.

Test No.	Brain Preparation	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Normal	46	27	17.5	16.5	13	11	11	11	11
	Centrifuged	51	29	17	16	13	11	11	11	11
2	Normal	29.5	21	15	12	10.5	10.5	10.5	10.5	11
	Centrifuged	27	23	15	12	10.5	10.5	10.5	10.5	10.5
3	Normal	51	33	27	21	17	12.5	12	12	12
	Centrifuged	50	33	25	18	14	12	12	12	13

when thromboplastin formation was much reduced, any small amount of residual anti-thromboplastin in serum might accelerate the breakdown of thromboplastin. To confirm and expand some of these findings two further experiments were undertaken.

Experiment 3I. To determine the effects of centrifugation on suspensions of brain residue.

Reagents: All normal reagents prepared as before.
Technique: As in experiment 30, the suspension of brain residue was divided into two equal portions after incubation. One of these was set aside for use as a control, the other was centrifuged at 500r.p.m. for 5 minutes, thus removing all coarse and moderate sized particles from the suspension, and reducing the surface available for platelet disintegration. The standard procedure was followed using the control and centrifuged specimens. The results of 3 such experiments are shown in Table 3I.
Results: In all three tests there was no difference in the minimum substrate clotting times whether normal or centrifuged specimens of residue had been used.
Discussion: Reduction of the foreign surface available for platelet disintegration had no effect on the reaction, and thus confirmed the findings of the previous experiment, namely that a purely passive role for a suspension of brain residue was of no significance.

Experiment 32. To determine the effects of substituting saline for a suspension of brain residue.

Reagents: I. All normal reagents prepared as before.
2. Normal saline.
Technique: A control test was first performed using all normal reagents and the standard technique, after which the procedure was repeated, I.0 ml. of saline having been substituted for the same volume of brain residue. The results of 5 experiments are shown

TABLE 32. To show the effects of substituting saline for a suspension of brain residue.

Test No.	Brain residue or saline	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Brain residue	40	22.5	16.5	14.5	13.5	13	12	12	12
	Saline	40	21	17	17.5	20	26	31	34	37
2	Brain residue	40	27.5	17	14.5	13.5	13	12	12	12.5
	Saline	36.5	17.5	18	19.5	22.5	32	39	44.5	47
3	Brain residue	44	30	17.5	15.5	14	12	11.5	12	12.5
	Saline	> 60	> 60	52	45	45.5	48	59	> 60	> 60
4	Brain residue	34.5	22.5	17.5	16.5	15.5	13.5	13	13	13.5
	Saline	56	27.5	18.5	20	22	27.5	33	35	36
5	Brain residue	22	11.5	11	11	11.5	12	12	11.5	12
	Saline	15	14.5	15	15.5	17.5	22	29	34	39

in Table 32.

Results: In every case the substitution of saline for suspensions of residue was accompanied by impaired thromboplastin formation. Despite this, however, when saline was used in lieu of brain residue, the minimum substrate clotting times were reached after much shorter periods of incubation than in the control mixtures. Once reached the minimum substrate clotting times in saline mixtures were poorly maintained and showed almost immediate and rapid decline.

Discussion: The first and most obvious feature of this experiment was the confirmation of the fact that brain residue supplied factors essential for maximum thromboplastin generation in this system. A second and fascinating feature was the marked difference in the "pattern" of the reaction between mixtures containing brain residue and those containing saline. In the presence of saline the incubation mixtures followed the behaviour of other thromboplastin generation mixtures in that the minimum substrate clotting times once reached, were poorly maintained and in very few minutes showed rapid and progressive lengthening. In the absence of brain residue the minimum substrate clotting times were reached after much shorter periods of incubation. The incubation times required before the minimum substrate clotting times were reached and the latter themselves, in the control tests in this experiment were comparable to the control tests in experiment 30. So too, the minimum substrate clotting times obtained with heat inactivated brain and saline in these two experiments were comparable. Despite heat inactivation of brain residue, however, it seemed highly likely that, although residue so treated lost its ability to influence the degree of thromboplastin generation itself, it did influence the reaction in other ways. This was indicated by the fact that when heated residue was used, the time of incubation required to attain maximum thromboplastin generation was intermediate between that of saline mixtures and those containing normal brain residue. Secondly,

TABLE 33. To show the effects of diluting a suspension of brain residue.

Test No.	Dilution of residue	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
1	Full strength	32	15	12.5	12	12	12
	1 in 2	24	16	13.5	13	13.5	13
	1 in 4	31	18	15	15.5	14.5	14.5
	1 in 10	32.5	21.5	19	19	19	19.5
2	Full strength	42	19.5	15.5	13.5	13	13
	1 in 2	35	20	15	14	14	14
	1 in 4	36.5	18.5	15.5	16	15.5	15.5
	1 in 10	26	19.5	19.5	20	21.5	21.5
3	Full strength	13	9	9	9.5	9.5	9.5
	1 in 2	13	10.5	10.5	10.5	10.5	11
	1 in 4	13.5	13	12.5	13	13	13
	1 in 10	-	-	-	-	-	-

TABLE 33. Cont'd.

Test No.	Dilution of residue	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
4	Full strength	20	12	12	12	12	12.5
	1 in 2	16	13	12.5	13	13.5	13
	1 in 4	16	14.5	14	13	14	14
	1 in 10	20.5	17.5	17	17.5	18	18
5	Full strength	35	14.5	12.5	12.5	12	12.5
	1 in 2	27	16	13.5	13	13.5	13.5
	1 in 4	22	16	15	15	15	15.5
	1 in 10	29.5	22.5	19.5	19.5	19	21.5

in the presence of heated residue maximum generation was maintained much longer than in the presence of saline but generally for shorter periods than when suspensions of normal residue were used. Furthermore, even in the presence of reduced thromboplastin formation, secondary to the use of heated residue, the decline of thromboplastic activity was much more gradual than in the presence of saline, although slightly faster than in normal control tests. On the basis of these findings it was reasonable to postulate that brain residue, as used in the system under consideration, had two functions. Of these the most obvious was to supply factors essential for the development of maximum amounts of thromboplastin in the presence of platelets, serum and calcium chloride. A second and altogether obscure function seemed to be an ability of brain residue to delay thromboplastin generation and to assist in stabilizing maximum generation at constant levels once this was attained.

Experiment 33. To determine the effect of diluting suspensions of brain residue.

Reagents: I. All normal reagents prepared as before.
2. Normal saline.

Technique: After incubation the suspension of brain residue was centrifuged at 500r.p.m. for 5 minutes to remove the coarsest particles, a process shown not to affect its activity. Subsequently aliquots of the fine supernatant suspension were diluted I in 2, I in 4 and I in I0 with normal saline, and the standard procedure was followed using undiluted residue suspension and each of the dilutions in turn. The results of 5 experiments are shown in Table 33.

Results: Increasing dilutions of brain residue suspension were accompanied by progressive lengthening of minimum substrate clotting times. Whatever dilution was used the usual plateau of maximum generation was evident, while the incubation times required for this to be reached did not alter significantly with

succeeding dilutions. However, when high dilutions of residue were used there was a slight tendency for the minimum substrate clotting times to decline before any such effect was apparent with other more concentrated suspensions.

Discussion: The progressive lengthening of substrate clotting times with increasing dilutions of the suspension of brain residue must have reflected progressive reduction of a factor or factors essential for thromboplastin formation. Furthermore, the fact that the speed of the reaction was not altered by progressive dilutions of the residue indicated that the essential components in brain residue did not act as accelerators of the reaction. Even at relatively high dilutions of brain residue, however, the "pattern" of the reaction was different from that found when saline was substituted for brain residue. It seemed therefore that even in high dilution brain residue exerted a stabilizing effect on thromboplastin generation. Alternatively the presence of reduced thromboplastin formation in "saline" mixtures may have permitted a more rapid decline of substrate clotting times through a neutralization or destruction of thromboplastin by any anti-thromboplastin present, for in these mixtures the latter would have been present in proportionately greater amounts than in mixtures containing "brain residue" with their resultant greater thromboplastin formation.

The last few experiments then, had established certain general features of the behaviour of brain residue, but it remained to identify the active principles present and possibly participating in the reaction. This was done in a composite group of tests considered as experiment 34. In these the ability of suspensions of brain residue to correct abnormal screening tests, classical thromboplastin generation tests and prolonged 'prothrombin' times in specific deficiency states was determined.

Experiment 34. To identify the active components in chloroform-treated, acetone-treated brain.

TABLE 34. The results of test to identify the active principles in brain residue.

Part (1).

REAGENT	"PROTHROMBIN" TIME
Factor VII deficient plasma.	49 seconds
Factor VII deficient plasma + 20% residue.	46 seconds
Factor VII deficient plasma + 20% serum.	13 seconds
Normal plasma.	12.5 seconds

TABLE 34. Part (2).

Test Nos. 1-4 using haemophilic plasma.

Test No.	Reagent Used	Results of Screening Test Substrate Clotting Time In Seconds					
1	Normal plasma diluted with buffer.	43	18	9	8	8	8
2	Brain residue suspension in lieu of plasma.	> 60	> 60	> 60	> 60	> 60	> 60
3	Haemophilic plasma diluted with buffer.	32.5	30	26	23	21.5	19
4	Haemophilic plasma diluted with brain residue.	34	12.5	9.5	8.5	8.5	8.5

TABLE 34. Part (2). Cont'd.

Test Nos. 5-8 using P.T.A. deficient plasma.

Test No.	Reagent Used	Results of Screening Test Substrate Clotting Time In Seconds					
5	Normal plasma diluted with buffer.	33	10.5	9.5	9.5	9.5	10.5
6	Brain residue suspension.	>60	>60	>60	>60	>60	>60
7	P.T.A. deficient plasma diluted with buffer.	42	25	16	17	17	17
8	P.T.A. deficient plasma diluted with brain residue suspension.	-	-	50	22	16	16

TABLE 34. Part (2). Cont'd.

Test No.	Reagent Used	Results of Screening Test Substrate Clotting Time In Seconds					
9	Haemophilic plasma in Owrens buffer.	> 60	> 60	41	32	27	27.5
10	Haemophilic plasma diluted with brain residue.	45	18	12	10.5	9.5	10

TABLE 34. Part (3).

Test No.	Reagent substituted for corresponding normal reagent.	Results of Classical Tests Substrate Clotting Time In Seconds					
1	All normal reagents.	>60	47	20	11	10.5	10.5
2	Brain residue for serum.	>60	51	47.5	39	40	47
3	Brain residue for treated plasma.	>60	35	31	28	33	41
4	Brain residue for serum and plasma.	>60	>60	>60	>60	>60	>60
5	Factor IX deficient serum in buffer.	60	50	38	27	22.5	20
6	Factor IX deficient serum in residue.	60	29	19.5	14.5	13	12.5
7	Factor X deficient serum in saline.	>60	>60	>60	>60	>60	>60
8	Factor X deficient serum in residue.	>60	>60	>60	>60	>60	44
9	Haemophilic treated plasma in buffer.	>60	>60	45	27	26	26
10	Haemophilic treated plasma in residue.	>60	18	12	9.5	9	9

Part (I) Factor VII - like activity.

Deficiencies of factor VII do not affect thromboplastin generation in either the screening or classical tests. The criterion of factor VII activity chosen was the ability of reagents containing factor VII to correct the prolonged prothrombin time of a factor VII deficient plasma.

Reagents: I. Plasma specifically lacking factor VII.

2. A suspension of acetone-treated brain prepared as for use in Quick's one-stage 'prothrombin' time estimation.
3. A suspension of chloroform and acetone-treated brain prepared in the usual way.
4. Calcium chloride.
5. Normal serum.

Technique: "Prothrombin" time estimations were performed on factor VII deficient plasma and repeated on the same plasma after the addition of 20 percent (volume for volume) of brain residue suspension. The results are shown in Table 34 (I).

Results: The prolonged one-stage "prothrombin" time of a factor VII deficient plasma was not corrected to any degree by the addition of brain residue suspension.

Discussion: It was concluded that brain residue had no factor VII - like activity.

Part (2) P.T.A. and factor VIII activity.

The presence of activity comparable to that of anti-haemophilic globulin and P.T.A. was assessed by the ability of brain residue suspension to correct the abnormal screening test for thromboplastin generation in patients with haemophilia and in one known case of P.T.A. deficiency.

Reagents: I. Lipid platelet substitute diluted I in 20 with Owren's buffer.

2. Normal platelet poor plasma diluted I in IO with Owren's buffer.
3. Suspension of brain residue prepared as before.
4. Haemophilic plasma diluted I in IO with Owren's buffer.
5. P.T.A. deficient plasma diluted I in IO with Owren's buffer.
6. Haemophilic plasma diluted I in IO with brain residue suspension.
7. P.T.A. deficient plasma diluted I in IO with brain residue suspension.
8. Platelet "poor" normal plasma as substrate.
9. Calcium chloride.

Technique: The screening test for thromboplastin generation was performed using lipid platelet substitute, calcium chloride and the reagents 2,3,4,5,6 and 7 above, in turn. The results obtained in two cases of haemophilia and one P.T.A. deficiency are shown in Table 34 (2).

Results: When normal plasma was used the minimum substrate clotting times ranged between 8 and 9 seconds, but when a suspension of brain residue was substituted for normal plasma no substrate clotting time was less than sixty seconds. When the plasma from two cases of haemophilia and the case of P.T.A. deficiency, all diluted in buffer, were tested, the minimum substrate clotting times were I9.5, 27 and I7 seconds respectively. The screening tests of the same haemophilic plasmas diluted in suspensions of brain residue were normal, the minimum substrate clotting times then being 8.5 and 9.5 seconds. The abnormal screening test of the P.T.A. deficient plasma was not corrected by dilution in brain residue suspension, the minimum substrate clotting time remaining at I6 seconds.

Discussion: Brain residue suspension in combination with lipid and calcium chloride was quite inert. On the other hand the ability of such residue to correct the abnormal screening tests in haemophilic plasma but not in a deficiency of P.T.A. could only be

interpreted as indicating that such residue retained activity like that of factor VIII but had no component equivalent to P.T.A.

Part (3) Factors VIII, IX and X.

While plasma lacking factor VIII was readily available, plasma specifically deficient in either factor IX or factor X was not to hand for use in the screening tests. Serum from patients with both the latter defects was available, however, and this was used in classical thromboplastin generation tests in further attempts to define the active principles in brain residue. At the same time the classical test was used to confirm the presence of factor VIII - like activity in brain residue.

Reagents: I. Lipid platelet substitute diluted I in IO with Owren's buffer.

2. A suspension of brain residue.
3. A I in 5 dilution of aluminium hydroxide-treated normal plasma in Owren's buffer.
4. A I in IO dilution of normal serum in Owren's buffer.
5. A I in IO dilution of factor IX deficient serum in Owren's buffer.
6. A I in IO dilution of factor IX deficient serum in brain residue suspension.
7. A I in IO dilution of factor X deficient serum in Owren's buffer.
8. A I in IO dilution of factor X deficient serum in brain residue suspension.
9. A I in 5 dilution of aluminium hydroxide-treated haemophilic plasma in brain residue suspension.
IO. A I in 5 dilution of aluminium hydroxide-treated haemophilic plasma in Owren's buffer.
II. Normal platelet poor plasma as substrate.

I2. Calcium chloride.

Technique: In the first instance a classical thromboplastin generation test was performed using all normal reagents. Additional control tests in which a suspension of brain residue was substituted for aluminium hydroxide-treated normal plasma, normal serum, and both reagents, in turn, followed. Following this, tests were performed in which, in turn, factor IX deficient serum diluted in buffer and brain residue suspension, factor X deficient serum diluted in buffer and residue suspension and aluminium hydroxide-treated haemophilic plasma diluted in buffer and in brain residue suspension, were substituted for the corresponding normal reagents. The results are shown in Table 34 (3).

Results: When all normal reagents were used the minimum substrate clotting time was I0.5 seconds. When a suspension of brain residue was substituted for either the plasma or serum preparation or both, thromboplastin formation was greatly reduced. Abnormal results were obtained when the dilution of factor IX or factor X deficient serum in buffer, or the haemophilic treated plasma in buffer were substituted for the corresponding normal reagents. If factor IX deficient serum and aluminium hydroxide-treated haemophilic plasma were diluted in the suspensions of brain residue, however, the classical tests were corrected to normal. No such correction was found when factor X deficient serum diluted in residue suspension was used.

Discussion: Correction of the defect in haemophilic plasma in this experiment confirmed the earlier findings of experiment 34 (2), that brain residue retained factor VIII - like activity. So too, marked correction of the defect in factor IX deficient serum indicated that residue retained activity comparable to that of factor IX, but there was no evidence of activity comparable to factor X. It was concluded therefore that brain residue prepared by chloroform treatment of acetone-treated brain, retained active principles equivalent to the plasma factors VIII and IX but there was no evidence of equivalent activity in respect of factors VII and X

TABLE 35. To show the effects of serial chloroform treatments on the inherent activity of brain residue.

Test No.	Number of Extractions	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Prothrombin time with residue
		4	10	15	20	25	30	
1	1	12	10.5	10.5	10.5	10.5	10.5	18
2	2	21	13	12.5	12.5	12.5	12.5	28
3	3	30	12	12	12	12	13	49
4	4	27	12	12	12	12.5	13	53
5	5	37	15	11.5	11.5	11.5	11.5	63
6	6	29	12	12	12	12	12	110

or P.T.A. Unfortunately no specimen deficient in Hageman factor was available and this factor could not be investigated.
A final experiment in relation to the activity of brain residue was experiment 35. It had been shown earlier that the degree of substrate clotting times obtained with any particular batch of residue was dependant on the inherent activity of the particular batch of residue being used. Whether such differences between succeeding preparations of residue depended on inherent variations in acetone-treated brain, prior to extraction, or whether it might be dependant on varying degrees of "inactivation" of acetone-treated brain, during chloroform treatment was not known. Experiment 35 was undertaken to clarify the problem.

Experiment 35. To determine whether chloroform treatment affects the inherent activity of brain residue.

Reagents: I. Acetone-treated brain prepared as for use in Quick's 'prothrombin' time estimation.
2. Chloroform.
3. Platelets, serum and calcium chloride prepared as for use in the standard technique.

Technique: Acetone-treated brain was further treated with chloroform as outlined in experiment 2. After each chloroform treatment a small aliquot of residue was set aside, dried in air at room temperature and ground to a coarse powder in a mortar and pestle. The individual aliquots were kept separate, and after the final extraction, each was used to prepare a suspension of residue in the usual way. Each of these suspensions in turn was used as the residue in the standard technique, each being tested with the one platelet preparation and the same serum. The results are shown in Table 35.

Results: The minimum substrate clotting time obtained with the suspension of brain residue treated but once with chloroform was I0.5 seconds. The minimum substrate clotting times obtained after 2,3,4,5 and 6 treatments were I2.5,I2,I2,II.5 and I2

seconds respectively.

Discussion: The faster minimum substrate clotting times obtained with the initial preparation may have reflected greater factor VIII - like and factor IX - like activity in this residue. With all succeeding preparations, the range of minimum substrate clotting times, II.5 - I2.5 seconds was within the range of technical error. Substrate clotting times did not lengthen progressively with increasing chloroform treatments. It seemed more probable, therefore, that the faster times obtained with the first preparation represented a composite effect of retained thromboplastic activity and added platelet thromboplastic factor. The slower and reasonably constant substrate clotting times obtained with all succeeding preparations on the other hand, were dependant on added platelet thromboplastic factor alone, inherent thromboplastic activity in brain residue having been reduced to negligible proportions by the extraction of tissue phospho-lipid. The constancy of the substrate clotting times in the last 5 suspensions indicated that inherent factor VIII and factor IX activity in brain residue was unaffected by repeated chloroform treatments. Thus it seemed that the variations in different batches of brain residue were related to initial differences in the activity of individual samples of acetone treated brain and did not follow from the preparation of residue.

Earlier in this thesis an hypothesis had been proposed, albeit in the most general terms to explain the mechanism of thromboplastin formation in an incubation mixture containing a suspension of brain residue, platelets, serum and calcium chloride. It was suggested that platelet thromboplastic factor reacted with brain residue to form thromboplastin but that this reaction could only proceed in the presence of a factor or factors supplied by normal serum.

Subsequent work would seem to have confirmed this theory and to have clarified the active components in each reagent. One could now postulate that platelet thromboplastic factor reacted with factors VIII and IX (or their equivalent) provided by brain residue,

under the influence of factor X and, possibly, factor VII, supplied by serum, to generate thromboplastin. Of the serum components factor X was of major importance and its prime function was to accelerate the reaction. Only at very low concentrations did factor X cause a reduction in the amount of thromboplastin formed. The evidence had shown that factor VII played a relatively unimportant part in the reaction, and that its concentration had to be reduced to very low levels before it affected either the rate or degree of thromboplastin formation. It was likely that under the usual test conditions its action could be largely ignored.

An interesting feature of the reaction is the fact that once thromboplastin formation has reached a maximum it is maintained at this level for a variable length of time, usually 10 to 60 minutes. This is well shown in experiment 26 in those tests using dilutions of normal serum, and is in sharp contrast to other tests based on thromboplastin generation. In the latter, minimum substrate clotting times are usually poorly maintained and show a rapid decline once the peak has been reached. The explanation for this difference must be that the system containing brain residue, serum, platelets and calcium chloride, as standardized, contains little anti-thromboplastic activity and that, for this reason, maximum thromboplastin formation is enabled to be maintained for a protracted period of time.

Whether factor V introduced into the system adsorbed onto platelets played any significant part in the process was obscure. Some evidence suggested that factor V might play a small role, possibly non-essential, in accelerating thromboplastin formation, but it seemed that under the normal conditions of the test it did not influence the ultimate yield of thromboplastin. Furthermore, if factor V did accelerate the reaction it was not interchangeable with factor X, for the former introduced in aluminium hydroxide-treated plasma was accompanied by negligible thromboplastin formation if serum was absent. In view of the doubt

surrounding any possible functions of factor V experiments were undertaken to clarify its position. These are described in the next section.

TABLE 36. To show the effects of adding aluminium hydroxide treated normal plasma to incubation mixtures.

Test No.	Mixture	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Control	28.5	15.5	11.5	11	11	11	11	11	11
	+ saline	32.5	14	12	11	11	11	11	11	12
	+ treated plasma	36	14	11	10	9	9	10.5	10.5	11.5
2	Control	16	12	10.5	10	10	9.5	10	10	11
	+ saline	26.5	15	12	11	10	10	10	10	10
	+ treated plasma	27.5	19	12	10.5	10	8	8.5	10.5	12
3	Control	35.5	20.5	15	13	12	12	12	12	12
	+ saline	33	20	16	14	12	12	12	12	12
	+ treated plasma	28	15.5	13.5	11.5	10.5	9.5	9.5	9.5	9.5
4	Control	30	17.5	14	12.5	12.5	11.5	11.5	11.5	12.5
	+ saline	20	14	13	12.5	11.5	11.5	12	13	13.5
	+ treated plasma	17	14	11.5	10.5	9.5	10	11	10.5	11
5	Control	35.5	19.5	14.5	13	11.5	11.5	11.5	11	11.5
	+ saline	23	14	12.5	12	11.5	11	11	11	11.5
	+ treated plasma	25	14	12.5	12	10	9.5	10.5	11	11

Part III. Experiments concerning factor V.

Aluminium hydroxide-treated plasma retains factors V and VIII, P.T.A. and Hageman factor. In experiment 36 aluminium hydroxide-treated plasma, as a source of factor V, was added to the standard incubation mixture, to gain some idea of what effect it might have on thromboplastin generation.

Experiment 36. To determine the effects of adding aluminium hydroxide-treated plasma to the standard incubation mixture.

Reagents: I. All normal reagents prepared as before.
2. Aluminium hydroxide-treated normal plasma.

Technique: As controls for this experiment the standard procedure was performed initially using all normal reagents, and secondly with the addition of 0.I ml. of normal saline. In the tests 0.I ml. of aluminium hydroxide-treated plasma was added to the usual volumes of all other reagents, and the usual technique followed. The results of 5 experiments using all different reagents on each occasion are shown in Table 36.

Results: The minimum substrate clotting times were the same in the control mixtures whether saline had been added or not. In each case, when aluminium hydroxide treated plasma had been added to incubation mixtures, the minimum substrate clotting times were I - 2.5 seconds faster than those of the control tests. These faster times were attained after much the same incubation times as in the control series, but in general they were poorly maintained, and in 4 cases they showed progressive lengthening some 5-I0 minutes after maximum generation was evident.

Discussion: Since four factors known to participate in intrinsic thromboplastin formation were added to the incubation mixtures in aluminium hydroxide-treated plasma, the faster substrate clotting times produced in this experiment could not be attributed to a function of factor V without reservation. Indeed the relative

TABLE 37. To show the effects of serial dilutions of aluminium hydroxide treated normal plasma on their addition to incubation mixtures.

Test No.	Dilution of + Treated Plasma	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Control	22	10.5	10	9.5	9.5	10.5	9.5	9.5	9.5
	FS*	14	12.5	10.5	11	7.5	8	9	10	12
	1:2	20	9.5	8.5	8.5	8	8	9.5	10	11
	1:4	13.5	11.5	10	10	9.5	9.5	9.5	10	11
	1:10	12.5	10	9.5	9.5	9.5	9.5	10.5	11	12
2	Control	34.5	16	12	11.5	11	11.5	11.5	11.5	11.5
	FS*	9	9	9	9.5	10	9.5	10.5	11.5	13
	1:2	18	10.5	11.5	10.5	10	10	10	10.5	11.5
	1:4	13.5	11	11	11	11	11.5	11.5	13	14
	1:10	27	13.5	11.5	11.5	11.5	11.5	11.5	11.5	13
3	Control	25.5	16.5	13.5	11.5	11	11	11	11	11
	FS*	26.5	14	10.5	9.5	9.5	9	10.5	11.5	12
	1:2	17.5	13.5	12.5	10.5	10.5	10.5	11	12.5	13
	1:4	22	14	14	11.5	11.5	11	12.5	13.5	15
	1:10	23	13.5	12	12	11	11	12.5	14	14.5

FS* = undiluted aluminium hydroxide treated plasma.

\+ = 0.1 ml. volumes of each preparation of aluminium hydroxide treated plasma were used.

importance of each of the four factors could not be differentiated. The precise function of factor V is, perhaps, still incompletely understood although there is evidence to suggest that it has a dual action, to accelerate thromboplastin formation on the one hand, and to enter into a stoichiometric reaction with the first intermediate product of thromboplastin formation on the other, and thereby to influence the ultimate yield of thromboplastin. Should this be so, there was no evidence in this experiment, that additional factor V, over and above that adsorbed onto platelets, had any influence on the rate of thromboplastin formation in this reaction. It was possible to attribute the greater yield of thromboplastin to this factor, however, but as stated above, with some reservation. One fact was quite plain from this experiment. It was apparent that some factor introduced with the treated plasma, was capable of initiating rapid neutralization or breakdown of formed thromboplastin. There could have been no other explanation for the rapid lengthening of minimum substrate clotting times, once maximum generation had been reached. In an attempt to clarify some of these points, experiment 37 was undertaken.

Experiment 37. To determine the effect of serial dilutions of aluminium hydroxide-treated plasma when introduced into the standard incubation mixture.

Reagents: I. All normal reagents prepared as before.
2. Aluminium hydroxide-treated normal plasma.

Technique: Aliquots of the aluminium hydroxide-treated plasma were diluted I in 2, I in 4 and I in 8 with Owren's buffer. The technique was the same as that in experiment 34, except that in addition to incubation mixtures containing 0.I ml. of full strength aluminium hydroxide-treated plasma, further tests were performed with 0.I ml. volumes of each of its dilutions. The results of 3 such experiments are shown in Table 37.

Results: When either undiluted or the I in 2 dilution of aluminium hydroxide-treated plasma was added to the incubation mixture, the

minimum substrate clotting times were faster than the control times. Those obtained with the I in 2 dilution of plasma were slower than those obtained with the undiluted preparation. When I in 4 or I in 8 dilutions of treated plasma were added to incubation mixtures, the minimum substrate clotting times obtained with both of these dilutions were identical and the same as those of the control tests. The incubation times required for the development of maximum thromboplastin varied even within the one group of tests, and sometimes the highest dilutions of treated plasma were associated with the fastest development of maximum thromboplastin. No matter what dilution of treated plasma was used, thromboplastin formation was poorly maintained, and in each group of tests minimum substrate clotting times declined quickly.

Discussion: Had added factor V played any part to accelerate thromboplastin formation in this system one would have expected progressive dilution of aluminium hydroxide-treated plasma to be accompanied by lengthening of the incubation times required for maximum thromboplastin generation to be reached. Such was not the case and it was felt that any significant function of added factor V in this respect did not exist. It was thought reasonable to suppose, as had been suggested earlier, that sufficient factor V was introduced into the system with platelets to accomplish the reaction under consideration. Only when large amounts of factor V were lost by vigorously washing platelets was the reaction likely to be slowed. A significant feature of this experiment, however, was the fact that while the effect of aluminium hydroxide adsorbed plasma in enhancing thromboplastin generation was rapidly removed by dilution, the promotion of rapid disappearance of formed thromboplastin persisted at relatively high dilutions. This indeed, was indicative of the presence of a powerful destructive or neutralizing mechanism in normal aluminium hydroxide-treated plasma.

TABLE 38. To show the effects of aluminium hydroxide treated normal plasma and P.T.A. deficient plasma when added to incubation mixtures.

Test No.	Reagent Used	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Control mixture	38	21	15	13	12.5	11	11	11	11
2	+ treated normal plasma	40	14	10	10.5	9	9	9	9.5	11
3	+ test plasma	34	10	10	9	9	9	10	12	13

In an endeavour to identify the factor or factors in aluminium hydroxide treated plasma responsible for improving thromboplastin generation, experiments 38 and 39 were performed.

Experiment 38. To determine the effect of adding P.T.A. deficient, aluminium hydroxide-treated plasma, to the present system.

Reagents: 1. All normal reagents prepared as before.
2. Aluminium hydroxide-treated normal plasma.
3. Aluminium hydroxide-treated P.T.A. deficient plasma.

Technique: This was basically the same as in experiment 36 and 37. Following a control experiment in which 0.1 ml. of saline was added to the usual incubation mixture, tests were performed in which, in turn, 0.1 ml. of each of the reagents 2 and 3 above was substituted for the saline, and the routine procedure followed. The results are shown in Table 38.

Results: The minimum substrate clotting times obtained with the use of either aluminium hydroxide-treated normal plasma or P.T.A. deficient plasma were the same, and, in both cases, faster than the times obtained with the control mixture containing saline.

Discussion: It was concluded that P.T.A. played no part in the production of the faster minimum substrate clotting times obtained on the addition of aluminium hydroxide-treated plasma to the usual incubation mixture. Although plasma specifically lacking Hageman factor was not available for testing, it seemed reasonable to concentrate attention on the two major factors in treated plasma, the factors V and VIII. The next experiment was identical with experiment 38, save that aluminium hydroxide-treated haemophilic plasma was compared with the corresponding normal reagent.

Experiment 39. To determine the effects of adding aluminium hydroxide-treated haemophilic plasma to the present system.

TABLE 39. To show the relative effects of aluminium hydroxide treated normal plasma and haemophilic plasma when added to incubation mixtures.

Test No.	Reagents Used	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Control	18.5	13	11.5	10.5	10	10	10	10	10
	+ N plasma*	19.5	12	9.5	9	9	9.5	9.5	9.5	9.5
	+ H plasma+	13	13	11	11	11	11	11	11.5	13
2	Control	24	14.5	12.5	12	12	12	12	12	12
	+ N plasma*	24	15	14	11.5	11	10.5	10	10.5	10.5
	+ H plasma+	31.5	20	20	19.5	17.5	15	13.5	12	12.5
3	Control	24	14.5	12.5	12	12	12	12	12	12
	+ N plasma*	24	15	14	11.5	11	10.5	10	10.5	11.5
	+ H plasma+	27.5	18.5	16.5	15	13.5	12.5	13	13.5	13.5
4	Control	46	26	17.5	15	13	12	12	12	12
	+ N plasma*	14	13	13.5	12.5	11	11.5	11	11.5	13
	+ H plasma+	32	24.5	24	17.5	17	15.5	15	13	13

TABLE 39. Cont'd.

Test No.	Reagents Used	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
5	Control	>60	39	19	15	12	10.5	10.5	10.5	10.5
	+ N plasma*	30	13	11	10.5	9.5	9.5	9.5	9	9.5
	+ H plasma+	46	24	14.5	15	15	13	12	10.5	10
6	Control	21.5	15	13	12	12	12	12	12	13.5
	+ N plasma*	20.5	11.5	11.5	10.5	10	10	11	11.5	12.5
	+ H plasma+	27	18	14.5	13	12.5	11.5	11	11.5	11.5
7	Control	>60	50	33.5	21	17	12.5	12.5	12.5	12.5
	+ N plasma*	>60	22	14	13	11.5	11.5	13	13.5	14.5
	+ H plasma+	>60	40	21	19.5	16	15	14	13.5	14
8	Control	44	30	21.5	17.5	15.5	13.5	13	12.5	13
	+ N plasma*	40.5	22	9	8.5	9.5	9.5	10.5	11	13.5
	+ H plasma+	19.5	-	17	15.5	14	13.5	12.5	13	13.5

Reagents: I. All normal reagents prepared as before.
2. Aluminium hydroxide-treated normal plasma.
3. Aluminium hydroxide-treated haemophilic plasma.

Technique: As for experiment 38. The results of 8 successive experiments performed at different times as suitable subjects presented are shown in Table 39.

Results: In cases I,3,4 and 7, when aluminium hydroxide-treated haemophilic plasma was added to the usual incubation mixture, the minimum substrate clotting times were actually slower than the control tests containing either saline or treated normal plasma. In cases 2, 5 and 8 the minimum substrate clotting times obtained with mixtures containing treated haemophilic plasma were the same as those of the saline controls, but much slower than those of controls containing treated normal plasma. In case 6 the minimum substrate clotting times were slightly faster than those obtained with the saline control but slower than those obtained with normal treated plasma.

Discussion: The results obtained were surprising. Both normal and haemophilic plasma treated with aluminium hydroxide should contain comparable amounts of factor V. By comparison with the corresponding normal reagent, the addition of aluminium hydroxide-treated haemophilic plasma to incubation mixtures in this series of tests, was accompanied by reduced thromboplastin formation. This seemed to indicate that the improvement in thromboplastin generation on the addition of aluminium hydroxide-treated normal plasma to incubation mixtures was dependent on its content of factor VIII, and that factor V was not concerned in this process. However, the occurrence of minimum substrate clotting times slower than controls with added saline, in 4 cases when treated haemophilic plasma was added, was an unexpected finding. Two possible explanations were forthcoming. In the first instance it may have been that haemophilic plasma contained an excess of inhibitory factors capable of over-riding any improvement in thromboplastin generation due to the action of factor V, and causing rapid neutralization of formed thromboplastin, and thereby

slower substrate clotting times. The second possibility was based on the assumption that factor VIII was responsible for improving thromboplastin formation. Under these circumstances, when haemophilic treated plasma was used, the reduced content of factor VIII would result in proportionately less thromboplastin formation, while the normal content of inhibitory factors in plasma would be present in relative excess. The presence of the latter then would be more evident, and manifest in the greater neutralization of an initially lesser quantity of formed thromboplastin with correspondingly slower substrate clotting times. At a later stage this second concept was shown to be correct, and the relevant work is detailed in a later section devoted to antithromboplastins. At this stage, however, it was assumed that the second theory was correct, and there seemed little doubt that added factor V had no significant effect on thromboplastin formation.

Subsequently an ampoule of purified porcine anti-haemophilic globulin, supplied by Dr. Rosemary Biggs, enabled a relevant experiment to be undertaken with a single product, uncomplicated by the presence of factor V or inhibitory factors. This was experiment 40 in which the relative effects of pure factor VIII and aluminium hydroxide-treated normal plasma were compared. It was difficult to decide the precise concentration of factor VIII to be used in this experiment, but finally a concentration was arbitrarily decided in the following way. Screening tests for thromboplastin generation were performed with normal plasma and plasma obtained from a severe haemophiliac. A concentration of factor VIII in Owren's buffer was prepared and serially diluted until a minimum concentration was reached which, when contained in a volume of 0.1 ml., was capable of correcting the defect in the plasma of a severe haemophiliac using the screening test as a gauge. This strength of factor VIII solution was then used.

Experiment 40. To compare the effects of adding aluminium

TABLE 40. To show the relative effects of aluminium hydroxide treated normal plasma and purified anti-haemophilic globulin when added to incubation mixtures.

Test No.	Reagent added to mixtures*	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Saline	22	15.5	12.5	11	10.5	9.5	9	9.5	9.5
	Treated plasma	13	8.5	7.5	8	8	8	8.5	9	9.5
	AHG solution	24	14.5	11.5	10.5	10	9.5	8.5	8.5	8.5
2	Saline	33.5	25	19.5	17.5	15.5	13	11	11	11
	Treated plasma	29	20	15	12.5	10.5	9.5	9.5	9.5	9.5
	AHG solution	24	15.5	13	11.5	11.5	10.5	9.5	9.5	9.5
3	Saline	19.5	16	13	12.5	11.5	10.5	10.5	10.5	10.5
	Treated plasma	20	13	10.5	10	9.5	9.5	10	11	11.5
	AHG solution	18	12	11.5	10.5	10.5	9.5	9.5	9.5	9.5
4	Saline	22	15.5	13	12.5	11.5	11.5	10.5	10.5	10.5
	Treated plasma	20.5	15	12	10.5	10	10	10	10.5	11.5
	AHG solution	18.5	11	11.5	11.5	11	9.5	9	9.5	9.5

* 0.1 ml. volumes of each reagent to be tested were added to the usual incubation mixture.

hydroxide-treated normal plasma and purified factor VIII to the standard incubation mixture.

Reagents: I. All normal reagents prepared as before.
2. Aluminium hydroxide-treated normal plasma.
3. A solution of purified factor VIII prepared as above.

Technique: The standard technique was first performed, 0.I ml. of saline being added to the usual reagents. This was then repeated, first with the addition of 0.I ml. of aluminium hydroxide-treated normal plasma, and then with the same volume of the solution of purified factor VIII. The results of 4 tests performed on successive days are shown in Table 40.

Results: Incubation mixtures containing pure factor VIII always had faster minimum substrate clotting times than the saline controls. In this series the minimum substrate clotting times in mixtures containing treated plasma, tended to be attained slightly sooner than in other mixtures. On the other hand, mixtures containing saline or pure factor VIII needed much the same periods of incubation to attain maximum thromboplastin formation. As in other cases, the minimum substrate clotting times in mixtures containing treated plasma were less well maintained than in the other mixtures tested. On the other hand the behaviour of mixtures containing pure factor VIII was identical with that of saline controls, for both showed the formation of a typical plateau of maximum thromboplastin formation, which was equally well maintained in both.

Discussion: The improved thromboplastin generation in the presence of purified factor VIII, supported the concept that similar improvement in mixtures containing aluminium hydroxide treated plasma was due to the latter's content of this factor and not factor V. In relation to doubt surrounding a possible "accelerator" action of factor V, it was noted that in this experiment all mixtures containing treated plasma attained

their minimum substrate clotting times slightly sooner than the others. This feature was not marked and while it might have been dependant on factor V, equally well, it might have been related to factor VIII for there was some evidence that the purified preparation of factor VIII itself accelerated thromboplastin formation. In incubation mixtures containing pure factor VIII the substrate clotting times were always faster than those obtained with mixtures containing saline after equal times of incubation, although ultimately the minimum substrate clotting times in both were reached at the same time. The presence of inhibitory substances in aluminium hydroxide-treated plasma was again demonstrated by the faster decline of substrate clotting times after the minimum had been reached, in incubation mixtures containing this reagent. This process was not active in the presence of purified factor VIII.

Thus while it had been possible to experiment with plasma deficient in factor VIII, and with a pure preparation of the same factor, it was unfortunate that no plasma specifically lacking factor V was to hand, nor were facilities available to produce a pure preparation of it. It will be recalled, however, that rabbit serum contains large amounts of factor V, and it was thought that this fact might be utilized to confirm the findings of some of the foregoing experiments. Aluminium hydroxide treatment of rabbit serum should remove any factor VII and factor X activity leaving behind factor V and any possible residual P.T.A. and Hageman factor. It had already been shown that P.T.A. did not influence the reaction and although it had not been possible to prove experimentally that Hageman factor similarly did not influence the reaction, it seemed probable that such was the case. Assuming this to be so, rabbit serum could be used to assess directly any activity of factor V. Experiment 4I was based on this consideration.

Experiment 4I. To assess the effects of adding aluminium

TABLE 41. To show the effects of aluminium hydroxide treated rabbit serum on incubation mixtures.

Test No.	Reagent added to standard incubation mixture*	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Saline	50	31.5	21	16.5	14	12	11.5	11.5	11.5
	Treated rabbit serum	49	33	22.5	20	16	14.5	13.5	14	14.5
	Treated plasma	24.5	18	14	12	11	10	10	10.5	11
2	Saline	65	-	-	-	27	16	12	10	10
	Treated rabbit serum	81	-	-	-	60	41.5	19	15	14.5
	Treated plasma	22	11	10	9.5	9	9	8.5	9	10
3	Saline	49	19	15	13	12.5	11.5	11.5	11.5	11.5
	Treated rabbit serum	37	16	14	13.5	13	13	13	13	14.5
	Treated plasma	16.5	10	9.5	9.5	10	10.5	11	14	14

* 0.1 ml. volumes of saline or each reagent to be tested were added to incubation mixtures.

hydroxide-treated rabbit serum to the incubation mixture.

Reagents: I. All normal reagents prepared as before.
2. Rabbit serum incubated on the clot for 4 hours at 37°C.
3. Aluminium hydroxide-treated normal plasma.

Technique: After incubation the rabbit serum was treated with aluminium hydroxide in the same manner as normal plasma. Following this all the normal reagents with the addition of 0.I ml. of saline were incubated in the usual way as a control test. The procedure was then repeated firstly with the addition of 0.I ml. of treated rabbit serum and secondly with 0.I ml. of treated plasma in lieu of saline. The results of 3 experiments performed on successive days are shown in Table 4I.

Results: As in former experiments, the minimum substrate clotting times obtained with mixtures containing aluminium hydroxide-treated plasma were always faster than those obtained with saline controls. Whenever treated rabbit serum was added to incubation mixtures however, the minimum substrate clotting times were some seconds slower than those obtained with saline controls.

Discussion: It is reasonable to assume that rabbit serum, as used in this experiment, contained not inconsiderable amounts of factor V. This being so, this experiment demonstrated that added factor V by itself was unable to improve thromboplastin formation in the reaction being considered, and in the negative sense, confirmed the importance of factor VIII in this respect. The actual interference with thromboplastin generation in the presence of this reagent could only be explained on the basis that inhibitory factors were introduced at the same time. The similarity between this process and the action of aluminium hydroxide-treated haemophilic plasma was immediately apparent. It seemed likely that the effect of both normal and haemophilic aluminium hydroxide-treated plasma introduced into the usual incubation mixture, was dependent upon a state of equilibrium between increased thrombo-

plastin formation (due to their content of factor VIII) and neutralization secondary to the simultaneous introduction of inhibitory factors. When normal plasma was treated with aluminium hydroxide thromboplastin formation outweighed neutralization and faster minimum substrate clotting times resulted. When haemophilic plasma was used, however, thromboplastin generation was less and the presence of even normal amounts of inhibitory substances was made manifest in proportionately slower substrate clotting times, by comparison with the corresponding normal reagent. Where the levels of factor VIII were very low, the introduction of inhibitory factors might be sufficient to prolong the substrate clotting times beyond those obtained with saline controls.

The presence of inhibitory factors as demonstrated in rabbit serum, posed the question of the occurrence of such factors in human serum and their possible effects on the generation of thromboplastin in mixtures containing platelets, brain residue, calcium chloride and serum. Should such factors have been present in serum as used in the standard technique, here too, any minimum substrate clotting time would reflect a state of balance between thromboplastin formation and neutralization. In instances where thromboplastin generation was impaired, say, by the use of platelets defective in thromboplastic function, here again any inhibitory factors would have been present in relative excess, and falsely low estimations of platelet thromboplastic function would result. Earlier experiments, in which the formation of a plateau of maximum thromboplastin generation was discussed, had suggested that inhibitory factors were present in negligible amounts in the standard incubation mixture. The possible implications of these factors, however, warranted clarification. The problem was approached in experiment 42.

Experiment 42. To determine the effects of introducing fresh

TABLE 42. To show the effects of adding aluminium hydroxide treated "fresh" human serum to incubation mixtures.

Test No.	Reagent added to standard incubation mixture*	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Saline	24.5	14	11.5	10.5	10	10	10	10	10
	Treated serum	32.5	14.5	12.5	12.5	12	14	15.5	16	19.5
2	Saline	33	24	18	16	14	12	12	12	12
	Treated serum	38	23	16.5	14	13	12	12	13	13
3	Saline	23	15	12	11.5	11	11	11	11	11
	Treated serum	23	15	13	12	12	12	12	13	14
4	Saline	23	16	14	12.5	11	11	11	11	12
	Treated serum	30	17	14	13	12.5	12	13	14	14
5	Saline	40	21	15	13	12	11	10	10	10
	Treated serum	23	18	15	14	13	13	13.5	15	16.5

* As for Table 41.

human serum treated with aluminium hydroxide into the standard incubation mixture.

Reagents: I. All normal reagents prepared as for use in the standard technique.

2. Human serum incubated on the clot for 3 hours at 37°C.

Technique: After incubation the "fresh" serum was treated with aluminium hydroxide in the same way as plasma. As a control test 0.I ml. of saline was added to a standard incubation mixture and the usual procedure followed. This was followed by the test mixture in which 0.I ml. of the treated serum was substituted for the saline. The results of 5 successive tests are shown in Table 42.

Results: In four of the tests the addition of relatively small amounts of aluminium hydroxide-treated fresh human serum to the incubation mixture, was accompanied by reduced thromboplastin formation. So too, the usual "plateau" formation was lost, in so far as the minimum substrate clotting times, once reached, declined rapidly.

Discussion: The results left no doubt that, even after 3 hours incubation on the clot at 37°C., human serum retained factors capable of neutralizing thromboplastin directly or of accelerating its breakdown or both. In these tests the addition of treated serum prolonged the minimum substrate clotting times by I to 2 seconds, a degree of interference that certainly warranted further investigation. The principal concern was to see if serum after I8 hours incubation, that is, serum prepared as for use in the standard technique, retained any significant degree of "inhibitory" activity.

Experiment 43. To determine residual "inhibitory" activity in serum after incubation for I8 hours at 37°C.

TABLE 43. To show the effects of adding serum treated with aluminium hydroxide after 18 hours incubation at 37°C. to incubation mixtures.

Test No.	Serum * Preparation	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	Control	30	15	10	9	8.5	8.5	8.5	8.5	8.5
2	Serum 1	18.5	12	10.5	9	9	10	11.5	11.5	13
3	Serum 2	11.5	10	10	9	9	10	11.5	13	13.5
4	Serum 3	17	10.5	8.5	9.5	8.5	9	11.5	13.5	13.5
5	Serum 4	12.5	10.5	9	9	9.5	10.5	11.5	11.5	13.5

* 0.1 ml. volumes of saline or test serum were added to incubation mixtures.

Reagents: I. Platelets, brain residue suspension and calcium chloride prepared as for use in the standard technique.

2. Serum samples collected from 5 normal donors and incubated on the clot for I8 hours at 37°C.

Technique: Of the five serum preparations, one was set aside to be used as the routine specimen for the day's work. The remaining four were all treated with aluminium hydroxide. A control test was performed as in experiment 38, using the usual quantities of all normal reagents, plus 0.I ml. of saline. Subsequently, the procedure was repeated four times, 0.I ml. of one of the aluminium hydroxide-treated serum preparations being substituted for the saline in each test. The results are shown in Table 43.

Results: The minimum substrate clotting time of the control test was 8.5 seconds. This time was maintained as the usual plateau of maximum generation for at least 20 minutes. In mixtures containing added aluminium hydroxide-treated serum, the minimum substrate clotting times varied between 8.5 and 9 seconds, but in all cases these times were poorly maintained, and after I5-20 minutes incubation, progressive lengthening was evident.

Discussion: These results showed that, after I8 hours incubation at 37°C., human serum retained no, or at the most, very small amounts of inhibitory factors, and it was plain that any fallacies in the technique due to their presence would be negligible. The addition of aluminium hydroxide-treated serum to incubation mixtures, while not capable of inducing significant changes in the minimum substrate clotting times, was not without effect, however. Loss of the usual "plateau" formation suggested that this reagent retained a factor capable of accelerating thromboplastin breakdown. In the standard procedure, the use of 0.I ml. of serum introduced so little of this factor that "plateau" formation was possible. The increased amount of this factor, however, added with treated serum was sufficient to tip the balance in the direction of rapid destruction of formed thromboplastin. Indeed,

the reverse process was evident in experiment 26. In this it was found that the higher the dilution of serum used in the standard technique, the longer was the time during which maximum thromboplastin generation was maintained. Overall, the results of the last two experiments suggested that inhibitory factors might exist in two forms. One of these was capable of causing direct neutralization of formed thromboplastin, the other of accelerating its rate of breakdown. These findings opened an interesting and poorly understood field, the role of inhibitory factors in coagulation. Further preliminary work in this field will be outlined in a later section.

Part IV. The Behaviour of Pathological Platelets.

While the foregoing work had established and elucidated a mechanism of thromboplastin formation in a system containing a suspension of brain residue, platelets, serum and calcium chloride, and while possible sources of error had been investigated and minimised, it remained to be shown that platelets defective in thromboplastic function behaved in the same way as normal platelets in the same system. Of fundamental importance it was essential to determine whether the same principles established for the assay of thromboplastic function of normal platelets applied to pathological platelets. Since it was considered that the main application of this work would lie in the assessment of platelet thromboplastic function in primary and secondary thrombocytopaenic states, as suitable examples from each group presented, their platelet thromboplastic function was assayed.
Experiment 44 describes this work.

Experiment 44. Assays of thromboplastic function using defective platelets.

Reagents: I. All normal reagents prepared as before.

2. Preparations of defective platelets. The technique used to prepare concentrates of pathological platelets was identical with that used for normal platelets. The utmost care was taken to ensure the accuracy of the 'low' spun plasma platelet counts. Basically the technique of these counts was the same as that used for the corresponding normal counterparts, with the added precautions that, if from prior knowledge of the whole blood platelet counts, very low values were expected, the final dilution in the plasma counts was adjusted appropriately in an attempt to increase their accuracy.

TABLE 44. Assays of thromboplastic function of pathological platelets.

Test No.	Disease	Platelet Concentration ($X10^6$)	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
			4	10	15	20	25	30
1	Acute leukaemia	200	39	20	18.5	17	17	17
		100	39.5	25.5	21.5	19	19	19.5
		50	43.5	31.5	24.5	22	22	22.5
2	Aplastic anaemia	200	50	29.5	26.5	22.5	23	23
		100	> 60	33	-	25	25	25
		50	> 60	> 60	-	27	27	28
3	Aplastic anaemia	200	43.5	24	20.5	20.5	20.5	20.5
		100	43	26	24.5	22.5	23	23
		50	54	39	30	25.5	26	26
4	Acute idiopathic thrombocytopaenic purpura	200	34	18	14.5	14.5	15	14.5
		100	41	20	16.5	16	16	16.5
		50	48	24.5	20	19.5	19.5	19

TABLE 44. Cont'd.

Test No.	Disease	Platelet Concentration ($X10^6$)	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
			4	10	15	20	25	30
5	Chronic idiopathic thrombocytopaenic purpura	200	>60	29.5	25	25	25	25
		100	>60	27.5	27	27	27	27
		50	>60	46	33	30.5	30.5	30.5

Technique: Prior to any test using pathological platelets, the normal procedure was carried out using all normal reagents to ensure that the suspensions of residue and serum were behaving normally. After this, as in the assays of normal platelet function, the procedure was repeated using concentrates of $200xI0^6$, $I00xI0^6$ and $50xI0^6$ pathological platelets in turn. The results of 5 experiments using platelets from a representative group of primary and secondary thrombocytopaenic diseases are shown in Table 44.

Results: In the 5 cases studied the differences in minimum substrate clotting times obtained with $200xI0^6$ and $I00xI0^6$ platelets were 2, 2,2.5,I.5 and 2 seconds, and between $I00xI0^6$ and $50xI0^6$ platelets 3,2,3,3.5 and 3.5 seconds.

Discussion: In experiment 2I it was established that in this technique of assessing platelet thromboplastic function, if the proportions of all other reagents were kept constant, a definite constant relationship existed between the differences in substrate clotting times obtained with varying concentrations of platelets. This relationship was maintained no matter what preparations of brain residue and serum were used. The substrate clotting times to be expected with concentrations of $I00xI0^6$ and $50xI0^6$ platelets could be expressed by the equations $(X+2)$ and $(X+5)$ seconds respectively when X equalled the minimum substrate clotting time obtained with $200xI0^6$ of the same platelets. In the 5 tests shown in experiment 44, in their order of performance, the minimum substrate clotting times obtained with $200xI0^6$ platelets were I7,23,20.5,I4.5 and 25 seconds. Should the same relationships pertaining to the substrate clotting times in assays of normal platelets apply to pathological platelets, on theoretical grounds one would have expected the minimum substrate clotting times obtained with $I00xI0^6$ of these platelets to have been I9,25,22.5, I6.5 and 27 seconds and with $50xI0^6$ platelets 22,28,25.5,I9.5 and 30 seconds respectively. The corresponding times obtained in practice were I9,25,23,I6 and 27 seconds with $I00xI0^6$

platelets and, with $50xIO^6$ platelets, 22,27,26,I9.5 and 30.5 seconds. It was quite clear therefore that pathological platelets behaved in assay procedures in the identical way of normal platelets, and the same principles applied equally well to both. Furthermore, it was shown in this experiment that pathological platelets from a wide range of primary and secondary thrombocytopaenic states all conformed to the same pattern and it was assured that with every justification one could assess the relative function of any unknown sample of pathological platelets by reference to the appropriate calibration graph of a concentration of normal platelets tested in parallel with the unknown.

A second problem stemmed from the fact that normal platelets have considerable amounts of factor V adsorbed onto their surface. Earlier experiments suggested that if factor V played any part in the reaction being studied, that amount introduced into the system with platelets was more than adequate. While the addition of aluminium hydroxide-treated plasma to incubation mixtures improved thromboplastin generation, this was due to its content of factor VIII only. The possibility that defective platelets might adsorb less factor V than normal posed the problem that should factor V play any part in the reaction, impaired thromboplastin generation in the presence of pathological platelets might result from the dual effects of reduced thromboplastic factor and reduced factor V. Should this be so, any estimate of platelet thromboplastic function could be falsely low and indeed reflect the dual defects of thromboplastic factor and factor V. It was thought that should estimations of thromboplastic function of pathological platelets represent this dual defect, then relatively greater improvement in thromboplastin generation should be obtained on the addition of aluminium hydroxide-treated plasma to incubation mixtures containing pathological platelets than was evident in corresponding mixtures containing normal platelets, for in the latter only factor VIII would be utilized while in the former both factor VIII and factor V would participate. This

TABLE 45. To show the effects of adding aluminium hydroxide treated normal plasma to incubation mixtures containing either normal or pathological platelets.

Test No.	Normal or Pathological Platelets	Reagent added to Incubation Mixture	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
			2	4	6	8	10	15	20	25	30
1	Normal	Saline	28	21	15	14	13	11	11	11	11
		Treated plasma	14	10	10	10	10	9	9	9	9
	Pathological Ac.leukaemia	Saline	30	21	18	16	14.5	13.5	13	13	13
		Treated plasma	24	14.5	13	12	12	11	11	11	11
2	Normal	Saline	25	20	16	13.5	12.5	11.5	11.5	11.5	11.5
		Treated plasma	17.5	11.5	11.5	10.5	10	10	10	11	11.5
	Pathological Aplastic anaemia.	Saline	57	43.5	34	26	24	20.5	20.5	20.5	20.5
		Treated plasma	55	37.5	27	20.5	18.5	19.5	20	21.5	23
3	Normal	Saline	28.5	15.5	11.5	11	11	11	11	11	11
		Treated plasma	36	14	11	10	9.5	9.5	10.5	10.5	11.5
	Pathological Acute I.T.P.	Saline	37	18	15	15	14.5	14.5	14.5	14	15.5
		Treated plasma	27	16	13	12.5	12.5	12.5	13	14	15

TABLE 45. Cont'd.

Test No.	Normal or Pathological Platelets	Reagent added to Incubation Mixture	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
			2	4	6	8	10	15	20	25	30
4	Normal	Saline	52	25.5	15.5	13	12	12	12	12	12
		Treated plasma	35	15	12.5	11.5	10	9.5	9.5	9.5	10
	Pathological Chronic I.T.P.	Saline	>60	>60	45	38	29.5	25	25	25	25
		Treated plasma	>60	50.5	29	26	23	23.5	23	24.5	25
5	Normal	Saline	24.5	18	14	12	11	10	10	10	10
		Treated plasma	10	9	9	8.5	8	8	9	10	10
	Pathological Lympho - sarcoma.	Saline	31	15.5	15	13.5	12.5	12.5	12.5	12.5	13
		Treated plasma	43	18	15	12	11.5	11	11	12.5	14

problem was studied in experiment 45.

Experiment 45. To assess the relative effects of the addition of aluminium hydroxide-treated plasma to incubation mixtures containing normal and pathological platelets.

Reagents: I. All normal reagents prepared as before.
2. Concentrations of pathological platelets.
3. Aluminium hydroxide-treated normal plasma.

Technique: The usual control experiment using all normal reagents plus 0.I ml. of saline was first performed. The procedure was then repeated using in turn, $200xI0^6$ normal platelets plus 0.I ml. aluminium hydroxide-treated plasma, $200xI0^6$ defective platelets plus 0.I ml. of saline and finally $200xI0^6$ defective platelets plus 0.I ml. of aluminium hydroxide-treated plasma. The results of 5 experiments using defective platelets obtained from a range of primary and secondary thrombocytopaenic states are shown in Table 45.

Results: Whether normal or defective platelets were used in incubation mixtures, faster minimum substrate clotting times followed the introduction of aluminium hydroxide-treated plasma. In the order in which the tests were performed, the differences in minimum substrate clotting times between mixtures containing normal platelets and either saline or 'treated' plasma were 2, I.5,I.5,2.5 and 2 seconds respectively. In mixtures containing defective platelets the corresponding times were 2,I,2,2 and I.5 seconds.

Discussion: In every case the improvement in substrate clotting times on the addition of aluminium hydroxide-treated plasma to incubation mixtures containing defective platelets was of the same order as that obtained in the corresponding mixtures containing normal platelets. In no instance was the improvement in substrate clotting times considerably greater on the addition of 'treated' plasma to pathological platelets than that obtained

with the corresponding normal controls. The findings suggested that, as was the case with normal platelets, complete utilization of the thromboplastic component of pathological platelets was attained in this technique, without the need for additional factor V. These results could be interpreted in another way. In examples 2 and 4, although the minimum substrate clotting times obtained with pathological platelets were greatly in excess of those obtained with the normal controls, much the same degree of improvement was obtained in both on the addition of 'treated' plasma. This suggested that if an assay procedure was performed with varying concentrations of platelets, and then repeated with the addition of aluminium hydroxide-treated plasma to each concentration, the same degree of improvement in substrate clotting times would be obtained with each concentration. Two parallel calibration graphs would result. Applying this consideration to the results of this experiment, the relative thromboplastic functions of the pathological platelets in mixtures containing saline, interpreted from the appropriate calibration graph of the corresponding normal platelets in saline mixtures, would have been 48,I7,34,I2 and 42 percent respectively. If the function of the same platelets in mixtures containing 'treated' plasma was assessed from the revised calibration graphs appropriate to the substrate clotting times of the corresponding normal platelets in mixtures containing treated plasma, the results become 48,I6,37,I2 and 37 percent. This interpretation makes it even more apparent that the thromboplastic function of pathological platelets was not enhanced to any extent more than that of normal platelets by the addition of aluminium hydroxide-treated plasma. It was concluded therefore, that the function of pathological platelets, as measured by this technique was a true indication of their thromboplastic function alone, and did not reflect the combined defects of thromboplastic factor and reduced factor V. As a corollary of this it could be said that if factor V was required for the reaction, as had been the case with normal platelets, sufficient was introduced

into the system with pathological platelets to promote the maximum thromboplastin generation of which they were capable. Further, since the pathological platelets used in this experiment were representative of a range of both primary and secondary thrombocytopaenic states, the same principles must apply to all the common types of platelet dysfunction.

The standard technique used throughout this work had been based on the use of concentrates containing $200xI0^6$ platelets, this corresponding to a final concentration of approximately $I00xI0^3$ platelets per cubic millimetre of incubation mixture. With this dilution, convenient substrate clotting times usually of the order I0 to I3 seconds were consistently obtained. The application of the technique to the assessment of the thromboplastic function of pathological platelets necessitated consideration of the concentration of platelets used in relation to 3 factors. I). It was possible that increased accuracy could be attained by the use of higher concentrations of platelets. 2). As in other coagulation procedures, sensitivity would possibly be reduced if high concentrations of platelets were used. 3). In severely thrombocytopaenic patients it was often difficult to obtain sufficient platelets to perform single estimations even when $200xI0^6$ platelets were used. In experiment 46 attempts were made to clarify some of these points.

Experiment 46. To assess the effects of increasing the concentrations of platelets used in the standard procedure.

Reagents: All normal reagents prepared as before.

Technique: Volumes of 'platelet rich' plasma calculated to yield $200xI0^6$, $400xI0^6$, $600xI0^6$ and $I200xI0^6$ platelets were centrifuged at 3000r.p.m. for I5 minutes and washed in the usual way. Subsequently the standard volumes of all other reagents were added to the various platelet concentrates and the usual procedure

TABLE 46. To show the effects of using increased concentrations of platelets in incubation mixtures.

Test No.	Platelet Concentration ($X10^6$)	Time of Subsampling In Minutes Substrate Clotting Time In Seconds					
		4	10	15	20	25	30
1	200	12	9.5	9.5	8.5	8.5	8.5
	400	10.5	8.5	7.5	7.5	7.5	7.5
	600	10	7.5	7.5	7.5	7	7
	1,200	9	7	6.5	6.5	6.5	6.5
2	200	26	15	11.5	11	11	11
	400	19	11.5	10.5	9.5	9.5	9.5
	600	15	10	8.5	8.5	8.5	8.5
	1,200	14	9	7.5	7.5	7.5	7.5
3	200	18	12.5	11	11	11	11
	400	16	10	9	9	9	9
	600	10.5	8	7	7	7	7
	1,200	9	7	6	7	6	6
4	200	18	12	10.5	10.5	10.5	10.5
	400	12	9	9	9	9	9
	600	10	8.5	8	8	8	8
	1,200	9	7	7	7.5	7	7

followed. The results of 4 such test are shown in Table 46.

Results: In every test, the greater the concentration of platelets used, the faster were the minimum substrate clotting times obtained. The higher the concentration of platelets used, however, the differences in substrate clotting times in relation to the number of platelets used, became less marked until at concentrations of 1200×10^6 platelets, the substrate clotting times were only one second faster than when 600×10^6 platelets were used.

Discussion: Obviously at higher concentrations of platelets than those used routinely, sensitivity was greatly reduced. The difference in substrate clotting times obtained with an additional 600×10^6 platelets, between the concentrations 600×10^6 and 1200×10^6 platelets was only one second, whereas, when 200×10^6 and 100×10^6 platelets have been used, the difference of only 100×10^6 platelets has invariably been associated with differences in substrate clotting times in the order of 2 seconds. The use of platelet concentrations of 200×10^6 and less, then, was associated with the most sensitive part of the calibration graph wherein lesser reductions in platelet numbers (and thereby in thromboplastic function) were associated with relatively greater differences in substrate clotting times. It was much more likely, therefore, that small but significant differences in platelet thromboplastic function would be evident if concentrations of 200×10^6 platelets and less were used, and it was decided to continue to use the technique as formerly standardized, when pathological platelets were to be tested.

A second feature strongly in favour of using this concentration of platelets stemmed from the difficulty of obtaining higher concentrations of platelets in severely thrombocytopaenic subjects. For a single estimate by the new technique, if the plasma platelet count was 20,000 per c.mm., 10 ml. of plasma would be needed to obtain the necessary 200×10^6 platelets. In adults this usually poses no problem, but in children, considerable difficulty may be experienced in obtaining sufficient blood, and such difficulty would be greatly magnified should higher concentrations be needed.

TABLE 47. To show the effects of reducing all reagents in proportion in estimating the thromboplastic function of pathological platelets.

Test No.	Platelet Concentration ($X10^6$)	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Minimum Substrate Clotting Time of normal
		4	10	15	20	25	30	
1	200	25	15	13.5	13.5	13	13	
Chronic	100	25	15	13.5	13.5	13.5	13.5	9 secs.
I.T.P.	50	-	-	15	14	13.5	13.5	
2	200	18	13	12	12	12	12	
Uraemia	100	21	13.5	12	12	12	12	10 secs.
	50	-	-	12	12	12	-	
3	200	49	17	13	11.5	11.5	11.5	
Lympho-	100	30	13	11.5	11	11.5	11.5	10 secs.
sarcoma	50	-	-	12	11.5	11.5	11.5	
4	200	49	17	15	14	14	14	
Acute	100	49	19	17	15.5	14	14.5	11.5 secs.
Leukaemia	50	-	-	16.5	14.5	14	14	

Indeed, even in adults, repeated venipunctures for relatively large volumes of blood are unpleasant and worrying. Accordingly in experiment 47, tests were undertaken to assess the reproducibility of results when all reagents were reduced in proportion, in the assessment of the function of pathological platelets.

Experiment 47. To assess the reproducibility of the results of the present technique when all reagents are reduced in proportion.

Reagents: 1. All normal reagents prepared as before.
2. Platelet 'rich' plasma obtained from patients with known thromboplastic defects.

Technique: As usual, a control test, using all normal reagents was first performed. The procedure was then repeated using in turn $200xI0^6$, $I00xI0^6$ and $50xI0^6$ pathological platelets, all other reagents being reduced in proportion to the platelet concentration. This experiment was repeated 4 times using platelets obtained from 4 cases of thrombocytopaenia of differing aetiologies. The results are shown in Table 47.

Results: In each of the 4 preparations of defective platelets tested, the estimates of thromboplastic function did not differ significantly between the 3 concentrations of platelets used.

Discussion: These results demonstrated that in any instance where difficulty existed in obtaining $200xI0^6$ platelets, reliable results could be obtained by using lesser concentrations of platelets and reducing the volumes of all other reagents in proportion to the concentration of platelets available.

Two further experiments concluded this series. It has been thought that erythrocytes might have some thromboplastic activity. In this work, when platelet concentrates had been prepared from thrombocytopaenic subjects, it was often found that the final platelet concentrates contained many erythrocytes. It was thought essential to determine whether their presence contributed anything to the estimates of platelet thromboplastic function as determined

by this technique. Experiment 48 describes this work.

Experiment 48. To gauge any thromboplastic activity inherent in erythrocytes.

Reagents: I. All normal reagents prepared as before.

2. A suspension of normal red cells in saline. After separation of the platelet 'rich' plasma from the specimen of whole blood, the remaining concentrate of red cells in plasma was centrifuged for a further I5 minutes at 3000r.p.m. After the supernatant platelet 'poor' plasma had been removed, an aliquot of the red cell mass was washed 5 times with an excess of normal saline. After the final wash, the red cells were suspended in a volume of normal saline, evenly distributed by rotation on a mechanical mixer, and a red cell count was performed on the final suspension.

Technique: For this experiment two control tests were performed, the one being the standard procedure using all normal reagents, while in the other while the technique remained the same as for the usual procedure, the incubation mixture contained a suspension of brain residue, serum and calcium chloride only. To assess any thromboplastic activity of erythrocytes, two volumes of the red cell suspension, each calculated to contain 200×10^6 cells, were centrifuged at 3000r.p.m. for I5 minutes. The supernatant saline was removed from both concentrates after which, in one the erythrocytes were retained intact while in the other the cells were lysed by repeated freezing and thawing in an alcohol-dry ice mixture. Subsequently standard volumes of a suspension of brain residue, serum and calcium chloride were added to each red cell preparation, and incubation at 37°C and subsampling were carried out in the way adopted for similar mixtures containing platelets. The results of 3 such experiments are shown in Table 48.

TABLE 48. To show the thromboplastic activity of erythrocytes in the technique described.

Test No.	Incubation Mixture*	Time of Subsampling In Minutes Substrate Clotting Time In Seconds								
		2	4	6	8	10	15	20	25	30
1	1	>60	45	29	19	16	14	13.5	13	13.5
	2	77	65	64	60	54	50	45	45	45
	3	98.5	76	66	61	55	51	49	45	45
	4	79	68	65	57	51.5	45	39	38	37.5
2	1	-	46	-	-	17	12	11	11	11
	2	>120	>105	80	80	80	64	54	52	54
	3	>120	>120	85	77	77	60	55	52	52
	4	>120	>120	100	78	66	59	53.5	50	43
3	1	53	34	23	18.5	15.5	12	10.5	10.5	10.5
	2	>120	>120	87	81	73	62	62	52	50
	3	77	75	75	71	69	66	63	60	53
	4	>102	89	69	69	69	47.5	44	44	44

* 1 Brain residue, platelets, serum and calcium chloride.
* 2 Brain residue, serum and calcium chloride
* 3 Brain residue, intact red cells, serum and calcium chloride.
* 4 Brain residue, frozen and thawed red cells, serum and calcium chloride.

Results: In each case thromboplastin formation was rapid and marked in incubation mixtures containing platelets. In control mixtures containing brain residue, serum and calcium chloride, thromboplastin formation was negligible. In incubation mixtures containing intact red cells, thromboplastin formation was no greater than in the latter mixtures. In mixtures containing thawed red cells, the substrate clotting times were a few seconds faster than those obtained with the intact cells, but again thromboplastin formation was so slight as to be negligible.

Discussion: The concentration of erythrocytes used in the experiment was far in excess of the number one might expect to 'contaminate' a platelet concentrate. The thromboplastin generated by this relatively large number of red cells however, was so slight that there could be no doubt that in the estimations of platelet thromboplastic function by this technique no significant error was introduced by the presence of erythrocytes.

A final experiment stemmed from conversations with those who have had considerable experience with the modified thromboplastin generation test of Biggs and Douglas, as adapted by Bonnin to quantitate platelet thromboplastic function. These workers had found that the amount of thromboplastin generated in such a system, was sometimes increased if platelets were frozen and thawed prior to incubation with the other reagents. Should this be so, it would seem that such estimations using intact platelets were not measuring their total thromboplastic efficiency. It was decided therefore, to compare identical preparations of intact and thawed platelets, both normal and pathological, to determine whether the present technique as used to date, gave a true estimate of the total thromboplastic activity of platelets or whether additional latent activity might be made apparent by freezing and thawing.

Experiment 49. To assess the effects of freezing and thawing platelets prior to incubation with brain residue, serum and calcium chloride.

TABLE 49. To show the thromboplastic function of 'intact' and 'frozen and thawed" normal and pathological platelets.

Test No.	Normal or Pathological Platelets.	Intact or Frozen and Thawed Platelets.	Time of Subsampling In Mins. Substrate Clotting Time In Secs.						Percentage Function
			4	10	15	20	25	30	
1	Normal Control	Intact	25.5	14	12	12.5	12	12	100
		Frozen & Thawed	19	14	12.5	12	12	12	100
	Pathological Uraemia	Intact	27	20	13	13	13	13	62
		Frozen & Thawed	23	14	13	13	12.5	13	62
	Pathological Lympho - sarcoma	Intact	59	45	34.5	30.5	30	31	9
		Frozen & Thawed	62	35	28	28	28	29	10
2	Normal Control	Intact	24.5	13.5	12.5	12	11	11	100
		Frozen & Thawed	22	13	12.5	11	11	11	100
	Pathological Ac.leukaemia	Intact	57.5	26.5	19.5	18.5	18	18	20
		Frozen & Thawed	28	18	18.5	18	18	18	20

TABLE 49. Cont'd.

Test No.	Normal or Pathological Platelets.	Intact or Frozen and Thawed Platelets.	Time of Subsampling In Mins. Substrate Clotting Time In Secs.						Percentage Function
			4	10	15	20	25	30	
3	Normal Control	Intact	42	14.5	11.5	11.5	11.5	11.5	100
		Frozen & Thawed	37	14.5	11.5	11.5	11.5	11.5	100
	Pathological Chronic I.T.P.	Intact	27	15	13	13	13	13	53
		Frozen & Thawed	32	15.5	13	12.5	12.5	12.5	62
	Pathological Aplastic anaemia	Intact	53.5	31	25.5	24	24.5	24.5	12
		Frozen & Thawed	47	27	26	24.5	25	26	12

Reagents: I. All normal reagents prepared as before.

2. Platelet 'rich' plasma prepared from patients with known platelet defects.

Technique: Duplicate platelet concentrates of 200×10^6 platelets were prepared from both normal and pathological platelet samples in the usual way. One of each pair of concentrates was incubated with a suspension of brain residue, serum and calcium chloride as in other tests. The other concentrate of each pair was frozen and thawed several times in an alcohol-dry ice mixture, before being treated in the same way as its 'intact' counterpart. The results of 3 experiments in which 5 preparations of different pathological platelets were tested, are shown in Table 49.

Results: The substrate clotting times obtained with normal platelets were the same whether the platelet concentrates were frozen and thawed prior to use, or whether they were used in an intact state. In two examples faster minimum substrate clotting times were obtained when the pathological platelets were frozen and thawed prior to use, but overall this improvement made little difference to the final estimated thromboplastic function.

Discussion: The results of this experiment demonstrated beyond doubt that for practical purposes the present technique measured all the available thromboplastic component of the platelets used, and was therefore, a reliable estimate of total thromboplastic efficiency.

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Part V. The Assay of Factor X.

When it was first found that both factor VII and factor X influenced thromboplastin formation in the system being studied, it was thought that it would not be possible to modify the reaction to assay factor X. Subsequently a more detailed analysis of the data suggested that while it might not be a perfect system, the reaction might be used to obtain an approximate working estimate of factor X in serum when other methods were not available, and that perhaps such estimates might be a little less accurate than those obtained by other techniques.

It has been shown already (Section 2) that when 0.I ml. volumes of serum and subsequent dilutions of this volume are used it is only dilution of factor X that affects the reaction until the concentration of factor VII approaches I0 percent of that normally present. Furthermore, the major function of factor X is to accelerate the reaction and until its concentration is reduced to 20-30 percent of that normally present, the degree of thromboplastin formation is not affected but the rate of formation is markedly so. In other words, if one arbitrarily assumes 0.I ml. of serum to provide I00 percent "serum activity", in the range I00 to I0 or I5 percent serum concentration, any alteration in the reaction either in rate or degree, reflects the activity of factor X alone.

Furthermore, earlier experiments had suggested that in any modification of the reaction to assay factor X, an optimum concentration of serum was twice that used in the assay of platelet thromboplastic function. If then one considered 0.2 ml. of serum in combination with I.0 ml. of a suspension of brain residue, $200 \times I0^6$ platelets and I.0 ml. of calcium chloride as I00 percent "serum factor" activity, in a range of serum concentrations I00 to I5-I0 percent, only factor X would affect the reaction. So too, in this range of concentrations the effect of diluting serum would be or should be to slow the rate of thromboplastin formation, and only at lesser concentrations would the degree as well as the

rate of thromboplastin formation be affected.

It seemed a reasonable assumption therefore that with some justification the basic procedure could be modified to quantitate factor X and that to do this the volume of serum used should be doubled in proportion to the other reagents. A calibration graph could be constructed by testing a series of dilutions of normal serum and timing accurately the incubation times required to reach maximum thromboplastin generation in each. Such a calibration graph should be an accurate reflection of factor X activity until the dilution of serum and, therefore, factor VII reached approximately 10 percent after which inaccuracy would ensue from the combined effects of factor VII and factor X deficiencies.

While recognizing this error it was considered worthwhile to pursue this work, for at present there is no readily available method to assay factor X. "Prothrombin" and "Stypven" time estimations will both detect deficiencies of factor X but neither can be used to quantitate the defect unless a supply of plasma specifically deficient in this factor is available for use as a diluent in the construction of a calibration graph. Because congenital deficiencies of factor X are rare such plasma is usually not readily available nor is the artificial preparation of such a reagent a simple matter. For these reasons a quantitative assessment of factor X is a restricted procedure. In relation to the error that might ensue in the production of a calibration graph in the method proposed, it must be remembered that congenital deficiencies of factor X are rarely absolute and the use of such plasma in the production of calibration graphs must introduce an error in other techniques.

The first experiment in this section describes the technique for the estimation of platelet thromboplastic function as modified to quantitate factor X.

Experiment 50. Technique for the assay of factor X.

TABLE 50. Part (1). Assays of factor X using 0.1ml. volumes of normal serum.

Time of Sub-sampling in minutes.	Test Number And Serum Dilution							
	1				2			
	F.S.	1:2	1:4	1:5	F.S.	1:2	1:4	1:5
2	60	-	-	-	> 60	-	-	-
4	31	61	-	-	45	56	55	-
6	18	-	-	-	26	-	-	-
8	15	-	-	-	21	-	-	-
10	12	23	30	33	17	30	36.5	43
15	11	17	-	-	12.5	19	30	-
20	11	14	19	22	12.5	18	26.5	33
25	11	13.5	-	-	12.5	14	21.5	-
30	11.5	12	16.5	18	12.5	12.5	19.5	27
35	12	12	-	-	12.5	12	17.5	-
40	13	12	14.5	16	13.5	12	15	21
45	-	11.5	-	-	13.5	12	14	-
50	13.5	12	13	14.5	13.5	12	14	18
55	-	12	12	-	-	-	12.5	15
60	14.5	12	12	13	14	12	12	13.5
65	-	12	12	13	-	-	12	13
70	-	12	12	13	-	-	12	13
75	-	-	-	12.5	15	12	12	12.5
80	-	-	12	12	-	-	12	12
85	-	-	-	12	-	-	-	12.5
90	-	-	12	12	-	-	12	12.5
95	-	-	-	-	-	-	-	-
100	-	-	12	12	-	-	-	-
105	-	-	-	-	-	-	-	12
110	-	-	12	12	-	-	-	12.5
120	-	-	12	12	-	-	-	12.5
130	-	-	-	-	-	-	-	-
140	-	-	-	-	-	-	-	-
150	-	-	-	-	-	-	-	-

TABLE 50. Part (1). Cont'd.

Test Number And Serum Dilution							
3				4			
F.S.	1:2	1:4	1:5	F.S.	1:2	1:4	1:5
67	-	-	-	-	-	-	-
33	48	66	60	35	39	-	-
21.5	-	-	-	-	-	-	-
19	-	-	-	-	-	-	-
17	29	45	45	14	21	35	44
13	22	-	-	12.5	15.5	-	-
11.5	19	34	35	11.5	14	25	32
11	18	-	-	11	14	-	-
11.5	16	27	31	11.5	12.5	20	26.5
11	15	-	-	12	11.5	-	-
11.5	14	28	36	12.5	11	18.5	21
12.5	13.5	-	-	13.5	11	17	-
-	13	23	27	-	11	16.5	20
-	13	-	-	-	11	16	-
-	13	22	25	-	11	16	18.5
-	13	-	-	-	-	16	18
-	-	20	21.5	-	-	16	18
-	13	-	-	-	-	15.5	18
-	-	18	19	-	-	-	17.5
-	13	-	-	-	-	15	17.5
-	-	17	19	-	-	15	-
-	-	-	-	-	-	15	16
-	-	16.5	18.5	-	-	-	-
-	-	-	-	-	-	15	-
-	-	16.5	18.5	-	-	-	16
-	-	16	17	-	-	15	15
-	-	16.5	17.5	-	-	-	15
-	-	16.5	17	-	-	-	15
-	-	-	17	-	-	-	15

TABLE 50. Part (1). Cont'd.

Test Number And Serum Dilution			
5			
F.S.	1:2	1:4	1:5
42	-	-	-
24	43	-	-
17	-	-	-
14.5	-	-	-
13	21	39	44
12	17	-	-
11	13	24	30
11	12	-	-
11	11	20	24.5
12.5	11	-	-
-	11	17	19
-	11	15	18
-	11	14	17
-	-	14	-
-	-	13	15.5
-	-	13	15.5
-	-	13	14.5
-	11	-	14
-	-	13	14
-	-	-	13
-	-	13	13.5
-	-	-	-
-	-	-	-
-	-	13	13
-	-	-	-
-	-	-	13
-	-	-	-
-	-	-	13
-	-	-	-

TABLE 50. Part (2). Assays of factor X using 0.1ml. volumes of normal serum.

Time of Sub-sampling in minutes.	Test Number And Serum Dilution							
	6				7			
	F.S.	1:2	1:4	1:5	F.S.	1:2	1:4	1:5
4	58	>60	-	-	43	48	-	-
8	27	36	43	51	26	30	59	61
12	15	19.5	-	-	17	21	-	-
16	12	15	-	-	13.5	16	31	40
20	12	13	21	29	13.5	14	-	-
24	12	11	-	-	14	13	23.5	32.5
28	12.5	10.5	14	19.5	14	12	-	-
32	13	10.5	12.5	-	14.5	11.5	18	24
36	-	10.5	12.5	-	15	11.5	-	-
40	-	10.5	12	16.5	-	12	16.5	22
44	-	10.5	11.5	15	-	12	-	-
48	-	11	11.5	14	-	12	15	19
52	-	-	11	14	-	12	14.5	18
56	-	-	11	13	-	12	14.5	18
60	-	-	10.5	13	-	-	13.5	16
64	-	-	11	12	-	-	13	15.5
68	-	-	10.5	12	-	-	13.5	15.5
72	-	-	11	12	-	-	13.5	14.5
76	-	-	10.5	12	-	-	13	14
80	-	-	11	12	-	-	13.5	14.5
84	-	-	11	12	-	-	13	14.5
88	-	-	11	12	-	-	-	14.5
92	-	-	11	12	-	-	13.5	15
96	-	-	-	-	-	-	-	-
100	-	-	-	12	-	-	-	-
104	-	-	-	-	-	-	-	14
120	-	-	-	-	-	-	-	-
132	-	-	-	-	-	-	-	-
150	-	-	-	-	-	-	-	14
210	-	-	-	-	-	-	-	-

TABLE 50. Part (2). Cont'd.

Test Number And Serum Dilution							
8				9			
F.S.	1:2	1:4	1:5	F.S.	1:2	1:4	1:5
37	23	-	-	41	67.5	-	-
16	17	62.5	61	14	34	51	57
14	14	-	-	13	18.5	-	-
13	13	34.5	41	13	15	25.5	34
14	12	-	-	13	12	-	-
14	12	21.5	27	13	11.5	19.5	23
14.5	12	-	-	13.5	11	-	-
14.5	12.5	18	23	13.5	11.5	16	18
16	12	17	-	14	11.5	-	-
-	13	15.5	19	14.5	12.5	13	15
-	-	13.5	16	-	-	13.5	-
-	-	13.5	15.5	-	-	12	13.5
-	-	13	15.5	-	-	12	13
-	-	13.5	15	-	-	12	13
-	-	12	15	-	-	11	13
-	-	12	14.5	-	-	-	-
-	-	13.5	14	-	-	11	12.5
-	-	12.5	14	-	-	-	12
-	-	12	13.5	-	-	11	12
-	-	12	13.5	-	-	-	11.5
-	-	12.5	13	-	-	10.5	12
-	-	12	13.5	-	-	11	-
-	-	-	-	-	-	-	12
-	-	-	-	-	-	-	-
-	-	-	-	-	-	11	12
-	-	12	13.5	-	-	-	-
-	-	-	-	-	-	11	12
-	-	-	14	-	-	-	-
-	-	-	-	-	-	-	-
-	-	-	-	-	-	-	-

TABLE 50. Part (2). Cont'd.

Test Number And Serum Dilution			
10			
F.S.	1:2	1:4	1:5
43	52	-	-
14	23.5	49	58
12.5	14.5	-	-
12.5	14	24	32
13	12	-	-
14	12	18	23
15	12	-	-
-	12	16	17.5
-	12	-	-
-	13.5	13.5	13.5
-	13.5	-	-
-	-	13.5	13.5
-	-	12.5	13.5
-	-	11.5	13
-	-	12	12.5
-	-	11.5	12.5
-	-	-	12
-	-	12	12
-	-	-	-
-	-	12	12
-	-	-	-
-	-	11.5	12
-	-	-	-
-	-	12	12
-	-	11.5	12
-	-	-	-
-	-	-	-
-	-	-	-
-	-	12	12
-	-	12	12

TABLE 50. Part (3). Assays of factor X using 0.2ml. volumes of normal serum.

Time of Sub-sampling in minutes.	Test Number And Serum Dilution											
	1				2				3			
	F.S.	1:2	1:4	1:5	F.S.	1:2	1:4	1:5	F.S.	1:2	1:4	1:5
2	31.5	41	-	-	47	-	-	-	61	-	-	-
4	12	23.5	50	54	19	53	-	-	33	62	-	-
6	11	15.5	-	-	14	30	-	-	18	-	-	-
8	10	12	27	40	12	20.5	45.5	39	15	33.5	57	45
10	10.5	11	-	-	12	17.5	-	-	13.5	-	-	-
12	10	9	18	28	13	14	-	-	12.5	17	-	-
14	10	9.5	-	-	12.5	12.5	-	-	13	-	-	-
16	10.5	9	15	22	13.5	12	17.5	19.5	13	14	30	26
18	11	9	-	-	14.5	11	-	-	13	12.5	-	-
20	12	9	13	18	-	10.5	15	15.5	13	12.5	-	-
22	-	-	-	-	-	10.5	-	-	14	11.5	-	-
24	-	9	13	18	-	10.5	15	15.5	14.5	11	21	19
26	-	-	-	-	-	10.5	-	-	-	10.5	-	-
28	-	9.5	12.5	16	-	10.5	-	-	-	10.5	-	-
32	-	10	12	14	-	10.5	12.5	13.5	-	11	17	15
36	-	-	10	12	-	10.5	12	14	-	10.5	-	-
40	-	-	10	11	-	10.5	12	13	-	10.5	14	14.5
44	-	-	10	11	-	-	12	13	-	11.5	14	14.5

TABLE 50. Part (3). Cont'd.

Time of Sub-sampling in minutes.	Test Number And Serum Dilution											
	1				2				3			
	F.S.	1:2	1:4	1:5	F.S.	1:2	1:4	1:5	F.S.	1:2	1:4	1:5
48	-	-	10	10	-	-	11.5	12	-	-	13.5	13.5
52	-	-	10	10	-	-	11	12	-	-	13	13.5
56	-	-	10	10	-	-	11	12	-	-	12.5	13
60	-	-	10	10	-	-	-	11.5	-	-	12.5	13
64	-	-	10	10	-	-	11.5	11	-	-	12.5	12.5
68	-	-	10	10	-	-	-	-	-	-	12.5	12
72	-	-	-	-	-	-	11	11	-	-	12	12.5
76	-	-	-	-	-	-	-	-	-	-	12	12.5
80	-	-	10	10	-	-	11	11.5	-	-	12	11.5
84	-	-	-	-	-	-	-	-	-	-	12	11.5
88	-	-	-	-	-	-	11	11	-	-	12	11.5
92	-	-	-	-	-	-	-	-	-	-	12	11.5
96	-	-	-	-	-	-	-	-	-	-	12	11
100	-	-	-	-	-	-	-	-	-	-	12	11.5
110	-	-	-	-	-	-	11.5	11	-	-	12.5	11.5
120	-	-	-	-	-	-	12	11	-	-	12.5	11
130	-	-	-	-	-	-	-	-	-	-	12.5	11.5
140	-	-	-	-	-	-	-	-	-	-	12	11.5

TABLE 50. Part (4). Assays of factor X using 0.2 ml. volumes of normal serum.

Time of Sub-sampling in minutes.	Test Number And Serum Dilution							
	4				5			
	F.S.	1:2	1:4	1:5	F.S.	1:2	1:4	1:5
2	26	29.5	40	-	20	25	54	-
4	11.5	14.5	32	34	10	19	45	45
6	9.5	10.5	22	-	10	13	26	-
8	9.5	10	17	18.5	10	11	18	26
10	10	9.5	13.5	-	11	11	15	-
12	10	9.5	11.5	13	11	11	12	17
14	11.5	9.5	11	-	12	11.5	11	14
16	12	9.5	11	11.5	-	11	10.5	13.5
18	-	9.5	10.5	11	-	11	10.5	12.5
20	-	9.5	10	11	-	11	10	11.5
22	-	9.5	10	10	-	11.5	9.5	11
24	-	9.5	10	10	-	12	9.5	11.5
26	-	10	9.5	10	-	-	10	10.5
28	-	10.5	9.5	10	-	-	10	10.5
30	-	12	9.5	10	-	-	10	10.5
32	-	-	9.5	9.5	-	-	10	10.5
34	-	-	9	9.5	-	-	10	10.5
36	-	-	9	9	-	-	10	10.5
38	-	-	9	9.5	-	-	10	10.5
40	-	-	9	9	-	-	10	10.5
42	-	-	9.5	9	-	-	10.5	10.5
44	-	-	9.5	9.5	-	-	11	10.5
46	-	-	9.5	9.5	-	-	-	-
48	-	-	10	9	-	-	-	-
50	-	-	9.5	9.5	-	-	-	-
52	-	-	10	9.5	-	-	-	-
54	-	-	10	10	-	-	-	-
56	-	-	10	10	-	-	-	-
58	-	-	10	10.5	-	-	-	-
60	-	-	10	10.5	-	-	-	-
62	-	-	10.5	10.5	-	-	-	-
64	-	-	-	-	-	-	-	-

TABLE 50. Part (4). Cont'd.

Test Number And Serum Dilution							
6				7			
F.S.	1:2	1:4	1:5	F.S.	1:2	1:4	1:5
33	-	58	-	30	22	52	-
14	22.5	43	-	15	16	36	44
12	15	30	-	11.5	12	23	-
12	13	20	-	11.5	11	17	28
12	12	18	-	11.5	10.5	14	-
13.5	12	15	-	11	10.5	13	19.5
13.5	11.5	14.5	-	12	10	11.5	-
14	11.5	13	-	12.5	10	11	16
-	11.5	11	-	12.5	10	11.5	14
-	11.5	10.5	-	14	10	11	14
-	11	11	-	-	10	10	13.5
-	11.5	10	-	-	10	10	13
-	11.5	10.5	-	-	10	9.5	12.5
-	12	10.5	-	-	10	9.5	12.5
-	12.5	10.5	-	-	10	9	12
-	-	10.5	-	-	10	9	12
-	12.5	10.5	-	-	-	9	12
-	12.5	10.5	-	-	-	9.5	11.5
-	13	10.5	-	-	-	9.5	11.5
-	13	10.5	-	-	-	9.5	11.5
-	-	11	-	-	-	9.5	11.5
-	-	10.5	-	-	-	9.5	11.5
-	-	10.5	-	-	-	9.5	11.5
-	-	10.5	-	-	-	10.5	11.5
-	-	11	-	-	-	10	11.5
-	-	10.5	-	-	-	9.5	11.5
-	-	11	-	-	-	10	11.5
-	-	10.5	-	-	-	10.5	11.5
-	-	10.5	-	-	-	10	11.5
-	-	10.5	-	-	-	10	11.5
-	-	-	-	-	-	10	11.5
-	-	-	-	-	-	-	-

TABLE 50. Part (4). Cont'd.

Test Number And Serum Dilution			
8			
F.S.	1:2	1:4	1:5
25.5	-	41	-
11.5	19	31	36
10	12.5	26	-
9.5	10.5	20	25
10	9	16	-
10	9.5	14	17
10	9.5	13	-
11	9.5	12.5	14
11.5	9.5	11.5	-
13	9.5	11	13
-	10	10.5	12.5
-	10.5	10.5	12
-	11	10.5	10.5
-	11.5	10.5	10.5
-	-	10.5	10.5
-	-	10.5	10.5
-	-	10.5	10
-	-	10.5	10.5
-	-	10.5	10.5
-	-	10.5	10.5
-	-	10.5	10.5
-	-	10.5	10.5
-	-	10.5	10.5
-	-	10.5	10.5
-	-	10.5	10.5
-	-	10.5	10.5
-	-	11	10.5
-	-	11	11
-	-	11	11.5
-	-	12	12
-	-	-	12

Reagents: I. A 5 percent suspension of brain residue in saline incubated for I5 minutes.

2. Platelet rich plasma prepared as formerly described.

3. Normal serum incubated on the clot for I8 hours at 37°C.

4. Calcium chloride.

Technique: Platelet concentrates calculated to contain 200 x 10^6 platelets may be prepared and washed as described for the estimation of platelet thromboplastic function. For the purposes of this procedure, however, the number of platelets need not be strictly defined and provided one is sure that the platelet rich plasma contains an average normal number of platelets it is sufficient to take equal aliquots of this (e.g. 0.4 - 0.5 ml.) and to concentrate and wash the platelets contained therein. After washing, the supernatant saline is removed and 0.2 ml. of undiluted serum is added to the button of platelets and the two reagents are mixed with a wooden probe. I.0 ml. of brain residue suspension is added and finally the whole is recalcified with I.0 ml. of calcium chloride. Coincident with recalcification a master stop watch is started and the mixture is incubated in a water bath at 37°C. At 2 minute (or other convenient short intervals) 0.I ml. volumes of the mixture are subsampled into 0.I ml. volumes of a plasma substrate with coincident recalcification with 0.I ml. of calcium chloride, and the substrate clotting times recorded. Subsampling is continued until a minimum substrate clotting time is reached and maintained. The time interval between initial recalcification of the incubation mixture and the first record of the minimum substrate clotting time is recorded. To obtain a calibration graph the test is repeated using dilutions of the normal serum in saline, and the percentage concentration of serum (i.e. the percentage of factor X) is plotted against the corresponding incubation times on arithmetic graph paper.

In Table 50, parts I and 2, are shown the results of assays performed in the way outlined using 0.I ml. volumes of serum.

In Table 50, parts 3 and 4, are shown the results of assays conducted using 0.2 ml. volumes of serum.

It can be seen that in every case whether the initial volume of serum used was 0.I ml. or 0.2 ml., progressive dilution was accompanied by the need for longer incubation to attain minimum substrate clotting times. When 0.I ml. volumes were used, however, the minimum substrate clotting times showed more variation than assays in which 0.2 ml. volumes were used. In the group in which 0.I ml. of serum was used the minimum substrate clotting times obtained with any sample of serum did not vary widely with dilution in the assays I,2,6,7,8,9 and I0. More significant changes were present in assays 3,4 and 5. In the latter, however, marked differences were not noted until the concentration of serum was reduced to 25 percent, a level at which earlier work had shown some marked changes might occur. Why such changes should be evident at this concentration with some sera and not others is probably related to inherent variations in the level of factor X in different individuals, and, as has been shown, once the critical concentration has been reached, further small reductions cause a disproportionate prolongation of substrate clotting times. It is significant that in the assays showing this feature the incubation times required to reach maximum thromboplastin generation were considerably longer for each dilution of serum than those required for corresponding dilutions in other assays where differences in substrate clotting times were not remarkable. These differences became more evident as dilution of the serum increased. As might have been anticipated, in none of the 8 assays using 0.2 ml. volumes of serum were marked variations in substrate clotting times present. In these assays the minimum concentration of serum used, exceeded the critical concentration below which both the degree and rate of thromboplastin formation might be affected. In every assay and independent of the volume of serum, when the reciprocal of the serum concentration was plotted against the corresponding incubation time on arithmetic graph paper the points always fell on or

TABLE 51. Part (1). The 'prothrombin' and 'Stypven' times of the plasma specimens corresponding to the sera (obtained from normal individuals and those having Dindevan therapy) used in the assays of factor X shown in Table 51 (2).

Group	Control or Test No.	'Prothrombin' time	'Stypven' time
A	Control	11 secs.	10 secs.
	Test 1	12 secs.	10 secs.
	2	17.5 secs.	10.5 secs.
	3	11.5 secs.	14 secs.
	4	22 secs.	15 secs.
	5	31 secs.	20 secs.
B	Control	11 secs.	8.5 secs.
	Test 1	14.5 secs.	9.5 secs.
	2	24 secs.	11 secs.
	3	17 secs.	11 secs.
	4	21 secs.	13 secs.
	5	48 secs.	10 secs.
C	Control	11 secs.	8 secs.
	Test 1	15 secs.	10 secs.
	2	18.5 secs.	11 secs.
	3	18 secs.	11 secs.
	4	17.5 secs.	10.5 secs.
	5	23 secs.	12.5 secs.

TABLE 51. Part (2). Assays of factor X using sera obtained from patients having Dindevan therapy.

Time of Sub-sampling in minutes.	Group And Test Number											
	A						B					
	C	1	2	3	4	5	C	1	2	3	4	5
2	22	12	37	41	55	47	12	22.5	32	22	51	-
4	12	11	21	20	46	42	10	14.5	25	15.5	38	60
6	10	10	12	12	33	40	9.5	11.5	17	12	32	53
8	10	10	10	12	26	35	9.5	10	12	10.5	24	45
10	10	10	10	11.5	25	35	10.5	10	11	10	18	40
12	10.5	11	10	11	17	35	11.5	10	10	9.5	14	37
14	10.5	12.5	10.5	11	16	33	-	10	9	9.5	11	35
16	11	13	11.5	10.5	15	32	-	10	9	9.5	10.5	33
18	-	-	-	10.5	14	28	-	10	9	10.5	10.5	31
20	-	-	-	10.5	13	25	-	10	9	10	9.5	31
22	-	-	-	11	12	23	-	10	9	10.5	9	30.5
24	-	-	-	12.5	11.5	21	-	10	9	11	9	30
26	-	-	-	-	11.5	20	-	11	9	-	9.5	27
28	-	-	-	-	11.5	19	-	11	9	-	9	27
30	-	-	-	-	12	18	-	11.5	9	-	9.5	26
32	-	-	-	-	11.5	17	-	12	10	-	9	27

TABLE 51. Part (2). Cont'd.

Time of Sub-sampling in minutes.	Group And Test Number											
	A						B					
	C	1	2	3	4	5	C	1	2	3	4	5
34	-	-	-	-	12.5	16	-	-	11	-	10	25
36	-	-	-	-	13	15	-	-	-	-	-	25.5
38	-	-	-	-	13	14.5	-	-	-	-	-	24
40	-	-	-	-	15	15	-	-	-	-	-	23
42	-	-	-	-	-	14.5	-	-	-	-	-	22
44	-	-	-	-	-	15	-	-	-	-	-	22
46	-	-	-	-	-	14	-	-	-	-	-	21
48	-	-	-	-	-	14	-	-	-	-	-	21
50	-	-	-	-	-	13.5	-	-	-	-	-	20.5
52	-	-	-	-	-	13.5	-	-	-	-	-	21
54	-	-	-	-	-	14	-	-	-	-	-	20.5
56	-	-	-	-	-	14	-	-	-	-	-	20.5
58	-	-	-	-	-	14	-	-	-	-	-	21
60	-	-	-	-	-	15.5	-	-	-	-	-	20.5
62	-	-	-	-	-	15	-	-	-	-	-	21
64	-	-	-	-	-	16	-	-	-	-	-	21
Factor X concentration	100	100	72	37	25	14	100	70	42	50	30	13

TABLE 51. Part (2). Assays of factor X using sera obtained from patients having Dindevan therapy.

Time of Sub-sampling in minutes.	Group And Test Number					
	C					
	C	1	2	3	4	5
2	25.5	27	40	45	39	60
4	17	13.5	27	34	30	49
6	11	9	15	22	24	33
8	9	9	12	15	15	25
10	9	8.5	10	12	11.5	18
12	9	8.5	9	10.5	10	13
14	9	8.5	8.5	10	10	11.5
16	9	10	8.5	9.5	9	11.5
18	10	11	8.5	9	9	10
20	10.5	-	9	9	9	9.5
22	-	-	9	9.5	9.5	9
24	-	-	9.5	9.5	9.5	9
26	-	-	10	10	10	9
28	-	-	-	-	-	9
30	-	-	-	-	-	9.5
32	-	-	-	-	-	10

TABLE 51. Part (2). Cont'd.

Time of Sub-sampling in minutes.	Group And Test Number					
	C					
	C	1	2	3	4	5
34	-	-	-	-	-	12
36	-	-	-	-	-	-
38	-	-	-	-	-	-
40	-	-	-	-	-	-
42	-	-	-	-	-	-
44	-	-	-	-	-	-
46	-	-	-	-	-	-
48	-	-	-	-	-	-
50	-	-	-	-	-	-
52	-	-	-	-	-	-
54	-	-	-	-	-	-
56	-	-	-	-	-	-
58	-	-	-	-	-	-
60	-	-	-	-	-	-
62	-	-	-	-	-	-
64	-	-	-	-	-	-
Factor X concentration	100	100	72	37	25	14

close to a straight line. Furthermore, this relationship was maintained in those assays where the degree as well as the rate of thromboplastin formation was reduced. These calibration graphs are shown in Figs. 2-I9, included as an appendix at the end of this thesis.

Experiment 5I. Assays of factor X in patients having Dindevan therapy.

Since no serum specifically deficient in factor X was to hand at this stage, recourse was made to pathological sera obtained from patients having anti-coagulant (Dindevan) therapy, and although these could be assumed to be deficient in both factor VII and factor X, it was thought that it might prove interesting to perform factor X assays on some few cases.

Accordingly, on each of 3 days, 5 patients were chosen from those having daily routine prothrombin time estimations. These and a normal donor were each bled of I0 ml. of blood within 30 minutes of each other, 4.5 ml. of which was immediately anti-coagulated with 0.5 ml. of 3.8 percent sodium citrate. The remainder was allowed to clot in a centrifuge tube and the serum was incubated on the clot for I8 hours at 37°C. "Prothrombin" and "Stypven" time estimations were performed immediately on the citrated specimens. On the next day, after the prescribed incubation period, factor X assays were performed on the serum. The normal 0.2 ml. volumes of undiluted normal serum and appropriate dilutions were used in the construction of a calibration graph as described. The procedure was then repeated using 0.2 ml. of undiluted serum from each of the patients in turn. The results are shown in Tables 5I (I) and 5I (2).

Results: In each of the tables the results are shown in 3 groups - A, B and C, each group corresponding to the test and control specimens studied on any one day. Table 5I (I) shows the "prothrombin" and "Stypven" times and Table 5I (2) the results of the assays. For clarity, in the latter, only the figures obtained with undiluted normal serum are shown together with the

corresponding figures for the test specimens. At the end of each assay the percentage concentration of factor X as calculated from the appropriate calibration graph is shown.

In the first group the "prothrombin" times ranged between II.5 and 3I seconds (normal - II seconds) and the "Stypven" times between I0 and 20 seconds (normal - I0 seconds). In the assays when 0.2 ml. undiluted normal serum was used a minimum substrate clotting time of I0 seconds was reached after 6 minutes incubation. With the test sera minimum substrate clotting times of I0,I0,I0.5, II.5 and I4 seconds were reached after 6,8,I6,24 and 46 minutes incubation respectively, the corresponding "Stypven" times being I0,I0.5,I4,I5 and 20 seconds. In this same order the relative concentrations of factor X were calculated to be I00,72,37,25 and I6 percent.

In group B the "prothrombin" times ranged from I4.5 to 48 seconds (normal - II seconds) and the "Stypven" times between 9.5 and I3 seconds (normal - 8.5 seconds). Using 0.2 ml. of undiluted normal serum a minimum substrate clotting time of 9.5 seconds was reached after 6 minutes incubation. In the test specimens minimum substrate clotting times of I0,9,9.5,9 and 2I seconds were reached after 8,I4,I2,20 and 46 minutes incubation respectively, the corresponding "Stypven" times being 9.5,II,II,I3 and I0 seconds. In the same order the relative concentrations of factor X were estimated to be 70,42,50,30 and I3 percent respectively.

In group C the "prothrombin" times ranged from I5 to 23 seconds (normal - II seconds) and the "Stypven" times between I0 and I2.5 seconds (normal - 8 seconds). Using 0.2 ml. of undiluted normal serum a minimum substrate clotting time of 9 seconds was reached after 8 minutes incubation. In the test specimens minimum substrate clotting times of 8.5,8.5,9.5,9 and 9 seconds were reached after I0,I4,I6,I6 and 22 minutes incubation respectively. The corresponding "Stypven" times were I0,II,II, I0.5 and I2.5 seconds respectively and in the same order the

relative concentrations of factor X were estimated to be 66,51, 47,47 and 37 percent.

Discussion: These tests using serum obtained from patients having Dindevan therapy would seem to have established the technique described as a satisfactory means of assaying factor X. The majority of the tests behaved as might have been expected from the theoretical considerations based on earlier experimental work, while the occasional unexpected result could be readily explained on facts already established.

In group A there was good correlation between the estimated levels of factor X and the "Stypven" times but as might be expected less correlation with the "prothrombin" times. In 4 of the tests the minimum substrate clotting times did not differ greatly from those obtained with normal serum, while in the fifth the content of factor X was calculated to be in the range where interference with both the rate and degree of thromboplastin formation might be expected.

In group B there was good correlation between the "Stypven" times and the estimated factor X concentration in the first 4 tests and, again, in these tests the minimum substrate clotting times did not differ significantly from those obtained with the control serum. In the remaining test the concentration of factor X was estimated to be I3 percent despite a "Stypven" time only I.5 seconds slower than the normal. This apparent discrepancy can be readily explained, however. The patient's "prothrombin" activity had been less than 5 percent for several days, and despite cessation of Dindevan therapy it was still at this level at the time his serum was tested. Since his "Stypven" time was not grossly abnormal it was reasonable to assume that his low "prothrombin" activity was dependent on low levels of factor VII and perhaps prothrombin. It was quite probable therefore that his factor VII level was within that very low range where the reaction might be influenced by factor VII and that the estimated factor X concentration of I3 percent reflected the combined effects of a

severe factor VII deficiency and a minor factor X reduction.

In group C there was good correlation with the "Stypven" times and the minimum substrate clotting times did not vary by more than 0.5 seconds from that obtained with the control serum. In these tests the factor X concentration was always estimated to be greater than the critical concentration below which the degree of thromboplastin formation might be impaired.

To date it has not been possible to compare the results of assays performed in this way with those based on the use of factor X deficient reagents. Until this is done the accuracy of the method must remain in doubt, but the work already done would seem to have established the technique as a satisfactory method of obtaining approximate levels of factor X in serum.

It is not claimed that the method is ideal but it does present some advantages. It is a time consuming procedure and technically it is not as simple or convenient as estimations of the "Stypven" time. The calibration graph is not as easily prepared as those based on a modification of the one-stage test when suitable factor X deficient plasma is available. Denson (1961) has described a method for the artificial preparation of a diluent in which the factor X and factor VII activity is markedly reduced while reasonable quantities of prothrombin remain. Such a diluent is satisfactory for use in the "Stypven" time estimations, but the preparation of asbestos or charcoal filtrates is neither straight forward or simple. Thus the present method would seem to involve less time and work than other methods when a plasma congenitally deficient in factor X is not to hand.

Since reagents congenitally deficient in factor X have a very restricted availability, the technique described has the distinct advantage that it brings a method of quantitating factor X within the scope of any laboratory able to undertake coagulation investigations. As indicated earlier, technically it is not the simplest of procedures and is time consuming. These disadvantages can be minimised in two ways however. Firstly, in

contradistinction to the assay of platelet thromboplastic function it is not essential to adhere to a set number of platelets, and it is possible to dispense with platelet counts on the platelet rich plasma. Secondly, in preparing a calibration graph it is pointless to commence subsampling from any given incubation mixture until just before the end point has been reached of the mixture immediately preceding it, and containing a higher concentration of serum. Incubation mixtures with all serum dilutions can therefore be prepared and tested concurrently.

A further difficulty was evident when 0.I ml. volumes of serum were used to construct calibration graphs. In these a minimum substrate clotting time was often gradually approached and it was sometimes difficult to determine the precise time at which they were first reached. When 0.2 ml. volumes of serum were used the plateau of maximum thromboplastin generation was approached much more rapidly and abruptly, and it was easy to determine the end points. The same considerations apply to unknown specimens but in these the problem can be overcome by prior determinations of the "Stypven" time. From this, one can gain a rough idea of the severity of the defect and in manifestly severe deficiencies the volume of serum used can be increased. In this way the testing of unknown specimens can be accelerated and accuracy increased.

Two final points need to be considered. Theoretically any assay technique based on the use of plasma deficient in factor X as a diluent, is open to the criticism that congenital deficiencies are rarely absolute and the diluent will contain small unknown quantities of factor X. This criticism cannot apply to the method under consideration. A second source of error lies in the fact that normal individuals, no doubt, have considerable variation in the factor X content in their plasmas. Thus the comparison of unknown blood samples against those from random normal donors may introduce a variable in any such assay. In the present technique this error can be minimised by pooling

sera from several donors. Such sera may be stored in multiple small aliquots at -20°C or stored in the lyophilised state for periodic use as a standard.

The technique described is therefore proposed as an alternative means of assaying factor X. It is recognized that while there are disadvantages attending its use, it has other advantages to commend it. Foremost among these is its ready availability attending its independence of reagents congenitally deficient in factor X or the need to prepare such a reagent artificially. Finally it must be accepted that while it may not find wide routine application, the technique does provide a new approach and a new means to study the behaviour of the relatively poorly defined factor X.

Part VI. Inhibitory Factors

When one ponders the mass of literature that has accumulated and is ever increasing dealing with those factors which for the want of a better term may be described as "positive" coagulation factors and which interact to secure haemostasis and coagulation, one is at once struck by the singular lack of information concerning those factors which limit this primary objective and secure localization of the process to an area requiring immediate repair. That such a "negative" system exists, is beyond doubt, but the scant attention it has received is perhaps out of proportion to its potential importance. Certain observed effects in earlier experiments were ascribed to the presence of "negative" or "inhibitory" factors in serum or aluminium hydroxide-treated plasma, and while some evidence was adduced to support this, further clarification seemed necessary.

Briefly to recapitulate the findings which led to this particular work being undertaken, it will be recalled that in experiment 25 the use of serum specifically lacking factor X caused both slowed and reduced thromboplastin formation in the reaction being studied. The reduced thromboplastin formation was not in accord with the suggested function of factor X as an "accelerator" of the reaction only, and it was thought at that time that the likely explanation depended on the presence of inhibitory factors in the serum as used, although it was subsequently shown that at low concentrations factor X might influence the degree as well as the rate of thromboplastin formation. In experiment 39 it was found that the addition of aluminium hydroxide-treated haemophilic plasma to the usual incubation mixture produced less improvement in thromboplastin generation than the corresponding normal reagent. Indeed, in some instances the minimum substrate clotting times were slower than those obtained with saline controls. Again it was suggested that these effects might have resulted from the presence of excess inhibitory activity in haemophilic plasma either absolute or relative. In subsequent experiments evidence was

adduced to show that the improved thromboplastin generation on the addition of aluminium hydroxide-treated normal plasma to incubation mixtures was dependent on the factor VIII content and that factor V played no significant part in the process. Experiment 41 demonstrated that aluminium hydroxide-treated fresh rabbit serum had a marked inhibitory effect on thromboplastin generation, while experiment 42 showed that fresh human serum, similarly treated had the same effect. Subsequently it was shown in experiment 43, that human serum treated with aluminium hydroxide after incubation on the clot for 18 hours at 37°C had lost its ability to prolong the minimum substrate clotting times obtained with the standard mixture, yet residual inhibitory action seemed to be retained for the rate of deterioration of such substrate clotting times was accelerated.

The experiments to be described in this section are some of those which have been undertaken in the field of "negative" coagulation and which are relevant to the experiments mentioned above. They are included to support the assumptions mentioned, that certain observed facts concerning the behaviour of "fresh" serum and aluminium hydroxide-treated haemophilic plasma depended upon the activity of "inhibitory" factors. In particular an attempt has been made to show that the effects of haemophilic plasma treated with aluminium hydroxide depended upon a relative imbalance of "inhibitors" rather than an absolute increase in such activity.

The first experiment in this series, experiment 52, was undertaken to gain some idea of the rate of deterioration of inhibitory factors in serum.

Experiment 52. To determine the rate of deterioration of anti-thromboplastins in serum.

Reagents: I. All normal reagents for thromboplastin generation prepared as before.

TABLE 52. The rate of deterioration of anti-thromboplastins in serum.

Control or Test	Age of Serum	Substrate Clotting Time In Seconds Time of Subsampling In Minutes									
		2	4	6	8	10	15	20	25	30	35
Control	18 hrs.	62	42	32	22	16	13	12.5	11	11	11
Test 1	1 hr.	63.5	36.5	24.5	19	15.5	13.5	12.5	15	14.5	18
Test 2	2 hrs.	60.5	34	23	17.5	15	13.5	12.5	13.5	14	15
Test 3	4 hrs.	53	32	21.5	17	15	13.5	13	14.5	15	16.5
Test 4	6 hrs.	46	25.5	17.5	15.5	14	12	12	13	14	-
Control	18 hrs.	-	26	-	16.5	15	11	11	11	11	-

2. Serum. A normal donor was bled of 20 ml. of blood and I.0 ml. volumes were placed in a series of Wassermann tubes and allowed to clot at 37°C.

Technique: At intervals of I,2,4 and 6 hours after collection, the serum from 4 tubes was pooled and treated with aluminium hydroxide. Immediately after this preparation, 0.I ml. of each sample was added to the standard incubation mixture and the usual procedure followed. Control tests using all normal reagents plus 0.I ml. of saline were performed at the beginning and completion of the experiment. The results are shown in Table 52.

Results: The minimum substrate clotting times obtained with both control tests were II seconds. In the test mixtures, the minimum substrate clotting times obtained with serum treated with aluminium hydroxide after I and 2 hours incubation were I2.5 seconds, after 4 hours incubation I3 seconds and after 6 hours I2 seconds. While the control mixtures showed the usual plateau of maximum thromboplastin generation, the minimum substrate clotting times were poorly maintained in all test mixtures and rapid decline was evident. This effect was slightly less marked in the mixture containing treated serum that had been incubated for 6 hours.

Discussion: It appeared that the activity of inhibitory factors in serum remained reasonably constant for some hours after collection of the specimen, but by 6 hours some decline was evident. In conjunction with experiment 43, it was reasonable to conclude that this process continued after this time, and that after I8 hours incubation the level of inhibitory factors in serum capable of affecting the minimum substrate clotting times themselves was negligible, while the process of accelerated breakdown of formed thromboplastin was still evident. It was reasonable to state therefore, that the impaired thromboplastin generation evident in experiment 25 may have had a dual basis. In part it may have been due to a concentration of factor X below a critical level wherein the degree as well as the rate of thromboplastin formation might be impaired. On the other hand the impairment may well have been exaggerated by, if not wholly, the result of, using a serum

retaining appreciable inhibitory activity, for it was recognized that this serum had not been prepared in the standard way prescribed.

It was thought that serum levels of inhibitory factors were probably a poor indication of the normal levels in plasma, for without doubt a considerable utilization must occur in normal coagulation. Methods were sought, therefore, to free plasma from all factors known to influence the system being used, principally factors VIII and X, and yet leave inhibitory factors intact. Serum factors could be easily removed by aluminium hydroxide treatment but the removal or destruction of AHG posed a problem. Experiments in which whole normal plasma was incubated with powdered asbestos, however, showed that this process was accompanied by a complex removal of many factors, and that plasma so treated was quite inert in the thromboplastin generation screening test and was unable to correct the defect in haemophilic plasma. These findings were not in accord with those of Tocantin's (1944,45,46,49,51) who has claimed that an anti-thromboplastin present in whole blood is removed by contact with asbestos fibres but were confirmed in an independent investigation in the Department of Medical Research at the Institute of Medical and Veterinary Science. To determine whether inhibitory factors were retained in this product, small volumes of plasma treated with asbestos and aluminium hydroxide were added to incubation mixtures as described in experiment 53.

Experiment 53. To determine whether plasma treated with asbestos and aluminium hydroxide retained inhibitory activity.

Reagents: 1. All normal reagents prepared as before.
2. Normal platelet poor plasma.

Technique: A 15 ml. centrifuge tube was lightly packed to one half of its capacity with powdered asbestos. 5 ml. of normal plasma was added to this and the whole mixture was incubated at 37°C for 30 minutes. After separation from the asbestos by centrifugation at 3000 r.p.m. for 10 minutes, 1.8 ml. of the supernatant asbestos-

TABLE 53. To show the presence of "inhibitory" factors in plasma treated with asbestos and aluminium hydroxide.

Control or Test	Reagent	Substrate Clotting Time In Seconds Time of Subsampling In Minutes									
		2	4	6	8	10	15	20	25	30	35
Control 1	0.1ml. saline	36	19.5	15.5	14	13.5	13	13	14	13	-
Control 2	0.1ml. Al(OH)3 treated plasma	25	12	11	11	10.5	11.5	12.5	13.5	12.5	-
Test 1	0.1ml. asbestos and Al(OH)3 treated plasma	20	17	16.5	17	16.5	16.5	16	16	16	-
Control 1	0.1ml. saline	75	47	35	26	24	15	12	10.5	10	10
Control 2	0.1ml. Al(OH)3 treated plasma	38	12.5	13	12	10	9	9	9.5	9	9
Test 1	0.1ml. asbestos and Al(OH)3 treated plasma	> 60	39	29	27	23.5	19.5	17	16.5	16.5	17.5

treated plasma was further treated with 0.2 ml. of aluminium hydroxide in the usual way. Subsequently 0.I ml. of the plasma so treated was added to the usual incubation mixture and the standard procedure followed. The usual control tests were performed in parallel with this. In these in turn, 0.I ml. of saline and 0.I ml. of aluminium hydroxide-treated plasma were added to the usual incubation mixture and the standard procedure followed. The results are shown in Table 53.

Results: As in other experiments the minimum substrate clotting times obtained on the addition of aluminium hydroxide-treated plasma to the standard incubation mixture were faster than those obtained with saline controls. On the other hand, the addition of plasma treated with aluminium hydroxide and asbestos resulted in minimum substrate clotting times seconds slower than the saline controls.

Discussion: Obviously the factor in aluminium hydroxide-treated plasma responsible for increasing thromboplastin generation in the standard incubation mixture was removed by asbestos treatment of plasma. Accompanying this removal potential inhibitory activity in plasma so treated became evident. It seemed possible then that plasma treated with both asbestos and aluminium hydroxide might be a suitable reagent to use for the assessment of inhibitory activity in whole blood. On the other hand, in view of Tocantin's work, it was unknown whether asbestos might not have removed some inhibitory activity and whether the effects produced in this experiment represented the activity of residual inhibitory factors and not the full potential of these factors. Experiments 54 and 55 clarified this problem.

It has been shown that inhibitors in serum are demonstrable by the addition of aluminium hydroxide-treated serum to the usual incubation mixture. It was thought that if a specimen of serum was treated with both aluminium hydroxide and asbestos, any reduction in inhibitor activity between this preparation and the same serum treated with aluminium hydroxide alone would indicate

TABLE 54. To show the effect of asbestos treatment on the inhibitory activity of serum.

Test Number	Reagent	Substrate Clotting Time In Seconds Time of Subsampling In Minutes								
		2	4	6	8	10	15	20	25	30
1	0.1ml. saline	24.5	15.5	13	12.5	11.5	11	11	11	11
	Al(OH)3 treated serum	-	15.5	-	13	14	14.5	15	16	16
	Asbestos and Al(OH)3 treated serum	-	15	-	13.5	13	13	13.5	15	14.5
2	0.1ml. saline	36	19.5	15.5	14	13.5	13	13	13.5	13
	Al(OH)3 treated serum	-	18	-	14.5	14	15	16	17	18.5
	Asbestos and Al(OH)3 treated serum	-	16	-	14	14	15	16	16	17

that asbestos removed inhibitor activity in part at least. This concept was tested in experiment 54.

Experiment 54. To determine the stability of inhibitory factors in the presence of asbestos.

Reagents: I. All normal reagents prepared as before.
2. Fresh serum.

Technique: 20 ml. of blood collected from a normal donor was allowed to clot and incubated at 37°C for one hour. The serum was separated from the clot and divided into two equal parts. One of these was treated with asbestos in the way outlined in experiment 53 and incubated for 30 minutes at 37°C. The control sample of serum was simply incubated at 37°C for 30 minutes. Subsequently both preparations were treated with aluminium hydroxide, and, in turn, 0.I ml. volumes of each preparation were added to a standard incubation mixture in the routine way. The usual control test using normal reagents plus saline was conducted in parallel with the test mixtures. The results are shown in Table 54.

Results: While both serum preparations caused longer substrate clotting times than were obtained with the saline control, the change was the same in both and the pattern of the whole reaction was similar in the two tests.

Discussion: This experiment proved that asbestos did not alter the inhibitor activity of serum and it was assumed that this was almost certainly so when plasma was treated similarly. To prove this point, and to determine the effects of varying the incubation times with asbestos on plasma, experiment 55 was undertaken.

Experiment 55. To assess the effects of varying the incubation times on the inhibitor action of plasma treated with asbestos.

Reagents: I. All normal reagents prepared as before.

TABLE 55. To show the effect on plasma inhibitory activity of asbestos treatment of plasma of varying duration.

Control or Test	Duration of asbestos treatment (In Minutes)	Substrate Clotting Time In Seconds Time of Subsampling In Minutes								
		2	4	6	8	10	15	20	25	30
Control	4	25	15	13	11	11	11	11	11	11
Test 1	15	31	15	16	15.5	15	15	16	18	18.5
Test 2	30	17	17	15.5	15.5	16	16.5	17.5	20	20
Test 3	45	28	19	19	19	16	14.5	15	16.5	18.5
Test 4	60	27	22	18	17.5	16	15	15	17	18

2. Normal plasma.

Technique: 5 ml. volumes of a specimen of normal plasma were added to four, I5 ml. centrifuge tubes containing powdered asbestos as outlined in experiment 53. The four tubes were incubated at 37°C and at intervals of I5,30,45 and 60 minutes, the plasma was recovered from one of the tubes by centrifugation and treated with aluminium hydroxide. After such treatment 0.I ml. volumes of each preparation in turn were added to the usual incubation mixture and the standard procedure followed. The usual control test was performed. The results are shown in Table 55.

Results: Inhibition of thromboplastin formation was of the same degree in the four tests.

Discussion: This experiment showed that inhibitory factors in plasma treated with asbestos and aluminium hydroxide were not reduced by asbestos treatment of plasma for intervals of I5-60 minutes. In conjunction with experiment 54 it was reasonable to assume that asbestos did not remove inhibitor activity from either plasma or serum. The preparation of plasma for assessment of inhibitor activity was then standardized on the technique outlined in experiment 53, namely treatment for 30 minutes at 37°C.

The range of variation in the normal levels of some coagulation factors is wide, for example factor VIII. Earlier in this thesis, however, it was shown that the daily range of variation in thromboplastic function of normal platelets was quite small. Experiment 56 was performed to gain some preliminary indication of the range of activity of inhibitory factors in the plasma of normal individuals.

Experiment 56. To determine the range of inhibitor activity in plasma obtained from 5 normal individuals.

Reagents: I. All normal reagents prepared as before.
2. Plasma collected from 5 normal donors.

TABLE 56. To show a range of "inhibitor" activity in 5 normal plasmas tested concurrently.

Control or Test	Substrate Clotting Time In Seconds Time of Subsampling In Minutes								
	2	4	6	8	10	15	20	25	30
Control	-	16.5	11	10	9	9	9	9	9
Test 1	28	16	13	11	11	11	12	13	14.5
Test 2	16	13.5	11	10.5	10.5	11	11	12	13
Test 3	30	12	10.5	12	10.5	10.5	10.5	12	13
Test 4	22	17	12	11	10.5	10.5	12	13	15
Test 5	40	18.5	15	11	11	11	11	12.5	13

Technique: The 5 preparations of plasma were treated with asbestos and aluminium hydroxide in the usual way. Subsequently 0.I ml. of each preparation was added to standard amounts of the usual incubation mixture and the routine procedure followed. A control experiment using saline in lieu of treated plasma was performed in parallel with these tests - the results are shown in Table 56.

Results: The minimum substrate clotting time obtained with the control test was 9 seconds. In mixtures containing added treated plasma, the minimum substrate clotting times ranged between I0.5 and II seconds.

Discussion: These results, albeit obtained in a very small group of subjects, suggested that the range of inhibitor activity in normal plasma did not vary greatly between different normal individuals. After this experiment 57 was proposed to compare the activity of inhibitory factors in plasma and serum obtained from the same individual.

Experiment 57. To compare the inhibitor activity in plasma and serum obtained from the one donor.

Reagents: I. All normal reagents prepared as before.
2. Normal plasma.
3. Fresh serum incubated on the clot for one hour.

Technique: A normal donor was bled of an appropriate quantity of blood, part of which was anticoagulated with 3.8 percent sodium citrate, while the remainder was allowed to clot. Serum was incubated on the clot for one hour and then treated with aluminium hydroxide, while the plasma obtained from the anticoagulated specimen was treated with asbestos and aluminium hydroxide in the standard way. 0.I ml. volumes of the plasma and serum preparations were added to standard incubation mixtures in the usual way. A control test was performed in parallel with the experimental mixtures. The results of four such experiments are shown in

TABLE 57. The relative concentrations of "inhibitor" activity in plasma and serum obtained from the same donor.

Test Number	Treated Plasma or Serum	Substrate Clotting Time In Seconds Time of Subsampling In Minutes									
		2	4	6	8	10	15	20	25	30	35
1	Control	27	14	11	10.5	10	10	10.5	11	11	-
	Plasma	27	18	16	15.5	15.5	15.5	15.5	17	19	-
	Serum	25	15.5	12.5	13	13	13	15	15.5	16.5	-
2	Control	27	14	11	10.5	10	10	10.5	11	11	-
	Plasma	27	18.5	18.5	17	17	17.5	17	15.5	15.5	17.5
	Serum	21	14.5	13	13	12.5	14	15.5	16	16	16.5
3	Control	34	16.5	11.5	10.5	10	10	10	10.5	11.5	-
	Plasma	24	16	15	14	14	14	14	16.5	18	-
	Serum	23	15	11.5	12	12	13	15	17	18.5	-
4	Control	-	16	12	10.5	10	10	10.5	10	10	-
	Plasma	24	18	16	16	16	18	18.5	19.5	22	-
	Serum	14	10	10	10	10	10	11	13	14.5	17

Table 57.

Results: Except for one mixture containing treated serum, both treated plasma and serum caused reduced thromboplastin formation in the incubation mixtures to which they were added. Treated plasma produced greater inhibition than the corresponding serum in each case.

Discussion: These results were no more than what was anticipated for it was only to be expected that some inhibitor activity would be consumed during coagulation, and that serum therefore would contain less of the factors concerned than the corresponding plasma. The surprising feature, however, was the fact that even after normal coagulation, serum still contained sufficient inhibitor activity to cause obvious reduction of thromboplastin formation in a potent incubation mixture. This was an adequate pointer to the potency inherent in the system obviously always present in amounts more than adequate to neutralize a normal coagulation process.

The foregoing experiments had established a basis for the comparison of inhibitory activity in different samples of serum and plasma. It remained to make such a comparison between normal and haemophilic plasma and serum.

On occasions reports have appeared citing the occurrence of anticoagulant activity in haemophilia. These reports have included descriptions of factors said to behave as anti-thromboplastins and, on occasions they have been incriminated as being responsible in part at least, for the haemorrhagic manifestations of this disease. Earlier in this thesis work was described wherein haemophilic plasma treated with aluminium hydroxide was added to the standard incubation mixture. When this was done thromboplastin formation was less than when the corresponding normal reagent was used. It was shown that in part at least, this was due to differences in the content of anti-haemophilic globulin between the two reagents. However, such an explanation would not suffice for all of the observed changes, for in some cases, in the presence of treated haemophilic plasma,

TABLE 58. To show the substrate clotting times obtained on the addition of asbestos and aluminium hydroxide treated normal and haemophilic plasma to identical incubation mixtures.

Reagent added to Incubation Mixture	Substrate Clotting Time In Seconds Time of Subsampling In Minutes								
	2	4	6	8	10	15	20	25	30
Saline Control	-	40	19.5	16.5	14	14	13	13	13
Al(OH)3 treated normal plasma	49	12.5	11	10.5	10	10	10	11	11.5
Al(OH)3 treated haemophilic plasma	90	25	22	16	14	13	12	13	13
Asbestos and Al(OH)3 treated normal plasma	68	34	20	19.5	22	22	22	22	27.5
Asbestos and Al(OH)3 treated haemophilic plasma	41	24	21	19	19	18.5	18	19	20.5

TABLE 58 cont'd.

Reagent added to Incubation Mixture	Substrate Clotting Time In Seconds Time of Subsampling In Minutes								
	2	4	6	8	10	15	20	25	30
Saline Control	73.5	57	36	26	18	14	13	13	13
Al(OH)3 treated normal plasma	18	11.5	9.5	8.5	8	8	9	10	12.5
Al(OH)3 treated haemophilic plasma	58	28	22.5	19	18	12	11.5	11.5	13.5
Asbestos and Al(OH)3 treated normal plasma	68	45	27	20	18.5	19	19	19.5	21.5
Asbestos and Al(OH)3 treated haemophilic plasma	69	44	26	20	18.5	19	20.5	21	25

TABLE 58 cont'd.

Reagent added to Incubation Mixture	Substrate Clotting Time In Seconds Time of Subsampling In Minutes								
	2	4	6	8	10	15	20	25	30
Saline Control	31.5	14	11	9.5	9.5	9.5	9.5	10	10
Al(OH)3 treated normal plasma	31	12	10	8.5	8	8	8	9	10
Al(OH)3 treated haemophilic plasma	17.5	15	12	11	10	9	9.5	9.5	11
Asbestos and Al(OH)3 treated normal plasma	25	15	15	14	13.5	13	13	13.5	15
Asbestos and Al(OH)3 treated haemophilic plasma	25	15	13	12	12	13	15	15	16

thromboplastin generation was actually less than in corresponding incubation mixtures containing saline in lieu of treated plasma. It was suggested that this latter effect stemmed from an absolute or relative excess of inhibitory activity in haemophilic plasma. The last experiments to be described were used to confirm this suggestion.

Experiment 58. To compare the levels of anti-thromboplastins in normal and haemophilic plasma.

Reagents: I. All normal reagents prepared as for the estimation of platelet thromboplastic function.
2. Normal plasma.
3. Haemophilic plasma.

Technique: Aliquots of both the normal plasma and haemophilic plasma were treated with aluminium hydroxide in the usual way. Further aliquots were incubated for 30 minutes at 37°C with asbestos and then adsorbed with aluminium hydroxide as outlined earlier. Control tests were performed first using in every case standard volumes of the usual incubation mixture plus, in turn, 0.I ml. of saline, 0.I ml. of aluminium hydroxide-treated normal plasma and finally 0.I ml. of aluminium hydroxide-treated haemophilic plasma. Tests followed in which 0.I ml. of either normal or haemophilic plasma treated with both asbestos and aluminium hydroxide was added to the usual incubation mixture. In every mixture the pattern of thromboplastin generation was followed in the usual way. The results of this experiment using three samples of plasma obtained from three haemophilic subjects at different times are shown in Table 58. The three haemophiliacs were examined when actively bleeding and prior to any treatment.

Results: In every experiment the behaviour of incubation mixtures containing either saline or normal or haemophilic plasma treated with aluminium hydroxide conformed to an expected pattern. When compared with mixtures containing saline, thromboplastin generation

was greater in those mixtures containing normal plasma treated with aluminium hydroxide. In mixtures containing haemophilic plasma treated with aluminium hydroxide, thromboplastin generation was less than that in the presence of the corresponding normal reagent and was either equal to or slightly greater than that developed in the control mixture containing saline. When either normal or haemophilic plasma treated with both asbestos and aluminium hydroxide was added to the usual incubation mixture, thromboplastin generation was impaired, but the degree of this impairment was much the same with both reagents and the patterns of the complete tests were very similar. In no case was thromboplastin generation impaired to a greater extent in the presence of haemophilic plasma than in the presence of normal plasma.

Discussion: It could only be concluded that in this series, at least, no excess inhibitory activity could be demonstrated and the levels in haemophilic plasma treated with asbestos and aluminium hydroxide were, for all practical purposes the same as those in normal plasma treated similarly. It seemed reasonable to assume therefore that the differences in thromboplastin generation between mixtures containing normal plasma treated with aluminium hydroxide and haemophilic plasma similarly treated, were dependent upon the differences in their content of factor VIII and the effects of a relative imbalance of anti-thromboplastins, dependent upon this lack of factor VIII in haemophilic plasma. When factor VIII was added with normal "treated" plasma the improvement in thromboplastin generation from this cause would over-ride any neutralization by anti-thromboplastins and faster minimum substrate clotting times resulted. In the case of "treated" haemophilic plasma, however, with the reduced levels of factor VIII, the improvement of thromboplastin generation would be proportionately less while approximately the same amount of anti-thromboplastin as was present in normal plasma would be added to incubation mixtures. The effect of the latter would tend to over-ride any improvement due to factor VIII and the minimum substrate clotting times would be

TABLE 59. To show the substrate clotting times obtained with the use of haemophilic serum as a source of factor X for the estimation of platelet thromboplastic function.

Test Number	Normal or Haemophilic Serum	Substrate Clotting Time In Seconds Time of Subsampling In Minutes						
		4	10	15	20	25	30	35
1	Normal	43	18.5	13.5	11.5	10.5	10.5	10.5
	Haemophilic	23	14	12	11	10.5	10.5	10.5
2	Normal	36	15.5	11.5	10.5	10.5	10.5	10.5
	Haemophilic	23	16.5	12	11	10.5	10.5	11
3	Normal	50	21	18	13.5	12	12	12
	Haemophilic	57	22	16	14	12	12	12
4	Normal	23	11	10	10	10	10	10
	Haemophilic	18	13	10	10	10.5	10	10
5	Normal	35	12.5	12	12	12	12	12
	Haemophilic	21	18	13	12	12	12	12

the same, slightly slower or at the most slightly faster than those in control mixtures containing saline in lieu of treated plasma.

In a second experiment the effects of normal and haemophilic serum both prepared as for use in the standard technique used to assess platelet thromboplastic function were compared. This is described in experiment 59.

Experiment 59. To compare the relative effects of normal and haemophilic serum used for the estimation of platelet thromboplastic function.

Reagents: I. All normal reagents prepared as for use in the estimation of platelet thromboplastic function.
2. Normal plasma. } Collected from the same donor.
3. Normal serum. }
4. Haemophilic plasma. } Collected from the same donor.
5. Haemophilic serum. }

Technique: Using all usual normal reagents plus 0.I ml. of saline the standard procedure was followed as a control test. Subsequently the procedure was repeated substituting in turn 0.I ml. of normal aluminium hydroxide-treated plasma, and then 0.I ml. of haemophilic plasma treated with aluminium hydroxide for the saline. This established that the latter reagents were behaving in the usual way. The normal serum and haemophilic serum collected at the same time as the corresponding plasma samples were incubated on the clot at 37°C for I8 hours, after which each was used as a source of serum in a standard procedure for the estimation of platelet thromboplastic function. The results of 5 such experiments are shown in Table 59.

Results: In these experiments the behaviour of haemophilic serum and normal serum prepared in the same way was identical, and in every case the same minimum substrate clotting times were obtained with both reagents.

Discussion: The fact that normal serum and haemophilic serum

incubated for I8 hours and subsequently used as a source of factor X in tests for the estimation of platelet function, behaved in an identical manner is not surprising, for, earlier experiments had demonstrated that the level of anti-thromboplastins in serum decreases progressively and that after I8 hours, their activity in normal serum is negligible. It was thought, however, that in haemophiliacs with their impaired coagulation, excessive amounts of anti-thromboplastins might be demonstrable in haemophilic serum after shorter periods of incubation. To test this possibility experiment 60 was undertaken.

Experiment 60. To compare the levels of anti-thromboplastins in fresh normal serum and haemophilic serum.

Reagents: I. All normal reagents prepared as for the estimation of platelet thromboplastic function.

2. Normal plasma. } Collected from the same donor.
3. Normal serum. }

4. Haemophilic plasma. } Collected from the same donor.
5. Haemophilic serum. }

Technique: The two serum preparations were incubated on the clot for 2 hours at 37°C after which the serum was separated by centrifugation and treated with aluminium hydroxide. The two plasma samples were treated with aluminium hydroxide in the usual way after which the following incubation mixtures were prepared and tested in the standard way.

I. All normal reagents plus 0.I ml. of saline.
2. All normal reagents plus 0.I ml. of normal plasma treated with aluminium hydroxide.
3. All normal reagents plus 0.I ml. of haemophilic plasma treated with aluminium hydroxide.
4. All normal reagents plus 0.I ml. of normal serum treated with aluminium hydroxide.
5. All normal reagents plus 0.I ml. of haemophilic serum treated with aluminium hydroxide.

TABLE 60. The substrate clotting times obtained on the addition of aluminium hydroxide treated normal and haemophilic serum to the usual incubation mixture.

Test Number	Reagent Used	Substrate Clotting Time In Seconds Time of Subsampling In Minutes									
		2	4	6	8	10	15	20	25	30	35
1	Saline	42	24	17	14.5	13	12	11	11	11	11
	$Al(OH)_3$ treated normal plasma	16	12	10.5	10.5	10	9.5	9.5	10	11	12.5
	$Al(OH)_3$ treated haemophilic plasma	18	15	12.5	12	11.5	11	11	12	12	13
	$Al(OH)_3$ treated normal serum	34	15	13	13	13	13	14.5	16	16.5	-
	$Al(OH)_3$ treated haemophilic serum	29	15.5	14.5	13.5	13.5	15.5	16	17.5	17.5	-

TABLE 60 cont'd.

Test Number	Reagent Used	Substrate Clotting Time In Seconds Time of Subsampling In Minutes									
		2	4	6	8	10	15	20	25	30	35
2	Saline	>60	45	29	19	16	14	13.5	12	12.5	12
	Al(OH)3 treated normal plasma	>60	35	16	15.5	14	11.5	10	10	10	10
	Al(OH)3 treated haemophilic plasma	26.5	21	17	14.5	12.5	12	11.5	11.5	11.5	12
	Al(OH)3 treated normal serum	22	15	14	13	13	13	14.5	15.5	16	-
	Al(OH)3 treated haemophilic serum	24	13.5	13	13	13	13.5	14	16	16	-

TABLE 60 cont'd.

Test Number	Reagent Used	Substrate Clotting Time In Seconds Time of Subsampling In Minutes									
		2	4	6	8	10	15	20	25	30	35
3	Saline	57	33	22	19	15	12	11	10	10	10
	Al(OH)3 treated normal plasma	13.5	14	11	10	9	8	8	8	9	10
	Al(OH)3 treated haemophilic plasma	50	25.5	26	21	18	13	12	10	10	10
	Al(OH)3 treated normal serum	48	29	22	17	16	15	14	15.5	15	-
	Al(OH)3 treated haemophilic serum	39	21	18	17.5	16.5	15	14	15	14.5	-

TABLE 60 cont'd.

Test Number	Reagent Used	Substrate Clotting Time In Seconds Time of Subsampling In Minutes									
		2	4	6	8	10	15	20	25	30	35
4	Saline	50	31.5	21	16.5	14	12	11.5	11.5	11.5	-
	Al(OH)3 treated normal plasma	10	9	9	8.5	8	8	8	8.5	9	-
	Al(OH)3 treated haemophilic plasma	35	14	13	12	10.5	10	9	9	9.5	-
	Al(OH)3 treated normal serum	49	25	19	14.5	13	12.5	13.5	15	16	-
	Al(OH)3 treated haemophilic serum	47	20	16.5	16	13.5	13	13.5	14	14.5	-

TABLE 60 cont'd.

Test Number	Reagent Used	Substrate Clotting Time In Seconds Time of Subsampling In Minutes									
		2	4	6	8	10	15	20	25	30	35
5	Saline	60	42	24.5	16.5	14.5	11.5	11.5	11.5	11.5	-
	Al(OH)3 treated normal plasma	53	15	12	10.5	10	9	9	9.5	10	-
	Al(OH)3 treated haemophilic plasma	60	20	17	14	12.5	12	11	11	11	-
	Al(OH)3 treated normal serum	73	44.5	25	21	19	17.5	17.5	19.5	22	-
	Al(OH)3 treated haemophilic serum	47	27.5	20	18	16	14	13.5	13	15	-

The results of five such experiments are shown in Table 60.

Results: The behaviour of those mixtures containing saline or treated plasma (normal or haemophilic) was the same as that expected from the use of those reagents in previous tests. When either normal or haemophilic serum treated with aluminium hydroxide was added to incubation mixtures there was interference with thromboplastin generation, but this was of the same degree in both instances. The effects of haemophilic serum were no greater than those of normal serum treated with aluminium hydroxide.

Discussion: It was expected on theoretical grounds that greater residual inhibitory activity would be retained and demonstrated in "fresh" haemophilic serum. Such was not the case. This was an unexpected result and no simple explanation has been forthcoming. However, for the purposes of this thesis the last three experiments had shown that there was no absolute increase in the activity of those inhibitory factors affecting the reaction being considered, in haemophilic plasma. It could be stated therefore, that the unusual results of experiment 39 depended on a relative imbalance of inhibitory factors consequent upon a reduced factor VIII level and a normal content of inhibitors.

Distinct from their clarification of the points mentioned above, the experiments described in this section, and others in similar vein not included herein, have served to demonstrate the "inhibitory" potency inherent in normal plasma and serum. It has been amply shown that the addition of relatively small volumes of appropriately treated plasma or serum to a powerful thromboplastin generation system is capable of producing detectable and often marked interference with thromboplastin generation within that system. As mentioned earlier, the possible importance of such a system is out of proportion to the attention it has received. Perhaps in no small way neglect of this phase of coagulation has stemmed from a lack of suitable techniques for quantitating and studying in any simple way the behaviour of inhibitory factors. The experiments described may, with further investigation provide one avenue of approach to this problem.

SECTION III

Application of Technique

SECTION III. APPLICATION OF TECHNIQUE.

This final section illustrates the application of the technique developed in this thesis to measure platelet thromboplastic function, in the investigation of various diseases either of a primary haemorrhagic nature or in which haemorrhagic manifestations are a common occurrence. Where the investigations undertaken have included procedures other than estimations of platelet thromboplastic function, the techniques used have been those outlined earlier as those used routinely in the laboratory in which this work was undertaken. Examples of the following diseases have been investigated and are described in the order in which they are set out below.

I. Von Willebrand's disease, thrombocytopathia and thrombocytopaenia associated with haemangiomata.
2. Henoch-Schonlein purpura.
3. Scurvy.
4. Dysproteinaemia.
5. Acute and chronic idiopathic thrombocytopaenic purpura.
6. Aplastic anaemia.
7. Acute leukaemia.
8. Thrombocythaemia - including chronic myeloid leukaemia, haemorrhagic thrombocythaemia and thrombocythaemia associated with malignant disease.
9. Polycythaemia and myelofibrosis.
10. Chronic lymphatic leukaemia and lymphosarcoma.
11. Haemolytic anaemia and thrombocytopaenia.
12. An investigation of the coagulation mechanism in uraemia.

.....

I. Von Willebrand's disease, thrombocytopathia and thrombocytopaenia associated with haemangiomata.

This section includes the results of platelet function studies undertaken in certain patients whose haemorrhagic diathesis has been defined as one appropriate to the categories listed above. Historically, Von Willebrand's disease, thrombocytopathia and thrombocyto-asthenia have been considered in the introduction to this thesis. The syndrome of thrombocytopaenic purpura with extensive haemangioma has not been mentioned.

The latter association has been described in several patients, all infants. (Bogin and Thurmond, I95I; Kasabach and Merritt, I940; Rhodes and Borelli, I944; Silver, Aggeler and Crane, I948; Southard, De Sanctis and Waldron, I95I; Weisman and Tagnon, I953). In these cases thrombocytopaenia and haemorrhagic manifestations were found only when the haemangioma was fully developed, and when the tumour regressed spontaneously or by radiation therapy, the thrombocytopaenia promptly remitted. The bone marrow varied considerably, megakaryocytes being variously reported as normal or decreased. Two major hypotheses have been advanced to explain the thrombocytopaenia. The first envisages the utilization of large numbers of platelets during thrombosis within the tumour while the second is based upon the supposed development of an antibody against platelets in an undetermined way in the presence of a severe vascular lesion. The haemorrhagic manifestations have been attributed to the associated thrombocytopaenia. One such case has been seen by the author and is described in this section. However, in this instance, strong presumptive evidence is produced to suggest that the haemorrhagic manifestations were based on a platelet thromboplastic defect.

(I). Von Willebrand's Disease.

8 patients classified as manifesting Von Willebrand's disease have been studied. In all the history of bleeding has been

TABLE 61. Part (1). Von Willebrand's Disease.

Results of routine investigations and platelet function.

TEST	CASE NUMBER			
	1(B.H.)	2(B.W.)	3(R.G.)	4(L.C.)
Bleeding time (mins.)	3	4	5	4
Clotting time (mins.)	7.5	5.0	8	6
Tourniquet test	+	-	-	+
Prothrombin time (secs.)	10.5 (N=10.5)	11 (N=11)	11 (N=11)	10.5 (N=10.5)
Screening test	Normal	Normal	Normal	Normal
Clot Retraction	Normal	Normal	Normal	Normal
Fibrinolysis	Normal	Normal	Normal	Normal
Platelet Count ($\times 10^3$)	400	282	260	200
Platelet Function (%)	100	100	100	100
Capillary Microscopy	Abnormal	Abnormal	-	-

TABLE 61. Part (1). Cont'd.

TEST	CASE NUMBER			
	5(B.M.)	6(J.A.)	7(H.P.)	8(H.D.)
Bleeding time (mins.)	2	7	4	8
Clotting time (mins.)	5.5	7	5	8.5
Tourniquet test	-	+	+	+
Prothrombin time (secs.)	12 (N=12)	12 (N=12)	11 (N=11)	12 (N=12)
Screening test	Normal	Abnormal	Normal	Normal
Clot Retraction	Normal	Normal	Normal	Normal
Fibrinolysis	Normal	Normal	Normal	Normal
Platelet Count (X 10^3)	314	220	190	354
Platelet Function (%)	100	100	100	100
Capillary Microscopy	Abnormal	Abnormal	Abnormal	Abnormal

TABLE 61. Part (2). Von Willebrand's Disease.

Details of the estimations of platelet thromboplastic function.

Case No.	Control or Patient	Time of Subsampling In Minutes Substrate Clotting Times In Seconds					
		4	10	15	20	25	30
1	Control	20	12	10.5	10.5	10.5	10.5
B.H.	Patient	22	11	10.5	10.5	10.5	10.5
2	Control	31	13	10.5	10.5	10.5	10.5
B.W.	Patient	22	12.5	10.5	10.5	10.5	10.5
3	Control	19	12	10	10	10	10
R.G.	Patient	21	12	10.5	10	10	10
4	Control	24	13	12	11	11	11
L.C.	Patient	21	12	11.5	11	11	11
5	Control	31	14.5	12	11	11	11
B.M.	Patient	27	14	12	11	11	11
6	Control	23	13	11	11	11	11
J.A.	Patient	23	13	11	11	11	11
7	Control	41	22	13	10.5	10	10
H.P.	Patient	33	20	12	10	10	10
8	Control	12.5	10	9.5	9.5	9.5	9.5
H.D.	Patient	14.5	10	9.5	9.5	9.5	9.5

similar, recurrent spontaneous bruising and epistaxes featuring prominently, together with excessive blood loss following surgery, dental extraction or minor trauma. In most patients there was a family history of similar but, usually, less severe bleeding. Every patient was investigated by the routine procedures adopted in this laboratory for the detection of a suspected bleeding tendency. In addition estimations of platelet thromboplastic function and capillary microscopy were performed. The results are shown in Table 6I, parts (I) and (2).

The diagnosis of Von Willebrand's disease is not always simple. In all of these patients the personal history, and, in most, the family history, was compatible with the diagnosis, and in all an essential laboratory feature, an abnormal bleeding time, was present. The abnormality of the latter was not so much in duration, for only in 6 was it prolonged beyond the normal and, in none, markedly so, but in the volume of blood lost during the procedure. In every patient there was an immediate excessive blood loss as soon as the skin was punctured, and this persisted throughout the duration of the bleeding time. The author agrees with Willoughby and Allington (I96I) that this is a significant feature and regards it and the actual duration of a bleeding time estimation of comparable importance. In 5 patients the tourniquet test was positive and in 6 direct microscopy demonstrated capillary abnormalities. In all of the latter there was an abnormally high proportion of widely dilated and tortuous capillaries while in cases 6 and 8 transverse communications between adjacent capillary loops were visualized. In case 6 some capillaries appeared to have pseudopodial attachments. The same patient had a slightly abnormal "screening" test secondary to a reduced level of factor VIII. The clotting times, platelet numbers, clot retraction and fibrinolytic activity were normal, in all, and platelet thromboplastic function was I00 percent in every case.

It was concluded therefore that in these examples of Von Willebrand's disease, the underlying defect was a capillary

TABLE 62. Thrombocytopathia - Case 1.

Patient V.D. and her daughters S.D., M.D. and B.D.

Part (1). Routine Investigations.

TEST	PATIENT			
	1(V.D.)	2(S.D.)	3(M.D.)	4(B.D.)
Bleeding time (mins.)	2.25	2	1	2
Clotting time (mins.)	7	6	8	8
Tourniquet test	-	-	-	-
Prothrombin time (secs.)	10.5 (N=10.5)	11 (N=11)	12 (N=11)	11 (N=11)
Screening test	Normal	Normal	Normal	Normal
Clot Retraction	Normal	Normal	Normal	Normal
Fibrinolysis	Normal	Normal	Normal	Normal
Platelet Count (X 10^3)	238	360	340	394
Platelet Function (%)	42	100	100	100

TABLE 62. Part (2). Thrombocytopathia - Case 1 (V.D.)

Details of platelet thromboplastic function estimations.

Case No.	Control or Patient	Time of Subsampling In Minutes Substrate Clotting Times In Seconds					
		4	10	15	20	25	30
1	Control	23	11	10	10	10	10
V.D.	Patient	19	15	13	12.5	12.5	13.5
2	Control	12	10.5	10	10.5	10.5	11
S.D.	Patient	12	10.5	10.5	10.5	11.5	12
3	Control	12	10.5	10	10.5	10.5	11
M.D.	Patient	12	10.5	10.5	10.5	10	11.5
4	Control	12	10.5	10	10.5	10.5	11
B.D.	Patient	12	10.5	10.5	11	12	12.5

abnormality and that there was no platelet thromboplastic defect.

(2). Thrombocytopathia.

Three patients have been studied. In the first two the history was almost identical with those outlined above while the third presented with severe, prolonged haemorrhage following surgery.

Case (I). V.D., a 38 year old female was admitted to hospital with recurrent severe epistaxes. There was a history of bruising tendency throughout her life, prolonged bleeding following dental extraction and haemorrhage necessitating blood transfusion after appendectomy and each of her 3 pregnancies. There was a similar but less marked bleeding tendency among isolated female relatives but the patient's own 3 daughters were apparently normal. Former routine coagulation studies had been quite negative. She was re-investigated during her present admission and, despite adequate platelet numbers, her platelet thromboplastic function was studied. The results are shown in Table 62, parts (I) and (2).

There was no demonstrable abnormality by routine screening procedures, but a marked platelet thromboplastic defect was found. Subsequently the patient's daughters were studied but there was no similar defect in them, although, at the same time, the maternal abnormality was confirmed.

Case (2). L.K., a 42 year old male had a very definite bleeding history manifest as recurrent epistaxes and bruising and prolonged bleeding following slight trauma. Dental extractions and minor surgical procedures had necessitated transfusion. Several prior investigations of his coagulation mechanism had been negative. He was referred to the laboratory for re-assessment in anticipation of future major surgery. At this time the routine procedures formerly used were repeated but, in addition, his platelet thromboplastic function was estimated. The only demonstrable and quite unexpected defect, a reduced platelet thromboplastic function (37%)

TABLE 63. Thrombocytopathia.

Case 2. - L.K.

Part (1). Routine Investigations.

Bleeding Time	Clotting Time	Tourniquet Test	Prothrombin Time	Screening Test	Clot Retraction	Fibrinolysis	Platelet Count
2 mins.	7.5mins.	Negative	12.5 secs. (N=11secs.)	Normal	Normal	Normal	$174X10^3$

TABLE 63. Part (2). Thrombocytopathia.

Details of platelet thromboplastic function estimations.

Time of	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes — Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
17.2.61	174	Control	13.5	10	9	10	9	9	37
		Test	22.5	12	12	12	12.5	12	
19.2.61	190	Control	40	15.5	12	11	10	10	48
		Test	49	16	13	12	12	12	
25.2.61 Base level	180	Control	16.5	11.5	9.5	9	9	9	42
		Test	19	13.5	12	11.5	11.5	12	
25.2.61 First transfusion	174	Control	16.5	12	10	9.5	9	9	37
		Test	19.5	12.5	12.5	12	12	12	
25.2.61 Second transfusion	126	Control	17	11	9.5	9	9	9	62
		Test	19	11	10.5	10	10	10	
26.2.61	170	Control	18.5	10.5	10	10	10	10.5	55
		Test	22	14	12	11.5	11.5	11.5	
27.2.61	184	Control	24	12	10	10	10	10.5	48
		Test	23	14	13.5	12	12	12	

was confirmed in a second estimation two days later.

It was thought that surgery, albeit major, might be safely undertaken at a future date if his platelet defect could be corrected, but, that steroids would be contra-indicated under the circumstances. Accordingly the patient was admitted to hospital to assess the value of the transfusion of platelet "rich" fresh blood. Immediately prior to transfusion, blood was collected for a base level platelet count and function. He was then bled of one pint of blood and immediately and rapidly transfused with one pint of fresh platelet rich blood collected into a plastic bag. I5 minutes after transfusion, blood was collected for platelet studies, and a second identical transfusion was performed. After this and subsequently I8 and 42 hours after the first transfusion further estimations were made. The results of all relevant investigations are shown in Table 63, parts (I) and (2).

Prior to transfusion the patient's platelet thromboplastic function was 42%. After the first transfusion, there was little change and a value of 37% was obtained. Mild thrombocytopaenia was evident after the second transfusion, a not unusual finding. The platelet thromboplastic function was markedly improved, however, and had risen to 62%. After I8 hours the platelet numbers had returned to normal and improved platelet thromboplastic function was still evident, but after 42 hours the latter had declined but was still a little above its pre-transfusion level.

Case (3). M.K., an 8 year old boy was admitted to hospital for tonsillectomy. Adenoidectomy some 4 years before had been complicated by profuse and continued haemorrhage, necessitating transfusion. He was stated to bruise easily and bleed excessively from small abrasions. The family history was of doubtful significance. The routine coagulation investigations undertaken prior to surgery were negative. Tonsillectomy was undertaken and again there was profuse and continued bleeding from both tonsillar fossae, this persisting for hours, and necessitating transfusion with two pints of whole blood, despite drastic local

TABLE 64. Thrombocytopathia.

Case 2 - M.K.

Part (1). Routine Investigations.

Bleeding time (min.)	Clotting time (min.)	Tourniquet test	Prothrombin time (sec.)	Screening test	Clot Retraction	Fibrinolysis	Platelet Count (X 103)
2.25	7	Negative	13.5 (N = 13)	Normal	Normal	Normal	394

Part (2). Details of platelet thromboplastic function estimations.

	Control or Patient	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Function Percent
		4	10	15	20	25	30	
	Control	24.5	13.5	9.5	9	9.5	9.5	33
	Patient	34	17	15.5	13	13	13	

measures. Prior to transfusion and while active bleeding was in progress, the "prothrombin" time, screening and classical thromboplastin generation tests were normal and there was no excess fibrinolytic activity. Seven days after tonsillectomy, his platelet thromboplastic function was found to be 33% of normal. The results are shown in Table 64, parts (I) and (2).

These 3 cases have been described at some length to illustrate the importance of estimations of platelet thromboplastic function in any patient in whom there is a definite history of abnormal bleeding and in whom routine screening procedures have failed to reveal any defect. In each of these patients apparently adequate platelet numbers were accompanied by marked defects of their thromboplastic function. The fallacy of relying on platelet numbers as a sufficient guide to a normal coagulation mechanism was well shown, for the first two patients had had prior investigations but in the face of normal platelet numbers it had been assumed that these were functionally sound. The second case illustrates a further important point, namely, the usefulness of estimations of platelet thromboplastic function in assessing the value of platelet transfusions in certain selected cases. A purely clinical assessment would have been of no use in the case described, while the occurrence of mild thrombocytopaenia following the second transfusion would have suggested an exaggerated haemorrhagic tendency had one to rely on platelet numbers alone. Definite quantitative information provided by the platelet function estimations demonstrated that such was not the case and provided valuable information to guide pre- and post-operative management in any future surgery.

(3). Purpura, thrombocytopaenia and haemangioma.

M.A., the first born male child of a 27 year old woman had extensive purpura and ecchymoses at birth. An extensive haemangioma involved both legs from the knee to the ankle. The whole blood platelet count was 30 x 10^3 per c.mm. The mother stated that she had bruised easily all her life and had, on

TABLE 65. Hereditary thrombocytopaenia with a functional platelet defect.

Part (1). Routine Investigations.

TEST	PATIENT			
	Mrs. A	Mrs. Z	Victor Z	Peter Z
Bleeding time (mins.)	¾	3	1	1.5
Clotting time (mins.)	5	4.5	5.5	7
Tourniquet test	++	+	-	-
Prothrombin time (secs.)	12.5 (N=11.5)	12 (N=11.5)	11 (N=11)	11 (N=11)
Screening test	Normal	Normal	Normal	Normal
Clot Retraction	Normal	Normal	Normal	Normal
Fibrinolysis	Normal	Normal	Normal	Normal
Platelet Count ($X\ 10^3$)	70	110	324	220
Platelet Function (%)	42	54	100	100

Mrs. A = mother of Mark A.
Mrs. Z = grandmother of Mark A.
Victor Z = uncle of Mark A.
Peter Z = uncle of Mark A.

TABLE 65. Part (2). Hereditary thrombocytopaenia with a functional platelet defect.

Details of platelet thromboplastic function estimations.

Case No.	Control or Patient	Time of Subsampling In Minutes Substrate Clotting Times In Seconds					
		4	10	15	20	25	30
1	Control	13	10	9.5	10	10	10
	Patient	21	16	12.5	12.5	12.5	12.5
2	Control	13	10	9.5	10	10	10
	Patient	17	13	12	11.5	11.5	11.5
3	Control	30	17	14.5	13	13	13
	Patient	34	17	14.5	14.5	13	13
4	Control	30	17	14.5	13	13	13
	Patient	29	16	14	13	13	13

Case No. 1 = Mrs. A.
Case No. 2 = Mrs. Z.
Case No. 3 = Victor Z.
Case No. 4 = Peter Z.

occasions developed crops of small red "spots" on her chest, legs and arms. These had not concerned her, because her own mother was affected similarly but to a lesser extent.

Bone marrow examination in the child was essentially normal except for an apparent reduction in megakaryocytes, and while insufficient blood could be obtained from him for platelet function studies, full coagulation investigations were carried out on the mother, grandmother and two maternal brothers. The results are shown in Table 65, parts (I) and (2).

No defect was found in the males, and in both the platelet thromboplastic function was I00 percent. The two females however, were both thrombocytopaenic, both had positive tourniquet tests and both had reduced platelet function. All defects were more marked in the mother than in the grandmother as was the bleeding tendency, but the latter and the thrombocytopaenia were less marked than in the child.

In the opinion of radiologists the child's haemangioma was not suitable for irradiation. Because of this and the reported tendency to spontaneous remission a conservative approach was adopted, and during twelve months close observation there was indeed a progressive and marked reduction in the size of both legs. There was no concomitant improvement in either platelet numbers or the haemorrhagic tendency. The former was consistently between $20\text{-}50 \times I0^3$ per c.mm., while the child continued to bruise, frequently and extensively, spontaneously or as a result of minimal trauma.

This child then differs from similar cases reported, in that a marked regression of the vascular tumours has not resulted in a rising platelet count or improved haemorrhagic diathesis. Secondly, what appears to be a congenital and inherited thrombocytopaenia and platelet functional defect has been demonstrated in 2 other members of the family. Furthermore, both defects and the haemorrhagic tendency seen to be becoming progressively worse with succeeding generations and it will be interesting to confirm this when adequate platelet function studies are undertaken on the child. The progress

and family studies in this case suggest that the same coagulation defects would have been found in this child in the absence of the haemangioma. Be that as it may it would be interesting to perform platelet function studies and perhaps familial studies in further cases manifesting the syndrome of thrombocytopaenic purpura and haemangioma.

2. Henoch-Schonlein Purpura.

Acute vascular purpura, according to Dameshek (I953) may be considered a generalized disturbance of small blood vessels in which purpura is only one, albeit a striking manifestation. Although the pathogenetic mechanism is not clear there is considerable evidence to suggest an immunologic basis (Bartley and Bell, I936; Dameshek, I953; Glanzmann, I920; Clark and Jacobs, I950). The acute purpuric reaction often follows in the wake of an infection, the ingestion of drugs, an insect bite (Siegel, Brown and Di Leo, I954) or the ingestion of a food to which the patient has become sensitized (Alexander and Eyermann, I927; Brown, I946). In adults a more chronic form of vascular purpura is sometimes seen in the presence of chronic infection. Specifically, in I950, Dalgleish and Ansell described the occurrence of anaphylactoid purpura in pulmonary tuberculosis. In two of the three patients studied by the author acute vascular purpura occurred during the course of pulmonary tuberculosis.

The first patient (F.P.) had long standing chronic tuberculosis for which he had recently completed a course of anti-tuberculous therapy (streptomycin, isoniazid, and para-amino-salicylic acid). During the last few weeks of therapy he had complained of recurrent joint-pains for which no cause could be found. Subsequently, he developed a widespread purpuric rash and melaena. He was investigated during a second such episode some 6 weeks after the first. The second patient (A.P.) was admitted to hospital with vague "rheumatic" pain, purpura, haematemesis and melaena. He, too, had chronic tuberculosis but had not had any chemotherapy for some

TABLE 66. Henoch-Schonlein Purpura.

Part (1). Routine Investigations.

TEST	PATIENT		
	1(F.P.)	2(A.P.)	3(E.L.)
Bleeding time (mins.)	3.5	3	5
Clotting time (mins.)	7	8¼	8
Tourniquet test	+	+	++
Prothrombin time (secs.)	12 (N=11)	11 (N=11)	12 (N=11)
Screening test	Normal	Normal	Normal
Clot Retraction	Normal	Normal	Normal
Fibrinolysis	Normal	Normal	Normal
Platelet Count ($X\ 10^3$)	470	200	246
Platelet Function (%)	100	100	100

TABLE 66. Part (2). Henoch-Schonlein Purpura.

Details of platelet thromboplastic function estimations.

Case No.	Control or Patient	Time of Subsampling In Minutes Substrate Clotting Times In Seconds					
		4	10	15	20	25	30
1	Control	17	12.5	12	12	11.5	12
F.P.	Patient	14.5	13	12	12.5	12	12
2	Control	24	12	11.5	11	11	11
A.P.	Patient	21	11.5	11	11	11	12
3	Control	31	12	10	10	10	10
E.L.	Patient	27	11	10	9.5	10	10

time. In both of these patients the history was suggestive of Henoch-Schonlein purpura. In the first patient an hypersensitivity reaction to one of the chemotherapeutic agents was considered but seemed unlikely in view of the fact that the second episode during which he was investigated occurred after all drugs had been withdrawn and their subsequent use was uneventful.

The third patient (E.L.) a boy of I2 years was seen after his history of swollen and tender joints, recurrent epistaxes, purpura and bruising, of recent onset had suggested Henoch-Schonlein purpura. The findings in all 3 patients are shown in Table 66, parts (I) and (2).

In so far as this thesis is concerned the important feature was the presence of normal platelet thromboplastic function in each of these patients, all of whom were studied in the active phase of their disease. It was concluded that in Henoch-Schonlein purpura the underlying defect was increased capillary permeability of undetermined aetiology, and that platelet thromboplastic defects did not contribute to the haemorrhagic signs.

3. Scurvy.

Since ascorbic acid is apparently essential for the synthesis of the cement substance of the capillary wall, the bleeding tendency of scurvy may be assumed to be typical of increased vascular permeability. However, the bleeding tendency may not always be due exclusively to increased capillary permeability for in some few cases thrombocytopaenia may occur. (Stefanini and Dameshek, I955; Smith, I960). Three children with clinical and radiological evidence of advanced scurvy have been studied by the author. The results are shown in Table 67, parts (I) and (2). All had positive tourniquet tests and one a markedly prolonged bleeding time. In each case the platelet thromboplastic function was found to be I00 percent. None, however, was thrombocytopaenic and it remains to be determined as suitable cases present whether thrombocytopaenic cases have an associated thromboplastic defect. None the less it appears

TABLE 67. Scurvy.

Part (1). Routine Investigations.

TEST	CASE NUMBER		
	1	2	3
Bleeding time (mins.)	2	$2\frac{3}{4}$	9.5
Clotting time (mins.)	$5\frac{1}{2}$	4	9
Tourniquet test	++	++	+++
Prothrombin time (secs.)	12 (N=11)	11 (N=11)	12.5 (N=11)
Screening test	Normal	Normal	Normal
Clot Retraction	Normal	Normal	Normal
Fibrinolysis	Normal	Normal	Normal
Platelet Count ($\times 10^3$)	280	256	318
Platelet Function (%)	100	100	100

TABLE 67. Part (2). Scurvy.

Details of platelet thromboplastic function estimations.

Case No.	Control or Patient	Time of Subsampling In Minutes Substrate Clotting Times In Seconds					
		4	10	15	20	25	30
1	Control	24	12.5	11	11	11	11
	Patient	24	12.5	11	11	11	11
2	Control	45	32	21	15	13	13
	Patient	40	27	18	14	13	13
3	Control	15	9	8.5	9	9	9
	Patient	17	10.5	9.5	9	9	9

that in advanced, non-thrombocytopaenic, scurvy platelet thromboplastic function is not impaired, and the haemorrhagic manifestations have a vascular origin.

4. Dysproteinaemia.

A bleeding tendency of varying severity is found in a number of conditions which have in common an abnormality of the plasma proteins. Included in this group are cases of multiple myeloma, idiopathic hyperglobulinaemia, cryoglobulinaemia and macroglobulinaemia. It has been said that thrombocytopaenia is the most important cause of bleeding in multiple myeloma (James et al, I953). However, in some instances where platelet numbers and, apparently, their function have been normal, other mechanisms have been invoked. These have included infiltration of the vascular wall by the abnormal protein (Uehlinger, I949; Ranstrom, I946), interference with the formation of thromboplastin and fibrin by the high concentration of abnormal protein (Craddock, Adams and Figueroa, I953; Luescher and Labhart, I949; Uehlinger, I949) and the binding of calcium by the abnormal protein (Rawson and Sundermann, I948; Craddock, Adams and Figueroa, I953). The same mechanisms have been invoked to explain haemorrhage in idiopathic macroglobulinaemia and cryoglobulinaemia, the latter being almost always symptomatic of some other fundamental disease process such as multiple myeloma, lymphoma etc. In addition, there have been several reports of the presence of inhibitors of coagulation in various disease states all associated with an abnormal plasma protein (de Nicola, I950; Bernard et al, I952; Andre et al, I952; Craddock et al, I953; Mueller, Ratnoff and Heinle, I95I). In these the main defect seems to have been an interference with thrombin formation.

Among the dysproteinaemias, the haemorrhagic manifestations of Waldenstrom's macroglobulinaemia have received widespread attention. (Hule and Wiedemann, I954; Long et al, I955; Jim and Steinkamp, I956; Quattrin et al, I956-57; Henstall and Kligermann, I958;

TABLE 68. Dysproteinaemias.

Part (1). Routine Investigations.

TEST	CASE NUMBER			
	1*	2**	3+	4++
Bleeding time (mins.)	2½	3	5¼	2¼
Clotting time (mins.)	8	6	7¾	8
Tourniquet test	+	++	++	-
Prothrombin time (secs.)	12 (N=11)	12.5 (N=11)	13 (N=10.5)	12.5 (N=11)
Screening test	Normal	Normal	Normal	Normal
Clot Retraction	Normal	Normal	Reduced	Normal
Fibrinolysis	Normal	Normal	Normal	Normal
Platelet Count ($\times 10^3$)	250	196	84	132
Platelet Function (%)	100	37	28	48

1* Hyper-gammaglobulinaemia - recurrent purpuric rash on legs.

2** Macro-globulinaemia - recurrent spontaneous bruising. Epistaxes.

3+ Myelomatosis - spontaneous bruising. Purpura. Epistaxes.

4++ Cryo-globulinaemia - spontaneous bruising.

TABLE 68. Part (2). Dysproteinaemias.

Details of platelet thromboplastic function estimations.

Case No.	Control or Patient	Time of Subsampling In Minutes Substrate Clotting Times In Seconds					
		4	10	15	20	25	30
1	Control	20	9	9	9	9	9
	Patient	19	10	9	9	9	9
2	Control	24	12	11	10.5	10.5	10.5
	Patient	23.5	14	13.5	13.5	13.5	13.5
3	Control	15	11	9.5	9	9	9
	Patient	27	18	15	13.5	13.5	14
4	Control	13.5	10	9	10	9	9
	Patient	16.5	11.5	11	10.5	11	11

Kappeler et al, I958). No consistent pattern has evolved. Henstall and Kligermann postulated lowering of plasma coagulation factors by their quantitative adsorption onto the abnormal proteins, while Long and others believed that the reduced prothrombin values they had found were the direct cause of the bleeding. Pachter et al (I959), however, did not consider that the prothrombin values reported were sufficiently low to account for the bleeding and after extensively investigating two patients of their own, they postulated that the bleeding tendency was due to impaired platelet function secondary to coating of platelets by an abnormal protein. Moreover, two earlier reports had suggested that platelets might be integrally involved in the bleeding tendency. Braunsteiner and his associates (I954) reported morphologically abnormal platelets in macro-molecular cryoglobulinaemia, while in I956, Jurgens using the thromboplastin generation test and other techniques, had postulated that the abnormal proteins interfered with platelets.

The author has had an opportunity to study the coagulation mechanism in four patients with dysproteinaemia, all of whom had been recently haemorrhagic or were actively haemorrhagic when studied. The results of the investigations are shown in Table 68, parts (I) and (2).

The first two patients had hyperglobulinaemia and macroglobulinaemia respectively. Both had normal platelet numbers, but despite this there was an obvious platelet thromboplastic defect in one (37%). The only other demonstrable defect was an increased capillary fragility in both patients.

The remaining two patients were both thrombocytopaenic. One of these had multiple myeloma and in the other cryoglobulins were present but no underlying disease could be discovered. In the patient with myeloma, a prolonged bleeding time, positive tourniquet test, a slightly prolonged 'prothrombin' time, reduced clot retraction and a marked platelet thromboplastic defect (28%) were revealed. In the remaining patient the mild thrombocytopaenia was accompanied by a platelet thromboplastic defect, as the only demonstrable abnormalities.

These results confirmed the suggestion of Pachter et al (1959) that platelet functional defects might be present in the dysproteinaemias, while the severity of the defects demonstrated in patients 2 and 3 might indicate that they play a significant, if not dominant, role in the haemorrhagic manifestations. Furthermore a platelet functional defect was present in the second case despite platelet numbers within the normal range. The demonstration of such defects in three of the four cases studied suggests that abnormalities of platelet function are of common occurrence in the dysproteinaemias, but whether they occur invariably at some stage in all cases remains to be determined as further patients are followed with serial examinations.

5. Acute and Chronic Thrombocytopaenic Purpura.

The historical implications of haemorrhage in thrombocytopaenic states have been considered fully in the introduction to this thesis. In several papers Bonnin (1956,1957,1961) has described the uses of estimations of platelet thromboplastic function in the assessment and management of haemorrhage in such conditions. The cases to be described in this section (7 of acute and 4 of chronic thrombocytopaenic purpura) are included for two reasons.

1. To illustrate the general use of estimations of platelet thromboplastic function in cases of acute and chronic thrombocytopaenia as an important adjunct to clinical care in the assessment and management of the haemorrhagic state.
2. To illustrate how estimations of platelet function may be used to clarify special problems and considerations in individual cases.

It is not intended to define the drug treatment of such cases in any detail or to formulate any strict relationship between levels of platelet function and the occurrence or severity of haemorrhage. Too few cases have been studied by the author using the technique described to draw any definite conclusions in the latter respect.

TABLE 69. Acute Thrombocytopaenic Purpura.

Case 1 - K.R.

Part (1). Routine Investigations.

Bleeding Time	Clotting Time	Tourniquet Test	Prothrombin Time	Screening Test	Clot Retraction	Fibrinolysis
10mins.	9mins.	++++	12 secs. (N = 11.5)	Normal	Absent	Normal

TABLE 69. Part (2). Acute Thrombocytopaenic Purpura.

Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count ($\times 10^3$)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30.	
24.6.60 On admission	22	Control	16.5	10	10	10	10	10	14
		Test	> 60	32	21.5	22	21.5	22	
27.6.60	6	Control	21	13	11	9.5	9.5	9.5	48
		Test	22.5	17	12.5	11.5	11.5	12	
29.6.60	16	Control	27	15	13	10.5	10	10	62
		Test	25	14	12	11	11	11	
1.7.60	38	Control	12.5	10	9.5	9.5	9.5	9.5	100
		Test	14.5	10	9.5	9.5	9.5	9.5	
3.7.60	138	Control	14	11	10	10	10	10	100
		Test	11	10	10	10	10	11	
5.7.60	260	Control	22	11.5	11	11.5	11	11	100
		Test	32	13	11.5	11	11	11	
7.7.60	422	Control	16.5	12	10.5	10.5	10.5	10.5	100
		Test	27	12	11	10.5	10.5	11	

(I). Acute thrombocytopaenic purpura.

Case I. K.R., a 20 year old female.

Two weeks after an attack of 'flu the patient developed purpura and spontaneous bruising followed by haematuria and menorrhagia. Except for the haemorrhagic signs the physical examination was negative. The whole blood platelet count was 22 x 10^3 per c.mm. and the diagnosis of acute thrombocytopaenic purpura was confirmed by the typical marrow findings. The coagulation studies on admission and subsequently are shown in Table 69, parts (I) and (2).

In addition to the thrombocytopaenia, on admission, the bleeding time was prolonged, the tourniquet test strongly positive and the platelet thromboplastic function was only I4 percent. Steroid therapy was begun (Prednisolone 60mgs/day) and progress was followed by serial platelet counts and estimations of platelet thromboplastic function.

All haemorrhagic manifestations had ceased within 24 hours and did not recur. On the third day however, the platelet count was only 6 x 10^3 per c.mm. and on the fifth I6 x 10^3 per c.mm. Despite this the corresponding platelet thromboplastic function was 48 and 62 percent respectively. Obviously then, clinical improvement had coincided with improvement in platelet thromboplastic function (and probably capillary repair) rather than rising platelet numbers. By the seventh day the platelet count had risen to 38 x 10^3 per c.mm. and their thromboplastic function was I00 percent. Slow reduction in steroid dosage was begun and, despite this, during the next week the platelet numbers on alternate days were I38 x 10^3, 260 x 10^3 and 422 x 10^3 per c.mm. respectively and on each occasion their function was I00 percent.

Since there had been no recurrence of haemorrhage, thrombocytopaenia or thromboplastic defects while steroids were being reduced the patient was discharged, and was seen thereafter weekly for one month, fortnightly for 2 months and thereafter monthly for a total of I2 months. On each occasion her platelet numbers and function were normal, despite a severe bout of 'flu 5 months

TABLE 70. Acute Thrombocytopaenic Purpura.

Case 2 - B.H. Part (1). Routine Investigations.

Bleeding Time	Clotting Time	Tourniquet Test	Prothrombin Time	Screening Test	Clot Retraction	Fibrinolysis
$2\frac{1}{2}$ mins.	6mins.	Negative	11secs. (N = 11)	Normal	Normal	Normal

Part (2). Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes — Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
5.10.59	66	Control	22	13	12	12	11.5	12	100
		Test	16.5	12	12	12	12	12	
13.10.59	110	Control	12.5	9.5	9.5	9.5	9.5	9.5	100
		Test	13	9.5	9.5	9.5	9.5	9.5	
23.10.59	40	Control	15.5	11.5	10	10	10	10.5	100
		Test	15	13	12.5	11	10	10	

TABLE 70. Part (2). Cont'd.

Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes — Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
6.11.59	110	Control	17.5	14.5	12	12.5	13	12	100
		Test	14.5	12	12	12	12.5	12	
4.12.59	72	Control	22	13	12	11	11	11	100
		Test	25	15.5	14	11.5	11	11	
15.1.60	118	Control	24	14	10.5	10	10	10	100
		Test	25	12.5	10	9.5	10	10	
12.2.60	80	Control	31	12.5	12	12	12	12	100
		Test	29	13.5	12.5	12	12	12	
18.3.60	164	Control	24.5	17	14	12	12	12	100
		Test	21	14.5	12	12	12.5	12	
12.4.60	220	Control	22.5	19	14	11.5	11	11	100
		Test	24	17.5	13	11	11	11	
14.5.60	270	Control	43	27	18	14	12	12	100
		Test	41.5	23	14	12.5	12	12	

after her original admission.

Case 2. B.H., a 23 year old male clerk.

The patient was referred to the laboratory for a complete blood examination and Paul Bunnell test to confirm a suspected diagnosis of infectious mononucleosis. The presence of characteristic lymphocytes and a high and subsequently rising titre (I in 64 to I in I024) in the Paul Bunnell reaction confirmed the diagnosis. In the initial examination of the blood film, platelets were thought to be markedly reduced and indeed the whole blood count was only 60 x $I0^3$ per c.mm. The patient had no haemorrhagic signs apart from a few purpuric spots on his palate. He returned to the laboratory on the day following the first examination for coagulation investigations. The results of these and subsequent studies are shown in Table 70, parts (I) and (2).

The only significant finding throughout was thrombocytopaenia, this persisting for 4 months. Despite this his platelet function was normal on all occasions, and, in view of this and the complete absence of any haemorrhagic signs no specific therapy was given. After 4 months the platelet count returned relatively quickly to normal, while his platelet thromboplastic function was always I00 percent. There was no recurrence over a period of I2 months observation.

Case 3. L.S., a I7 year old typiste.

I0 days prior to admission to hospital the patient had had an acute upper respiratory tract infection after which she noted a tendency to bruise easily. Menstruation commencing five days prior to admission was exceptionally profuse and continued unabated beyond its usual three days. Coincident with this, she developed purpura and spontaneous bruising on her legs, arms and trunk, and, on the morning of admission she had had an epistaxis. The whole blood platelet count was 22 x $I0^3$ per c.mm. and the diagnosis of acute idiopathic thrombocytopaenic purpura was confirmed by marrow examination. The initial and subsequent coagulation studies are

TABLE 71. Acute Thrombocytopaenic Purpura.

Case 3 - L.S.

Part (1). Routine Investigations.

Bleeding Time	Clotting Time	Tourniquet Test	Prothrombin Time	Screening Test	Clot Retraction	Fibrinolysis
4mins.	6½ mins.	++	11secs. (N = 11)	Normal	Absent	Normal

TABLE 71. Acute Thrombocytopaenic Purpura.

Case 3 - L.S. Part (2). Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count ($\times 10^3$)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
16.11.60	22	Control	55	21	14.5	12	12	12	30
		Test	72	27	17	15.5	16	16	
17.11.60	16	Control	33	17	13	11.5	11	11.5	100
		Test	25	16.5	13	11.5	11.5	11.5	
21.11.60	24	Control	37	18	13	10.5	10	10	100
		Test	39	20.5	13	11	10	10	
23.11.60	8	Control	65	26.5	16	12	11	11	> 100
		Test	45	22	14	12	10	10	
24.11.60	10	Control	18.5	10.5	10	10	10	10.5	> 100
		Test	24	12	9.5	9.5	9.5	10	
25.11.60	11	Control	21	11.5	10	10	10	10	77
		Test	21	11	10.5	10.5	10.5	10.5	
28.11.60	18	Control	33	17	12.5	11	11	11	100
		Test	27	17.5	12	10.5	11	11	
6.12.60	38	Control	17	10.5	10	10	10	10.5	77
		Test	17	12	11	10.5	10.5	10.5	

TABLE 71. Part (2). Cont'd.

Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
9.12.60	28	Control	35	16.5	11.5	10	10	10	62
		Test	34.5	16	11.5	11.5	11	11	
12.12.60	45	Control	21	13	11.5	10	10	10	100
		Test	19	12	10.5	10	10	10	
17.12.60	58	Control	33	21.5	13	12.5	10.5	10.5	100
		Test	29	23	14.5	12	10.5	10.5	
19.12.60	78	Control	24	18	10	10	10	10	100
		Test	21	16	10.5	10	10	10	
24.12.60	98	Control	20	13	10.5	10.5	10.5	10.5	100
		Test	19	14	11	10.5	10.5	11	
2.1.61	270	Control	27	13	12	11	11	11	100
		Test	23	11.5	11.5	11	11	11	
9.1.61	560	Control	43	20.5	17	13	11	11	100
		Test	31	15	12	11	11	11	
16.1.61	420	Control	18	12	10	10	10	10.5	100
		Test	20	11.5	10.5	10	10	10	

shown in Table 7I, parts (I) and (2).

On admission she had numerous bruises on her legs, arms and trunk, purpura on her palate and dried fresh blood in her nose and naso-pharynx. Her bleeding time was slightly prolonged, the tourniquet test positive and her platelet thromboplastic function 30 percent. Steroid therapy (prednisolone 60mgs/day) was commenced and her progress assessed by serial platelet counts and platelet function estimations.

24 hours after admission her platelet count was only I6 x $I0^3$ per c.mm. Their thromboplastic function had returned to I00%, however, and all haemorrhagic signs had ceased to progress. During the next two weeks there was no significant change in platelet numbers, and, despite this steroids were being reduced to a small maintenance dose. Continuing thrombocytopaenia was a disconcerting feature, and, because of this, and other social and economic considerations splenectomy was seriously considered. This was opposed on the grounds that despite thrombocytopaenia, the platelet thromboplastic function had remained I00 percent and, indeed, on two successive occasions was found to be greater than this, and because there was no recurrence of haemorrhage.

Accordingly she was discharged from hospital on a small daily maintenance dose of prednisolone and seen regularly as an outpatient. One week later the platelet count had risen to 38 x $I0^3$ per c.mm. but their function was estimated to be 77 percent. 3 days later and coincident with menstruation the platelet count had fallen to 28 x $I0^3$ per c.mm. and their thromboplastic function to 62 percent. Menstruation was not abnormal, however, and within a few days the platelet count began to rise rapidly to reach thrombocythaemic levels and finally to become stabilized within the normal range. Coincident with this improvement platelet function returned to I00 percent and remained at this level. Steroids had been discontinued. There has been no relapse in I0 months follow up despite two severe respiratory infections similar to those preceding the initial illness.

This case demonstrated two interesting features. It is one

of only two instances throughout this work wherein the platelet function of a patient under treatment has been estimated to be greater than the normal. This may have been due to technical error but since it happened on two successive occasions this is, perhaps, unlikely. The other interesting observation is the possible influence of an hormonal mechanism on the progress of the disease, instanced by the exacerbation of the haemorrhagic state coincident with menstruation prior to admission and the slight relapse with menstruation during the course of treatment, and the rapid improvement when menstruation ceased. A probable hormonal influence is evident in the next case to be described but active at a much later stage.

Case 4. D.C., a 32 year old housewife.

4 months prior to the present admission the patient had been admitted to hospital with acute idiopathic thrombocytopaenic purpura, presenting with the sudden onset of purpura, bruising, haematuria and epistaxis. At this time her platelet count was 26×10^3 per c.mm., the bleeding time prolonged, the tourniquet test positive and her platelet thromboplastic function markedly reduced. Steroid therapy was begun and progress followed by serial platelet counts and estimations of platelet thromboplastic function. During this admission the latter was assessed by Bonnin's modification of the thromboplastin generation test. For 3 weeks there was no significant change in either platelet numbers or function, and the patient continued to bruise spontaneously, had frequent epistaxes and developed menorrhagia. Finally transfusion was needed to correct the resulting anaemia. Because of the poor response to high steroid dosage splenectomy was decided upon but refused by the patient. During the next 4 weeks the platelet count rose steadily finally becoming stabilized at approximately 100×10^3 per c.mm. During this time the platelet function returned to normal and remained thus. Steroids had been discontinued meanwhile.

4 months following the first admission and following an attack

TABLE 72. Acute Thrombocytopaenic Purpura.

Case 4 - D.C. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count ($X 10^3$)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
29.7.61	16	Control	31	17	12.5	11	11	11	5
		Test	120	45	36	37	36	36.5	
31.7.61	20	Control	17	13	10.5	9.5	9.5	9.5	9
		Test	62	34	32	28.5	28.5	29.5	
3.8.61	50	Control	21	13	10	10	10	10.5	< 5
		Test	64	47	39	39	39	39	
/.8.61	30	Control	31	17	12	10.5	10.5	11	< 5
		Test	66	51.5	46	46	48	47	
10.8.61	96	Control	43	15	12	12	12.5	12	25
		Test	33.5	21	18	17.5	17.5	18	
11.8.61	150	Control	21	11	11	11.5	11	11	48
		Test	23	15	13	12.5	13	13	

TABLE 72. Acute Thrombocytopaenic Purpura. Cont'd.

Date of Examination	Platelet Count ($\times 10^3$)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
12.8.61	140	Control	24	17	13	11	10.5	11	77
		Test	27	16.5	13	11.5	11.5	11.5	
19.8.61	136	Control	26.5	18	13	10.5	10.5	10.5	100
		Test	24	16	12	10.5	10.5	10.5	
25.8.61	84	Control	19	12	10	9	9	9	42
		Test	27	15	11.5	11.5	11.5	11	
28.8.61	58	Control	35.5	18	12.5	9.5	10	10	100
		Test	37	15	11	10	10	10	
7.9.61	100	Control	-	-	16	12.5	11	11	100
		Test	-	-	11.5	11	11	11	
18.9.61	134	Control	12.5	10	9.5	9.5	9.5	9.5	100
		Test	15.5	11	10	9.5	9.5	9.5	
16.10.61	120	Control	17	14	12.5	12	12	12	100
		Test	14.5	13	12	12	12.5	12	

TABLE 72. Acute Thrombocytopaenic Purpura. Cont'd.

Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
13.11.61	190	Control	13	10	10	10	10	10	100
		Test	13	10	10	10	10.5	11	
17.5.62 Mid pregnancy	240	Control	30	17	13.5	11	11	11	100
		Test	29	18	14	11	11	11	
23.9.62 1 week prior to confinement	100	Control	22	11.5	11	11	11	11	100
		Test	21	12	11	11	11	11	
30.9.62 1 day prior to confinement	50	Control	33	13	11.5	11	11	11	100
		Test	32	13	11.5	11	11	11	
1.10.62 confinement	40	Control	12.5	10	9.5	9.5	9.5	9.5	100
		Test	14	10	9.5	9.5	9.5	9.5	

of influenza there was a sudden recurrence of spontaneous bruising, epistaxis and menorrhagia. The platelet count had fallen to I6 x $I0^3$ per c.mm. and the patient was readmitted for further steroid therapy. Progress was followed in the usual way but during this admission the technique described in this thesis was used to estimate platelet thromboplastic function. The results are shown in Table 72.

As in the former admission and despite large doses of steroids (Prednisolone I50mgs./day) there was virtually no change in platelet numbers or their function for I0 days, the latter ranging between 5 and I0 percent of normal. Throughout this time the patient was actively haemorrhagic, menorrhagia persisted, and she had frequent epistaxes. As before, transfusion was needed to correct the resulting anaemia and again the patient was urged to consider splenectomy. Two days later she agreed to this and operation was arranged for the following afternoon. On the morning before surgery, a pre-operative assessment showed rising platelet numbers and improved thromboplastic function. Splenectomy was delayed pending reappraisal of the situation and during the next two weeks the platelet numbers rose to a value just below the normal range and their function returned to normal. All haemorrhagic signs had disappeared, and coincident with this general improvement, steroids were gradually reduced and finally discontinued. Coincident with the next menstrual period both platelet numbers and function declined again, (42%) but within a few days the latter returned to normal without the need for steroids. A normal platelet thromboplastic function (I00 percent) was maintained during the next three months and, during this time the platelet count returned to values within the normal range.

Despite advice to the contrary, the patient became pregnant within a few weeks. Throughout the major part of pregnancy the platelet count remained normal and her platelet thromboplastic function I00 percent. However, one week prior to confinement the platelet count had fallen to I00 x $I0^3$ per c.mm. On the day before delivery it was 50 x $I0^3$ per c.mm., and, a few hours before delivery 40 x $I0^3$ per c.mm. On each occasion however, the platelet thrombo-

TABLE 73. Acute Thrombocytopaenic Purpura.

Case 5 - AA

Part (1). Routine Investigations.

Bleeding Time	Clotting Time	Tourniquet Test	Prothrombin Time	Screening Test	Clot Retraction	Fibrinolysis
1 min.	6mins.	++	11.5secs. (N = 10.5)	Normal	Reduced	Normal

TABLE 73. Acute Thrombocytopaenic Purpura.

Case 5 - A.A. Part (2). Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count ($\times 10^3$)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
7.6.60	40	Control	31	20	14	12	12	12	20
		Test	39	27	21	18.5	19	19	
9.6.60	38	Control	25	18	12	11.5	11.5	11.5	25
		Test	37	24	18.5	17	17	17.5	
14.6.60	46	Control	19	13	11.5	11.5	11.5	12	77
		Test	20	12.5	12	12	12	12	
17.6.60	36	Control	38	16	12.5	12.5	12	12.5	42
		Test	47	22	16	15.5	15	15	
24.6.60	32	Control	27	18.5	12	11	11	11	48
		Test	33	22	13.5	13	13	13	
1.7.60	44	Control	24	15	12.5	11	11.5	11.5	22
		Test	29	21	19	18	18	18.5	
3.7.60	40	Control	17	13	10	10	10	10	37
		Test	22	17	14.5	13	13	13	

plastic function was estimated to be 100 percent and on the last occasion the bleeding time was normal and the tourniquet test negative. Considerable alarm was felt because of the progressive thrombocytopaenia, but in consideration of the normal function of the platelets, labour was allowed to progress normally. A normal birth followed without blood loss being any more than that usually encountered. The child had no purpura or ecchymoses, and within a few days the maternal platelet count had returned to normal.

In this patient it was possible that a hormonal influence was active on two occasions. Coincident with menstruation during recovery from the acute phase of the disease there was a decline in both platelet numbers and function, and later, during the last week of pregnancy thrombocytopaenia was evident but platelet function remained normal.

Two further patients to be described presented as surgical problems.

Case 5. A.A., aged 70 years, retired traveller.

This patient developed a "cold" followed a few days later by acute urinary retention. An emergency prostatectomy was attended by profuse bleeding and the wound margins continued to ooze for some hours. On the next day purpura and spontaneous bruising were evident and coagulation investigations were requested. The results of these (and subsequent studies) are shown in Table 73, parts (I) and (2).

The bleeding time was normal but the tourniquet test was positive and clot retraction was reduced. The platelet count was only 40 x 10^3 per c.mm., and, in view of this, platelet thromboplastic function studies were performed and bone marrow examination undertaken. The latter was consistent with the diagnosis of acute idiopathic thrombocytopaenic purpura, while the platelet thromboplastic function was found to be 20 percent. Initially it was thought perhaps wise to adopt a conservative attitude towards the bleeding tendency but the defect was quite severe and clinically becoming worse. Therefore, despite recent surgery, and, with some reluctance, steroid

therapy (Prednisolone 40mgs./day) was commenced under an apparently adequate antibiotic cover. Progress was followed by serial platelet counts and estimations of platelet thromboplastic function.

During the next seven days there was no significant change in the platelet count but their function improved to 77 percent with prompt cessation of all haemorrhage. At this stage wound infection was apparent and it was deemed advisable to reduce the dose of steroids as quickly as possible. When this was done, while the platelet count remained virtually unchanged, their thromboplastic function began to fall. Despite antibiotic therapy under laboratory control the wound infection worsened and after some days, at which stage the platelet thromboplastic function was 22 percent, there was a recurrence of purpura, spontaneous bruising, and haemorrhage from the wound. The problem of management was complex but it was decided to re-introduce the smallest doses of steroids needed to keep the patient outside the actively haemorrhagic range, and to treat the infection with intense local and general means. After two days on smaller doses of prednisolone, (20mgs./day) the platelet thromboplastic function was 37 percent and there was no further haemorrhage, but on the next day the patient died from combined effects of toxaemia and exhaustion.

Case 6. M.O'D., aged 74 years, pensioner.

One week prior to admission the patient developed purpura and spontaneous bruising, and these she cheerfully accepted as a normal accompaniment of her age. On the day before admission she fell, hitting her thigh and causing the development of a massive haematoma. On arrival at hospital she was admitted to a surgical ward where attention was centred on the haematoma and the presence of other bruises and purpura was either ignored or regarded as the result of her fall. Surgical incision of the haematoma was accompanied by almost uncontrollable haemorrhage to such a degree that 3 pints of blood were needed to correct the resulting anaemia. Prior to transfusion however, and in consideration of the belated diagnosis of thrombocytopaenic purpura, bone marrow and coagulation

TABLE 74. Acute Thrombocytopaenic Purpura.

Case 6 - M.O'D.

Part (1). Routine Investigations.

Bleeding Time	Clotting Time	Tourniquet Test	Prothrombin Time	Screening Test	Clot Retraction	Fibrinolysis
2½ mins.	8mins.	++	11.5secs. (N = 11)	Normal	Normal	Normal

TABLE 74. Acute Thrombocytopaenic Purpura.

Case 6 - M.O'D Part (2). Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
On admission	96	Control	31	15.5	13.5	12.5	12	12	18
		Test	72	23	20	19.5	20	20	
27.3.61	90	Control	25.5	17	13	11	11	11	54
		Test	27	19	13	12.5	12.5	12.5	
29.3.61	86	Control	16.5	9	9	9	9	9	62
		Test	22	10.5	10	10	10	10	
11.4.61	60	Control	19	12.5	10	10	10	10.5	54
		Test	19.5	14	11.5	11.5	11.5	13	
18.4.61	40	Control	21	14	10.5	10	10	10	54
		Test	27	14.5	11.5	11.5	12	11.5	

studies were undertaken. The marrow showed a generalized hyperplasia of all elements but especially of megakaryocytes most of which appeared non-functional. The results of initial coagulation studies and subsequent investigations are shown in Table 74, parts (I) and (2). The tourniquet test was positive but the bleeding time was normal in three sites (both ear lobes and right forearm). The whole blood platelet count was 96 x 10^3 per c.mm. and their thromboplastic function 18 percent. Steroid therapy (Prednisolone 40mgs./day) was commenced under antibiotic cover.

The response in this patient was much the same as in the previous case. After 24 hours of steroids all haemorrhagic manifestations had ceased, and, after two days the platelet thromboplastic function had risen to 54 percent and, on the fifth was 62 percent. Despite this improvement there was no significant change in platelet numbers. It was thought reasonable to reduce steroids as quickly as possible to promote wound healing and to minimise the likelihood of wound infection supervening. Two weeks later the wound was well healed but the platelet count had fallen to 60x10^3 per c.mm. and the platelet function to 54 percent. One week later the platelet count was 40x10^3 but their thromboplastic function was static at 54 percent. There was no obvious haemorrhagic state and the only significant feature was a tendency to bruise easily on trauma. The patient was discharged home to the care of her private practitioner and asked to report back in a week's time, but failed to do so. She was not seen by the author again.

Case 7. A 4 year old boy.

R.K., was admitted to hospital with extensive bruising, purpura and haematuria. There was no obvious antecedant, relevant history and the present illness had appeared suddenly as a bruising tendency which had progressed rapidly over a period of 3 days. The physical findings were those of the haemorrhagic state only. Macroscopic and microscopic haematuria was present. The relevant initial and subsequent coagulation studies are shown in Table 74a, parts (I) and (2). The bleeding time was 13 minutes and the tourniquet test

TABLE 74 (a). Acute Thrombocytopaenic Purpura.

Case 7 - R.K. Part (1). Routine Investigations.

Bleeding Time	Clotting Time	Tourniquet Test	Prothrombin Time	Screening Test	Clot Retraction	Fibrinolysis
13 mins.	6mins.	++++	13 secs. (N=13secs.)	Normal	Absent	Normal

Part (2). Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
On admission	< 1000								
4.4.62	20	Control	29	13	12	10.5	10.5	10.5	100
		Test	27	12	11	10.5	10.5	10.5	
7.4.62	250								
9.4.62	506								
11.4.62	400								

TABLE 74 (a). Part (2). Cont'd.

Date of Examination	Platelet Count ($X 10^3$)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
14.4.62	250	Control	13	9.5	9.5	9.5	10	10.5	100
		Test	14	10	9.5	9.5	9.5	10	
21.4.62	234								
28.4.62	200	Control	22.5	11.5	10	10	10	10	54
		Test	27	13	12	12	11.5	11.5	
30.4.62	130								
2.5.62	38								
4.5.62	22	Control	16	11	11	11	11	11	33
		Test	26	16	14.5	14.5	14.5	15	
7.5.62	140								
14.5.62	300	Control	31	15	13	11	11	11	100
		Test	30	15	13	11	11	11	
28.5.62	200								
11.6.62	210								
18.6.62	200								

strongly positive. The platelet count was less than 1000 per c.mm. and, regrettably insufficient blood could be obtained for platelet thromboplastic function estimations at this stage. Bone marrow examination confirmed the diagnosis of acute idiopathic thrombocytopaenic purpura, and steroid therapy was begun forthwith. (Prednisolone 40mgs./day). Progress was followed by serial platelet counts and occasional estimations of platelet thromboplastic function.

All haemorrhage had ceased within 24 hours and there was an immediate and rapid rise in platelet numbers. Within 2 days this had reached 20 x 10^3 per c.mm. The improvement in platelet function was more dramatic than the increase in numbers, however, and at this stage was found to be 100 percent. After four days, daily reduction in steroid dosage was begun, and despite this, after seven days the platelet count had reached thrombocythaemic levels. The patient was discharged from hospital and steroids were discontinued within a few days.

During the next two weeks the platelet count became stabilized between 200-250 x 10^3 per c.mm., and, on the one occasion during this time when it was assessed, the platelet thromboplastic function was found to be still 100 percent. At the end of the third week the platelet count was virtually unchanged but an estimation of their thromboplastic function for demonstration purposes rather than for any positive indication gave the surprising value of 54 percent. Because of this the patient was seen more frequently. Within a few days the platelet count had fallen to 38 x 10^3 per c.mm., and two days later to 22 x 10^3 per c.mm. with a platelet function of 33 percent. Small doses of steroids (Prednisolone 20mgs./day) were given and again there was a prompt return of both platelet numbers and function to normal. Slow withdrawal of steroids over the next four weeks was accomplished without relapse and over three months subsequent observation there has been no recurrence of thrombocytopaenia.

Discussion:

These 7 cases illustrate the use of estimations of platelet thromboplastic function in an assessment of the initial severity and

progress of acute idiopathic thrombocytopaenic purpura. The author is convinced that such estimations have an important place in the management of such cases.

Many years ago, Duke warned of the dangers inherent in relying on purely clinical assessment of the severity of the disease and certainly the poor correlation between platelet numbers and the severity of haemorrhage in many instances is general experience. Indeed in the cases described above, when first seen, the second case had absolute platelet numbers of 60×10^3 per c.mm. but no haemorrhagic signs whatsoever, while case 6 had 96×10^3 platelets per c.mm. but a severe haemorrhagic tendency.

Bonnin's concept (Bonnin 1956,57) that the severity of haemorrhage which might accompany primary thrombocytopaenia was related not at all to platelet numbers but rather to their thromboplastic function was a major advance in an understanding of the underlying mechanism governing the severity of haemorrhage in this condition. His initial work and subsequent papers verifying his concept have been convincing, but, to date, lack independent confirmation. Although his theory may eventually need revision and some modification there seems to be small doubt that estimations of platelet thromboplastic function provide the most accurate guide yet of the severity of the coagulation defect, and it is in such an assessment that the technique described finds an important application.

It is not unreasonable to anticipate a disocciation between platelet numbers, their function and the haemorrhagic state during the treatment of primary thrombocytopaenia, similar to that seen initially. Indeed such has been found to be the case and it is a common occurrence for there to be a complete cessation of haemorrhage and dramatic improvement in platelet function before any marked change in platelet numbers is evident. The correlation between clinical improvement or otherwise and platelet function seems to be more complete than between the former and platelet numbers, and it is only reasonable, therefore, to control and adjust therapy in relation to platelet function and not platelet numbers.

In primary thrombocytopaenia one is confronted with a complex

defect of both haemostasis in which capillaries and platelets are involved, and coagulation in which, principally, platelet functions are affected. It may be, that the prompt improvement in the haemorrhagic state with therapy, reflects an immediate reparatory process primarily involving capillaries. However, it is surely the case that both capillary damage and platelet abnormalities have a common basis and the one process is responsible for both. Thus from one direct cause say, an antibody, both capillary and platelet damage ensue. Added to probable initial direct capillary damage one can postulate secondary impairment of capillary function following damage to essential platelet functions related to the maintenance of an intact capillary bed, and thereby a dual defect of haemostasis. Added to this is impairment of coagulation resulting from damaged platelet functions.

Bonnin (1956,57) has postulated that capillary damage and platelet thromboplastic defects run in parallel and that by assessing the latter one gains a mirror image of the degree of capillary damage. This may be so, but at present it is beyond the scope of experimental confirmation. The author feels that in essentials this theory may be true in so far as anything likely to damage platelets may directly injure capillaries or secondarily involve them as a consequence of platelet damage, but it is possible that in degree the two, capillary and platelet damage, may be quite disocciated, and, in any one case, the greater effect may be felt on the one or the other.

Accepting this modification then, one prefers to be less dogmatic in relating levels of platelet function to the degree of capillary damage and to say that while both may be affected by the one injurious process, estimations of platelet thromboplastic function are a direct measure of impaired coagulation only and are a guide to the possible severity of haemorrhage which might occur should haemostasis be impaired to the extent that spontaneous escape of blood from the vascular tree is possible. With reservation one can postulate that the more severe is platelet thromboplastic damage, the more likely is there to be more advanced capillary damage, and the likelihood of haemorrhage being manifest is increased. Therefore

indirectly estimations of platelet thromboplastic function perhaps give one some indication of the likelihood of spontaneous haemorrhage occurring, but one cannot at this stage formulate strict rules to say that at a certain level of platelet thromboplastic function haemorrhage of certain severity will invariably occur.

Since platelets play an important role in haemostasis and since defective haemostasis is likely to be produced directly or indirectly in primary thrombocytopaenia, improvement in platelet thromboplastic function is likely to be accompanied by improvement in haemostasis, although not necessarily of the same degree. This is a logical conclusion if one accepts that both platelet damage and capillary lesions may follow the one direct cause, for should this be so, therapy able to protect platelets from the damaging agent is surely able to protect capillaries in like manner. So too, coincident with improvement in platelet thromboplastic function, capillary integrity must benefit by the restoration to a normal or improved state of those platelet functions essential to the maintenance of an intact capillary network. Serial estimations of platelet thromboplastic function then provide a direct measure of the integrity of coagulation and therefore of the severity of haemorrhage possible at various stages and indirectly may provide a rough guide to the effectiveness of haemostasis, but there is no proof that the two run in parallel. At best, one can say that coincident with variation in platelet thromboplastic function there is likely to be associated alteration in haemostasis but the latter cannot be quantitated.

Thus, from what has been said and in the face of the recurrent problem of disocciation between platelet numbers and the severity of haemorrhage, it is only reasonable to assess progress and regulate therapy by serial estimations of platelet thromboplastic function wherever possible, recognizing that such estimations are an accurate assessment of impaired coagulation and are probably a useful but indirect and less reliable indication of the effectiveness of haemostasis. The rationale of this approach is well shown by cases 3 and 4. In case 3 there was no change in platelet numbers for two weeks after therapy was begun and in consideration of this splenectomy was

contemplated. Opposed to this, however, was the complete cessation of haemorrhage and return of platelet thromboplastic function to normal within 24 hours. This improvement was maintained throughout and was associated with an eventual restoration of platelet numbers to normal. In case 4, at the end of pregnancy, there was a profound thrombocytopaenia and grave concern was felt, yet in consideration of normal platelet thromboplastic function, it was confidently predicted that labour could proceed without fear of haemorrhage and such was the case.

So too in those instances where response to therapy is not good or where usual doses of steroids are contra-indicated serial estimations of platelet thromboplastic function have proved most useful. The hormones used in the treatment of primary thrombocytopaenia are not without undesirable side effects and one's aim is to keep the dose as small as is compatible with a definite aim, namely, to keep the patient free from haemorrhage. Since the latter is related to platelet function and not numbers it is by serial estimations of platelet function that one can determine with confidence, the minimum dose of steroids compatible with one's purpose. Cases 5 and 6 illustrate this point. In these, a possible, prolonged use of large doses of steroids was contra-indicated but by serial estimations of platelet thromboplastic function one could quickly reach a compromise, and employ the dose capable of maintaining the patient free from haemorrhage but retaining the minimum of unwanted side-effects.

Bonnin (I96I), (personal communication) has stated that in patients who respond well initially to therapy, relapse is often heralded by a decline in platelet thromboplastic function before any dramatic change in platelet numbers is evident. It is only by following patients through with serial estimations of function that imminent relapse can be detected at an early stage and corrective measures taken. Case 7 is an example of this occurrence, for here, quite by chance, a decline in platelet function was found before there was a significant change in platelet numbers. This enabled closer clinical supervision to be given with added safety to the patient and therapy was begun before haemorrhagic phenomena recurred.

TABLE 75. Chronic Thrombocytopaenic Purpura.

Case 1 - M.R. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
First Examination	60	Control	18.5	11	9	9	9	9	31
		Test	25.5	14.5	13	13	13	13	
7 days later	60	Control	14	10	10	10	10	10	100
		Test	14.5	11	10	10	10	10	

The author feels that a strong case has been made for the introduction of serial estimations of platelet thromboplastic function in the assessment and control of cases of acute idiopathic thrombocytopaenic purpura, for in this way the possible fallacies of a purely clinical assessment and the uncertainty surrounding the significance of absolute platelet numbers are overcome.

In chronic thrombocytopaenic purpura, too, such estimations have an important place, as the following cases illustrate.

2. Chronic idiopathic thrombocytopaenic purpura.

Case I. M.R., 22 years, housewife.

M.R. had had acute thrombocytopaenic purpura five years ago with a poor response to hormone therapy. Splenectomy had produced marked symptomatic improvement but thrombocytopaenia had persisted and the patient continued to bruise easily and had frequent episodes of purpura. The haemorrhagic state did not warrant continuous steroid therapy. She was referred to the laboratory for assessment prior to dental extraction, since a previous extraction performed by an unsuspecting dentist, not alerted by the patient, had been attended by profuse haemorrhage. When seen, her platelet count was 60 x 10^3 per c.mm., her platelet thromboplastic function 31 percent and she had a few purpuric spots on her upper arms and neck. She began steroid therapy and was seen one week later. On this occasion her platelet count was unchanged but their function was 100 percent. Dental extraction performed on the next day was uneventful. The results are shown in Table 75.

Case 2. S.M., aged 42 years, housewife.

Her first episode of acute idiopathic thrombocytopaenic purpura two years ago had responded well to steroid therapy but had subsequently relapsed into a state of chronic thrombocytopaenia with platelet numbers ranging between 50 and 80 x 10^3 per c.mm. She tended to bruise easily but not spontaneously and purpura was not evident. Steroid therapy was not indicated. In January 1961, the

TABLE 76. Chronic Thrombocytopaenic Purpura.

Case 2 - S.M. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count (X 103)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
25.1.61	60	Control	42	14.5	11.5	11.5	11.5	11.5	29
		Test	27	19	16	16	16	16	
8.2.61	68	Control	17	12	11.5	11	11	11	62
		Test	19	14	13	12	12.5	12	
17.2.61	54	Control	37	14.5	11.5	11.5	11.5	11.5	54
		Test	27	15	13	13	13	13	
24.2.61	60	Control	31	15.5	13.5	12.5	12	12	48
		Test	33	18	16	15	14	14	
28.2.61	54	Control	16	10	10	10	10.5	12	48
		Test	22	12	12	12	12	13	

TABLE 76. Case 2 Cont'd.

Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
15.3.61	46	Control	27	13	11	11	11.5	11	48
		Test	31	17	14	13	13	13	
29.3.61 Respiratory Infection	34	Control	31.5	14	12	11.5	11.5	11.5	37
		Test	30.5	16	14.5	14.5	15	14.5	
13.4.61	48	Control	13.5	10.5	8.5	9	9	9	54
		Test	16	13.5	12	10.5	10.5	10.5	
28.4.61	40	Control	27	15	13	12	12	12.5	48
		Test	25	14.5	14.5	14	14	14	

patient returned to the laboratory complaining that since an attack of 'flu some three weeks before she had begun to bruise spontaneously and had developed purpura. Her platelet numbers were not significantly different from prior estimations but her platelet function was only 29 percent. She was given small doses of steroids and her progress was followed by serial platelet counts and estimations of platelet function. Two weeks later her platelet count was $68xIO^3$ per c.mm. and their function 62 percent. Symptomatically she was much improved and steroids were discontinued. During the next few weeks she was seen frequently and although her platelet thromboplastic function fell a little when steroids were stopped it became stabilized at approximately 50 percent of normal. Coincident with a second respiratory infection there was again a drop in platelet function (37%) but this was followed by a spontaneous return to values of approximately 50 percent of normal. The results of these investigations are shown in Table 76.

Case 3. A.T.M., aged 43 years, male, insurance assessor.

The patient had undergone splenectomy for acute idiopathic thrombocytopaenic purpura in I956, but had remained thrombocytopaenic and was rarely without some bruises (produced by minimal trauma or spontaneously) or purpura or both. He was seen at approximately three monthly intervals for assessment or more frequently during any exacerbation of his symptoms. He was first seen by the author in November, I959 and the results of his platelet function studies are shown in Table 77.

On the first three occasions he was seen, his platelet function was assessed at 42, 33 and 42 percent respectively. On each occasion he had ecchymoses and purpura on his upper arms, chest, neck and legs. When next seen he had had an epistaxis the day before, and his platelet function was only I5 percent. Because of this he was admitted to hospital for observation. No specific therapy was given and when after a few days there had been no further epistaxes, he was discharged. Three months later his platelet function was estimated to be 42%, apparently his normal

TABLE 77. Chronic Thrombocytopaenic Purpura.

Case 3 - A.T.M. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
18.11.59	80	Control	27.5	17	15	13	13.5	13	42
		Test	21	17	15.5	15.5	15.5	15.5	
17.12.59	40	Control	20	10.5	10.5	10.5	10.5	10.5	33
		Test	44	16	14	14	14	14	
22.12.59	72	Control	13	10.5	9.5	9.5	10	9.5	42
		Test	36	14.5	12	12	12	12	
21.3.60	26	Control	28	14	14	14	14	14	15
		Test	60	27	25	25	25	26	
18.7.60	46	Control	21	13	11.5	11	11	11	42
		Test	26	18	15	13.5	13.5	13.5	
21.9.60	28	Control	14	10.5	10.5	10.5	10.5	10.5	18
		Test	31	20	19	18.5	19	18.5	
14.11.60	16	Control	23	12.5	12	11	11	11	37
		Test	28	15.5	14	14	14	14	

TABLE 77. Case 3. Cont'd.

Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
13.12.60	28	Control	41	27	15	11	10.5	10.5	42
		Test	35	25	17.5	13.5	13	13	
18.1.61	20	Control	-	16.5	12.5	11	11	11	100
		Test	31	14.5	12	11	11	11	
2.2.61	42	Control	41	18.5	13	12	11	11	100
		Test	31	15	12.5	12	11	11	
15.2.61	34	Control	65	25.5	16	12	11	11	77
		Test	45	22	14	11.5	11.5	11.5	
15.3.61	40	Control	21.5	18.5	12.5	11	10.5	10.5	100
		Test	26.5	21.5	13	11	10.5	10.5	
11.4.61	50	Control	21	14.5	13	13	12	12	77
		Test	19	13	13	12.5	12.5	12.5	
4.5.61	46	Control	12	8.5	8.5	8.5	8.5	8.5	100
		Test	11.5	10	8.5	8.5	8.5	8.5	

stable value. When seen in September, I960, he stated that he had recently had another epistaxis and that since that time he had noticed an exacerbation of his bruising tendency. His platelet function on this occasion was I8 percent. Two months later it was 37 percent. In December, I960, the patient reported that his ever present bruises and purpura were becoming an embarrassment in his work and asked if anything could be done to limit their occurrence. On this occasion his platelet thromboplastic function had returned to its usual levels of approximately 40 percent, but in consideration of the patient's wishes he was given small doses of steroids. While there was no significant change in platelet numbers, this resulted in a marked improvement in his platelet thromboplastic function which was estimated to be I00, I00, 77, I00, 77 and I00 percent in 6 estimations performed during the next 6 months. Throughout this time small doses of steroids were being given, and while his haemorrhagic tendency was less, it was by no means completely absent. In relation to this it is interesting to record that on 4.5.6I when his platelet thromboplastic function was I00 percent, his bleeding time was I0 minutes with a grossly excessive blood loss throughout this time. One may speculate then, that at this time the improvement in platelet thromboplastic function was disocciated from a similar improvement in capillary function either direct or indirect, or possibly in deference to Hellem's work, there were insufficient adhesive platelets available to secure normal haemostasis. Whatever the true explanation, the persistence of a bruising tendency and purpura would find solution in the presence of a "capillary" defect, despite an improved coagulation mechanism.

Case 4. D.H., aged 53 years, housewife.

Not strictly a case of chronic thrombocytopaenic purpura this patient, none the less, was an interesting study. Two years before she had responded well to steroid therapy for acute thrombocytopaenic purpura with prompt return of platelet numbers and function to normal and prompt cessation of all haemorrhage. She was discharged to the care of her private practitioner, and was not seen again until

TABLE 78. Chronic Thrombocytopaenic Purpura.

Case 4 - D.H. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count ($\times 10^3$)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
3.3.61	360	Control	11	9	9	9	9	9.5	100
		Test	11.5	9	9	9	9	11	
21.3.61	298	Control	48	17.5	14	11.5	11	11	100
		Test	40	19	13	11	11	11	
10.4.61	304	Control	42	14.5	11.5	11.5	11.5	11.5	100
		Test	43	15	11.5	11.5	12	11.5	
13.4.61	290	Control	41	18.5	13	12	11	11	77
		Test	31	15	12.5	13	11.5	11.5	
18.4.61	314	Control	23	19.5	14	13	10	10	48
		Test	27	19.5	13	12.5	12	12	

TABLE 78. Case 4. Cont'd.

Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
27.4.61	260	Control	12	10.5	10.5	10.5	10.5	-	48
		Test	14	12.5	12.5	12.5	13	12.5	
2.5.61	320	Control	55	23	11	11	11	11	48
		Test	-	13	12.5	13	13	13.5	
4.5.61	330	Control	13.5	10	10	10	10	10	62
		Test	13	12	11	11	11	11	
11.5.61	340	Control	14	10	10	10	10	10	100
		Test	11	10	10	10	10	11	

she was referred to hospital for investigation of severe back pain. This was found to be due to generalized osteoporosis and in seeking a cause for this it was discovered that since her discharge she had been maintained on prednisolone therapy in the mistaken belief that this was essential to prevent a recurrence of her former disease. It was decided to withdraw steroids carefully and during the process to watch her platelet numbers and function. The results of this study are shown in Table 78. It will be seen that during the whole of the period of observation there was no significant change in platelet numbers. After four weeks, however, when steroids had been stopped altogether, the patient's thromboplastic function had fallen to 48 percent, and was maintained at about this level for a further four weeks after which there was a return to previous levels of 100 percent. During this phase of reduced function the patient volunteered that she was bruising more easily than before, but later, when a normal thromboplastic function was restored, this tendency was lost.

Discussion: These cases demonstrate the usefulness of estimations of platelet thromboplastic function in the care of patients with chronic thrombocytopaenic purpura.

In the first case described, it would have been impossible to be assured that surgery, even minor, could be safely undertaken if one had to rely on platelet numbers alone, for these did not alter during a week of steroid therapy. On the other hand, during this week their thromboplastic function was restored to normal and one could advise that surgery could be safely undertaken.

The second and third examples are representative of the unfortunate few whose acute disease passes into a chronic state punctuated by exacerbations of the haemorrhagic tendency. In these two patients such relapses were usually not associated with any dramatic change in platelet numbers and it was only by estimations of platelet thromboplastic function that the severity of relapses could be accurately assessed, and the response to corrective therapy and its subsequent withdrawal gauged. Indeed it is only thus by observing changes in platelet thromboplastic function in serial estimations that

a temporary or continuing downward trend can be detected and appropriate corrective measures taken at an early stage, for as these cases demonstrate such relapses not infrequently occur without a corresponding alteration in platelet numbers. In the fourth case a definite decline in platelet function was evident and persisted for four weeks when steroids were finally stopped. This happened despite the fact that there was no significant change in platelet numbers which remained well within normal limits. Any deviation from the normal would have passed unnoticed in the absence of estimations of platelet thromboplastic function. In this instance the change was not severe, although definite and continued, and nothing untoward happened. Under other circumstances, however, had the decline continued and had one relied on platelet numbers alone as an index of the patient's condition, one might have been lulled into a state of false security until confronted with an actively haemorrhagic patient.

It is for these reasons that estimations of platelet thromboplastic function have a definite place in the supervision of chronic thrombocytopaenic purpura.

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6. Aplastic Anaemia.

Historically, thrombocytopaenia secondary to a primary disease process has been discussed in the introduction to this thesis. Aplastic anaemia has been considered in this way.

Few would doubt that to the patient with aplastic anaemia, haemorrhage is one of the most distressing and dangerous symptoms. It is certainly one of the most difficult problems confronting the physician. For these reasons any technique that may be used to assess any facet of the disease process has a place in the management of aplastic anaemia. To this end the following eight cases are used to illustrate the application and uses of estimations of platelet thromboplastic function in the assessment of the coagulation

TABLE 79. Aplastic Anaemia.

Case 1 - H.W. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
25.9.59	66	Control	17	13	12.5	12.5	12.5	14.5	16
		Test	44.5	23.5	22	22	22.5	24	
28.9.59	34	Control	18	13.5	12.5	12.5	13	12.5	15
		Test	44.5	24.5	23	23	24	24	
29.9.59	40	Control	13.5	12.5	12	12.5	12.5	12	22
		Test	33.5	19	19.5	19	18.5	19	
2.10.59	42	Control	17.5	13	12.5	12	12.5	12	17
		Test	52.5	20.5	21	21	20.5	23.5	
5.10.59	22	Control	22	13	12	12	11.5	12	28
		Test	-	16.5	16	16.5	16.5	17	
7.10.59	12	Control	14	11	9.5	9.5	9.5	9.5	13
		Test	25.5	23	21.5	21.5	21.5	21	

TABLE 79. Case 1. Cont'd.

Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
9.10.59	18	Control	13	12	12	12	11.5	12	18
		Test	-	22	20	20	20.5	21	
13.10.59	10	Control	19.5	13	12.5	12.5	12	12.5	32
		Test	-	16	16.5	16	16	-	
4.11.59	90	Control	22.5	15.5	13.5	13	13	13.5	26
		Test	35	19.5	18	18	18.5	18	
9.11.59	60	Control	24	13.5	13	12.5	12.5	12.5	< 5
		Test	49	43	38	39	38	39	
12.11.59	45	Control	26	17	15	13.5	13	13	5
		Test	> 60	50.5	41.5	38	38	38.5	
16.11.59	48	Control	24	15.5	13	12.5	12.5	12.5	15
		Test	51.5	25	22.5	22.5	23.5	22	

TABLE 79. Aplastic Anaemia.

Case 2 - D.M. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count ($X 10^3$)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
29.9.59	20	Control	13.5	12.5	12	12.5	12.5	12	29
		Test	-	16.5	16.5	17	16.5	17	
2.10.59	28	Control	17.5	13	12.5	12	12.5	12	22
		Test	-	21	18.5	18.5	20.5	20	
4.10.59	32	Control	23	13	12	12	11.5	12	77
		Test	15	12.5	12	12.5	12.5	17	
9.10.59	20	Control	13	12	12	12	11.5	12	15
		Test	-	26	22.5	22.5	22	23	

TABLE 79. Aplastic Anaemia.

Case 3 - M.D. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count (X 10³)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
12.11.59	40	Control	21	15	13.5	12.5	12.5	12.5	5
		Test	> 60	>60	46	37	36.5	36.5	
16.11.59	32	Control	24	15.5	13.5	12.5	12.5	12.5	< 5
		Test	> 60	55	41	38	38	38	
26.11.59	22	Control	21.5	14.5	13.5	13	13	13	12
		Test	-	29.5	26.5	26.5	26.5	26.5	

Case 4 - J.P. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count (X 10³)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
19.10.60	26	Control	23	13	11	11	11	11	13
		Test	44.5	26	24.5	23	23	23	

TABLE 79. Aplastic Anaemia.

Case 5 - E.H. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
31.8.60	20	Control	22	13.5	12	12.5	12.5	12.5	16
		Test	50	29.5	26.5	22.5	23	23	
6.9.60	40	Control	20	12.5	11.5	11.5	11.5	11.5	17
		Test	43.5	24	20.5	20.5	20.5	20.5	
13.9.60	14	Control	25	15.5	13	11	11	11	42
		Test	31	16.5	13.5	13.5	13.5	13.5	

TABLE 79. Aplastic Anaemia.

Case 6 - C.D. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
6.7.60	32	Control	14	10.5	10.5	10.5	10.5	10.5	10
		Test	49	33	26	26.5	26	26.5	
-	28	Control	19	12	10.5	10.5	10.5	10.5	12
		Test	43	25	23	23.5	23	24	

TABLE 72. Aplastic Anaemia.

Case 7 - M.H. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count (x 10³)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
16.9.60	60	Control	19.5	14	12.5	12	12.5	12	26
		Test	-	18.5	17	17	17	18	
12.10.60	58	Control	33	14	12	11.5	11.5	11.5	19
		Test	37	27.5	20.5	19	19	19	
20.10.60	70	Control	31	14.5	12	11	10.5	10.5	< 5
		Test	> 60	> 60	53	40	41	41	
22.10.60	54	Control	37	21	13	11	11	11	7
		Test	60	54	35	32	32.5	32	

TABLE 79. Aplastic Anaemia.

Case 8 - M.B. Details of platelet thromboplastic function estimations.

Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
			4	10	15	20	25	30	
27.2.61	22	Control	21	12	11	10	10	10	10
		Test	> 60	49	33	26	26	26.5	
4.3.61	20	Control	31.5	15	11.5	10	10.5	10	12
		Test	43	37	26	23	23	23	
14.3.61	24	Control	33	14.5	12	12	12	12	9
		Test	57	42	31	29.5	29	29.5	

defect in aplastic anaemia.

In these cases, all of which were ultimately fatal, chloromycetin was considered to be the likely if not definite causative agent in six (cases I,2,3,4,5 and 8) and Butazolidine was incriminated in the remaining two. Of the chloromycetin induced cases, in retrospect, it was considered that all too often repeated and prolonged courses of the drug had been given without just cause.

Except case 4 who died 24 hours after admission from a terminal pharyngeal and laryngeal haemorrhage, all patients had serial estimations of platelet thromboplastic function. The latter patients all received intense steroid therapy (prednisolone, ACTH or both) and some were given testosterone in addition. The results of their investigations are shown in Table 79.

As Table 79 shows, there was a poor response to drug therapy in all patients save case 5, and at the same time in general they remained haemorrhagic to a greater or lesser degree throughout. These results stand in sharp contrast to the results of therapy with the same drugs in primary idiopathic thrombocytopaenia as illustrated in the preceding section. In case 2 there was a temporary amelioration of the haemorrhagic manifestations (ecchymoses, purpura, haematuria), and a marked improvement in platelet function following platelet transfusion, but a prompt return to the former state within five days. In this case and all the others save case 5, haemorrhage figured prominently as a terminal event and in cases I,2,3,6,7 and 8 cerebral haemorrhage was the immediate cause of death. In some cases there was a mild but significant symptomatic improvement and temporary lessening of the grosser haemorrhagic signs during steroid therapy but as the results show this was not related to any significant change in platelet function, and probably was dependent on improvement in capillary resistance. Case 5 was the only case to show any heartening change in platelet function and really sustained lessening of haemorrhagic signs with hormone therapy. He was a 78 year old man who had had three courses of chloromycetin within 6 months for recurrent bouts

of bronchopneumonia associated with congestive cardiac failure. He presented with extensive ecchymoses, purpura, haematemesis, melaena and haematuria. His platelet thromboplastic function on admission was 16%. Prednisolone therapy for two weeks did not improve the level of platelet function and the patient remained haemorrhagic. At this stage ACTH was substituted for prednisolone, and despite the fact that the platelet count was less at the time of the third estimation than on the two previous occasions, their function had improved to 42% and the patient's haemorrhagic state was much less. The patient died during the next week from a fulminating pneumonia, but his progress prior to this illustrates an important use of platelet function studies in aplastic anaemia.

It has been shown that in some conditions (aplastic anaemia, thrombocytopaenic purpura) a poor or no improvement may follow the use of one particular hormone preparation. On the other hand, a change to a similar product of different manufacture or an entirely different preparation of similar activity may produce a marked improvement both subjectively and objectively. In so far as haemorrhagic manifestations are concerned, clinical improvement may follow despite an unchanged platelet count, and positive evidence of improved coagulation may only be found in an altered platelet thromboplastic function. Some clinical improvement may be evident when hormone therapy is begun, but as the patients described herein have shown, this may be due to an improved capillary resistance and thereby better haemostasis while the coagulation defect remains unchanged. It is only by estimating platelet thromboplastic function that one can distinguish between apparent improvement due to an isolated effect on capillary integrity or a more general improvement of both haemostasis and coagulation together. In this way estimations of platelet thromboplastic function may help to decide whether the hormone being used is being of general benefit and may assist in the determination of the most effective drug for any specific case. Case 5 is a good example of this concept, for his initial prednisolone therapy was associated with some subjective improvement and his haemorrhagic state was ameliorated to a certain

degree but was still evident. Throughout prednisolone therapy his platelet thromboplastic function was unaltered. The introduction of ACTH however, produced a significant improvement in the latter and a dramatic improvement in his haemorrhagic state, but at the same time there was a decline rather than an elevation of his platelet numbers.

The results of these cases also illustrate that a severe coagulation defect reflected in a remarkably low platelet thromboplastic function and accompanied by impaired haemostasis, while figuring prominently in the termination of such conditions, is not necessarily fatal within a short time of its development. Indeed some of these patients survived for weeks in the face of grossly impaired coagulation mechanisms. So too, some patients and especially children, may appear remarkably active and well, despite a profound anaemia and haemorrhagic diathesis. In the knowledge that many patients have a limited life expectancy and with the desire to make their remaining time as happy and as satisfying as is possible, it is sometimes difficult to be sure on purely clinical grounds, what restrictions should be placed on their activity. Consideration of the haemorrhagic tendency must play an important part in any decision, and in this, estimations of platelet thromboplastic function as an indication of the severity of the coagulation defect, may play a helpful role in deciding what course of action should be adopted.

7. Acute Leukaemia.

A bleeding diathesis varying from a tendency to bruise after mild trauma to the spontaneous development of purpura and ecchymoses and haemorrhage from the mucous membranes and elsewhere is frequently seen in acute leukaemia. This bleeding tendency has often been attributed primarily to an accompanying thrombocytopaenia (Freeman and Hyde, 1952: Soulier and Dausset, 1950: Wintrobe, 1942). On occasions other haematologic abnormalities have been described. These have included excessive fibrinolytic activity (Cooperberg and

Nieman, I955: Pisciotta and Schulz, I955), circulating anti-coagulants (Freeman, I952: Volkert and Hertel, I943), hypo-fibrinogenaemia (Cooperberg and Nieman, I955) and thrombocytopathia (Bigelow, I954), Stefanini and Dameshek, I955).

In an extensive investigation Freeman and Hyde (I952) tried to relate haemorrhage in acute leukaemia and other conditions to platelet numbers, with limited success. In 35I examinations they found that 94 non-bleeding subjects had platelet counts above $55xI0^3$ per c.mm., while 22I with clinical signs of bleeding had platelet numbers below this level. However, in 36 instances wherein the platelet count was less than $55xI0^3$ per c.mm. there was no clinical evidence of haemorrhage. Thus, while thrombocytopaenia appeared to be essential if haemorrhage was to occur, it was not uncommon to find examples of critically low platelet counts with no sign of bleeding. The same authors were unable to produce conclusive evidence that the development of a circulating anti-coagulant was of importance.

The rarity of hypofibrinogenaemia, increased fibrinolysis, anti-coagulants etc. as causes of haemorrhage in acute leukaemia and the lack of any consistent or satisfying explanation for such a frequent and serious complication, caused Lewis and her associates (I957) to undertake a battery of tests of haemostatic function in 39 patients with various forms of leukaemia. They compared the results with similar data obtained from patients with thrombocytopaenia from other causes. In the leukaemic group, thrombocytopaenia and reduced factor V levels were the most consistent findings. The prothrombin consumption was abnormal using patient's platelets and normal plasma in every case in which the platelet count was less than $50 \times I0^3$ per c.mm. and in some instances when higher counts were present, indicating a platelet functional defect. The results of bleeding time estimations and the tourniquet test were abnormal less frequently than in idiopathic thrombocytopaenic purpura. None of the abnormalities correlated well with the degree of haemorrhage and the authors concluded "that even after this exhaustive and pains-taking study, no simple answer is apparent for the question: Why

do leukaemic patients bleed?"

In similar vein to his studies on primary thrombocytopaenia, Bonnin (I96I) has shown a similar although less strict relationship between haemorrhage and platelet thromboplastic function in secondary thrombocytopaenia including acute leukaemia. In these conditions, however, the picture was frequently complicated by deficiencies of coagulation factors other than platelet defects. This work has done much to resolve outstanding questions but, quite apart from this, the author feels that estimations of platelet thromboplastic function can play an important part in the successful routine management of acute leukaemia.

While there can be small doubt of the urgency surrounding the development of haemorrhage at any stage in the progress of leukaemia, the need for active corrective measures is, perhaps, all the more important in the initial stages of the disease than later. A distinction such as this stems, not from a disregard for the old, known case, but from the realization that in the terminal stages, when remission cannot be induced it is rarely possible to improve the haemorrhagic state except, perhaps, very temporarily by platelet transfusion. On the other hand, with the use of modern chemotherapy there is an excellent chance that the patient first presenting can be given a few more weeks or months of symptom free, happy life if the initial acute phase can be overcome and a complete remission induced. Indeed, insofar as the Adelaide Children's Hospital is concerned, the average duration of life from diagnosis to death is now II.7 months, a sharp contrast to the average of two months existing in the years I935-55.

This somewhat brighter outlook, however, can only be fulfilled if the initial period of the acute disease is successfully negotiated and survival during this time is sufficient to permit the drugs commonly used to exert their full suppressive action. Among the foremost causes of death, early or late, is haemorrhage, and consideration of the patient's haemorrhagic state must play a prominent part to determine therapy. That such should be the case must be evident when one recognizes that the action of some of the

TABLE 80. Acute Leukaemia.

Part (1). Routine Investigations.

Case No.	Bleeding time (mins.)	Clotting time (mins.)	Tourniquet test	Prothrombin activity (%)	Screening test
1 D.J.	5.5	9.5	++++	47	Normal
2 O.W.	2.75	7	++	91	Normal
3 V.L.	3.25	7.5	+	50	Normal
4 E.B.	2	8	+	100	Normal
5 J.M.	7	6	++	67	Normal
6 M.M.	15	11	+++	15	Abnormal
7 M.G.	9	7.5	++	55	Normal
8 L.F.	2	7	+	100	Normal
9 T.E.	1	6	-	91	Normal
10 E.U.	3.5	5	+	67	Normal
11 B.O.	13	9	+++	50	Normal

Clot Retraction	Fibrinolysis	Platelet Count ($\times 10^3$)	Platelet Function %	Haemorrhagic State
Absent	Normal	25	20	Purpura, ecchymoses, haematuria, epistaxes.
Normal	Normal	34	29	Menorrhagia
Normal	Normal	50	34	Fading ecchymoses.
Normal	Normal	70	77	None
Absent	Normal	22	20	Extensive purpura. Ecchymoses.
Absent	Normal	30	12	Spontaneous bleeding from gums. Epistaxes. CVA.
Reduced	Normal	46	18	Purpura. Haematemesis.
Normal	Normal	48	29	Purpura.
Normal	Normal	104	34	Purpura.
Normal	Normal	32	25	Purpura. Ecchymoses.
Absent	Normal	22	13	Epistaxes. Haematemesis. Subarachnoid haemorrhage. Purpura.

TABLE 80. Acute Leukaemia.

Part (2). Details of platelet thromboplastic function estimations.

Case No.	Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
1 D.J.	26.9.59	25	Control	23	14	12	11	10.5	10.5	20
			Test	69	18	17.5	17.5	17.5	17.5	
	30.9.59	25	Control	34	15.5	12	10.5	10.5	10.5	22
			Test	40	23	18.5	17	17	17	
	3.10.59	20	Control	20.5	12	11	10.5	10.5	11	25
			Test	28.5	19.5	16	16	16	16	
	14.11.59	270	Control	37	18	11	11	11	11	77
			Test	39	19.5	11.5	11.5	11.5	11.5	
	18.3.60	24	Control	13.5	10	10	10	10	10	20
			Test	25.5	18	17	17	17	17	

TABLE 80. Part (2). Cont'd.

Case No.	Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes — Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
2 O.W.	7.6.61	34	Control	19	12	11.5	11.5	11.5	11.5	29
			Test	31	17	16.5	16	16	16.5	
	9.7.61	450	Control	21	17	12	12	12.5	12	100
			Test	19	14	12	12	12	12.5	
	18.10.61	185	Control	18	12.5	11	11	11	11	31
			Test	23	16	15	15	15	15	
	30.10.61	84	Control	30	16	13	11	11	11	48
			Test	30	20	14.5	13.5	13	13	
3 V.L.	31.8.60	50	Control	22	13.5	12	12.5	12.5	12.5	34
			Test	24.5	17	16.5	16	16	16.5	
	23.9.60	35	Control	18	11	10	10	10	10	11
			Test	36	24	24.5	24	24.5	24.5	

TABLE 80. Part (2). Cont'd.

Case No.	Date of Examination	Platelet Count ($X 10^3$)	Control or Test	Time of Subsampling In Minutes — Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
4 E.B.	5.10.60	70	Control	16.5	11	11	10.5	10.5	10.5	77
			Test	15	11.5	11.5	11	11	11	
	19.10.60	80	Control	23	13	11	11	11	11	13
			Test	43	28	24	23	23	23	
	24.10.60	26	Control	20	12	10.5	10.5	10.5	10.5	20
			Test	37.5	23	19.5	17.5	17.5	17.5	
	4.11.60	32	Control	16	10	9	9	9	9	33
			Test	16.5	12.5	12.5	12.5	12.5	12.5	
	21.12.60	40	Control	15	10.5	9	9	9	9	37
			Test	22	15	13.5	12	12	12	
5 J.M.	2.3.61	22	Control	57	18.5	13	12	12	12	20
			Test	42	22.5	20.5	18.5	19	19	
6 M.M.	-	30	Control	20	11.5	10	10	10	10	12
			Test	33	24.5	23.5	23.5	23	23.5	

TABLE 80. Part (2). Cont'd.

Case No.	Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
7 M.G.	26.9.60	46	Control	24	11	10	10	10	10	18
			Test	44	21	18.5	18	18	18	
	12.10.60	60	Control	33	14	12	11.5	11.5	11.5	29
			Test	31	20	17	16	16	16	
8 L.F.	27.1.61	48	Control	23	11	10	10	10	10	29
			Test	55	19	16	14.5	14.5	14.5	
	9.3.61	40	Control	27	12	10.5	10	10	10	62
			Test	31	12.5	11	11	11	11	
	12.4.61	40	Control	19	13	11.5	11	11	11.5	42
			Test	24	15	14	13.5	14	13.5	

TABLE 80. Part (2). Cont'd.

Case No.	Date of Examination	Platelet Count (X 10³)	Control or Test	Time of Subsampling In Minutes — Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
9 T.E.	14.1.62	104	Control	65	19.5	14	13	12.5	12.5	34
			Test	71	27.5	18	16.5	15.5	16	
	23.2.62	40	Control	24.5	13.5	12.5	12	11	11	20
			Test	57.5	26.5	19.5	18.5	18	18	
10 E.U.	22.12.59	32	Control	12	9.5	9.5	9.5	9.5	9.5	25
			Test	17.5	15.5	15	15.5	15	16	
11 B.O.	12.4.62	22	Control	31	12.5	11	10	10	10	13
			Test	> 60	37	23.5	22	22.5	22	
	24.4.62	110	Control	27	13	11	11	11	11	77
			Test	31	15	13	11.5	11.5	11.5	
	7.5.62	226	Control	53	27	14	10.5	10	10	100
			Test	47	22	12	10	10	10	
	13.7.62	34	Control	17	11	10.5	10.5	10.5	10.5	18
			Test	-	21	19	18.5	18.5	19	

most useful drugs is not a selective inhibition of any one cell element, but a general marrow suppression, and that their use without full consideration of the whole disease process, might easily accentuate an already existing haemorrhagic state by further suppressing platelet production and function. Thus one could readily precipitate a fatal haemorrhagic crisis in what, with more careful assessment, might have been a much more satisfactory case.

If, then, one accepts a determination of a patient's haemorrhagic tendency as a pre-requisite by which early therapy may be determined, how does one assess this? In Table 80, part (I) are shown the initial coagulation investigations in II cases of acute leukaemia. These results include the values of platelet thromboplastic function obtained in each of these cases as determined by the technique described in this thesis. In Table 80, part (2) are shown the actual figures upon which the latter initial estimates are based and, in some cases, additional estimations performed on subsequent occasions. These results show that the commonest and most severe defects were those of platelet thromboplastic function and of tests reflecting abnormalities of the haemostatic mechanism in which platelets are concerned. Thus a prolonged bleeding time and a positive tourniquet test were frequently found, but whether these were dependent on primary vascular involvement or followed secondarily upon thrombocytopaenia or platelet functional defects is not clear. The latter, perhaps, is more likely, but, whatever the cause these investigations left no doubt that in acute leukaemia there is a disruption of both haemostasis and coagulation, the former predisposing to the onset of spontaneous haemorrhage and the latter playing a major role to determine its possible severity. Defects of other specific coagulation factors, almost certainly prothrombin and factor VII, were demonstrated in many cases by the presence of prolonged "prothrombin" time but in no case were these defects severe, and, as Wintrobe has suggested, they in themselves could not produce the haemorrhagic state of an acute leukaemia. Nevertheless, in general although their severity is much less than the platelet defects found, they do add to the overall coagulation

defect and contribute to the severity of any haemorrhage that might occur. It seems likely therefore, that by estimating platelet thromboplastic function one can gain the clearest indication of the overall haemorrhagic state, such values reflecting the first and most severe defects, and being a direct measure of the major component of impaired coagulation, and probably reflecting secondarily the degree of impairment of haemostasis.

In what way can studies such as these guide therapy? The drugs used in therapy can be grouped as the anti-metabolites, the purine antagonists, the cytotoxic agents and hormones, (ACTH, cortisone and related compounds). The first three groups act as general marrow suppressants and thus are likely to potentiate a pre-existing haemorrhagic tendency, and while the mode of action of the hormonal agents has not been clarified there is no doubt that they can induce excellent remissions in a high proportion of cases, (especially acute lymphatic leukaemia in childhood) but in contra-distinction to the other agents they seem to exert a beneficial action on capillary resistance and platelet function. Different workers vary in their choice for initial treatment, some preferring to use either hormones or, the anti-metabolites or purine antagonists alone, initially, and others to use both hormones and a second agent together from the inception of therapy. In a recent review Colebatch (I96I) has shown that if one drug is used to commence therapy remissions can be obtained in some 50-66 percent of cases, the remission rate depending in large part on the drug used. If hormones and one of the marrow suppressants are combined in initial therapy remissions can be obtained in some 80 percent of cases or more. It seems the best policy, therefore, to use one of the anti-metabolites or similar agents as soon as possible in acute leukaemia, but one can use assessments of the haemorrhagic state to decide the safety of such an approach at the commencement or at any subsequent stage of therapy. Thus if initially one finds a severe impairment of platelet thromboplastic function, it is probably wiser to commence therapy with hormones alone and follow the haemorrhagic state with serial estimations of platelet function with a view to introducing additional therapy as soon as satisfactory

improvement in the haemorrhagic state is evident. In similar vein, if the haemorrhagic state is such that dual therapy is begun at once, serial estimations of platelet function can be used to detect an early potentiation of an haemorrhagic tendency and one can adjust or interrupt therapy appropriately and continue with hormone treatment alone until improvement is evident.

Such an approach has proved helpful in the management of acute leukaemia but it must be said that in a proportion of cases it is difficult to know whether an exacerbation of thrombocytopaenia and haemorrhage during treatment is due to drug action or leukaemic relapse. If the cytotoxic agent is responsible withdrawal of the drug will be accompanied by an improvement in the haemorrhagic state and platelet function shortly. Should such not happen it is reasonable to assume that an exacerbation of the leukaemic process is responsible. At this juncture, re-introduction of intense therapy with cytotoxic agents combined with the judicial use of platelet transfusions as determined by the results of platelet function studies may tide the patient over a critical haemorrhagic phase and permit sufficient time for a remission to be induced.

Among the patients studied by Lewis and her colleagues there was a striking correlation between the general clinical status and the tendency to bleed. Clinical deterioration in both acute and chronic leukaemias was invariably found when bleeding occurred, while patients in good remission did not bleed. This observation underlies the importance of regarding bleeding in leukaemia as an integral part of the disease process and is born out by this study. In patients I,2, and II, in each of whom adequate serial studies were performed, coincident with a complete remission there was a restoration of platelet numbers and their function to normal. When relapse was evident, both platelet numbers and their function had declined.

8. Thrombocythaemia.

In 1934, Epstein and Goedel suggested that haemorrhagic

thrombocythaemia was a suitable name for a condition characterized by repeated haemorrhages chiefly from the mucous membranes and a remarkable increase in circulating platelets. Nonetheless the individuality of haemorrhagic thrombocythaemia has been challenged, some authors regarding it not as a distinct disease but merely as a symptom complex which could arise in the course of a variety of conditions such as chronic myeloid leukaemia, polycythaemia vera and myelofibrosis (McCabe et al, 1955; Mallarme and Auzepy, 1957). While acknowledging that such "secondary" cases do occur, others have recognized haemorrhagic thrombocythaemia as a distinct entity (Hardisty and Wolf, 1955; Wasserman and Vroman, 1958). In 1960, Gunz published a critical review of the literature and added five cases of his own. He concluded that there were sufficient individual features, both clinical and haematological, to consider some cases presenting with thrombocythaemia and a picture dominated by haemorrhage as a distinct entity, (haemorrhagic thrombocythaemia) and quite separate from "secondary" cases occurring in association with chronic myeloid leukaemia and so on. Probably intimately related to "secondary" thrombocythaemia, yet sufficiently unique to be considered in a third group are those cases of thrombocythaemia arising in the course of a malignancy not of primary marrow origin, for example carcinoma, localized or disseminated.

While Gunz (1960) has stressed a picture dominated by haemorrhage as a distinguishing feature of haemorrhagic thrombocythaemia, haemorrhage is by no means absent in secondary cases. The exact basis for haemorrhage in either primary or secondary cases is not fully understood, and, as has been the case with acute leukaemia and dysproteinaemia, numerous defects have been described. According to Gunz (1960) while capillary resistance has almost without exception been reported as normal, the bleeding time has been variously described as normal or increased, and there have been numerous reports of minor deficiencies of prothrombin, factor V, factor VII or fibrinogen. A deficiency of factor VIII has been recorded in one case by Wasserman and Vroman (1958).

Although the relationship between platelets and haemorrhage has

received considerable attention, again, no consistent pattern has emerged. Whenever bleeding has occurred high platelet levels have been present but there has been no correlation between the platelet count and the severity of bleeding (Fanger et al I954). Furthermore not every patient with a high platelet count has bled and there have been several cases reported where thrombosis was the leading symptom and haemorrhage was absent (Epstein and Richter, I948: Monto et al, I952: Moolten et al, I949). Studies of platelet serotonin content in several cases have demonstrated low values (Gunz, I960: Bigelow, I954: Hardisty and Stacey, I957: Hardisty and Wolf, I955: Zucker and Borrelli, I956) but the incidence of bleeding was unrelated to mean serotonin values. Various authors have studied platelet thromboplastic function by means of the thromboplastin generation test. Soulier et al (I957) studied 27 cases to find their thromboplastic function normal, increased or decreased. Not only did it vary between different patients but also in the same patient at different times. Of their results the most interesting were perhaps those in which platelet thromboplastic function was subnormal when "in vivo" concentrations of platelets were used but normal on dilution to normal levels. This they attributed to the liberation of an anti-coagulant by high concentrations of platelets. The existence of such an anti-coagulant has been shown by others (Klein et al, I956: Klein and Fiorentino, I957: Larrieu et al, I955: Spaet, I956: Spaet et al, I956), with high in vitro concentrations of both normal and pathological platelets. Using extremely high concentrations of normal platelets ($3{,}000 \times 10^6$) in the technique described in this thesis, the author has found thromboplastin generation to be reduced below that obtained with lesser concentrations of the same platelets. While not excluding the possibility that an anti-coagulant may be liberated, the author feels that a more acceptable explanation is to be found in one of the fundamental principles governing the kinetics of enzyme action. It is well known that, in enzyme actions, if a substrate is increased out of proportion to the available enzyme far less of a final product is obtained than may be derived with the same amount of

enzyme but less of the substrate. Various explanations have been forthcoming but one of the most plausible suggests that if normal enzyme activity is to proceed all the reactive sites in the active centre of a single enzyme molecule must be occupied by a single substrate molecule. In the presence of high substrate concentrations it is likely that more than one substrate molecule becomes attached to each active centre and so normal enzyme activity is blocked. Limitations in the availability of suitable enzymes in proportion to the substrate would not apply in vivo to the extent that such limitations are provided by in vitro tests. Therefore, although it has been suggested that the presence of an anticoagulant may play a part in the production of haemorrhagic manifestations, this seems unlikely. A more plausible explanation, in part at least, probably lies in the presence of thromboplastic defects as demonstrated with normal platelet concentrations in some of Soulier's cases and confirmed by Gunz (I960).

The author has had an opportunity to study platelet thromboplastic function in I2 patients with thrombocythaemia of diverse aetiology. Of these, one may be considered a control subject in that a pronounced thrombocytosis developed after splenectomy for traumatic rupture of the spleen. The remaining cases are readily divided into three groups.

In the first group are considered 3 patients with chronic myeloid leukaemia, each of whom had two estimations of platelet thromboplastic function at an interval of one to four months.

The second group consists of four patients who were considered to be true examples of haemorrhagic thrombocythaemia when first seen. The first two of these patients presented with hepatosplenomegaly, a moderate hypochromic microcytic anaemia, a leucocytosis of some 20-30 x 10^3 cells per c.mm. of blood with a few primitive cells only, and markedly elevated platelet numbers with varied and bizarre platelet morphology. In both the bone marrow was generally hypercellular but the outstanding feature was a disproportionate increase in megakaryocytes. Both cases progressed to an ultimately fatal termination and throughout the outstanding feature was the persistence

of thrombocythaemia. The remaining cases presented initially with severe haemorrhage during surgery. The first patient (J.T.) was a man 26 years of age who bled profusely during and after submucous resection of the nasal septum, after which he was found to have a normochromic, normocytic anaemia (Hb. 9.8gms.) presumed to be due to blood loss, a leukaemoid reaction and pronounced thrombocythaemia. At this stage it was uncertain whether the peripheral blood findings represented a reaction to his acute blood loss or not. Despite correction of his anaemia by transfusion the leukaemoid reaction persisted and his platelet count rose steadily. In view of this, and the finding of a slightly enlarged spleen, bone marrow examination was undertaken. This was reported as being hypercellular with a marked diminution of normal fat spaces and a remarkable increase in megakaryocytes. At this time the diagnosis of haemorrhagic thrombocythaemia was made and the platelet function study reported herein was made. Twelve months later, however, the patient developed a terminal acute myeloid leukaemia. The final patient in this group (M.J.) was a middle-aged woman who had undergone an hysterectomy for menorrhagia. There was profuse and continued haemorrhage during operation and post-operatively of such a degree to necessitate a laparotomy some hours later in quest of an isolated bleeding site. None was found but there was a diffuse oozing from the operation site and an extensive intra-abdominal haematoma. At the same time an enlarged spleen was noted. On the next day, and following transfusion the haemoglobin value was normal but in the stained film the erythrocytes appeared as a mixture of hypochromic and normochromic, transfused cells. There was a leuco-erythroblastic reaction and an intense thrombocythaemia with varied and bizarre platelet morphology. Bone marrow examination was undertaken and again the significant feature was the remarkable increase of megakaryocytes, suggesting a diagnosis of haemorrhagic thrombocythaemia. Coagulation studies, including platelet function were performed and myeleran therapy commenced. The latter produced a prompt disappearance of the leuco-erythroblastic picture but the high platelet count persisted for some weeks. Eventually this, too, responded

TABLE 81. Thrombocythaemia.

Part (1). Post-traumatic splenectomy.

Case No.	Date of Examination	Platelet Count ($\times 10^3$)	Control or Test	Time of Subsampling In Minutes — Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
1 J.M.	-	3,050	Control	31	15.5	13.5	12.5	12	12	> 100
			Test	-	14	13	11.5	11.5	11.5	

TABLE 81. Part (2). Chronic Myeloid Leukaemia

Case No.	Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
1 A.M.	14.2.61	1,040	Control	18	13	12	11	11	11	23
			Test	46	20	17	17	17	17	
	18.3.61	1,600	Control	31	15.5	13.5	12.5	12	12	29
			Test	-	22.5	18.5	16.5	16.5	16.5	
2 M.F.	5.10.60	2,000	Control	16.5	11	11	10.5	10.5	10.5	26
			Test	24	17.5	17	15.5	15.5	15.5	
	3.2.61	1,700	Control	13	10	9.5	10	10	10	62
			Test	20	14	11	11	11	11	
3 A.T.	13.1.61	1,200	Control	21	12.5	11.5	11	11	11	48
			Test	22	15	14	13	13	13	
	20.3.61	920	Control	27.5	13	13	13.5	13.5	15	48
			Test	36.5	15	14	14	15	15	

TABLE 81. Thrombocythaemia.

Part (3). Haemorrhagic Thrombocythaemia.

Case No.	Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
1 C.L.	-	1,860	Control	15	10	10	10	10	10	33
			Test	19	15	13.5	13.5	13.5	13.5	
2 T.R.		1,350	Control	37	16	12.5	11	10	10	48
			Test	35	16	12.5	11.5	12	12	
3 J.T.		1,550	Control	15.5	14	10	9	9	9	31
			Test	21	16	14	13.5	13	13	
4 M.J.		2,100	Control	17.5	13	11	11	11	11	25
			Test	24	18	16.5	16	16.5	16.5	

TABLE 81. Part (4). Carcinoma with thrombocythaemia.

Case No.	Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
1* M.H.	-	1,500	Control	11	9	9	9	9	9	31
			Test	13.5	13	13	13	13	13	
2** C.McK	-	920	Control	23	15	13	12	12	12	42
			Test	27	18.5	16	15	14.5	14.5	
3+ C.R.	-	940	Control	28	14.5	13	12.5	12.5	12.5	54
			Test	24	15	14	14	13.5	14	
4++ I.H.		1,020	Control	27	13	10	10	10	10	100
			Test	29	14	10.5	10	10	10	

1* Carcinoma stomach.

2** Carcinoma pancreas.

3+ Carcinoma stomach with pleural secondaries.

4++ Carcinoma jejunum.

with a return of platelet numbers to normal, and, with maintenance myeleran therapy a complete remission has been maintained for more than three years.

The final group includes four patients admitted to hospital for investigation of a probable intra-abdominal carcinoma. Each was found to have a markedly raised platelet count and in every case the bone marrow was found to be generally hypercellular but with a disproportionate increase in the number of megakaryocytes. At laparotomy cases I and 3 were shown to have carcinoma of the stomach and cases 2 and 4 carcinoma of the pancreas and small bowel respectively. The lesions were inoperable in cases 2 and 3 and the patients died shortly after laparotomy.

The results of the studies of platelet thromboplastic function in each of these patients are shown in Table 8I.

In the "control" subject the thrombocythaemia following splenectomy was associated with a normal platelet thromboplastic function, and it seemed reasonable to assume that a greatly increased platelet production independent of a primary or secondary marrow disorder was not productive of an impaired thromboplastic function of those platelets.

Platelet thromboplastic defects were invariably present in the cases of chronic myeloid leukaemia studied, but in general, these were less severe than those found in acute leukaemia. It is interesting to consider these results in parallel with those of case 4 (E.B.) described in the acute leukaemia group. This patient had chronic myeloid leukaemia that had been in excellent remission prior to her present investigations. The latter were undertaken early in an acute relapse, heralded by the appearance of and increasing numbers of, blast cells in the peripheral blood and a falling platelet count. Prior to this her platelet numbers had been at the upper limit of normal and she had been free from haemorrhage. At the first investigation her platelet count was 70×10^3 per c.mm. and their thromboplastic function was 77%. After two weeks while there had been little change in platelet numbers, their function had fallen to a very low level (13%) and she was actively haemorrhagic. Overall,

from the results found in both acute and chronic leukaemia, it seems that the haemorrhagic state follows levels of platelet function quite well but is unrelated to platelet numbers. During an acute phase and when patients are markedly haemorrhagic low platelet function is evident but with the onset of a complete remission this returns to normal (cases I, 2 and II, acute leukaemia). Subsequent acute relapse is accompanied by a recurrence of thrombocytopaenia, platelet functional defects and haemorrhage. If an acute myeloid leukaemia proceeds into a chronic state or if such is present from the outset, even despite thrombocythaemia, platelet defects are common, but in general less severe than in the acute disease, and, at the same time, haemorrhagic manifestations are less severe.

The demonstration of platelet thromboplastic defects in the four cases considered at the time of the estimations to be examples of haemorrhagic thrombocythaemia was not an unexpected finding. This confirmed the work of earlier authors (Soulier et al, 1957: Gunz, 1960), and there can be little doubt that this abnormality must play a significant role in the haemorrhagic manifestations of this disease. To the best of the author's knowledge this is the first time that platelet thromboplastic function has been quantitated in haemorrhagic thrombocythaemia. Again the relative unimportance of platelet numbers in relation to haemorrhage was well shown and the danger of surgery in patients with apparently adequate platelet numbers but impaired function could receive no better illustration, for two of the cases presented following alarming haemorrhage at operation.

The association between malignancy, thrombocythaemia and platelet functional defects in the remaining cases is obscure but provides an interesting topic for further study. In all of these cases the marrow appearances were consistent with those of haemorrhagic thrombocythaemia, but it would have been an extraordinary coincidence to find four patients with this relatively rare disease and an unrelated malignancy in so short a time. That such an association was not purely chance was underlined further when successful removal of the primary tumour was achieved in two cases. In these, following surgery, there was a prompt reversal of the

marrow appearances and platelet levels to normal, and, in the first case, a formerly reduced platelet thromboplastic function was restored to normal. The platelet thromboplastic function was normal both before and after surgery in the second operable case, and abnormal in both patients with inoperable lesions. The findings were intensely interesting but await further study and clarification.

9. Polycythaemia and Myelofibrosis.

As indicated in the preceding section both polycythaemia vera and myelofibrosis may be accompanied by thrombocythaemia. In consideration of the findings described in the previous section it is not unreasonable to suggest that when this happens platelet thromboplastic defects may be present. The two cases of polycythaemia vera and the two of myelofibrosis studied by the author had either low normal or reduced platelet numbers and they are therefore considered under a separate heading. The results are shown in Table 82.

Although the incidence has varied considerably with different series, Calebresi and Meyer (I959) concluded that, overall, 30 percent of patients with polycythaemia develop an haemorrhagic tendency, excluding easy bruising. Despite this frequency no consistent abnormality has been described. At various times poor clot retraction, thrombocytopaenia, a prolonged 'prothrombin' time, fibrinogenopaenia, fibrinolysis (Lawrence, I955: Bjorkman et al, I956: Stefanini and Dameshek, I955), deficiencies of factors VII, V and X (Prasad, I960: Aggeler et al, I96I), and an anti-coagulant effect dependent on a high platelet concentration have all been incriminated. Results of platelet function studies have been controversial. In some cases (especially in association with thrombocythaemia) the thromboplastin generation test has revealed an abnormality (Biggs and McFarlane, I962) but Teodorovich and Beznosekov (I962) after an investigation of the coagulation properties and morphology of platelets in 20 cases of polycythaemia vera, concluded that the platelets were functionally normal.

In a recent excellent review Bourancle and Doan (I962) have

TABLE 82. Polycythaemia Vera and Myelofibrosis.

Part (1). Polycythaemia Vera.

Case No.	Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes — Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
1 C.M.	21.12.60	120	Control	17	12	11	10	10	10	29
			Test	26	17.5	17	14.5	14.5	14.5	
	13.1.61	160	Control	46	17	12	11	11	11	37
			Test	> 60	19.5	16	14.5	14	14	
2 J.S.	3.10.60	150	Control	21	11.5	10	10	10	10	20
			Test	39	19.5	17	17	17	17	

TABLE 82. Polycythaemia Vera and Myelofibrosis.

Part (2). Myelofibrosis.

Case No.	Date of Examination	Platelet Count ($X\ 10^3$)	Control or Test	Time of Subsampling In Minutes — Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
1 N.L.	22.12.59	18	Control	13	9.5	10	9.5	9.5	9.5	15
			Test	48	28	21	19.5	19.5	19.5	
	24.12.59	10	Control	16	10	9.5	9.5	9.5	9.5	16
			Test	54	20	19	19.5	19	19	

TABLE 82. Part (2). Cont'd.

Case No.	Date of Examination	Platelet Count ($X 10^3$)	Control or Test	Time of Subsampling In Minutes — Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
2 B.T.	24.2.61	10	Control	42	14.5	11.5	11.5	11.5	11.5	12
			Test	53.5	31	25.5	24	24.5	24.5	
	27.2.61	14	Control	21	12	11	10	10	10	11
			Test	58	24	24	23	24	24	
	8.3.61	24	Control	31.5	15	11.5	10	10.5	10	14
			Test	25	22	21	21	22	24	
	14.3.61	24	Control	33	14.5	12	12	12	12	15
			Test	42	28	24	22	22.5	24	
	17.3.61	22	Control	31	14.5	12	11	11	11	20
			Test	46	24	18	18	18	18	
	20.3.61	28	Control	31	12.5	12	12	12	12.5	11
			Test	58	32	27	27	27.5	27	
	22.3.61	20	Control	27.5	13	13	13.5	13	13.5	9
			Test	-	37	29	30.5	30	32	
	24.3.61	20	Control	27	12	9	9	9.5	11.5	12
			Test	42	29	23	23	23	27.5	

considered the clinical, haematologic and pathologic findings in 110 cases of myelofibrosis. Of these 51 had platelet numbers in excess of 400,000 per c.mm. at diagnosis and in 23 the count was greater than 1×10^6 per c.mm. Nevertheless thrombocytopaenia was not infrequent at the onset and often ensued during the progress of the disease. So too, haemorrhage was common as a presenting symptom or at some stage in the disease and cerebral haemorrhage was the immediate cause of death in 18.1 percent of cases. The exact basis for the haemorrhagic manifestation is not fully known, but broadly speaking, the same abnormalities as have been demonstrated in polycythaemia vera and 'thrombocythaemia' have been described.

Both polycythaemic patients studied by the author had been troubled by the development of spontaneous bruising, and the second had had several recent epistaxes. Platelet thromboplastic function was estimated twice on the first patient (C.M.) at an interval of three weeks. On the first occasion his platelet count was 120×10^3 per c.mm. and their thromboplastic function was 29%. On the second occasion the corresponding values were 160×10^3 per c.mm. and 37% respectively. The second patient (J.S.) was examined only once, when it was found that although his platelet count was at the lower limit of normal (150×10^3 per c.mm.) their thromboplastic function was markedly reduced being only 20%. The author did have the opportunity to study further cases himself, but since these initial investigations a colleague has continued an investigation of additional cases with high or normal platelet numbers using the technique described in this thesis. In these a reduced platelet thromboplastic function has been the common finding.

The two patients with myelofibrosis were both markedly haemorrhagic when first seen and remained so throughout the period they were studied. The first patient (M.L.) had massive bruising and purpura, and he had had an epistaxis on the day of his first examination. At this time his platelet count was 18×10^3 per c.mm. and their thromboplastic function was 15%. Two days later the latter was 16% and spontaneous bleeding from the gums had begun, to

TABLE 83. Lymphosarcoma and Chronic Lymphatic Leukaemia.

Case No. Diagnosis	Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
1 (H.M.) Lympho-sarcoma. Steroids.	18.1.61	8	Control	40	15.5	12	11	10	10	18
			Test	60	28	20	19	18	18	
	22.1.61	22	Control	24.5	14	12	12.5	12	12	9
			Test	59	45	34.5	30.5	30	31	
	15.3.61	24	Control	24	12	11	10.5	10.5	10.5	22
			Test	-	-	17.5	17	17	17	
	7.4.61	18	Control	12.5	9	9	9	9	10	22
			Test	27	16	15.5	15.5	15.5	17	
	13.4.61	10	Control	17	11	9.5	9	9	9	48
			Test	23	13	12	11.5	11	11	
	27.4.61	18	Control	39.5	16	13	12.5	11	11	37
			Test	32.5	16.5	15.5	15.5	14	14	

TABLE 83. Cont'd.

Case No. Diagnosis	Date of Examination	Platelet Count (X 103)	Control or Test	Time of Subsampling In Minutes Substrate Clotting Time In Seconds						Percent Functio
				4	10	15	20	25	30	
2 (G.T.) Lympho-sarcoma.	23.9.60	50	Control	18	11	10	10	10	10	42
			Test	38.5	12.5	12.5	12.5	12.5	13	
	24.10.60	100	Control	20	12	10.5	10.5	10.5	10.5	34
			Test	30	16.5	14	14	14	14	
	14.11.60	112	Control	33	16.5	13	12	12	12	48
			Test	23	15	14	14	14	14	
	30.11.60	140	Control	30	17	14.5	13	13	13	48
			Test	38	20	15.5	15	15	15	
	11.1.61	80	Control	16	10.5	9	9	9	10	48
			Test	17	12	12	11	11	11	

TABLE 83. Cont'd.

Case No. Diagnosis	Date of Examination	Platelet Count (X 10³)	Control or Test	Time of Subsampling In Minutes — Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
3 (O.M.) Lympho-sarcoma.	7.4.61	44	Control	12.5	9	9	9	9	10	37
			Test	15	12	12	12	12	12.5	
4 (J.W.) Lympho-sarcoma.	17.10.60	190	Control	53	18	14	12	12	12	62
			Test	47	18	14.5	13	13	13	
5 (E.L.) Chronic Lymphatic Leukaemia	6.6.60	234	Control	17	12.5	12	11	11	11	100
			Test	15	11.5	11	11	11	11	
	18.12.60	80	Control	12.5	10	9.5	9.5	9.5	9.5	100
			Test	15.5	10.5	9.5	9.5	9.5	9.5	
6 (T.W.) Chronic Lymphatic Leukaemia	7.3.61	180	Control	43	17	13	12	12	12	62
			Test	41	18	14.5	13	13	13	

be followed on the third day by a massive terminal haemoptysis. The second patient had haematuria, and like the first, extensive ecchymoses and purpura. During the course of a month's study neither her platelet count nor their function altered significantly, the former varying between 10 and 30×10^3 per c.mm. and the latter between 11 and 20 percent. During this time large doses of prednisolone and, later, ACTH had slight effect to relieve her haemorrhagic state; she continued to bruise spontaneously, developed epistaxes in addition to gross haematuria, and finally died from cerebral haemorrhage on the afternoon of her final examination. Obviously then, platelet thromboplastic defects must play a major role to determine the haemorrhagic complications of myelofibrosis, and, if the last case is a typical example, the response of these defects to steroid therapy is similar to that in most cases of aplastic anaemia, examples of which were described earlier.

10. Chronic Lymphatic Leukaemia and Lymphosarcoma.

Haemorrhagic manifestations are usually less frequent and less severe in chronic lymphatic leukaemia than in chronic myeloid leukaemia. According to Hayhoe (1960) spontaneous haemorrhage, as a mode of onset, occurs in approximately five percent of cases of chronic lymphatic leukaemia but is present in some 20-30 percent of cases of chronic myeloid leukaemia. In similar vein, Wintrobe (1961) states that haemorrhagic symptoms are unusual in lymphosarcoma; but when such ensue thrombocytopaenia is usually present, the bleeding time is prolonged and clot retraction is reduced. The latter two findings would suggest a platelet functional defect.

The author has estimated platelet thromboplastic function on one or more occasions in two cases of chronic lymphatic leukaemia and in four patients with lymphosarcoma. The results are shown in Table 83.

The first of the patients with lymphosarcoma (H.M.) was actively haemorrhagic when first seen. He had extensive purpura and ecchymoses, persistent haematuria and recurrent epistaxes. At this time his bleeding time was 17 minutes, the tourniquet test strongly

positive, and his platelet count was $8xIO^3$ per c.mm. with an estimated I8% thromboplastic function. In serial studies the latter remained low for six weeks, there was little change in platelet numbers and the patient remained actively haemorrhagic. Subsequently steroid therapy was begun and although there was no change in platelet numbers, their function was markedly improved, values of 48 and 37% being obtained on two occasions. Coincident with this improvement the most severe haemorrhagic symptoms (haematuria and epistaxes) ceased, and the occurrence of spontaneous bruising and purpura was lessened but by no means completely absent. This improvement with its lessening of the patient's distress was maintained until his death shortly after the last examination.

In the second patient (G.T.) the malignancy was more benign than in the first and except for an occasional epistaxis, invariably associated with an allergic rhinitis, he was free from haemorrhage at all times. Over a period of four months his platelet count varied between $50xIO^3$ and $I40xIO^3$ per c.mm., but quite independent of these fluctuations his platelet thromboplastic function was consistently in the region of 48%. This and thrombocytopaenia were the only demonstrable coagulation defects.

The third patient was seen following the development of purpura and bruising over the abdominal wall after palpation of his spleen by a number of students. Again the only demonstrable defects were thrombocytopaenia ($44xIO^3$ platelets per c.mm.) and a reduced platelet function (37%).

The fourth patient was investigated prior to the commencement of radiotherapy because of the abnormalities detected in earlier patients with lymphosarcoma. He had no haemorrhagic manifestations. His platelet count was in the low normal range and their thromboplastic function was slightly reduced (62%).

Of the two cases of chronic lymphatic leukaemia the first (E.L.) had two estimations of platelet thromboplastic function six months apart. During this time his platelet count fell from $234xIO^3$ per c.mm. to $80xIO^3$ per c.mm., but on both occasions their function was I00% and he was quite free from any haemorrhagic signs. The second patient

(T.W.) was seen only once, and, on this occasion although his platelet count was normal, a minor functional defect was present. At this time he too was quite free of haemorrhage.

These results would seem to indicate that while manifest haemorrhagic signs may be rare in lymphosarcoma, coagulation defects are apparently common for in each of the four patients examined platelet functional defects were present, three had thrombocytopaenia and one a markedly prolonged bleeding time and a positive tourniquet test. In three patients haemorrhagic signs were minimal or absent, but in the fourth a severe continued haemorrhagic diathesis was present. The significant feature seems to be that in these cases platelet functional defects and related abnormalities (a prolonged bleeding time etc.) were the only significant findings and this would seem to suggest that these contribute most to the haemorrhagic state.

No definite conclusion can be drawn in relation to chronic lymphatic leukaemia but it is suggested that here, too, platelet functional defects may be present and that they are quite independent of platelet numbers. The two cases described illustrate this point, for in the first patient, despite the development of thrombocytopaenia during a six month interval, his platelet function remained unchanged, while the second patient who was not thrombocytopaenic had a mild functional defect.

II. Haemolytic Anaemia with Thrombocytopaenia.

The brilliant concept of Evans and Duane (1949) and Evans et al (1951) that fundamentally acquired haemolytic anaemia and thrombocytopaenic purpura might have a common aetiology, and that in any particular case either haemolysis or thrombocytopaenia might so dominate the picture that the occurrence of other abnormalities might be missed, has received ample confirmation since their first report (Gasser and Hollander, 1951: Loeb, Seaman and Moore, 1952: Dausset and Colombani, 1959: Harris-Jones et al, 1958: Dacie and De Gruchy, 1951). The author has had an opportunity to study two

TABLE 84. Acquired Haemolytic Anaemia with Thrombocytopaenia.

Case No.	Date of Examination	Platelet Count (X 10^3)	Control or Test	Time of Subsampling In Minutes / Substrate Clotting Time In Seconds						Percent Function
				4	10	15	20	25	30	
1 H.P.	26.10.60	20	Control	19.5	12.5	12	12	12.5	12	42
			Test	17	15	14.5	14.5	15	15.5	
2 B.L.	30.3.61	18	Control	16	10	10	10	10	10.5	7
			Test	40	35.5	35	32	32	33	
	3.4.61	14	Control	15	9.5	9.5	9.5	9.5	11	18
			Test	-	21	18	18	18	19	

cases presenting with the dual defect, haemolysis and thrombocytopaenia. The results of the relevant investigations are shown in Table 84.

The first patient was a 58 year old woman who presented with a typical acute auto-immune haemolytic anaemia, and while the features of this dominated the picture, the presence of purpura and a few scattered ecchymoses on her arms and legs suggested a more widespread involvement of her haemopoietic tissue. This was confirmed by a marked thrombocytopaenia ($20xIO^3$ platelets per c.mm.) and a reduced platelet thromboplastic function (42%), abnormalities assumed to be quite sufficient to explain her haemorrhagic state.

The second patient a I2 year old boy had had an earlier admission to hospital with an initial acute haemolytic anaemia unaccompanied by thrombocytopaenia. He responded satisfactorily to steroid therapy and remained well for some months until there was a sudden recurrence of severe haemolysis, and, at this time, profound thrombocytopaenia and haemorrhagic manifestations were present. The latter included extensive purpura and ecchymoses, haematuria and epistaxes. During this admission and prior to his death the patient had two estimations of platelet thromboplastic function at an interval of five days. On both occasions he was markedly thrombocytopaenic ($I8xIO^3$, and $I4xIO^3$ platelets per c.mm. respectively) and his platelet function was very low (7 and I8%). Apart from a positive tourniquet test and a prolonged bleeding time no other coagulation abnormalities were found.

It is apparent then that the thrombocytopaenia accompanying an acquired haemolytic anaemia may be associated with platelet function defects of the same character as those found in primary idiopathic thrombocytopaenic purpura.

THE COAGULATION MECHANISM IN URAEMIA

This section describes a final but most significant application of the technique developed herein, as used in a detailed investigation of the coagulation mechanism in uraemia.

While earlier work was in progress it occurred to the author that while haemorrhagic manifestations of greater or lesser severity are common in uraemia, there had been no completely satisfactory explanation for their occurrence. Most often they have been considered to result primarily from capillary defects, the latter in themselves being secondary to a generalized toxaemia. However, reports have appeared describing deficiencies of specific coagulation factors in the absence of capillary lesions and others have demonstrated platelet functional defects.

Larrain and Adelson (1956) found prolonged bleeding and clotting times in three patients with acute renal insufficiency and two had an associated, impaired, prothrombin consumption. Despite this, and the fact that all three developed significant clinical bleeding, the authors were unable to demonstrate any defect of plasma factors, platelet numbers or platelet function, nor was any circulating anticoagulant apparent. Later, Larrain and Langdell (1956) investigated 15 dogs rendered uraemic by ureteral ligation. Serial studies were made as the uraemia progressed. Throughout, the only significant finding was a prolonged clotting time in siliconized glassware, the "prothrombin" times, prothrombin consumption, fibrinogen levels, bleeding times and platelet numbers remaining basically normal. Similar methods were used by Lewis, Zucker and Ferguson (1956) in a study of 12 uraemic patients. Their results were more significant. While but three patients developed mild thrombocytopaenia, eleven were found to have a platelet defect either in thromboplastic component or accelerator factor. They found no evidence of specific vascular abnormalities. In 1958, Cahalane, Johnson, Monto and Caldwell described platelet thromboplastic defects in uraemia, as did Alexander in 1960. In the latter case such defects were only apparent when the sensitivity of the thromboplastin generation test

was increased by reducing the number of platelets used. In contradistinction to these reports, however, Altschuler, Marcus and Ullman (1960) repeatedly failed to confirm the presence of platelet thromboplastic defects. Similarly while some of the authors cited above had reported thrombocytopaenia to be a common occurrence others had maintained that such a finding was unusual. Thus, overall, there was little agreement on the presence or absence of platelet dysfunction or its importance in relation to the occurrence or severity of haemorrhage in uraemia.

The development of a new and sensitive method of quantitating platelet thromboplastic function provided a unique opportunity to review this problem, and, since it was apparent that there had been no comprehensive study of all the coagulation abnormalities that might occur in uraemia, it was decided to extend the investigations undertaken to include a broader field of possible defects. Thirty five uraemic subjects were studied, all chosen at random from patients admitted to hospital with elevated blood urea nitrogen levels. Prior to any investigation there was no knowledge of either the patient's primary disease or of the presence or absence of haemorrhagic complications. In each case the following techniques were used in the first instance:- bleeding time: clotting time: tourniquet test: one-stage "prothrombin" time: whole blood platelet count: clot retraction and fibrinolysis: estimation of platelet thromboplastic function: screening test for thromboplastin generation. Details of the techniques used for these procedures have been given earlier. In addition, in any case where a defect of a specific coagulation factor was suggested by a prolonged "prothrombin" time or an abnormal "screening" test or both, an attempt was made to specify the defect by means of the thromboplastin generation test described by Biggs and Douglas (1953) (and, as detailed earlier) and by "correction" experiments wherein the ability of certain sera to correct the defect in the thromboplastin generation test and the one-stage "prothrombin" test were studied. The sera available for these purposes were obtained from

TABLE 85. The primary diseases, haemorrhagic signs, blood urea nitrogen levels and results of blood coagulation studies in 33 uraemic patients.

Case	Disease	BUN mg. %	Bleeding time (min.)	Tourniquet test.
1	Chronic nephritis	75	1	++
2	Chronic renal failure	165	1.5	-
3	Anuria following repair of ruptured peptic ulcer	79	1	-
4	Severe congestive cardiac failure	170	18	-
5	Periarteritis nodosa	140	2	+
6	Severe head injuries	85	2.5	-
7	Post-prostatectomy uraemia	165	1	++
8	Carcinoma of stomach, dehydration	123	1.75	-
9	Systemic lupus erythematosus	105	2	-
10	Marfan's syndrome, amyloidosis	110	10	+
11	Cardiac failure	48	1.5	-
12	Chronic renal failure	143	2	++
13	Chronic nephritis	75	2	+
14	Benign prostatomegaly	95	2	++
15	Hypertension, renal failure	105	2	-
16	Hypertension, renal failure	120	15	++++

	One-stage prothrombin time (secs.)	Platelets (X 10^3 per c.mm.)	PTF+ (%)	Specific coag. defect	Haemorrhagic manifestations
	11	160	55	Nil	Nil
	12.5	150	48	Factor 7	Nil
	16	500	23	Factor 7	Mild purpura
	13.5	160	25	Factor 7	Widespread purpura
	10	20	35	Nil	Mild purpura
	11	385	100	Nil	Nil
	12	140	16	Factor 7	Moderate purpura, spontaneous bruising
	10.5	200	55	Nil	Nil
	11	220	42	Nil	Nil
	18	560	21	Factor 10	Haematemesis, epistaxis, haematuria, mild purpura
	10	210	100	Nil	Nil
	12.5	350	37	Factor 7	Mild purpura
	11	210	48	Nil	Nil
	11.5	166	48	Nil	Nil
	12.5	220	43	Factor 7	Bruising tendency on trauma
	11	164	12	Nil	Ecchymoses

+ Each patient was compared with a randomly selected normal on the assumption that the normal value was 100 per cent.

TABLE 85. Cont'd.

Case	Disease	BUN mg. %	Bleeding time (min.)	Tourniquet test.
17	Rheumatoid arthritis, acute papillary necrosis	75	1.5	++
18	Hypertension, cardiac failure	125	8.5	-
19	Unknown	80	1.5	-
20	Chronic nephritis	85	1	+
21	Pyelonephritis, diabetes, cardiac failure	79	1.5	-
22	Rheumatoid arthritis, pyelonephritis, renal failure	37	2.5	-
23	Fractured femur, dehydration	76	1	-
24	Post-prostatectomy uraemia	100	2	-
25	Pneumonia, meningitis	50	2	-
26	Chronic nephritis	76	2	+
27	Myxoedema, pyelonephritis	34	1	-
28	Renal failure	249	2	++
29	Hypertension, chronic nephritis	124	1	-
30	Chronic nephritis	148	2	-
31	Chronic renal failure	123	2	+
32	Renal failure	65	2.5	-
33	Pyelonephritis	95	2	-
34	Acute nephritis	130	3.25	-
35	Acute nephritis	110	15	+++

One-stage prothrombin time (sec.)	Platelets ($X\ 10^3$ per c.mm.)	PTF+ (%)	Specific coag. defect	Haemorrhagic manifestations
11	100	21	Nil	Ecchymoses, purpura
12.5	60	38	Factor 7	Mild purpura
13	134	25	Factor 7	Nil
12	214	35	Factor 7	Nil
13.5	174	38	Factor 7	Minimal purpura
12.5	134	50	Factor 7	Nil
11.5	350	66	Nil	Nil
12.5	470	48	Factor 7	Nil
11	226	100	Nil	Nil
10.5	124	66	Nil	Nil
11	110	42	Nil	Minimal purpura
14	320	18	Factor 7	Extensive ecchymoses
11	245	31	Nil	Nil
11	200	42	Nil	Bruising tendency on trauma
11	250	42	Nil	Mild purpura
11	240	100	Nil	Nil
10	340	100	Nil	Nil
12	304	33	Nil	Purpura, ecchymoses
11.5	250	17	Nil	Extensive purpura, bruising, epistaxis

two patients known to be deficient in factor VII only and from a patient with Christmas disease. Serum specifically lacking factor X was not available and such a deficiency was presumed to be present in one patient in whom appropriate tests demonstrated in addition to a deficiency of factor VII, a defect in the serum factors other than factor IX. The results of this study together with the blood urea nitrogen levels, primary diseases and the haemorrhagic state of each patient at the time of investigation are shown in Table 85. This table excludes the results of the clotting times, clot retraction and fibrinolytic activity, none of which were judged to be abnormal in any patient.

The bleeding time was prolonged in only five patients and the tourniquet test was abnormal in I4. A deficiency of factor VII resulting in a prolongation of the "prothrombin" time, was present in I4 cases, but, in only two (cases 3 and I0), was the defect marked. In only one case (I0) was there any other specific defect. In this patient, the investigations suggested a complex abnormality involving both factors VII and X. Pronounced thrombocytopaenia was a rare occurrence, and in only two instances (cases 5 and I8) was the platelet count less than I00,000 per c.mm. Six patients had mildly reduced platelet numbers (between $I00xI0^3$ and $I50xI0^3$ per c.mm.) but in 27 the platelet numbers were either normal or in excess of the normal range. The most consistent abnormality was a reduced platelet thromboplastic function, a defect found in 30 of the 35 patients studied and varying in severity from 66 to I2 percent of normal.

If one attempts to relate these findings to the clinical haemorrhagic state of these patients some interesting facts emerge. The five patients with a prolonged bleeding time were among those who had the most severe haemorrhagic manifestations and all had an associated severe platelet thromboplastic defect. However, I2 other patients had haemorrhagic signs sometimes of equal severity, but normal bleeding times, and in five of these (cases 3,7,I7,I9 and 28) the platelet thromboplastic defects were of a comparable degree to those found in the patients whose bleeding times were prolonged. Although the tourniquet test was positive in I4 cases this did not

correlate as well as might have been expected with either the levels of platelet function, the clinical manifestations of haemorrhage, or the bleeding time. This is illustrated by the fact that while cases 3,4,I8 and 2I had low levels of platelet function and definite, albeit mild, purpura, the tourniquet test was negative. In addition, cases 4 and I8 were among the few with a prolonged bleeding time. The most consistent relationship existed between levels of platelet function and haemorrhage. All patients who had haemorrhagic signs had a platelet function defect and certainly the more obvious the former, the more severe was the latter. Purpura was not present whenever the platelet function was estimated to be greater than 43 percent but haemorrhagic signs were present in all but four of 20 patients whose platelet function was less than this. Of the I7 patients manifesting haemorrhagic signs a defect of a specific coagulation factor in addition to platelet dysfunction was present in only 9, and, of these, in only two (cases 3 and I0) was the defect considered to be of a degree sufficient to cause spontaneous haemorrhage by itself. Five patients had the same specific defects but no haemorrhagic signs. All patients who had the dual defects and haemorrhage had platelet function less than 43 percent. The rarity of a significant thrombocytopaenia was very interesting, even more so when one notes that of the five patients with a prolonged bleeding time in only one (case I8) was the platelet count below the accepted normal range. Consideration of table 85 demonstrates a complete lack of any correlation between platelet numbers and levels of platelet thromboplastic function, and equally so there was no correlation between haemorrhagic signs and platelet numbers.

The relationship between the levels of blood urea nitrogen and platelet thromboplastic function was subjected to statistical analysis. This suggested a significant linear relationship between these data ($p < 0.0I$). The equation for the straight line was calculated and the slope was found to be 0.29. However, in all instances it was impossible to determine the duration of the elevation of blood urea nitrogen prior to these investigations but some evidence

acquired during the experimental studies suggested that the duration of the uraemic state might well influence the above relationship. Case 26, a patient with chronic nephritis, illustrates this point. When first studied his blood urea nitrogen was 76mgs.%., his platelet count I24xI0^3 per c.mm. and their thromboplastic function 66%. During the next two weeks frequent blood urea nitrogen estimations varied little, ranging between 70 and 80mgs.%. At the end of this time, however, the platelet numbers had fallen to a value of I04xI0^3 per c.mm. and their function showed an even greater change being only 29%.

In the present series of cases the effect of cortico-steroids in promoting an improvement in platelet function was not clearly or adequately demonstrated. In most cases possible adverse effects on the primary disease or the nitrogen load contravened their use. In three patients nonetheless, worthwhile observations were made. Cases I3 and 22 were given doses of prednisolone (40mgs. daily) for four and six days respectively, after which the platelet thromboplastic function in both was found to be I00 percent compared with initial values of 48 and 50 percent. At the same time the levels of blood urea nitrogen and platelet numbers were virtually unchanged. Case I6 was a woman, extremely ill and approaching the terminal phase of renal failure. Clinically she had developed widespread ecchymoses and purpura over the body, arms and legs. Her tourniquet test was markedly positive (+++), her bleeding time in excess of I5 minutes and her platelet thromboplastic function but I2 percent despite a normal platelet count. She was given 40mgs. of prednisolone daily and after four days her bruising had begun to fade and no new lesions were apparent. At this stage her platelet function was 27%. The patient died two days later before further investigations were performed.

Discussion: Bonnin has stressed a distinct relationship in thrombocytopaenic states between the occurrence and severity of haemorrhage and levels of platelet function. This relationship was closest in idiopathic thrombocytopaenic purpura and related types of secondary thrombocytopaenia, but was not as strict in acute leukaemia and

aplastic anaemia where coincident deficiencies of other coagulation factors were more common. The latter may have contributed to and complicated the mechanism. The relative unimportance of platelet numbers was stressed and emphasis was placed on the parallel between the haemorrhagic state of a patient and the average thromboplastic function per platelet. Whether the platelet thromboplastic factor was directly concerned or whether the levels of platelet function reflected the degree of vascular damage was not known.

The present study would certainly lend strong support to the concept that platelet numbers are of little importance. Indeed, several patients had platelet numbers above normal yet in the presence of a sufficiently reduced platelet thromboplastic function, purpura or other haemorrhagic manifestations were present. In general, however, the relationship between the latter and platelet function was not as strict as Bonnin has claimed exists in idiopathic thrombocytopaenic purpura. In part, at least, this may be explained by the frequent occurrence of factor VII deficiencies as a complicating factor in uraemia. So too, in primary thrombocytopaenia there is often more consistent evidence of vascular abnormalities than was found in this study. In the former it is usual for the bleeding time to be prolonged and the tourniquet test abnormal when low levels of platelet function are present, that is to say there is an obvious defect of both haemostasis and coagulation, a combination conducive to active haemorrhage. Lewis, Zucker and Ferguson (1956), too, noted the discrepancy between the high incidence of a normal bleeding time and reduced platelet function in their series. However, if we look at the results of this study more carefully, the importance of a vascular lesion (a defect of haemostasis) as well as a coagulation defect in the production of haemorrhagic manifestations is probably apparent, for 11 of the 17 haemorrhagic cases had evidence of a possible vascular defect in that the bleeding time was prolonged, the tourniquet test abnormal, or both. In the remainder the haemorrhagic signs were minimal, a tendency to bruise easily or minimal purpura being the manifestations of haemorrhage. Furthermore in two of the latter cases the platelet function defect was as

severe as that found in some of the cases with the most marked haemorrhage but evidence of impaired haemostasis in addition.

If one returns to earlier discussions pertaining to haemostasis and the facts that platelets play a role in maintaining an intact vascular tree and a normal bleeding time, and that platelets possibly carry a protein factor essential to the maintenance of normal capillary integrity, what explanation is there for the fact that while 30 of 35 patients had evidence of impaired platelet thromboplastic function only 5 had evidence of vascular damage reflected in a prolonged bleeding time and I4 a positive tourniquet test? In consideration of the bleeding times, one could perhaps postulate a selective impairment of different platelet functions, support for such a suggestion being found in the fact that none of the patients studied was judged to have an impaired clot retraction. On the other hand the recent work of Hellem (I960) and Hellem, Borchgrevink and Ames (I96I) would seem to explain the unexpected finding that the bleeding time was normal in so many patients and so poorly related to the degree of platelet thromboplastic dysfunction. These authors have demonstrated that the bleeding time is related to the number of adhesive platelets and that when these are in excess of $40xI0^3$ per c.mm. the bleeding time is normal. It may well be that in this study the bleeding time was not abnormal more frequently because so few patients had reduced platelet numbers, and that because of this, reduction of the number of adhesive platelets below this critical level was a rare occurrence. On this basis it is readily appreciated that the bleeding time may be more frequently abnormal in truly thrombocytopaenic platelet dysfunction and the dissociation between the frequency of platelet thromboplastic abnormalities and abnormal bleeding times in uraemic patients is clear.

If one attempts to relate bleeding times, positive tourniquet tests and platelet dysfunction as linked and to some extent inter-dependent abnormalities, however, no such simple or acceptable explanation is forthcoming. The greater incidence of a positive tourniquet test might suggest at first consideration, a closer relation between this and platelet abnormalities than would seem to

exist between the latter and the bleeding time had not Hellem's work clarified the latter. The facts that in this series the bleeding time was prolonged in some patients in whom the tourniquet test was negative and vice versa, suggests that these two are independent and not necessarily related phenomena. This concept was discussed earlier in this thesis when it was suggested that the bleeding time and tourniquet test might reflect quite different aspects of capillary physiology, the former their ability to retract or otherwise respond to direct and deliberate trauma from without and the latter their inherent strength or their competence to withstand internal stress. If, as Luscher and Asper (I960) have suggested, platelets do convey a protein essential for the maintenance of capillary integrity, it may be that the normal function of this factor is to maintain a normal tourniquet test and its decrease or inability to function may be responsible for the development of a positive tourniquet test. This function of platelets may be quite distinct from their function in relation to the bleeding time the latter being related as it is to absolute numbers of adhesive platelets. Thus it is possible that there may be a close association between the occurrence of a positive tourniquet test in uraemic subjects and the presence of undoubted platelet abnormalities demonstrated by their reduced thromboplastic function. Rather against this explanation is the fact that there was little correlation between the degree of abnormality in the tourniquet test and the degree of platelet damage as indicated by their thromboplastic function. One could perhaps minimise this objection by invoking again varying degrees of selective damage to different platelet functions, or, even more basic, varying degrees of capillary integrity in different patients prior to the advent of the damaging agent. If the explanation given should approach the truth, again, it is readily appreciated that a positive tourniquet test is likely to be present more often in thrombocytopaenic platelet dysfunction than in non-thrombocytopaenic cases.

A more simple and therefore more likely explanation of the

positive tourniquet test stems from the reasonable assumption that the platelet abnormalities in uraemia result from the prolonged retention of abnormally high concentrations of potentially toxic nitrogenous products. These same products may well be directly responsible for capillary damage. Humble (I949) has shown that because of certain unusual characteristics, the arteriolar end of a capillary loop is peculiarly vulnerable to selective or more severe damage than the rest of the capillary. He has shown that purpuric lesions of whatever cause, always arise from this restricted area. One can conceive that while the mechanism behind the tourniquet test revolves around an isolated segment of the capillary, the bleeding time may involve any part or the whole of one or more capillaries. One can postulate that while the whole capillary loop may be damaged by the toxaemia in uraemia this generalized damage is rarely, if ever, sufficient to cause grave impairment of the capillary's function in relation to the bleeding time. On the other hand more severe localized damage in that area of the capillary loop from whence purpuric leaks arise, and which must therefore be intimately associated with the processes underlying the mechanism of the tourniquet test, would readily explain the higher incidence of positive tourniquet tests and the closer association between the incidence of these and platelet thromboplastic dysfunction. In consideration of this explanation one cannot but conclude again that in uraemia, in most cases platelet adhesiveness is the main determinant behind a prolonged bleeding time. While there is no doubt that an abnormal capillary function alone may produce a prolonged bleeding time, should this be the fundamental process in uraemia, such capillary defects must be the result of toxic damage, and the most severe damage would exist at the arteriolar end of the capillary. Under these circumstances one would expect a positive tourniquet test to be an invariable accompaniment of a prolonged bleeding time but such has not been the case.

There can be little doubt that in like manner the deficiencies of specific coagulation factors as described arise through the action

of retained toxic metabolites on their tissue of origin.

This study then, has clarified some consistent and recurrent abnormalities upon which the haemorrhagic complications of uraemia must depend. In order of frequency these have been platelet thromboplastic defects, vascular abnormalities and deficiencies of specific coagulation factors, especially factor VII. The findings have suggested that when significant haemorrhage occurs dual defects of haemostasis and coagulation are apparent. The defects of haemostasis have been less frequent than defects of coagulation and are probably of complex aetiology depending in part on a primary toxic effect on the capillary network exaggerated by varying degrees of damage to those platelet functions essential to the maintenance of an intact vascular network. In this hypothesis haemostatic defects in uraemia are produced in similar fashion to the production of such defects in primary thrombocytopaenia. Vascular damage produced in this way, if of sufficient degree, may determine the onset of spontaneous haemorrhage, the severity of which will depend on the extent to which coagulation is impaired. The frequency of platelet thromboplastic defects and the fact that these have been consistently the most severe abnormalities suggests that they are the major factor in deciding the severity of haemorrhage should it occur. It is true to say that while an assessment of "total thromboplastic efficiency" has been complicated by the presence of deficiencies of specific coagulation factors (especially factor VII) together with platelet thromboplastic defects, the latter have provided the most useful guide in assessing a patient's potential haemorrhagic state. This claim is well supported by the important point that in several of the patients studied, a marked platelet thromboplastic defect was the only demonstrable abnormality.

As has been stressed before in this thesis, the danger of relying on platelet numbers, the bleeding time and the clotting time as a sufficient guide to a patient's possible haemorrhagic state (specifically in relation to renal biopsy in this context), could receive no better illustration. It is disturbing how often the bleeding and clotting times alone are requested and apparently

accepted as adequate pre- and, sometimes, post-operative checks.

Finally, the patients studied suffered from a variety of acute and chronic diseases, and while in many instances these were of primary renal origin, in others renal involvement could only be regarded as a late, secondary development. As far as could be ascertained the one feature common to all was a raised blood urea nitrogen level. It may be said therefore, that the abnormalities described could not in any way be attributed to the primary disease process, and that one can anticipate their occurrence in any condition accompanied by a prolonged elevation of blood urea nitrogen.

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SUMMARY AND CONCLUSIONS

The material presented in this thesis can be said to have advanced both medical knowledge and practice.

The technique developed to assay platelet thromboplastic function has been shown to be a sensitive and reliable method to detect and measure defects of platelet thromboplastic factor. This claim is illustrated by the case reports described in the final section of the thesis and has been substantiated by the adoption of the technique in other laboratories and by the fact that it has been included in the latest editions of Professor J.V. Dacie's standard text "Practical Haematology".

When compared with the thromboplastin generation test as modified to assay platelet thromboplastic function, the present method has distinct advantages of a technical nature. Both procedures require the combination of four reagents before complete thromboplastin generation proceeds. The modified thromboplastin generation test requires that three of these, platelets, serum, and aluminium hydroxide-treated plasma be prepared each day, calcium chloride being the only reagent that may be prepared in advance and stored without fear of deterioration. Three of the essential reagents used in the new technique, brain residue, serum and calcium chloride are simple to prepare and each may be stored for prolonged periods without loss of activity. Thus it is, that the preparation for any test is simple, and with the daily preparation of one rather than three reagents, the likelihood of errors being introduced is minimized. So too, the comparison of successive tests would seem to be far more satisfactory wherein one rather than three of the reagents varies with each test.

While it is true that the actual performance of the new technique is a longer procedure than the performance of the modified thromboplastin generation test, the increased time interval is not so great as to render the new method a time wasting and impractical

procedure. Indeed, additional technical advantages are provided by this feature. Thus with the increased intervals between subsampling less technical experience and dexterity is required to perform a satisfactory test. So, too, there is ample time to repeat any individual step should an error have been made. The latter is often difficult if not impossible when the modified thromboplastin generation test is used.

A further but definite advantage of the new procedure stems from the limited availability of the modified thromboplastin generation test in markedly thrombocytopaenic subjects. Unless sufficient platelets are available to allow an incubation mixture to be prepared sufficient in quantity to permit subsampling to be carried out at minute intervals for up to ten minutes it is difficult to perform a completely satisfactory and reliable test. It has been shown, however, that in similar circumstances the number of platelets used in the new procedure may be reduced by half or even more without loss of accuracy.

It is confidently stated therefore that the technical improvements, reliability, sensitivity and the reproducibility of results obtained by the new technique make it a distinctly superior procedure for the estimation of platelet thromboplastic function.

In addition to its major use as described, it has been shown that the technique has further exciting possibilities. The few relevant experiments included have illustrated that, with minor modifications, it can be used to assay factor X when the non-availability of serum or plasma specifically lacking factor X precludes the use of more conventional techniques. Perhaps of more significance, the extension of the technique to study the behaviour of anti-thromboplastic activity in plasma and serum has provided a new and promising approach to clarify some aspects of this neglected subject. Based on this work, this subject is now the topic for an extensive investigation by a colleague.

As outlined, therefore, it can be said that this study has contributed to the practice of medicine in that it has resulted in the development of three technical procedures. Of these, one has

an important practical, clinical application in the assessment and management of haemorrhage in diseases affecting blood platelets. The other two have more limited use but they may provide useful research techniques if not of routine practical application.

The final section of this thesis may be said to have advanced medical knowledge in so far as it has confirmed or denied defects of platelet thromboplastic function as causative or contributing agents in the production of haemorrhage in various diseases. Thus it has been shown that platelet thromboplastic function is normal in Von Willebrand's disease, Henoch-Schonlein purpura and scurvy. The controversial question of the presence of platelet defects in dysproteinaemia, thrombocythaemia, polycythaemia vera and myelofibrosis has been resolved by the frequent demonstration of such defects in appropriate cases. Indeed they were often of such a degree to be sufficient to explain the haemorrhagic state by themselves. It has been shown that estimations of platelet thromboplastic function have an important place in the management of thrombocytopaenic purpura (both acute and chronic), acute leukaemia and aplastic anaemia. Furthermore, apart from a routine application in these diseases, it has been shown that in these and other conditions, for example, thrombocytopathia, estimations of platelet thromboplastic function are unequalled in the solution of special problems. Among the latter one may include determinations of the extent and duration of improvement in coagulation following platelet transfusions, adjustment of steroid dosages in haemorrhage complicated by infection, and to assess the relative merits of different steroid preparations in promoting improved platelet function in cases refractory to some preparations. The danger inherent in the assumption that normal platelet numbers reflect a normal platelet function has been stressed and amply illustrated throughout this thesis. It has been shown that not infrequently a definite but formerly unexplained haemorrhagic tendency has its origin in a persistent abnormality of platelet thromboplastic function. Unless the latter is estimated whenever there is a positive bleeding tendency but all other coagulation factors have been found to be normally

present, diagnoses will be missed.

It is thought that the detailed investigation of the coagulation mechanism in uraemia described in this thesis has made a significant contribution to medical knowledge and that it has an important practical application. The assumption formerly widely held and perpetuated that the haemorrhagic signs in uraemia are based primarily on capillary defects has been proved false. While such defects do occur and while deficiencies of specific coagulation factors are not uncommon, it has been shown that by far the most frequent and often the most severe defects are abnormalities of platelet thromboplastic function. Furthermore the latter are present despite normal platelet numbers in the majority of cases. On the other hand, the bleeding time is not often prolonged in association with the platelet defect. This study has again emphasized the danger inherent in relying on platelet numbers as a sufficient guide to an intact coagulation mechanism and has illustrated the futility of assuming estimations of the bleeding and clotting times to be adequate pre-operative investigations prior to renal biopsy. Indeed this study has left no doubt that an estimation of platelet thromboplastic function is an essential pre-requisite to any operative procedure on any patient with a raised blood urea nitrogen level.

APPENDIX I

Figures 2 - I9 Assays of factor X

FIGURE 2: ASSAY OF FACTOR $\bar{X}$

DRAWN FROM TABLE 50 PART (1) TEST 1

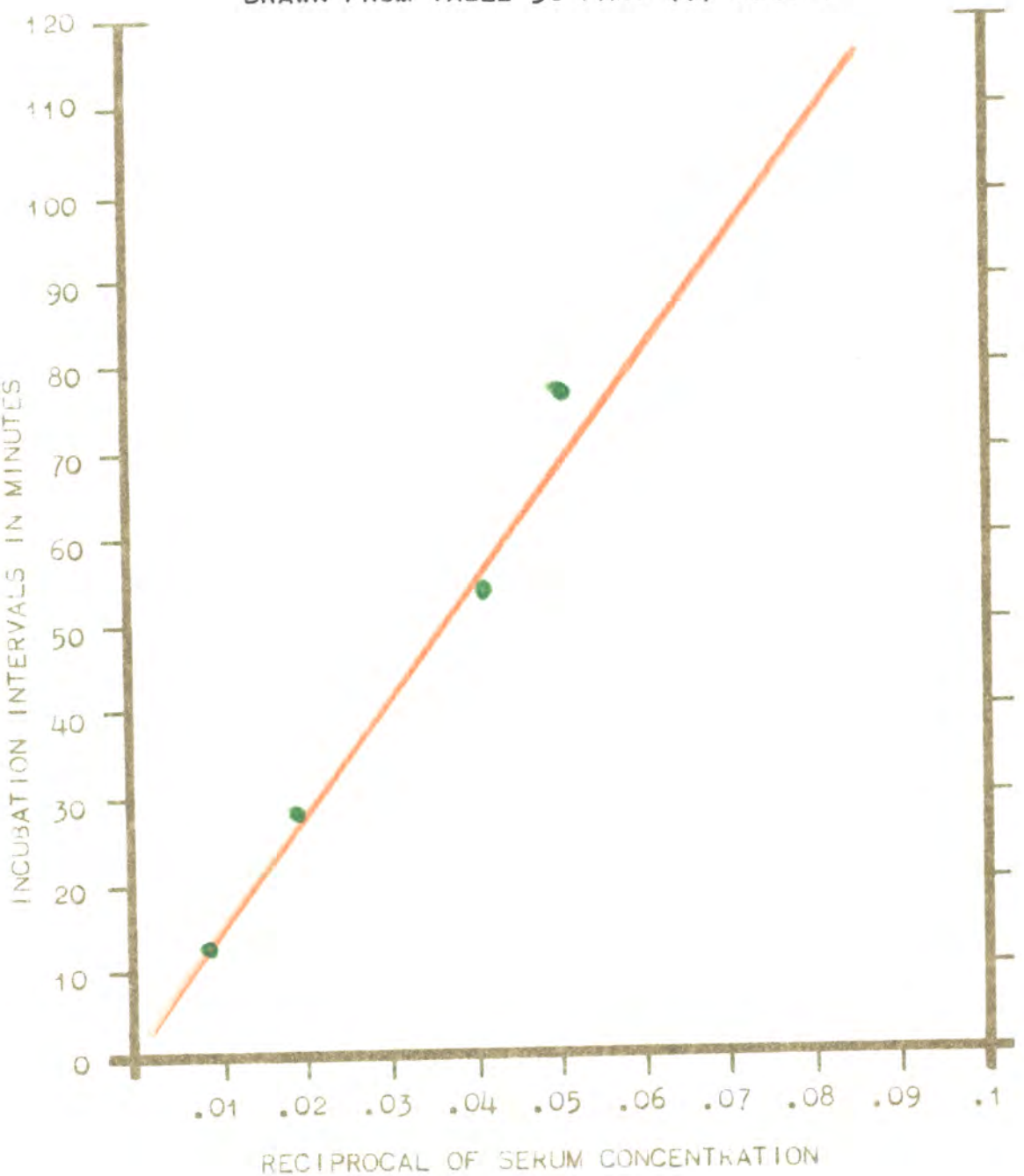


FIGURE 3: ASSAY OF FACTOR $\bar{X}$

DRAWN FROM TABLE 50 PART (1) TEST 2

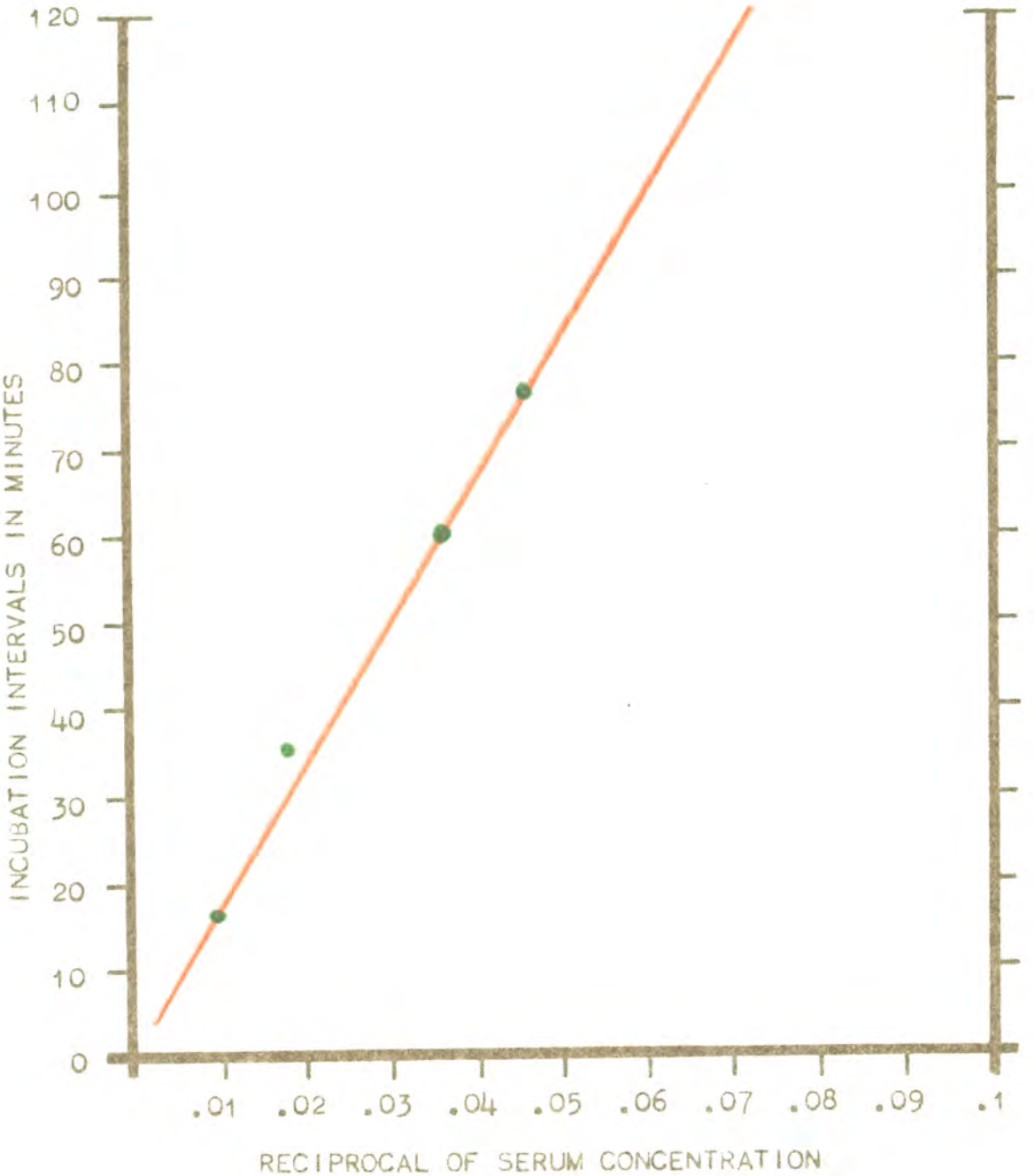


FIGURE 4: ASSAY OF FACTOR $\underline{\bar{X}}$

DRAWN FROM TABLE 50 PART (1) TEST 3

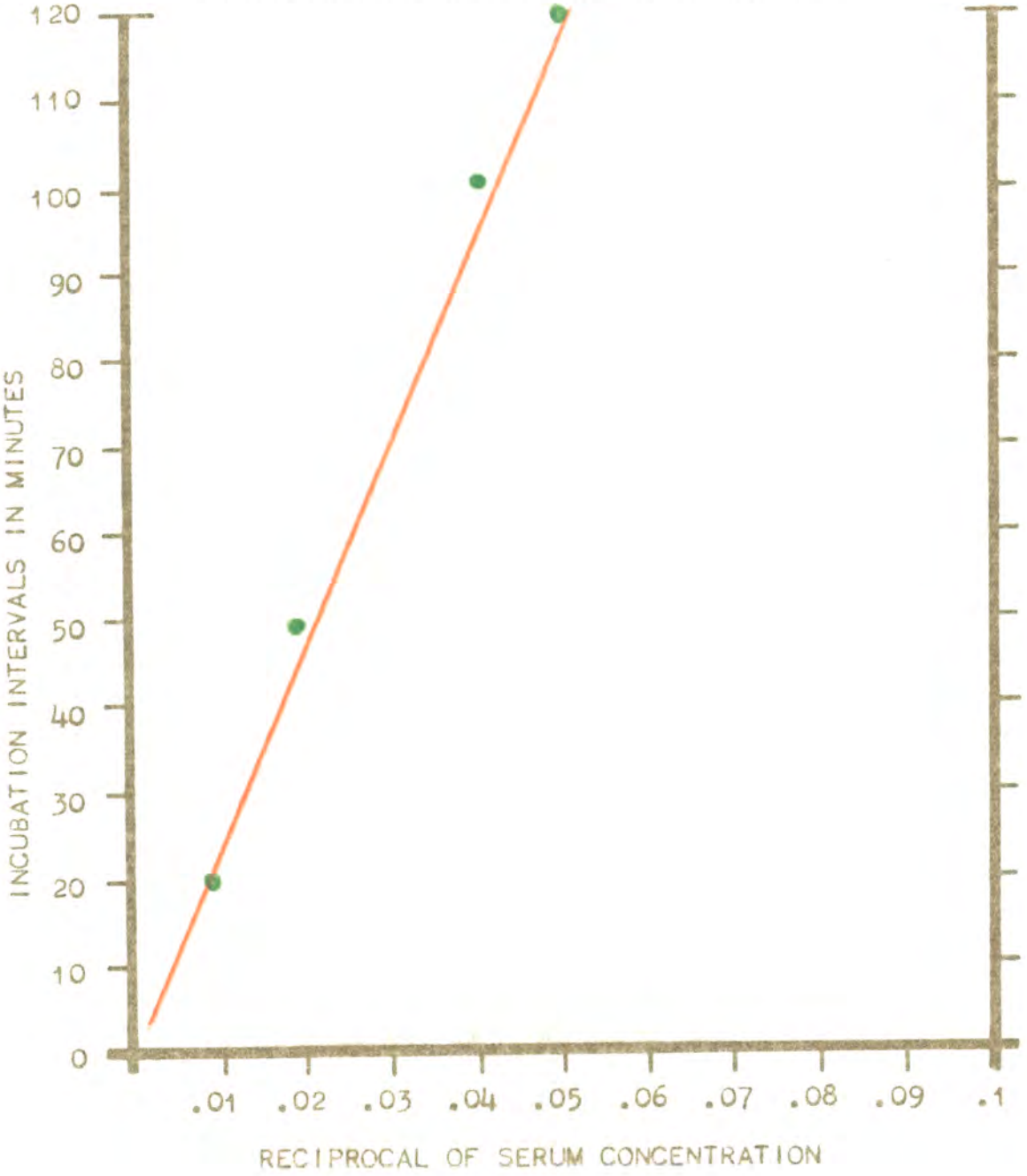


FIGURE 5: ASSAY OF FACTOR $\bar{X}$

DRAWN FROM TABLE 50 PART (1) TEST 4

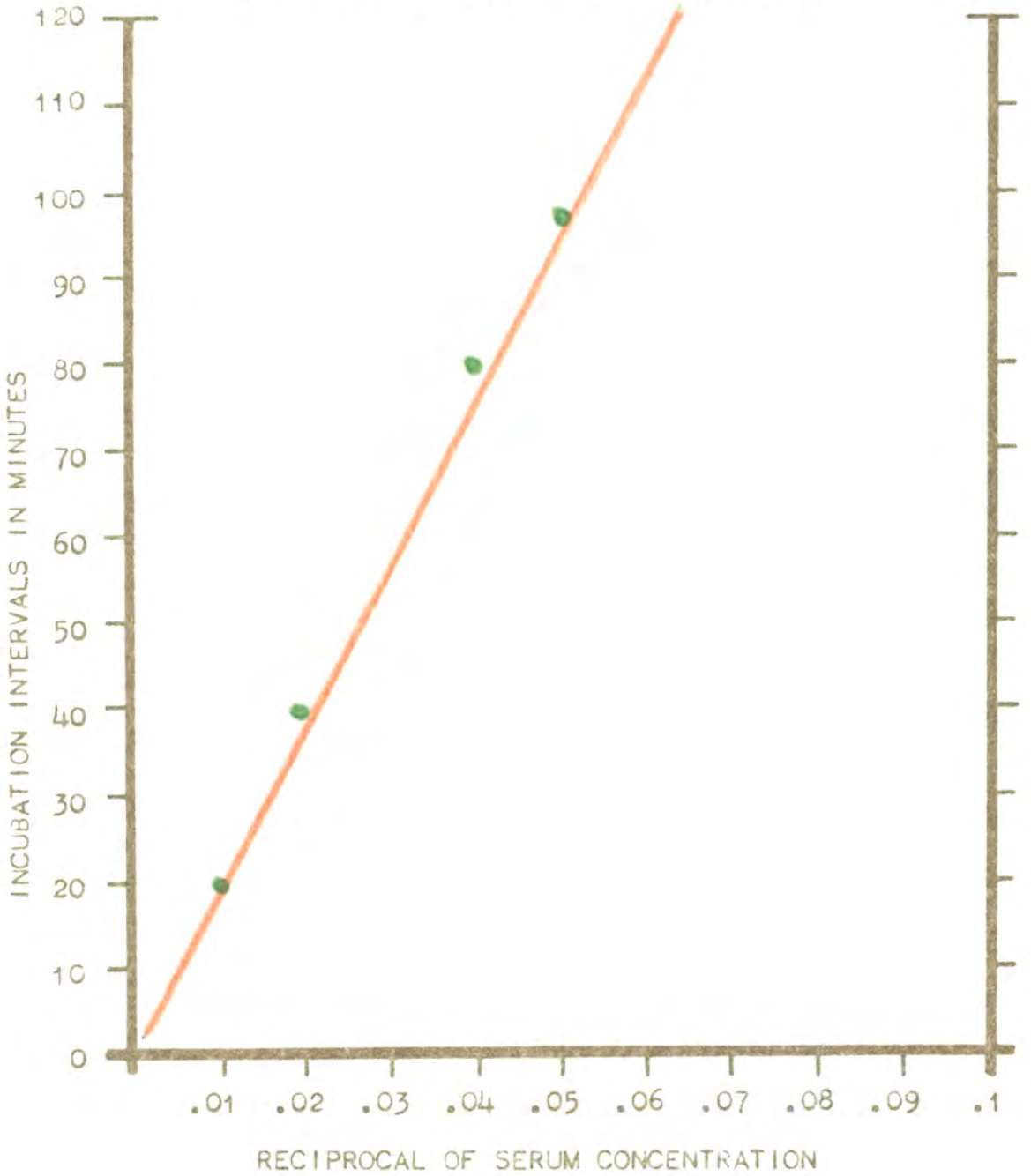


FIGURE 6: ASSAY OF FACTOR $\bar{X}$

DRAWN FROM TABLE 50 PART (1) TEST 5

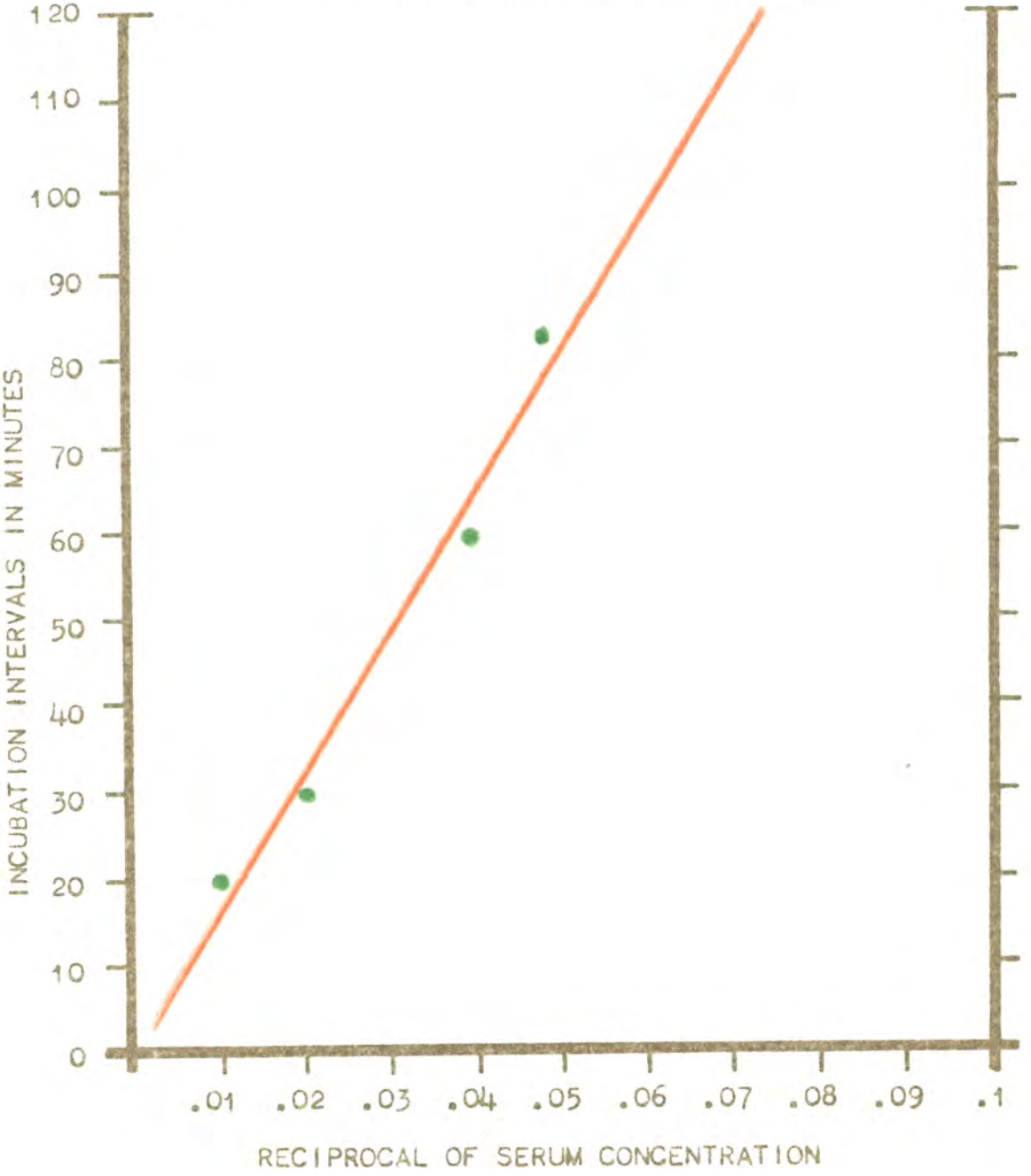


FIGURE 7: ASSAY OF FACTOR $\bar{X}$

DRAWN FROM TABLE 50 PART (2) TEST 6

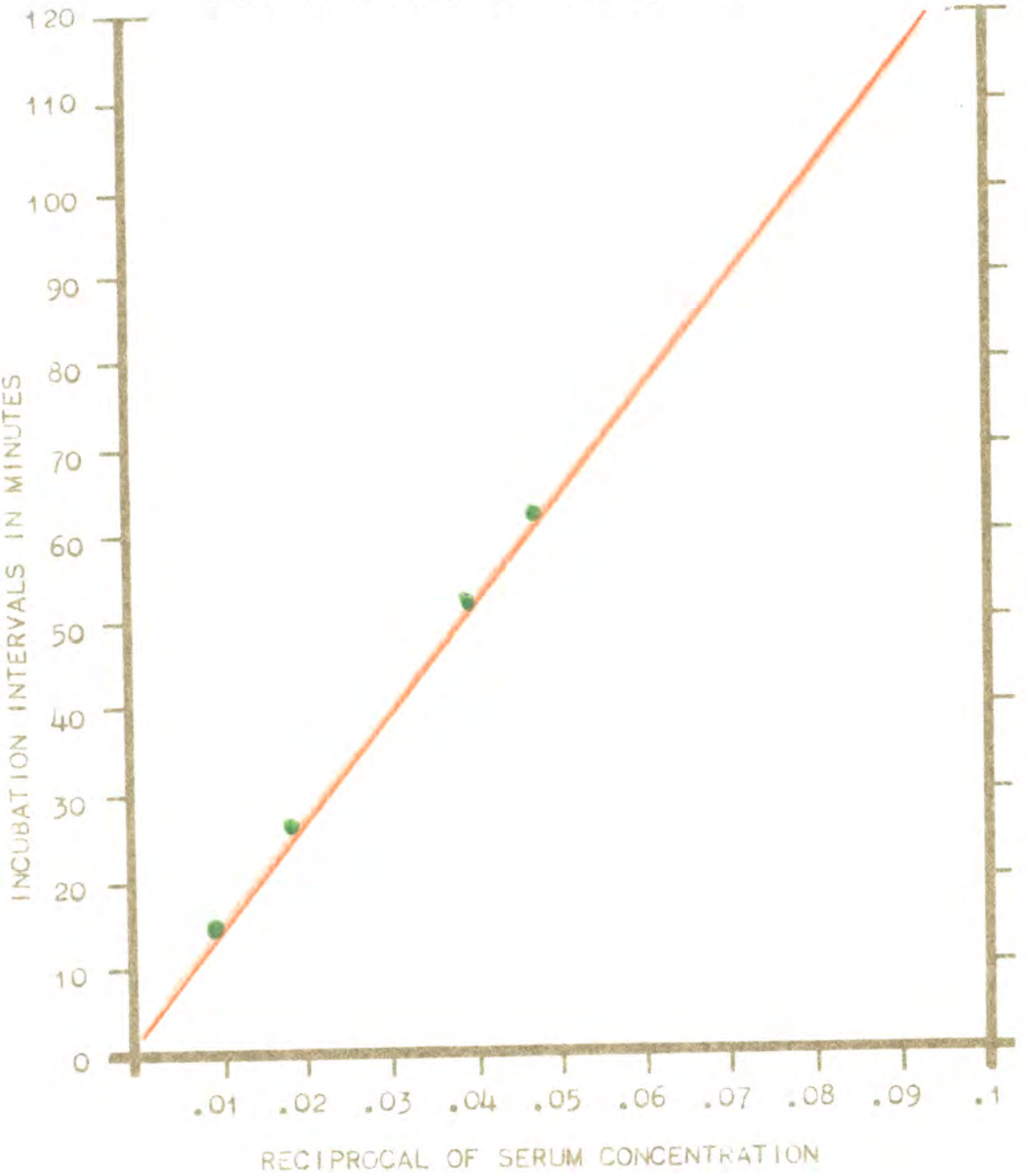


FIGURE 8: ASSAY OF FACTOR $\bar{X}$

DRAWN FROM TABLE 50 PART (2) TEST 7

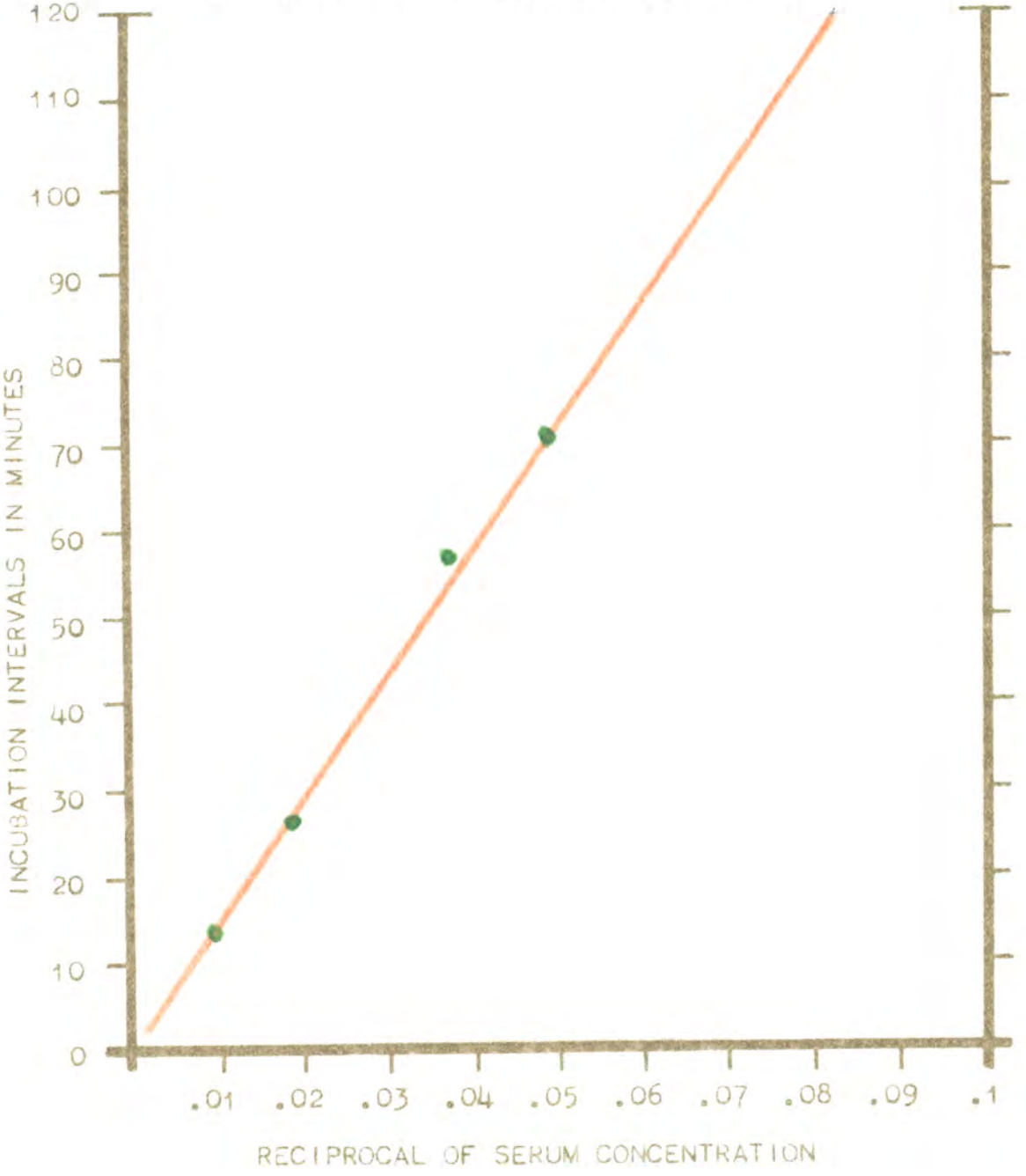


FIGURE 9: ASSAY OF FACTOR $\overline{X}$

DRAWN FROM TABLE 50 PART (2) TEST 8

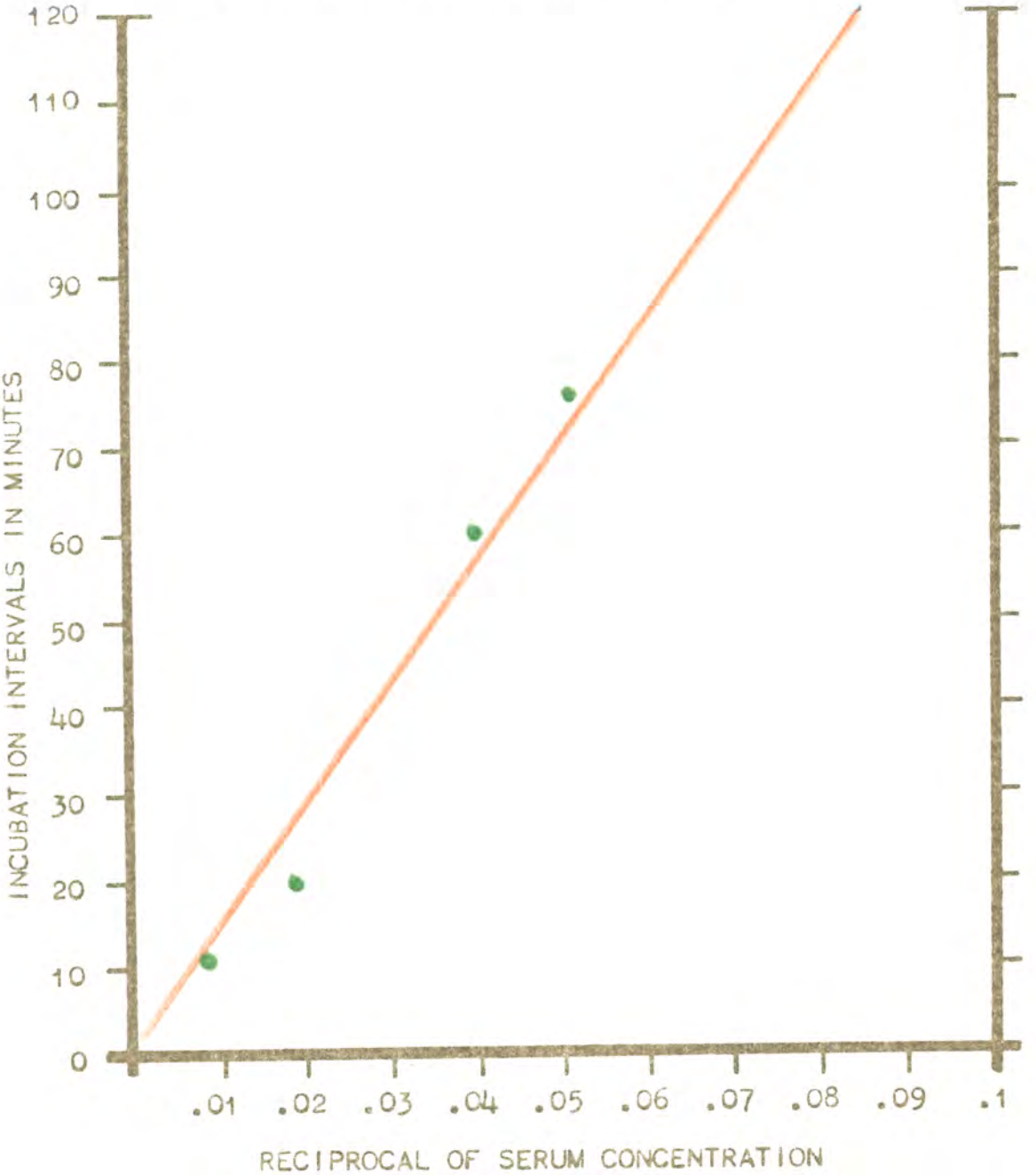


FIGURE 10: ASSAY OF FACTOR $\bar{X}$

DRAWN FROM TABLE 50, PART (2) TEST 9

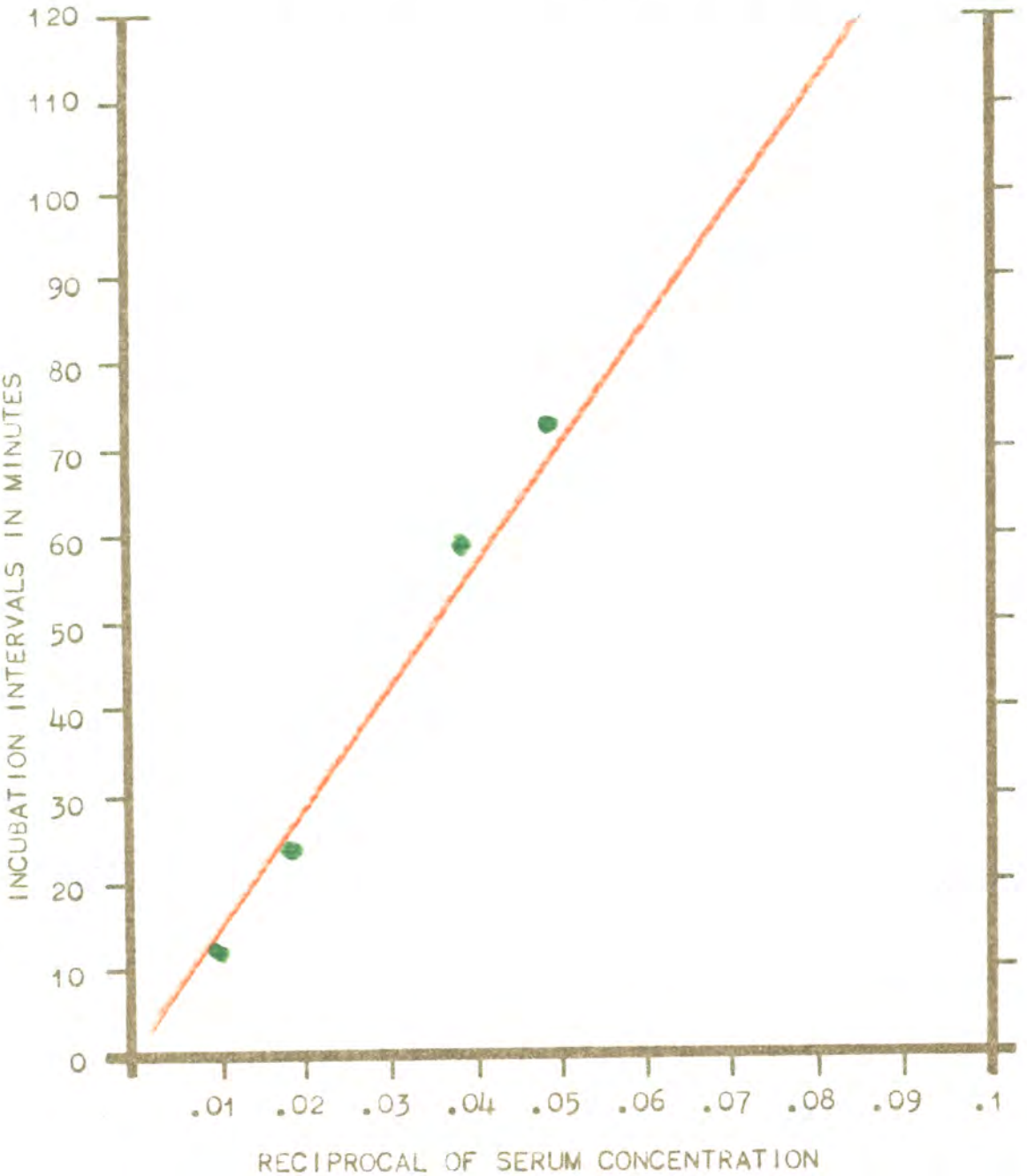


FIGURE 11: ASSAY OF FACTOR $\underline{\bar{X}}$

DRAWN FROM TABLE 50 PART (2) TEST 10

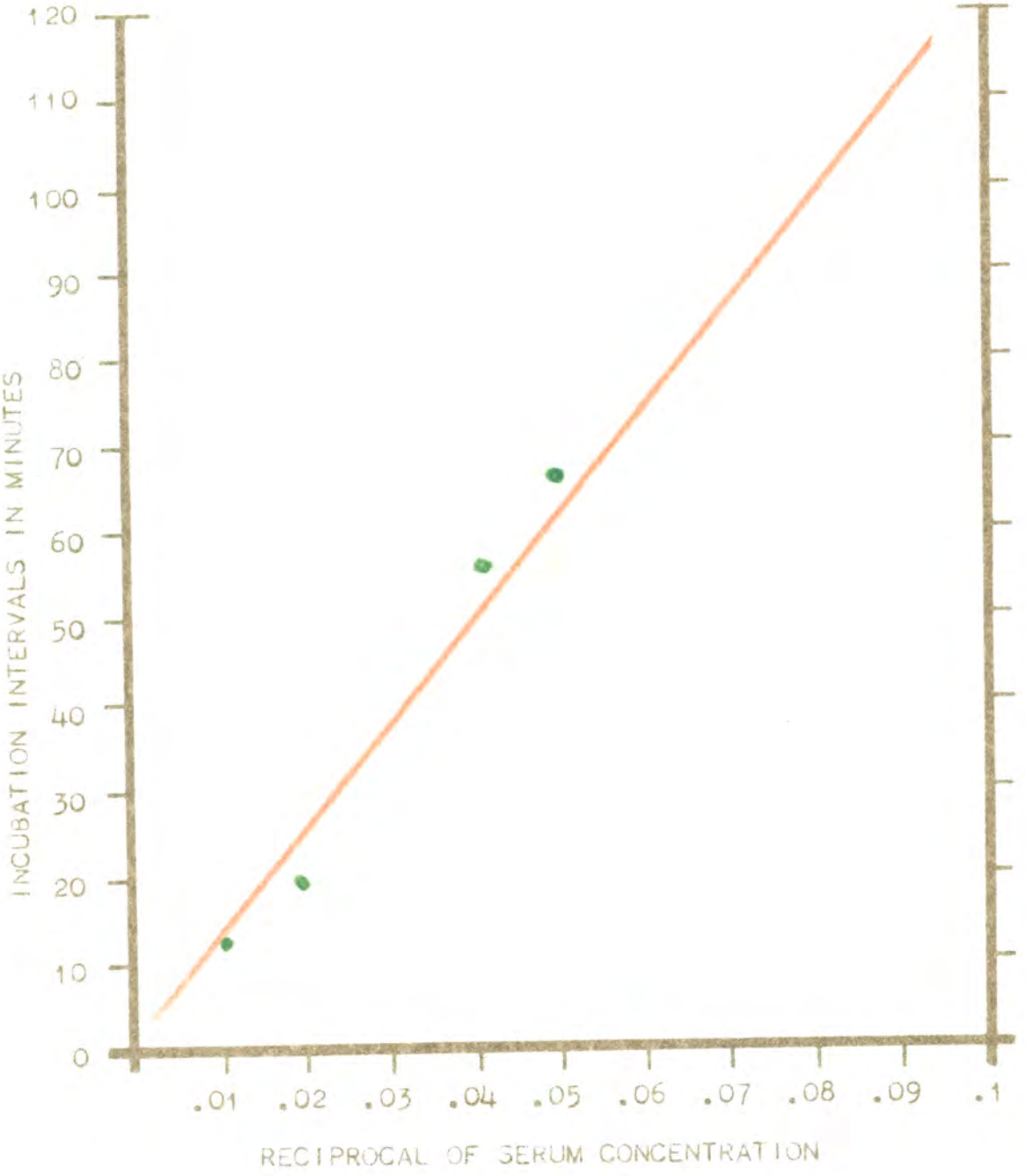


FIGURE 12: ASSAY OF FACTOR X
Drawn from Table 50, Part (3), Test 1.

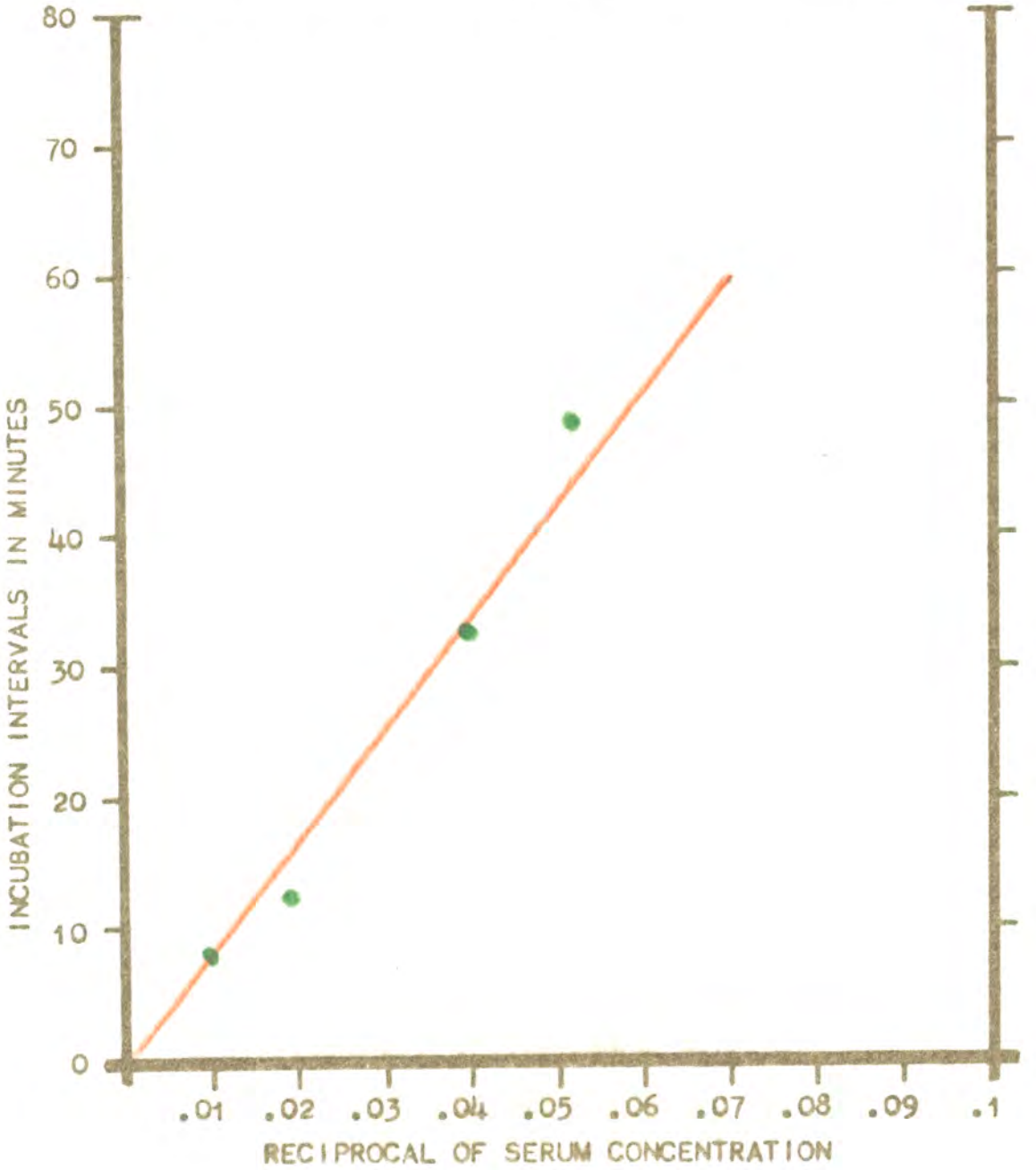


FIGURE 13: ASSAY OF FACTOR X
Drawn from Table 50, Part (3), Test 2.

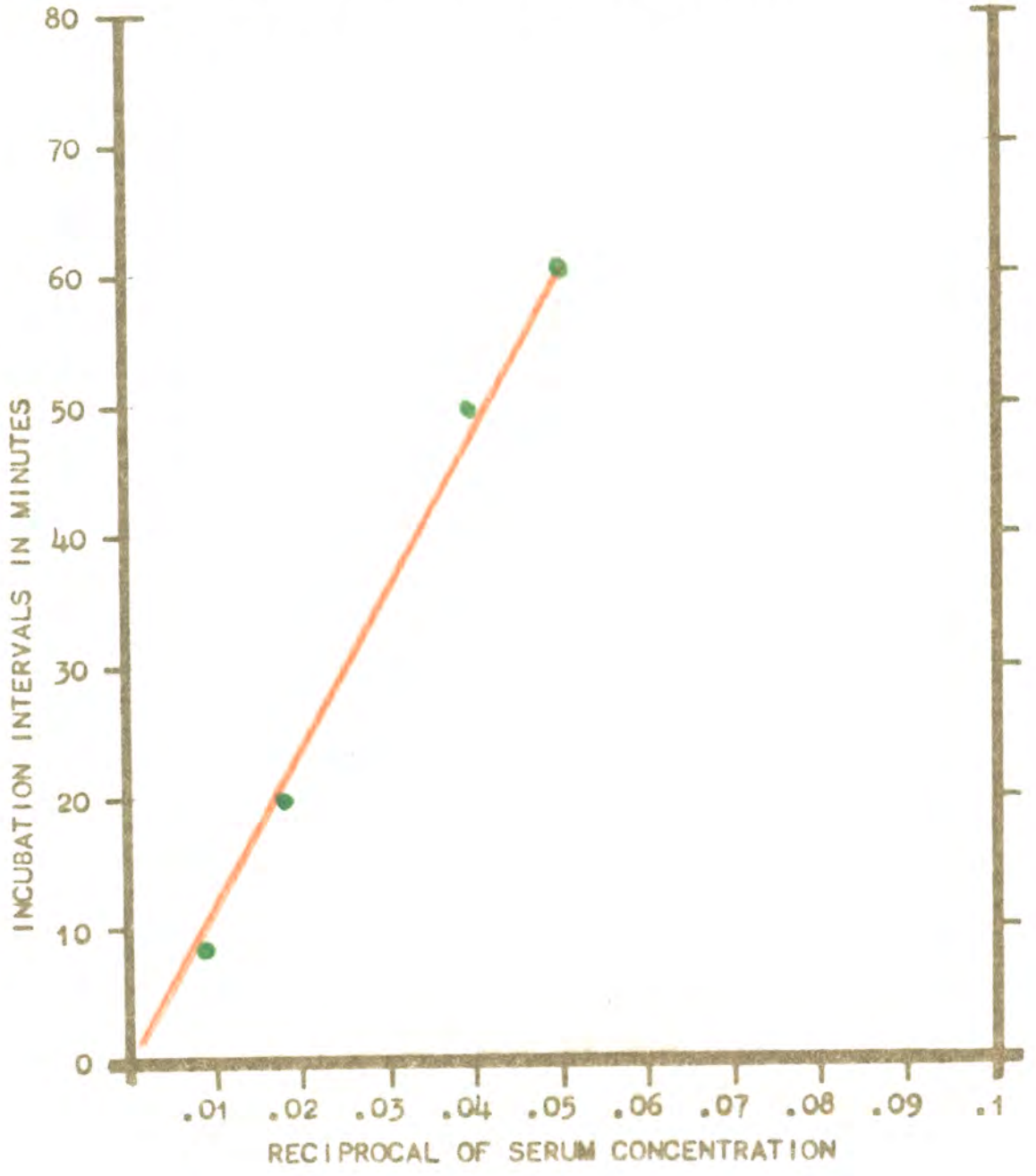


FIGURE 14: ASSAY OF FACTOR X
Drawn from Table 50, Part (3), Test 3.

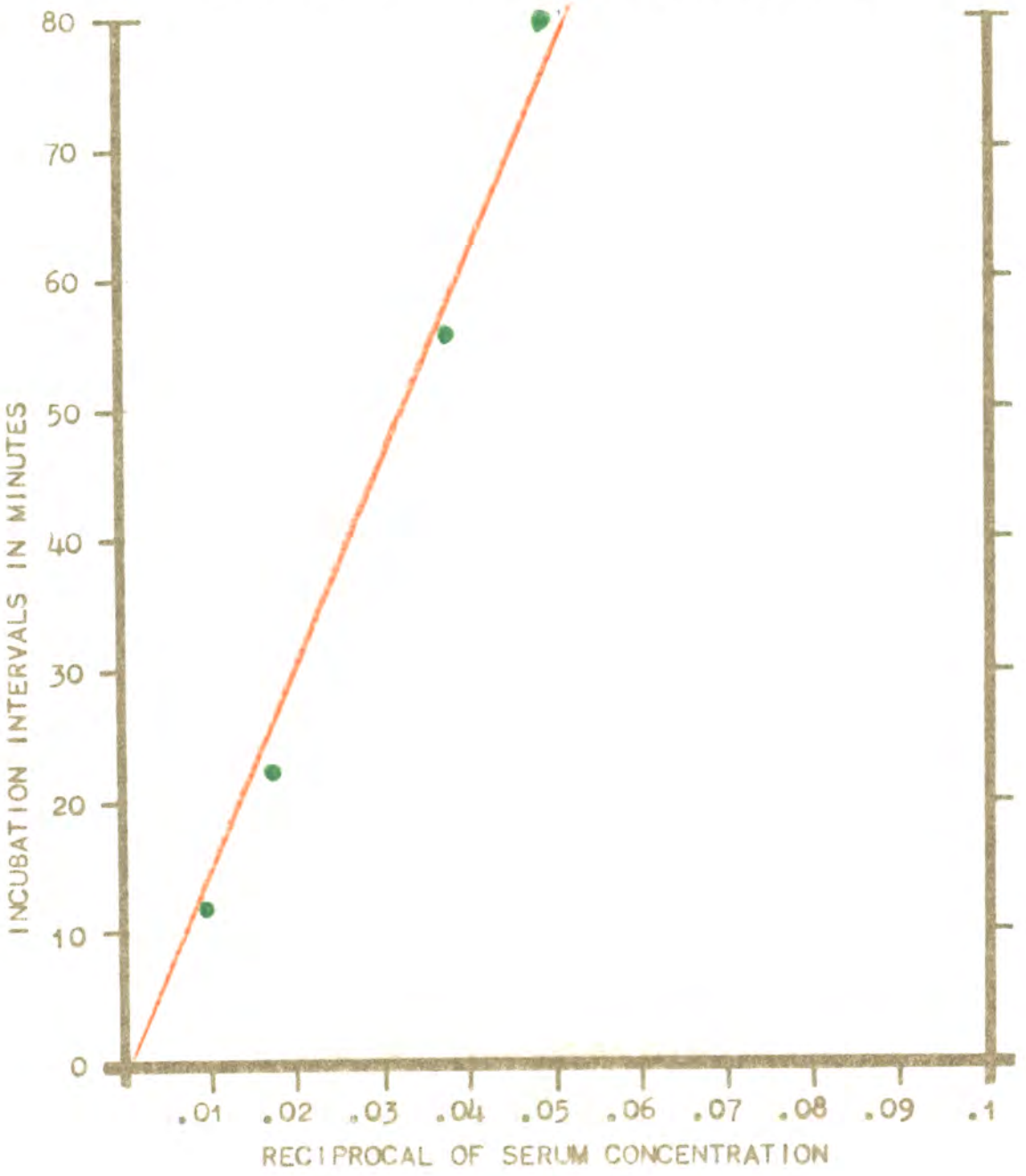


FIGURE 15: ASSAY OF FACTOR X
Drawn from Table 50, Part (4), Test 4.

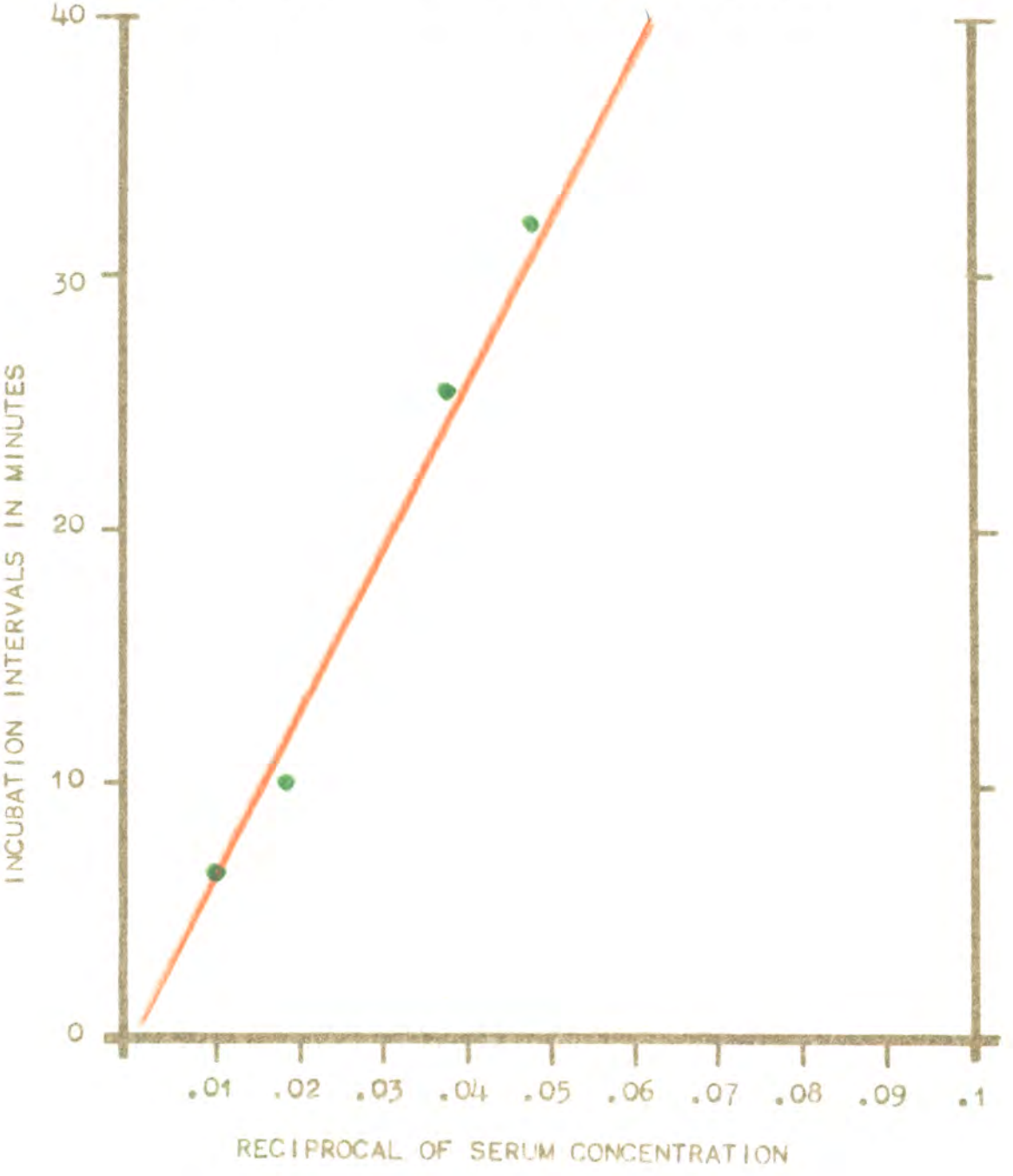


FIGURE 16: ASSAY OF FACTOR X
Drawn from Table 50, Part (4), Test 5.

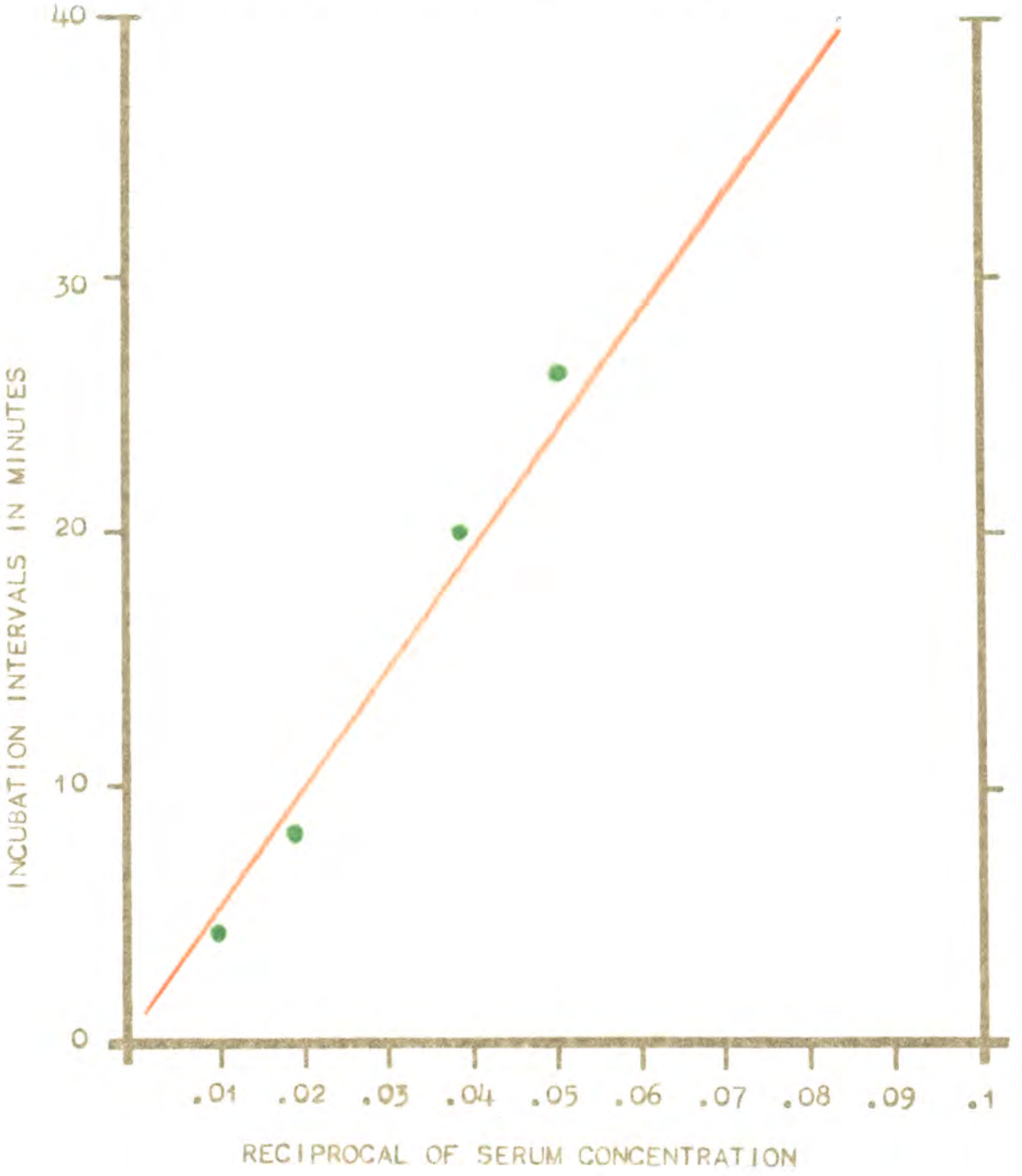


FIGURE 17: ASSAY OF FACTOR X
Drawn from Table 50, Part (4), Test 6.

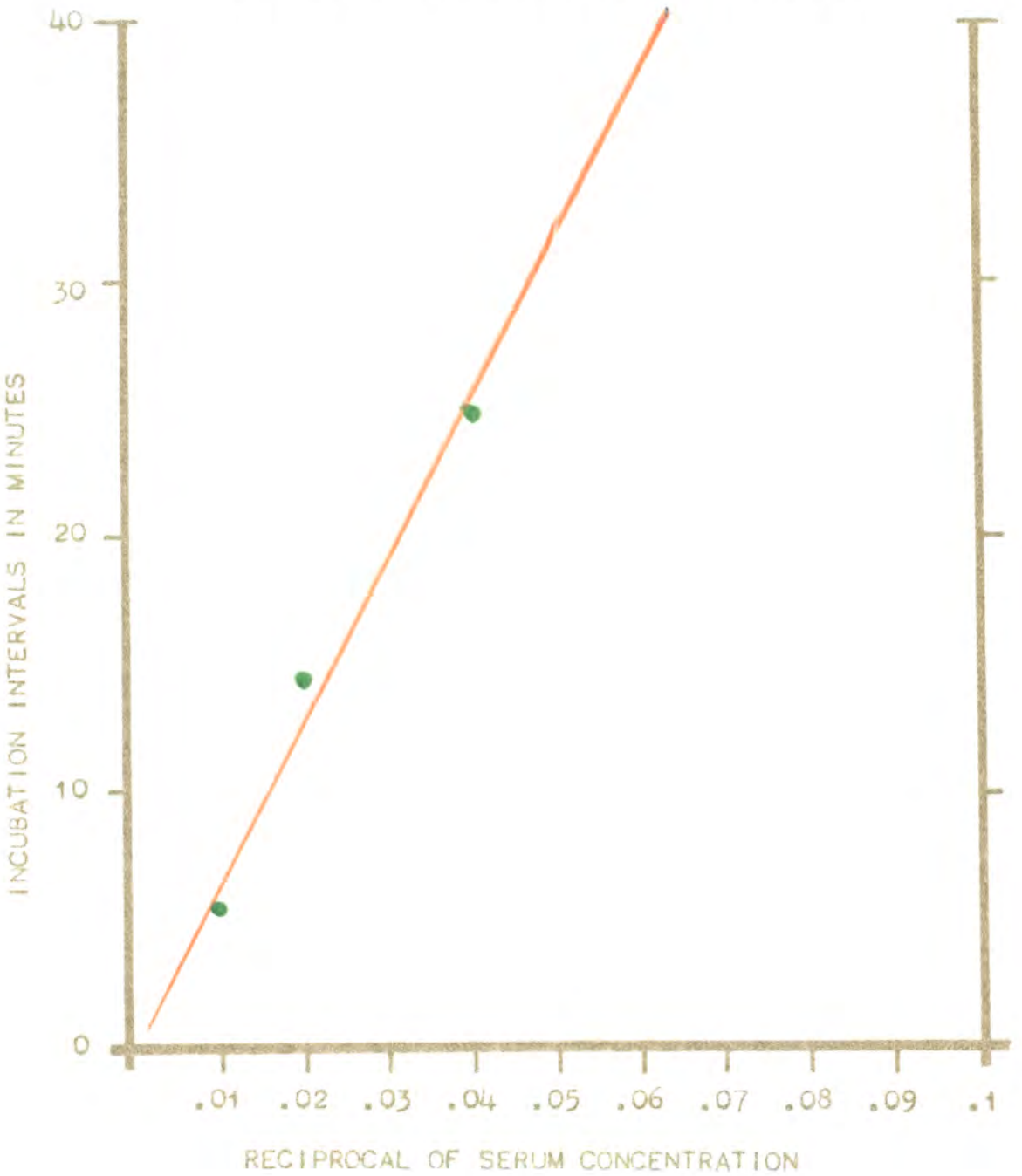


FIGURE 18: ASSAY OF FACTOR X
Drawn from Table 50, Part (4), Test 7.

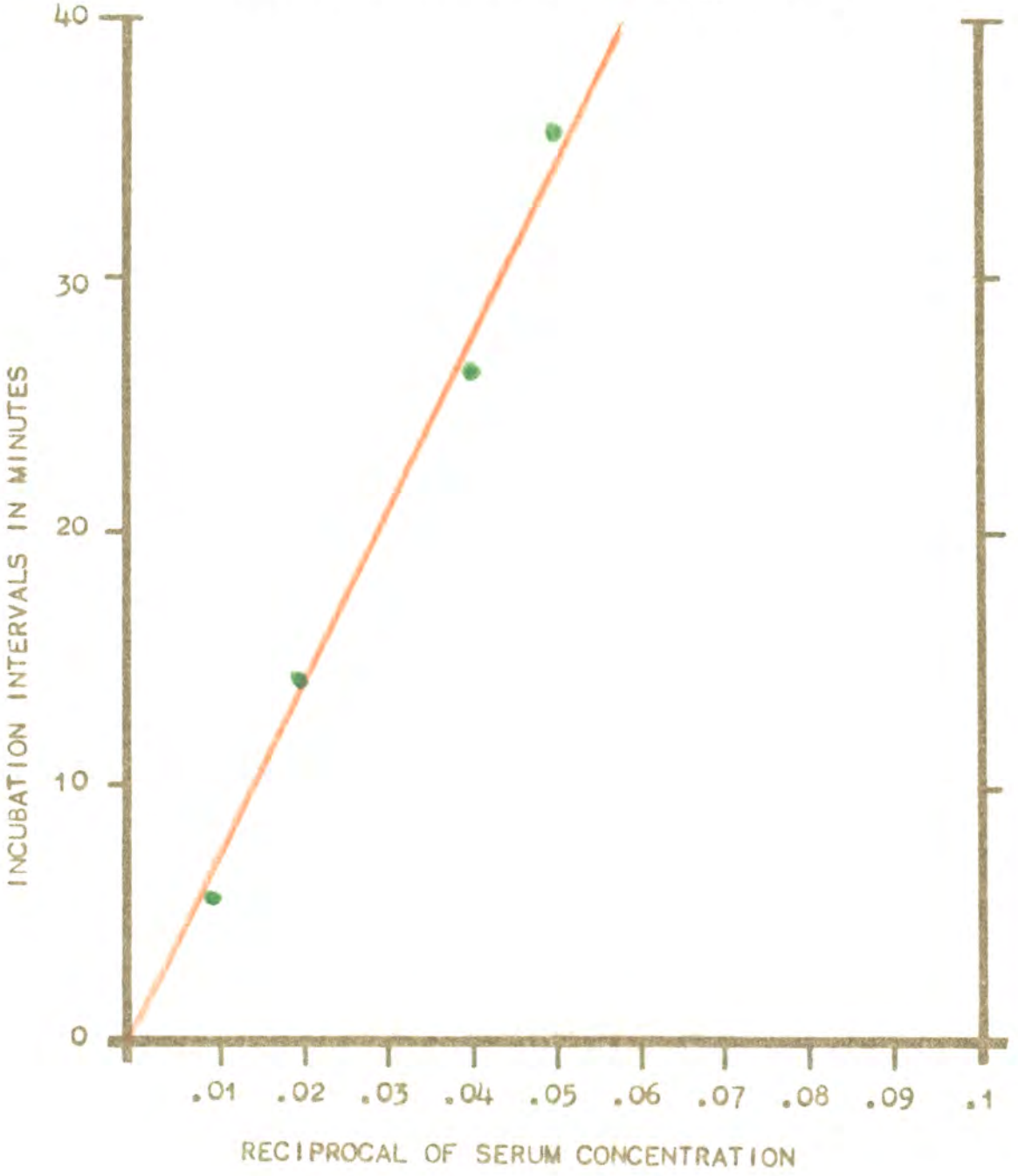
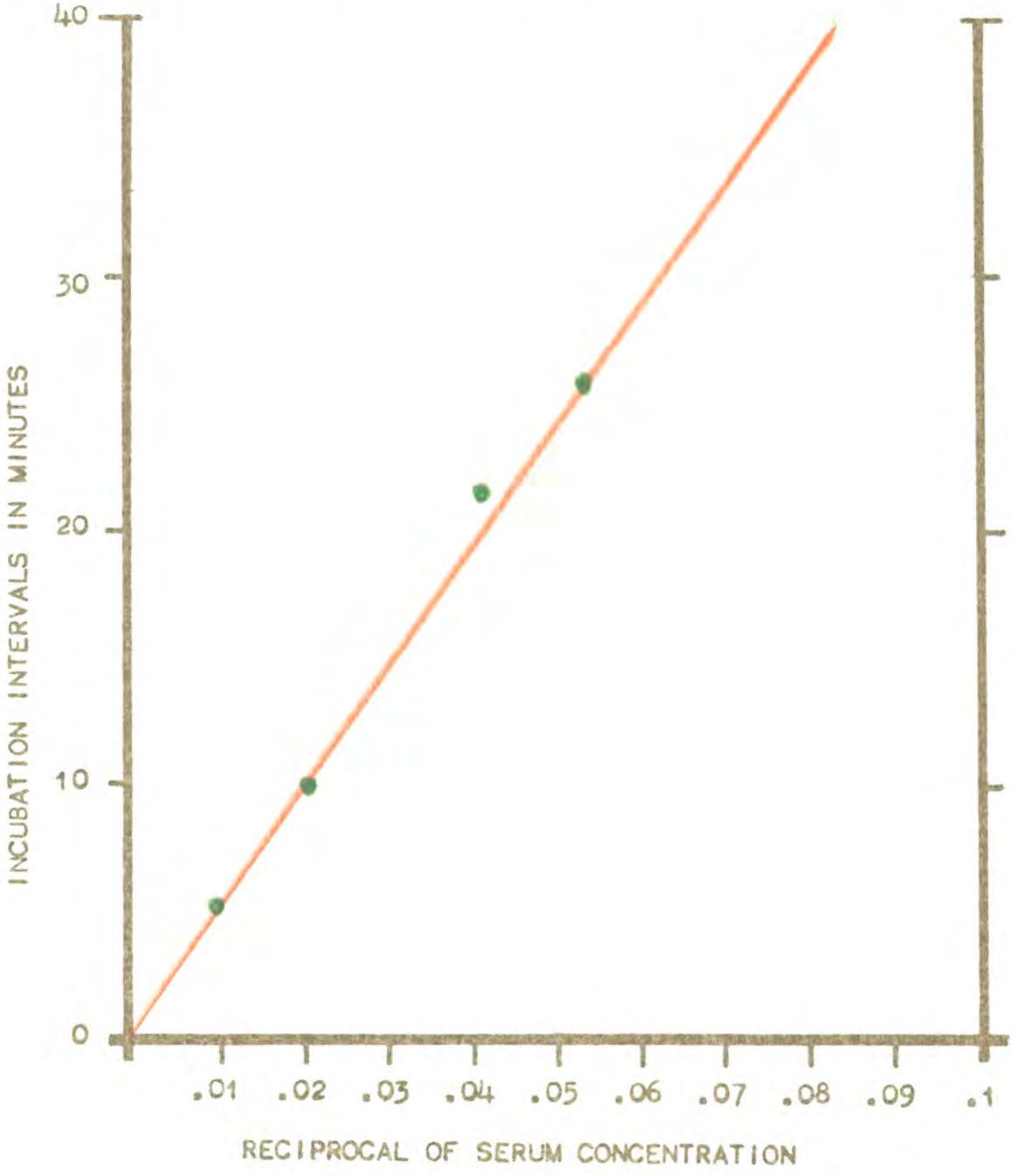


FIGURE 19: ASSAY OF FACTOR X
Drawn from Table 50, Part (4), Test 8.



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