BLOOD PLATELETS AND PROTEOLYTIC ENZYMES

by

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PREFACE.

The work presented in this thesis was carried out in the laboratory of Professor A.S. Douglas, in the University Department of Medicine, Glasgow Royal Infirmary. While performing this work I held the post of biochemist. I was directed in the experimental work for this thesis by Professor E.M. McGirr, Professor A.S. Douglas and Dr G.P. McNicol. Specific mention must be made of some portions of the work which I did not personally perform. Mrs S. Beaton and Miss H. Moss carried out the ultracentrifugation runs on the Spinco, Model E, Ultracentrifuge (chapter 9) and Dr A.H.C. Uttley examined one of the fibrinogen degradation products in the Chandler tube (chapter 10). Some of the work described in this thesis has been published, as described below, and some further portions are being prepared for publication:-

Wilson, P.A., McNicol, G.P., Douglas, A.S., (1966),

The effect of streptokinase and trypsin on platelet aggregation in vitro. Thrombos. Diathes haemorrh. (Stuttg.), Suppl. 20, 297.

Wilson, P.A., McNicol, G.P., Douglas, A.S., (1967),

Some effects of trypsin and streptokinase on platelet aggregation. Thrombos. Diathes haemorrh. (Stuttg.), In press.

CONTENTS

VOLUME 1

ABBREVIATIONS.

ADP, adenosine - di - phosphate

AMP, adenosine - mono - phosphate

DEAE cellulose, diethylaminoethyl cellulose.

REFERENCES.

Where more than one reference has been used the references are given in alphabetical order of the principal author.

CHAPTER 1

INTRODUCTION

Interest in platelets has been stimulated in the last two decades with the increased recognition of the importance of their physiological and pathological role. It is now well established that normal haemostasis, after vascular injury, is mainly achieved by the formation of a 'haemostatic plug', consisting of densely packed platelets, which seals the injured vessel, thus preventing excessive loss of blood. It has also been established that the formation of thrombi, leading to the occlusion of blood vessels, is, in large part due to the formation of platelet masses which slow the blood flow until fibrin formation occurs in the stagnant portion of the occluded vessel.

For twenty years anticoagulant drugs have been widely used in the treatment of thrombo-embolic disease; these drugs are administered with the intention of limiting fibrin deposition. These conventional anticoagulant drugs have been shown to interfere with platelet aggregation in vitro (Cunningham, McNicol and Douglas, 1965). More recently activators of the fibrinolytic enzyme system have become available for use in human therapeutics for the purpose of dissolving intravascular fibrin; the use of these agents is referred to as thrombolytic therapy. The present study was undertaken to investigate the relationships between the fibrinolytic enzyme system and certain aspects of platelet aggregation.

A number of standard in vitro techniques are available which enable quantitative assessment of the ability of platelets to clump. Three

such techniques have been modified to determine the effect of fibrinolytic agents on platelet function. The first method, using recalcified plasma, measures platelet aggregation, which precedes fibrin formation, in an artificial circulation (Chapter 4). Within the last ten years one chemical agent, adenosine-di-phosphate (ADP), has been identified which is currently thought to play a fundamental role in initiating platelet aggregation both *in vivo* and *in vitro*. Advantage has been taken of the fact that, with ADP, platelet aggregation may be induced in platelet rich plasma without subsequent fibrin formation and consequently without interference by coagulation reactions leading to clot formation (Chapter 5). The third method used in the present study enables platelet function to be assessed in the presence of red cells by measuring the ability of platelets to adhere to glass and to each other on passage of blood through a column of glass beads (Chapter 6).

There is still some doubt as to the specific aspect of platelet function which is measured by any given *in vitro* technique. Accordingly, an attempt was made to correlate the results obtained from the use of the three different techniques of measuring platelet aggregation (Chapter 7).

From the investigations of the effects of fibrinolytic agents on platelet function it was found that in vitro the enzymes could accelerate platelet aggregation (Chapters 4, 5 and 6) : the effects of the products of proteolysis of fibrinogen were investigated as the possible agents by which this effect

might be mediated. Fibrinogen proteolysis products were fractionated by column chromatography (Chapter 8), their physical and chemical characteristics were determined (Chapter 9) and their effect on platelet aggregation investigated (Chapter 10).

This thesis is presented in two volumes. The first contains the text and references and the second figures and tables. The text begins with an historical introduction followed by an account of some current concepts of the components and function of the platelet and an outline of possible mechanisms of platelet aggregation. An outline is also presented of some of the in vitro tests available to study platelet aggregation. There follows a description of the methods used in the work for this thesis and thereafter the presentation and discussion of the investigative work mentioned above. The figures are numbered in order of their introduction in the text and the tables are set out in two appendices. The first appendix contains independent tables in order as they are referred to in the text and the second appendix contains the tables from which the figures were drawn as indicated in the text and in the figure legends.

CHAPTER 2

PLATELETS AND HAEMOSTASIS

HISTORICAL INTRODUCTION

It was not until the middle of the nineteenth century that microscopists recognised corpuscles in blood which we know today as platelets. The first description of a platelet was reported by Donne in 1842 and was confirmed by Zimmerman in 1845 who also remarked on the tendency of the cells to clump together. During the course of the next fifty years a number of workers observed platelets in blood and gradually came to recognise their functional significance in haemostasis and thrombus formation. In 1865 Schultze reported that platelets exhibited a tendency to clump and to form granular masses. This observation was confirmed by Riess (1872) and extended by Osler (1874), who stated that the 'granular masses' of Schultze resulted from the agglutination of small bodies which occurred as single units in the circulation. The clumping phenomenon of platelets in vitro was also remarked upon by Vulpian (1873), who noted the presence in blood of colourless corpuscles which had the properties of sticking to and accumulating in clumps on a cover glass, and by Ranvier (1873), who observed microscopic changes in platelets and the formation of fibrin in a drop of shed blood. In 1882, Bizzozero, extending the work of Hayem (1877; 1878) recognised the platelet as a distinct element in blood within the vessels and indicated the part it plays in

thrombosis since he was able to show that white thrombi, which had previously been thought to consist solely of leukocytes (Zahn, 1875), consisted almost exclusively of platelets. The observations of Bizzozero were confirmed and extended by a number of authors, Howell (1884), Eberth and Schimmelbusch (1885) and Laker (1889). Eberth and Schimmelbusch (1885) made the important discovery that blood corpuscles run in the centre of the blood stream and that slowing or stasis of the circulation is followed by migration of leukocytes and platelets to the periphery of the stream. They also recognised that platelet changes occur during blood coagulation but, unlike Bizzozero (1882) who believed that platelet changes preceded blood coagulation, they believed the two processes were concurrent. Thus by the 1880's the function of the platelet and its importance in thrombus formation was well recognised. It was at this time that the importance of the role of platelet thrombi in the arrest of bleeding was proposed by Hayem (1882) and by Lubnitzky (1885). But it was not until 1912, however, that this role of the platelet in spontaneous haemostasis was convincingly demonstrated by Duke.

While these early workers recognised the functions of platelets in haemostasis and thrombus formation there was some confusion concerning the origin of these cells in blood. In 1865, Schultze had reported that platelets were formed from leukoctyes and this view was supported by Riess (1872).

Hayem (1878), however, believed that the platelets were precursors of

erythrocytes and it was not until the beginning of the twentieth century that Wright (1910) found the true origin of platelets from the megakaryocytes.

Thus, by the beginning of the nineteenth century, much of our present knowledge of the platelet and its haemostatic function had been documented. There was scant information concerning the platelet until the early 1940's when the advent of refined microscopic techniques stimulated a renewal of interest in the platelet. Since then, the platelet has been investigated at many biochemical levels and its structure, biochemical composition and function have been extensively documented.

The Platelet

Electronmicroscopy has enabled the ultrastructure of the platelet to be precisely defined and it has been shown to consist of osmophilic α granules, vacuoles and a few mitochondria randomly distributed within the platelet hyaloplasm which is enclosed within a unit cell membrane (Castaldi et al., 1962; Firkin et al., 1965; Movat et al., 1965; White et al., 1965). Closely associated with the surface of the platelet, in what has been referred to as the 'plasmatic atmosphere' of the platelet (Roskam, 1923) are many if not all the coagulation factors (Adelson et al., 1961; Iatridis and Ferguson, 1965). In addition to its ability to contribute to coagulation by the presence of a microcosm of coagulation factors associated with the platelet surface, the platelet can contribute coagulation factors which are normally contained within the cellular membrane. These factors include a

phospholipid which contributes to the interaction of plasma coagulation proteins in the formation of the intrinsic prothrombin activator (Ferguson, 1960; Horowitz and Marcus, 1964; Macfarlane, 1964; Marcus and Spaet, 1958; Marcus and Zucker, 1965a; Seegers, 1961) and a protein or high molecular weight polypeptide which has the ability to directly neutralize heparin (Deutsch and Kain, 1961; Godal, 1962; Marcus and Zucker, 1965b). Disrupted platelets can also exhibit antifibrinolytic activity (Alkjaersig, 1961; Johnson and Schneider, 1953). Another intracellular protein of the platelet which has been identified is one which can be clotted by thrombin and which exhibits a similar immunological pattern to that of fibrinogen (Castaldi and Caen, 1965; Gokcen and Yunis, 1963; Grette, 1962; Seligmann et al., 1957; Sokal, 1962; Ware et al., 1948). One of the striking features of platelet biochemistry is the very high energy store, in the form of adenosine-triphosphate (ATP) which this cell possesses (Born 1956a and 1958: Fantl and Ward, 1956). The capacity to act as a store of certain biologically active compounds appears to be one of the functions of the platelet since it has been shown that normally all the serotonin of the blood is contained within the platelet (Zucker, 1962). These platelet components all contribute, in their various ways, to the role of the platelet in haemostasis.

The functions of the platelet

In the last two decades, interest in the function of the platelet in haemostasis has been renewed. Progress has been made along two complementary

lines of investigation; *in vivo* techniques have been developed by, among others, Hughues (1962), Roskam (1961) and Zucker (1947), which have confirmed and extended the observations of the nineteenth century workers, and several useful *in vitro* techniques have been devised (Born, 1962a; Hellem, 1960; Salzman, 1963; Wright, 1941). Indeed, it has been from the application of these two modes of investigation that some understanding of how platelets clump and the mechanisms which enable them to exhibit this phenomenon has been obtained.

In figure 1 is shown an outline of the functions currently ascribed to platelets in haemostasis.

Platelets in thrombus formation. Outlined in figure 2 is a diagrammatic representation of the currently accepted reaction sequence of thrombus formation (Glynn et al., 1966; Hellem and Owren, 1964; Lüscher, 1967; Mustard et al., 1964a; Owren, 1966; Poole and French, 1961; Roskam, 1961; Spaet, 1964). The platelet is the first and most important formed element demonstrable at a break in the vascular lining, (French and Poole, 1963; Jorgensen and Borchgrevink, 1963; Kjaerheim and Hovig; 1962; Roskam, 1961; Zucker, 1947). The adhesion of platelets to subendothelial tissue appears initially to be independent of blood coagulation (Jorgensen and Borchgrevink, 1964; Mustard et al., 1964a) and is thought to be initiated by adenosine-di-phosphate (ADP) release since ADP may be liberated from damaged tissue (Born et al., 1964; Honour and Mitchell, 1963) and it has been shown to emerge from erythrocytes under certain

conditions (Gaarder et al., 1961; Hellem 1960; Käser-Glanzmann and Lüscher, 1962). Since Hovig and Holmsen (1963) have shown that ADP is released from platelets when they come into contact with collagen and that the ATP content of platelets diminishes during clotting (Born 1956b and 1958) or when they begin to lose their morphological integrity (Zucker and Borrelli, 1961) it is thought that a platelet plug develops round the platelets already adhering to the site of lesion, under the stimulus of ADP in the surrounding medium (Hellem and Owren, 1964; Mustard et al., 1964; Spaet, 1964). The platelets in the plug gradually lose their morphological integrity (Castaldi et al., 1962; Rosenthal and Vyas, 1961; Sharp, 1961) and ADP, serotonin and possibly phospholipid are released from the cells (Grette, 1962; Hardisty and Hutton, 1966; Mustard et al., 1964a; Spaet and Cintron, 1965). These platelet alterations are rapidly followed by fibrin formation round the platelet plug (Jorgensen and Borchgrevink, 1963; Kjaerheim and Hovig, 1962; Sharp, 1961) and a thrombus is thus formed. Platelet aggregation produced by ADP is reversible (Born, 1962b and 1965; Born and Cross, 1963b; Mitchell and Sharp, 1964) and consequently the initial stage of thrombus formation is thought to be reversible and it is not until platelets begin to lose their morphological integrity and the plug becomes impermeable to blood flow that the process becomes irreversible (Hellem and Owren, 1964).

Platelets in coagulation. The main function of platelets in coagulation, apart from supplying a microcosm of coagulation factors in their 'plasmatic atmosphere', is the release of a phospholipid, platelet factor 3, which acts

as a cofactor in the formation of blood thromboplastin (Macfarlane, 1964; Seegers et al., 1962). Unlike the role of the platelet in thrombus formation, the platelet contribution to coagulation is made at a relatively late stage in the reaction sequence and platelet clumping is not an essential step in the reaction sequence since traces of thrombin are sufficient to release the phospholipid to the coagulation system (Macfarlane, 1966; Marcus and Zucker, 1965a).

Platelets and vasoconstriction. The blood serotonin, which is transported in the platelets, may be released from these cells by the action of thrombin (Grette, 1962) or by contact with connective tissue particles (Spaet and Zucker, 1964). This process may facilitate haemostasis by stimulating contraction of the vessel wall.

Platelets and clot retraction. The way in which platelets organise a fibrin clot has been studied extensively (Marcus and Zucker, 1965c) and has been demonstrated to be dependent on the presence of a contracile protein, thrombosthenin, within the platelet (Bettex-Galland and Lüscher, 1961; Grette 1962). Thus platelets are able to contribute to the maintenance of vascular patency.

Capillary integrity. Platelets have been demonstrated to play an essential role in the maintenance of vascular integrity but their precise function in this process has not been firmly established (Marcus and Zucker 1965d).

Although the role of the platelet in haemostasis is now well established and some insight into the manner in which platelets become

organized within a thrombus has been achieved, the precise mechanism involved in the clumping of platelets has not as yet been fully elucidated.

Mechanism of platelet aggregation

In 1962 it was proposed that the adenosine nucleotide, ADP, might be the compound responsible for the formation of haemostatic plugs (Born, 1962b). This possibility was suggested by the discovery of a factor, present in erythrocytes, which could increase platelet adhesiveness (Hellem, 1960), by the subsequent identification of this factor as ADP (Gaarder et al., 1961) and by the demonstration that the ATP content of platelets was diminished during clotting (Born, 1956b) and as platelets became fused during the formation of a platelet plug (Zucker and Borrelli, 1961). The ability of ADP to induce platelet aggregation both in vivo (Born and Cross 1963c; Davey and Landler, 1964; Nordoy and Chandler, 1964; Regoli and Clark, 1963) and in vitro (Born, 1962b; Hellem et al., 1963; Mitchell and Sharp, 1964; O'Brien, 1962a; Ollgaard, 1961; Zucker and Borrelli, 1962) is now firmly established. Furthermore, it has been shown that aggregation by ADP is inhibited by AMP, adenosine and 2-chloro-adenosine and that this inhibition is competitive and highly specific (Born, 1964; Born and Cross, 1963a; Born et al., 1965; Clayton et al., 1963; Skalhegg et al., 1964). The inhibition of ADP induced aggregation by adenosine has also been demonstrated to operate in vivo (Regoli and Clark, 1963). In addition, adenosine can inhibit aggregation brought about by other agents; eg, the actions of thrombin,

serotonin and adrenaline can be blocked by adenosine (O'Brien, 1964). These observations support the hypothesis that ADP plays a key role in the formation of platelet aggregates and implies that aggregation brought about by substances other than ADP is mediated by ADP. Evidence to substantiate this view has been found by Haslam (1964) who has shown that, in the presence of an enzyme system which catalyses the phosphorylation of ADP to ATP, neither thrombin nor fatty acids are able to induce platelet aggregation.

Platelet aggregation is dependent on viable platelets, calcium (Born and Cross, 1963b; Hellem 1964; Mitchell and Sharp, 1964) and on the presence of a plasma factor (Born and Cross, 1964). The plasma factor has characteristics in common with fibrinogen and with the factor deficient in the plasma of patients with Von Willebrand's disease (Born and Cross, 1964; McLean et al., 1964; Skalhegg et al., 1964). These various factors involved in platelet aggregation have led to the development of two hypothetical models for the mechanisms of platelet aggregation. The first hypothesis is based on the contention that ADP participates in a binding reaction, by means of hydrogen bonds, through the 6-amino group of the nucleotide, to a specific receptor molecule on the surface of the platelet and that calcium ions and the plasma factor are necessary for forming bridges between the negatively charged phosphate groups of two molecules of ADP (Born, 1965; Clayton et al., 1963; Gaarder and Laland,

1964; Skalhegg et al., 1964). This suggestion has been extended by Gaarder and Laland (1964) who have pointed out that all the nucleotides so far known to bring about aggregation have, at physiological pH, an uneven number of negative charges, whereas the inhibitors have no charge at all or an even number of charges. Because of the double charge of the calcium ion, nucleotides with an uneven number of negative charges would, when attached to the platelets, provide more opportunities for calcium ion bridges between the nucleotides. The second hypothesis stems from recent studies with radioactive labelled ADP which have shown that ADP induced platelet aggregation is accompanied by dephosphorylation of the nucleotide (Kerby, 1966; Salzman et al., 1966). It has been suggested that this process releases energy, necessary for unidentified binding reactions and that the protein factor is involved in a secondary bridging phenomenon (Salzman et al., 1966; Spaet and Lejnieks, 1966). According to this scheme, platelet clumping by ADP is a metabolic reaction dependent on energy transfer.

Thus, although the precise mechanism of platelet aggregation has not been fully elucidated the importance of the phenomenon in coagulation is well recognised.

Platelets and Fibrinolysis.

Platelets have been shown to possess antiplasmin activity (Alkjaersig, 1961; Holemans and Gross, 1961; Johnson and Schneider, 1953;

Stefanini and Murphy, 1956) and indeed, it has been demonstrated that intact platelets may concentrate the protein,antiplasmin, from plasma (Stefanini and Murphy, 1956). However, platelets have also been shown to possess a protein which exhibits proactivator activity (Holemans and Gross, 1961; Lewis et al., 1962) and which has been identified in washed platelets, by immunological methods, with plasminogen (Nachman, 1966), and it would appear that platelets possess enzymes whose activity can either inhibit fibrinolysis or enhance the process.

One hypothesis proposed to account for the maintenance of vascular integrity suggests that a dynamic balance between coagulation and fibrinolysis exists within the vascular tree (Astrup, 1958; Flute, 1965; Lüscher, 1967; Sherry et al., 1959). Fibrinolytic agents have been demonstrated to be capable of dissolving thrombi both in vivo (Douglas and McNicol, 1964) and in vitro (Flute, 1965; McNicol, et al., 1965). It has been shown, however, that in the process of destruction of a thrombus by the digestion of fibrin, platelet emboli are released which appear to be resistant to further fibrinolytic activity (McNicol et al., 1965). Platelets may play a role in maintaining the dynamic balance between fibrinolysis and coagulation by influencing the rate of fibrinolysis although there is still some controversy as to whether the presence of platelets inhibits or enhances the lysis of plasma clots formed by thrombin (Alkjaersig, 1961;

Bickford and Taylor 1963).

Fibrinolytic Agents.

Streptokinase. Streptokinase is present in culture filtrates of certain strains of haemolytic streptococci. The enzyme first acts stoichiometrically with blood proactivator (Troll and Sherry, 1955). The activator so formed may then convert plasminogen to plasmin (Alkjaersig et al., 1958).

Urokinase. Urokinase is the physiological activator of plasminogen which has been isolated from human urine (Ploug and Kjeldgaard, 1957; Sobel et al., 1952). The activation of plasminogen by urokinase has been shown to be an enzymatic reaction (Alkjaersig et al., 1958; Kjeldgaard and Ploug, 1957).

Plasmin is the proteolytic enzyme which can be produced from its inactive precursor, plasminogen, by various activation procedures (Sherry et al., 1959) which can hydrolyse arginine-lysine linkages (Sherry et al., 1959). In vitro the enzyme has been shown to have equal affinity for fibrinogen and fibrin (Ratnoff and Colopy 1953). Other plasma proteins susceptible to digestion by plasmin are prothrombin (Alagille and Soulier, 1956), factor V (Greenwalt and Triantaphyllopoulos, 1954) and antihaemophilic globulin (Lewis et al., 1949). Platelets have also been shown to be a substrate for plasmin activity. Unlike trypsin, plasmin does not remove clottable protein from the platelet or cause release of serotonin and ATP (Morse et al., 1965). The enzyme does appear to remove clottable protein from the platelet surface, however, since plasmin treated platelets

cannot be aggregated by thrombin (Morse et al., 1965). Platelet factor 4, the factor which exhibits antiheparin activity (Marcus and Zucker, 1965b), can be inactivated by plasmin, (Niewiarowski et al., 1963).

Trypsin. Trypsin is an enzyme present in pancreatic juice which can hydrolyse peptide linkages involving the carboxyl groups of the basic amino acids lysine and arginine (Dixon and Webb 1960a). This enzyme can digest the plasma proteins fibrinogen (Mihalyi and Godfrey, 1963a) and fibrin (Fletcher et al., 1962) and it has been shown to activate plasminogen in vitro (Alkjaersig et al., 1958). In addition to its fibrinogenolytic and fibrinolytic activities, trypsin may enhance blood coagulation by participating in the conversion of prothrombin to thrombin (Ferguson et al., 1960; Stormorken, 1956; Yin, 1964). Furthermore low concentrations of the enzyme have been shown to promote platelet aggregation (Haslam, 1964). It has been shown, however, that high concentrations of trypsin abolish the ability of platelets to aggregate by thrombin (Schmid et al., 1962) or by ADP (Haslam, 1964). This may be accounted for by the observations that platelets or factors associated with platelets, may act as substrates for the proteolytic activity of the enzyme. Trypsin can remove clottable protein from the platelet (Morse et al., 1965) and, like thrombin, can cause release of serotonin and ADP from platelets (Grette, 1962; Morse et al., 1965). Platelet factor 1 (adsorbed plasma factor V) can be destroyed by the enzyme (Hjort et al.,

1955) which can also inactivate platelet factor 4 (Deutsch and Kain, 1961).

Chymotrypsin. Chymotrypsin, like trypsin, is present in pancreatic juice and can hydrolyse peptide linkages but, unlike trypsin, its preferred linkages are those involving aromatic amino acid residues (Dixon and Webb 1960a). Its direct action on the platelet appears to be similar to that of plasmin in that it does not remove clottable protein from the platelet or cause the release of serotonin and ATP (Morse et al., 1965).

Ficin. Ficin is an enzyme obtained from fig latex which can, like trypsin and plasmin, hydrolyse arginine-lysine linkages (Dixon and Webb 1960b).

As has been mentioned, the work for this thesis has involved the investigation of the effect of fibrinolytic agents and the effect of products of their fibrinogenolytic activity on platelet aggregation in vitro. The two agents principally investigated were streptokinase and trypsin. As discussed above streptokinase is a cofactor involved in the conversion of plasminogen to plasmin which is the naturally occurring proteolytic enzyme of the fibrinolytic system. Plasmin is difficult to prepare in a form free from contaminating plasma proteins. Consequently, trypsin was selected as a suitable substitute since in vitro it exhibits similar substrate specificity to plasmin and it has the advantage of being commercially available in a purified form.

CHAPTER 3

MATERIALS AND METHODS

In this chapter is presented an account of the materials and methods used in the work for this thesis. Experiments designed to clarify some detail of the work in a particular chapter are described in the relevant chapter. A brief outline of the basis of each method and a short discussion of some of the possible sources of error in interpretation of results are discussed for the major assay systems.

Standard methods are described for the assessment of platelet aggregation and platelet adhesiveness. These include a technique for the estimation of the ability of platelet clumps to form in a recalcified plasma system, a turbidimetric method for the detection of platelet clumps in plasma on the addition of a stimulant of platelet aggregation and a method which can utilize whole blood for the estimation of platelet adhesiveness. A short outline of the historical background to these techniques is also presented.

A method is described for the preparation of proteolysis products of the plasma protein, fibrinogen. Various methods used for the characterization of the fibrinogen degradation products are outlined, namely, ultracentrifugation, electrophoresis and reaction kinetic studies. Mention is also made of a method for the detection of defective fibrin polymerization caused by some of these products.

The biochemical assay techniques used to estimate the plasminogen and fibrinogen content of plasma and the concentration of streptococcal antibodies present in the plasma are presented. Other biochemical methods discussed include a method for the estimation of protein content of a sample and an enzymatic assay for the estimation of adenosine - 5' - diphosphate and adenosine - 5' - monophosphate in blood and plasma.

Some details of the methods of collection and handling of blood specimens used during the work for this thesis are described.

Measurement of platelet aggregation in vitro.

There are a number of terms which are currently in use to describe various stages in the process of platelet clumping in thrombus formation. In this thesis, the term platelet adhesion refers to the initial stage in haemostasis, in which platelets adhere to damaged endothelium and which is mimicked *in vitro* by the interaction between platelets and a foreign surface. The process of platelet to platelet interaction or cohesion of platelets which occurs during the enlargement of the platelet plug is referred to as platelet aggregation. The term viscous metamorphosis is used to describe the stage in the sequence of reactions leading to thrombus formation, which precedes fibrin formation, when platelet aggregation is irreversible and the platelets have fused to form an impermeable plug.

Platelet adhesion. One of the first successful methods for measuring platelet adhesiveness *in vitro* was devised by Wright in 1941 and was dependent on the ability of platelets to adhere to a glass surface. Anticoagulated blood was rotated in a glass chamber and serial platelet counts performed at various time intervals. The percentage of platelets lost during the rotation process was assessed from the difference between the platelet counts before and after rotation. The principle of this method was applied to a more rapid test by Moolten and Vroman (1949). Anticoagulated blood was passed through a length of glass wool, the residual blood removed with saline and platelet counts performed on the blood before and after passage through the filter. Hellem (1960) also used this principle to develop an elegant method of measuring platelet adhesiveness by performing direct counts on anticoagulated blood before and after it passed through a column of glass beads. A number of modifications of this technique have been devised (Hirsh et al., 1966a; O'Brien, 1961; Salzman, 1963) and one of these (Hirsh et al., 1966a) is outlined in this chapter and discussed in chapter 6.

Platelet aggregation. By comparing the adhesiveness of whole blood and platelet rich plasma, Hellem (1960) demonstrated that a factor, present in erythrocytes, was essential for platelet adhesion to glass beads. The subsequent identification of this factor as ADP has led to the development of a method of estimating platelet aggregation in

platelet rich plasma (Born, 1962a; Cuthbertson and Mills, 1963a; O'Brien, 1962b). The method depends on the observation that platelet aggregation may be induced when ADP is added to platelet rich plasma which is being stirred (Born, 1962a). The progress of platelet aggregation can be followed quantitatively by recording changes in light transmission through the plasma sample as the platelet clumps form (Born, 1962a). This method is outlined in this chapter and described more fully in Chapter 5. Platelet aggregation in platelet rich plasma can also be measured using an artificial circulation system (Cunningham et al., 1965). This method depends on the ability of platelets to clump together to form macroscopic aggregates before fibrin formation occurs and a thrombus forms as recalcified platelet rich plasma is rotated in a loop. The method is described in this chapter and in Chapter 4.

Materials.

Adenosine - 5' - diphosphate was used in the form of the sodium salt as supplied by the Sigma Chemical Company, St. Louis. This compound was prepared at regular intervals by dissolving the salt in a barbitone - saline buffer, pH 7.2 and was stored at - 20°C in small amounts. Each aliquot was used once only.

Barbitone - Saline buffer. The barbitone - saline buffer was made up as follows:- 0.1 M barbitone sodium, 57 ml; 0.1 N HCl, 43 ml; sodium

chloride, 0.56 g; 0.9 per cent sodium chloride to 200 ml.

Fibrinogen. Grade L (human, lyophilized) fibrinogen was obtained from A.B. Kabi, Stockholm. It was stored in the powdered form at - 20°C and was reconstituted in 0.1 M phosphate buffer, pH 7.6 immediately before use.

Phosphate buffer. The phosphate buffer of 0.1 molar, pH 7.6 was made up as follows:- NaH_2PO_4, 2.028 g; Na_2HPO_4, 14.964 g; NaCl, 9.0 g; distilled water to 1,000 ml.

Streptokinase was used in the purified form, Kabikinase, supplied by A.B. Kabi, Stockholm. The enzyme was reconstituted with 0.9 per cent sodium chloride to give a solution containing 10,000 N.I.H. units/ml which was then divided into small amounts and stored at - 20°C. A fresh aliquot was used for each assay.

Trypsin. Trypsin was used as the preparation of twice recrystallized, salt free powder supplied by Koch-Light Laboratories Ltd. The enzyme was dissolved in 0.9 per cent sodium chloride to give solutions of the required concentrations of protein per ml solution. The enzyme was stored at 4°C.

Soybean Trypsin Inhibitor. Type 1 - S, was obtained from the Sigma Chemical Company, St. Louis. The powder was dissolved in 0.9 per cent sodium chloride and was used at concentrations comparable to those of the various trypsin solutions studied. The enzyme was stored at 4°C.

Adenosine was obtained from British Drug Houses Ltd. The compound was

dissolved in distilled water as required and the solutions stored at 4°C.

Thrombin was used in the form of Thrombin Topical (Parke, Davis and Co., Detroit). A stock solution of concentration 100 N.I.H. units per ml was stored in 50 per cent glycerol - saline at - 20°C and diluted in saline to the required strengths.

Carbonate Buffer. The carbonate buffer of 0.1 molar, pH 8.9 was made up as follows:- $Na_2 CO_3$, 10.599 g; distilled water to 1,000 ml; pH adjusted to 8.9 with CO_2. A fresh buffer solution was made up for each experiment.

Casein solution was prepared as described by McNicol and Douglas (1964c). 25 g of casein (L. Light and Co., Ltd.) were boiled for 20 minutes in 500 ml, 0.1 M phosphate buffer, pH 7.6 while still hot. The solution was then dialysed for 24 hours at 4°C with constant stirring against 4 litres of 0.1 M phosphate buffer, pH 7.6. The dialysate was changed once. The casein solution was stored at 4°C.

Tubing. Portex transparent vinyl tubing NT/13 and NT/17 was supplied by Portland Plastics Ltd., Kent. Esco (Rubber) Ltd.,(London) supplied translucent silicone rubber tubing.

Radioactive Iodine. $^{131}I_2$ was supplied by the Radiochemical Centre, Amersham, England.

Tagged Fibrinogen was prepared with human fibrinogen and $^{131}I_2$ by the

method of Clement and McNicol (1959) as described by McNicol and Douglas (1964b).

Radioactive iodine ($^{131}I_2$) was prepared from 1 mcu carrier-free radioactive iodine (as NaI) in 0.1 ml dilute sodium thiosulphate solution by the addition of one drop of $^{127}I_2$ in methanol and one drop of 0.01N H_2SO_4. One drop of heparin (25 units/ml) was added to the radioactive iodine followed by 5 ml of human fibrinogen in citrate saline solution (4mg/ml). The fibrinogen iodine mixture was then passed through a Cl phase resin column (Amberlite IRA 400 Cl) with 5 ml, 0.1 M phosphate buffer, pH 7.6. The fibrinogen was stored in small aliquots at -20°C.

Methods.

(a) Estimation of platelet aggregation and adhesiveness.

Chandler Tube technique. This method is based on the observation that, when whole blood is made to flow round a closed plastic loop, a thrombus forms which exhibits the same histological features as a thrombus formed in vivo (Chandler, 1958). The histological appearance of a Chandler thrombus is shown in figures 3 and 4. When platelet rich plasma replaces whole blood in this system, in the presence of a physiological concentration of calcium ions, a 'snowstorm' of platelet aggregates becomes visible before the appearance of fibrin strands and the formation of a white thrombus (Cunningham et al., 1965).

The method used was a modification of that described by Cunningham et al., (1965). The transparent vinyl tubing, 12.3 mm bore, 71 cm in length was made into a loop by means of a nylon adaptor. The loop was washed in cold water and rinsed in 0.9 per cent NaCl. 6 ml of platelet rich plasma was added to the loop and the volume made up to 15 ml with 0.9 per cent NaCl. The system was then recalcified with one-tenth plasma volume, i.e. 0.6 ml, 0.25 M $CaCl_2$, and the loop and contents rotated on the turntable of a blood cell suspension mixer, (Matburn Ltd., London), rotating at 28.5 rpm in a glass fronted incubator at 37°C. Platelet aggregation was assessed by the length of time taken for the 'snowstorm' of platelet aggregates to appear after recalcification.

The method was designed to resemble as closely as possible the in vivo conditions for blood coagulation. Unfortunately, the method fails to achieve this ideal situation. The flow round the loop is laminar whereas, in vivo blood flow is turbulent in nature. The divergence from in vivo conditions is further increased by the use of platelet rich plasma. During centrifugation, it has been shown that some of the platelets most susceptible to aggregation appear to be lost (Stormorken et al., 1965). Consequently the platelet rich plasma sample may not contain the full platelet population of the initial sample. In addition, the red cells contain adenosine-di-phosphate (ADP) which is a potent initiator of platelet aggregation (Gaarder et al., 1961). Any hemolysis of the red cells which

may occur during the process of centrifugation will release ADP into the system. This may cause the aggregation and consequent loss of a number of platelets from the plasma sample.

The Chandler tube method involves the estimation of platelet aggregation in a recalcified plasma system in which platelet clumping is followed by fibrin formation. It may be assumed, therefore, that this method is assessing the first visible stage, but relatively late stage of the haemostatic process and that any alteration in the rate of formation of the platelet aggregates may indicate either, a true change in platelet aggregation ability or, may reflect some change in an earlier stage of haemostasis.

The limitations inherent in this technique have to some extent been overcome or minimized by the limited use and rigid standardisation of the method, (Chapt. 4). Each plasma sample examined was divided into two equal portions. One portion was treated with the compound under investigation and the second portion with saline so that the dilution of both the test and control plasma samples was the same. The plasma samples were added to identical Chandler tubes, recalcified simultaneously and rotated together on the mixer. In this way it was possible to compare the effect of the compound on the rate of formation of platelet aggregates with that for an identical control sample.

Turbidimetric Method. (Born, 1962 a, b; Born and Cross, 1963b; O'Brien,

1962b). The basis of this method for the assay of platelet aggregation was the observation (Born, 1962a) that platelets can be induced, by chemical stimulants, to form platelet aggregates in a platelet rich plasma system when the plasma is kept in motion. The apparatus used in this study consisted of an EEL titrator connected to a galvanometer (EEL type 20, Evans Electroselenium Ltd., England). A perspex cuvette was fitted onto the titrator above a magnetic stirrer and in the light path from the photoelectric cell. 2 ml, platelet rich plasma were added to the cuvette together with a small stirring rod. Once the stirring rod was rotating at a uniform speed, the optical density reading of the plasma sample on the galvanometer scale was adjusted to an arbitrary value of 0.600. As platelet aggregation occurs in the plasma, the light transmission through the sample is increased and is recorded as a fall in optical density on the galvanometer scale. Before the addition of the platelet aggregation inducing agent, the plasma was agitated for at least 60 seconds to ensure that no change in optical density occurred. If a change was observed, indicating that spontaneous aggregation was occurring in the plasma, the sample was discarded. Changes in light transmission, at a wavelength of 492 mμ were recorded at 30 second intervals over a period of ten minutes from the time of addition of the stimulating agent.

There are a number of sources of possible error embodied in this method. The use of platelet rich plasma introduces similar discrepancies

as were considered above for the Chandler tube system. In the titrator magnetic stirrer system there is no accurate gauge of the speed at which the stirring rod rotates. As it has been demonstrated (O'Brien, 1962b), that the degree of agitation of the plasma influences the extent to which platelet aggregation occurs, this is an important disadvantage of the system as used in the work for this thesis. In the present system, the photoelectric light beam was not shielded from incident light under the experimental conditions used. This may result in some discrepancy in the readings of optical density recorded during a period of months.

As with the Chandler tube system, some of the drawbacks of the assay system were overcome by rigid standardization in the use of the system. Two titrators and galvanometers were used simultaneously for each experiment. Each platelet rich plasma sample was divided, one portion treated with a volume of the test substance and a second portion with an equal volume of 0.9 per cent saline as a control. The platelet aggregation occurring in the control and test samples was assayed simultaneously, the two samples being randomly allocated between the two sets of instruments. It was only possible to standardize the speed of rotation of the stirring rod in the plasma by observation. A minimum fixed volume of plasma, 2 ml, was selected so that the meniscus of the fluid in the cuvette lay immediately above the light path. As the stirring rod rotated, a vortex formed in the plasma and the speed of rotation was arbitrarily fixed as the

maximum speed which produced a vortex just insufficient to interfere with the light path through the plasma sample.

Glass Bead Column Method. Hellem (1960) has shown that when whole blood, containing an anticoagulant, is pumped through a column of glass beads, a number of platelets adhere to the surface of the glass beads and to each other. The technique used in this thesis was a modification of Hellem's original method (Hirsh et al., 1966a). The glass bead column was made by filling a length of vinyl tubing (NT/13, Portland Plastics, Kent) with 2.5 g Ballotini glass beads (0.57 mm diameter) to give a column 6 cm in length. The glass beads were held in the column by a filter of fine nylon gauze fitted at each end of the column. 2 ml of citrated whole blood were drawn into a graduated plastic syringe which was then fitted to an electrically operated mechanical pump. The blood was pushed through the standardized column of glass beads at a constant rate. The rate was assessed by the time taken for the leading edge of the column of blood to pass through the column of glass beads. The pump and glass bead columns used in the present study gave a mean contact time between blood and glass of 30 ± 1 seconds. Any observation lying outside this range was discarded. Platelet counts were performed on the blood samples before and after passage through the column. The difference between the two counts was expressed as a percentage of the initial platelet count and this value was taken as an index of platelet adhesiveness in the sample.

With his original technique Hellem (1960) has shown that platelet adhesiveness increases as the glass surface area increases, as the contact time between the blood and the glass surface increases and as the packed cell volume of the blood sample increases. With the method described above the glass surface area and the contact time have been standardized and only the packed cell volume can vary. For most of the experimental work in this thesis it has not been necessary to make allowance for this in the final analysis of the results since each blood sample has been divided into two portions each of 2 ml, one portion treated with a volume of the substance under test and the second with an equal volume of 0.9 per cent saline. Each sample was treated identically and the percentage adhesiveness of each sample compared. Where this procedure was not used allowance was made for the packed cell volume of the sample as described in Chapter 7.

The main source of error with this technique stems from the fact that the result is dependent on accurate platelet counting. In an attempt to minimize this, four independent platelet counts were performed on each blood sample before and after passage through the column and the percentage platelet adhesiveness calculated from the mean of the four readings. Platelet counts were performed using formol citrate as the diluting fluid (Dacie, 1956c). 10 ml, freshly filtered diluting fluid was added to a clean glass test tube together with 0.1 ml blood. The tube was inverted gently until the contents were thoroughly mixed. A sample was then run

onto a Neubauer improved counting chamber and allowed to stand in a damp atmosphere for twenty minutes before the platelets were counted with a microscope using a white light source.

(b) Preparation of fibrinogen degradation products.

Four degradation products were prepared, three by tryptic digestion of fibrinogen and one by the enzymatic action of urokinase-activated plasminogen. The tryptic fibrinogen degradation products were each prepared by incubating 0.3 ml of the various trypsin solutions with a solution containing 200 mg fibrinogen dissolved in 4 ml, 0.1 M phosphate buffer, pH 7.6 at 37°C for 10 minutes. At the end of incubation, 0.3 ml of the appropriate concentration of soybean trypsin inhibitor was added to the system. The urokinase degradation product was prepared by incubating 0.1 ml of the enzyme preparation with a solution composed of 200 mg fibrinogen dissolved in 4 ml, 0.1 M phosphate buffer, pH 7.6 and 1.0 ml of plasminogen solution containing 17.5 Sgouris units per ml.

Column Chromatography of the fibrinogen degradation products was carried out by a modification of the method of Nussenzweig et al., (1961). A separate column was prepared for each degradation product. The mixture obtained by incubating fibrinogen with the enzyme preparation was layered onto a DEAE cellulose column, 37.5 cm long and 2.5 cm in diameter (DE II, Whatman, W. & R. Balston Ltd., England). The fibrinogen incubation mixture was

eluted with a linear concentration gradient within the limits of 0.1M carbonate buffer, pH 8.9 to 0.1M carbonate buffer, pH 8.9 plus 0.2 M sodium chloride in a total volume of 800 ml. The chromatography was carried out at 4°C and the flow rate for each column lay within the range 80-100 ml per hour. The eluate was collected in 10 ml amounts using an Aimer fraction collector with a drop counter attachment. The eluate samples were screened for protein content by estimations of optical density measurements at 280 m/u on a spectrophotometer (SP 500, Unicam Instruments, Cambridge). The samples found to contain protein were concentrated with Sephadex G - 25 (coarse) using Seitz filters and the precise protein concentrations estimated by the method of Lowry et al., (1951).

(c) Biochemical Assays.

Streptokinase Sensitivity Test. This assay was carried out by the method of Johnson et al., (1957) as described by McNicol and Douglas (1964c). This test is designed to determine the concentration of streptokinase required to neutralise both plasma antibody to streptokinase and other inhibitors of the enzyme.

Streptokinase was added to 0.2 ml plasma in a series of test tubes at concentrations ranging from 100 - 500 N.I.H. units per ml plasma. The plasma - streptokinase mixture was then clotted with 0.1 ml thrombin (20 N.I.H. units/ml) and the samples incubated at 37°C. The time of clot lysis was recorded. The concentration of streptokinase used in subsequent

experiments was the concentration which produced maximum lytic activity.

Thrombin Clotting Time. This test is used as an index of defective fibrin polymerization.

The method used was that described by Fletcher et al., (1959). To 0.1 ml plasma was added 0.3 ml of a 'thrombin titration mixture' (Seegers and Smith, 1942) as described by McNicol and Douglas, 1964a). 0.1 ml thrombin (10 N.I.H. units/ml) was added and the clotting time at 37°C recorded. The 'thrombin titration mixture' was made as follows:- 0.9 per cent sodium chloride, 6 ml; 0.7 per cent calcium chloride, 2 ml; 15 per cent acacia, 2 ml; 0.1 M tris buffer, pH 7.5, 1 ml.

Fibrinogen Assay. The method used was that of Ratnoff and Menzie (1951) as described by McNicol and Douglas (1964a). In this method plasma fibrinogen is converted to fibrin by thrombin. The fibrin is washed free of contaminating proteins and then subjected to alkaline hydrolysis. The tyrosine released is estimated colorimetrically. The initial concentration of fibrinogen is calculated from the assumption that the ratio of tyrosine residues to each fibrinogen molecule is constant and is 11.7 to 1.0.

Into a 15 ml test-tube were placed '0.2 ml' glass beads (diameter not more than 0.15 mm); 6 ml, 0.9 per cent saline; 0.2 ml, 2.5 per cent $CaCl_2$; 0.2 ml, plasma and 0.1 ml, thrombin (100 N.I.H. units/ml). The contents of the tube are thoroughly shaken and allowed to stand at 4°C

for 30 minutes. The tube was then shaken and centrifuged at 1,000 g for 10 minutes. The supernatant was discarded and the glass beads washed three times with 0.9 per cent saline with centrifugation and decantation of the supernatant between each wash. 0.4 ml, 10% sodium hydroxide were then added to the glass beads and fibrin and the tube boiled for 20 minutes, the top of the tube being sealed with tin foil to minimize evaporation. After cooling the tyrosine released was estimated by the addition of 2.0 ml, 0.5N sodium hydroxide, 0.6 ml, 5 per cent trichloroacetic acid and 0.6 ml, Folin Ciocalteu reagent diluted 1:2 with distilled water. The mixture was thoroughly shaken and allowed to stand for 10 minutes to permit maximum colour development. The optical density was read at 650 mμ on a spectrophotometer (SP 500, Unicam Instruments) against a reagent blank. Optical density readings were converted to mg fibrinogen per 100 ml plasma from a standard tyrosine curve.

Plasminogen Assay. The method used was the caseinolytic assay of Remmert and Cohen (1949) as modified by Alkjaersig et al., (1959). The initial steps of the assay involve the destruction of plasma antiplasmin with acid, the neutralization of the plasma with alkali and buffer, and the conversion of plasma plasminogen to plasmin by the addition of streptokinase. Casein is used as a substrate for the plasmin. The

enzyme reaction is stopped by the precipitation of the proteins with trichloroacetic acid (TCA). The tyrosine residues released remain in the supernatant and are measured colormetrically. The amount of tyrosine released from the casein is a measure of the amount of plasmin present.

0.5 ml, plasma was incubated with 0.5 ml, 1/6 N HCl for 15 minutes at room temperature. 0.5 ml, 1/6 N NaOH was then added followed by 1.0 ml, phosphate buffer, 0.1 molar, pH 7.6 and 0.5 ml streptokinase (2,000 units/ml). 2.0 ml of a 5 per cent casein solution were added and the mixture thoroughly shaken. After the addition of casein the mixture was incubated at 37°C for 62 minutes. 2 ml samples were removed from the incubation mixture at 2 minutes and 62 minutes and each added to 2 ml of a 10 per cent TCA solution. The supernatant was obtained by centrifugation at 1,000 g for 10 minutes and the tyrosine measured by the addition of 1 ml supernatant to 5.0 ml, 0.5 N NaOH and 1.5 ml, 5 per cent TCA. After the addition of 1.5 ml Folin Ciocalteu reagent (diluted 1:2 with distilled water) the tubes were allowed to stand for 20 minutes to allow for the maximum colour development. The optical density was recorded at 650 m/u on a spectrophotometer (SP 500, Unicam Instruments Ltd.) using the 2 minute sample as blank for the 62 minute sample. The optical density was equated to tyrosine release from a standard curve. One casein unit of plasminogen released 180 mg tyrosine per hour.

Estimation of Protein. The method used was that devised by Lowry et al.,

(1951). This test is based on the reaction between the phenolic residues of a protein moiety and Folin Ciocalteu's reagent and may be used over the range of protein concentration 20 to 400 mg/ml.

A volume of protein solution not exceeding 1 ml was added to a test tube and the final volume made up to 1 ml with distilled water. 5 ml of the alkaline copper reagent were then added to the test tube and the mixture allowed to stand at room temperature for at least 10 minutes. After the addition of 0.5 ml, Folin Ciocalteu reagent (diluted 1:1 with distilled water) the tube was allowed to stand for 30 minutes to allow the maximum colour development to occur. The optical density was read on a spectrophotometer (SP 500, Unicam Instruments Ltd.) at 750 mμ against a reagent blank. The optical density was equated to protein concentration from a standard curve. The standard curve was prepared with bovine albumin (Armour Pharmaceutical Co. Ltd., Eastbourne) over a range of concentration 0-400 mg/ml.

Alkaline Copper reagent was prepared as follows:- 1 ml, 1 per cent $CuSO_4 . 5H_2O$ solution; 1 ml, 2 per cent Na,K tartrate; 2 per cent Na_2CO_3 in 0.1 M NaOH to 100 ml.

Adenosine-di-phosphate (ADP) and adenosine-mono-phosphate (AMP), were estimated enzymatically using a commercially prepared assay kit (TCK - Art. No. 15980, C.F. Boehringer and Soehne, Mannheim, Germany).

In this procedure ADP is phosphorylated with phosphoenol pyruvate and pyruvate kinase. The pyruvate formed in this reaction is reduced to lactate with diphosphopyridine nucleotide and lactic acid dehydrogenase. The change in adsorption spectrum which occurs as the diphosphopyridine nucleotide is oxidized is followed at 366 mμ on a spectrophotometer and is used to monitor the reaction. After the ADP in the solution is consumed and the reaction stopped, the AMP present in the solution is measured by the addition of myokinase which phosphorylates AMP to ADP with the ATP which was formed in the first reaction. The ADP formed in this reaction is estimated in the same way as that formed in the original reaction.

The preparation of the extracts for assay was carried out in the manner described by Holmsen et al., (1965) and the samples analysed for ADP and AMP by the enzymatic method given with the Boehringer test kit. 0.5 ml of the whole blood or plasma was added to 2.0 ml, 0.5 M perchloric acid at 4°C. After thorough mixing, the sample was centrifuged at 1,600 g for 5 minutes at 4°C and 2.0 ml of the supernatant added to 1.0 ml, 1.3 M K_2CO_3 in 1.0 M triethanolamine hydrochloride at 4°C. The solution was maintained at 4°C for at least 5 minutes, thoroughly mixed and again centrifuged at 1,600 g for 5 minutes. If not immediately required for assay the supernatant was stored at -20°C for not more than 3 days.

For the determination of ADP and AMP, 2.0 ml of the supernatant was diluted with 4.0 ml, distilled water. 2.0 ml of this mixture was added to a glass cuvette (1 cm light path, 4 ml capacity) together with 0.15 ml, 0.01 M phosphoenolpyruvate containing 1.3 M KCl and 0.4 M Mg SO_4. 0.10 ml, 0.009 M diphosphopyridine nucleotide and 0.02 ml, lactic acid dehydrogenase (1 mg/ml) were also added to the cuvette. The solution was thoroughly mixed, allowed to equilibrate at room temperature for 5 minutes and the optical density recorded at 366 mμ on an SP 500. The optical density was recorded at 30 second intervals until three consecutive readings were constant (E_1). 0.02 ml, pyruvate kinase (1 mg/ml) was then added to the solution in the cuvette. After thorough mixing and 5 minutes equilibration at room temperature the optical density was recorded. The recordings were repeated at 1 minute intervals until three consecutive readings were obtained (E_2). The difference between E_1 and E_2 was used to calculate the concentration of ADP in the solution (Adam, 1965). To determine the concentration of AMP in the solution, 0.02 ml, myokinase (2 mg/ml) was added to the reaction mixture in the cuvette and after thorough mixing the solution was allowed to stand at room temperature for 10 minutes. The optical density was recorded at minute intervals until a constant value was obtained (E_3). The difference between E_2 and E_3 was used to calculate the concentration of AMP present in the solution (Adam, 1965).

Molecular weight determinations. The approximate molecular weights of

some of the fibrinogen degradation products were estimated and compared to that of the pure fibrinogen preparation with a sucrose density gradient technique. Linear sucrose density gradients over the range 15 per cent to 1 per cent sucrose solution were prepared with the Buchler Density Gradient Sedimentation System (Buchler Instruments Inc., N.J.). When protein is layered onto the surface of the sucrose solution and the system centrifuged, the protein moves through the sucrose solution until it reaches a point of equilibrium, approximately equivalent to its sedimentation value, with the sucrose solution. By careful elution of the resulting gradient the position of the protein in relation to the density of the sucrose solution may be determined by examination of the absorption spectrum of the gradient at 280 mμ. 0.2 ml of the protein sample, of approximate concentration 5 mg per ml, was layered onto the surface of a freshly prepared gradient which was then centrifuged at 38,000 rpm for 6 hours at 4^{o}C on a Spinco Model L Ultracentrifuge (Beckmann Instruments Inc., California) using head No. S W 39. After centrifugation the gradient was analysed by eluting samples from the bottom of the gradient with a Buchler 'piercing unit'. The samples were collected in a series of tubes, 8 drops per tube and after the addition of 0.5 ml, distilled water to each tube, the samples were screened for protein content by analysis at 280 mμ on an SP 500.

Reaction Kinetics. The rate of the reaction between trypsin and fibrinogen

was followed on a pH stat consisting of titrator (Type TTIc) connected to an autoburette (Type ABU1b) and a titrigraph (Type SBR 2c), (Radiometer Electronic Measuring Instruments, Copenhagen). As trypsin digests fibrinogen, peptide bonds are broken with the release of protons. When the reaction is carried out in the pH stat a volume of alkali, sufficient to neutralize the free protons in the system, is added to the reaction mixture. The amount of alkali required to maintain a constant pH is recorded on the titrigraph and under carefully standardized conditions this volume may be equated to the number of peptide bonds broken during the reaction.

20 mg Kabi human fibrinogen dissolved in 1 ml, 0.9 per cent saline were transferred to the reaction flask surrounded by a water bath at 37°C and allowed to equilibrate for at least ten minutes. When equilibration had occurred, 0.2 ml of trypsin (at an appropriate concentration) was added, a stop watch started and a stream of nitrogen passed over the reaction mixture. The reaction was followed for 10 minutes on the titrigraph, 0.0335 M NaOH being transferred from the autoburette to the reaction flask to maintain a constant pH of 8.5. Since the rupture of one hydrogen bond utilizes 0.998 meq alkali, and the volume of alkali used during the reaction was known, it was possible to calculate the number of peptide bonds broken during the reaction process.

Electrophoresis. Electrophoretic studies were performed by horizontal

paper electrophoresis for 17 hours at 200 volts using a Shandon electrophoretic tank and power pack (Shandon Scientific Company, London) (Smith, 1960). The buffer used was 0.1 M barbitone buffer, pH 8.6 (Flynn and De Mayo, 1951), and the strips (2.5 cm x 20 cm) of Whatman filter paper (No. 1) were soaked in the buffer and blotted before the application of the protein samples. 20 µl samples of the protein solutions were applied to the paper with the current passing through the paper. Six strips were run during each experiment. The strips were stained with Bromophenol blue, destained with 1 per cent acetic acid and the colour of the dried strips developed by exposure to ammonia vapour.

Barbitone buffer. The barbitone buffer of 0.1 molar, pH 8.6 was made up as follows:- 10.3 g, sodium diethyl barbitone; 1.84 g, barbitone; distilled water to 1,000 ml.

(d) Collection of Blood.

All blood samples used in this thesis were collected by clean venepuncture with plastic disposable syringes. The samples were collected from three groups of donors (a) healthy colleagues, (b) hospital outpatients and (c) hospital inpatients. The patients were suffering from a variety of diseases, none of which are known to affect blood coagulation or platelet aggregation.

With the minimum delay, the blood was transferred from the syringe to siliconed glass centrifuge tubes containing 3.8 per cent sodium citrate, the relative proportions of blood to citrate being 9 to 1.

Siliconed glassware was used for all experiments which involved the examination of platelets. Two methods were used to siliconize the glassware. The first method used a water insoluble commercial silicone preparation, Silicone M441 (Imperial Chemical Industries Ltd.) as described by Dacie (1956d). For the later work in this thesis a water soluble commercial silicone preparation was used, Siliclad (Clay Adams Inc., N.J.).

Platelet rich plasma was prepared from citrated whole blood by centrifugation at 600 g for 5 minutes at 4°C.

CHAPTER 4

STUDIES ON THE EFFECT OF STREPTOKINASE AND TRYPSIN ON PLATELET AGGREGATION IN THE CHANDLER TUBE SYSTEM

In this chapter is presented the results of the investigations of the effect of two enzymes, streptokinase and trypsin on platelet aggregation in the Chandler tube system. Some of the factors influencing platelet aggregation in this system have been investigated and are discussed.

The possibility of the existence of a haemostatic balance mechanism has been discussed in chapter 2. Previous work reported from this laboratory on the effect of anticoagulant drugs on the formation of artificial thrombi and on the effect of fibrinolytic enzymes on the lysis of artificial thrombi appeared to offer a means of investigating the possible role of such a mechanism. The anticoagulant drugs, phenindione and warfarin, and heparin have been shown to cause significant prolongation for the time taken for platelet aggregates to form *in vitro* (Cunningham et al., 1965). It has also been shown that perfusion of an artificial thrombi with the fibrinolytic enzyme, streptokinase, results in the lysis of the thrombi but with release of platelet emboli into the system which are resistant to further destruction by the enzyme (McNicol et al., 1965). Accordingly, the effect of streptokinase on the formation of artificial thrombi was investigated in the Chandler tube system.

Streptokinase owes its proteolytic and fibrinolytic activity to its

ability to activate the fibrinolytic enzyme system by bringing about the conversion of plasminogen to plasmin (Sherry et al., 1959). Plasmin, a proteolytic enzyme, can digest many proteins, including fibrinogen, fibrin, antihaemophilic globulin, factor V and prothrombin. Under physiological circumstances in vivo its preferred substrate is fibrin, but in the presence of excess plasmin, fibrinogen is also readily digested (Sherry et al., 1959). The effect of streptokinase on the lysis of artificial thrombi is caused by the production of plasmin in close association with fibrin which is digested.

Trypsin can also digest fibrinogen and fibrin (Fletcher et al., 1962; Mihalyi and Godfrey, 1963a), and since trypsin is readily available commercially in a purified form, it was also selected for investigation of its effect on the formation of platelet aggregates in the Chandler tube system.

Materials.

Platelet poor plasma was obtained from platelet rich plasma by centrifugation at 1600 g for 15 minutes at 4°C.

0.25 M $CaCl_2$ was made up from anhydrous calcium chloride (Analar, British Drug Houses Ltd.) and was titrated against 0.02M potassium thiosulphate (KCNS) before use (Douglas, 1962e). A few grains of ferric alum were added to 1 ml of the calcium chloride solution. After the addition of 3 ml, 0.05N Ag NO_3 in concentrated HNO_3 the solution was run under a cold water tap and shaken until the solution was milky white. The solution was titrated with 0.02M KCNS until a red end point was obtained. A reagent blank containing no calcium

chloride was treated similarly.

Human fibrinogen. This was prepared by the phosphate buffer method of Jaques (1943) as described by Biggs and Macfarlane (1962b) to give a product which contained 90-95 per cent coagulable protein.

Human prothrombin was prepared by adsorption with alumina and elution with phosphate buffer pH 8.0 as described by Biggs and Macfarlane (1962c).

Details of the preparations of streptokinase, trypsin, ADP, adenosine, thrombin and radioactive fibrinogen are described in chapter 3.

Methods.

Platelet rich plasma. 36 ml of venous blood were withdrawn from a volunteer by clean venepuncture into a plastic syringe and 9 ml added to four siliconed glass centrifuge tubes, each containing 1 ml of 3.8% sodium citrate at 4°C. With minimum delay, the tubes were centrifuged at 600 g for 5 minutes at 4°C and the resulting platelet rich plasma pooled. The plasma was maintained at 4°C until the experiment was started. It has been shown that cooling increases the tendency of platelets to aggregate (Born, 1962b). However, without a constant temperature laboratory this was found to be the most satisfactory method of handling the plasma samples to obtain reproducible results.

Estimation of platelet aggregation. Two identical Chandler loops were prepared as described in Chapter 3. 6 ml, platelet rich plasma were added to each loop, to one loop was added a volume of the compound under investigation and to the second loop an equal volume of 0.9 per cent saline. The volume in

both loops was made up to 15 ml with 0.9 per cent saline and both loops and their contents incubated at 37°C for 10 minutes. At the end of the incubation period, both loops were recalcified with 0.6 ml of 0.25 M $CaCl_2$ and the time for the appearance of the 'snowstorm' of platelet aggregates to occur was recorded.

Fibrinogen clotting times. The ability of the proteolytic enzyme, trypsin, to convert plasma prothrombin to thrombin was assayed by the following method. 0.3 ml, human prothrombin and 0.3 ml of the enzyme preparation were incubated together at 37°C for 10 minutes. 0.2 ml of the incubation mixture was then added to 0.2 ml of human fibrinogen (4 mg/ml) at 37°C and the clotting time recorded. 0.3 ml, distilled water replaced the enzyme preparation in the control experiment.

Plasma recalcification times. The effect of the enzymes trypsin and streptokinase on plasma recalcification times was estimated by the following method. Blood was collected by clean venepuncture and added to centrifuge tubes containing 0.077M sodium EDTA in the ratio of 9.25 volumes of blood to 0.75 volumes of EDTA. Plasma was obtained by centrifugation at 600g for 10 minutes. 0.1 ml of the enzyme preparation was added to 0.2 ml plasma. After 10 minutes incubation at 37°C the mixture was recalcified with 0.2 ml, M/40 $CaCl_2$ and the clotting time recorded.

The incorporation of fibrinogen into the Chandler thrombus was investigated with radioactive iodine tagged fibrinogen. 0.5 ml of the fibrinogen solution

was added to 6 ml, platelet rich plasma in the Chandler loop and the final volume made up to 15 ml with 0.9 per cent saline. The test loop was treated similarly. Both loops and their contents were incubated at 37°C for 10 minutes. A 1.0 ml sample of the fluid in each loop was taken and the samples counted for radioactive content on a scintillation counter (Scaler 1700, Isotope Developments Ltd.). The remaining samples in both loops were recalcified simultaneously with 0.56 ml, 0.25 M $CaCl_2$. After platelet aggregation and any subsequent fibrin formation had occurred the fibrin clot or the platelet clumps were separated from the supernatant and washed twice with 0.9 per cent saline. The material remaining was then counted. A 1.0 ml sample of the supernatant from each loop was also counted at the end of the experiment. The results were expressed as percentages of the radioactivity present in the initial 15 ml sample.

The one stage prothrombin time assay was performed by the method of Quick (1935) as described by Douglas (1962d). 0.1 ml, human brain extract, 0.1 ml, plasma and 0.1 ml, adenosine or distilled water were incubated at 37°C for 10 minutes. The system was recalcified by the addition of 0.1 ml, M/40 $CaCl_2$ and the time for clot formation to occur recorded. The preparation of brain was made from a human brain by saline extraction as described by Douglas (1962f).

The thrombin clotting time assay was carried out as described in chapter 3.

The streptokinase sensitivity test was carried out as described in chapter 3.

Results.

Effect of Streptokinase. The effect of streptokinase on platelet aggregation was investigated with eleven plasma samples. A streptokinase sensitivity test was performed on each sample before platelet aggregation was assayed. Streptokinase was used in the Chandler loop at the concentration which produced maximum lytic activity. It was found that, at this concentration, streptokinase produced a marked acceleration of platelet aggregation in every plasma sample investigated. The results are illustrated in figure 5 and are tabulated in table 26. The mean time for platelet aggregation to occur in the control loops was 382 $\pm$ 110 seconds and in the test loops 276 $\pm$ 81 seconds ($t = 6.602$, $p < 0.001$). It was also noted that streptokinase caused a marked decrease in fibrin formation, with in some cases, complete failure of fibrin formation to occur as a sequel to platelet aggregation.

Effect of Trypsin. The effect of trypsin at a concentration of 10 µg/ml plasma was investigated with seven plasma samples. As with streptokinase, trypsin produced significant acceleration of platelet aggregation in every plasma sample investigated. The mean time for platelet aggregation in the control loops was 457 $\pm$ 115 seconds and in the test loops 151 $\pm$ 41 seconds

($t = 7.764$, $p < 0.001$; figure 6, table 27). Trypsin did not appear to be as effective as streptokinase in preventing fibrin formation since a fibrin clot formed in every plasma sample. This difference was eliminated when the concentration of trypsin was increased to 1 mg/ml. Recalcification was carried out in both the test and control loops immediately after the addition of trypsin to the test loop. Under these conditions, trypsin produced marked acceleration of platelet aggregation in each of the seven plasma samples investigated. The mean time for platelet aggregation in the control loops was 666 ± 64 seconds and in the test loops, 49 ± 20 seconds ($t = 26.491$, $p < 0.001$; figure 7, table 28). In addition it was noted that, as with streptokinase, trypsin caused impaired fibrin formation to such an extent that fibrin formation failed to occur in some of the trypsin treated plasma samples.

To further investigate the observation of defective fibrin formation, 0.5 ml, $^{131}I_2$ tagged fibrinogen was added to the plasma in both the test and control loops in a series of four plasma samples, and equilibration allowed to proceed at $37^{\circ}C$ for 10 minutes. 0.3 ml, trypsin was added to the test loop to give a concentration of 1 mg/ml plasma, 0.3 ml, 0.9 per cent saline added to the control loop, the contents of each loop thoroughly mixed and a 1.0 ml sample taken from each loop. The loops were immediately recalcified and the experiment carried out as described above. The results were expressed as percentages of radioactivity present

in the initial 15 ml samples in each loop and are shown in table 1. The figures confirm the qualitative observation of impaired fibrin formation in the presence of trypsin; in the control samples the major portion of the radioactivity was incorporated into the thrombus, while in the trypsin treated samples the bulk of the radioactivity remained in the supernatant and only a small percentage was incorporated into the platelet clumps.

An important activity of trypsin in plasma has been shown to be the conversion of prothrombin to thrombin (Ferguson et al., 1960; Ferguson and Ennis, 1963; Stormorken, 1956; Yin, 1964). Thrombin is the highly specific proteolytic enzyme which brings about blood coagulation by the conversion of fibrinogen to fibrin and in addition it has been shown that trace quantities of thrombin are sufficient to promote platelet aggregation (Haslam, 1964; Schmid et al., 1962; and Shermer et al., 1961). To further investigate the effect of trypsin on platelet aggregation the following experiments were carried out.

The effect of trypsin, at a concentration of 10 μg/ml, on platelet aggregation in a non-recalcified plasma system was investigated with two plasma samples. As the diluted plasma in the loops was rotated at 37°C the plasma took on a 'ground glass' appearance but no overt platelet snowstorm effect was seen. Microscopic examination revealed small platelet clumps consisting of approximately 10 to 50 platelets to be present in both the control and trypsin treated plasma specimens. There appeared to be no

significant difference between the control and the trypsin treated plasma specimens, an observation suggesting that during the incubation of trypsin and plasma, under the experimental conditions of the system, no significant conversion of prothrombin to thrombin occurred. In an attempt to substantiate this hypothesis, the effect of trypsin on human prothrombin was investigated with a fibrinogen clotting time assay system. Human prothrombin and trypsin were incubated at 37°C for 10 minutes and the presence of any thrombin generated during this time was detected by the ability of a sample of the incubation mixture to convert human fibrinogen to fibrin. The results are shown in table 2. It would seem that trypsin at a concentration of 10 µg/ml does not cause significantly greater conversion of prothrombin to thrombin than that which occurs in the saline control. From the results it would seem that trypsin at a concentration of 1 mg/ml is causing some inhibition of the formation of thrombin. An explanation for the grossly prolonged clotting time observed would be that sufficient trypsin is being carried over from the incubation mixture to cause the digestion of fibrinogen. In view of these findings, the effect of trypsin in promoting platelet aggregation cannot be convincingly explained solely on the basis of its action on the conversion of prothrombin to thrombin. Consequently further clarification of the results was sought by the examination of the effect of trypsin on platelets.

The effect of trypsin on platelets was investigated by comparing the

alteration in the speed of formation of platelet aggregates resulting from the incubation of trypsin and platelet poor plasma with that produced by incubation of trypsin and platelet rich plasma. In this series of experiments platelet rich plasma was divided into two equal portions. One sample was centrifuged at 1600 g for 15 minutes to yield platelet poor plasma which was divided into two, 3 ml samples. Each sample was added to a Chandler loop, to one sample was added 0.3 ml, trypsin to give a final concentration of 10 μg/ml plasma and to the control sample was added 0.3 ml, 0.9 per cent saline. After 10 minutes incubation at 37°C, 0.3 ml, soybean trypsin inhibitor (to give a final concentration of 10 μg/ml plasma) was added to each loop together with 3 ml of the initial platelet rich plasma sample. The final volume in both loops was made up to 15 ml with 0.9 per cent saline and the loops recalcified in the usual way. The experiment was performed on seven plasma samples and the results are shown in figure 8 and table 29. Incubation of trypsin with platelet poor plasma appeared to produce no significant enhancement of platelet aggregation. The mean time for platelet aggregation in the control loops was 504 $\pm$ 236 seconds and in the test loops 307 $\pm$ 124 seconds ($t = 2.970$, < 0.025). The experiment was repeated with a further seven plasma samples but with the timing of addition of platelet rich and platelet poor plasma reversed. There appeared to be significant acceleration of platelet aggregation produced by the incubation of trypsin with platelet rich plasma (figure 9, table 30). The mean time for platelet aggregation in the control

loops was 462 ± 114 seconds and in the test loops 208 ± 122 seconds ($t = 4{,}500$, $p < 0.01$). These observations would seem to suggest that platelets themselves make some contribution to the effect of trypsin on platelet aggregation.

Though in the Chandler tube system of estimating platelet aggregation, platelet aggregation is followed by fibrin formation, the results presented above seem to indicate that the appearance of platelet aggregates can be accelerated in the absence of direct enhancement of the initial enzymatic reactions which promote fibrin formation and hence the clotting of plasma. Further suggestive evidence for this view was obtained by the examination of the effect of trypsin and streptokinase in a recalcification system. Plasma was incubated with the enzyme preparation for 10 minutes at 37°C, the system recalcified and the clotting times recorded. The results are presented in table 3 and show that as the concentration of trypsin increased the recalcification time became longer; the higher the concentration of trypsin added to the system, the less effect it had on the system. This is the reverse of the situation found in the Chandler tube system where higher concentrations of trypsin produce greater acceleration in the rate of formation of platelet aggregates. The effect of streptokinase (2,000 N.I.H. units/ml) was particularly striking. At a concentration which can enhance platelet aggregation (Chapt. 5), streptokinase produced gross prolongation of the time taken for clot

formation to occur.

Of the many phsiological compounds studied, adenosine-di-phosphate (ADP) appears to be the most potent stimulator of platelet aggregation both in vitro (Born, 1962b; Gaarder et al., 1961; Hellem, 1960; Hellem et al., 1963; Mitchell and Sharp, 1964; O'Brien, 1962a; Ollgaard, 1961; Zucker and Borrelli, 1962) and in vivo (Born and Cross, 1963c; Davey and Landler, 1964; Regoli and Clark, 1963). ADP has also been shown to produce transient platelet thrombi in rats (Nordoy and Chandler, 1964). Thus it was considered likely that ADP might influence the formation of platelet aggregates in the Chandler tube system.

Effect of Adenosine-di-phosphate. The effect of ADP on platelet aggregation was investigated with ten plasma samples. 0.3 ml of ADP (10 µg/ml) was added to the test loop and 0.3 ml, 0.9 per cent saline added to the control loop immediately before recalcification. ADP produced a marked acceleration of platelet aggregation in every sample. The mean time for platelet aggregation to occur in the control loops was 655 $\pm$ 152 seconds and in the test loops 583 $\pm$ 117 seconds, ($t = 4.824$, $p < 0.001$; figure 10 and table 31). In the test loop, to which ADP had been added, small discrete platelet clumps became visible some time before the actual 'snowstorm' effect was apparent. This result suggested that platelet aggregation could be accelerated by a biochemical pathway not dependent on the enzymatic cascade sequence (Biggs and Macfarlane, 1962a), which leads to the formation of

thrombin and fibrin clot formation. Corroborative evidence was obtained when the effect of ADP was examined in recalcified and non-recalcified plasma specimens. ADP (0.5 µg/ml plasma) was added to plasma in both the test and the control loops but only the control loop was recalcified. In the seven plasma samples investigated no significant difference was found between the rate of formation of platelet aggregates in the recalcified and non-recalcified samples (figure 11 table 32). The mean time for platelet aggregates to form in the control loops was 85 $\pm$ 66 seconds and in the test loops 59 $\pm$ 20 seconds, ($t = 0.938$, $p < 0.4$). Further confirmation of the suggestion that the appearance of platelet aggregates in the Chandler tube is to some extent at least independent of the forces promoting clot formation came from the investigation of adenosine, a specific inhibitor of the adenine nucleotides.

Effect of Adenosine. The effect of adenosine was investigated with seven plasma samples. 0.6 ml, adenosine (1 mg/ml) was added to the test loop, 0.6 ml, 0.9 per cent added to the control loop and both loops incubated for 10 minutes at 37°C before recalcification. As was anticipated, adenosine was found to cause retardation of platelet aggregation in every plasma sample (figure 12 and table 33). The mean time for platelet aggregation to occur in the control loops was 377 $\pm$ 62 seconds and in the test loops 460 $\pm$ 65 seconds, ($t = 6.606$, $p < 0.001$). It was noted that in some of the adenosine treated samples the normal 'snowstorm' of platelet aggregates did not occur but a large clump of platelets appeared immediately before fibrin formation

occurred.

The effect of adenosine on the conversion of prothrombin to thrombin and on the reaction between thrombin and fibrinogen was investigated in a one stage prothrombin time assay and in a thrombin clotting time assay. In the one stage prothrombin time assay, adenosine was incubated with tissue thromboplastin and plasma at 37°C for 10 minutes before the system was recalcified. Table 4 shows that adenosine, over a range of concentration 100 µg/ml plasma to 1 mg/ml plasma, caused no inhibition of the conversion of plasma prothrombin to thrombin. Similarly, in the thrombin clotting time assay, incubation of adenosine, over the range of concentrations 100 µg to 1 mg per ml plasma, with plasma and 'thrombin time mixture' (Chapt. 3) at 37°C for 10 minutes produced no inhibition of the thrombin fibrinogen reaction (table 5). These results suggest that in the Chandler tube system, platelet aggregation per **se** and not the complete coagulation process, is being inhibited by adenosine.

In view of the results which suggested that the action of trypsin on platelet aggregation might be mediated through a pathway which did not involve thrombin, it was considered pertinent to investigate the possibility that the ADP mechanism of platelet aggregation might be involved.

Effect of trypsin and ADP. The effect of the combined action of ADP and trypsin on platelet aggregation was investigated in a series of seven plasma samples. For this experiment 54 ml of blood were withdrawn from each

subject. Three Chandler loops were prepared, 6 ml of platelet rich plasma added to each loop, 0.3 ml, trypsin (200 µg/ml) added toone loop, the volume in each loop made up to 14.7 ml with 0.9 per cent saline and all the loops incubated at 37°C for 10 minutes. After the addition of 0.3 ml, 0.9 per cent saline to the control loop, 0.3 ml ADP (10 µg/ml) was added to the other two loops and each loop was recalcified. The results are shown in figure 13, table 34. The combined action of trypsin and ADP appeared to be more effective in promoting acceleration of platelet aggregation than the action of ADP alone. The mean time for aggregation to occur in the control loops was 590 $\pm$ 194 seconds, in the ADP treated loops 349 $\pm$ 67 seconds and in the ADP plus trypsin treated loops 137 $\pm$ 70 seconds. The results of statistical analysis of these differences were, for the control and ADP treated loops, $t = 2.970$, $p < 0.025$ and for the ADP and ADP plus trypsin treated loops, $t = 8.194$, $p < 0.001$. During this experiment the time between the appearance of platelet clumps and of fibrin strands was recorded in the ADP and ADP plus trypsin treated loops. There was found to be no significant difference between the two loops (table 6). The mean time interval between platelet aggregation and fibrin formation in the ADP treated loops was 46 $\pm$ 16 seconds and in the ADP plus trypsin treated loops 58 $\pm$ 31 seconds, ($t = 1.704$, $p < 0.20$).

Additional evidence in support of the suggestion that the ADP mechanism for platelet aggregation might be involved in the enhancement of aggregation by trypsin was obtained from the investigation of the combined action of

trypsin and adenosine.

Effect of trypsin and adenosine. The effect of the combined action of trypsin and adenosine on platelet aggregation was investigated with sixteen plasma samples. 0.3 ml of trypsin (200 µg/ml) was added to 6 ml plasma in two Chandler loops, 0.6 ml of adenosine (1 mg/ml) was added to the test loop and the volume in both loops made up to 15 ml with 0.9 per cent saline. After 10 minutes incubation at 37°C, both loops were recalcified. The results are shown in figure 14 and table 35. At the concentrations of trypsin and adenosine used there appeared to be only slight inhibition of platelet aggregation in the adenosine treated sample. The mean time for platelet aggregation to occur in the control loops was 96 $\pm$ 28 seconds and in the test loops 139 $\pm$ 71 seconds, ($t = 2.284$, $p < 0.05$). The experiment was repeated with a further series of seven plasma samples in which the concentration of trypsin was kept at 10 µg/ml plasma but the concentration of adenosine was increased to 200 µg/ml plasma. In every plasma sample, platelet aggregation was significantly retarded in the adenosine treated loop (figure 15, table 36). The mean time for platelet aggregation to occur in the control loops was 96 $\pm$ 35 seconds and in the adenosine treated loops 135 $\pm$ 42 seconds, ($t = 4.289$, $p < 0.01$).

Discussion.

The investigation of the effect of the enzymes trypsin and streptokinase shows that these enzymes can cause acceleration of platelet aggregation and,

in consequence, acceleration of the rate of formation of a thrombus in an artificial circulation. This observation together with results of the investigations of the effect of the adenine nucleotides on platelet aggregation in vitro suggest that the formation of the thrombus in the Chandler tube system is not solely dependent on the generation of thrombin in the system.

Platelet aggregation and fibrinolytic enzymes.

Trypsin has been shown to promote blood coagulation in plasma (Douglas and Colebrook, 1916; Eagle and Harris, 1937; Northrop and Kunitz, 1932; Stormorken, 1956). The coagulative action of trypsin has been demonstrated to be due to its ability to convert prothrombin to thrombin (Ferguson et al., 1960; Ferguson and Ennis, 1963; Stormorken, 1956; Yin, 1964). Many workers have demonstrated the ability of thrombin to promote platelet aggregation (Bounameux, 1956 and 1957; Haslam, 1964; Lüscher, 1956; O'Brien, 1964; Schmid et al., 1962; Shermer et al., 1961; Zucker and Borrelli, 1959). Thus, it might be supposed that the effect of trypsin on platelet aggregation is an effect which is secondary to its action on prothrombin. The results presented above, however, indicate that the action of trypsin cannot be accounted for solely on this basis and that its activity as a fibrinogenolytic agent may also be of considerable importance. This view is supported by the similarity of the results obtained for the fibrinolytic enzyme, streptokinase which possesses both fibrinolytic and fibrinogenolytic activities but has no coagulation properties.

The examination of the effect of trypsin in a recalcification system (table 3) indicates that coagulation can be enhanced even in the absence of calcium ions, a result in agreement with the findings of Stormorken (1956) who has also shown that the effect of trypsin on coagulation is increased as the concentration of calcium is increased. The action of trypsin on platelet aggregation, however, appears to be independent, at least within the physiological range, of the concentration of calcium ions since no difference could be detected between the effect of trypsin on platelet aggregation in citrated platelet rich plasma in a recalcified and non-recalcified system. A further difference between the effect of the enzyme on coagulation and on platelet aggregation becomes apparent when its actions over a range of concentrations are compared. As the concentration of trypsin is increased so its effect on the acceleration of platelet aggregation is increased (table 27 and table 28) but its effect on the recalcification time is decreased (table 3). Thus, platelet aggregation may be enhanced by trypsin under conditions in which the coagulative properties of the enzyme are diminished. The two systems are not directly comparable, however, since in the recalcification system after 10 minutes incubation at 37°C with trypsin (10 µg/ml) a clot formed in the plasma but was not observed to form in the Chandler loop until after recalcification. This difference may, at least in part, be accounted for by the use of non-siliconed glass tubes for the collection of the blood and the assay of the plasma in the recalcification system as opposed to the

use of siliconed glassware and 'non-wettable' surfaces in the Chandler tube system.

In addition to its coagulative properties, trypsin may act as a fibrinogenolytic enzyme (Fletcher et al., 1962 and Mihalyi and Godfrey, 1963a). As the concentration of trypsin is increased in plasma, its fibrinogenolytic activity may gain ascendency over its coagulative properties. Some evidence to support this view is obtained from the investigation of the fate of radioactive tagged fibrinogen in the Chandler tube system (table 1). Additional evidence is also obtained from the investigation of the ability of trypsin to convert prothrombin to thrombin (table 2). In this experiment clear cut conclusions may not be drawn from the results, however, since while trypsin may be carried over from the incubation mixture which may then rapidly digest the fibrinogen, trypsin may also digest thrombin (Eagle and Harris, 1937) formed in the reaction mixture.

The evidence obtained from the experiments in which the effect of trypsin on platelet aggregation after incubation with platelet poor plasma was compared to that after incubation with platelet rich plasma (figures 8, 9 and tables 29, 30), suggests that platelets make some contribution to the tryptic effect. Several workers have studied some of the effects of trypsin digestion of platelets. Schmid et al., (1962) have demonstrated that the treatment of washed platelets with high concentrations of trypsin (50 µg per ml platelet suspensions) abolished the ability of platelets to aggregate with thrombin and

caused irreversible damage to the platelets. Haslam (1964) has shown that treatment of washed platelets with high concentrations of trypsin (0.2 mg/ml) abolished their ability to aggregate with ADP while low concentrations of trypsin (0.2 µg/ml) resulted in the rapid and complete aggregation of platelets in the presence of calcium ions. From the results presented above it would appear unlikely that incubation of trypsin with platelet rich plasma produces irreversible damage to the platelets since not only is there no visible diminution of the 'snowstorm' of platelet aggregates but also the formation of the platelet clumps is accelerated. This contention is also supported by results presented in Chapter 5.

One possible explanation of the enhancement of platelet aggregation by trypsin may be found by consideration of the observations of Grette (1962) who has shown that the treatment of platelets with trypsin may result in a release of phospholipid, serotonin and ADP from the platelet, an effect which is similar to that produced by the action of thrombin on platelets. Since trypsin can promote the acceleration of platelet aggregation under conditions in which coagulation is not enhanced, it is unlikely that phospholipids or serotonin play a primary role in the action of the enzyme on platelet aggregation. Hence it is possible that the effect of trypsin may, at least in part, be mediated through ADP. This hypothesis is substantiated by the evidence for the additive effect of ADP and trypsin produced by their combined action on platelet aggregation (figure 13 and table 34) and by the observation that the effect

of trypsin can be blocked by adenosine, the specific inhibitor of the adenine nucleotides (figure 15 and table 36).

Platelet Aggregation in the Chandler tube system.

The Chandler tube system assesses platelet aggregation by virtue of the ability of platelets to adhere to each other with the formation of platelet clumps in an artificial circulation system. The mechanism by which the phenomenon of platelet aggregation occurs in this system has not been fully elucidated but some factors which may contribute to thrombus formation are known.

The results presented above for the action of streptokinase on plasma coagulation in a simple recalcification system and on thrombus formation in the Chandler tube (figure 5 tables 3, 36), offer strong circumstantial evidence that the stimulus to the formation of platelet aggregates is not due solely to the generation of thrombin. Supporting evidence is found in the similar observations for trypsin in both coagulation systems and in the Chandler tube system (figures 6, 7, tables 3, 27, 28). Further, it has been shown that the appearance of platelet aggregates in this system may be enhanced by ADP (figure 10, table 31) and inhibited by adenosine (figure 12, table 32). Gaarder and Laland (1964) have proposed a hypothesis to account for the ability of ADP to enhance platelet aggregation. They suggest that ADP may stimulate the adhesion of platelets to each other by the formation of hydrogen bonds between the platelets and ADP molecules. It is possible that a similar mechanism operates in the Chandler tube system. The initial

molecules of ADP necessary to promote the first steps in the adhesion of platelets may come from the plasma by virtue of ADP released from any red cells hemolysed during the centrifugation process to produce the plasma or from any platelets damaged during the subsequent handling process. Hovig and Holmsen (1963) have shown that collagen fibres may release ADP from the platelets. The mechanical stress produced by the rotation of plasma in contact with a foreign surface may also be sufficient to promote ADP release from platelets. It has been demonstrated that during the process of platelet adhesion further ADP is released from the platelets (Born, 1956b; Zucker and Borrelli, 1961) which enables a large platelet clump to form. Hence, once the initial stimulus to platelet aggregation is present the chain reaction of formation of platelet clumps and thrombus formation (Hellem and Owren, 1964) follows. Thus it is possible that, in the Chandler tube system, the initial stimulus to platelet aggregation may be ADP.

Conclusions.

From the results presented in this chapter it would appear that the enzymes streptokinase and trypsin possess the ability to accelerate the formation of platelet aggregates in the Chandler tube system. The effect of trypsin cannot be accounted for solely by its coagulative action or its action on platelets per se. However, its action does appear to be mediated in some way through the ADP mechanism of platelet aggregation. It would also appear that the formation of platelet aggregates in the Chandler tube system cannot be accounted for solely on the basis of thrombin generation and that the ADP mechanism is implicated in the generation of platelet aggregates.

CHAPTER 5.

STUDIES ON THE EFFECT OF STREPTOKINASE AND TRYPSIN ON PLATELET AGGREGATION IN THE TURBIDIMETRIC SYSTEM.

In this chapter are presented the results of the investigations of the effect of the enzymes, streptokinase and trypsin, on platelet aggregation in platelet rich plasma.

One of the disadvantages encountered in the artificial circulation system (Chapter 4), is that fibrin formation invariably occurs as a sequel to platelet aggregation. As a result, it is sometimes difficult to define whether a compound under investigation in this system, is acting specifically on platelet aggregation or is causing some alteration of the general haemostatic process since both reactions could be reflected by a change in the rate of formation of platelet aggregates. Trypsin has been demonstrated to be able to promote coagulation by its ability to convert prothrombin to thrombin (Ferguson et al., 1960; Ferguson and Ennis, 1963; Stormorken, 1956; Yin, 1964). From the results presented in Chapter 4 it was concluded that the apparent ability of trypsin to cause acceleration of the rate of formation of platelet aggregates in an artificial circulation might be mediated through the action of ADP. Consequently, in an attempt to further elucidate the relationship between trypsin and platelet aggregation, the action of trypsin on ADP induced aggregation was investigated under conditions in

which fibrin formation does not normally occur.

In the Chandler tube system (Chapter 4) it was demonstrated that streptokinase appeared to be able to enhance platelet aggregation and it was suggested that this effect might be mediated through its ability to activate plasminogen with the formation of plasmin (Troll and Sherry, 1955). This has been further investigated in the turbidimetric system.

Trypsin is a proteolytic enzyme and although streptokinase is not itself directly proteolytic, plasmin which is produced by its activity can digest many proteins. In an attempt to determine whether the ability of trypsin and streptokinase to enhance platelet aggregation is specific to these enzymes or is common to other proteolytic enzymes, the effects of urokinase, ficin and chymotrypsin on ADP induced platelet aggregation have also been investigated.

Heparin, although not a fibrinolytic agent, has been included with the proteolytic enzymes because it can influence the dynamic balance between coagulation and fibrinolysis. It can suppress coagulation by interfering in the interaction of thrombin and fibrinogen and by the prevention of the conversion of prothrombin to thrombin by the blood thromboplastin system (Douglas, 1962a). Heparin was first isolated from liver by Howell and Holt (1918) and

has been identified as an acidic mucopolysaccharide consisting, of a polymer of D-glucuronic acid and D-glucosamine (Douglas, 1962b). This compound has also been shown to affect the behaviour of platelets. Large doses of heparin administered intravenously prevent platelet plug formation in cut vessels (Zucker, 1947) but high concentrations of heparin applied locally are not as effective (Hugues, 1959) and will not prevent platelet clumping induced with mesenteric fibres (Hugues, 1962; Spaet and Zucker, 1964). In vitro heparin also inhibits thrombin induced platelet clumping (Clayton and Cross, 1963; Cuthbertson and Mills, 1963b; Zucker and Borrelli, 1959) and can delay thrombus formation in vitro by causing an inhibition of the initial stages of the process which involves platelet aggregation (Cunningham et al., 1965). The effect of this anticoagulant drug on ADP induced platelet aggregation in vitro has been investigated and the results are presented in this chapter.

Materials.

Nor-Adrenaline was obtained in the form of bitartrate from Koch Light Laboratories Ltd. It was made up to the required concentration with 0.9 per cent saline and stored at $4^{o}C$.

Chymotrypsin was used in the form of chymotrypsin, type II, prepared

from bovine pancreas as supplied by the Sigma Chemical Company, St. Louis. The enzyme was dissolved in 0.9 per cent saline to the required concentration and stored at $4^{o}C$.

Ficin prepared from fig latex was obtained from Koch Light Laboratories Ltd. The enzyme was diluted with 0.9 per cent saline to the required concentration and stored at $4^{o}C$.

Urokinase was supplied by Leo Pharmaceutical Products, Denmark. The enzyme was dissolved in distilled water to give a concentration of 2,000 Sgouris units per ml and this stock solution was stored at $-20^{o}C$. The preparation was further diluted as required.

Heparin. The preparation of heparin used was supplied by Weddel Pharmaceuticals, London at a concentration of 50 mg per ml (5,000 units per ml). It was diluted with 0.9 per cent saline to the required concentrations.

Fibrinogen in the form of human lyophilized, Grade L, was supplied by A.B. Kabi, Stockholm. The protein was dissolved in 0.9 per cent saline to the required concentrations and was stored at $-20^{o}C$.

Details of the preparations of streptokinase, trypsin, ADP, adenosine, thrombin and radioactive fibrinogen are described in Chapter 3.

Calcium Chloride was made up to give solutions of 0.25 M, 0.11 M,

0.01 M and 0.0025 M and the molarity of these solutions was checked by titration before use as described in Chapter 4.

Phosphate Buffer. Phosphate buffer of 0.1 molar and pH 7.6 was prepared as described in Chapter 3.

Barbitone saline was prepared as follows:- 0.9 g, NaCl; 0.1 g, $NaHCO_3$ made up to 1,000 ml with distilled H_2O.

Bicarbonate saline citrate was prepared as follows:- 0.9 g, NaCl; 0.1 g, $NaHCO_3$; 2.94 g, Nacitrate; distilled water to 1,000 ml.

Tris buffers. Tris buffer, 0.1M and pH 7.5 was made up as follows:- 25 ml, 0.2 M tris (hydroxy-methyl) aminomethane (24.2 g per litre); 20 ml, 0.2N HCl made up to 100 ml with distilled H_2O.

Tris buffer, 0.154 M and pH 7.2 was prepared as follows:- 25 ml, 0.308 M tris (hydroxy-methyl) aminomethane (37.3 g per litre); 22.1 ml, 0.308N HCl made up to 100 ml with distilled H_2O.

Tris buffer, 0.154 M and pH 8.2 was prepared as follows:- 25 ml, 0.308 M tris (hydroxy-methyl) aminomethane (37.3 g per litre); 11.0 ml, 0.308N HCl made up to 100 ml with distilled H_2O.

'Platelet suspension mixture' was prepared by the addition of sodium chloride ($1.4X10^{-1}M$), potassium chloride ($2.3 \times 10^{-3}M$) and calcium

chloride (1.7×10^{-3}M) to 0.1M tris buffer, pH 7.5.

It was made up as follows:- NaCl, 0.817g; KCl, 0.0164g; $CaCl_2$, 0.0182g; 0.1M tris buffer, pH 7.5 to 100 ml.

Potassium chloride was prepared at a concentration of 0.154 molar by dissolving 1.15g, KCl in 100 ml distilled H_2O.

Magnesium Sulphate was prepared by dissolving 1.85 g $MgSO_4$ in 100 ml distilled water to give a concentration of 0.154 molar.

Sodium chloride was prepared at a concentration of 0.154 molar by dissolving 0.886 g NaCl in 100 ml distilled H_2O.

Sodium EDTA of 0.077M was prepared from 2.867g, ethylene diaminotetra-acetic acid, disodium salt and distilled water to 100 ml.

Platelet rich plasma was prepared and stored as described in Chapter 4 except that only 18 ml of blood were collected from each volunteer.

Platelet poor plasma was prepared as described in Chapter 4.

Methods

Estimation of platelet aggregation. Two EEL titrator and galvanometer systems were used simultaneously in all the experiments described in this chapter. Each platelet rich plasma sample was divided, to one

ml sample was added the substance under investigation and to a second 2 ml sample an equal volume of 0.9 per cent saline as a control. The control and test plasma samples were randomly allocated between the two titrator systems. Unless otherwise stated, the cuvettes containing the control and test plasma samples were incubated with a volume of saline and an equal volume of test substance respectively, at 37°C for 10 minutes before being transferred to the titrators. The actual estimation of platelet aggregation was carried out simultaneously on each plasma sample at room temperature.

Preparation of washed platelet suspensions. The first method used was that described by Born and Cross, (1964). 12 ml of citrated platelet rich plasma was prepared as described in Chapter 4 and from this platelet poor plasma obtained by centrifugation at 1,600 g at 4°C for 15 minutes. The platelet pellet was resuspended in 6 ml of bicarbonate saline citrate and centrifuged once more at 1,600 g for 15 minutes. This procedure was repeated once with bicarbonate saline citrate and once with bicarbonate saline. The platelets were finally resuspended in 6 ml of the 'platelet suspension mixture' at 4°C and were maintained at 4°C until the assay of platelet aggregation was performed.

The second method used was that described by Haslam, (1964). For this experiment 92.5 ml of blood were withdrawn by clean venepuncture from a volunteer and anticoagulated by addition to 0.077 M sodium EDTA

in siliconed glass centrifuge tubes at 4°C in the ratio, 9.25 ml of blood to 0.75 ml EDTA. The blood was centrifuged at 300 g for 15 minutes at 4°C to obtain platelet rich plasma which was then centrifuged at 750g for 15 minutes at 4°C. The platelet pellet was resuspended in a volume, equal to that of the initial platelet rich plasma, of a washing mixture composed of 90 vols, 0.154 M NaCl : 8 vols, 0.154 M tris buffer, pH 7.2: 2 vols, 0.077 M EDTA. This platelet suspension was then centrifuged at 250g for 12 minutes at 4°C and the washed platelets finally resuspended in approximately 20 ml of a solution composed of 9 vols, 0.154 M NaCl : 1 vol, 0.154 M tris buffer, pH 7.2, to give a platelet count of the order of 5×10^5 per mm^3. This platelet suspension was stored at 4°C until platelet aggregation was assayed. For the assay of platelet aggregation, to each cuvette was added 1.76 ml of the platelet suspension and 0.2 ml, 0.154 M KCl, 0.02 ml, 0.154 M $MgSO_4$ and 0.02 ml, 0.11 M $CaCl_2$. This mixture was then incubated with either saline or the compound under investigation at 37°C for 10 minutes before the cuvette containing the sample was transferred to the titrator and ADP added to the system.

Platelet counting was carried out as described in Chapter 3.

Streptokinase sensitivity tests were performed as described in Chapter 3. Urokinase sensitivity tests were carried out in a similar manner with

urokinase in place of streptokinase.

Destruction of the antiplasmin content of plasma was carried out by acid-alkali treatment of the plasma (Alkjaersig et al., 1959). Platelet rich plasma was centrifuged at 1,600 g for 15 minutes at 4°C and the platelet poor plasma obtained decanted from the platelet pellet. 5 ml of the platelet poor plasma were treated with 5 ml, 0.167 M HCl at room temperature for 15 minutes to destroy the acid labile plasmin inhibitor, antiplasmin. 5 ml, 0.167 M NaOH was then added to neutralize the acid and the plasma was rebuffered by the addition of 5 ml, 0.1M phosphate buffer, pH 7.6. The platelets were then resuspended in 5-6 ml of this acid - alkali treated plasma to give a platelet count of the same order as that of the initial platelet rich plasma sample.

Radioactive iodine labelled fibrinogen was used to monitor the digestion of fibrinogen occurring in the presence of streptokinase and trypsin. 0.1 ml, $^{131}I_2$ tagged fibrinogen was added to the plasma samples in the control and test cuvettes before incubation at 37°C. After thorough mixing 0.2 ml samples were removed from the control and test cuvettes and clotted with 0.1 ml thrombin (100 N.I.H. units per ml). The clot which formed was transferred with a clean wooden spatula to a test-tube and the radioactive content assessed on a scintillation counter (Scaler 1700, Isotope Developments Ltd.). 0.2 ml samples were again removed from the control

and test cuvettes after platelet aggregation in the samples had been monitored for 10 minutes and treated similarly. The clottable protein present after incubation and platelet aggregation was expressed as a percentage of that present in the initial sample.

Fibrinogen clotting times were used to assay the ability of ficin and chymotrypsin to convert human prothrombin to thrombin. The method used is described in Chapter 4.

Results.

Effect of Streptokinase. The effect of streptokinase on ADP induced platelet aggregation was estimated on ten plasma samples in the turbidimetric system. Streptokinase was studied at the concentration which produced maximum lytic activity as determined for each plasma sample in the streptokinase sensitivity test. The results are shown in figure 16 and table 37. It would appear that at this concentration, incubation of plasma with streptokinase before the examination of platelet ADP reactivity can cause a significant decrease in the degree to which platelet aggregation occurs. $4\frac{1}{2}$ minutes after the addition of ADP to the plasma samples the mean optical density reading of the control samples was 0.366 ± 0.208 and of the streptokinase treated samples 0.499 ± 0.129, ($t = 2.413$ $p < 0.05$). This result is contrary to the observations made for the effect of a

similar concentration of streptokinase on platelet aggregation in the Chandler tube system (Chapter 4). Two possible explanations for these converse observations may be found if the mechanism by which platelets aggregated differed in the two systems or if streptokinase was made to exert its enzymatic action under different sets of conditions.

From the observations made for the effect of ADP and adenosine in the Chandler tube system, (Chapter 4), it was concluded that ADP is involved in the formation of platelet aggregates in this system. In Chapter 4 it was also suggested that the action of the proteolytic enzyme trypsin on platelet aggregation might, at least in part, be mediated through the action of ADP. It was therefore, considered most improbable that the different actions of streptokinase observed in the two systems could be accounted for by the postulation of the operation of two different mechanisms of platelet aggregation.

In the Chandler tube system, streptokinase was incubated with plasma diluted in the ratio 1 volume plasma to 1.5 volumes, 0.9 per cent saline, whereas in the turbidimetric system streptokinase was incubated with undiluted plasma. In chapter 4 it was suggested that streptokinase might exert its action on platelet aggregation by virtue of its ability to activate plasminogen to plasmin. Plasma contains a protein,

antiplasmin, which is the specific inhibitor of the enzyme plasmin (Sherry et al., 1959). In the diluted plasma the relative concentrations of the enzyme and its inhibitor may become less important and antiplasmin may be less effective in inhibiting the action of any plasmin formed in the presence of streptokinase. Thus, in an attempt to explain the unexpected observation for the effect of streptokinase in the turbidimetric system the action of this enzyme was re-examined with diluted plasma.

Streptokinase at a concentration of 100 N.I.H. units per ml plasma was added to seven plasma samples which were diluted with 0.9 per cent saline in the proportions, 1 ml plasma to 1.5 ml saline. After 10 minutes incubation at 37°C, ADP was added to the samples and the platelet aggregation which ensued followed by monitoring the changes of optical density of the plasma samples. Under these conditions no pattern emerged for the action of streptokinase on platelet aggregation in diluted plasma, figure 17 and table 38. There was no statistically significant difference between the optical density readings in the control and test samples. 3 minutes after the addition of ADP the mean optical density reading of the control samples was 0.266 ± 0.119 and of the test samples 0.298 ± 0.077, ($t = 1.122$, $p < 0.40$). This result would seem to suggest that, while the effect of streptokinase on platelet aggregation is not in statistical agreement with that for the Chandler tube system, there is some support for the concept

of antiplasmin playing a role in the apparent inhibition of platelet aggregation by the enzyme in undiluted plasma; in two of the seven plasma samples investigated, platelet aggregation was enhanced by streptokinase. Another factor which must contribute to the interpretation of the results is the use of a fixed concentration of streptokinase for all the plasma samples. From the previous experiments in which streptokinase sensitivity tests were carried out, 100 N.I.H. units of streptokinase per ml of plasma was accepted as the average concentration of the enzyme which produced maximum lytic activity. Thus in the above experiment this concentration of enzyme might be insufficient to neutralize all the streptococcal antibodies present in the sample and hence the action of streptokinase could be inhibited not only by antiplasmin but also by non-neutralized antibodies. In an attempt to clarify the situation, the effect of streptokinase, at a very much increased concentration, on platelet aggregation in plasma in which the antiplasmin had been destroyed was investigated.

The effect of streptokinase at a concentration of 2,000 N.I.H. units per ml plasma was studied with 17 plasma samples. Each platelet rich plasma sample was centrifuged at 1600 g for 15 minutes, the platelet pellet removed and the remaining platelet deficient plasma treated with 0.167 M HCl for 15 minutes followed by neutralization with 0.167 M NaOH

and pH readjustment with 0.1M phosphate buffer, pH 7.6. The platelets were then resuspended in a sufficient volume of the antiplasmin-free plasma to give a platelet count similar to that of the initial platelet rich plasma. One portion of the platelet suspension was incubated with streptokinase and a second portion with saline as a control. Under these conditions streptokinase caused significant enhancement of platelet aggregation (figure 18, table 39). 10 minutes after the addition of ADP the mean optical density of the control samples was 0.499 ± 0.066 and of the test samples 0.430 ± 0.129, ($t = 2.574$, $p < 0.025$). Thus it would appear that, in the turbidimetric system, streptokinase can only enhance ADP induced platelet aggregation when used in a sufficiently high concentration to neutralize all the streptococcal antibodies of antiplasmin deficient plasma.

It has been shown that nor-adrenaline is one of many physiological compounds which may induce platelet aggregation in vitro (Mitchell and Sharp, 1964; O'Brien, 1963 and 1964). The view is currently held that while physiological compounds, other than ADP, may induce platelet aggregation it is likely that their action is mediated through ADP (Haslam, 1964). Thus in an attempt to elucidate the physiological significance of the effect of streptokinase on platelet aggregation, the effect of the enzyme at a concentration of 100 N.I.H. units per ml

plasma on nor-adrenaline induced aggregation was investigated in 10 plasma samples. At this concentration streptokinase appeared to have no significant effect on nor-adrenaline induced platelet aggregation (figure 19, table 40). 10 minutes after the addition of nor-adrenaline (1.0 μg per ml plasma), the mean optical density reading of the control plasma was 0.371 $\pm$ 0.224 and of the test plasma 0.351 $\pm$ 0.219, ($t = 0.623$, $p < 0.60$).

In the Chandler tube system some evidence was obtained to suggest that during the incubation of plasma and streptokinase, fibrinogen was digested (Chapter 4). This observation has been confirmed in the turbidimetric system by the use of radioactive fibrinogen. To five of the plasma samples used in the investigation of the effect of streptokinase in diluted plasma and to four of the samples in which platelet aggregation was induced by nor-adrenaline, 0.1 ml, $^{131}I_2$ tagged fibrinogen was added to both the control and test plasma samples before they were incubated with saline and streptokinase respectively. 0.2 ml plasma was removed from both the test and control samples before incubation and clotted with 0.1 ml thrombin (100 N.I.H. units/ml). The clot was then counted for radioactive content on a scintillation counter (Scaler 1700, Isotope Developments Ltd.). Following incubation and after platelet aggregation had been monitored for 10 minutes, further 0.2 ml plasma

samples were removed and treated similarly. The percentage clottable protein present after aggregation was calculated from the two results. The figures are presented in table 7. It can be seen that, unlike the Chandler tube system in which fibrinogen appeared to be markedly digested in every plasma sample, in the turbidimetric system streptokinase appeared to have little effect on the fibrinogen molecule in some plasma samples. This observation supports the suggestion that, in some of the plasma samples, the action of streptokinase may be inhibited and that this may account for the inconsistent results obtained with streptokinase in this system.

Thus the effect of streptokinase on platelet aggregation appears to be due to its ability to activate plasminogen to plasmin. In the Chandler tube system the effect of the proteolytic enzyme trypsin was investigated as an alternative to further investigation of plasmin because of the difficulties in obtaining plasmin sufficiently free from contaminating plasma constituents. The effect of trypsin on platelet aggregation was studied in the turbidimetric system.

Effect of Trypsin. The effect of trypsin, at a concentration of 10 µg per ml, was investigated with ten plasma samples. At this concentration, the enzyme not only failed to enhance platelet aggregation but in some samples appeared to cause inhibition of aggregation (figure 20 and

table 41). One minute after the addition of ADP (0.5 μg per ml plasma) the mean optical density reading of the control plasma was 0.513 $\pm$ 0.061 and of the test plasma 0.544 $\pm$ 0.033, ($t = 2.119$, $p < 0.10$). This result does not agree with the observations made with the Chandler tube system (Chapter 4) in which trypsin, at a similar concentration produced an acceleration of platelet aggregation in every plasma sample investigated. However, the same ten plasma samples were used to examine the effect of trypsin at a concentration of 100 μg per ml plasma. At this concentration trypsin treatment of plasma caused enhancement of aggregation in every sample (figure 20 and table 42). 1½ minutes after the addition of ADP (0.5 μg per ml plasma) to the system the mean optical density reading of the control samples was 0.529 $\pm$ 0.041 and of the test samples 0.501 $\pm$ 0.041, ($t = 3.925$, $p < 0.005$). The effect of trypsin was even more dramatic when it was studied at a concentration of 1 mg per ml plasma. In a series of seven plasma samples, trypsin, at this concentration, was added to the plasma which was immediately agitated. Under these conditions trypsin produced a marked enhancement of aggregation in every plasma sample even although the plasma and enzyme had not been incubated together at 37°C for 10 minutes and no ADP was added to the system (figure 21 and table 43). 2 minutes after the onset of agitation the mean optical density readings

of the control samples was 0.540 ± 0.041 and of the test samples 0.263 ± 0.204, ($t = 3.080$, $p < 0.025$). In three of the trypsin treated plasma samples small fibrin strands appeared towards the end of the 10 minute period during which platelet aggregation was monitored. This may have been due to the fact that in the trypsin treated plasma samples no platelet disaggregation was observed and thrombus formation was occurring following the formation of irreversible platelet aggregates. In an attempt to determine whether trypsin could cause enhancement of platelet aggregation in the absence of fibrin formation and in the absence of added ADP, the experiment with trypsin (1 mg per ml plasma) was repeated with seven plasma samples which were incubated at 37°C for 10 minutes with streptokinase (1,000 M.I.H. units per ml plasma) before the addition of trypsin. Under these conditions the addition of trypsin again resulted in platelet aggregation as soon as agitation was commenced (figure 22, table 44). Thirty seconds after the onset of agitation the mean optical density of the control samples was 0.525 ± 0.009 and of the test samples 0.345 ± 0.060, ($t = 6.485$, $p < 0.001$). No fibrin strands were visible in any of the plasma samples investigated.

As with streptokinase, it was thought that the difference between the effects of trypsin, at a concentration of 10 µg per ml, on platelet aggregation in the Chandler tube and turbidimetric systems might be due

to the different conditions under which the enzyme was incubated with the plasma. Consequently, the effect of trypsin on platelet aggregation in plasma diluted in the same proportions as in the Chandler tube system was investigated. Trypsin (10 µg per ml plasma) was added to seven plasma samples which were diluted with 0.9 per cent saline in the proportions, 1 ml plasma to 1.5 ml saline. After 10 minutes incubation at 37°C, ADP (1.0 µg per ml plasma) was added to the control and test samples. 4 minutes after the addition of ADP the mean optical density reading of the control plasma was 0.254 ± 0.118 and of the test plasma 0.273 ± 0.110, ($t = 0.965$, $p < 0.40$). Under these conditions no pattern became apparent for the action of trypsin on platelet aggregation (figure 17, table 45).

The action of trypsin is dependent on the presence of calcium ions, although the concentration of calcium is not normally critical for its proteolytic action. Calcium ions are present in normal citrated plasma since the anticoagulant, sodium citrate, does not effectively chelate all the calcium ions present in whole blood. In an attempt to confirm that the action of trypsin, at low concentration, on platelet aggregation is independent of the concentration of calcium it was decided to investigate the effect, on ADP induced platelet aggregation, of incubation of trypsin (10 µg/ml

plasma) and plasma to which calcium had been added at the same concentration as used for the Chandler tube system. In order to prevent clot formation during the incubation period, heparin was to be added to the system. A pilot experiment was carried out to determine the minimum concentration of heparin required to prevent clot formation in the plasma within the period of the experiment. To 0.5 ml normal citrated plasma was added 0.05 ml heparin solution and 0.05 ml, 0.25 M $CaCl_2$. The time for clot formation to occur was recorded and is tabulated (table 8). A concentration of 10 units heparin per ml plasma was selected as appropriate for the experiment. A second pilot experiment was carried out in which heparin (10 units per ml plasma) was incubated with plasma at 37°C for 10 minutes before the ADP reactivity was estimated in the turbidimetric system. It was found that under these conditions heparin appeared to cause enhancement of platelet aggregation. The effect of heparin on platelet aggregation has been further investigated and will be discussed below. Thus it was not possible to investigate the effect of trypsin on platelet aggregation in the presence of a relatively high concentration of calcium. As a compromise, the effect of trypsin on platelet aggregation in plasma to which a low concentration of calcium ions had been added was investigated.

In a series of seven plasma samples calcium chloride (0.25 m mole per ml plasma) was added to both the control and test samples and trypsin (10 µg per ml plasma) to the test sample. After 10 minutes incubation at 37°C platelet aggregation was induced by the addition of ADP. At this concentration the presence of calcium chloride did not substantially alter the result from that obtained for trypsin at the same concentration in plasma to which calcium chloride had not been added (figure 23, table 46). The mean optical density readings of the control and test samples 1 minute after the addition of ADP were 0.372 $\pm$ 0.115 and 0.331 $\pm$ 0.118 respectively, ($t = 1.363$, p 0.30). The experiment was repeated with a further series of seven plasma samples and a higher concentration of calcium. Incubation of trypsin with plasma to which calcium chloride, at a concentration of 1.0 m mole per ml plasma, had been added resulted in no significant alteration of platelet aggregation from that of the control sample (figure 24, table 47). 3 minutes after the addition of ADP, the mean optical density reading of the control sample was 0.371 $\pm$ 0.196 and of the test sample 0.446 $\pm$ 0.143, ($t = 1.690$, $p < 0.20$). Thus in the turbidimetric system the action of trypsin on platelet aggregation does not appear to depend on the concentration of calcium in the system.

Certain of the observations made on the action of trypsin on the acceleration of platelet aggregation in the Chandler tube system

(Chapter 4), it was suggested that the presence of platelets enhanced the effect of the enzyme. This possibility has been further examined with the turbidimetric system. In a series of seven plasma samples, platelet rich plasma was divided and one portion centrifuged at 1600g for 15 minutes to obtain platelet poor plasma. The platelet poor plasma samples were subdivided, one portion used as a control and the other portion treated with trypsin (100 µg per ml plasma) at 37°C for 10 minutes. After incubation soybean trypsin inhibitor (100 µg per ml plasma) was added to both the control and test samples together with a volume of platelet rich plasma equal to that of the incubated platelet poor plasma sample. Under these conditions trypsin appeared to have no significant effect on platelet aggregation (figure 25, table 48). 10 minutes after the addition of ADP, the mean optical density reading of the control plasma was 0.480 ± 0.133 and of the test plasma 0.453 ± 0.121, ($t = 1.170$, $p < 0.30$). However, when the experiment was repeated with the times of addition of platelet rich plasma and platelet poor plasma reversed, trypsin at a similar concentration appeared to cause significant enhancement of platelet aggregation even in the presence of soybean trypsin inhibitor (figure 26, table 49). 10 minutes after the addition of ADP to the system the mean optical density reading of the control samples was 0.484 ± 0.104 and of the test samples 0.448 ± 0.083, ($t = 2.768$, $p < 0.05$).

It would therefore appear that platelets themselves or some factors closely related to platelets may act as the substrate for trypsin. In order to determine whether platelets themselves acted as the substrate for the enzymatic action of trypsin, the effect of trypsin on platelet aggregation in a suspension of washed platelets was investigated.

In a series of seven citrated plasma samples, the plasma was centrifuged at 1600g for 15 minutes, the supernatant platelet poor plasma decanted and the platelet pellet washed twice with bicarbonate saline citrate and finally with bicarbonate saline (Born and Cross, 1964). The platelet suspension was centrifuged at 1600 g for 10 minutes between each wash. After a series of pilot experiments 0.1 M tris buffer, pH 7.5 was chosen as a suitable medium for the platelet suspension and thus after washing the platelet pellet was resuspended in a volume of the buffer, containing additional sodium, potassium and calcium ions, sufficient to give a platelet count similar to that of the initial platelet rich plasma sample. Trypsin (10 μg per ml) appeared to have little effect on platelet aggregation in this platelet suspension (figure 27, table 50). 10 minutes after the addition of ADP (0.5 μg per ml suspension) to the system, the mean optical density of the control samples was 0.503 ± 0.058 and of the test samples 0.516 ± 0.025, ($t = 0.291$, $p < 0.80$). This experiment was rather disappointing in that even in the control

sample platelet aggregation occurred only to a limited extent. This system could not be improved by resuspending the washed platelet pellet in a volume of its own platelet poor plasma. This observation together with the apparent loss of a considerable number of platelets during the washing procedure seemed to indicate that this was not an ideal system for the investigation of platelet aggregation in a washed platelet suspension. Accordingly the experiment was repeated with a different washing regimen (Haslam, 1964). Blood was collected from a volunteer and anticoagulated by the addition of 9.25 ml whole blood to 0.75 ml, 0.077 M sodium EDTA, pH 7.2. The blood was centrifuged at 600 g for 5 minutes to obtain platelet rich plasma which was then centrifuged as above to obtain platelet poor plasma. The platelet pellet was then washed as set out in the methods section and was resuspended finally in a medium containing 9 volumes, 0.154 M sodium chloride and 1 volume 0.154 M tris buffer pH 7.2, to give a final platelet count of approximately 5×10^5 per cumm. For the estimation of platelet aggregation, 0.2 ml, 0.154 M KCl, 0.02 ml, 0.154 M $MgSO_4$ and 0.02 ml, 0.11 M $CaCl_2$ were added to 1.76 ml of this platelet suspension. The effects of trypsin at the concentrations of 10 µg, 100 µg and 1 mg per ml suspension on platelet aggregation in this suspension were investigated. The results for trypsin at the concentrations 10 µg and 1 mg per ml suspension are

shown in figure 28. For clarity, the readings obtained with trypsin at a concentration of 100 µg per ml suspension have been omitted as they lay between the readings for the other concentrations. It would appear that trypsin can almost completely inhibit platelet aggregation in this platelet suspension. The action of trypsin at 100 µg per ml and 1 mg per ml was not altered by the addition of fibrinogen (400 mg per ml) to the system (tables 9, 10), although fibrinogen, at this concentration, when added alone to the system could produce enhancement of platelet aggregation (figure 29). The effect of streptokinase (2,000 N.I.H. units per ml suspension) on platelet aggregation in the washed platelet suspension has also been investigated. The results are shown in figure 30 and it would appear that streptokinase has no effect on platelet aggregation in this system. The effect of trypsin and streptokinase on platelet aggregation in a washed platelet suspension was unchanged when the pH of the tris buffer of the suspension medium was increased to pH 8.2. From the results it would seem that the effect of trypsin and streptokinase in enhancing platelet aggregation in platelet rich plasma is not mediated through a direct action on the platelets.

An attempt was made to investigate the action of thrombin on platelet aggregation in a washed platelet suspension. Thrombin (0.04 N.I.H. units per ml platelet suspension) was incubated with 2 ml platelets

suspended in medium (tris buffer 0.1 M, pH 7.2 plus additional ions). Unfortunately platelet aggregation in this sample could not be assayed since a small fibrin clot had formed before the incubation period was over. This observation would seem to indicate that the washing schedule carried out for these experiments did not remove the coagulation factors which are believed to be closely associated with the platelets in blood (Adelson et al., 1961; Iatridis and Ferguson, 1965).

From the observations made on the effect of ADP and adenosine on platelet aggregation in trypsin treated plasma it was suggested that the action of trypsin might be mediated through the ADP mechanism. Evidence to support this view has been obtained from the study of the action of trypsin on ADP induced platelet aggregation. Additional supporting evidence has been obtained from the investigation of the effect of adenosine on trypsin treated plasma. In a series of seven plasma samples, one portion of the plasma was incubated with trypsin (100 µg per ml) and a second portion with trypsin (100 µg per ml) and adenosine (100 µg per ml). In every plasma sample investigated platelet aggregation was inhibited in the test sample (figure 31, table 51). One minute after the addition of ADP (0.5 µg per ml plasma) to the system, the mean optical density reading of the control samples was 0.491 ± 0.054 and of the

test samples 0.567 $\pm$ 0.039, ($t = 2.724$, $p < 0.05$). The ability of adenosine to inhibit the action of trypsin on platelet aggregation appeared to be dependent on the relative concentrations of trypsin and adenosine. In a series of seven plasma samples, trypsin (1 mg per ml) was added to the control sample and trypsin (1 mg per ml) and adenosine (0.5 mg per ml) to the test sample. At these concentrations adenosine was only partially able to inhibit the action of trypsin (figure 21, table 53); in three of the plasma samples investigated adenosine caused marked inhibition in the degree of platelet aggregation and in the remaining four samples aggregation was virtually unchanged from that occurring in the absence of adenosine. 4 minutes after the addition of trypsin to both the control and test samples the mean optical density reading of the control plasma was 0.177 $\pm$ 0.182 and of the test plasma 0.303 $\pm$ 0.086, ($t = 1.818$, $p < 0.20$). In this series of experiments the plasma was not preincubated with either trypsin or adenosine before agitation of the plasma was commenced and changes in optical density monitored. As ADP was not added to the system the partial inhibiting action of adenosine would appear to be due to its action at some stage in the sequence of changes produced by trypsin during the initiation of platelet aggregation.

During the investigation of the effect of streptokinase on platelet aggregation it was observed that the action of streptokinase did not

appear to be changed if aggregation was induced by ADP or by nor-adrenaline. A similar observation was made with trypsin. The effect of trypsin (10 µg per ml) was investigated in a series of 10 plasma samples in which aggregation was induced by nor-adrenaline (figure 32, table 53). 2 minutes after the addition of nor-adrenaline (1.0 µg per ml) to the system, the mean optical density reading of the control plasma was 0.440 ± 0.141 and of the test plasma 0.395 ± 0.207, ($t = 1.691$, $p < 0.20$). Thus, just as in the series of experiments in which aggregation was induced with ADP, trypsin at a concentration of 10 µg per ml plasma appeared to have no significant effect on nor-adrenaline induced aggregation.

The effect of trypsin at a concentration of 10 µg per ml on platelet aggregation in the turbidimetric system does not agree with the effect observed for trypsin at the same concentration in the Chandler tube system (Chapter 4). From the use of radioactively tagged fibrinogen in the Chandler tube system it was observed that as a result of incubation of plasma with this concentration of trypsin marked digestion of fibrinogen occurred. In an attempt to determine whether digestion of fibrinogen also occurred during incubation and platelet aggregation in the turbidimetric system, a sample of I^{131} fibrinogen was added to the control and test plasma samples before incubation in five of the samples used for the investigation of trypsin (10 µg per ml) on diluted plasma and in

five of the samples used in the study of nor-adrenaline induced aggregation. 0.2 ml amounts of both the control and test samples were removed before incubation and after platelet aggregation, 0.1 ml thrombin (100 N.I.H. units per ml) added and the amount of radioactivity present in the clot which formed counted on a scintillation counter (Scaler 1700, Isotope Developments Ltd.). The percentage clottable protein present after aggregation was calculated from the two results. The figures are presented in table 7. It would appear that in neither the undiluted nor in the diluted plasma samples is there any significant reduction in the amount of clottable protein present before and after treated with trypsin at this low concentration. This is in complete contrast to the results obtained from the use of radioactive fibrinogen in the Chandler tube system and may in part explain the different results obtained for the effect of trypsin, at this concentration, on platelet aggregation in the two systems.

From the results presented above it may be concluded that the proteolytic enzymes, trypsin and streptokinase, can, under certain conditions, result in enhanced platelet aggregation in plasma samples with which they have been incubated. In an attempt to determine whether platelet aggregation could be enhanced by other proteolytic enzymes, the effect of urokinase, chymotrypsin and ficin on ADP

induced platelet aggregation was investigated.

Effect of proteolytic enzymes. The effect of urokinase on ADP induced aggregation was investigated in a series of ten plasma samples. Urokinase was studied at the concentration which produced maximum lytic activity as determined for each plasma sample in a urokinase sensitivity test. The results are shown in figure 33 and table 54. It would appear that at this concentration, incubation of plasma with urokinase results in some inhibition of platelet aggregation. 3 minutes after the addition of ADP (0.5 µg per ml plasma), the mean optical density of the control plasma was 0.400 ± 0.264 and of the test plasma 0.499 ± 0.128, ($t = 3.134$, $p < 0.02$). Urokinase, like streptokinase, is an activator of plasminogen (Sherry et al., 1959) and it would seem that at an equivalent concentration these enzymes have a similar effect on platelet aggregation in the turbidimetric system.

Chymotrypsin and ficin were studied as enzymes with which the action of trypsin could be compared. Both these proteolytic enzymes have pH optima in a similar range to that of trypsin but as shown in table 2, neither of these enzymes appeared to be able to convert prothrombin to thrombin.

Chymotrypsin like trypsin is a pancreatic enzyme and like trypsin it is able to hydrolyze tyrosylglycine linkages. Its effect on platelet aggregation was studied in a series of seven plasma samples. The results are shown in figure 34 and table 55. It would appear that chymotrypsin

at a concentration of 100 μg per ml plasma has no effect on ADP induced platelet aggregation. 5 minutes after the addition of ADP, the mean optical density reading of the control samples was 0.364 ± 0.156 and of the test samples 0.377 ± 0.144, ($t = 0.412$, $p < 0.070$).

Ficin is a proteolytic enzyme obtained from fig latex and is able to hydrolyze certain synthetic esters such as benzoylarginine amide. The enzyme was incubated, at a concentration of 10 μg per ml plasma, with a series of 12 plasma samples. It would appear that ficin at this concentration has no significant effect on ADP induced platelet aggregation (figure 35, table 56). 5 minutes after the addition of ADP, the mean optical density reading of the control plasma was 0.360 ± 0.160 and of the test sample 0.335 ± 0.165, ($t = 0.996$, $p < 0.40$).

Further experiments with higher concentrations would have to be carried out before it would be possible to assess the full significance of the effect, if any, on platelet aggregation of the proteolytic enzymes chymotrypsin and ficin.

During a pilot experiment described above it was observed that heparin at a concentration of 10 units per ml plasma appeared to cause enhanced aggregation. This observation was further investigated together with the effect of heparin on ADP induced aggregation at the concentrations 0.05 units per ml and 500 units per ml.

Effect of heparin. Following the single observation with heparin (10 units per ml) a further seven plasma samples were investigated. The incubation of plasma with heparin at this concentration appeared to result in some enhancement of aggregation (figure 36 table 57). 4 minutes after the addition of ADP to the system the mean optical density reading of the control plasma was 0.196 ± 0.146 and of the test plasma 0.096 ± 0.091, ($t = 2.594$, $p < 0.05$). This result was contrary to the effect of heparin on platelet aggregation in the Chandler tube system observed by Cunningham et al., (1964). However, these workers had used heparin at a concentration of 0.1 to 0.2 units per ml plasma. Thus the effect of heparin at a concentration of 0.05 units per ml plasma on ADP induced aggregation was investigated in an attempt to demonstrate the anticoagulant action of heparin, in therapeutic concentrations, on platelet aggregation. The effect of heparin (0.05 units per ml) was investigated in a series of 14 plasma samples. Heparin, at this concentration, appeared to cause marked enhancement of platelet aggregation, figure 37 and table 58. 10 minutes after the addition of ADP, the mean optical density of the control plasma was 0.436 ± 0.139, and of the test plasma 0.376 ± 0.167, ($t = 3.297$, $p < 0.010$). Thus the effect of heparin on the enhancement of platelet aggregation appeared to be even greater at a concentration of 0.05 units per ml than at 10 units

per ml. Thus it was observed that, contrary to expectations, at low concentrations heparin appears to enhance platelet aggregation in the turbidimetric system. The effect of heparin, at a high concentration, on ADP induced aggregation was investigated. Heparin (500 units per ml plasma) was incubated with a series of 8 plasma samples before platelet aggregation was induced by the addition of ADP (0.5 µg per ml). In every sample investigated heparin appeared to cause some inhibition of platelet aggregation (figure 38, table 59). 10 minutes after the addition of ADP the mean optical density reading of the control plasma was 0.422 ± 0.184 and of the test plasma 0.508 ± 0.140, ($t = 2.563$, $p < 0.05$). It would seem, therefore, that heparin must be present in a relatively high concentration before it can inhibit ADP induced platelet aggregation.

A series of experiments was described above in which the action of trypsin on platelet aggregation in a suspension of washed platelets in tris buffer, pH 7.5. The same series of seven bicarbonate-citrate solution washed platelet suspensions was used to determine whether heparin caused enhancement of platelet aggregation by direct action on the platelet. Over the series of experiments heparin (10 units per ml) appeared to have no significant effect on platelet aggregation in this suspension (figure 27, table 60). 10 minutes after the addition of ADP the mean optical density reading of the control plasma was $0.503 \pm$

0.058 and of the test plasma 0.512 $\pm$ 0.053, ($t = 0.756$, $p < 0.50$). Thus it would appear that heparin does not enhance platelet aggregation by direct action on the platelet and that some plasma factors must therefore be essential for its 'coagulant' action.

Discussion.

The investigations of the effect of the enzymes streptokinase and trypsin in the turbidimetric system show that under certain conditions these enzymes appear to be able to enhance the extent to which platelet aggregation can occur in platelet rich plasma. It would appear, however, that these enzymes do not exert their action directly on the platelets but their ability to enhance platelet aggregation depends on the alteration of one or more plasma constituents of which one may be fibrinogen since the results seem to indicate that the effect of the enzymes on platelet aggregation may be related to their fibrinogenolytic activity.

Effects of Streptokinase and Trypsin. The turbidimetric system for the measurement of platelet aggregation appears to be less sensitive to the action of streptokinase and trypsin, at low concentrations than the Chandler tube system. The results of the investigation of some of the differences in the conditions under which the enzymes react with the plasma in the two systems suggest that the apparent different effects of the enzymatic action on platelet aggregation cannot be explained solely

on a difference in experimental conditions. When trypsin and streptokinase were incubated with plasma, diluted in the same proportions as for the Chandler tube system, there was no significant difference in the effects observed compared to those for the undiluted samples. The effect of trypsin, at the relatively low concentration of 10 µg per ml plasma, did not appear to be influenced by the concentration of calcium ions present in this system (figures 23 and 24). Consequently, some other explanation must be sought for the apparent difference of the effect of low concentrations of trypsin and streptokinase on platelet aggregation when measured in the Chandler tube and the turbidimetric systems.

As discussed in chapter 2, during the process of thrombus formation a sequence of reactions, involving platelets, occurs (Glynn et al., 1966; Hellem and Owren, 1964; Käser-Glanzmann and Lüscher, 1962; Lüscher, 1967; Mustard et al., 1964a; Poole and French, 1961; Roskam, 1961; Sharp, 1961). It is thought that after the adhesion of one or more platelets to the damaged tissue, ADP, which is released from the adhering platelets, stimulates the adhesion of more platelets leading to the formation of a permeable platelet plug. At this stage in the reaction sequence it has been shown that platelets maintain their morphological integrity (Hellem and Owren, 1964; Sharp, 1964) and

aggregation is thought to be reversible (Hellem and Owren, 1964). During the formation of a thrombus, however, the platelets in this permeable platelet plug gradually undergo viscous metamorphosis with the loss of morphological integrity and the formation of a platelet plug impermeable to blood flow. The turbidimetric method estimates reversible platelet aggregation (Born and Cross, 1963b). This is confirmed by the examination of the mean control curves in which plasma was incubated with saline before ADP was added to the system and it can be seen in figures 16 and 20 that after the initial aggregation in response to the challenge by ADP the platelet aggregates are rapidly dispersed. With the turbidimetric system, microscopic platelet aggregation is sufficient to alter the turbidity of the plasma which is recorded as a change in optical density on the galvanometer scale but with the Chandler tube system, the appearance of macroscopic platelet aggregates is taken as the end point of the effect of the substance under investigation on the rate of formation of platelet clumps. In addition, in the Chandler tube system, the appearance of platelet clumps is followed by fibrin formation. Thus, it is likely that in the Chandler tube system the end point of the reaction is assessed at a point at which platelet aggregation has become irreversible and hence the Chandler tube and turbidimetric systems are measuring different stages in the reaction sequence. This may, at least in part, account

for the apparent different effect of low concentrations of trypsin and streptokinase on platelet aggregation in the two systems since it would appear that the enzymes do not act directly on the platelets (figures 28 and 30) but on certain of the plasma proteins. Evidence to support this view may be drawn from figure 20 in which it would appear that when platelet aggregation is enhanced by the action of the enzymes on the plasma, platelet disaggregation is impaired over that observed in the control samples.

Recently it has been shown that a wide range of physiological compounds can induce platelet aggregation in vitro of which one is nor-adrenaline (Mitchell and Sharp, 1964, O'Brien, 1963b and 1964). The effects of streptokinase and trypsin at low concentrations are shown in figures 19 and 32 and it can be seen that, as for ADP induced aggregation, these enzymes exert no significant effect on nor-adrenaline induced aggregation. Platelet aggregation initiated by nor-adrenaline is similar to that induced by ADP in that it is reversible (Mitchell and Sharp, 1964). Consequently, this observation tends to support the view that the apparent conflict in the results obtained for the action of low concentrations of the enzymes streptokinase and trypsin in the Chandler tube and turbidimetric systems is dependent on the stage in the reaction sequence of the formation of platelet aggregates that is measured

by the system rather than on the experimental conditions under which the enzymes react with the plasma in the two systems.

A further point, which may be relevant to the consideration of results obtained with the Chandler tube and turbidimetric systems is the different stimuli causing aggregation in the two systems. The Chandler tube system reflects the speed of platelet aggregation in an artificial circulation in which fibrin formation occurs. As discussed in chapter 4, the explanation for the appearance of platelet aggregates in this system is not clear but aggregation may be initiated by trace amounts of ADP released into the system during the procedure of centrifugation to obtain platelet rich plasma and the subsequent handling of the samples. On the other hand, in the turbidimetric system, relatively large amounts of ADP are added to plasma to initiate platelet aggregation. The concentration of ADP is probably much greater than that actually required to cause the initial stimulation of platelet aggregation and consequently there may be masking of the effect of low concentrations of streptokinase and trypsin on platelet aggregation in this system.

In chapters 2 and 4, it was suggested that, as in the fibrinolytic enzyme system, the action of streptokinase on platelet aggregation may be mediated through its ability to convert plasminogen to plasmin.

Some evidence which would seem to support this has been obtained from the investigation of streptokinase and antiplasmin in the turbidimetric system. When plasma was incubated with streptokinase, at a concentration which produced maximum lytic activity, ADP induced aggregation appeared to be impaired (figure 16); when the plasma was diluted and streptokinase added, at a concentration of a similar order, the enzyme appeared to have little effect on aggregation (figure 17); but when the enzyme, at a relatively high concentration was incubated with plasma in which the antiplasmin had been destroyed, streptokinase appeared to produce a significant increase in the extent to which aggregation occurred (figure 18). Thus, it may be concluded that streptokinase is not able to produce enhancement of platelet aggregation in the presence of antiplasmin, indicating that the effect of the enzyme is mediated through plasmin.

The effect of trypsin at the relatively high concentrations of 100 µg/ml and 1 mg/ml on ADP induced platelet aggregation appears to be similar to that observed for the effect on the initial stages of thrombus formation as measured in the Chandler tube system. (figures 6, 7, 20 and 21). As in the Chandler tube system in which trypsin (1 mg/ml) could cause significant acceleration of platelet aggregation without prior incubation of the enzyme and plasma, so trypsin (1 mg/ml) could induce aggregation in the turbidimetric system without either prior

incubation before the plasma was stirred or the addition of ADP to the system (figure 21). In this series of experiments, however, the enhancement of aggregation cannot be accounted for solely by the action of trypsin since in three of the plasma samples investigated, small fibrin strands appeared towards the end of the ten minute period during which platelet aggregation was monitored. In these three plasma samples at least, thrombin generation may have been a contributory factor. This concentration of trypsin (1 mg/ml) could be made to enhance platelet aggregation in the absence of fibrin formation by prior incubation of the plasma samples with streptokinase (2,000 N.I.H. units/ml) before trypsin was added to the system (figure 22). No fibrin strands became visible in any of the seven plasma samples during the period of the experiment, perhaps because the clottable protein had been digested by plasmin, formed under the action of streptokinase, before the addition of trypsin to the system. Some of the prothrombin present in the plasma may also have been digested but as data is not available on the rate of destruction of prothrombin under these particular experimental conditions, the possibility that thrombin was being generated in this system cannot be excluded. The in vitro investigations of the effect of trypsin on the coagulation system described in chapter 4 (tables 2 and 3) seemed to suggest, however, that the effect of trypsin on platelet aggregation was not entirely

due to its action on coagulation as estimated by its ability to convert prothrombin to thrombin but that its fibrinogenolytic activity was also involved in the process.

The results obtained in the investigation of the clottable protein present after ADP and nor-adrenaline induced aggregation in streptokinase and trypsin treated plasma support the hypothesis that fibrinogenolysis is critical to the action of streptokinase and trypsin in enhancing platelet aggregation (table 7). In this system, in which these enzymes had no significant effect on platelet aggregation (figures 17, 19 and 32), the percentage clottable protein did not appear to differ significantly between the control and enzyme treated plasma samples. In the Chandler tube, however, similar concentrations of these enzymes not only produced marked acceleration of platelet aggregation (figures 5 and 6) but also appeared to cause a significant decrease in the amount of clottable protein incorporated into thrombi (table 1).

In chapter 4 it was suggested, from the results of the investigations of the effect of incubation of trypsin with platelet poor plasma and platelet rich plasma in the Chandler tube system, (figures 8 and 9), that the presence of platelets appeared to enhance the tryptic effect. Similar results have been obtained with the turbidimetric system (figures 25 and 26). During this investigation, soybean trypsin

inhibitor was added to the trypsinized plasma before platelet aggregation was assayed. The results suggest that trypsin is not acting directly on the platelet aggregation mechanism but that the enzyme produces some change either on the plasma proteins or the platelets themselves which can subsequently influence platelet aggregation. However, from the investigation of the effect of streptokinase and trypsin on a washed platelet suspension it would appear that the enhancement of platelet aggregation apparently produced by these enzymes is not mediated through direct enzymatic action on the platelets.

Incubation of streptokinase (2,000 N.I.H. units/ml) with a washed platelet suspension appeared to have little effect on ADP induced platelet aggregation in the sample (figure 30). Morse et al., (1965) have demonstrated that incubation of washed platelets with streptokinase alone, unlike the results with trypsin, plasmin or thrombin, produced no detectable alterations in the platelets. Hence, it seemed unlikely that the apparent slight enhancement of the aggregation of washed platelets produced by streptokinase could be explained on the basis of the enzyme causing any alteration of the platelets themselves. The action of streptokinase on fibrinolysis has been demonstrated to be due to its ability to convert plasminogen to plasmin (Sherry et al., 1959) and it has also been shown from the results presented above that

its effect on platelet aggregation may also be mediated through the same reaction. Thus, if this mechanism were to operate in washed platelet suspensions, either exogenous plasminogen would have to be added to the system or plasminogen, or a precursor, which had not been removed by the washing procedure would have to be present in association with the platelets. It has been demonstrated that platelets are surrounded by an atmosphere, sometimes referred to as the 'plasmatic atmosphere', which contains a number of coagulation factors (Adelson et al., 1961; Iatridis and Ferguson, 1965). However, not only may plasminogen be present, but antiplasmin may also be adsorbed onto the platelet surface and in addition platelets themselves possess relatively high antiplasmin activity (Johnson and Schneider, 1953; Alkjaersig, 1961). In the present system it is unlikely that platelet antiplasmin may play a significant role since, presumably, some rupture of the platelet membrane would have to occur before it could be released into the system. On the basis of one experiment it is not possible to draw more than tentative conclusions from the result. Plasma factors may be present in the washed platelet suspension since incubation of the suspension with thrombin resulted in the formation of a fibrin clot. However, with the present limited available information it is not possible to define whether the fibrinogen was present in association

with the platelet membrane or, since it has been shown that thrombin can release and inactivate clottable protein from platelets (Morse et al., 1965; Schmid et al., 1962), was released from the platelets by thrombin.

The results of the investigations of trypsin, at the concentrations 10 µg, 100 µg and 1 mg per ml platelet suspension, show that the enzyme appears to cause impaired aggregation of ADP induced platelet aggregation in a washed platelet suspension (figure 28). This result does not agree with the effect of trypsin on washed platelets observed by Haslam (1964). The discrepancy may be explained by the fact that Haslam used a lower concentration of trypsin (2 µg per ml platelet suspension) and that he did not incubate the trypsin and washed platelet suspensions together before investigating platelet aggregation. Schmid et al., (1962) have shown that washed platelets treated with trypsin (50 µg per ml) remain morphologically intact although clottable protein had been removed from the platelets and they cannot subsequently be aggregated by thrombin. This result has been confirmed by Morse et al., (1965) who further demonstrated that platelets treated with trypsin, at concentrations less than 2 µg per ml, retain clottable protein and can be aggregated by thrombin. Fibrinogen has been demonstrated to be an integral part of the platelet structure (Castaldi and Caen, 1966; Gokcen and Yunis, 1963; Grette,

1962; Morse et al., 1965; Nachman et al., 1964; and Schmid et al., 1962), and it has also been shown to be closely associated with the platelet surface (Adelson et al., 1961; Iatridis and Ferguson, 1965). In addition, fibrinogen has been shown to be an important factor in ADP induced platelet aggregation (McLean et al., 1964). It would appear that the addition of fibrinogen alone to a washed platelet suspension is sufficient to promote enhancement of ADP induced platelet aggregation (figure 29). This result is in agreement with the observations for the effect of fibrinogen on platelet aggregation in platelet rich plasma (Cross, 1964; Solum and Stormorken, 1965) and of Kopec (1966) for washed platelet suspension, and gives support to the concept of the involvement of fibrinogen in the mechanism of platelet aggregation. The marked impairment of aggregation caused by trypsin could not be reversed by the addition of fibrinogen to the system although Schmid et al., (1962) have been able to demonstrate that the addition of fibrinogen to trypsinized platelet suspensions restored their ability to aggregate in the presence of thrombin. In the present experiment, however, trypsin and fibrinogen were present together in the washed platelet suspension during the incubation of the mixture so that the possibility exists that fibrinogen itself was digested before platelet aggregation was assayed.

In order to make the investigations of the effects of streptokinase and trypsin on washed platelets more comparable to the investigations in

platelet rich plasma, lower concentrations of the enzymes should be used to allow for the presence of antiplasmin (Sherry et al., 1959) and antitrypsin (Wu and Laskowski, 1960) present in plasma. Nevertheless, it may be concluded from the experiments that the apparent ability of the enzymes streptokinase and trypsin to enhance aggregation is not mediated through their direct action on the platelet but requires the presence of one or more plasma factors.

It was suggested from the results of the investigations of the combined action of trypsin and ADP and trypsin and adenosine in the Chandler tube system (figures 13 and 15) that theeffect of trypsin was mediated through the ADP mechanism of platelet aggregation (Gaarder and Laland, 1964; Salzman et al., 1966). This has been confirmed with the turbidimetric system in which not only can trypsin be made to cause significant enhancement of ADP induced aggregation (figure 20), but also it would appear that adenosine can inhibit the action of trypsin in both the presence and absence of ADP (figures 31 and 21).

Effects of proteolytic enzymes. At the concentrations studied, neither urokinase, ficin nor chymotrypsin appear to cause any enhancement of ADP induced platelet aggregation. Urokinase, at an equivalent concentration to streptokinase, appeared to exert a similar effect on ADP induced aggregation in that aggregation appeared to be impaired in some of the

samples investigated (figures 16 and 33). Urokinase is an activator of plasminogen (Astrup and Sterndoff, 1953; Sobel et al., 1952; Williams 1951), and consequently it is conceivable that its action in plasma like that of streptokinase, is mediated through the formation of plasmin.

Ficin and chymotrypsin, at a concentration of 100 µg per ml plasma, produced no apparent effect on ADP induced aggregation (figures 34 and 35). Table 2 shows that at this concentration, these proteolytic enzymes did not appear to be able to convert any plasma prothrombin to thrombin. Morse et al., (1965) have shown that papain and chymotrypsin, like thrombin, trypsin and plasmin, are able to bring about inactivation of the clottable protein of washed platelets with the release of serotonin and ATP into the system; such platelets are not then susceptible to aggregation by thrombin. However, the concentrations of papain and chymotrypsin required to produce a similar effect on washed platelets appeared to be somewhat greater than that for plasmin and trypsin. Consequently, the concentrations of ficin and chymotrypsin studied in the present series of experiments may be comparable to trypsin at a concentration of 10 µg/ml rather than at a concentration of 100 µg/ml. The effects of ficin and chymotrypsin at higher concentrations would have to be examined before reaching a conclusion that streptokinase and trypsin have a specific

effect not produced by other proteolytic enzymes.

Effect of Heparin. From the results presented in figures 36, 37 and 38 it would appear that the effect which heparin exerts on ADP induced platelet aggregation depends on the concentration of heparin in the system. At the relatively low concentrations of 0.05 units/ml and 10 units/ml plasma heparin appears to cause marked enhancement of ADP induced aggregation and that at a high concentration (500 units per ml) heparin may cause marked inhibition of platelet aggregation. These observations are in agreement with those of Hellem et al., (1963) on the action of heparin on platelet adhesiveness in platelet rich plasma. These workers demonstrated that platelets were more adhesive and more sensitive to ADP in the presence of low concentrations of heparin (4.5 - 180 units per ml) and that platelet adhesiveness was only inhibited by the presence of heparin at concentrations greater than 180 units per ml. Mustard et al., (1964b) have, however, demonstrated that heparin at a concentration of 100 units per ml plasma appears to cause no inhibition of ADP induced platelet aggregation. Thus it would appear that heparin has a coagulant action at low concentrations and an anticoagulant effect at high concentrations in vitro when platelet aggregation in the absence of fibrin formation is investigated. Cunningham et al., (1965) have shown that heparin at a low concentration (0.1 to 0.2 unit per ml) caused significant prolongation of the rate of formation of platelet aggregates

in an artificial circulation in which fibrin formation occurred as a sequel to platelet aggregation.

The results described for the action of heparin on ADP induced aggregation are not in agreement with observations made for the action of heparin on thrombin induced aggregation since it has been shown that heparin inhibits platelet aggregation in the presence of thrombin (Clayton and Cross, 1963; Mustard et al., 1964b; Zucker and Borrelli, 1959). The anticoagulant action of heparin in a system in which clot formation can occur may be accounted for by the observation that heparin can inhibit the action of thrombin on fibrinogen (Howell, 1928) and can also inhibit the conversion of prothrombin to thrombin (Howell and Holt, 1918; Howell, 1925).

From the series of experiments in which the effect of heparin, at a concentration of 10 units per ml, on washed platelets was examined it would appear that heparin does not cause enhancement of platelet aggregation by direct action on the platelets. Unfortunately, it would appear that the platelets had been damaged during the washing process since only minute traces of aggregation occurred in the control samples (figure 27) and so no conclusions may be drawn from this experiment. Hampton and Mitchell (1966) have shown that heparin appears to be able to alter the surface charge of human blood platelets as measured by their electrophoretic mobility. Heparin at concentrations up to 50 units per ml plasma appears to prevent the change in platelet electrophoretic mobility which

is usually observed after the addition of ADP and nor-adrenaline.

With the evidence at present available it is not clear why heparin appears to exert different effects on ADP induced aggregation depending on the concentration present in the system. The strong electronegative charge of heparin (Douglas, 1962c) may contribute to its antithrombotic properties since platelets themselves possess a strong negative surface charge (Abramson, 1928) and in addition it has been shown that dextrans exert a protective effect against thrombosis (Borgstrom et al., 1959).

Conclusions.

From the results presented in this chapter it would appear that the fibrinogenolytic agents streptokinase and trypsin exert an 'anticoagulant' effect on ADP induced aggregation when present in low concentrations but their effect becomes 'coagulant' when they are present in relatively high concentrations. This observation is in contrast to the effect of the anticoagulant agent, heparin, which appears to exert a 'coagulant' effect on ADP induced platelet aggregation at low concentrations and becomes 'anticoagulant' only at high concentrations.

From the investigations of the actions of trypsin and streptokinase on washed platelets, it appears that the enzymes do not exert their effect on aggregation by direct action on the platelets but require the presence of certain plasma factors. It would seem that the ability of streptokinase to enhance ADP induced platelet aggregation depends on the production of plasmin.

CHAPTER 6.

STUDIES ON THE EFFECT OF STREPTOKINASE AND TRYPSIN ON PLATELET ADHESIVENESS.

In this chapter are presented the results obtained from the investigations of the effect of streptokinase and trypsin on platelet adhesiveness. Platelet adhesiveness was assessed by the ability of platelets to adhere to glass beads and to each other in either citrated whole blood or platelet rich plasma during passage through a standard column of glass beads. The concentrations of ADP present in whole blood and plasma before and after the samples came into contact with the glass beads have been determined together with the apparent influence of trypsin digestion of whole blood and plasma on the concentration of nucleotide present in the samples.

The effect of the enzymes streptokinase and trypsin on platelet aggregation in platelet rich plasma has been described in chapters 4 and 5. Neither the Chandler tube system nor the turbidimetric system, however, is suitable for the investigation of platelet aggregation in the presence of red cells. This is a considerable disadvantage when an attempt is made to correlate in vitro findings with in vivo conditions. Hellem (1960) has devised a method for the investigation of platelet adhesiveness in the presence of red cells. A modification of this method (Hirsh et al., 1966a) has been used to investigate the effect of streptokinase and trypsin on platelet adhesiveness in both the presence and absence of red cells.

Materials.

Trypsin and Streptokinase were used as described in chapter 3.

Platelet rich plasma was prepared from citrated whole blood by centrifugation at 600g for 5 minutes at room temperature (approximately 20°C) and was kept at 20°C until platelet adhesiveness was assayed.

Glass Beads. These were Ballotini beads of 0.57 mm diameter. The beads were prepared for the columns by thoroughly washing with chromic acid followed by rinsing in 20 per cent sodium carbonate solution and finally in tap water. After the glass beads had been thoroughly rinsed in distilled water they were dried in a hot air oven.

Tubing. Portex transparent tubing (N.T. 13) was cut into lengths of 7.0 cm, the Esco translucent silicone tubing (3.0 mm bore, 2.0 mm wall) was cut into lengths of 1.5 cm and 1.0 cm and the Esco translucent silicone tubing (2.0 mm bore, 1.0 mm wall) was cut into lengths of 2.0 cm. All the lengths of tubing were prepared for use by thorough washing in tap water, followed by rinsing in distilled water and finally the tubing was thoroughly dried in a hot air oven.

Methods.

Estimation of platelet adhesiveness. The method was used as described in chapter 3. Citrated whole blood was allowed to stand at room temperature and was tested 30 to 60 minutes after collection. Platelet rich plasma was prepared from citrated whole blood which had been standing for 30 minutes at room temperature and was tested 40 to 60 minutes after collection. The

samples were pumped through a column of glass beads, 6 cm in length containing 2.5 gm washed glass beads, with a transit time lying between 29 - 30 seconds; observations lying outside this range were discarded. Duplicate platelet counts were set up on the samples before and after passage through the column and two platelet counts were read on each sample so that a total of four platelet counts were performed on each blood sample before and after passage through the column. The percentage platelet adhesiveness was calculated from the mean platelet counts.

Platelet counting was performed by the method of Dacie (1956c) as described in chapter 3.

White cell counts were performed as described by Dacie (1956b) 0.05 ml blood was added to 0.95 ml diluting fluid and the solution thoroughly mixed by gentle inversion. The counts were performed on an improved Neubauer counting chamber with a white light source. The diluting fluid was 2 per cent (V/V) acetic acid coloured pale violet with crystal violet.

ADP/AMP assays were carried out as described in chapter 3 with a commercially prepared test kit. The extracts of blood and plasma were prepared for analysis by the method of Holmsen et al., (1965) as described in chapter 3. The optical density readings E_1, E_2 and E_3 obtained during the oxidation of diphosphopyridine nucleotide (DPNH) in the indicator reaction were used to calculate the concentrations of ADP and AMP in the sample (Adam, 1965).

Calculation.

Extinction coefficient for DPNH at 25°C at 366 mμ is 3.30 ($cm^2/\mu moles$)

$$\Delta \frac{E\ ADP \times VA \times VE}{\varepsilon \times d \times Vp} = \mu moles\ ADP\ in\ extract$$

and $$\Delta \frac{E\ AMP \times VA \times VE}{\varepsilon \times d \times Vp \times 2} = \mu moles\ AMP\ in\ extract$$

Where $\Delta E\ ADP = E_1 - E_2$

$\Delta E\ AMP = E_2 - E_3$

VA = Volume in test mixture = 2.0 ml.

VE = Volume of extract = 0.5 ml.

ε = 3.30 ($cm^2/\mu mole$)

d = light path of the cuvette = 1 cm.

Vp = total volume of extract added to the cuvette

= 0.01106 ml

Under the stated conditions the calculation becomes

$\Delta E\ ADP \times 54.796 = \mu moles\ ADP$ per ml sample

and

$\Delta E\ AMP \times 27.398 = \mu moles\ AMP$ per ml sample

Results.

Effect of Streptokinase. The concentration of streptokinase used throughout

the series of experiments to investigate the effect of the enzyme on platelet aggregation was that concentration which produced maximum lytic activity. This was determined by performing streptokinase sensitivity tests on plasma obtained from a portion of each citrated blood sample before platelet adhesiveness in the sample was assayed. The citrated whole blood was divided into two samples, each of 2 ml; one sample was treated with streptokinase and the second sample with an equal volume of 0.9 per cent saline to act as a control. After incubation at 37°C for 10 minutes, the blood was pumped through a glass bead column and the percentage of platelets lost during the process calculated from the difference between the platelet counts before and after passage through the column. The value obtained was accepted as representing the percentage platelet adhesiveness of the sample. The results are shown in figure 39 and table 61 and it would appear that streptokinase treatment of blood results in a significant decrease in platelet adhesiveness. The mean percentage adhesiveness in the eight control samples was 47.9 ± 27.2 and in the eight test samples 21.3 ± 17.4, ($t = 2.402$, $p < 0.05$). This observation is in agreement with the results obtained with a similar concentration of enzyme in the turbidimetric system (figure 16) but does not agree with the findings for the effect of streptokinase in the Chandler tube system (figure 5). In both the Chandler tube and turbidimetric systems the plasma samples and the enzymes

under investigation were thoroughly mixed during the measurement of platelet aggregation; by rotation in an artificial circulation in one system and by vigorous stirring in the other system. This procedure may facilitate numerous collisions between the enzyme molecules and their substrate molecules and hence it may promote some enhancement of the enzymatic reaction. In the experimental design outlined above for the investigation of the effect of streptokinase on platelet adhesiveness, the enzyme and blood were not vigorously mixed. The experiment was therefore modified, in order that the conditions under which the enzyme could react with the blood were more closely comparable to the conditions in the turbidimetric and Chandler tube systems.

In a series of ten citrated blood samples, each sample was divided into two portions, each of 2 ml, which were added to small polystyrene stoppered containers (capacity 5 ml). To one sample was added streptokinase, at the concentration which produced maximum lytic activity in the sample, and to the second sample an equal volume of 0.9 per cent saline. Both test and control samples were then rotated, on the turntable of a blood cell suspension mixer (Mathurn Ltd., London), rotating at 28.5 rpm, at 37°C for 10 minutes before the blood was passed through a glass bead column. Under these conditions it appears that streptokinase treatment of blood results in a significant increase in the platelet adhesiveness of the sample (figure 40, table 62). The mean percentage adhesiveness of the ten control

samples was 52.0 $\pm$ 19.5 and of the ten test samples 63.9 $\pm$ 23.7, ($t = 2.780$, $p < 0.025$). No visible hemolysis of red cells was apparent in either the control or test blood samples after rotation and passage through the column. This result is in agreement with the observations made for the action of streptokinase on the rate of formation of platelet aggregates in the Chandler tube system (figure 5). Since it would appear that the action of streptokinase on platelet aggregation is mediated through plasmin (Chapter 5) and since trypsin has been selected as a proteolytic enzyme comparable to plasmin, the effect of trypsin on platelet adhesiveness was also investigated.

Effect of Trypsin. The ability of trypsin to influence platelet adhesiveness was investigated with a series of ten blood samples. Each sample was divided into two portions each of 2 ml, one of which was treated with trypsin (10 μg per ml blood) and the other with an equal volume of 0.9 per cent saline. Both samples were then incubated at 37°C for 10 minutes, without rotation, before the blood was passed through the column. The results are presented in figure 41 and table 63. Under these conditions of incubation of trypsin and blood it would seem that this enzyme has some tendency to produce an increase in platelet adhesiveness, but since in four of the ten plasma samples investigated, platelet adhesiveness was decreased in the presence of trypsin, the overall result is not statistically significant. The mean percentage platelet

adhesiveness of the ten control samples was 48.4 $\pm$ 30.1 and of the test samples 61.5 $\pm$ 18.6, ($t = 1.513$, $p < 0.20$). This result is in agreement with the observations for the effect of trypsin, at this concentration, on ADP induced aggregation in the turbidimetric system (figure 20) but differs from the findings in the Chandler tube system (figure 6). As mentioned above, however, the Hellem glass bead column method for the investigation of platelet adhesiveness differs from the other two systems used in this thesis to investigate platelet aggregation in that the enzyme and blood samples are not thoroughly mixed during the assay procedure. Consequently, the experiment was repeated with the modified system whereby the enzyme and blood were thoroughly mixed during the incubation period.

With a series of eight citrated blood samples trypsin (10 μg/ml) and the blood sample were rotated on the turntable of a blood cell suspension mixture for 10 minutes at 37^{o}C before platelet adhesiveness was assayed. Control samples were treated similarly. In this system trypsin appears to be able to produce a significant increase in the percentage platelet adhesiveness of the blood samples investigated (figure 42, table 64). The mean percentage adhesiveness of the eight control samples is 58.9 $\pm$ 11.7 and of the test samples 69.7 $\pm$ 8.9 ($t = 3.011$, $p < 0.02$). As in the investigation of streptokinase there was no visible red cell hemolysis produced either by the rotation of the blood or passage through the column.

It has recently been suggested that the Hellem glass bead column method of measuring platelet adhesiveness may reflect red cell behaviour rather than true platelet behaviour (Harrison and Mitchell, 1966). If this is the case, it may be argued that the apparent effect of streptokinase and trypsin on platelet adhesiveness may merely be indicative of the action of these enzymes on red cells. Consequently the effect of trypsin on platelet adhesiveness in platelet rich plasma was investigated. In a series of seven platelet rich plasma samples, each sample was divided into two portions each of 2 ml. One portion was incubated with trypsin (10 μg per ml plasma) at 37°C for 10 minutes and the second portion with an equal volume of saline to serve as a control before the samples were pumped through the columns. The results are shown in figure 43 and table 65. In each of the seven plasma samples investigated, platelet adhesiveness was increased in the presence of trypsin. The average percentage adhesiveness of the control samples was 10.6 $\pm$ 12.7 and of the test samples 44.0 $\pm$ 36.6, ($t = 2.801$, $p < 0.05$). It would therefore seem that the apparent ability of trypsin to increase platelet adhesiveness in citrated whole blood is not mediated through its action on red cells.

These experiments suggest that trypsin is less effective in its ability to cause an increase in platelet adhesiveness in whole citrated

blood than in platelet rich plasma since the enzyme and whole blood must be thoroughly mixed throughout the period of incubation before a significant increase in platelet adhesiveness is detected. The effect of red cells and white cells on platelet aggregation in platelet rich plasma has been investigated by Harrison et al., (1966), who have shown that red cells appear to exert no action on ADP and ATP induced aggregation but that white cells enhance disaggregation of ADP induced aggregation and cause initial enhancement followed by inhibition of ATP induced aggregation; the apparent difference between the effects of trypsin in whole blood as contrasted with platelet rich plasma might be accounted for by the influence of white cells on adhesiveness in the whole blood system.

Effect of white cells. The effect of the presence of white cells on platelet adhesiveness in platelet rich plasma was investigated with two samples. Blood was withdrawn from two hospital patients (A and B), known to have an elevated red cell sedimentation rate, citrated in the usual way and allowed to stand at room temperature until a minimum of 4 ml of plasma could be separated from the red cells. In neither sample did this take longer than 30 minutes. The white cell count in the platelet rich plasma samples was 1,530 per cu mm for subject A and 2,145 per cu mm for subject B. One portion of the platelet rich, white cell rich plasma was incubated with trypsin (10/ug/ml) at 37°C for 10 minutes and the second portion was incubated with saline before the platelet

adhesiveness was assayed. The percentage white cell adhesion in the control and trypsin treated samples was also estimated by performing white cell counts on the plasma before and after passage through the glass bead column. The results are shown in table 11. Platelet and white cell adhesiveness was assessed in a citrated whole blood sample from subject B treated with both saline and trypsin and the results are also shown in table 11. It can be seen that, as demonstrated above, trypsin appears to be able to cause an increase in platelet adhesiveness in the presence of platelets and white cells but that this effect is not always produced when red cells are also present in the system. The incubation of whole blood or plasma with trypsin also appears to result in an increase in the number of white cells lost during the passage of the sample through the column of glass beads. On the present experimental evidence, the apparent difference in the action of trypsin on platelet adhesiveness in whole blood and platelet rich plasma systems cannot be explained on the basis of the interference of white cells in the reaction sequence.

Hellem (1960) has shown that the adhesion of platelets to glass beads in whole blood is dependent on a factor released from the red cells. This factor has been identified as ADP by Gaarder et al., (1961). From the observations made concerning the action of trypsin on platelet

aggregation presented in chapters 4 and 5, it was suggested that the action of trypsin might be mediated through the ADP mechanism of platelet aggregation (Gaarder and Laland, 1964; Haslam, 1964; Salzman et al., 1966). This possibility has been investigated with the glass bead column method by assaying the concentration of ADP and AMP present in either blood or plasma, treated with trypsin, before and after passage through the column. Control samples treated with 0.9 per cent saline were assayed similarly.

ADP and AMP assays. In a series of eight citrated whole blood samples, 2.5 ml blood were incubated at 37°C for 10 minutes with trypsin (10 µg/ml) and 2.5 ml blood with an equal volume of saline. Aliquots (0.5 ml) of both the control and test samples were added to 2.0 ml, 0.5M perchloric acid at 4°C and the remaining blood was passed through a glass bead column and a further 0.5 ml sample added to perchloric acid as soon as all the blood had passed through the column. The concentration of ADP and AMP in the samples was estimated as described above, and the percentage platelet adhesiveness was also assayed. The same procedure was carried out on a series of seven platelet rich plasma samples. The results are shown in tables 12, 13, 14 and 15. It would appear that over the series of experiments there is no significant difference in either ADP or AMP concentrations in the control and trypsin treated samples. When citrated whole blood was studied, the mean concentration of ADP (µmoles per ml) in the control sample before passage was 1.103 ± 0.539 and after passage

1.055 $\pm$ 0.243, ($t = 0.242$, $p < 0.90$) and in the trypsin treated samples the values were 1.110 $\pm$ 0.582 and 1.123 $\pm$ 0.543 respectively, ($t = 0.063$, $p < 0.975$). In the series of platelet rich plasma samples, the mean concentration of ADP in the control sample before passage was 0.963 $\pm$ 0.298 and after passage 0.830 $\pm$ 0.434 ($t = 0.860$, $p < 0.50$) and in the trypsin treated samples the values were 0.712 $\pm$ 0.288 and 0.650 $\pm$ 0.319 respectively, ($t = 0.164$, $p < 0.90$). Further, in the citrated whole blood sample, the mean concentration of AMP (μmoles per ml) in the control series before passage was 1.870 $\pm$ 0.353 and after passage 1.781 $\pm$ 0.293, ($t = 0.930$, $p < 0.40$) and in the trypsin treated series the values were 1.976 $\pm$ 0.282 and 2.072 $\pm$ 0.337 ($t = 2.053$, $p < 0.10$). With the platelet rich plasma samples the values were, for the control series 0.669 $\pm$ 0.313 and 0.830 $\pm$ 0.548 ($t = 1.196$, $p < 0.30$) and for the trypsin treated series 0.654 $\pm$ 0.263 and 0.595 $\pm$ 0.156 ($t = 0.725$, $p < 0.50$). When the concentration of ADP present in the control samples after incubation was compared to that in the trypsin treated samples it was found that trypsin caused no significant change in the citrated whole blood system but produced a significant decrease in platelet rich plasma. The mean concentration of ADP (μmoles per ml) present in whole blood in the control sample after incubation and before passage was 1.103 $\pm$ 0.539 and in the trypsin treated samples 1.110 $\pm$ 0.582 ($t = 0.020$, $p < 0.09$) but in the

platelet rich plasma samples the values were 0.963 $\pm$ 0.298 and 0.712 $\pm$ 0.288 respectively ($t = 3.133$, $p < 0.025$). Different results were found when the concentrations of AMP present after incubation in the control and trypsin treated samples were compared since in neither the whole blood nor the platelet rich plasma series was any significant difference in AMP concentration detected. The mean AMP concentration (μmoles per ml) in the whole blood series in the control samples after incubation was 1.870 $\pm$ 0.353 and in the test samples 1.976 $\pm$ 0.282 ($t = 0.786$, $p < 0.50$) and in the plasma series the values were 0.669 $\pm$ 0.313 and 0.654 $\pm$ 0.263 respectively ($t = 0.289$, $p < 0.80$). In neither the control nor the trypsin treated samples did there appear to be any correlation between the percentage of platelets lost and the change in ADP concentration as the whole blood or plasma was passed through the column (tables 16 and 17). The percentage platelet adhesiveness of the control and trypsin treated samples did not appear to depend on the initial ADP concentration of samples (tables 16 and 17).

Discussion.

It has been shown that when platelet adhesiveness in whole blood is measured by the glass bead column method, there is a direct relationship between the packed cell volume of the sample and platelet adhesiveness in that sample (Hellem, 1960; Hirsh et al., 1966a; McClure et al., 1966).

In the present study no allowance has been made for the packed cell volume of the whole blood samples investigated. Since each sample was divided and one portion treated with the enzyme while the second was treated with saline to act as a control, each sample on which the action of either streptokinase or trypsin was examined, had its own individual control.

The investigation of the effect of the enzymes streptokinase and trypsin on platelet adhesiveness in citrated whole blood shows that these enzymes can cause an increase in platelet adhesiveness when they are thoroughly mixed with the blood during the period of incubation (figures 40 and 42 and tables 62 and 64). When the enzymes and whole blood are incubated together with initial mixing but without continued motion in the sample, trypsin appears to cause inhibition of platelet adhesiveness in some samples and streptokinase produces a significant decrease in platelet adhesiveness (figures 41 and 39 and tables 63 and 61). These results are in striking contrast to the findings obtained when the effect of trypsin on platelet rich plasma is examined. Trypsin can produce a significant increase in platelet adhesiveness in platelet rich plasma when the plasma and enzyme are incubated together without constant motion in the sample during the incubation period (figure 43, table 65). In an attempt to determine whether this difference between the apparent action of trypsin in citrated whole blood and platelet rich plasma could be accounted for by the

presence of cellular components present in whole blood but absent in plasma, the effect of the presence of white cells and red cells on the action of trypsin has been investigated.

It would seem that white cells do not interfere with the action of trypsin on platelet adhesion in platelet rich plasma (table 11). Confirmation for the suggestion that a factor other than white cells is involved in the apparent inhibition of the tryptic effect in whole blood was obtained from the observation that trypsin appeared to cause a decrease in platelet adhesiveness in a sample of citrated whole blood but could produce an increase in platelet adhesiveness in a sample of platelet plus white cell rich plasma prepared from the same citrated whole blood (table 11). Consequently the effect of the presence of red cells on the action of trypsin on platelet adhesiveness was investigated.

ADP has been shown to have a unique position in platelet aggregation in vitro (Born 1962a; Gaarder et al., 1961; Haslam, 1964; Hellem and Owren, 1964; Hovig and Holmsen, 1963; Käser-Glanzmann and Lüscher, 1962; Lüscher 1967; O'Brien, 1964). In addition, ADP may be liberated from red cells under certain conditions (Spaet, 1964) and it has been shown that its liberation from red cells during the passage of whole blood through a glass bead column stimulates the adhesion of platelets (Gaarder et al., 1961; Hellem, 1960). When the effect of trypsin on the concentration

of ADP present in blood or plasma before and after passage through a column of glass beads was investigated it was found that incubation of whole blood with trypsin appeared to cause no significant change in the ADP concentrations of the samples (table 12) but there appeared to be a significant decrease in the concentration in platelet rich plasma (table 14). This may account for the apparent different effect of trypsin on platelet adhesiveness in whole blood and plasma. It has been shown that platelets are rich in ATP and ADP (Born, 1956 a and b) and that during the process og aggregation the nucleotides are rapidly broken down (Born, 1956b and 1958; Salzman et al., 1966; Spaet 1964; Zucker and Borrelli, 1961). Thus the incubation of plasma with trypsin may result in platelet changes reflected by a fall in the concentration of ADP in the sample which facilitate platelet adhesion to a foreign surface as well as to each other. In whole blood, these changes may either be prevented or the action of trypsin may be inhibited in some way. The decrease in ADP concentration in trypsinized plasma is not reflected by an increase in AMP concentration (table 15). Since AMP is only one of the products of metabolism of ADP (Chen and Jorgensen, 1957; Ireland and Mills, 1966) it is not possible to draw any conclusion from this observation except that either some of the ADP present in the sample may be phosphorylated to ATP or that it is being degraded beyond ADP.

During the investigation of the action of trypsin on platelet adhesiveness in platelet rich plasma it was observed that in some samples a surprisingly high percentage of platelet adhesiveness was estimated in the control samples treated with saline. When the ADP concentration of of whole blood and plasma were measured, it was found that the mean ADP concentrations in both whole blood and platelet rich plasma were very similar (tables 12 and 14). This observation is in agreement with that of Born (1956 a and b) who demonstrated that platelets have a very large store of nucleotides. Further, when the apparent ADP concentrations of whole blood and plasma, in the presence and absence of trypsin, before and after passage through the glass bead columns were compared there appeared to be no significant difference in the amounts measured (tables 12 and 14). The percentage platelet adhesiveness observed in the control and trypsin treated plasma and whole blood samples did not appear to be related to either the initial ADP concentration or to the change in ADP concentration found in the samples. This result appeared somewhat surprising since it has been shown that in citrated platelet rich plasma, platelet adhesiveness appears to be closely related to the concentration of added ADP (Hellem et al., 1963; Holmsen et al., 1965). The concentration of ADP required to promote platelet adhesion may be so small that it is not detectable by the assay system used in this study. Spaet and Lejnicks

(1966) have suggested that the function of ADP in platelets is to supply energy for platelet aggregation and since ADP is a relatively high energy source it is conceivable that minute amounts are required for the initiation of and the process of platelet aggregation. It has also been suggested that the participation of a heat labile plasma factor is essential to the process of ADP induced aggregation (Born and Cross, 1964; Castaldi, 1966; Skalhegg et al., 1964). These observations may account for the apparent lack of correlation between platelet adhesiveness and ADP concentration found in this study.

Jorgensen (1956) found that the breakdown of ATP in whole blood was much more rapid than in plasma. This observation may account for the findings in the present series of experiments in which the concentration of AMP present in whole blood appeared to be much greater than that present in plasma (tables 13 and 15). Thus it may be the greater platelet adhesiveness observed in whole blood over that of platelet rich plasma may be accounted for, not by the concentration of ADP in the system but by its ability to be dephosphorylated since this process has been shown to occur as platelets aggregate (Born, 1956 b and 1958; Salzman et al., 1966; Spaet, 1964; Zucker and Borrelli, 1961). It is therefore difficult to come to any firm conclusions from this short series of experiments but it would seem that platelet adhesiveness in either whole blood or plasma is not dependent

solely on the concentration of nucleotide in the sample.

Conclusions.

From the results presented in this chapter it would appear that trypsin can increase platelet adhesiveness in platelet rich plasma and that both streptokinase and trypsin can produce an increase in platelet adhesiveness in whole blood under certain conditions of incubation. The percentage of platelets lost during the passage of either whole blood or plasma through a glass bead column does not appear to be related to the initial concentration of ADP in the sample.

CHAPTER 7.

COMPARISON OF CHANDLER TUBE, TURBIDIMETRIC AND HELLEM METHOD FOR THE ESTIMATION OF PLATELET AGGREGATION.

In chapter 3 was discussed the outline of the various techniques used to measure platelet aggregation and the various aspects of platelet aggregation on which each method was based. There is still some doubt as to the precise function of the platelet which is measured by each test and in an effort to determine whether there was any correlation between the tests used in chapters 4, 5 and 6, a comparison was made of the various methods of assessing platelet aggregation and adhesiveness.

Materials.

ADP was prepared as described in chapter 3.

Platelet rich plasma was prepared as described in chapter 3.

Chandler loops were prepared as described in chapters 3 and 4.

Glass bead columns were prepared as described in chapters 3 and 6.

Calcium chloride, 0.25M was prepared as described in chapter 4.

Methods.

Platelet aggregation was estimated in the Chandler tube system as described in chapters 3 and 4 and in the turbidimetric system as described in chapters 3 and 5.

Platelet adhesiveness was assessed by the Hellem glass bead column method as described in chapters 3 and 6.

Platelet counting was performed by the method of Dacie (1956 c) as described in chapter 3.

Packed cell volume. It has been shown that there is a direct relationship between packed cell volume and platelet adhesiveness (Hellem, 1960; Hirsh et al., 1966a; McClure et al., 1966). In order to define the relationship between packed cell volume and platelet adhesiveness, the effect of the addition of red cell concentrate to platelet rich plasma on platelet adhesiveness was investigated. To 45 ml blood, withdrawn from a volunteer by clean venepuncture, was added 5 ml of 3.8 per cent sodium citrate. The citrated blood was then centrifuged at 600g for 5 minutes at room temperature and the top two thirds of the plasma removed from each centrifuge tube. The red cell concentrate was then centrifuged at 1500 g for 20 minutes at room temperature and the bottom 1.0 ml of red cell concentrate harvested from each tube. The platelet poor plasma from each tube was also collected. A series of samples was then prepared in which the volume of platelet rich plasma was kept constant and the relative concentrations of platelet poor plasma and red cell concentrate added to each sample were varied so that, while maintaining a uniform dilution of the platelet rich plasma, the haematocrit of the samples varied over the range 9% - 43%. The percentage platelet adhesiveness was estimated by the glass bead column

method and the results are shown in figure 44.

The packed cell volume of the samples was measured by means of a Hawksley microhaematocrit centrifuge.

Fibrinogen assays were performed as described in chapter 3.

Plasminogen assays were carried out as described in chapter 3.

Results.

Twenty five normal control subjects were selected. From each volunteer, 22.5 ml blood were withdrawn by clean venepuncture and anticoagulated with 2.5 ml, 3.8% sodium citrate. 5 ml of the citrated whole blood was allowed to stand at room temperature for a minimum of 30 minutes and a maximum of 45 minutes while platelet rich plasma was prepared from the remainder of the blood, and tested simultaneously in the Chandler tube system and in the turbidimetric system. For the Chandler tube system 5 ml, platelet rich plasma were made up to 14.5 ml with 0.9 per cent saline and the plasma recalcified with 0.5 ml, 0.25M $CaCl_2$. For the turbidimetric system 2 ml, platelet rich plasma were added to a cuvette in the light path of a photoelectric cell, the plasma stirred for at least 1 minute to ensure that spontaneous aggregation did not occur and platelet aggregation induced by the addition of ADP (0.5 μg per ml plasma). The contact time between the citrated whole blood and the glass beads in the Hellem system was 30 ± 1 sec.

In addition to the citrated whole blood collected from each patient

a sample was anticoagulated by addition to disodium EDTA and the packed cell volume, of each subject, estimated on this sample. From the results of the graph of percentage packed cell volume against percentage platelet adhesiveness (figure 44) a linear correlation appears to exist between the two parameters. From this graph it was estimated that a change of one unit of packed cell volume corresponded to an alteration of 1.8 units of platelet adhesiveness. The mean value for the packed cell volume of the twenty five samples investigated was 40.3 with a standard deviation of $\pm$ 5.0 (table 18) which lies within the accepted range of normal (Dacie, 1956 a). In the present trial, the packed cell volume of the individual blood sample was not adjusted to 40 per cent by the addition of platelet poor plasma or red cell concentrate to the citrated whole blood because the packed cell volume of the samples lay within the physiological range of normal.

Mathematical correlation of the results of platelet function as assessed by each method, the Chandler tube, the turbidimetric method and the glass bead column method, have been determined and the results are shown in table 19. The 1 minute and 4 minute optical density readings obtained in the turbidimetric system were selected for comparison. The 1 minute reading was selected because by this time platelet aggregates

had begun to form in every sample so that this reading represents the initial stages of the reaction between platelets and exogenous ADP. The 4 minute reading was selected because by this time the maximum aggregation had occurred in the majority of the twenty five samples as was indicated by a graph drawn from the mean values obtained from the optical density readings. From the results presented in table 19 there appears to be no significant correlation between the results of the three methods examined, irrespective of the optical density readings selected for comparison. The platelet adhesiveness for each of the samples was then adjusted, to allow for the differences in the packed cell volume, from the calibration curve described above and the mathematical correlation between the results of each technique reinvestigated. The correlation coefficients obtained are shown in table 19. Once again there is no mathematical significance between the observations obtained for each of the three methods. No advantage appeared to have been gained by the correction of the platelet adhesiveness for packed cell volume since, as shown in table 18, the mean and standard deviations of the adhesiveness over the series of samples investigated was similar before and after adjustment.

The data from forty of the plasma samples examined in the control loop of the Chandler tube experiments described in chapter 4 was gathered. The mathematical correlation between the speed of formation of platelet aggregates and the platelet count in each sample was estimated and the result is shown in table 20. There appears to be no significant correlation

between the speed of formation of platelet aggregates and the platelet count. Similar mathematical treatment was carried out on 53 of the control specimens used in the turbidimetric experiments described in chapter 5. For this series of experiments the optical density reading 2 minutes after the addition of ADP to the sample was examined since over the series of experiments this was the time at which maximum aggregation invariably occurred. The result is shown in table 20 and indicates that there appears to be no mathematical correlation between the degree of platelet aggregation, as estimated from the optical density change occurring in the sample, and the platelet count of the sample. Mathematical correlation was also assessed between the platelet counts of the twenty-five citrated whole blood samples used in the trial described above and the percentage platelet adhesiveness of the samples. As shown in table 20 there appears to be no correlation between platelet count and percentage platelet adhesiveness either uncorrected or adjusted for packed cell volume.

The concentration of fibrinogen and plasminogen, present in the plasma samples used in the Chandler tube series of experiments and turbidimetric experiments on which mathematical studies were performed, was estimated. As shown in table 21 the results lay within the accepted range of normal for this laboratory.

Discussion.

From the results presented above there appears to be no correlation

between the results obtained from the three techniques used to investigate platelet aggregation and platelet adhesiveness (table 19). This observation throws some doubt on the validity of these tests as an index of platelet function. As was discussed in chapters 2 and 3, however, each of the methods used is based on a different aspect of the phenomenon of platelet clumping. The turbidimetric system of Born depends on the ability of platelets to react with or metabolise exogenous ADP, the Hellem glass bead column method is based on the ability of platelets to adhere to a glass surface and the Chandler tube system depends on the ability of platelets to form aggregates as a precursor to fibrin formation in an artificial circulation. The Chandler tube system, as mentioned in chapter 5, probably measures a much later stage in the sequence of platelet aggregation than either of the other two methods since macroscopic platelet clumping has to occur before it is detected in the rotating loop. In addition the platelet clumps which form in the Chandler tube system do not disaggregate, whereas, as indicated by the control curves in figures 16 and 20, the platelet aggregates which form in the turbidimetric system undergo spontaneous disaggregation (Born and Cross, 1963b; Mitchell and Sharp, 1964). The Hellem glass bead column method possibly measures a stage of aggregation intermediate between that of the turbidimetric method and the Chandler tube method since platelet adhesion to the glass surface is

initiated by the release of ADP from red cells damaged during the passage of blood through the column (Hellem, 1960; Stormorken et al., 1965). Thus it is possible that the apparent lack of correlation between each method is probably due to the fact that a different parameter is being measured in each case. This view is partially substantiated by the observation of Hirsh et al., (1966b) that a significant mathematical correlation exists between the results of platelet adhesiveness assessed in the Hellem glass bead column and the Wright 'rotator' since with both these techniques a similar parameter is being measured, namely, the ability of platelets to adhere to a damaged surface. Furthermore, the platelet populations which are assayed in the methods involving platelet rich plasma and those utilizing citrated whole blood are probably different. Stormorken et al., (1965) have shown that the most adhesive platelets within a sample are those which are most easily sedimented and accordingly a percentage of adhesive platelets within each sample may have been lost during the centrifugation procedure to prepare platelet rich plasma.

The Chandler tube method, the turbidimetric method and the Hellem method would each appear to measure some inherent property of the platelet population of the samples under investigation. In none of the methods was there any mathematical correlation between platelet count and the extent of platelet clumping or the rate of clumping within the sample

(table 20). A lack of correlation between platelet count and platelet adhesiveness has previously been reported (Hirsh et al., 1966a). This observation is contrary to that of Stormorken et al., (1965) who have shown that within a specific platelet population there exists a linear relationship between platelet count and platelet adhesiveness. These workers also claim that there is a linear relationship between the amount of ADP added to a sample platelet rich plasma and the adhesiveness of that sample. This is contrary to the results presented in chapter 6 where the data fail to reveal a correlation between the recorded platelet adhesiveness of a sample and the initial ADP concentration within the sample. From these observations it may be concluded that the various methods used in this trial measure some, as yet undefined, inherent property of the platelets and the results obtained are to some extent independent of the number of platelets present.

The difference between the results obtained with the three methods may be quantitative rather than qualitative since it would appear that alterations, in platelet rich plasma and blood, which influence platelet aggregation are reflected in all three methods although the quantitative changes are variable. In chapters 4, 5 and 6 have been presented and discussed the observations on the effect of the enzymes streptokinase and

trypsin on platelet function. Both enzymes could be made to enhance aggregation in the Chandler tube, turbidimetric and Hellem techniques but in some instances the concentration of enzyme required to produce this effect is higher than in others. Further investigations will have to be performed before the significance of the similarities and the differences of the various techniques currently available to investigate the ability of platelets to clump in vitro.

Conclusions.

From the results presented in this chapter it would appear that there is no mathematical correlation between observations made for platelet function in the Chandler tube method, the turbidimetric method and the glass bead column method. There is some evidence to suggest, however, that each method measures some inherent property of the platelet population of the samples under investigation and that the differences observed between each method may be quantitative rather than qualitative.

CHAPTER 8.

PREPARATION OF FIBRINOGEN DEGRADATION PRODUCTS.

The observations presented in chapters 4, 5 and 6 show that trypsin can produce significant enhancement of both platelet aggregation and platelet adhesiveness. In the platelet rich plasma systems described in chapters 4 and 5 it appeared that the effect of trypsin on platelet aggregation could not be accounted for wholely on the basis of the coagulative properties of the enzyme. In addition, in the Chandler tube system, in which fibrin formation occurs as a sequel to platelet aggregation it was found that, the presence of trypsin in the system resulted in significant impairment in fibrin formation. Further, as discussed in chapter 5, it appears that trypsin does not produce enhancement of platelet aggregation by direct action on the platelets and that the presence of plasma proteins are essential for its action. Accordingly, it was thought worth while to investigate the effect of fibrinogen digests on platelet aggregation. The fibrinogen digests were purified by column chromatography before appraisal of their physical and chemical characteristics and of their effect on platelet aggregation.

In this chapter is presented an account of the method of preparation of fibrinogen degradation products formed by the action of trypsin on fibrinogen or on the action of urokinase activated plasminogen on the protein.

Materials.

Fibrinogen. Human lyophilized fibrinogen, Grade L, was obtained from

A.B. Kabi, Stockholm. The protein was made up to the required concentration with 0.1M phosphate buffer, pH 7.6 immediately before use.

Plasminogen. Human lyophilized plasminogen, Grade B, was obtained from A.B. Kabi, Stockholm. The protein was reconstituted, immediately before use, with distilled water to give a concentration of 17.5 Sgouris units per ml.

Urokinase was obtained from Abbott Laboratories, Illinois. Immediately before use the enzyme was reconstituted with distilled water to give a concentration of 40,000 CTA units per ml.

Diethylaminoethyl cellulose (DE11) was obtained from W. and R. Balston Ltd., England. The DEAE cellulose was prepared for column chromatography by immersing DE11 in 0.5N HCl, in the proportion of 10 g cellulose to 150 ml acid, and allowing the suspension to stand for one hour. The cellulose was then washed with distilled water before it was immersed in 0.5 N, NaOH in the same proportions as with the acid. Finally the cellulose was washed with 1 M NaCl until the pH of the washings was 7 and most of the fine particles had been removed by decantion.

G-25 Sephadex (coarse) was obtained from Pharmacia, Sweden.

The preparations of trypsin and soybean trypsin inhibitor used are described in chapter 3 along with the methods of preparation of 0.1M phosphate buffer, pH 7.6 and 0.1M carbonate buffer, pH 8.9

Methods.

Preparation of Columns. The columns were packed at room temperature with DEAE cellulose in 2 M NaCl. The DEAE cellulose was held in the column by a thin layer of glass wool covered with a layer of glass beads to give a smooth surface to support the cellulose. The glass columns were of heavy duty glass, 45 cm long and 2.5 cm internal diameter, and were specially made to have a negligible dead space. The first few centimetres of cellulose were packed into the column under gravity and the rest of the column was packed under the pressure of a small hand pump, at approximately 5lbs pressure per sq. in. The final length of the cellulose column was 37.5 cm. After packing, the column was washed with distilled water until the pH of the eluant was 7.0 and then equilibrated with 0.1M carbonate buffer, pH 8.9 before a protein solution was eluted from the column. A fresh column was prepared for each experiment.

Buffer system. Fibrinogen degradation products were eluted from the DEAE cellulose column with a linear concentration gradient with the range of buffer from 0.1M carbonate buffer pH 8.9 to 0.1M carbonate buffer, pH 8.9 plus 0.2 M NaCl in a total volume of 800 ml. The head of pressure between the top of the column and the reservoir was adjusted in each experiment so that a flow rate of approximately 90 ml per hour could be achieved.

Concentration of proteins. The samples eluted from the column which had been shown to contain protein by measurement of optical density at 280 mμ were concentrated with G-25 Sephadex using Seitz filters. 10 ml of the protein solution were concentrated with 4 g Sephadex by centrifugation at 1600 g for 15 minutes at 4^{o}C.

Estimation of protein concentration was carried out by the method of Lowry et al., (1951) as described in chapter 3. The fibrinogen degradation products prepared by column chromatography were stored at -20^{o}C in small amounts for periods not greater than a few weeks.

Results.

Initial experiments were carried out to study the elution patterns of pure fibrinogen, plasminogen, trypsin, soybean trypsin inhibitor and urokinase from a DEAE cellulose column.

100 mg pure human fibrinogen dissolved in 2 ml, 0.1M phosphate buffer, pH 7.6 was layered onto the surface of a DEAE cellulose column. The protein was eluted from the column with the buffer system, 0.1M carbonate buffer, pH 8.9 to 0.1M carbonate buffer plus 0.5 M NaCl, in a total volume of 800 ml with a flow rate of 90 ml per hour. The fractions were collected in volumes of 10 ml and the pattern obtained is shown in figure 45. Fibrinogen appears to be eluted from the column at a sodium chloride molarity of 0.105M. A small shoulder appears on the main peak and this may either be an impurity in the fibrinogen solution

or some denatured protein.

0.5 ml, purified plasminogen solution (17.5 Sgouris units per ml) was eluted from a DEAE cellulose column with the buffer system 0.1M carbonate buffer, pH 8.9 to 0.1M carbonate buffer plus 0.2 M NaCl, in 350 ml with a flow rate of 100 ml per hour. The fractions were collected in 10 ml amounts and the pattern is shown in figure 46. Plasminogen appears to be eluted from the column in two fractions, the major fraction being eluted from the column at a sodium chloride molarity of 0.077 M and the smaller fraction at 0.159 M NaCl.

A solution composed of 1 ml trypsin (22.2 mg/ml) and 1 ml soybean trypsin inhibitor (22.2 mg/ml) was eluted from a column with the buffer system, 0.1M carbonate buffer, pH 8.9 to 0.1M carbonate buffer plus 0.4 M NaCl, in a total volume of 800 ml with a flow rate of 80 ml/hour. The eluant was collected in 10 ml samples and the pattern is shown in figure 47. Within the salt concentration studied, only one protein containing fraction appeared to be eluted from the column which is in keeping with concept of the formation of an irreversible stoichiometric compound in the presence of trypsin and soybean trypsin inhibitor (Northrop et al., 1948). The protein was eluted from the column at a sodium chloride molarity of 0.314M.

0.5 ml urokinase (40,000 CTA units per ml) were eluted from a

DEAE cellulose column with the buffer system, 0.1 M carbonate buffer, pH 8.9 to 0.1M carbonate buffer plus 0.2 M NaCl, in 400 ml with a flow rate of 84 ml per hour. The eluant was collected in 10 ml amounts and the elution pattern is shown in figure 48. It would seem from the elution pattern that the urokinase solution contains a number of components, none of which are removed from the column at a sodium chloride molarity of less than 0.075M. The main component appears to be eluted at a salt concentration of 0.120 M NaCl.

Preparation of degradation products. For the preparation of fibrinogen degradation products by tryptic digestion, conditions of incubation were selected which were to some extent comparable to those under which trypsin and platelet rich plasma were allowed to react. The average fibrinogen content of plasma was assumed to be 300 mg per 100 ml plasma. Thus the incubation of 2 ml plasma with 20 μg trypsin would be approximately equivalent to incubating 6 mg fibrinogen with 20 μg trypsin. Similarly, incubation of 2 ml plasma with 200 μg trypsin was assumed to be comparable to incubating 6 mg fibrinogen with 200 μg trypsin.

The first degradation product was made by the addition of 0.3 ml trypsin (2.22 mg per ml) to 200 mg fibrinogen in 4 ml, 0.1M phosphate buffer pH 7.6. After incubation at 37°C for 10 minutes, 0.3 ml soybean trypsin inhibitor (2.22 mg per ml) were added to the mixture and the resulting solution was layered onto a DEAE cellulose column. The protein

was eluted from the column with the buffer system described above. The flow rate was 88 ml per hour and the elution pattern is shown in figure 49. The major portion of the protein appeared to be eluted as a single fraction with a much smaller fraction separating at a slightly higher salt concentration. The main component was eluted from the column in a volume of 50 ml between the NaCl concentrations 0.032M and 0.042M, the sample containing the largest concentration of protein (as estimated by optical density measurements) being eluted at 0.036M NaCl. The pooled samples from this peak were concentrated with G-25 sephadex and the protein concentration of the final protein solution estimated by the method of Lowry et al., (1951). This product will be referred to in subsequent chapters as product 'a'.

The second fibrinogen degradation product was made by the incubation of 200 mg fibrinogen in 4 ml, 0.1 M phosphate buffer, pH 7.6 with 0.3 ml trypsin (22.2 mg per ml) at 37°C for 10 minutes. After incubation, 0.3 ml soybean trypsin inhibitor (22.2 mg per ml) was added to the incubation mixture and the products of digestion separated from the mixture by fractionation on a DEAE cellulose column. The protein was eluted with the standard buffer system with a flow rate of 90 ml per hour. The resulting elution pattern is shown in figure 50. As with the first degradation product prepared, the major portion of the protein was eluted as a single

well defined peak in a volume of 60 ml between the NaCl concentrations 0.033 M and 0.045 M and with a maximum optical density reading at the concentration 0.040 M NaCl. The samples forming this peak were pooled, concentrated with G-25 sephadex and the protein concentration of the resulting solution estimated. This protein will be referred to as product 'b'. A second sample of product 'b' has been made and, as shown in figure 51, the elution pattern is, within the limits of experimental error, identical to the first pattern obtained for this product. The major portion of the protein was eluted from the column in a volume of 40 ml between the concentrations 0.040M and 0.047M NaCl with a maximum at 0.045M NaCl.

A third degradation product was made by the incubation of 200mg fibrinogen in 4 ml, 0.1M phosphate buffer, pH 7.6 with 0.3 ml trypsin (22.2 mg per ml) at 37°C for 16 hours. After incubation, 0.3 ml soybean trypsin inhibitor (22.2 mg per ml) was added and the various protein fractions eluted from this mixture by column chromatography. The elution was carried out with the usual buffer system, with a flow rate of 95 ml per hour and the resulting elution pattern is shown in figure 52. The elution pattern is somewhat diffuse but a single well defined fraction was separated in a volume of 50 ml between 0.035M NaCl and 0.045 M NaCl with a maximum at 0.040M NaCl. The samples in this fraction were pooled,

concentrated with G-25 sephadex and the protein concentrations of the final solutions estimated. This product will be referred to as product 'c'.

Trypsin is an enzyme which is not normally found in circulating blood. It has been shown that plasmin digestion of fibrinogen can produce a variety of degradation products (Fletcher et al., 1966; Hirsh et al., 1965; Jerushalmy and Zucker, 1966; Nussenzweig et al., 1961). Thus, in order to compare degradation products prepared by tryptic digestion of fibrinogen, a degradation product which could be produced under physiological conditions was prepared by digestion of fibrinogen with plasmin formed by the activation of plasminogen by urokinase. Fibrinogen, 200 mg in 4 ml, 0.1M phosphate buffer, pH 7.6, was incubated with 0.1 ml urokinase (40,000 CTA units per ml) and 1.0 ml plasminogen (17.5 Sgouris units per ml) at 37°C for 10 minutes. After incubation, the mixture was immediately layered onto a DEAE cellulose column and the fractionation process started. The elution was carried out with the usual buffer system with a flow rate of 84 ml per hour. The elution pattern is shown in figure 53. The major portion of the protein was eluted a single broad peak in a volume of 80 ml between the NaCl concentrations 0.030M and 0.047M. The central sample of this peak was eluted at a NaCl concentration of 0.035 M. The samples forming this peak were pooled, concentrated with G-25 sephadex and the protein concentration of the final solution estimated. This protein will

be referred to as product 'd'.

For comparative purposes, in the subsequent study of the effect of the degradation products, prepared as described, on platelet aggregation, pure fibrinogen incubated at 37°C without the addition of an enzyme was fractionated by column chromatography. After incubation of 200 mg fibrinogen in 4 ml, 0.1M phosphate buffer, pH 7.6 at 37°C for 10 minutes the protein was eluted from a column with the usual buffer system at a flow rate of 92 ml per hour. The elution pattern is shown in figure 54. Fibrinogen was eluted from the column in a volume of 70 ml between the concentrations 0.085M and 0.010 M NaCl. The central sample of the peak was eluted with a concentration of 0.091 M NaCl. In view of the different concentration gradients used in the initial experiment and in the present experiment, the elution patterns for the two fibrinogen solutions are essentially similar. The samples comprising the peak were pooled, concentrated with G-25 sephadex and the protein concentration in the final solution estimated.

Discussion.

The degradation products formed by the digestion of fibrinogen with trypsin or plasmin appear to be quite separate and distinct entities from the original fibrinogen. The degradation products prepared by plasmin digestion and by short trypsin digestion of fibrinogen appear to be somewhat similar in that they are all eluted from the DEAE cellulose column

within the sodium chloride concentration range of 0.030 M and 0.047 M. With prolonged digestion of fibrinogen by trypsin a diffuse elution pattern was obtained (figure 52) but a definite peak of protein was visible within this concentration range. These observations would seem to suggest that the initial reaction of fibrinogenolytic enzymes on fibrinogen involves the formation of a smaller protein moiety which in turn may be degraded by prolonged proteolytic activity. This suggestion is supported to some extent by the observations of Mihalyi and Godfrey (1963b).

With the elution system described in this chapter minor variations in the elution patterns of the various degradation products may have been lost since the eluant samples were collected in the relatively large amounts of 10 ml. There is, however, a striking similarity between the elution patterns of the degradation products 'a', 'b' and 'd'.

CHAPTER 9.

CHARACTERIZATION OF FIBRINOGEN DEGRADATION PRODUCTS.

In this chapter is presented the results of investigations carried out on the various degradation products formed by proteolytic digestion of fibrinogen as described in chapter 8. The physicochemical parameters chosen to characterize the various protein fragments included molecular weight determinations, ultracentrifugation patterns and electrophoretic studies. The degree of digestion occurring in the formation of two of the products has also been investigated by estimation of the number of peptide bonds of the parent fibrinogen molecule ruptured during the enzymatic reaction.

Materials.

Sucrose, Analar grade, was obtained from British Drug Houses Ltd. 1 per cent and 15 per cent sucrose solutions were made up in 0.05M sodium phosphate buffer, pH 7.5.

Phosphate buffer. Sodium phosphate buffer of 0.05 molar and pH 7.5 was prepared as follows:- 16.0 ml, 0.2 M NaH_2PO_4 (27.80g per litre); 84 ml, 0.2 M Na_2HPO_4 (28.54g per litre); distilled water to 200 ml.

Ribonuclease (RNase) was a chromatographically prepared protein obtained from Koch Light Laboratories Ltd.

Tubing used for dialysis was Visking tubing 8/32" obtained from H.M.C. Scientific Instruments Centre, London.

Fibrinogen was obtained in the form human, lyophilized Grade L from A.B. Kabi, Stockholm.

Trypsin. The preparation used was that described in chapter 3.

Methods.

Sucrose density gradients were prepared with the Buchler Gradient Sedimentation System as described in chapter 3. The gradients were prepared immediately before centrifugation was carried out.

Ultracentrifugation studies were performed on a Spinco, Model E, Ultracentrifuge equipped with a Schliern optical system. The ultracentrifugal patterns for the sedimentation runs were photographed at zero, 8, 16, 24, 32 and 64 minutes from the start of the run. For the diffusion runs, the ultracentrifugal patterns were photographed 8, 16, 24, 32, 40 and 48 minutes from the start of the run.

Electrophoresis was carried out as described in chapter 3.

Reaction kinetics. The degree of digestion of fibrinogen occurring during the formation of the degradation products 'a' and 'b' was investigated as described in chapter 3.

Results.

Molecular weight determinations. The approximate molecular weights of the fibrinogen degradation products 'a', 'b', 'c' and 'd' were estimated by sedimentation in a sucrose solution with a linear concentration gradient. The proteins were eluted from the density gradient by collection of drops

from the bottom of the tube and the samples collected screened for protein content by measuring the optical density of the solutions at 280 m μ.

Pure fibrinogen (molecular weight 340,000) and RNase (molecular weight 12,700) were assayed in the sucrose density gradient system as references for the estimation of the molecular weights of the degradation products. The elution pattern obtained with fibrinogen is shown in figure 55. Fibrinogen sediments in the sucrose solution as two peaks. The main peak appeared to be sedimented in the range of sucrose concentrations from 9.0% to 5.0% sucrose with a maximum at 6.4%. The very much smaller, secondary peak was eluted with the sucrose solution in the concentration range 1.8% to 1.2% sucrose with a peak at 1.5% sucrose. From this pattern it would appear that the fibrinogen solution is not completely homogeneous. It is possible that the small secondary peak may represent plasminogen which is contaminating the fibrinogen preparation. The fractionation of fibrinogen on a DEAE cellulose column appears to remove the contaminating protein. The pattern obtained from the elution of the protein solution, obtained from fibrinogen by column chromatography, from the sucrose density gradient is shown in figure 56. The protein fraction bears close resemblance to the main fraction of the untreated fibrinogen (figure 55). The protein was sedimented in the range of sucrose concentrations 7.2% to 4.7% sucrose with a maximum at 5.3% sucrose. This fraction was used as a reference for the degradation products. The second protein studied as a reference for the

degradation products was RNase. This protein, of known molecular weight, was sedimented in the range of sucrose concentrations 4.25% to 1.4% sucrose with a maximum at 2.5% sucrose. The elution pattern is shown in figure 57.

The elution pattern obtained with product 'a' is shown in figure 58. The protein solution appeared to have some denatured protein which was eluted from the bottom of the gradient but the bulk of the protein was eluted as a somewhat diffuse band between the sucrose concentration of 8.25% and 2.50% with a maximum at 4.25% sucrose. There appeared to be a second fraction in this solution which was eluted with sucrose at a concentration of 2.2%. The molecular weight of the main fraction was estimated from the concentration of sucrose at which the maximum optical density reading of the protein fraction was obtained and was calculated as 217,263.

The elution pattern obtained for product 'b' in the sucrose density gradient system is shown in figure 59. The main fraction of the protein was removed from the gradient between the sucrose concentrations of 8.25% and 3.2% with a maximum at 4.6% sucrose. The molecular weight of the fraction, calculated from this point, was 258,175. As with product 'a', there appeared to be a second protein separated from the solution during the sucrose density gradient experiment at a sucrose concentration of 1.75% sucrose.

Product 'c' remained at the top of the sucrose solution and was

eluted with the sucrose solution at a concentration of 1%. Therefore it would appear that the molecular weight of this protein is less than 12,700 since the protein RNase which has this molecular weight was removed from the gradient with 2.5% sucrose.

Product 'd' was eluted from a sucrose density gradient in three fractions (figure 60). The main fraction was separated between the sucrose concentrations of 8.2% and 3.25% with a maximum at 4.9% sucrose. The molecular weight calculated from this point was 293,243. Two smaller fractions were visible at sucrose concentrations of 12.1% sucrose and 2.4% sucrose.

With the sucrose density gradient method, the molecular weights of the degradation products 'a', 'b' and 'd' appeared to be similar. The results are somewhat unsatisfactory, however, as the proteins separated in the sucrose solution as diffuse bands and it was difficult to determine precise molecular weights. The molecular weights of the two products 'a' and 'b' were further investigated by ultracentrifugation studies.

Ultracentrifugation. The ultracentrifugation studies were performed by Miss H. Moss and Mrs S. Beaton of the Biochemistry Department, University of Glasgow. Samples of products 'a' and 'b' were prepared and concentrated with G-25 sephadex until the protein concentration was 10 mg per ml. Both products were then dialysed overnight at 4°C, each sample against 1 litre of 0.15 M NaCl. The protein solutions were then stored at -20°C until they were analyzed on the ultracentrifuge. It was only possible to

perform one determination of the sedimentation and diffusion coefficients for each product. The sedimentation and diffusion patterns obtained for product 'b' are shown in figures 61 and 62. For product 'a' the sedimentation coefficient was calculated as 6.72×10^{-13} sec and the diffusion coefficient as 6.62×10^{-7} cm ^{2} per sec From these results the molecular weight of the product was calculated from the Svedberg equation as 82,427. For product 'b', the sedimentation coefficient was calculated as 4.37×10^{-13} sec and the diffusion coefficient as 1.44×10^{-6} cm per sec. The molecular weight of product 'b' was calculated from these values in the Svedberg equation as 24,642.

Electrophoresis. The degradation products were examined by electrophoretic analysis as described in chapter 3. For comparative purposes pure fibrinogen and fibrinogen which had been fractionated on a DEAE cellulose column were also investigated. The patterns are shown in figure 63. As all the proteins moved in diffuse bands it was not possible to draw precise conclusions for the electrophoretic characteristics of the degradation products. All the degradation products studied appear to behave similarly during electrophoresis and in a somewhat different fashion to fibrinogen. The fibrinogen samples tended towards the anode indicating that the molecule has a net negative charge while the degradation products remained as diffuse bands around the origin. The electrophoretic bands for fibrinogen before and after passage through a DEAE cellulose column are identical.

This suggests that the fractionation of the protein on the column does not cause degradation or denaturation of the protein.

An attempt to improve the electrophoretic bands by current reversal at the end of electrophoresis was not successful.

Reaction Kinetics. The degree of digestion of the fibrinogen molecule which occurred during the formation of products 'a' and 'b' was estimated with a pH stat as described in chapter 3. To simulate the conditions for the formation of product 'a', the reaction between 1 ml fibrinogen (20mg per ml) and 0.5 ml trypsin (0.1334 mg per ml) was followed at 37°C for 10 minutes. During this reaction 0.03 ml, 0.0335 N NaOH were required to maintain the pH of the fibrinogen solution at 8.6. This is equivalent to 1.005×10^{-6} moles of alkali and hence the number of moles of alkali required to maintain 340,000g fibrinogen at a constant pH would be 17.085. Since 0.998 meq of alkali are utilized for every hydrogen bond broken in the protein molecule, it would appear that 17 peptide bonds in the fibrinogen molecule are broken during the formation of product 'a'.

For the investigation of the reaction leading to the formation of product 'b', the reaction between 1 ml fibrinogen (20 mg per ml) and 0.5 ml trypsin (1.334 mg per ml) was followed at 37°C for 10 minutes. To maintain the pH of the fibrinogen solution at 8.6 during this reaction, 0.08 ml, 0.0335N NaOH were utilized. Since 2.680×10^{-6} moles of alkali would be required for 0.02g fibrinogen, 45.56 moles of alkali would be required for 340,000g fibrinogen. Thus it would appear that, in the formation of

product 'b', 45 peptide bonds in the fibrinogen molecule are broken.

Discussion.

From the results it would appear that the degradation products formed by enzymatic digestion of fibrinogen have different physico-chemical parameters from the parent molecule.

The molecular weights of the degradation products, as determined by sucrose density studies, indicated that the products were all smaller then fibrinogen. It was not possible to detect any significant difference between products 'a', 'b' and 'd'. This may partly be accounted for by the observation that all the proteins, including fibrinogen, were eluted from the gradient within a wide range of sucrose concentration indicating that, in addition to sedimentation, some diffusion of the proteins through the sucrose solution had occurred. Further, there are a number of sources of error in this system; the sampling system from the gradient is empirical and minor variations in the size of the drops collected may occur and minor variations may also occur in the dilution of the samples.

The ultracentrifuge studies show clearly that the products 'a' and 'b' are quite different. Table 22 illustrates the different sedimentation and diffusion coefficients and molecular weights for the two products and of fibrinogen. The ultracentrifugation data for fibrinogen was obtained from Caspary and Kekwick (1954) as quoted by Scheraga and Laskowski (1957).

Although it was not possible to carry out ultracentrifugation analysis on product 'd', formed by urokinase-activated plasminogen digestion of fibrinogen, there are a number of reports which suggest that degradation products formed by plasmin proteolysis are similar to these formed by tryptic digestion of fibrinogen (Beck and Jackson, 1966; Fletcher, et al., 1962). It has been shown that the enzyme plasmin degrades fibrinogen into three classes of fragment: the first has a sedimentation coefficient of 5.27×10^{-13} sec and a molecular weight of 88,000; the second has a sedimentation coefficient of 3.00×10^{-13} sec and a molecular weight of 30,000; and the third has a sedimentation coefficient of 1.4×10^{-13} sec (Alkjaersig et al., 1962; Fletcher, 1966; Fletcher et al., 1966). Similar values have also been obtained by Larrieu et al., (1966). These values are comparable to those found for the degradation products produced by trypsin digestion of fibrinogen (table 22). These observations together with the pattern obtained by sucrose density gradient sedimentation of product 'd' would seem to suggest that the product formed under the proteolytic action of urokinase-activated plasminogen is similar to those produced by tryptic digestion of fibrinogen.

Electrophoresis of the degradation products revealed no distinguishable difference between the patterns for the products formed under the action of trypsin to that of the product formed by urokinase-activated plasminogen although the products gave slightly different patterns to that of fibrinogen. Fibrinogen, both before and after passage through a DEAE cellulose column was found to be anionic. This is in agreement with the observations of

several authors using different electrophoretic media (Beck and Jackson, 1966; Mihalyi, 1950; Seegers et al., 1945). Further, by means of starch gel electrophoresis, it has been shown that trypsin digestion products also have an anodal tendency (Beck and Jackson, 1966) and that this tendency is also exhibited by plasmin digestion products (Beck and Jackson, 1966; Nussenzweig et al., 1961). The movement of the products towards the anode during paper electrophoresis was not demonstrated in the present study but such movement may have been masked by the diffusion of the products during the electrophoretic run.

The reaction kinetics of the degradation of bovine fibrinogen by trypsin have been extensively studied by Mihalyi and Godfrey (1963a). These workers demonstrated that the reaction proceeded in two stages; the first stage involved the splitting of 12 peptide bonds and the second stage involved the rupture of 88 peptide bonds. Subsequent examination of the product formed at the end of the first stage of the reaction showed that the protein had a sedimentation coefficient of 5.22×10^{-13} sec, a diffusion coefficient of 5.12×10^{-7} cm ^{2} per sec and a molecular weight of 95,000 (Mihalyi and Godfrey, 1963b). Further, they suggested that 3 such fragments were formed from every molecule of fibrinogen. The present results for the action of trypsin on human fibrinogen are in accord with those of Mihalyi and Godfrey (1963a and b). The degradation of the fibrinogen molecule results in a

product with a very much smaller molecular weight than fibrinogen although relatively few peptide bonds have been broken. The currently accepted concept for the structure of fibrinogen is that the molecule is made up of three peptide chains (Blomback and Yamashina, 1958; Blomback et al., 1966; Laki and Gladner, 1964). Thus it seems probable that the initial action of trypsin on fibrinogen is on the bonds between the three chains of the molecule since cleavage of the bonds between the peptide chains would result in a marked diminution in the molecular weight of the protein moiety. Consequently the degradation products may be molecules relatively simple in structure as compared to the parent fibrinogen molecule.

CHAPTER 10.

EFFECT OF FIBRINOGEN DEGRADATION PRODUCTS ON PLATELET AGGREGATION AND PLATELET ADHESIVENESS.

In this chapter is presented an account of the investigations of the effect of fibrinogen degradation products on platelet aggregation and platelet adhesiveness. The preparation and characterization of the degradation products studied, is presented in chapters 8 and 9. The effect of pure fibrinogen, from which the degradation products were made, on platelet aggregation and platelet adhesiveness has also been studied.

In chapter 4 it was demonstrated that the enzymes streptokinase and trypsin could cause a marked acceleration in the rate of platelet aggregation under conditions in which fibrin formation was severely impaired. The inhibitory actions of fibrinogen degradation products, formed either 'spontaneously' during substrate decay or by the enzymatic action of plasmin on the protein, on the conversion of fibrinogen to fibrin under the action of thrombin have been recognised for a number of years (Bang et al., 1962; Fletcher et al., 1962; Latallo et al., 1962; Latallo et al., 1964; Triantaphyllopoulos, 1958, 1959, 1960, 1961; Triantaphyllopoulos and Triantaphyllopoulos, 1962; 1964). The present study was undertaken to investigate whether the presence of fibrinogen degradation products in the system could account, not only for the occurrence of defective fibrin formation, but also for the acceleration of

the rate of formation of platelet aggregates. To ensure that the degradation products, produced by both tryptic digestion of fibrinogen and by the enzymatic action of urokinase - activated plasminogen on fibrinogen, behaved in the predicted fashion in the thrombin - fibrinogen reaction, the effect of these products on a thrombin clotting time system was studied.

Materials.

Fibrinogen was human lyophilized fibrinogen, Grade L, supplied by A.B. Kabi, Stockholm.

Fibrinogen degradation products were prepared as described in chapter 8.

ADP was the same preparation as that described in chapter 3.

Carbonate Buffer of 0.1 molar and pH 8.9 was prepared as described in chapter 3.

Methods.

Turbidimetric Method. ADP induced platelet aggregation was assessed in the turbidimetric system as described in chapters 3 and 5.

Chandler tube system. Platelet aggregation in an artificial circulation was studied by the method described in chapters 3 and 4.

Platelet adhesiveness was investigated in the modified glass bead column method of Hellem (1960) as described in chapters 3 and 6.

Thrombin clotting times. The effect of fibrinogen degradation products was investigated in the thrombin clotting time system described in chapter 5. To 0.05 ml plasma was added 0.05 ml, degradation product, 0.3 ml

'thrombin time mixture' and finally 0.1 ml thrombin (10 units per ml).

Results.

Effect of product 'a'. The effect of fibrinogen degradation product 'a' on platelet aggregation was investigated in the turbidimetric system and the Chandler tube system and its effect on platelet adhesiveness in whole blood was studied with the modified Hellem technique.

The effect of the product on platelet aggregation in the turbidimetric system was studied in a series of seven platelet rich plasma samples. Each plasma sample was divided into two portions each of 2 ml. To one portion was added 0.2 ml product 'a' to give a final concentration of 100 µg per ml plasma and to the second portion was added 0.2 ml, 0.9 per cent saline. Both samples were then incubated at 37°C for 10 minutes before platelet aggregation was induced in the samples by the addition of ADP (0.5 µg per ml plasma). The results are shown in figure 64 and table 66. Product 'a' appears to have no significant effect on ADP induced platelet aggregation. The mean optical density reading of the seven control samples, 1 minute after the addition of ADP, was 0.456 ± 0.090 and of the seven test samples 0.442 ± 0.103, ($t = 1.172$ $p < 0.30$).

In the turbidimetric system fibrin formation does not normally occur as a sequel to platelet aggregation. To study the effect of the degradation product in a situation in which platelet aggregation preceded thrombus formation, product 'a' was added to plasma in the Chandler tube

system. In a series of three plasma samples each sample was divided into two 5 ml amounts and to one portion was added product 'a' at a concentration of 200 µg per ml plasma while to the second portion was added an equal volume of 0.9 per cent saline. The volume in both loops was made up to 15 ml with 0.9 per cent saline and both loops incubated at 37°C for 10 minutes before recalcification with 0.5 ml, 0.25 M $CaCl_2$. In each of the three plasma samples investigated the presence of product 'a' appeared to cause acceleration of the rate of formation of platelet aggregates (table 23). No visible difference was detected between fibrin formation in the control and test loops. There may be some doubt, however, as to the validity of this result since the protein was stored in solution for several weeks before its effect in this sytem was studied and in addition to any spontaneous digestion which may have occurred some denaturation of the protein may also have taken place.

From these observations it would appear that, in platelet rich plasma, the effect of product 'a' appears to differ depending on the system used to investigate platelet aggregation and perhaps also on its concentration in the plasma. Thus, the effect of product 'a' on platelet adhesiveness has been investigated at the two concentrations used in the plasma studies, namely 100 µg and 200 µg per ml blood. With a series of eight citrated whole blood samples, each sample was divided into two portions, each of 2 ml. One portion was incubated at 37°C for 10 minutes with 0.1 ml product 'a'

(100 µg per ml blood) and the second with an equal volume of 0.1M sodium carbonate buffer, pH 8.9. After incubation, each sample was passed through a glass bead column and the platelet adhesiveness of each sample assessed from the number of platelets lost during passage. The results are shown in figure 65 and table 67. The average percentage platelet adhesiveness of the eight control samples was 41.9 ± 17.4 and of the eight samples treated with product 'a' 33.5 ± 16.7, ($t = 2.149$, $p < 0.010$). It would appear that, at this concentration, the presence of product 'a' tends to produce a slight decrease in platelet adhesiveness although the difference between the control and test samples is not significant. When the concentration of product 'a' was increased to 200 µg/ml blood, the protein appeared to exert an even less pronounced influence on platelet aggregation than at the lower concentration. The effect of product 'a' (200 µg per ml blood) was investigated with a series of eleven citrated whole blood samples and the results are shown in figure 66 and table 68. The average percentage platelet adhesiveness of the eleven control samples was 43.4 ± 17.8 and of the eleven samples treated with product 'a' the value was 40.6 ± 17.4, ($t = 0.826$, $p < 0.50$). Hence, at this concentration it would appear that product 'a' exerts no significant influence on platelet adhesiveness. While this result is in agreement with observations in the turbidimetric system it is contrary to those made with the Chandler tube system. As discussed in chapter 6, one of the differences between the

measurement of platelet adhesiveness in the modified Hellem technique and the measurement of platelet aggregation is that at no stage during the procedure is the blood thoroughly mixed with the substance under investigation for any length of time. With earlier studies on the action of trypsin and streptokinase on platelet adhesiveness (chapter 6) this difference was partly overcome by agitating the blood and the substance under investigation during the period of incubation. This was achieved by rotating the sample on the turntable of a blood cell suspension mixer (Matburn Ltd., London) sited in an incubator at 37°C. This experimental procedure was carried out with product 'a'. In a series of seven blood samples, blood and the protein (100 µg per ml) were agitated for 10 minutes at 37°C and a second sample of blood treated similarly with 0.1M sodium carbonate buffer, pH 8.9. The results are shown in figure 67 and table 69. From the results it would appear that, even with thorough mixing, product 'a' does not produce a significant effect on platelet adhesiveness. The average percentage platelet adhesiveness of the seven control samples was 72.5 ± 12.3 and of the seven test samples 70.4 ± 15.6 ($t = 1.084$, $p < 0.40$).

Effect of product 'b'. As with product 'a', the effect of fibrinogen degradation product 'b' on platelet clumping was investigated in the turbidimetric system, the Chandler tube system and with the modified Hellem technique.

The effect of product 'b' on ADP induced platelet aggregation in the turbidimetric system was studied in a series of seven platelet rich plasma

samples. The experimental procedure was similar to that described above for product 'a'. The results are presented in figure 68 and table 70. From the results it would appear that the presence of product 'b', at a concentration of 100 µg per ml plasma, produces a significant enhancement of platelet aggregation. The mean optical density reading of the seven control plasma samples, 2 minutes after the addition of ADP (0.5 µg per ml) was 0.378 ± 0.140 and the value for the seven test samples to which product 'b' had been added was 0.341 ± 0.141 ($t = 3.253$, $p < 0.02$).

A similar result was obtained for the effect of product 'b' on platelet aggregation in the Chandler tube system. The experimental procedure was the same as that for product 'a' as described above. In each of the three plasma samples investigated the presence of product 'b' at a concentration of 200 µg per ml resulted in an acceleration in the rate of formation of platelet aggregates (table 24). The presence of this fibrinogen degradation product did not appear to impede the formation of fibrin following platelet aggregation in this system.

When the effect of product 'b' on platelet adhesiveness in whole blood was investigated the protein did not appear to be as effective in influencing platelet adhesion as in the plasma systems. Product 'b' (100 µg per ml blood) was incubated with blood at 37°C for 10 minutes and over the series of eight samples investigated there appeared to be no significant difference in the percentage of adhesive platelets in the

control and test samples (figure 69 and table 71). The average percentage platelet adhesiveness of the eight control samples was 41.9 $\pm$ 17.4 and of the eight test samples to which product 'b' had been added the value was 39.6 $\pm$ 17.6 ($t = 0.594$, $p < 0.60$). Similar results were obtained when the concentration of product 'b' was increased to 200 μg per ml (figure 70 and table 72). The average percentage adhesiveness of the eleven control samples was 43.4 $\pm$ 17.8 and of the eleven test samples treated with product 'b' (200 μg per ml blood) the average value was 38.5 $\pm$ 13.7 ($t = 1.251$, $p < 0.30$). When the conditions of incubation were altered, as described above for product 'a', to make this technique more comparable to the Chandler tube and turbidimetric systems the apparent effect of product 'b' in this system was also altered. In a series of seven samples, blood and product 'b' (100 μg per ml) were agitated together at 37°C for 10 minutes before being pumped through a glass bead column. Control samples containing 0.1M carbonate buffer, pH 8.9 were treated similarly. The results are shown in figure 71 and table 73. The average percentage platelet adhesiveness of the seven control samples was 72.5 $\pm$ 12.3 and of the seven test samples 77.6 $\pm$ 11.7 ($t = 3.456$, $p < 0.02$). Thus it would seem that the presence of product 'b' can produce a statistically significant increase in the number of platelets lost from a sample during its passage through a glass bead column.

Effect of product 'c'. The effect of product 'c' on ADP induced platelet aggregation in the turbidimetric system was investigated with a series of

seven plasma samples. The experiments were carried out as described for product 'a'. The results are shown in figure 64 and table 74. From the results it would seem that product 'c' at a concentration of 100 µg per ml plasma does not impair platelet aggregation but produces a marked increase in the rate of disaggregation of platelet clumps which have formed. Two minutes after the addition of ADP (0.5 µg per ml), platelet aggregation is still proceeding in the control plasma samples but disaggregation has occurred to such an extent in the samples treated with the degradation product that over the series of seven plasma samples investigated the difference between the optical density readings in the control and test plasma samples is statistically significant. At this point, the mean optical density reading of the control samples was 0.445 ± 0.121 and of the test samples treated with product 'c' (100 µg per ml) the value was 0.473 ± 0.105 ($t = 3.233$, $p < 0.02$).

Effect of product 'd'. The effect of product 'd' on platelet aggregation was investigated in both the turbidimetric and Chandler tube systems.

The effect of product 'd' on ADP induced platelet aggregation was investigated in the turbidimetric system with a series of ten plasma samples. The experiments were carried out as described above for product 'a'. From the results it would appear that the presence of product 'd' (100 µg per ml) can cause a significant increase in the extent of platelet aggregation which occurs in the sample following the addition of ADP (figure 72 and table 75).

The mean optical density reading of the ten control samples, $1\frac{1}{2}$ minutes after the addition of ADP (0.5 µg per ml) was 0.305 ± 0.159 and in the ten test samples to which product 'd' was added, the mean value was 0.216 ± 0.112 ($t = 2.415$, $p < 0.05$).

A similar result was obtained when the effect of product 'd' on platelet aggregation was studied in the Chandler tube system. This experiment was performed by Dr A.H.C. Uttley. The effect of product 'd' (180 µg per ml) was studied in a series of seven plasma samples and the investigation was carried out as described above for product 'a'. The results are shown in figure 73 and table 76 where it can be seen that every sample investigated the presence of product 'd' resulted in an acceleration of the rate of formation of platelet aggregates. The mean time for the formation of platelet aggregates to form in the seven control samples was 477 ± 168 seconds and for the seven test samples 294 ± 45 seconds ($t = 3.874$, $p < 0.01$).

The results presented suggest that the various degradation products obtained by enzymatic digestion of fibrinogen, have slightly different effects on platelet aggregation. In an attempt to confirm that these observations were produced by the protein moieties added to the various systems and were not artefacts produced by the passage of fibrinogen through a DEAE cellulose column, the effect of fibrinogen on platelet aggregation

before and after passage through the column was investigated.

Effect of fibrinogen. The effect of fibrinogen (as supplied by the manufacturers) on ADP induced platelet aggregation was studied in a series of seven plasma samples. The experimental procedure was carried out as described above. The results are shown in figure 74 and table 77 and it would seem that fibrinogen (100 μg per ml) can cause a significant enhancement in the extent to which platelet aggregation occurs following the addition of ADP. The mean optical density readings of the seven control samples, 1½ minutes after the addition of ADP (0.5 μg per ml), was 0.406 $\pm$ 0.135 and of the seven test samples to which fibrinogen (100 μg per ml plasma) had been added 0.275 $\pm$ 0.198 ($t = 3.162$, $p < 0.025$).

The same series of seven plasma samples was used to study the effect, on platelet aggregation, of fibrinogen which had been 'purified' by passage through a DEAE cellulose column. The results are shown in figure 74 and table 78. It would appear that 'purified' fibrinogen tends to cause enhancement of ADP induced aggregation but the difference between the control and protein treated samples, over the series of seven samples, was not significant since in one of the seven samples the protein appeared to produce slight inhibition of aggregation. One and a half minutes after the addition of ADP (0.5 μg per ml) to the system, the mean optical density reading in the seven control samples was 0.406 $\pm$ 0.135 and the mean value in the seven samples treated with 'purified' fibrinogen (100 μg per ml) was 0.312 $\pm$

0.123 ($t = 2.361$, $p < 0.10$).

The effect of fibrinogen on platelet adhesiveness in citrated whole blood was also investigated. In a series of blood samples, blood and fibrinogen (100 µg per ml blood) were incubated together at 37°C for 10 minutes. Control samples to which 0.9 per cent saline had been added were treated similarly. The results are shown in figure 75 and table 79. Under these conditions, fibrinogen did not appear to cause an increase in the number of platelets lost during the passage of blood through a glass bead column. The average percentage platelet adhesiveness of the seven control samples was 37.0 ± 9.9 and of the seven samples treated with fibrinogen 38.5 ± 6.8 ($t = 0.359$, $p < 0.80$). However, when the Hellem system was further modified to ensure thorough mixing of the blood and protein during incubation, fibrinogen appeared to exert a different effect on platelet adhesiveness. The same seven blood samples were used to investigate the effect of fibrinogen (100 µg per ml) on platelet adhesiveness following thorough mixing during the 10 minute incubation period. The results are shown in figure 76 and table 80 and it can be seen that under these conditions fibrinogen produces a marked increase in the percentage platelet adhesiveness of the samples. The average percentage adhesiveness of the seven control samples was 61.7 ± 11.0 and of the seven fibrinogen treated samples 78.6 ± 9.0 ($t = 5.166$, $p < 0.005$).

From the observations of the effects of products 'a' and 'b' on platelet clumping it would appear that both these proteins may possess a

coagulant action since they can both cause enhancement of platelet aggregation. In order to determine whether this coagulant activity was also demonstrable in the conversion of fibrinogen to fibrin, the effects of products 'a' and 'b' in a thrombin clotting time system were investigated.

Thrombin clotting time. The effects of products 'a' and 'b' were studied over the range of concentration 100 µg per ml plasma to 2 mg per ml plasma in a thrombin clotting time system. The results are shown in table 25. Over the range of concentrations studied, it would appear that both degradation products inhibit the conversion of fibrinogen to fibrin which suggests that the presence of these products in the system can result in defective fibrin polymerization.

Discussion.

From the results presented above, it would seem that the various degradation products formed by enzymatic digestion of fibrinogen can exert a number of apparently different influences on platelet aggregation. Product 'a', formed by mild tryptic digestion of fibrinogen, exerts no significant effect on ADP induced platelet aggregation (figure 64, table 66) or on platelet adhesion (figures 65, 66 and 67, tables 67, 68 and 69) but can enhance platelet aggregation in an artificial circulation system (table 23). Product 'b', formed by moderate tryptic digestion of fibrinogen, can enhance ADP induced platelet aggregation (figure 68, table 70), aggregation in an artificial circulation system (table 24) and

platelet adhesiveness (figure 71, table 73). Product 'c', formed by prolonged tryptic digestion of fibrinogen, inhibits ADP induced platelet aggregation (figure 64, table 74). Finally, product 'd', formed by moderate urokinase-activated plasminogen digestion of fibrinogen, enhances both ADP induced platelet aggregation (figure 72, table 75) and aggregation in an artificial circulations system (figure 73, table 76).

Conflicting reports have appeared in the literature concerning the effect of fibrinogen degradation products on platelet aggregation. Some of the confusion has been caused by the fact that, for the most part, the degradation products have not been characterized according to their physicochemical properties. In many instances degradation products were not isolated from the fibrinogen incubation mixture so that the effect of a mixture of degradation products on platelet aggregation has been studied rather than an individual protein moiety. Degradation products prepared by digestion of fibrinogen with streptokinase-activated plasminogen have been shown to inhibit both ADP induced aggregation (Jerushalmy and Zucker, 1966; Kowalski et al., 1963) and fibrinogen induced aggregation (Kopec et al., 1966) <u>in vitro</u>. In addition, they have been shown to prolong the bleeding time in the presence of a normal platelet count following injection into dogs (Kowalski et al., 1964). However, degradation products prepared by plasmin digestion of fibrinogen have also been shown to exert

no influence on ADP induced platelet aggregation (Hirsh et al., 1965) and to exert no influence on ADP induced aggregation except when ADP is added in trace amounts when they inhibit platelet aggregation (Larrieu et al., 1966). Larrieu et al., (1966) have also demonstrated that degradation products, prepared by plasmin digestion of fibrinogen, inhibit the spreading of platelets on glass, inhibit spontaneous platelet aggregation and delay platelet aggregation after the addition of calcium chloride to plasma. The degradation products investigated by Hirsh et al., (1965) had sedimentation coefficients of 5.27×10^{-13} sec and 3.00×10^{-13} sec and molecular weights of 88,000 and 30,000 respectively (Fletcher et al., 1966). Two of the products studied by Larrieu et al., (1966) had very similar properties; the sedimentation coefficients were 5.2×10^{-13} sec and 3.0×10^{-13} sec. These values are sufficiently different from the coefficients, (Chapter 9), of the products 'a' and 'b' to conclude that the protein moieties examined by these workers were quite different from those examined in the present study. Further, the observation of the apparent variety of influences which the series of degradation products, prepared by tryptic digestion of fibrinogen appear to exert on platelet aggregation may account for the apparent divergence in the observations of earlier workers. Recently, there has been published a report of the discovery of a fibrinogen degradation product which possesses the ability to decrease the number of circulating platelets after infusion into dogs and which can not only

induce platelet aggregation *in vitro* but can also enhance ADP induced aggregation *in vitro* (Barnhart et al., 1966).

In the present study it has been demonstrated that fibrinogen can enhance ADP induced aggregation in platelet rich plasma (figure 74, table 77) and that it can also, under certain conditions, increase the percentage platelet adhesiveness in citrated whole blood (figure 76, table 80). This is in agreement with observations previously reported for the effect of the protein on ADP induced aggregation in platelet rich plasma (Kopec et al., 1966; McLean et al., 1964) and in suspensions of washed platelets (Cross, 1964; Solum and Stormorken, 1965). Fibrinogen, which had been further purified by passage through a DEAE cellulose column, did not appear to be quite as effective as the initial fibrinogen sample in the enhancement of platelet aggregation although it still tended to enhance ADP induced aggregation (figure 74, table 78). Figure 45 shows the fibrinogen was eluted from the DEAE cellulose column as one major peak and two smaller peaks. The heterogeneity of the initial fibrinogen solution is also confirmed from the pattern obtained following sedimentation in a sucrose density gradient system (figure 55). On the other hand the purified fibrinogen solution was sedimentated in the gradient system as a single peak suggesting that it was a homogeneous protein (figure 56). Thus it is possible that some other protein or proteins, closely associated with fibrinogen, are able to influence platelet aggregation. If this were the case, it would explain the

apparent quantitative difference between the actions of fibrinogen on platelet aggregation before and after it has been subjected to column chromatography.

Kopet et al., (1966) have shown that fibrinogen degradation products can be adsorbed onto the surface of washed platelets more firmly and in greater amounts than fibrinogen. It may be that products 'b' and 'd', which have been shown to enhance platelet aggregation, have similar spatial arrangements of charged groups in their molecular structure as has the complex fibrinogen molecule and this might enable them to behave like fibrinogen on the surface of the platelet. It has also been shown that those degradation products which inhibit platelet aggregation also inhibit the release of adenine nucleotides from platelets in the presence of agents which can stimulate platelet aggregation (Kopec et al., 1966). If, however, products 'b' and 'd' can substitute for fibrinogen on the platelet surface they may not possess the required configuration or electrophoretic properties to produce this inhibition.

The ability of fibrinogen degradation products to inhibit the thrombin-fibrinogen reaction has been extensively reported in the literature (Alkjaersig et al., 1962; Bang et al., 1962; Beck and Jackson, 1966; Fletcher et al., 1962; Godal and Helle, 1963; Hirsh et al., 1965; Jerushalmy and Zucker, 1966; Kowalski et al., 1964; Larrieu et al., 1966; Latallo et al., 1964; Lewis and Wilson, 1964). As shown in table 25,

products 'a' and 'b' also exhibit 'antithrombin like' activity since they prolong the time taken for thrombin to convert fibrinogen to fibrin. This observation would seem to suggest that the ability of product 'b' to enhance platelet aggregation depends on its effect on the platelets themselves since it produces inhibition of the later stages of coagulation and thrombus formation.

Platelet aggregation has been shown to play a critical role in the initial stages of haemostasis (Grette, 1962; Hellem and Owren, 1964; Hjort and Hasselback, 1961; Käser-Glanzmann and Lüscher, 1962; Marr et al., 1965; Poole and French, 1961; Russel, 1961). The attractive theoretical concept of a haemostatic balance mechanism suggests that fibrinolysis and coagulation may be continuously operating in parallel throughout the vascular bed (Astrup, 1958; Sherry et al., 1959) and if such is the case, products of fibrinogen proteolysis may have an important homeostatic role in displacing the equilibrium of the system towards coagulation.

The possibility of a physiological role for a fibrinogen degradation product which can enhance platelet aggregation is strengthened by the circumstance that it can be produced not only with trypsin but with the intravascular fibrinolytic enzyme plasmin, produced by activation of human plasminogen by human urokinase, and that its activity is not confined to platelet rich plasma but can also be demonstrated in citrated whole blood.

Conclusions.

Three degradation products have been prepared by tryptic digestion of fibrinogen and one by plasmin digestion. The product prepared with plasmin has been shown to enhance ADP induced platelet aggregation and to acceleration aggregation in an artificial circulation system. In addition to these actions, one of the products prepared with trypsin can enhance platelet adhesion in whole blood. The tryptic degradation product exhibits coagulant action on the early stage of the haemostatic process while possessing an antithrombic activity on the late stage of the reaction sequence.

CHAPTER 11

DISCUSSION.

The recognition of the key role of the platelet in normal and defective haemostasis has stimulated much research into platelet function. In view of the current concept that in vivo fibrinolysis and coagulation are in a state of dynamic equilibrium, the present studies were carried out to investigate the possible effect of fibrinolytic agents on one of the earliest steps in the haemostatic process, that is, platelet aggregation.

(1) The functions of the platelet.

The platelet contribution to the haemostatic mechanism includes a phospholipid, platelet factor 3, which participates in the formation of blood thromboplastin; serotonin which can induce vasoconstriction and thrombosthenin, a contractile protein which, in conjunction with the large store of ATP contained within the platelet membrane, facilitates clot retraction. Apparently the most complex and perhaps least understood aspect of platelet function is that of its role in the formation of haemostatic plugs and in thrombus formation. The first formed element demonstrate at a point of injury in the vascular lining is the platelet. The current concept of thrombus formation suggests that a clump of platelets gradually builds up round the platelets already adhering to damaged endothelium, so forming a plug which causes the slowing of the blood flow round the point of injury; fibrin

formation then occurs round the platelet mass. The plug becomes impermeable to blood flow and forms an effect seal over the damaged area. When this reaction sequence occurs to arrest bleeding from a vessel *in vivo* it is beneficial but when it occurs within a vessel and results in the occlusion of that vessel it is a great potential hazard.

(2) Mechanism of platelet aggregation.

It is at present considered that, both *in vivo* and *in vitro*, ADP plays a key role in the formation of platelet aggregates. The nature of the reaction involved in the process of platelet to platelet interaction has not as yet been fully elucidated but it is thought to be dependent on either the presence of ADP or on the dephosphorylation of ADP to supply energy for the reaction between platelets or between platelets and a foreign surface. Calcium ions and a plasma factor are also considered to be essential for the formation of bridges between platelets to hold them together, although neither the precise nature of such a binding reaction nor of the plasma factor involved is fully understood.

(3) Platelets and fibrinolysis.

Astrup (1958) was one of the first authors to suggest that, under physiological circumstances, a dynamic equilibrium may exist between the coagulation and fibrinolytic enzyme systems, the former leading to the deposition of fibrin to seal any defect in endothelium tissue and the latter to remove such fibrin deposits after they have served their useful function

and the integrity of the endothelium has been restored. However, the first stage in the repair of damaged endothelium is the adhesion of platelets to the site and it may be that a dynamic balance between the haemostatic and fibrinolytic mechanisms may also exist at an earlier stage in the reaction sequence than fibrin deposition, i.e. at the level of platelet aggregation and adhesion (Lüscher, 1967).

The studies for this thesis included an *in vitro* assessment of the possible existence for such a mechanism by the investigation of the effect of fibrinolytic agents on platelet aggregation and adhesion.

(4) Haemostasis and fibrinogenolysis.

I Effect of Streptokinase.

(a) Streptokinase and platelet aggregation and adhesion.

The incubation of either citrated whole blood or platelet rich plasma with streptokinase can lead to the enhancement of platelet adhesion and platelet aggregation of the samples (figures 40, 5 and 18). Streptokinase exerts its fibrinolytic activity by participating in the conversion of plasminogen to the active proteolytic enzyme plasmin (Sherry et al., 1959). The action of streptokinase on platelet function *in vitro* also appears to be mediated through this reaction. ADP induced aggregation can only be enhanced by streptokinase after the antiplasmin in the plasma had been destroyed (figure 18) and acceleration of the rate of formation of platelet aggregates in an artificial circulation system is accompanied by marked fibrinogenolysis (table 1) which is possibly the result of plasmin activity

since streptokinase alone does not exhibit proteolytic activity (Sherry and Alkjaersig, 1957).

(b) Streptokinase and washed platelets.

Streptokinase causes only slight enhancement of ADP induced aggregation of washed platelets resuspended in buffer (figure 30). The specificity of streptokinase towards plasminogen may account for this observation. Adelson et al., (1961) have postulated that virtually all the plasma proteins can be concentrated by the platelet to form a 'plasmatic atmosphere' round the surface of the cell. Since the washing procedure might be expected to remove most, if not all, the proteins from the system, the substrate for the enzyme, streptokinase, might no longer be present in the medium. The complete protein complement does not appear to be removed by the washing procedure, however, since a fibrin clot forms on the addition of thrombin to the platelet suspension (chapter 5). Wherever fibrinogen is present it is likely that some plasminogen may also be present. Thus the apparent slight enhancing effect of streptokinase on platelet aggregation of washed platelets may be due to the formation of plasmin.

(c) Platelet aggregation and fibrinogenolysis.

Uninhibited proteolytic activity appears to be a prerequisite for the enhancement of platelet aggregation in platelet rich plasma since the acceleration of the rate of formation of platelet aggregates in the Chandler tube (figure 5) is accompanied by a decrease in the amount of fibrin

incorporated into the Chandler thrombus (table 1) whereas if there is no enhancement of ADP induced aggregation (figure 17) neither is there a significant decrease in clottable protein (table 7). In vitro, it would seem, that at least one product formed by the proteolytic activity of plasmin on fibrinogen can cause enhancement of platelet aggregation (chapter 10).

(d) Streptokinase and coagulation.

Clot formation in recalcified plasma is delayed in the presence of streptokinase (table 3), probably as a result of fibrinogen digestion by plasmin formed in the plasma. In vitro the conversion of prothrombin to thrombin also appears to be inhibited by the presence of streptokinase (table 2) although the results may be explained on the basis of digestion of thrombin by plasmin or by the fact that fibrinogen, which acted as a substrate for formed thrombin in the assay system, may have been digested by plasmin before thrombin could exert its activity.

II Effect of trypsin.

(a) Trypsin and platelet aggregation and adhesion.

As with streptokinase, the incubation of platelet rich plasma or citrated whole blood with trypsin can produce enhancement of platelet aggregation and platelet adhesion of the treated samples (figures 6, 20, 42 and 43).

(b) Trypsin and washed platelets.

The ability of trypsin to cause enhancement of platelet aggregation does not appear to be due to its direct action on the platelet despite the

observations that the presence of platelets during the incubation of trypsin and plasma seems to contribute to its activity towards platelet clumping (figures 8, 9, 25 and 26). The platelet contribution to this phenomenon is most probably due to the presence of the plasma proteins in the 'plasmatic atmosphere' of the platelet since trypsin appears to abolish the ability of washed platelets to aggregate under the influence of ADP (figures 27 and 28). Although fibrinogen is still present in the washed platelet system as evidence by the formation of a fibrin clot as a result of addition of thrombin, (chapter 5) the fibrinogen of the system may be present as an integral part of the platelet structure and consequently its digestion by trypsin would probably lead to the loss of cellular integrity of the platelet with consequent inability to aggregate. It has previously been reported that trypsinized platelets fail to aggregate in the presence of thrombin when the clottable protein has been fully digested (Morse et al., 1965; Schmid et al., 1962).

(c) Platelet aggregation and fibrinogenolysis.

From the data at present available, it would appear that, as with plasmin, some degree of fibrinogenolysis is a prerequisite for the enhancement of platelet aggregation by trypsin; the acceleration in the rate of formation of platelet aggregates in the presence of trypsin in the Chandler tube (figure 7) is accompanied by a decrease in the amount of fibrin

incorporated into the ensuing thrombus, whereas when there is no significant enhancement of aggregation (figures 17 and 32) neither is there a significant decrease in the amount of clottable protein in the system (table 7). Further evidence to suggest that fibrinogenolysis may be of importance in the enhancement of platelet aggregation and adhesion was obtained from the observations that a product formed by tryptic digestion of fibrinogen can produce enhancement of platelet aggregation in vitro (chapter 10).

(d) Trypsin and coagulation.

In addition to its proteolytic activity towards fibrinogen and fibrin in vitro (Ratnoff and Colopy, 1953), trypsin can activate plasminogen, although relatively slowly compared to the specific inhibitors streptokinase and urokinase (Sherry et al., 1959). Since this enzyme is also able to contribute to coagulation by its apparent ability to act as a cofactor for the conversion of prothrombin to thrombin (Ferguson and Ennis, 1963; Ferguson et al., 1960; Stormorken 1956) it might be supposed that this could account for its ability to enhance platelet aggregation. However, in the present study its apparent ability to induce changes in plasma proteins which enhance platelet aggregation cannot be accounted for solely on the basis of thrombin generation. Under in vitro conditions, fibrinogen appears to be the preferred substrate for the proteolytic activity of trypsin when present in relatively high concentrations; the conversion of prothrombin to thrombin is apparently

impaired by trypsin either because thrombin itself is destroyed by the enzyme or more probably because trypsin digests the substrate for the action of thrombin, namely fibrinogen (table 2); the acceleration of the rate of formation of platelet aggregates by trypsin in an artificial circulation system is accompanied by significant fibrinogenolysis (table 1). Further evidence which suggests that the effect of trypsin on platelet aggregation is not related to its coagulant activity was obtained from the observations that concentrations of trypsin which enhance clot formation in recalcified plasma (table 3) can inhibit platelet aggregation (figure 20) while concentrations of trypsin which inhibit clot formation (table 3) can enhance platelet aggregation (figure 20).

III Other proteolytic enzymes and platelet aggregation.

(a) Urokinase.

If the hypothesis is valid that the action of streptokinase on platelet aggregation and fibrinogenolysis is mediated through plasminogen activation, on theoretical grounds and it would seem probable that urokinase would behave similarly since this enzyme is the naturally occurring in vivo activator of plasminogen. Although urokinase did not appear to enhance ADP induced platelet aggregation in the platelet rich plasma system examined (figure 33), antiplasmin was not removed or destroyed in the specimens examined. This may have masked any possible activity of the urokinase towards platelet function since plasma antiplasmin had to be destroyed before the ability of streptokinase to enhance ADP induced

platelet aggregation was observed (figure 18). Further, a fibrinogen degradation product formed under the action of urokinase-activated plasminogen on fibrinogen, in an antiplasmin free system, promotes platelet aggregation.

(b) Ficin and Chymotrypsin.

On the basis of the series of experiments performed with ficin and chymotrypsin it is not possible to state categorically whether they could enhance platelet aggregation or not, since they were not examined over a sufficiently wide range of concentrations. These enzymes have a similar action as trypsin on synthetic substrates but the results presented in table 2 seem to indicate that neither enzyme is capable of contributing to the activation of prothrombin to thrombin.

IV Relationship between platelet aggregation and fibrinogenolysis.

The observations presented above tend to support the concept of a relationship between platelet aggregation and fibrinogenolysis; fibrinogenolytic agents seem to be able to simultaneously support platelet aggregation and adhesion and fibrinogenolysis *in vitro*. The concept for the existence of such a mechanism *in vivo* is largely conjectural. Although it is now well recognised that platelets play an important part in haemostasis it is as yet not fully understood how accurately the ability of platelets to function in haemostasis is reflected by the *in vitro* tests currently available to assess platelet aggregation and adhesion.

The lack of knowledge in this area of haemostasis leads to difficulties when an attempt is made to collate *in vitro* and *in vivo* observations.

The evidence available from *in vivo* studies suggests that streptokinase and urokinase are dominantly fibrinolytic and 'antihaemostatic'. Furthermore enzymatic agents such as trypsin are not found under normal circumstances free in the circulating blood. *In vivo* plasmin normally has fibrin as a substrate and it is only under conditions of enhanced fibrinolysis in which plasmin is produced faster than it can be neutralized by antiplasmin, that fibrinogen may equally readily be digested (Sherry et al., 1959). However, it is possible that just as certain degradation products of fibrinogen exist which can enhance platelet aggregation, so there may be one or more products of fibrin degradation which possess the same potential activity. The existence of such activity might in fact contribute towards one of the potential hazards of fibrinolytic therapy, namely the formation of platelet emboli which are resistant to further fibrinolytic activity (McNicol et al., 1965). It would appear unlikely that the concentration of degradation products of either fibrinogen or fibrin, would be present in sufficiently high concentrations to enhance or promote platelet aggregation except in areas of localized fibrinolytic activity.

The *in vivo* observations do not exclude the possibility that a dynamic balance exists *in vivo*, at a subclinical level, between

haemostasis and fibrinolysis. Such a relationship could be reflected *in vitro* by the observations of enhanced platelet aggregation and adhesion in the presence of fibrinolytic agents and products of fibrinogenolysis.

(5) Mechanism of platelet aggregation and adhesion.

(a) Comparison of methods.

The results of the comparison of three methods of assessment of the ability of platelets to form aggregates show that there are no significant mathematical correlations between the observations made with each technique (table 19). However, as discussed in Chapter 7, the difference in the results obtained with each assay method, namely the Chandler tube, turbidimetric and glass bead column methods appears to be quantitative rather than qualitative. The effect of streptokinase and trypsin on platelet aggregation appears to differ according to the method of assay used. Once again however, the difference appears to be quantitative rather than qualitative since enhancement of platelet aggregation and adhesion can be produced in each system by the enzymes although the concentration of the enzymes required to produce this effect may vary.

(b) Role of ADP.

The formation of platelet aggregates in each system depends on the availability of the nucleotide, ADP; in the turbidimetric system ADP added to platelet rich plasma is the stimulus for aggregation, in the glass bead column method ADP is supplied to the platelets from the red cells

(chapter 6; Hellem, 1960) and as the formation of platelet aggregates in the Chandler tube can be enhanced by ADP and inhibited by adenosine (chapter 4) it may be concluded that ADP is involved in the reaction sequence. These observations are in agreement with the hypothesis of Haslam (1964) that ADP is involved in the aggregation of platelets irrespective of the stimulus to aggregation. The influence of ADP and adenosine on the action of trypsin on platelet aggregation also seem to support the concept that ADP is involved at some stage in the reaction sequence. The ability of trypsin to enhance platelet aggregation in platelet rich plasma to which no exogenous ADP has been added can be suppressed by adenosine, the specific inhibitor of ADP (figure 21). In the Chandler tube the influence of trypsin can be enhanced by ADP and inhibited by adenosine (figures 13 and 15). It has not been possible to demonstrate any significant alteration in ADP and AMP concentrations in either citrated whole blood or platelet rich plasma samples following the incubation of the samples with trypsin prior to the investigation of platelet adhesiveness or subsequent to the passage through the column of glass beads even though platelet adhesion was increased by trypsin treatment of the samples (chapter 6). Possible explanations include: (i) insensitivity of the method for the assay of ADP and AMP to minor changes which may occur in the system; (ii) the concentration of ADP necessary to trigger the mechanism of platelet adhesion and to keep it operating may be infinitessimal; (iii) ATP,

ADP and their derivatives are in a continual state of metabolic turnover and consequently the relative concentrations of each nucleotide may act as a more accurate guide of increased adhesiveness than absolute values.

(c) Effect of trypsin.

As discussed in Chapter 7, the various techniques used in this thesis to measure platelet aggregation and adhesion may possibly measure different stages in the reaction sequence leading from the presence of platelets as single cells in blood to their development into a solid mass, impermeable to blood flow, under conditions of trauma. This circumstance may, at least in part, account for the apparent differences in the action of an agent, such as trypsin, in the various in vitro systems. In the turbidimetric system it would appear that trypsin at concentrations which do not enhance platelet aggregation does not hinder platelet disaggregation when compared to that which occurs in the control samples (figures 17, 20, 23 and 24). As the concentration of trypsin is increased platelet aggregation is enhanced and platelet disaggregation impeded (figures 20 and 21). There is some confusion in the literature as to the extent of destruction caused by the incubation of platelets with trypsin. Grette (1962) has shown that trypsin, like thrombin, can cause the release of serotonin and ADP from the cells and this has been confirmed by Morse et al., (1965) who have also demonstrated that trypsin removes the clottable protein from the platelet. Schmid et al., (1962) found, however, that trypsinized human platelets remain morphologically intact. The extent of

digestion of platelet proteins by trypsin probably depends on the conditions under which the incubation occurs. When trypsin and washed platelets, resuspended in buffer, are incubated together it would seem that some degree of digestion of platelet proteins or proteins closely associated with the platelet occurs since the platelets apparently lose their ability to aggregate under the influence of ADP (figure 28). When the incubation is carried out in platelet rich plasma, plasma proteins may be the preferred substrate for the proteolytic activity of trypsin since, although platelet aggregation is enhanced, the platelet aggregates which are formed under the influence of trypsin are less readily disaggregated than those formed in the absence of trypsin (figures 21 and 25). This phenomenon is unlikely to be caused by the direct action of trypsin in the platelet since the same phenomenon is observed when fibrinogen or fibrinogen degradation products cause enhancement of aggregation (figures 68, 72 and 74). It may be that the presence of trypsin in platelet rich plasma not only enhances the extent to which aggregation occurs after the addition of ADP to the system but can promote the sequence of changes involved in the platelet contribution to thrombus formation to a stage beyond that of initial ADP reactivity i.e., to a stage in the sequence in which the platelet clumping is irreversible. It is this stage in the reaction sequence that is possibly being measured in the Chandler tube system and this may account for the apparent greater sensitivity of this technique to the action of trypsin and the other agents which can

promote platelet aggregation.

(d) Platelet to platelet interaction.

As outlined in chapter 2, it has been demonstrated that platelet aggregation requires a plasma factor which has characteristics in common with both fibrinogen and the protein, deficient in the plasma of patients suffering from Von Willebrand's disease (Born and Cross, 1964; McLean et al., 1964; Skalhegg et al., 1964). If such a factor is vital to the initial stages of aggregation it must be closely associated with the platelet and not readily removed or destroyed since washed platelets resuspended in buffer appear to be able to aggregate effectively in vitro on the addition of ADP (figures 28, 29 and 30). It has been suggested that the protein factor involved in platelet aggregation may participate in the formation of 'bridges' between platelets which would favour platelet aggregation (Born, 1965; Clayton et al., 1963; Gaarder and Laland, 1964; Salzman et al., 1966; Skalhegg et al., 1964; Spaet and Lejnicks, 1966). The suggested ability of fibrinogen to participate in the same 'binding' reaction as the plasma factor may explain the enhanced platelet aggregation found in the presence of fibrinogen; further, the failure of disaggregation may be due to the irreversible nature of the 'bridges' formed in the presence of added fibrinogen (figure 74). As certain fibrinogen degradation products can also enhance aggregation and impair disaggregation (figures 68 and 72), some indications as to possible characteristics of agents which may participate in the binding reactions may be inferred. The size of the protein involved

in the reaction would not appear to be significant since the degradation product 'b' which can enhance platelet aggregation or adhesion *in vitro* in each method studied has a molecular weight of approximately one tenth of that of fibrinogen (chapter 9). The availability of suitable groups on the surface of the protein e.g. negatively charged groups, may be of more importance in the 'binding' reactions than the size and shape of the molecule. Such a concept might possibly explain the apparent ability of agents, such as heparin, which would not normally be associated with any 'coagulant' action, to enhance platelet aggregation under certain conditions *in vitro* (figures 36 and 37).

CHAPTER 12

SUMMARY AND CONCLUSIONS.

The recognition of the importance of the physiological and pathological roles of the platelet has stimulated intensive study of the platelet and its mode of action within the haemostatic mechanism. This thesis gives an account of the investigation of the effect of the enzymes streptokinase and trypsin on the ability of platelets to adhere to a foreign surface and to form aggregates *in vitro*.

(1) Introduction

Described in chapter 2 are some of the functions currently ascribed to platelets in haemostasis and an outline of the mechanism thought to enable platelets to perform one such function, namely platelet aggregation. An account is also given of the relationship between platelets and fibrinolysis.

(2) Materials and methods

Chapter 3 contains a description of the materials and standard coagulation and biochemical methods used in the work for this thesis. The methods outlined in this chapter include the assay systems used to study the effect of substances on platelet aggregation and adhesion, the Chandler tube method, the turbidimetric method of Born and the Hellem glass bead column method. These three methods have been adapted to meet the special requirements for this thesis and the modifications are outlined in

chapters 4, 5 and 6 respectively. Also described in chapter 3 is a sensitivity test which gives an indication of the minimum concentration of the fibrinolytic enzymes streptokinase and urokinase which must be added to plasma in order that the inhibitors present in plasma may be overcome. A number of coagulation tests have been used to investigate particular aspects of the work and these include a thrombin clotting time assay described in chapter 3, a one-stage prothrombin time assay, plasma recalcification time and fibrinogen clotting time assays described in chapter 4. Chapter 3 also contains a description of a method of estimating the total protein content of a sample and of the methods used to estimate the concentration of two plasma, namely plasminogen and fibrinogen. The methods used to prepare and purify the proteins obtained by proteolytic digestion of fibrinogen are outlined in chapter 3 and described in detail in chapter 8. The methods used to characterize these proteins are detailed in chapter 3 and include methods of estimating molecular weight, electrophoretic charge and the degree of digestion involved in the formation of the fibrinogen degradation products. A further biochemical technique described in chapter 3 is the method of estimation of ADP and AMP concentrations in blood samples. The method of collection of blood for assay and for the preparation of plasma are presented in chapter 3 while the methods used to obtain suspensions of washed platelets are described in chapter 5.

(3) Haemostasis and fibrinogenolysis

An account of the effect on platelet aggregation and adhesion of the 'fibrinolytic' agents streptokinase and trypsin is presented and discussed in chapters 4, 5 and 6. The products of the fibrinogenolytic activity of trypsin and urokinase have been purified (chapter 8), their physico-chemical parameters studied (chapter 9) and their effect on platelet aggregation and adhesion examined (chapter 10).

(a) Enhancement of platelet aggregation and adhesion by streptokinase and trypsin

Addition of streptokinase or trypsin to citrated whole blood or platelet rich plasma can result in enhancement of platelet aggregation and adhesion in the samples. This effect of the two enzymes can be detected with each of the three in vitro methods used to investigate platelet function although the concentration of the enzymes required and the conditions under which enhancement is observed may vary for each method. The rate of formation of platelet aggregates in the artificial circulation of the Chandler tube is accelerated by streptokinase, at a concentration which produces maximum lytic activity, and by trypsin (10 μg/ml plasma). The ability of platelets to respond to the challenge of ADP is enhanced by streptokinase, only after plasma antiplasmin has been destroyed, and by trypsin (100 μg/ml plasma). Platelet adhesion to glass beads is

increased after citrated whole blood is thoroughly mixed with either streptokinase, at a concentration which produces maximum lytic activity or with trypsin (10 µg/ml blood).

(b) Action of streptokinase and trypsin on washed platelets

Streptokinase causes slight enhancement of ADP induced aggregation of washed platelets resuspended in buffer. Although the presence of platelets during the incubation of trypsin and plasma appears to contribute to its enhancement of platelet clumping in platelet rich plasma, trypsin causes almost complete inhibition of ADP induced aggregation of washed platelets resuspended in buffer.

(c) Platelet aggregation and fibrinogenolysis

The acceleration of the rate of formation of platelet aggregates in the Chandler tube by streptokinase and trypsin is accompanied by marked fibrinogenolysis. In plasma samples in which streptokinase and trypsin fail to cause enhancement of the reactivity of platelets towards ADP no significant fibrinogenolysis was detected.

(d) Other proteolytic enzymes and platelet aggregation

Urokinase, at a concentration which produced maximum lytic activity, was not found to cause any enhancement of ADP induced platelet aggregation in platelet rich plasma. At the concentrations studied neither ficin nor chymotrypsin were observed to exert any influence on the ADP reactivity of platelets in platelet rich plasma.

(d) Heparin and platelet aggregation

At the relatively low concentrations of 0.05 units and 10 units per ml plasma, heparin appears to enhance ADP induced platelet aggregation in platelet rich plasma. ADP reactivity of platelets was inhibited by heparin when present at a concentration of 500 units per ml plasma.

(e) Preparation of fibrinogen degradation products

Fibrinogen degradation products formed by the proteolytic activity of trypsin and urokinase-activated plasminogen on fibrinogen were prepared by incubating the enzyme preparations with fibrinogen. The proteolysis products were separated from the incubation mixture by chromatography on a DEAE cellulose column.

(f) Characterization of fibrinogen degradation products

(i) Molecular weight

The sedimentation patterns obtained for the various fibrinogen degradation products were examined in a sucrose density gradient system and compared to the patterns obtained for both the initial fibrinogen solution and fibrinogen which had been eluted from a DEAE cellulose column. From the results obtained it appeared that the degradation products were all smaller molecules than the parent fibrinogen but the technique did not reveal any significant difference between the various degradation products. When two of the degradation products were examined in the Spinco, Model E,

ultracentrifuge it was confirmed that the degradation products had much lower molecular weights and dissimilar sedimentation and diffusion coefficients than fibrinogen. The two degradation products were also found to be dissimilar to each other.

(ii) Electrophoresis

The degradation products were examined by paper electrophoresis. Fibrinogen and fibrinogen eluted from a DEAE cellulose column were also examined for comparative purposes. The electrophoretic patterns obtained with both fibrinogen preparations were identical and showed that the fibrinogen molecules migrate towards the anode. The degradation products appeared as diffuse bands round the origin and the patterns could not be improved by current reversal at the end of electrophoresis.

(iii) Reaction kinetics

The number of peptide bonds broken during the formation of two of the degradation products formed by tryptic digestion of fibrinogen was examined in a pH stat.

(g) Fibrinogen degradation products and platelet aggregation

The effect of the three degradation products prepared by tryptic digestion of fibrinogen on platelet aggregation was examined. The product prepared under mild conditions of proteolysis exerted no action on the reactivity of platelets towards ADP or on the ability of platelets to adhere to glass beads. The degradation product prepared under the influence of a higher concentration of trypsin was found to accelerate

the rate of formation of platelet aggregates in the Chandler tube, to enhance the ability of platelets to react to the challenge of ADP and to result in an increase in platelet adhesiveness to glass beads. The degradation product prepared by prolonged tryptic digestion of fibrinogen inhibited ADP induced platelet aggregation. The degradation product prepared by the action of urokinase-activated plasminogen on fibrinogen was found to accelerate the rate of formation of platelet aggregates in the Chandler tube and to enhance ADP induced platelet aggregation in platelet rich plasma. All the degradation products prepared were found to exhibit marked 'antithrombin' activity.

(4) Comparison of methods of assessing platelet aggregation

The three in vitro methods used in the assessment of the ability of platelets to form aggregates were compared. There were no significant mathematical correlations found between the observations made with the Chandler tube, turbidimetric and glass bead column methods.

Conclusion.

The important role at present ascribed to the platelet in haemostasis has stimulated much interest in the platelet, its mode of action within the haemostatic mechanism and factors which can influence the platelets either to form aggregates or to remain as discrete cells.

The work for this thesis included the investigation of the action on platelet aggregation and adhesion of 'fibrinolytic' agents, particularly streptokinase and trypsin. In vitro these enzymes appear to be able to

enhance platelet aggregation and adhesion and it would seem that they may do so by virtue of their fibrinolytic and fibrinogenolytic activity. Products of the fibrinogenolytic action of plasmin (produced by streptokinase) and trypsin can cause marked enhancement of platelet aggregation and platelet adhesion while exhibiting pronounced 'antithrombin' activity. The results presented in this thesis seem to indicate that *in vitro* there is a relationship between platelet aggregation and fibrinogenolysis.

A comparative study of the *in vitro* techniques used to investigate platelet aggregation and adhesion is also presented. No apparent correlation was found for the assessment of platelet function with each test but from the evidence obtained with the effect of the fibrinolytic agents in each system it may be concluded that apparent lack of correlation may be due to quantitative rather than qualitative differences.

Much work remains to be done before the *in vivo* significance of the actions of the fibrinolytic agents and their fibrinogenolytic products on the initial stages of haemostasis can be fully assessed. Clearly the factors involved in platelet aggregation are complex and elucidation of the mechanisms of aggregation and disaggregation still present many challenging problems.

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BLOOD PLATELETS AND PROTEOLYTIC ENZYMES

Summary of thesis submitted for degree of Ph.D. by
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This thesis gives an account of the investigation of the effect of a variety of proteolytic enzymes on platelet aggregation. The discovery of platelets in blood and the recognition of their importance in haemostasis and thrombus formation is described in an historical introduction, which is followed by an account of materials and methods used, including three *in vitro* tests designed to estimate the ability of platelets to aggregate. Techniques for the preparation, purification and characterization of degradation products formed by proteolytic digestion of fibrinogen are also described.

The experimental section of the thesis begins with an account of the assessment *in vitro* of the effect of fibrinogenolytic agents, particularly streptokinase and trypsin, and of products of fibrinogenolytic activity on platelet aggregation and adhesion. Platelet aggregation was assessed in two ways; by an artificial circulation system - the Chandler tube system, which measures the rate of formation of platelet aggregates in flowing recalcified citrated plasma, and by a turbidimetric method which measures the ability of platelets in response to adenosine-di-phosphate (ADP). As a measure of platelet adhesion, the diminution in platelet count as a result of passage of citrated whole blood or platelet-rich plasma through a column of glass beads was used (modified Hellem technique).

Platelet clumping, as estimated by all methods, was enhanced by both streptokinase and trypsin although the concentrations of each enzyme

required to produce this effect varied in the different assay systems. Streptokinase, at a concentration producing maximum lytic activity, accelerated the rate of formation of platelet aggregates in the Chandler tube and produced a marked increase in platelet adhesiveness in the Hellem-type technique. An increase in platelet ADP reactivity was only detected in the turbidimetric system after the plasma antiplasmin had been destroyed. Trypsin, at a concentration of 10 ug/ml sample produced a significant increase in the rate of formation of platelet aggregates in the Chandler tube system and an increase in platelet adhesiveness in the Hellem-type technique, but the concentration of enzyme had to be increased to 100 ug/ml sample before an increase in platelet ADP reactivity was detected.

Neither streptokinase nor trypsin influence platelet aggregation by a direct action on the platelet; streptokinase produces no significant enhancement and trypsin produces almost total inhibition of aggregation of washed platelets resuspended in buffer.

The effect on platelet aggregation of other proteolytic enzymes - urokinase, ficin, and chymotrypsin was also investigated, but none of these produced any enhancement of platelet reactivity to ADP in the turbidimetric system. These enzymes were however examined in less detail than streptokinase and trypsin.

Heparin was also studied and at the concentrations of 0.05 units and 10 units per ml plasma was found to cause significant enhancement of ADP induced aggregation in the turbidimetric system, but when present at a concentration of 500 units per ml plasma caused significant inhibition of ADP reactivity.

The enhancement of platelet aggregation by streptokinase and trypsin was associated with marked fibrinogenolysis; several fibrinogen degradation products were prepared and their effect on platelet aggregation and adhesion examined. The degradation products were prepared by incubation of fibrinogen with either trypsin or urokinase-activated plasminogen followed by fractionation of the protein components of the incubation mixture on a DEAE cellulose column. The ultracentrifugal and electrophoretic properties of the breakdown products were determined and the number of peptide bonds broken during formation of two of the products determined.

The effect on platelet aggregation and adhesion was examined of three products of tryptic digestion of fibrinogen; the product of least digestion (molecular weight apprximately 83,000) produced no significant effect on platelet ADP reactivity or platelet adhesiveness, the product of prolonged digestion (molecular weight less than 13,000) inhibited ADP induced platelet aggregation while the product of intermediate digestion (molecular weight 25,000) enhanced the rate of formation of platelet aggregates, ADP reactivity and platelet adhesiveness. A product prepared by digestion with urokinase-activated plasminogen enhanced both the rate of formation of platelet aggregates in the Chandler tube and ADP induced platelet aggregation. The two larger fragments produced by trypsin both exhibited a marked 'antithrombin' effect.

The three in vitro methods used to study platelet aggregation and adhesion were compared. No mathematical correlations were found between the results obtained with the different methods; however the effect of trypsin and

streptokinase could be detected with each method and the observations made with each method could to some extent be influenced by the concentration of ADP in the system.

The experimental data presented demonstrate that under certain circumstances the enzymes streptokinase and trypsin can produce enhancement of platelet aggregation. The evidence suggests that they may do so by digestion of fibrinogen; in purified form, one of the products of fibrinogen digestion would appear to augment platelet aggregation. However, other fibrinogen degradation products appear to impair aggregation. These products of proteolytic activity may be important in maintaining a haemostatic balance in vivo.

The thesis concludes with a summary of the work.

BLOOD PLATELETS AND PROTEOLYTIC ENZYMES

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Volume 2.

APPENDIX 1

Tables 1 - 25.

Table 1 Incorporation of fibrinogen into the Chandler thrombus

Subject No.	Percentage radioactivity in thrombus	
	Control	Test
1	68.5	12.5
2	74.1	39.9
3	65.3	7.7
4	50.0	10.1

Table 1 shows the percentage of radioactive fibrinogen incorporated into Chandler thrombi formed from saline treated platelet rich plasma and from trypsin (1 mg/ml) treated platelet rich plasma.

Table 2 Effect of proteolytic enzymes on thrombin generation

Reagent	Clotting time (seconds)
Control	5.8; 5.2
Trypsin 10 µg/ml	5.5; 5.2
" 100 µg/ml	162.0; 180.0
" 1 mg/ml	180+ ; 180+
Chymotrypsin 100 µg/ml	29.0; 28.5
Ficin 10 µg/ml	6.9; 6.9
" 100 µg/ml	5.2; 5.0
" 1 mg/ml	5.0; 7.5

Table 2 shows the effect of 0.2 ml of a series of incubation mixtures on the clotting time of 0.2 ml, fibrinogen. The incubation mixtures consisted of 0.3 ml of partially purified human prothrombin and 0.3 ml of the enzyme preparation which were maintained at 37°C for 10 minutes before a sample was added to partially purified human fibrinogen.

Table 3 Effect of trypsin and streptokinase on plasma recalcification times

Sample No.	Recalcification Time (seconds)			
	Control	Streptokinase 2,000 N.I.H. units/ml	Trypsin 10 µg/ml	Trypsin 100 µg/ml	Trypsin 1 mg/ml
1	249; 258	960	Samples	44	106
2	297; 308	808	clotted	47	203
3	256; 251	457	before recalcification	154	184

Table 3 shows the effect of streptokinase and trypsin on the rate of clot formation in recalcified plasma. 0.2 ml EDTA plasma and 0.1 ml of the enzyme preparation were incubated together at 37°C for 10 minutes before the addition of 0.2 ml M/40 $CaCl_2$ to the system.

Table 4 Effect of adenosine on one stage prothrombin time assay

System	Clotting time (seconds)
Control	27.0; 27.0
Adenosine 100 µg/ml	25.5; 25.0
" 200 µg/ml	25.5; 25.8
" 500 µg/ml	26.8; 27.0
" 1 mg/ml	27.0; 27.0

Table 4 shows the effect of adenosine, at various concentrations, on the one stage prothrombin time assay. 0.1 ml of a preparation of human brain, 0.1 ml plasma and 0.1 ml adenosine were incubated at 37°C for 10 minutes before the addition of 0.1 ml, M/40 $CaCl_2$ to the system.

Table 5 Effect of adenosine on the thrombin clotting time assay

System	Clotting time (seconds)
Control	9.0; 9.0
Adenosine 100 µg/ml	9.0; 9.0
" 200 µg/ml	9.0; 9.2
" 500 µg/ml	8.8; 9.0
" 1 mg/ml	9.0; 8.8

Table 5 shows the effect of adenosine, at various concentrations, on the thrombin clotting time assay. 0.1 ml plasma, 0.3 ml 'thrombin time mixture' and 0.1 ml adenosine were incubated at 37°C for 10 minutes before the addition of 0.1 ml thrombin (6 units /ml).

Table 6 Effect of ADP on fibrin formation

Subject No.	Rate of fibrin formation (seconds)	
	Control	Test
1	41	50
2	52	61
3	30	23
4	22	35
5	56	70
6	53	50
7	67	120
Mean ± SD	46 ± 16	58 ± 31

Table 6 shows the effect of ADP (0.5 µg/ml plasma) on the rate of formation of fibrin following platelet aggregation in the Chandler tube. There is no significant difference between the results of the control and ADP treated samples; $t = 1.704$, $0.10 < p < 0.20$.

Table 7 Percentage clottable protein present after aggregation

System	Subject No.	Percentage clottable protein		
		Control	Streptokinase	Trypsin
ADP induced platelet aggregation in diluted plasma	1	74.2	14.1	61.4
	2	78.3	57.7	89.3
	3	76.7	61.5	81.7
	4	69.4	68.8	68.6
	5	88.2	33.3	85.9
Nor-adrenaline induced aggregation	6	64.4	84.0	62.1
	7	77.2	-	81.2
	8	74.1	38.9	68.3
	9	62.7	53.6	64.1
	10	54.4	41.7	87.7

Table 7 shows the effect of incubation of platelet rich plasma with saline, streptokinase (100 N.I.H. units/ml) and trypsin (10 µg/ml) on the percentage clottable protein remaining in the samples after platelet aggregation.

Table 8 Effect of heparin on *in vitro* clot formation

Concentration of heparin units /ml plasma	Clotting time (minutes)
8	38
10	65
20	90
50	90

Shown is the effect of heparin on clot formation in vitro. The system consisted of 0.5 ml plasma, 0.05 ml heparin and 0.05 ml M/4 $CaCl_2$.

Table 9 Effect of trypsin on platelet aggregation of washed platelets suspended in a fibrinogen enriched solution

Time after addition of ADP (min)	Optical Density Readings	
	Control	Test
1	0.568	0.582
2	0.562	0.580
3	0.560	0.579
4	0.560	0.579
5	0.552	0.578
6	0.550	0.577
7	0.548	0.572
8	0.545	0.572
9	0.545	0.570
10	0.545	0.570

Shown are the optical density readings, obtained after the addition of ADP (0.5 µg/ml suspension), of a control sample of washed platelets incubated at 37°C for 10 minutes with trypsin (100 µg/ml suspension) and a test sample of washed platelets incubated with trypsin (100 µg/ml suspension) and fibrinogen (400 mg/ml suspension). The washed platelets were suspended in a buffer medium (Haslam, 1964).

Table 10 The effect of trypsin on platelet aggregation of washed platelets

Time after addition of ADP (min)	Optical Density Readings	
	Control	Test
1	0.548	0.562
2	0.540	0.560
3	0.535	0.555
4	0.535	0.548
5	0.540	0.542
6	0.539	0.542
7	0.542	0.541
8	0.535	0.540
9	0.538	0.540
10	0.538	0.540

Shown is the effect of addition of fibrinogen to a suspension of washed platelets on the action of trypsin on ADP induced aggregation. The optical density readings, obtained after the addition of ADP (0.5 µg/ml suspension), to a control sample of washed platelets incubated with trypsin (1 mg/ml suspension) at 37°C for 10 minutes and a test sample of washed platelets incubated with trypsin (1 mg/ml suspension) and fibrinogen (400 mg/ml suspension), are presented.

Table 11 Effect of the presence of white cells on the action of trypsin on platelet adhesiveness

Subject	System	Control		Test	
		% fall in platelet count	% fall in white cell count	% fall in platelet count	% fall in white cell count
A	Platelet rich and white cell rich plasma	13.1	2.3	25.3	24.8
B	Platelet rich and white cell rich plasma	21.5	20.5	34.6	33.1
	citrated whole blood	62.3	1.7	29.5	21.4

Shown is the effect of the presence of white cells on the action of trypsin (10 µg/ml) on platelet adhesiveness of platelet and white cell rich plasma and of citrated whole blood.

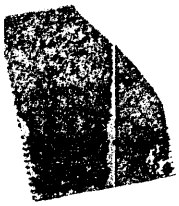

Table 12 ADP concentrations in citrated whole blood

Subject	μmoles per ml ADP			
	Control		Test	
	a	b	a	b
1	0.068	0.082	0.225	0.068
2	0.116	0.191	0.136	0.150
3	0.184	0.130	0.041	0.048
4	0.041	0.136	0.239	0.259
5	0.130	0.136	0.150	0.109
6	0.130	0.116	0.041	0.136
7	0.177	0.123	0.136	0.136
8	0.252	0.136	0.136	0.205
Mean ± S.D.	0.121 ± 0.083	0.131 ± 0.030	0.138 ± 0.023	0.139 ± 0.022

Shown are the A.D.P. concentrations of citrated whole blood samples before (a) and after (b) passage through a column of glass beads. The control samples were treated with saline and the test samples with trypsin (10μg/ml blood). There is no significant difference between the results of either the control and trypsin treated samples before and after passage; $t = 0.243$, $0.80<p<0.90$ and $t = 0.033$, $0.995<p<0.999$ respectively.

Table 13. AMP concentrations in citrated whole blood

Subject	μ moles per ml AMP			
	Control		Test	
	a	b	a	b
1	0.249	0.249	0.263	0.297
2	0.273	0.252	0.310	0.324
3	0.222	0.191	0.218	0.252
4	0.263	0.280	0.273	0.276
5	0.201	0.171	0.218	0.218
6	0.273	0.222	0.225	0.211
7	0.239	0.211	0.208	0.211
8	0.143	0.198	0.252	0.273
Mean	0.233	0.222	0.246	0.258
± S.D.	± 0.044	± 0.036	± 0.035	± 0.042

Shown are the AMP concentrations of citrated whole blood samples treated with 0.9% sodium chloride and trypsin (10 μg/ml blood) before (a) and after (b) passage through a glass bead column. The differences in concentration before and after passage are not significant in either the control or trypsin treated samples; $t = 0.929$, $0.30 < p < 0.40$ and $t = 1.980$, $0.05 < p < 0.10$ respectively.

Table 14 ADP concentrations in platelet rich plasma

Subject	μ moles per ml ADP			
	Control		Test	
	a	b	a	b
1	0.143	0.123	0.068	0.068
2	0.082	0.027	0.068	0.123
3	0.123	0.170	0.123	0.126
4	0.061	0.055	0.027	0.034
5	0.136	0.123	0.095	0.034
6	0.170	0.068	0.123	0.068
7	0.123	0.157	0.126	0.123
Mean	0.120	0.103	0.090	0.082
± S.D.	± 0.037	± 0.054	± 0.037	± 0.041

Shown are the ADP concentrations of platelet rich plasma samples treated with 0.9% sodium chloride or trypsin (10 μg/ml plasma) before (a) and after (b) passage through a glass bead column. The differences between the results before and after passage are not significant in either the control or trypsin treated samples; $t = 0.854$, $0.30 < p < 0.40$ and $t = 0.515$, $0.60 < p < 0.70$ respectively.

Table 15 **AMP concentrations in platelet rich plasma**

Subject	μ moles per ml AMP			
	Control		Test	
	a	b	a	b
1	0.133	0.246	0.126	0.116
2	0.075	0.102	0.089	0.068
3	0.061	0.068	0.058	0.058
4	0.041	0.068	0.061	0.061
5	0.061	0.044	0.072	0.072
6	0.068	0.068	0.041	0.075
7	0.143	0.126	0.123	0.068
Mean	0.083	0.103	0.081	0.074
± S.D.	±0.039	±0.069	±0.033	±0.019

Shown are the AMP concentrations of platelet rich plasma samples treated with 0.9% sodium chloride or trypsin (10 μg/ml plasma) before (a) and after (b) passage through a column of glass beads. The differences between the results before and after passage are not significant in either the control or trypsin treated samples; $t = 1.184$, $0.10 < p < 0.20$ and $t = 0.728$, $0.40 < p < 0.50$ respectively.

Table 16 Percentage platelet adhesiveness and ADP concentrations in citrated whole blood and platelet rich plasma.

System		% Platelet adhesiveness	Initial ADP concentration μ moles/ml	Change in ADP concentration μ moles/ml
Citrated whole blood	1	93.1	0.068	+0.014
	2	67.6	0.116	+0.075
	3	89.3	0.184	-0.054
	4	37.3	0.041	+0.095
	5	5.6	0.130	+0.006
	6	15.2	0.130	-0.014
	7	24.6	0.177	-0.054
	8	35.6	0.252	-0.116
Platelet rich plasma	1	13.2	0.143	-0.020
	2	29.8	0.082	-0.055
	3	25.9	0.123	+0.047
	4	0	0.061	-0.006
	5	0	0.136	-0.013
	6	0	0.170	-0.102
	7	5.4	0.123	+0.034

Table 17 Percentage platelet adhesiveness and ADP concentrations in citrated whole blood and platelet rich plasma treated with trypsin

System		% Platelet adhesiveness	Initial ADP concentration μ moles/ml	Change in ADP concentration μ moles/ml
Citrated whole blood	1	76.8	0.225	-0.157
	2	90.7	0.136	+0.014
	3	78.6	0.041	+0.007
	4	62.1	0.239	+0.020
	5	73.8	0.150	-0.041
	6	39.7	0.041	+0.095
	7	61.6	0.136	0
	8	41.8	0.136	+0.069
Platelet rich plasma	1	98.6	0.048	0
	2	89.5	0.068	+0.055
	3	25.9	0.123	+0.003
	4	3.2	0.027	+0.007
	5	18.2	0.095	-0.061
	6	45.6	0.123	-0.055
	7	26.7	0.126	-0.003

Table 18 Packed cell volume and platelet adhesiveness

	% packed cell volume	% platelet adhesiveness (uncorrected)	% platelet adhesiveness (corrected)
Mean	40.3	49.8	50.4
S.D.	± 5.0	± 18.3	± 14.6

Shown are the mean and standard deviations of the packed cell volume and platelet adhesiveness estimated on a series of twenty-five subjects. The percentage platelet adhesiveness was corrected, for each sample, from the calibration curve shown in figure 44 to allow for the individual variations of packed cell volume from the mean value of 40%.

Table 19 Comparison of results of various methods of measuring platelet aggregation

Methods	Correlation coefficient	Significance
Chandler and Born (1 minute reading)	+ 0.15	$p < 0.1$
Chandler and Born (4 minute reading)	- 0.13	$p < 0.1$
Chandler and Hellem (no correction)	+ 0.19	$p < 0.1$
Born (1 minute reading) Hellem (no correction)	- 0.18	$p < 0.1$
Born (4 minute reading) Hellem (no correction)	- 0.21	$p < 0.1$
Chandler and Hellem (corrected)	+ 0.25	$p < 0.1$
Born (1 minute reading) Hellem (corrected)	- 0.39	$0.1 < p < 0.5$
Born (4 minute reading) Hellem (corrected)	- 0.46	$0.5 < p < 0.01$

Shown are the mathematical correlations determined from the results of observations of platelet function estimated on a series of twenty-five subjects with each of three methods. In no instance is there significant correlation between the results obtained with the Chandler tube, turbidimetric (Born) or glass bead column (Hellem) methods.

Table 20 Comparison of platelet aggregation and platelet count

Method	No. of observations	Correlation coefficient	Significance
Chandler	40	- 0.04	$p < 0.1$
Born (2 minute reading)	53	- 0.09	$p < 0.1$
Hellem (uncorrected)	25	- 0.23	$p < 0.1$
Hellem (corrected)	25	- 0.11	$p < 0.1$

Shown are the mathematical correlations determined from the results of observations on platelet aggregation and from platelet counts. The readings obtained with the three methods have been compared to their respective platelet counts. In no case is there significant correlation between the platelet count of a sample and the extent to which platelet aggregation occurs within the sample.

Table 21 Plasma protein concentrations

Method	No. of observations	Plasminogen casein units per ml	Fibrinogen mgm per 100 ml
Chandler System	40	2.7 ± 0.7	300 ± 120
Turbidimetric system	53	3.1 ± 1.3	345 ± 122

Shown are the means and standard deviations of the plasminogen and fibrinogen concentrations of the platelet rich plasma samples investigated in the Chandler tube series of experiments (Chapter 4) and in the turbidimetric system (Chapter 5).

Table 22 The ultracentrifugation data for fibrinogen and the fibrinogen degradation products 'a' and 'b'

Protein	Sedimentation coefficient x 10^{-3} sec.	Diffusion coefficient x 10^{-7} cm per sec.	Molecular Weight
Fibrinogen	7.6	1.97	341,000
Product 'a'	6.72	6.62	82,427
Product 'b'	4.37	14.40	24,642

Shown are the values for the sedimentation coefficients, diffusion coefficients and molecular weights of fibrinogen, product 'a' and product 'b'. The values quoted for fibrinogen are from the data of Caspary and Kekwick (1954) as quoted by Seheraga and Laskowski (1957).

Table 23 Effect of product 'a' on platelet aggregation in the Chandler tube

Subject No.	Time of platelet aggregation (seconds)	
	Control	Test
1	522	464
2	447	392
3	420	386

Shown is the effect of product 'a' (200 µg per ml plasma) on the rate of formation of platelet aggregates in the Chandler tube. The product and plasma were incubated at 37°C for 10 minutes before the system was recalcified.

Table 24 Effect of product 'b' on platelet aggregation in the Chandler tube

Subject No.	Time of platelet aggregation (seconds)	
	Control	Test
1	522	438
2	447	362
3	420	361

Shown is the effect of product 'b' (200 µg/ml plasma) on platelet aggregation in platelet rich plasma in the Chandler tube. The product and plasma were incubated together at 37°C for 10 minutes before the system was recalcified.

Table 25 Effect of products 'a' and 'b' on thrombin clotting time

Sample	Thrombin clotting times (seconds)
Saline control	16.5; 16.0
Product 'a' 2 mg/ml	30.2; 30.0
1 mg/ml	25.2; 25.4
500 µg/ml	25.5; 25.5
200 µg/ml	25.0; 25.5
100 µg/ml	25.5; 25.2
Product 'b' 2 mg/ml	28.5; 28.8
1 mg/ml	26.2; 26.2
500 µg/ml	24.5; 25.0
200 µg/ml	23.2; 23.8
100 µg/ml	23.4; 23.8

Shown are the effects of products 'a' and 'b' in a thrombin clotting time system. To 0.05 ml, plasma was added to 0.05 ml, product and 0.3 ml, 'thrombin time mixture'. The sample was clotted with 0.1 ml thrombin (10 units per ml).

FIGURES.

FUNCTION OF PLATELETS IN HAEMOSTASIS

1. Platelet plug
2. Vasoconstriction - 5-HT release
3. Contribution to coagulation
4. Clot retraction
5. Capillary integrity

Figure 1 shows the function of the platelet in haemostasis.

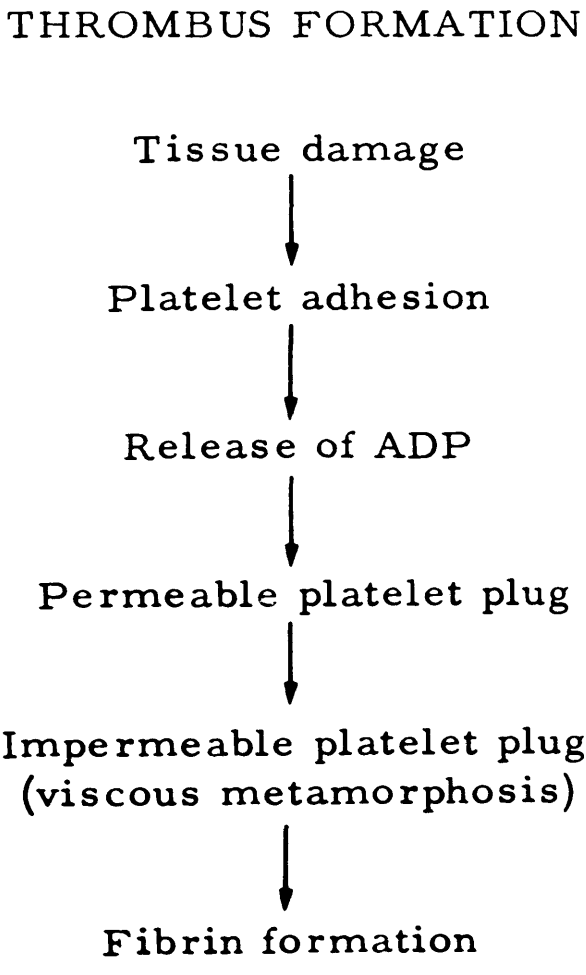

Figure 2 illustrates schematically the sequence of reactions leading to thrombus formation as postulated by Hellem and Owren (1964).

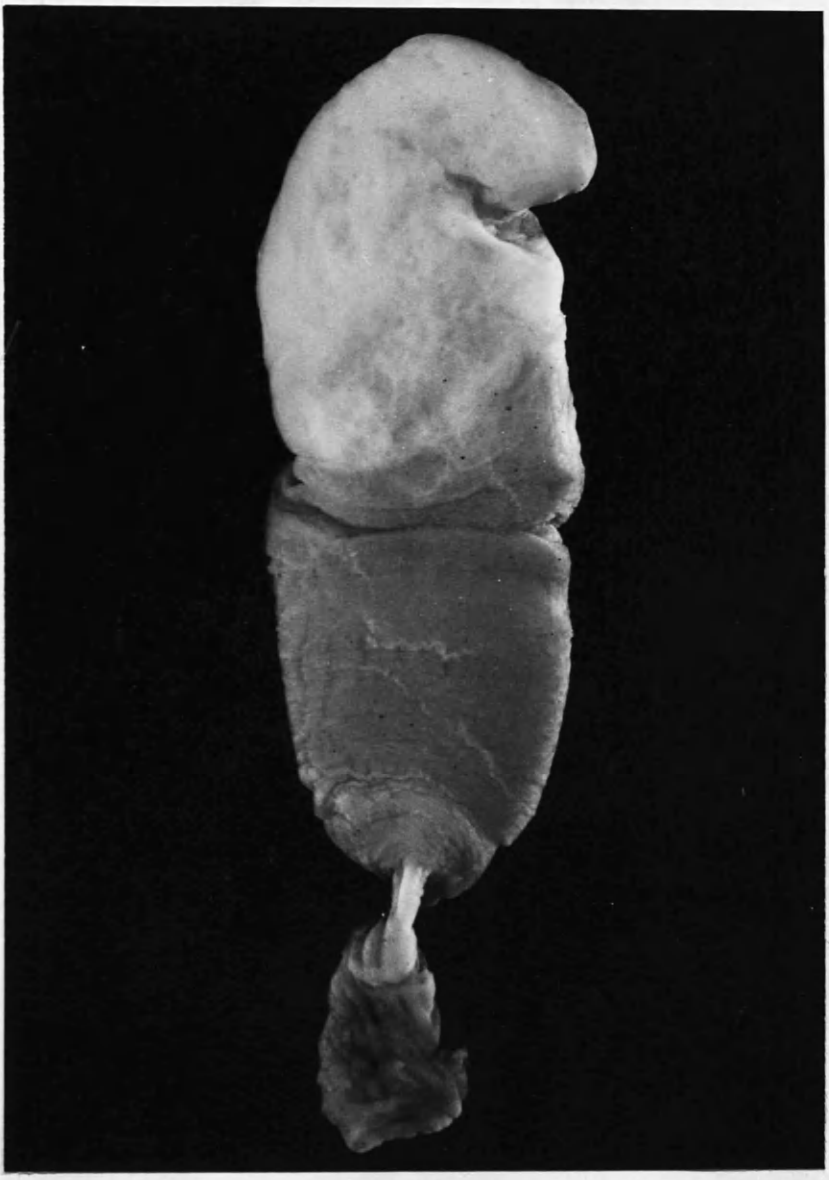

Figure 3 shows an artificial thrombus made in a Chandler's tube by rotation of 15 ml whole blood for 1 hour. The white head and the fibrin tail can be seen.

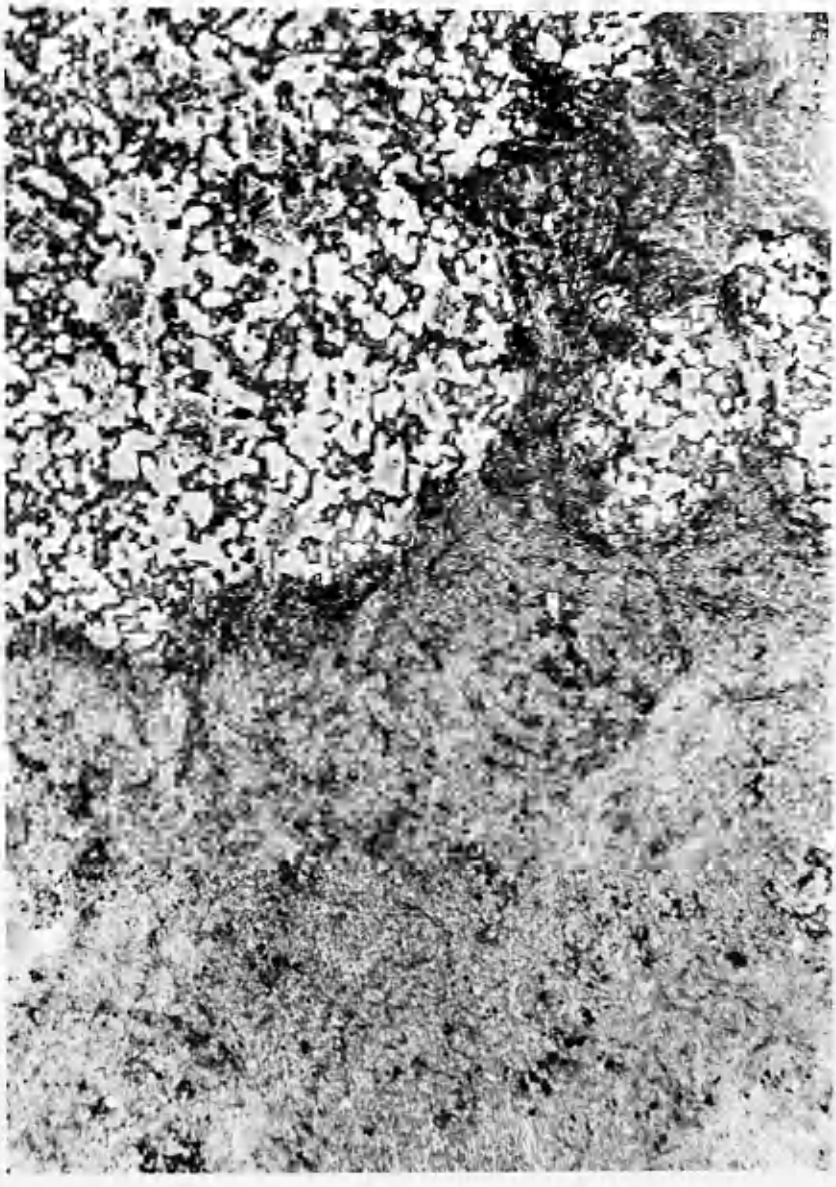

Figure 4 shows a longitudinal section through a Chandler thrombus. The contrast between the platelet head and the fibrin tail can be seen. Original magnification x 500. Stain - Picro Mallory. (Photomicrograph prepared by Dr. F. Walker).

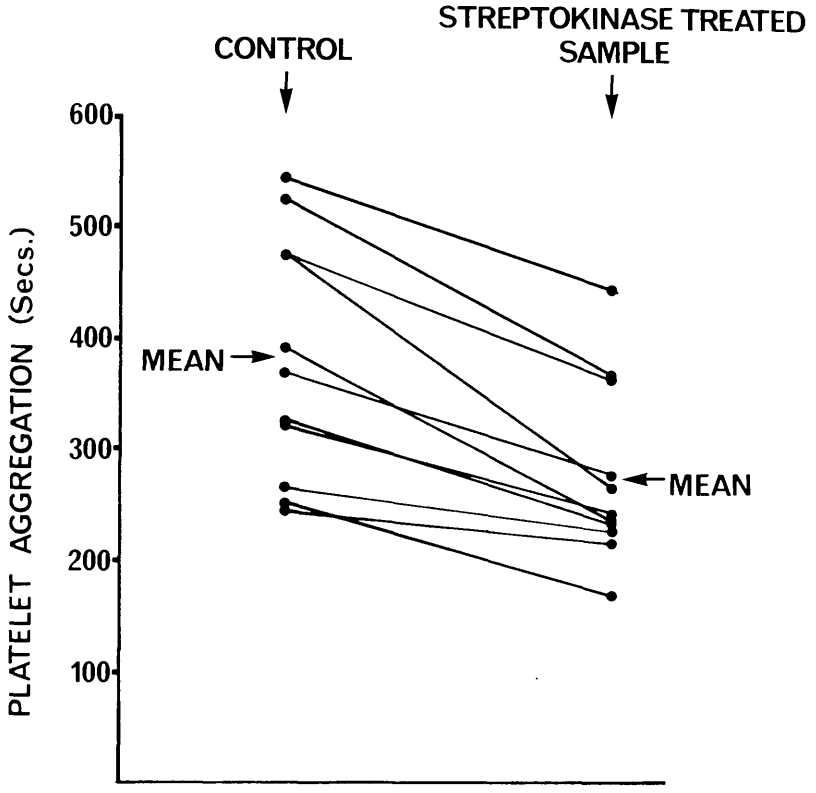

Figure 5 illustrates the influence of streptokinase, at a concentration which can produce maximum lytic activity, on the rate of formation of platelet aggregates in the Chandler tube. The speed of platelet aggregation is accelerated in each of the eleven plasma samples investigated following incubation of plasma with streptokinase at 37°C for 10 minutes. The mean time for platelet aggregation to occur in the control loops is 382 $\pm$ 110 seconds and in the test loops 276 $\pm$ 81 seconds, $t = 6.602$, $p < 0.001$. The figure is constructed from data in table 26.

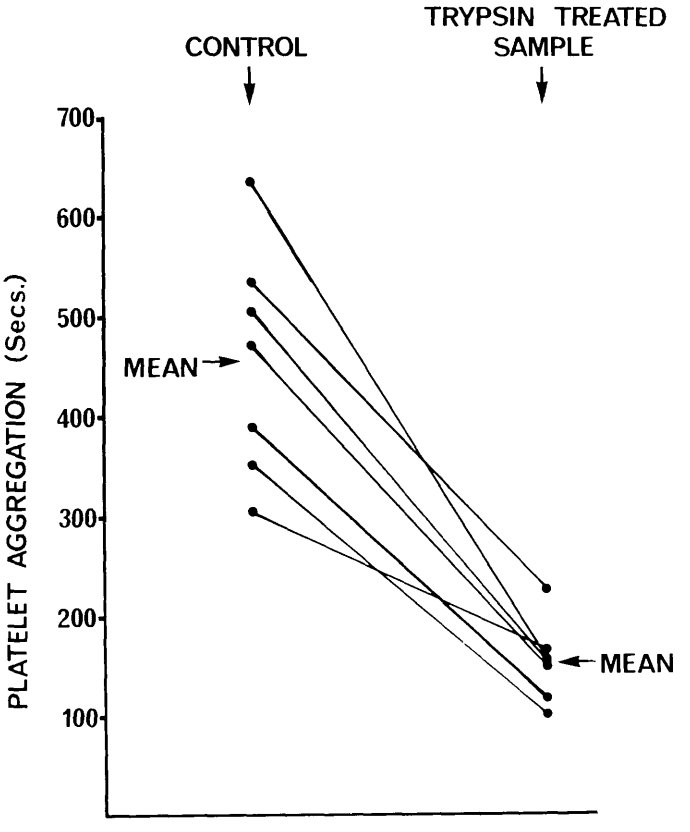

Figure 6 illustrates the effect of trypsin (10 µg/ml plasma) on the rate of formation of platelet aggregates in the Chandler tube. The speed of aggregation is accelerated in each of the seven plasma samples investigated as a result of incubating plasma with trypsin at 37°C for 10 minutes. The mean time for platelet aggregation to occur in the control loops is 457 $\pm$ 115 seconds and in the test loops 151 $\pm$ 41 seconds; $t = 7.764$, $p < 0.001$. The figure is compiled from data presented in table 27.

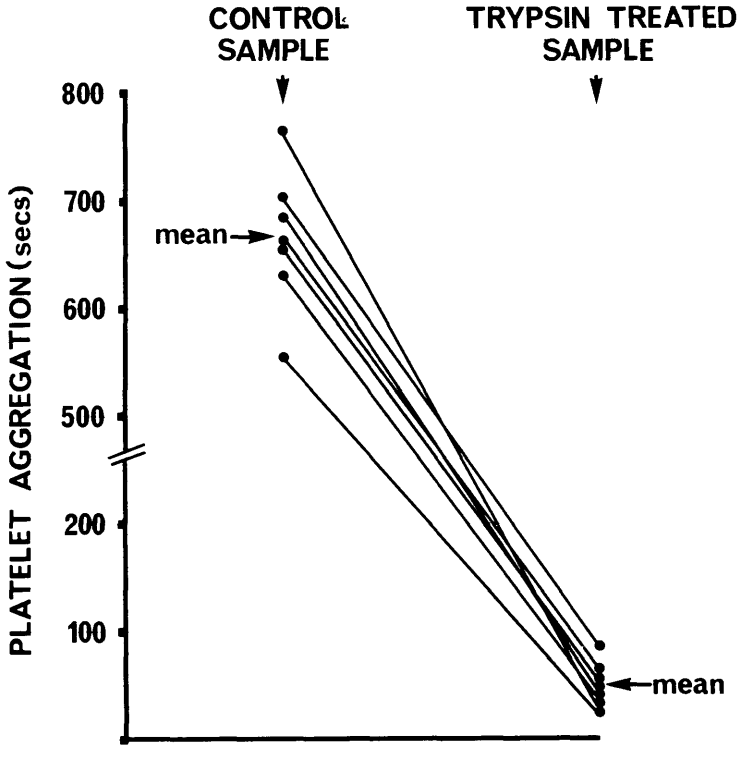

Figure 7 shows the effect of trypsin (1 mg/ml plasma) on the rate of formation of platelet aggregates in the Chandler tube. The speed of platelet aggregation is accelerated in each of the seven plasma samples to which trypsin was added. The mean time of platelet aggregation in the control loops is 666 ± 64 seconds and in the trypsin treated loops 49 ± 20 seconds; $t = 26.491$, $p < 0.001$. The figure is prepared from table 28.

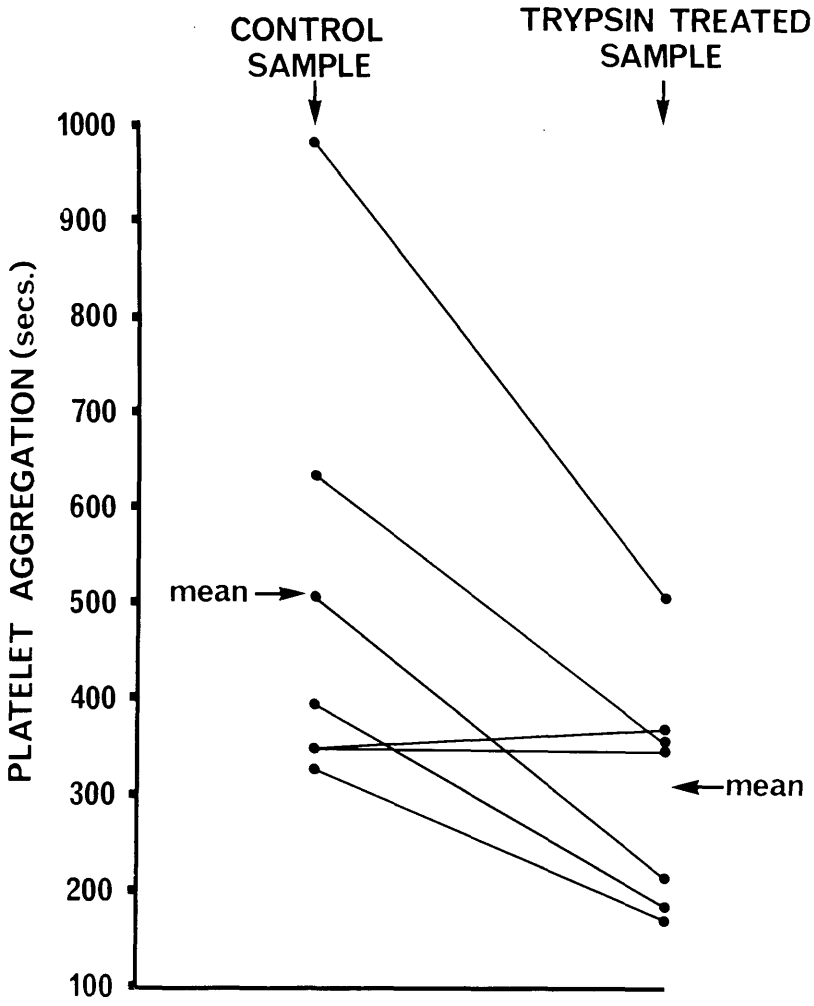

Figure 8 illustrates the effect of incubating platelet poor plasma with trypsin (10 µg/ml plasma) at 37°C for 10 minutes, before the addition of platelet rich plasma, on the rate of formation of platelet aggregates in the Chandler tube. The mean time of platelet aggregation in the test loops is 307 ± 124 seconds which is significantly less than the mean time of the control loops 504 ± 236 seconds; $t = 2.970$, $0.020 < p < 0.025$. The data from which this figure was compiled is shown in table 29.

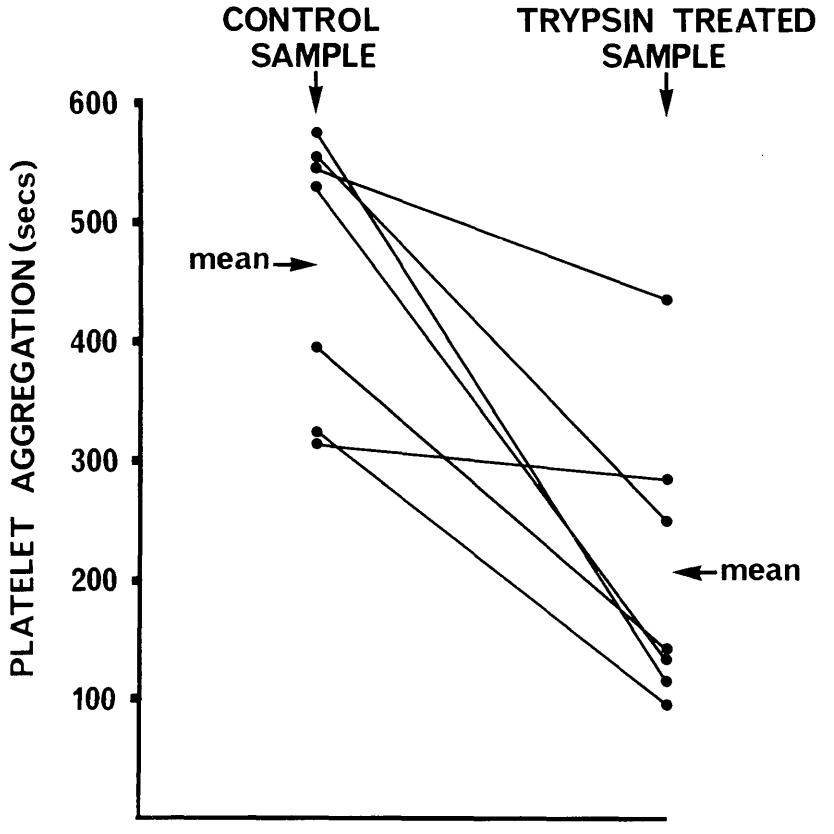

Figure 9 shows the effect, on the rate of formation of platelet aggregates in the Chandler tube, of incubating platelet rich plasma with trypsin (10 µg/ml plasma) at 37°C for 10 minutes. The speed of aggregation is accelerated in each of the seven plasma samples investigated. The mean time of platelet aggregation in the control loops is 462 ± 114 seconds and in the trypsin treated loops 208 ± 122 seconds. The difference between the two results is significant; $t = 4.500$, $0.001 < p < 0.005$. The figure is prepared from table 30.

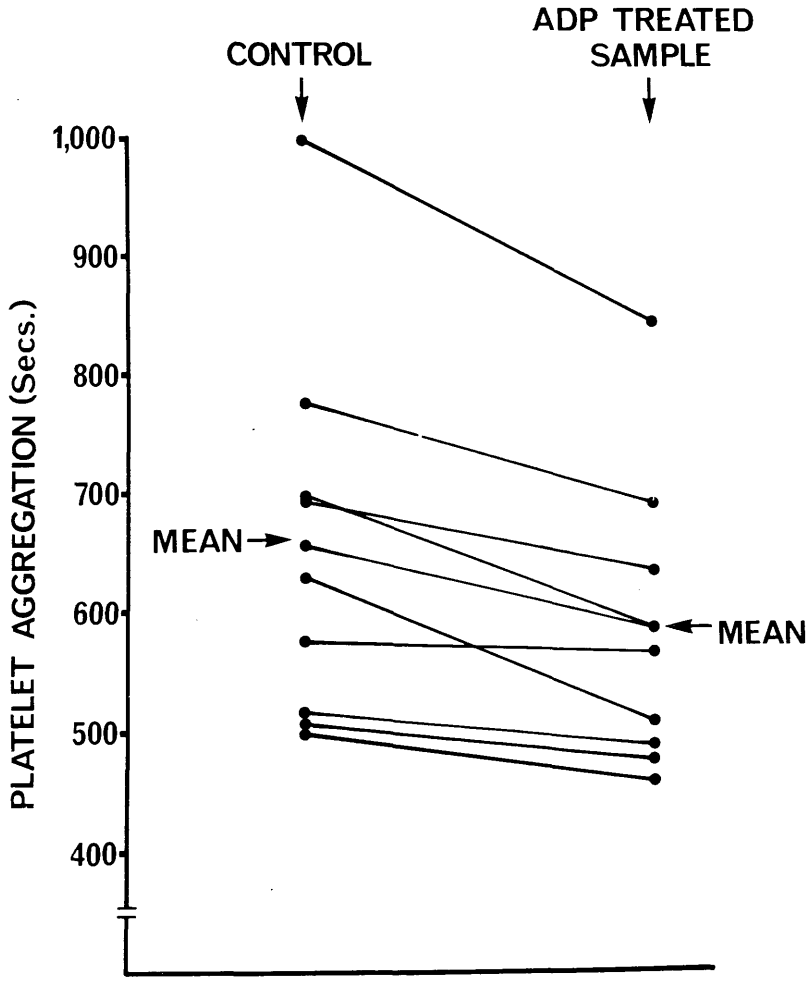

Figure 10 illustrates the effect of ADP (0.5 µg/ml plasma) on the rate of formation of platelet aggregates in the Chandler tube. Platelet aggregation is accelerated in each of the ten plasma samples investigated. The mean time of platelet aggregation of the ADP treated plasma is 583 ± 117 seconds which is significantly less than the mean time of the control samples 655 ± 152 seconds; $t = 4.824$, $p < 0.001$. The figure is constructed from data in table 31.

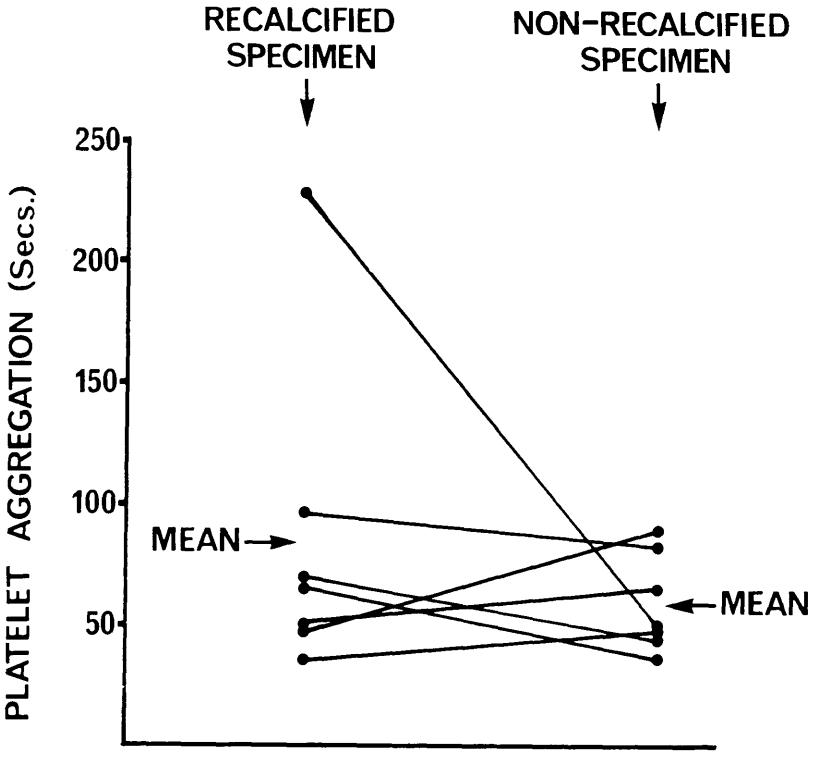

Figure 11 illustrates the effect of ADP (0.5 µg/ml plasma) on the rate of formation of platelet aggregates in recalcified and non-recalcified plasma in the Chandler tube. Over the series of seven plasma samples investigated there is no significant difference between the effect of ADP in the two systems. The mean time of platelet aggregation in the recalcified loops is 85 $\pm$ 66 seconds and in the non-recalcified loops 59 $\pm$ 20 seconds; $t = 0.938$, $0.30 < p < 0.40$. The figure is constructed from the data in table 32.

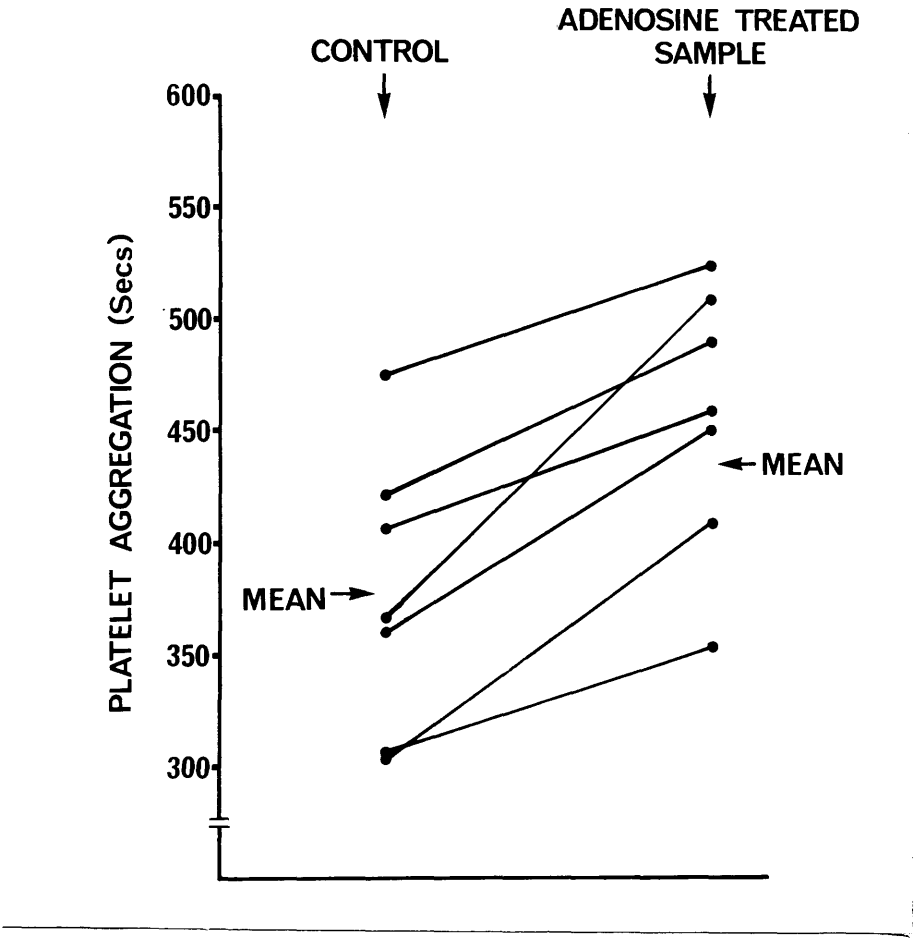

Figure 12 shows the effect of adenosine (100 µg/ml plasma) on the rate of formation of platelet aggregates in the Chandler tube. The speed of platelet aggregation is retarded in each of the seven plasma samples treated with adenosine. The mean time of platelet aggregation in the control loops is 377 ± 62 seconds and in the adenosine treated loops 460 ± 65 seconds; $t = 6.606$, $p < 0.001$. The figure is compiled from table 33.

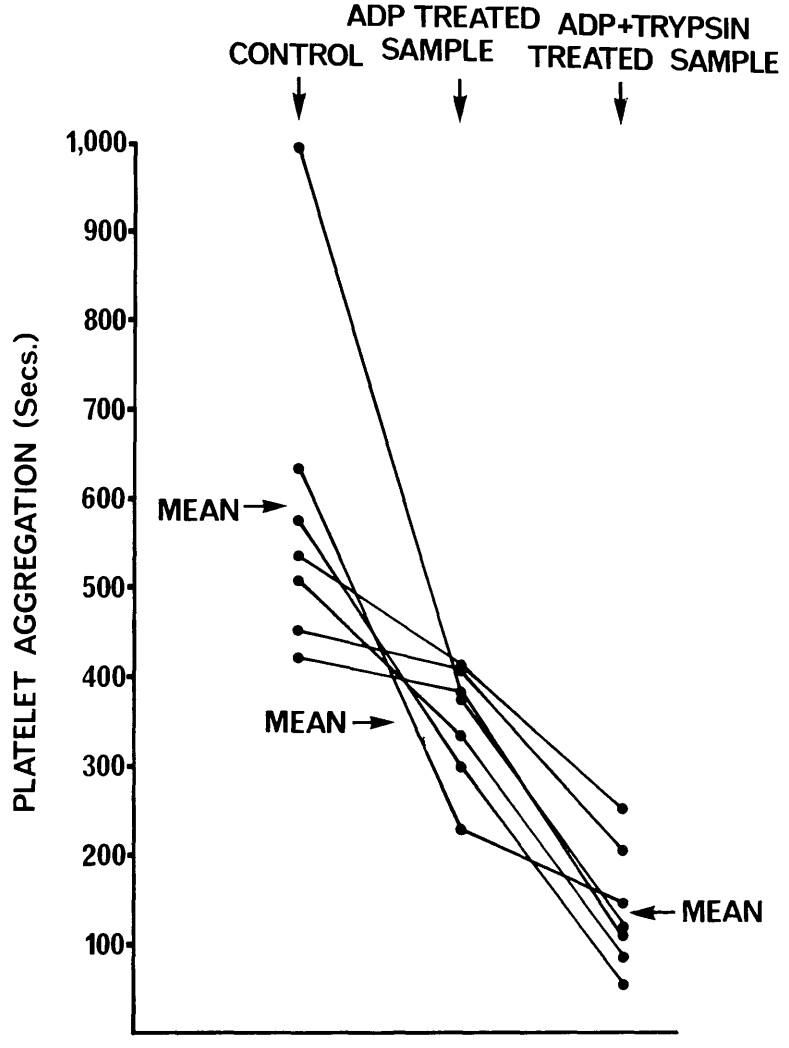

Figure 13 illustrates the combined effect of trypsin (10 µg/ml plasma) and ADP (0.5 µg/ml plasma) on the rate of formation of platelet aggregates in the Chandler tube compared to the rate in plasma treated with 0.9% sodium chloride and the rate in plasma treated only with ADP (0.5 µg/ml plasma). In each of the seven plasma samples investigated the speed of platelet aggregation is most rapid in the sample treated with trypsin and ADP. The mean time of platelet aggregation in the control loops is 590 ± 194 seconds, in the ADP treated loops 349 ± 67 seconds and in the ADP plus trypsin treated loops 137 ± 70 seconds. The differences between the means of the control and ADP treated loops and of the ADP and ADP plus trypsin treated loops are significant; $t = 2.970$, $0.020 < p < 0.025$ and $t = 8.194$, $p < 0.001$ respectively. The figure is constructed from the data of table 34.

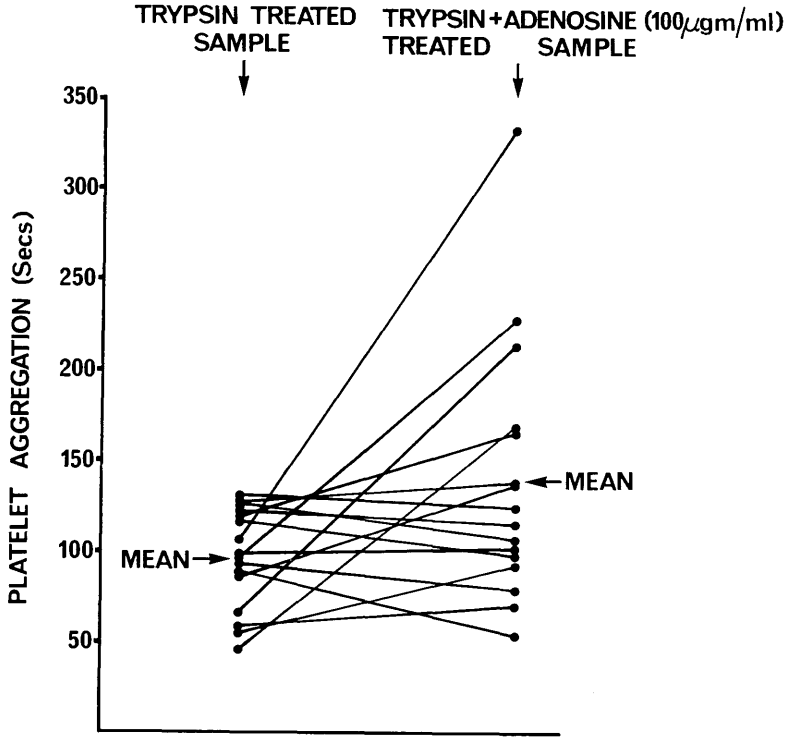

Figure 14 shows the effect of adenosine (100 μg/ml plasma) on the rate of formation of platelet aggregates in plasma treated with trypsin (10 μg/ml plasma). Over the series of ten plasma samples investigated the speed of platelet aggregation is significantly retarded in the adenosine treated samples. The mean time of platelet aggregation in the trypsin treated samples is 96 ± 28 seconds and in the trypsin and adenosine treated samples 139 ± 71 seconds; $t = 2.284$, $0.025 < p < 0.050$. The data of table 35 was used to compile this figure.

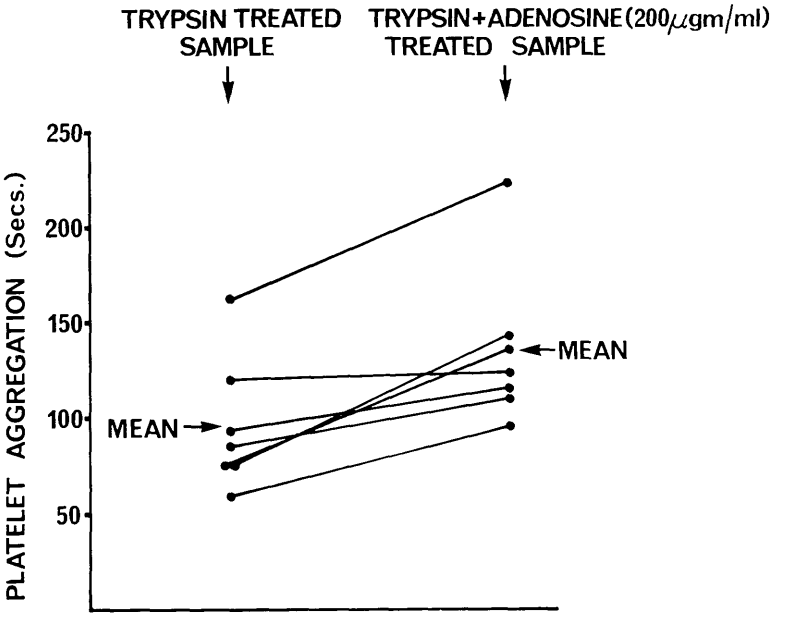

Figure 15 illustrates the effect of adenosine (200 μg/ml plasma) on the rate of formation of platelet aggregates in plasma treated with trypsin (10 μg/ml plasma). The speed of platelet aggregation is retarded in each of the seven plasma samples treated with adenosine. The mean time of platelet aggregation in the control loops is 96 ± 35 seconds and in the adenosine treated loops 135 ± 42 seconds; $t = 4.289$, $0.005 < p < 0.010$. The figure is constructed from the data of table 36.

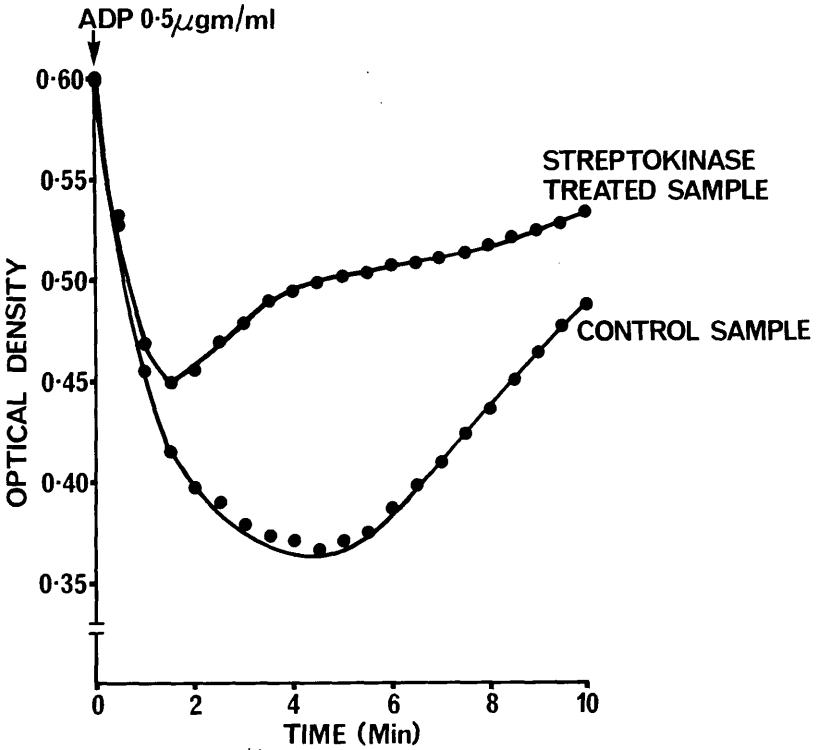

Figure 16 shows the effect of streptokinase on ADP induced platelet aggregation. The concentration of streptokinase was the concentration which produced maximum lytic activity as determined for each plasma sample. Over the series of ten plasma samples investigated streptokinase causes significant inhibition of platelet aggregation. 4½ minutes after the addition of ADP (0.5 μg/ml plasma), the mean optical density reading of the control samples is 0.366 ± 0.208 and of the streptokinase treated samples 0.499 ± 0.129; $t = 2.413$, $0.025 < p < 0.050$. The values of the individual samples at this time interval are presented in table 37.

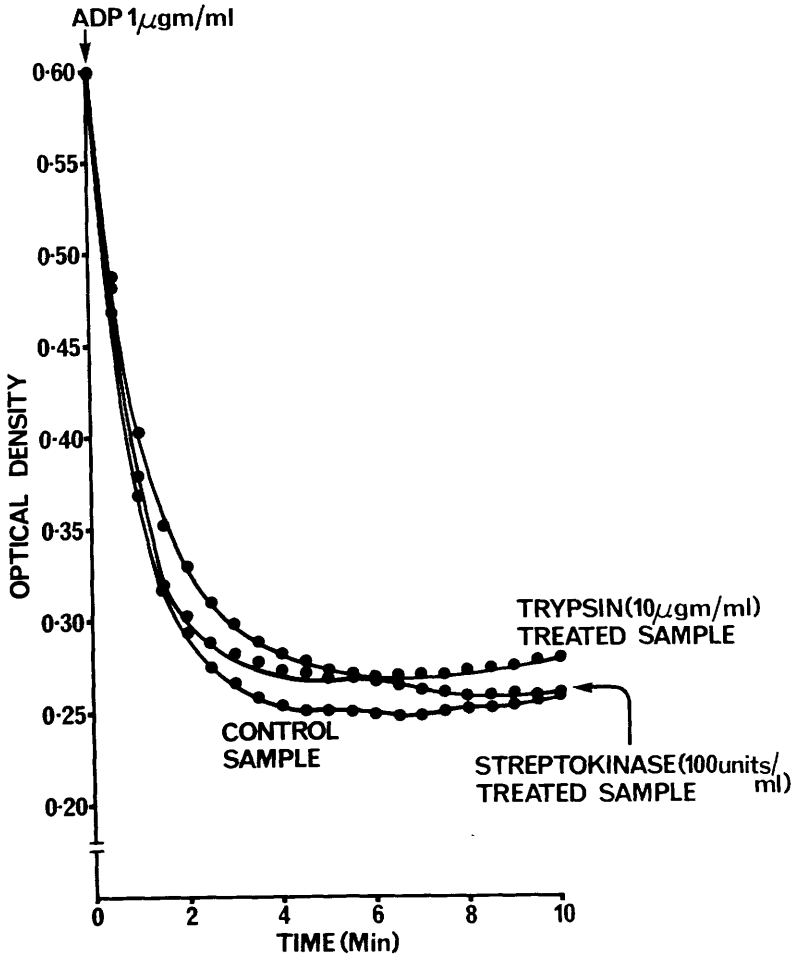

Figure 17 illustrates the effect of streptokinase (100 M.I.H. units/ ml sample) and trypsin (10 ug/ml sample) on ADP induced platelet aggregation in platelet rich plasma diluted 1: 2.5 with 0.9% sodium chloride. Neither streptokinase nor trypsin treatment of diluted plasma results in a significant change in the extent of platelet aggregation. 3 minutes after the addition of ADP (1.0 μg/ml sample), the mean optical density readings of the control samples is 0.266 $\pm$ 0.119 and of the streptokinase treated samples 0.298 $\pm$ 0.077; t = 1.122, $0.30 < p < 0.40$. 4 minutes after the addition of ADP, the mean optical density reading of the control samples is 0.254 $\pm$ 0.118 and of the trypsin treated samples 0.273 $\pm$ 0.110; t = 0.965, $0.30 < p < 0.40$. The individual readings, for each of the seven plasma samples investigated, at these time intervals, are tabulated in tables 38 and 45.

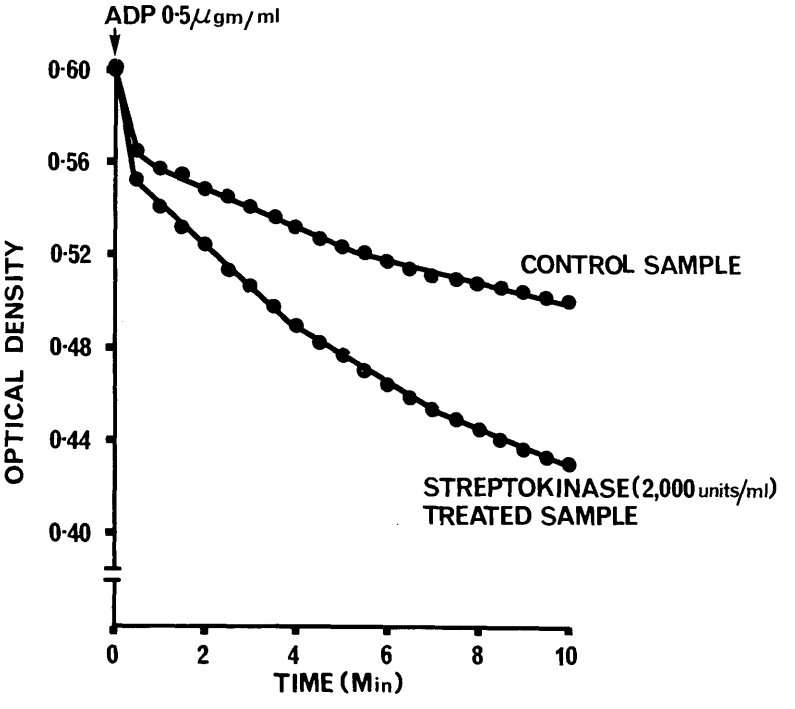

Figure 18 shows the effect of streptokinase (2,000 I.U.H. units/ml plasma) on the ADP induced aggregation of platelets suspended in plasma pre-treated with acid and alkali to destroy antiplasmin. 10 minutes after the addition of ADP (0.5 μg/ml plasma), the mean optical density reading of the control samples is 0.499 ± 0.066 and of the streptokinase treated samples 0.430 ± 129. The difference between these results is significant; $t = 2.574$, $0.20 < p < 0.025$. The optical density readings at this time interval of each of the plasma samples investigated are presented in table 39.

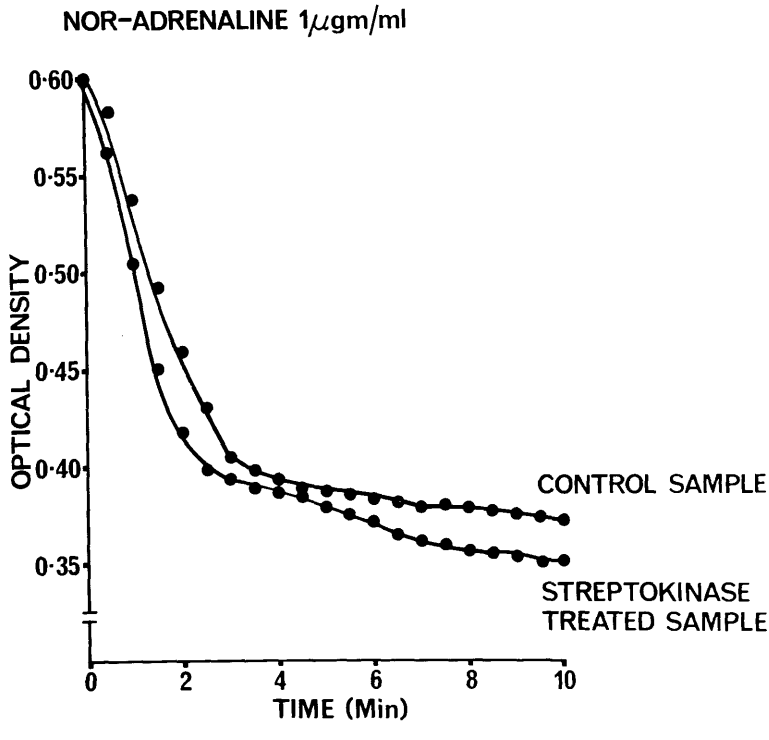

Figure 19 illustrates the effect of streptokinase on nor-adrenaline induced platelet aggregation. There is no significant difference between the extent of aggregation which occurs in the control samples treated with 0.9% sodium chloride and the test samples treated with streptokinase (100 N.I.H. units/ml plasma). 10 minutes after the addition of nor-adrenaline (1.0 μg/ml plasma), the mean optical density of the control samples is 0.371 ± 0.224 and of the streptokinase treated samples 0.351 ± 0.219; $t = 0.623$, $0.50 < p < 0.60$. The optical density readings of the individual plasma samples, at this time interval, are shown in table 40.

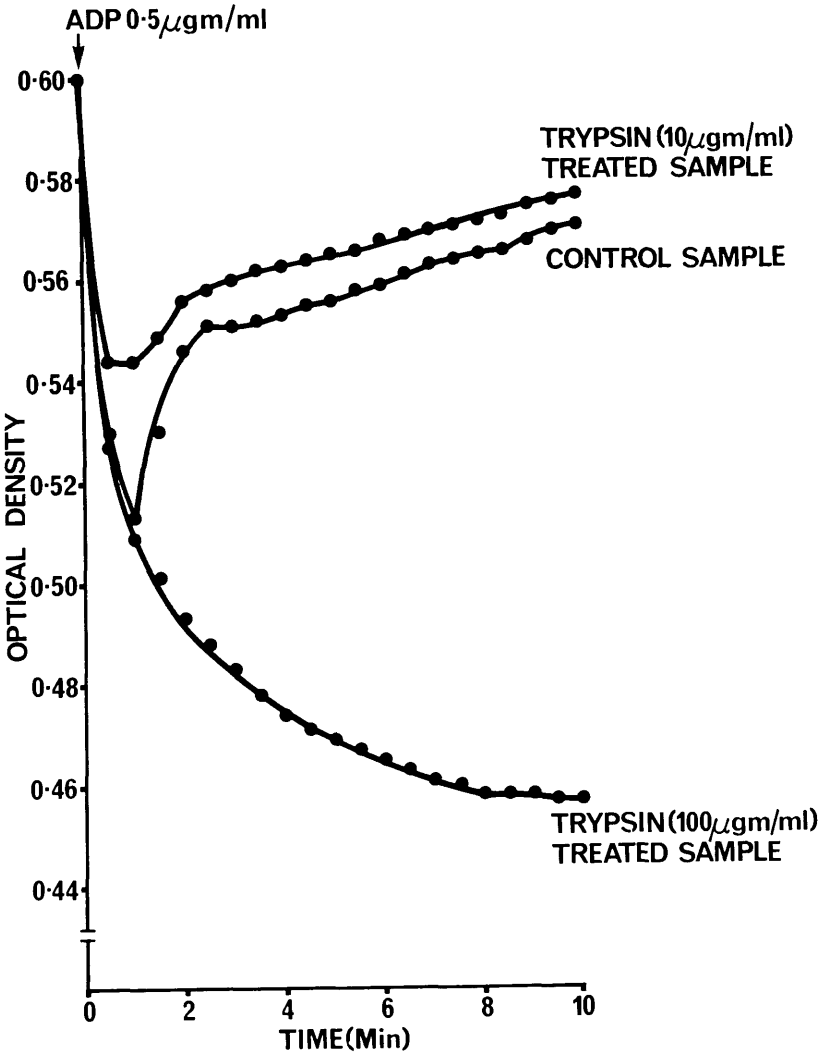

Figure 20 shows the effect of trypsin, at concentrations of 10 μg/ml plasma and 100 μg/ml plasma, on ADP induced platelet aggregation. Trypsin at a concentration of 10 μg/ml plasma appears to produce no significant change in the extent of aggregation from that of the saline treated control samples; 1 minute after the addition of ADP (0.5 μg/ml plasma), the mean optical density reading of the control samples is 0.513 ± 0.061 and of the test samples 0.544 ± 0.033; $t = 2.119$, $0.05 < p < 0.10$. Platelet aggregation is significantly enhanced by trypsin at a concentration of 100 μg/ml plasma; 1½ minutes after the addition of ADP (0.5 μg/ml plasma), the mean optical density reading of the control samples is 0.529 ± 0.041 and of the trypsin treated samples 0.501 ± 0.041; $t = 3.925$, $0.001 < p < 0.005$. The optical density readings of the individual plasma samples, at these time intervals, are presented in tables 41 and 42.

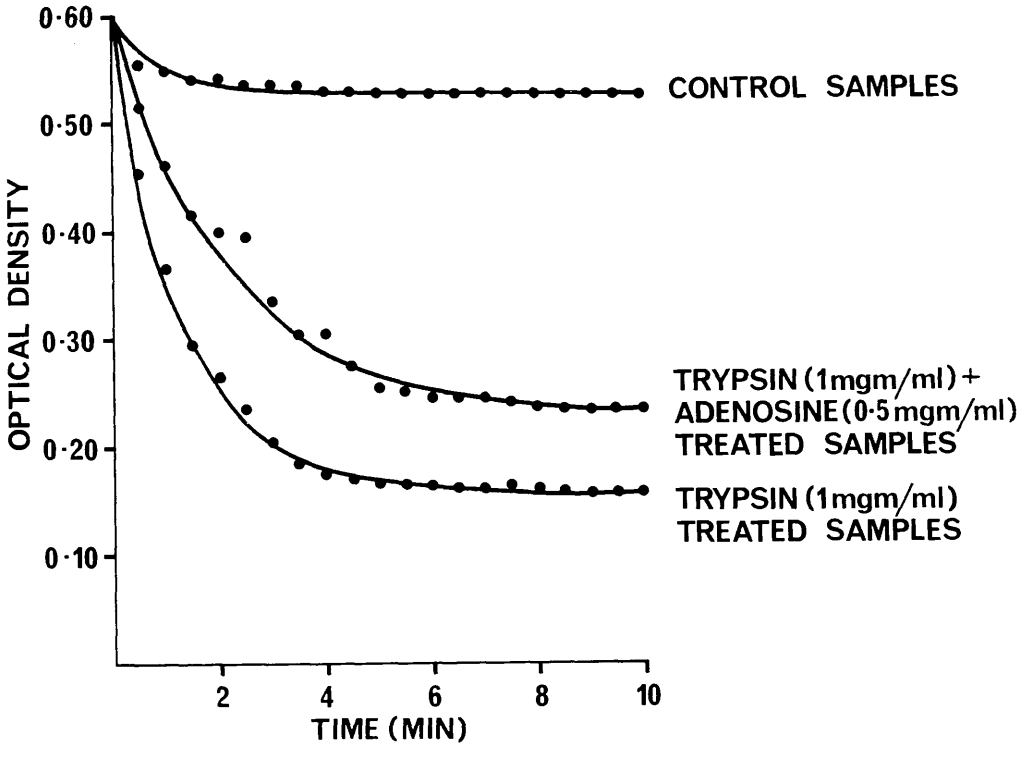

Figure 21 illustrates the ability of trypsin (1 mg/ml plasma) to promote platelet aggregation in the absence of ADP and the ability of adenosine (0.5 mg/ml plasma) to partially block this action. 2 minutes after the addition of 0.9% sodium chloride to the control samples and trypsin to the test sample, the mean optical density reading of the trypsin treated sample is 0.263 $\pm$ 0.204 which is significantly less than the control value of 0.540 $\pm$ 0.041; $t = 3.030$, $0.020 < p < 0.025$. The mean optical density reading of the trypsin treated samples is 0.177 $\pm$ 0.182 and of the trypsin plus adenosine samples 0.303 $\pm$ 0.086, 4 minutes after the additions were made to these samples. The difference between the results is not significant; $t = 1.818$, $0.10 < p < 0.20$. The optical density readings of the seven plasma samples, at these time intervals, are shown in tables 45 and 52.

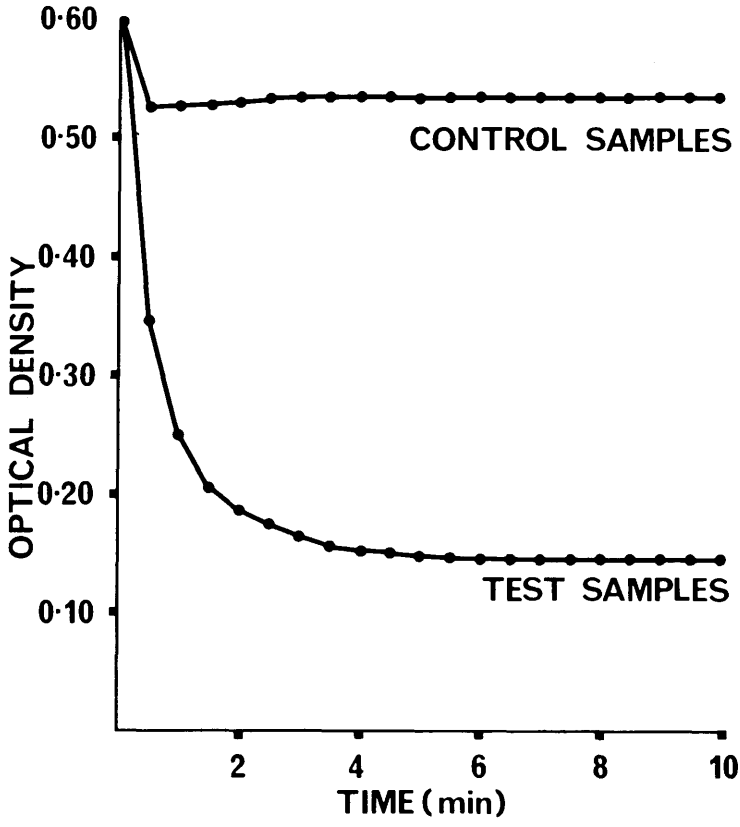

Figure 22 shows the effect of trypsin (1 mg/ml plasma) on ADP induced aggregation in plasma pre incubated ith streptokinase (1,000 M.I.U. units/ml plasma) at 37°C for 10 minutes. Platelet aggregation was enhanced in each of the seven plasma samples investigated. 30 seconds after the addition of 0.9% sodium chloride to the control samples and of trypsin to the test samples, the mean optical density reading of the control samples is 0.525 $\pm$ 0.009 and of the test samples 0.345 $\pm$ 0.060; $t = 6.485$, $p < 0.001$. The optical density readings of each of the seven plasma samples, at this time interval, are shown in table 44.

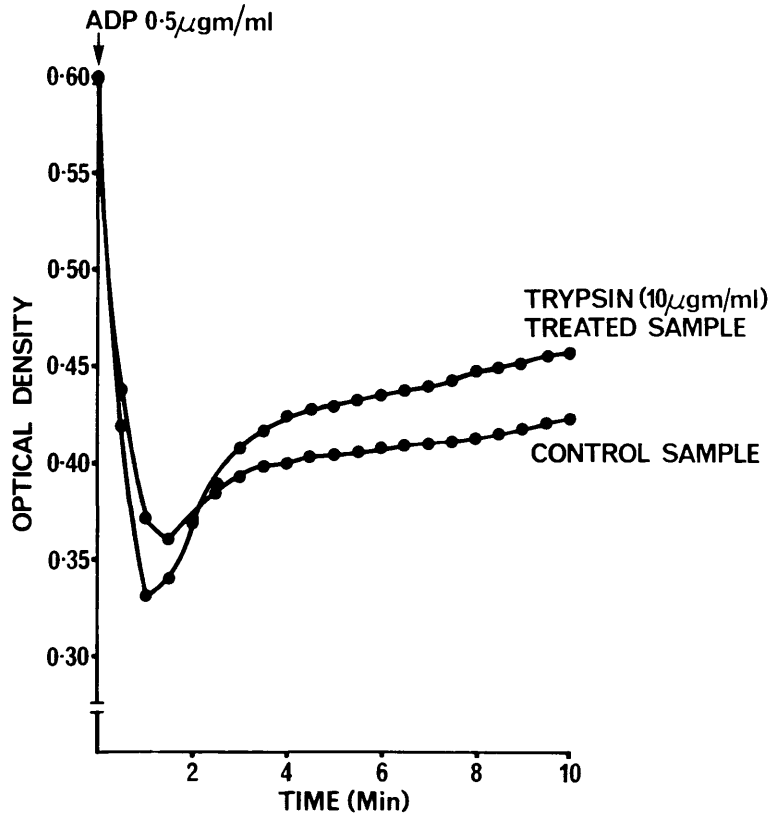

Figure 23 illustrates the effect of trypsin (10 μg/ml plasma) on ADP induced platelet aggregation in platelet rich plasma to which calcium chloride (0.25 mmole/ml plasma) had been added. Trypsin has no significant effect on platelet aggregation in this system. 1 minute after the addition of ADP (0.5 μg/ml plasma), the mean optical density reading of the control samples is 0.372 ± 0.115 and of the trypsin treated samples 0.331 ± 0.118; $t = 1.363$, $0.20 < p < 0.30$. The optical density readings of the seven plasma samples, at this time interval, are shown in table 46.

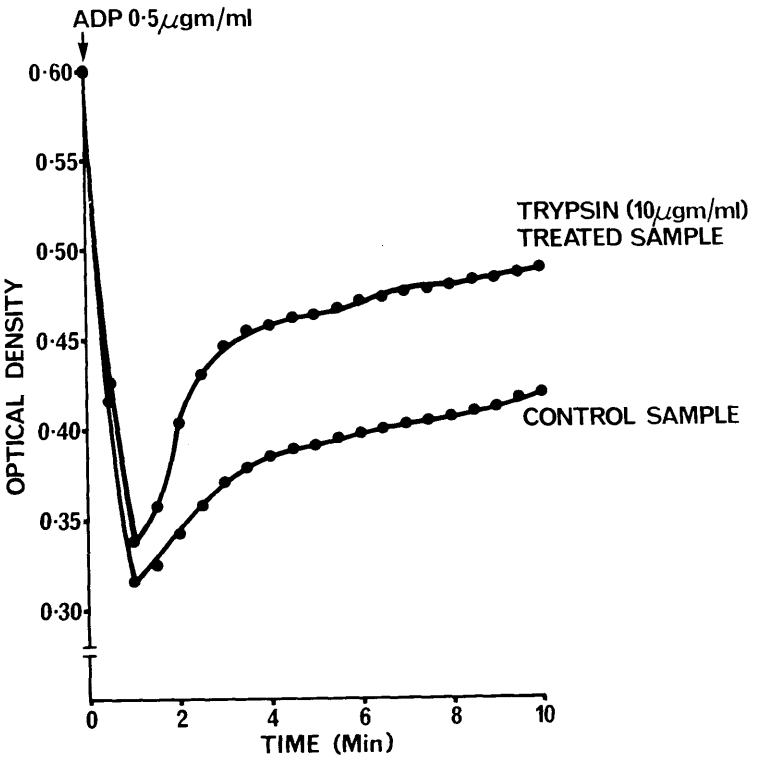

Figure 24 shows the effect of trypsin (10 μg/ml plasma) on ADP induced platelet aggregation in platelet rich plasma to which calcium chloride (1.0 mmole/ml plasma) had been added. Trypsin has no significant effect on platelet aggregation in this system. 3 minutes after the addition of ADP (0.5 μg/ml plasma), the mean optical density reading of the control samples is 0.371 ± 0.196 and of the trypsin treated samples 0.446 ± 0.143; $t = 1.690$, $0.10 < p < 0.20$. The optical density readings of the seven plasma samples, at this time interval, are presented in table 47.

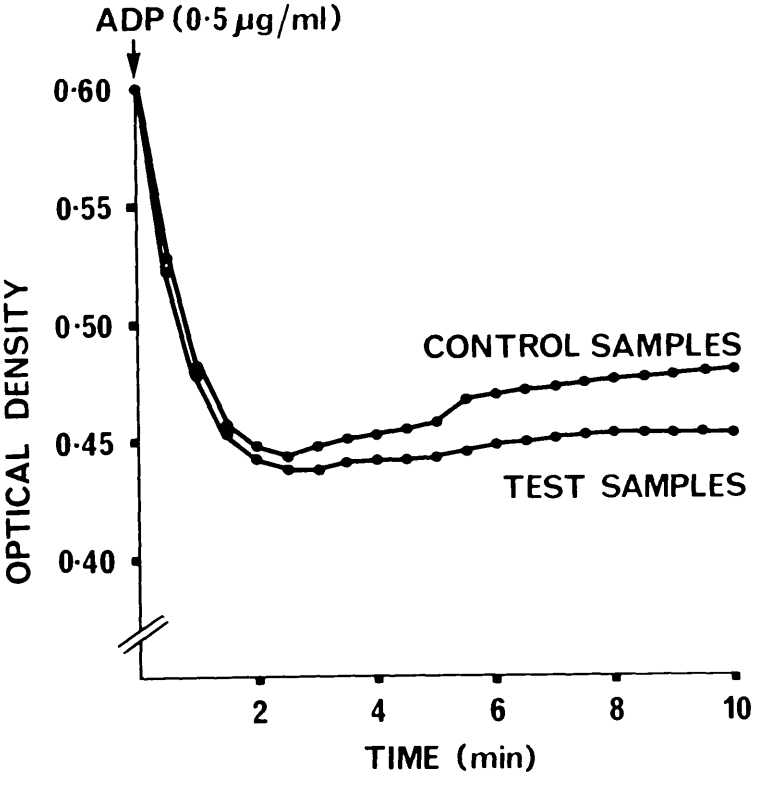

Figure 25 illustrates the effect of incubation of trypsin (100 µg/ml plasma) with platelet poor plasma, before the addition of platelet rich plasma, on ADP induced platelet aggregation. There is no significant difference between the platelet aggregation of the control and trypsin treated samples; 10 minutes after the addition of ADP (0.5 µg/ml plasma), the mean optical density reading of the control samples is 0.480 ± 0.133 and of the trypsin treated samples 0.453 ± 0.121, $t = 1.170$, $0.20 < p < 0.30$. The optical density readings of each of the seven plasma samples, at this time interval, are shown in table 48.

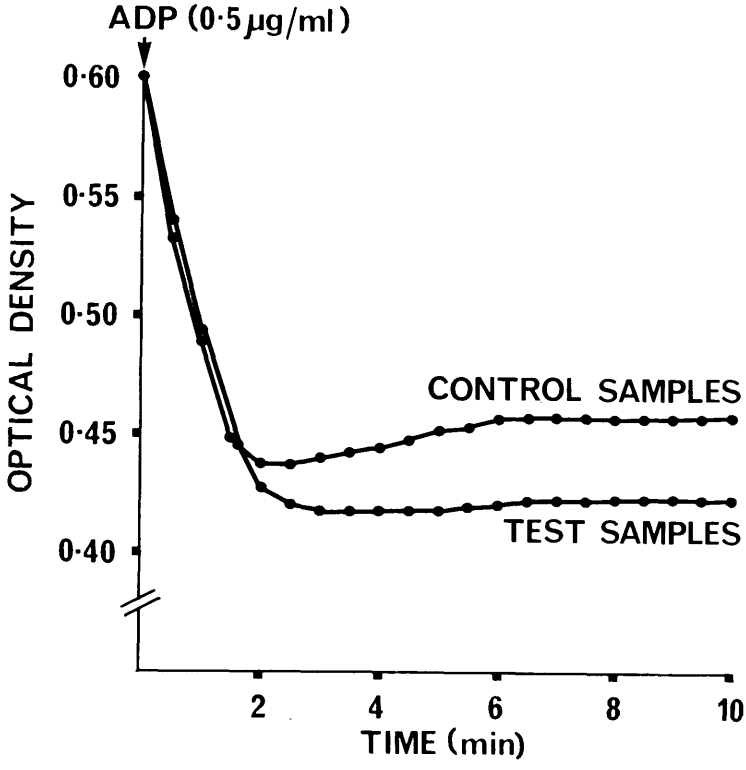

Figure 26 shows the effect of incubation of trypsin (100 µg/ml plasma) with platelet rich plasma, before the addition of platelet poor plasma, on ADP induced platelet aggregation. Trypsin treatment of the plasma samples results in significant enhancement of platelet aggregation; 10 minutes after the addition of ADP (0.5 µg/ml plasma), the mean optical density reading of the control samples is 0.484 ± 0.104 and of the test samples 0.448 ± 0.085, $t = 2.768$, $0.025 < p < 0.050$. The optical density readings, at this time interval, of the seven plasma samples investigated are presented in table 49.

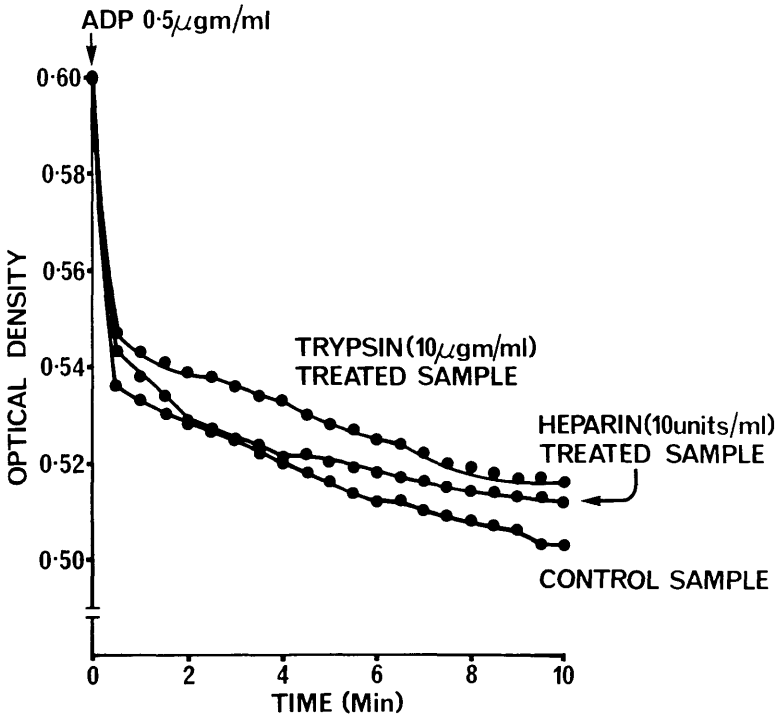

Figure 27 illustrates the effect of trypsin (10 μg/ml suspension) and heparin (10 units /ml suspension) on ADP induced platelet aggregation of washed platelets suspended in 0.1 M tris buffer, pH 7.5. Neither trypsin nor heparin has a significant effect on platelet aggregation in this system; 10 minutes after the addition of ADP (0.5 μg/ml suspension), the mean optical density readings of the control samples is 0.503 ± 0.058, of the trypsin treated samples 0.516 ± 0.025 and of the heparin treated samples 0.512 ± 0.053, ($t = 0.291$, $0.70 < p < 0.80$ and $t = 0.756$, $0.40 < p < 0.50$ respectively). The optical density readings, at this time interval, of the seven plasma samples investigated are shown in tables 50 and 60.

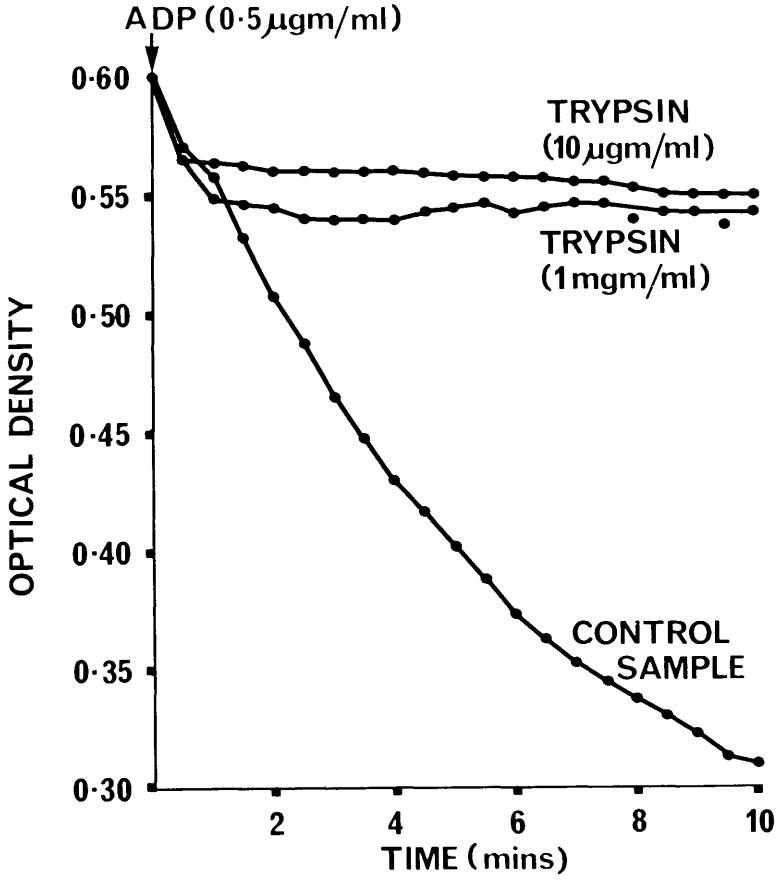

Figure 28 shows the effect of trypsin, at concentrations of 10 µg/ml suspension and 1 mg/ml suspension, on ADP induced platelet aggregation of a sample of washed platelets suspended in a buffer medium (Haslam, 1964).

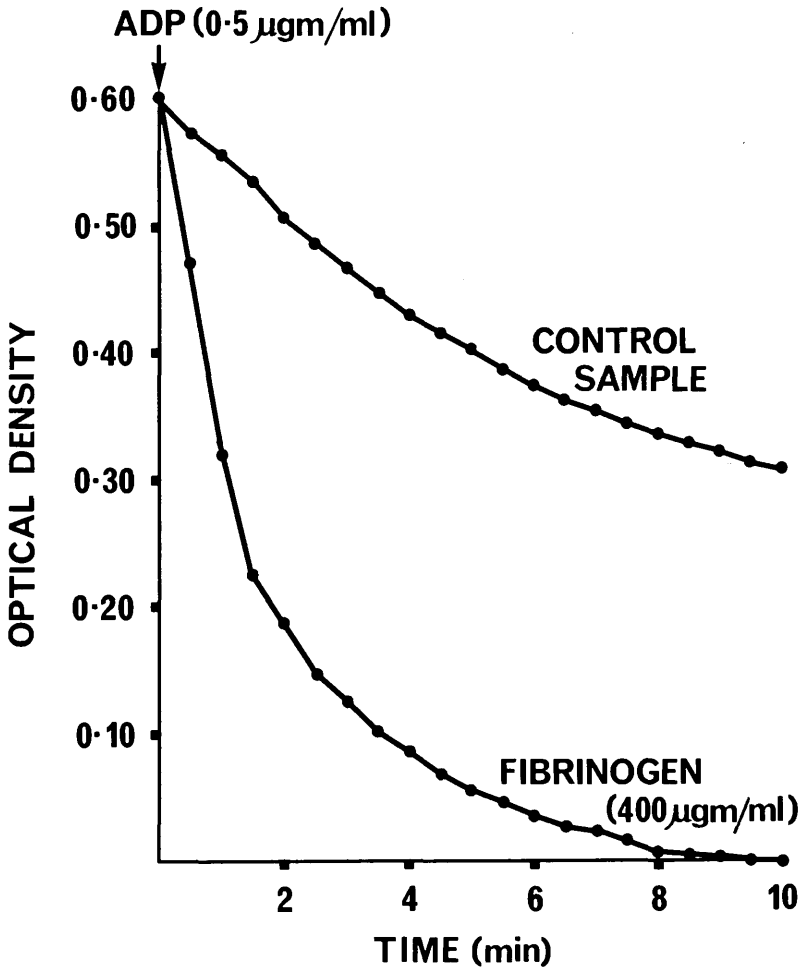

Figure 29 illustrates the effect of fibrinogen (400 µg/ml suspension) on ADP induced platelet aggregation of a sample of washed platelets suspended in a buffer medium (Haslam, 1964).

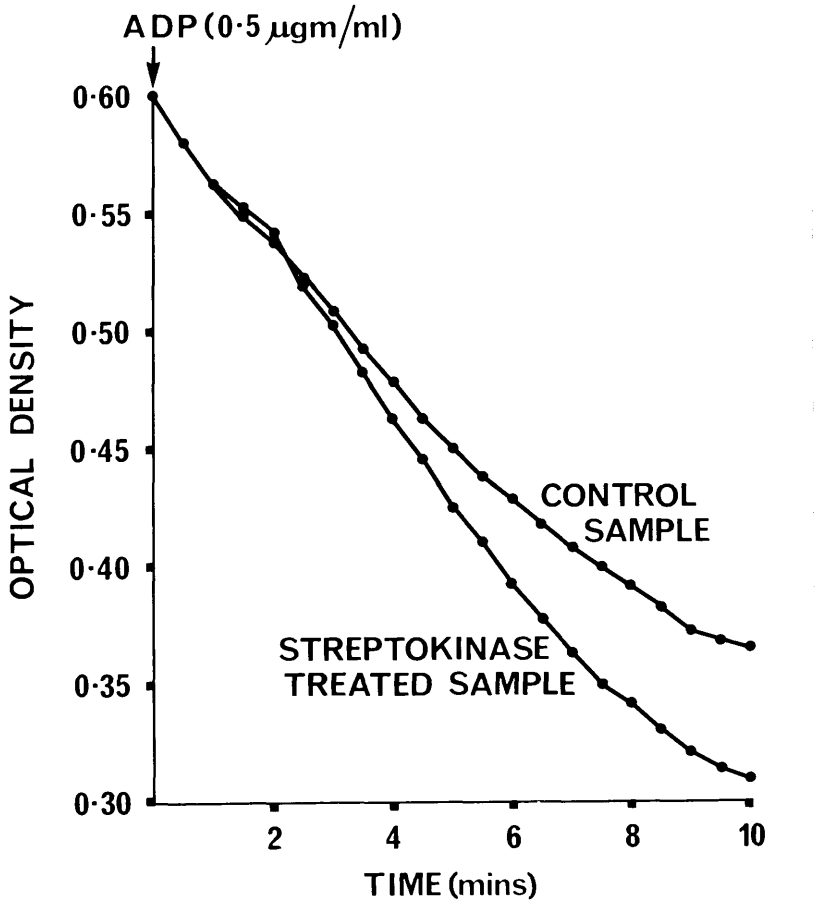

Figure 30 shows the effect of streptokinase (2,000 N.I.H. units/ml suspension) on ADP induced platelet aggregation of a sample of washed platelets suspended in a buffer medium (Haslam, 1964).

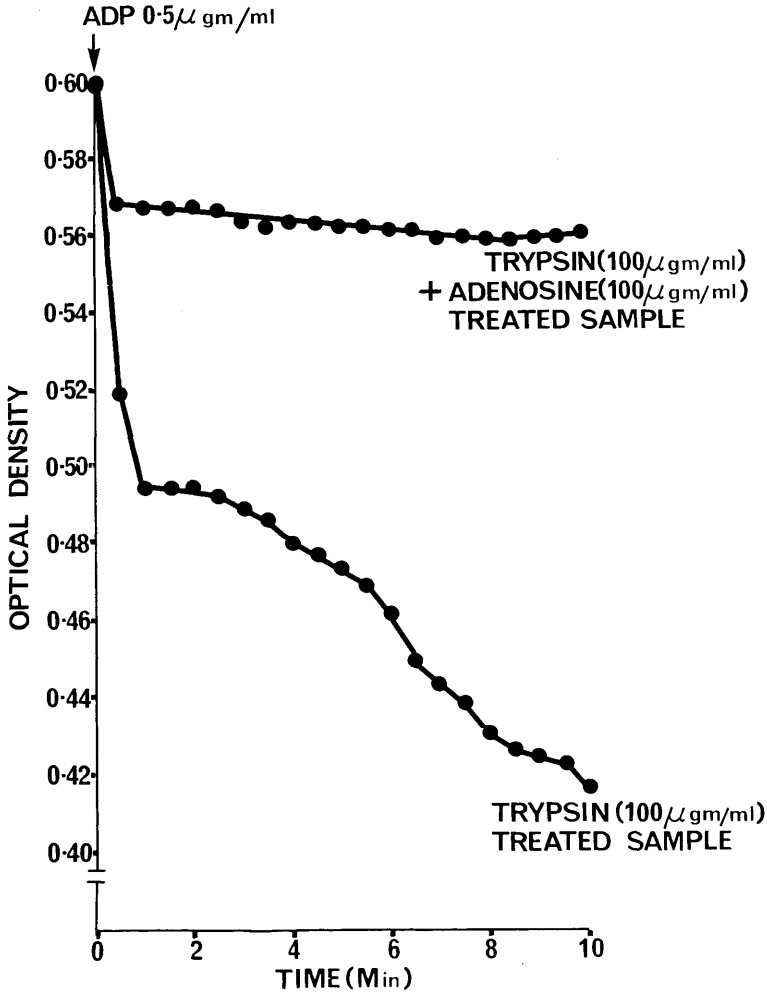

Figure 31 illustrates the effect of adenosine (100 μg/ml plasma) on ADP induced platelet aggregation of platelet rich plasma treated with trypsin (100 μg/ml plasma). 1 minute after the addition of ADP (0.5 μg/ml plasma), the mean optical density readings of the trypsin treated samples is 0.491 ± 0.054 which is significantly less than the mean value of the samples treated with trypsin and adenosine 0.567 ± 0.039; $t = 2.724$, $0.025 < p < 0.050$. The optical density readings, at this time interval, of the seven plasma samples investigated are shown in table 51.

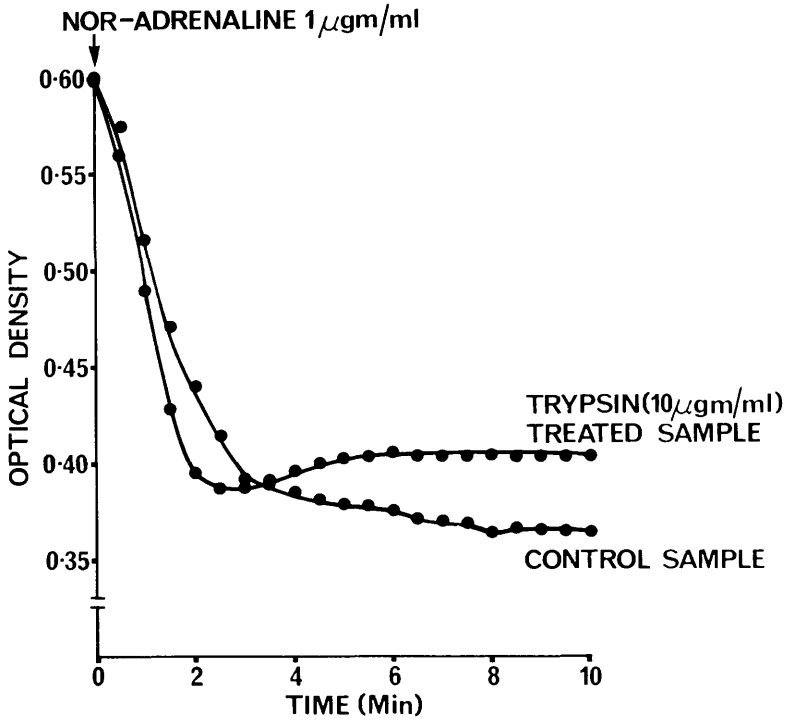

Figure 32 shows the effect of trypsin (10 µg/ml plasma) on nor-adrenaline induced platelet aggregation. Over the series of 10 plasma samples investigated, trypsin has no significant effect on platelet aggregation; 2 minutes after the addition of nor-adrenaline (1.0 µg/ml plasma), the mean optical density readings of the control samples is 0.440 ± 0.141 and of the trypsin treated samples 0.395 ± 0.207, ($t = 1.691$, $0.10 < p < 0.20$). The optical density readings, at this time interval, of the ten plasma samples investigated are presented in table 53.

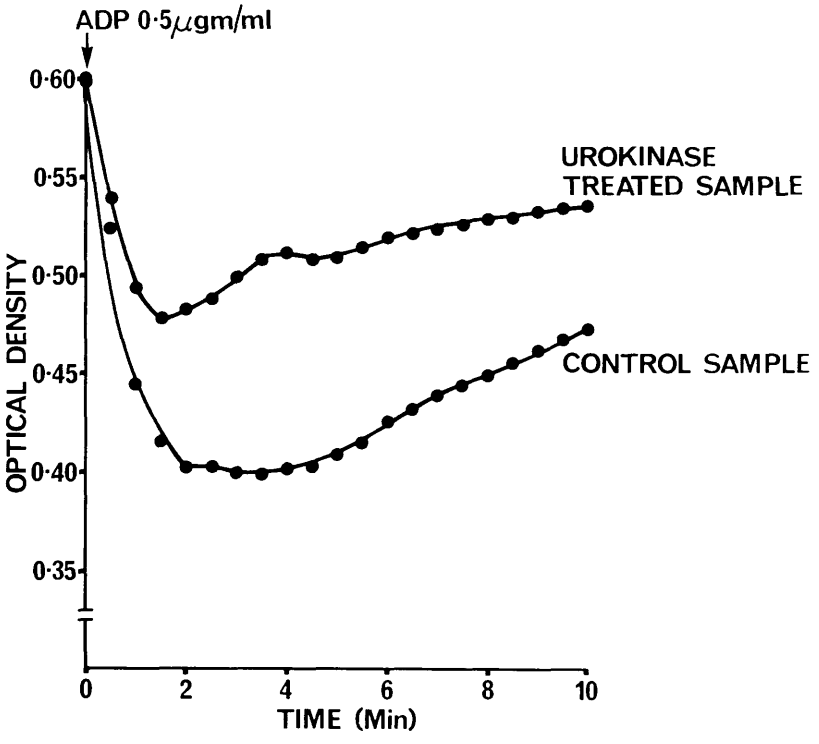

Figure 33 illustrates the effect of urokinase, at a concentration which produces maximum lytic activity, on ADP induced platelet aggregation. Urokinase produces significant inhibition of platelet aggregation; 3 minutes after the addition of ADP (0.5 μg/ml plasma), the mean optical density reading of the control samples is 0.400 ± 0.264 and of the trypsin treated samples 0.499 ± 0.128, ($t = 3.134$, $0.01 < p < 0.02$). The optical density readings, at this time interval, of the ten plasma samples investigated are shown in table 54.

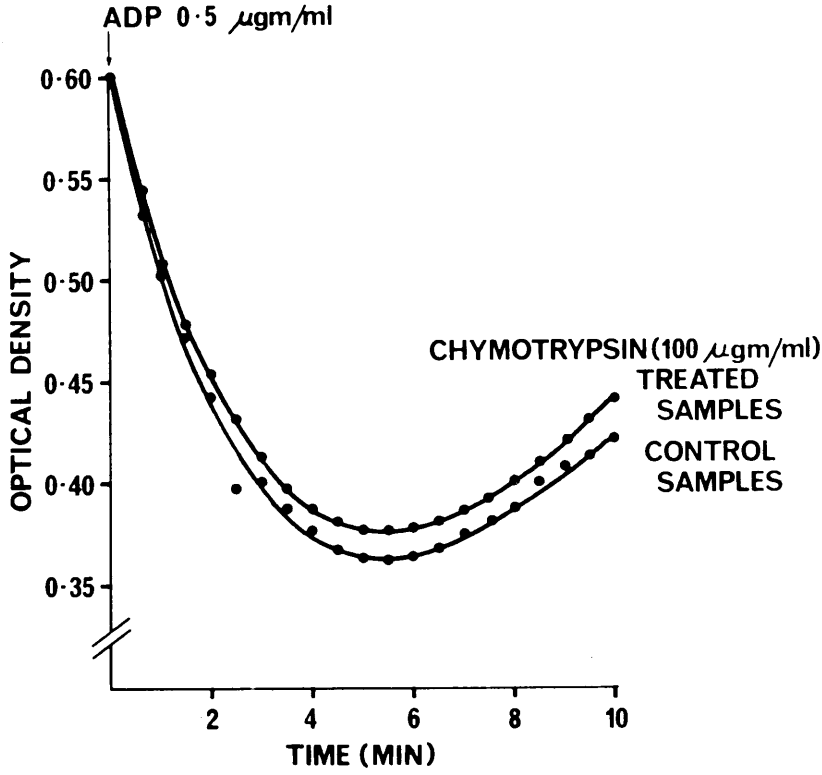

Figure 34 shows the effect of chymotrypsin (100 μg/ml plasma) on ADP induced platelet aggregation. There is no significant difference between the platelet aggregation which occurs in the control and chymotrypsin treated samples; the mean optical density reading of the control samples, 5 minutes after the addition of ADP (0.5 μg/ml plasma), is 0.364 ± 0.156 and of the chymotrypsin treated samples 0.377 ± 0.144, ($t = 0.412$, $0.60 < p < 0.70$). The optical density readings, at this time interval, of the seven plasma samples investigated are presented in table 55.

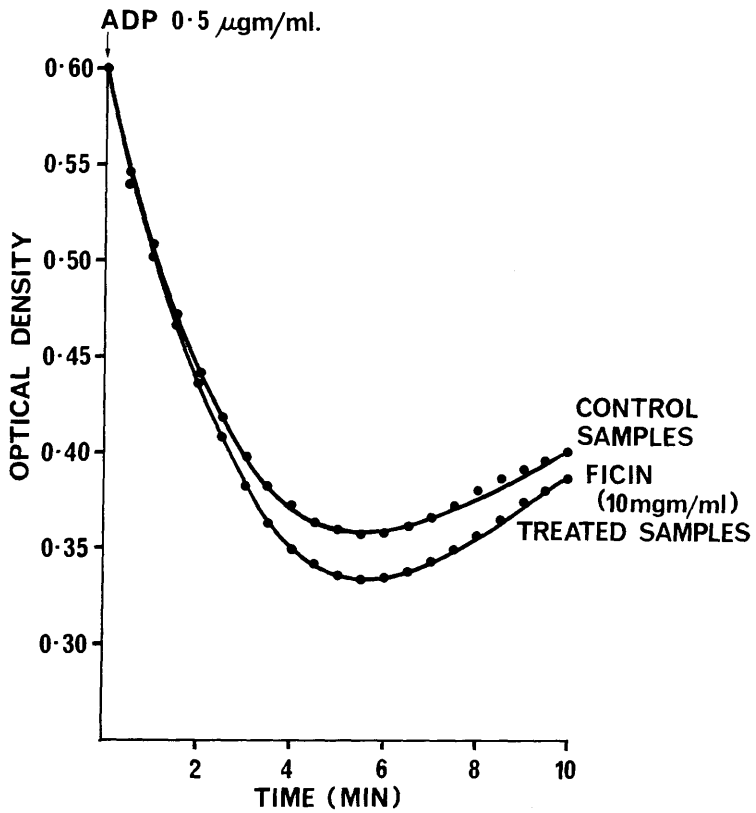

Figure 35 illustrates the effect of ficin (100 μg/ml plasma) on ADP induced platelet aggregation. There is no significant difference between the platelet aggregation which occurs in the control and ficin treated samples; 5 minutes after the addition of ADP (0.5 μg/ml plasma), the mean optical density reading of the control samples is 0.360 ± 0.160 and of the ficin treated samples 0.335 ± 0.165, ($t = 0.996$, $0.30 < p < 0.40$). The optical density readings, at this time interval, of the twelve plasma samples investigated are shown in table 56.

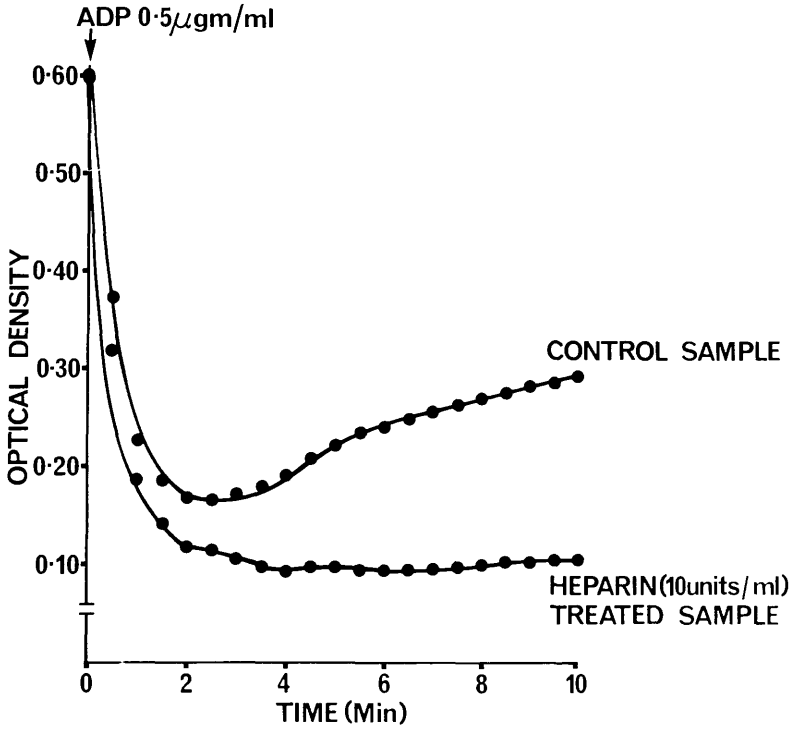

Figure 36 shows the effect of heparin (10 units/ml plasma) on ADP induced platelet aggregation. Platelet aggregation is significantly enhanced in the samples treated with heparin; 4 minutes after the addition of ADP (0.5 μg/ml plasma), the mean optical density reading of the control samples is 0.196 ± 0.146 and of the heparin treated samples 0.096 ± 0.091, ($t = 2.594$, $0.025 < p < 0.020$). The optical density readings, at this time interval, of the eight plasma samples investigated are presented in table 57.

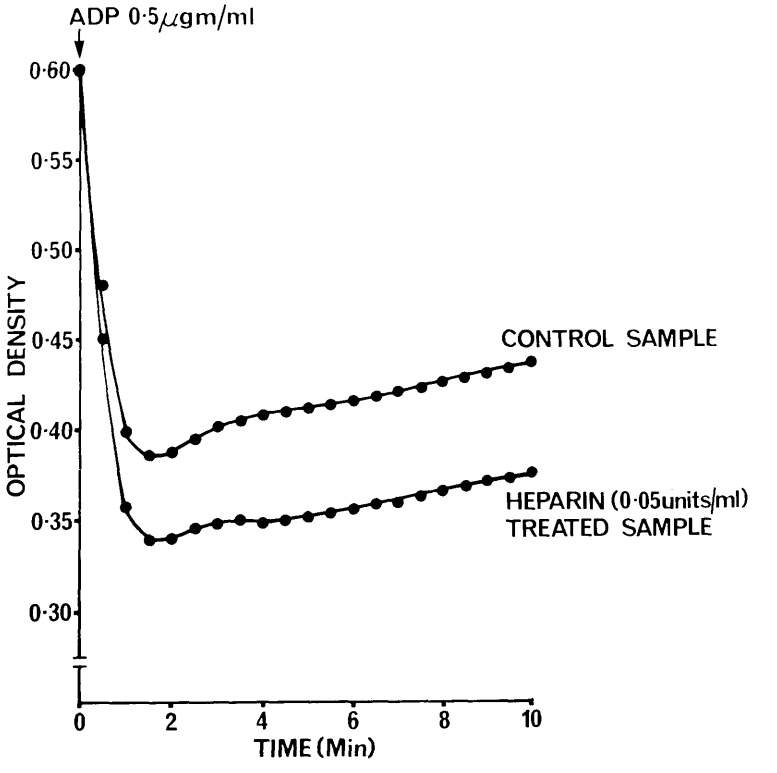

Figure 37 illustrates the effect of heparin (0.05 units/ml plasma) on ADP induced platelet aggregation. The presence of heparin at this concentration results in significant enhancement of platelet aggregation; 10 minutes after the addition of ADP (0.5 μg/ml plasma), the mean optical density reading of the control samples is 0.436 $\pm$ 0.139 and of the heparin treated samples 0.376 $\pm$ 0.167, ($t = 3.297$, $0.005 < p < 0.010$). The optical density readings, at this time interval, of the fourteen plasma samples investigated are shown in table 58.

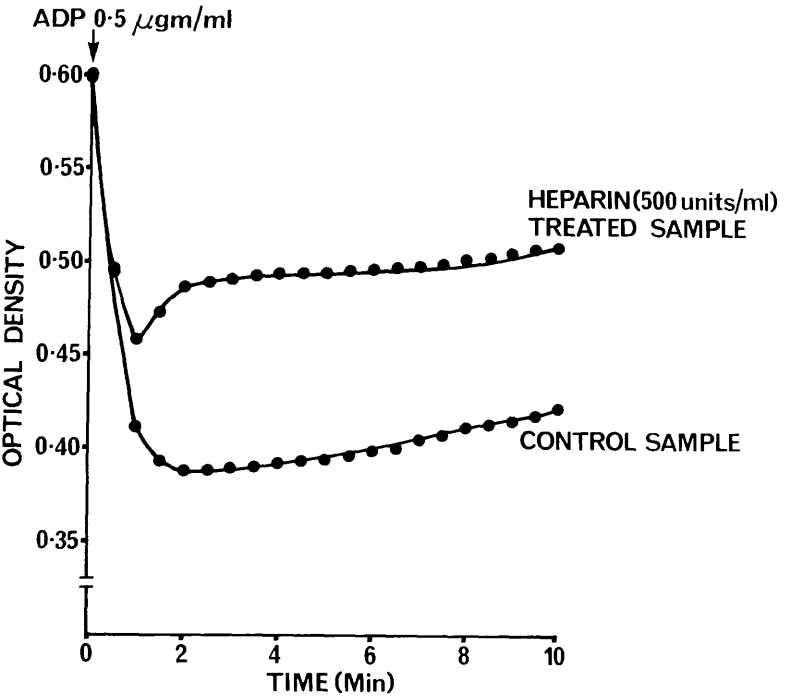

Figure 58 shows the effect of heparin (500 units/ml plasma) on ADP induced platelet aggregation. Platelet aggregation is significantly inhibited by the presence of heparin, at this concentration; 10 minutes after the addition of ADP (0.5 μg/ml plasma), the mean optical density reading of the control samples is 0.422 ± 0.184 and of the heparin treated samples 0.508 ± 0.140, ($t = 2.563$, $0.025 < p < 0.050$). The optical density readings, at this time interval, of the eight plasma samples investigated are shown in table 59.

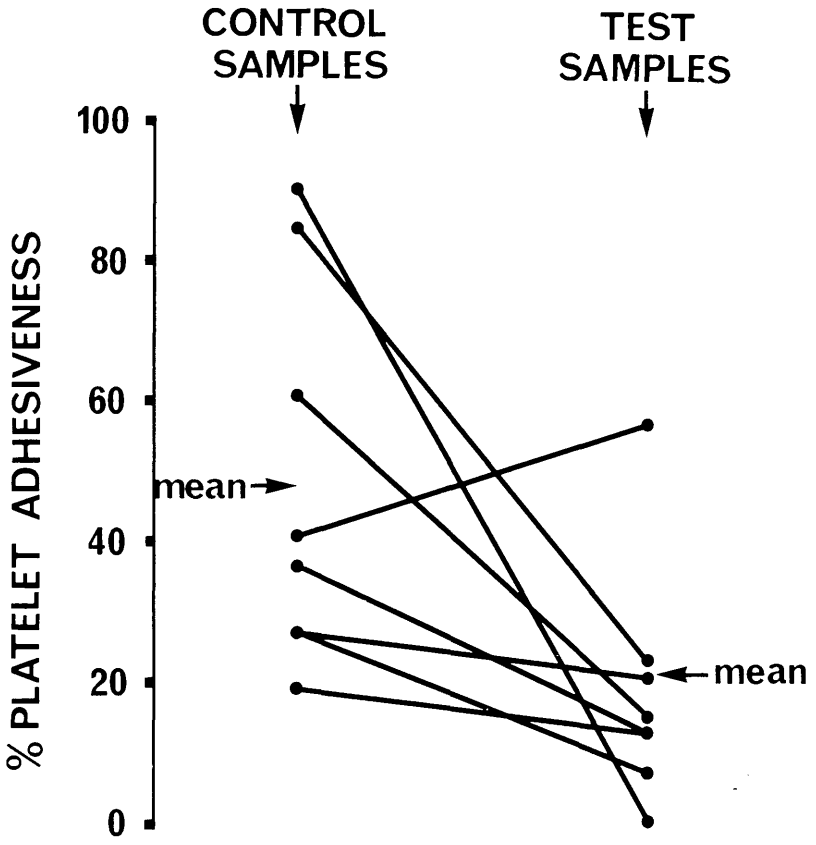

Figure 59 shows the effect of streptokinase, at the concentration which produces maximum lytic activity, on platelet adhesiveness of citrated whole blood. The presence of streptokinase, at this concentration, produces a significant decrease in the number of platelets lost during the passage of blood through the glass bead column. The mean percentage platelet adhesiveness of the control samples is 47.9 ± 27.2 and of the streptokinase treated samples 21.3 ± 17.4, ($t = 2.402$, $0.025 < p < 0.050$). The figure is constructed from the data of table 61.

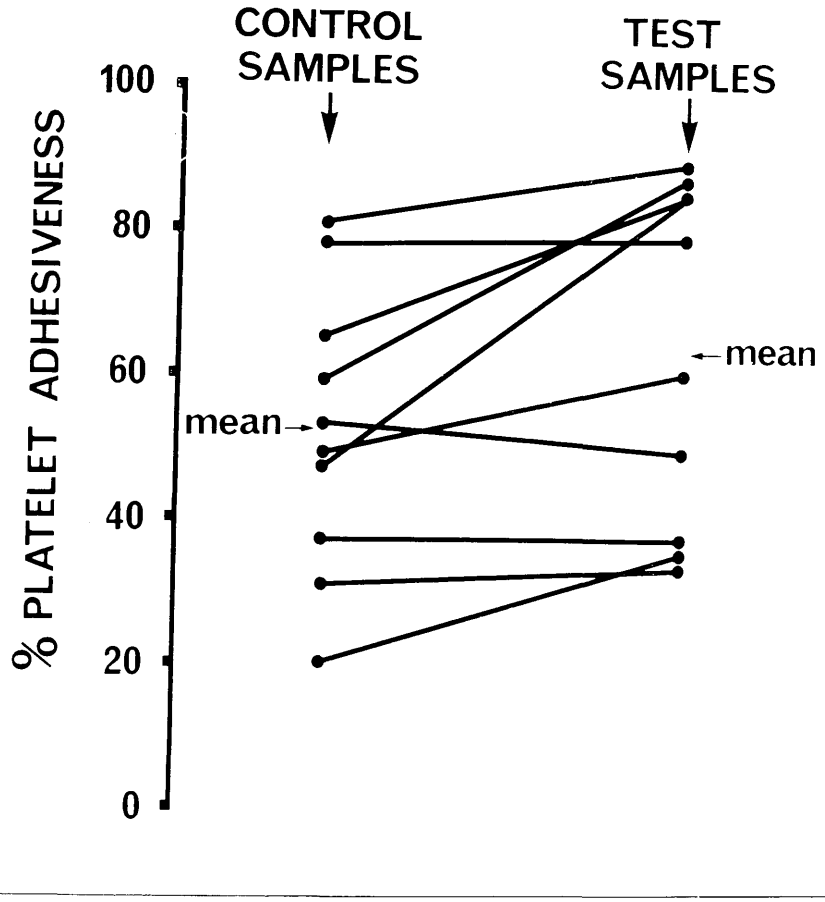

Figure 40 illustrates the effect of streptokinase, at the concentration which produces maximum lytic activity, on platelet adhesiveness of citrated whole blood after agitating the blood and streptokinase together at 37°C for 10 minutes. The number of platelets lost, during the passage of blood through the glass bead column, is significantly greater in the streptokinase treated samples than the control samples; the mean percentage platelet adhesiveness of the control samples is 52.0 ± 19.5 and of the test samples 63.9 ± 23.7, ($t = 2.780$, $0.020 < p < 0.025$). The figure is compiled from the data of table 62.

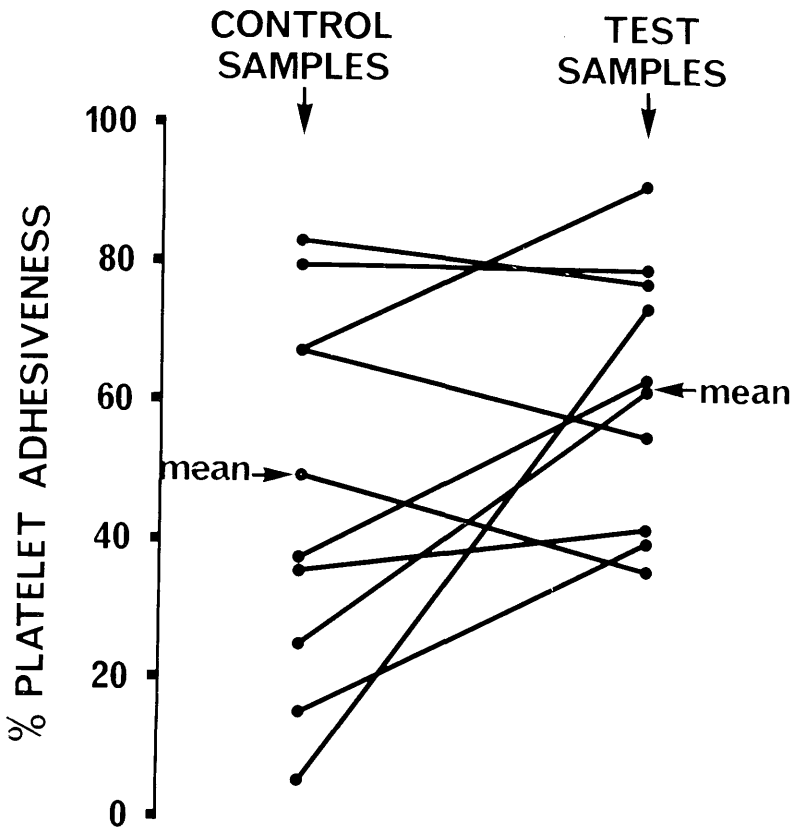

Figure 41 shows the effect of trypsin (10 µg/ml blood) on platelet adhesiveness of citrated whole blood. There is no significant difference between the mean percentage platelet adhesiveness of the control samples, 48.4 ± 30.1, and of the trypsin treated samples, 61.5 ± 18.6; $t = 1.515$, $0.10 < p < 0.20$. The data presented in table 63 was used to compile this figure.

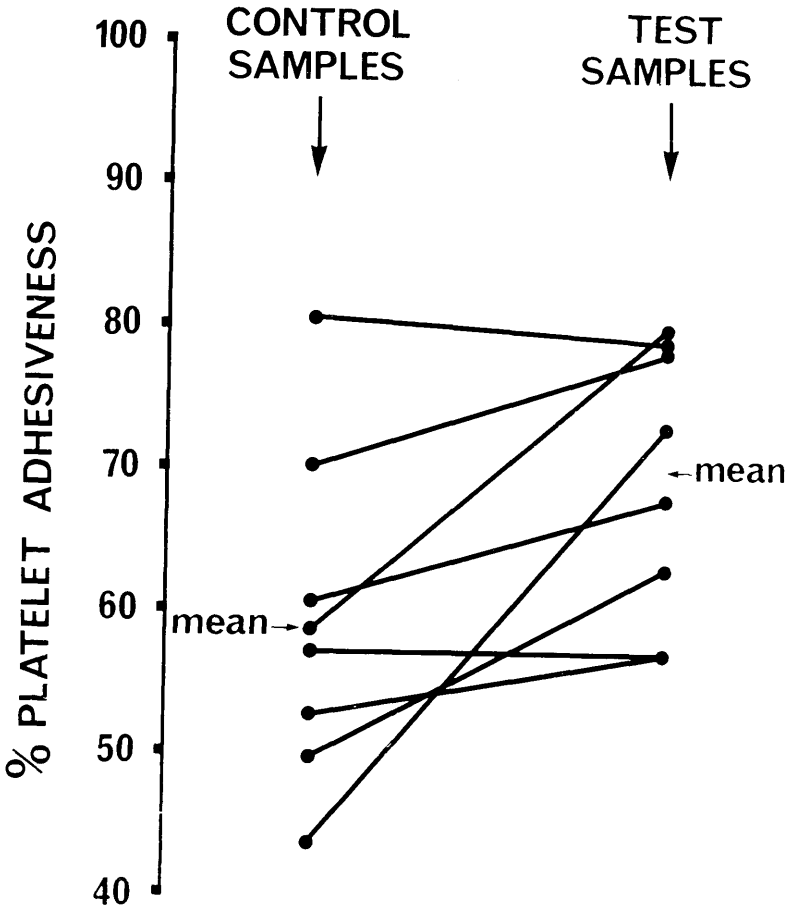

Figure 42 illustrates the effect of trypsin (10 µg/ml blood) on platelet adhesiveness in citrated whole blood after the trypsin and blood had been agitated together at 37°C for 10 minutes. The mean percentage platelet adhesiveness of the control samples is 58.9 ± 11.7 which is significantly less than the mean value of the trypsin treated samples 69.7 ± 8.9; $t = 3.011$, $0.01 < p < 0.02$. The figure was constructed from the data of table 64.

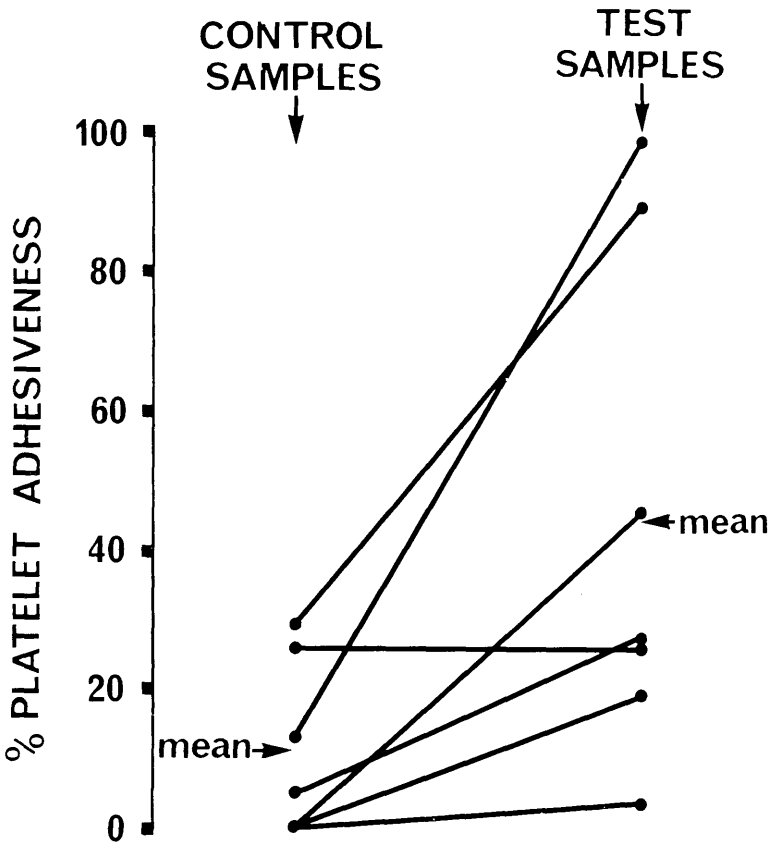

Figure 43 shows the effect of trypsin (10 µg/ml plasma) on platelet adhesiveness in platelet rich plasma. Trypsin results in a significant increase in the percentage platelet adhesiveness of the plasma; the mean value of the control samples is 10.6 ± 12.7 and of the trypsin treated samples 44.0 ± 36.6, ($t = 2.801$, $0.025 < p < 0.050$). The figure is compiled from data presented in table 65.

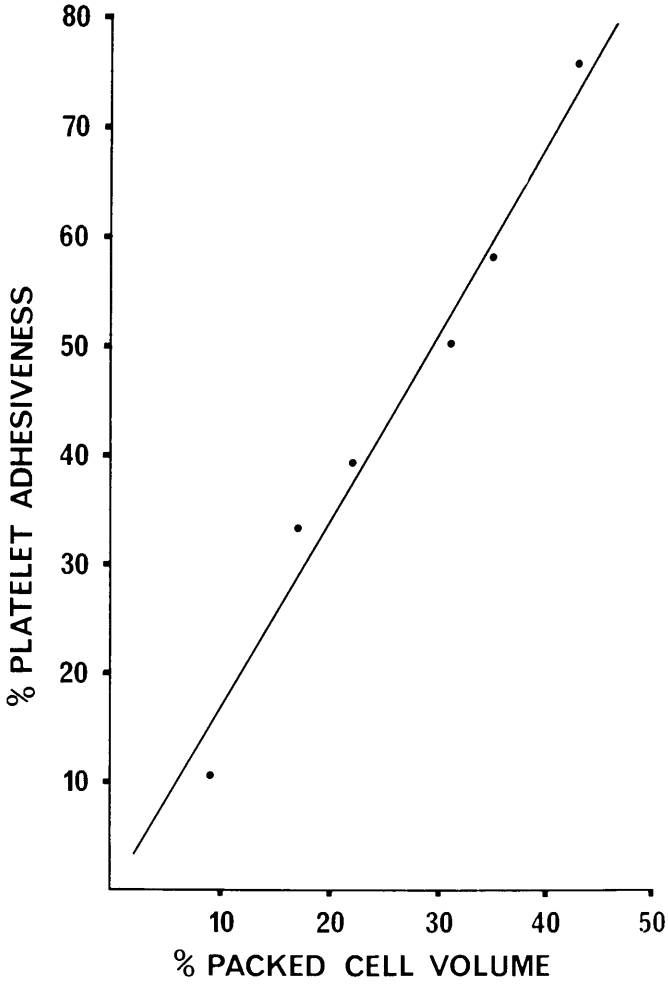

Figure 44 shows the effect of the packed cell volume on platelet adhesiveness.

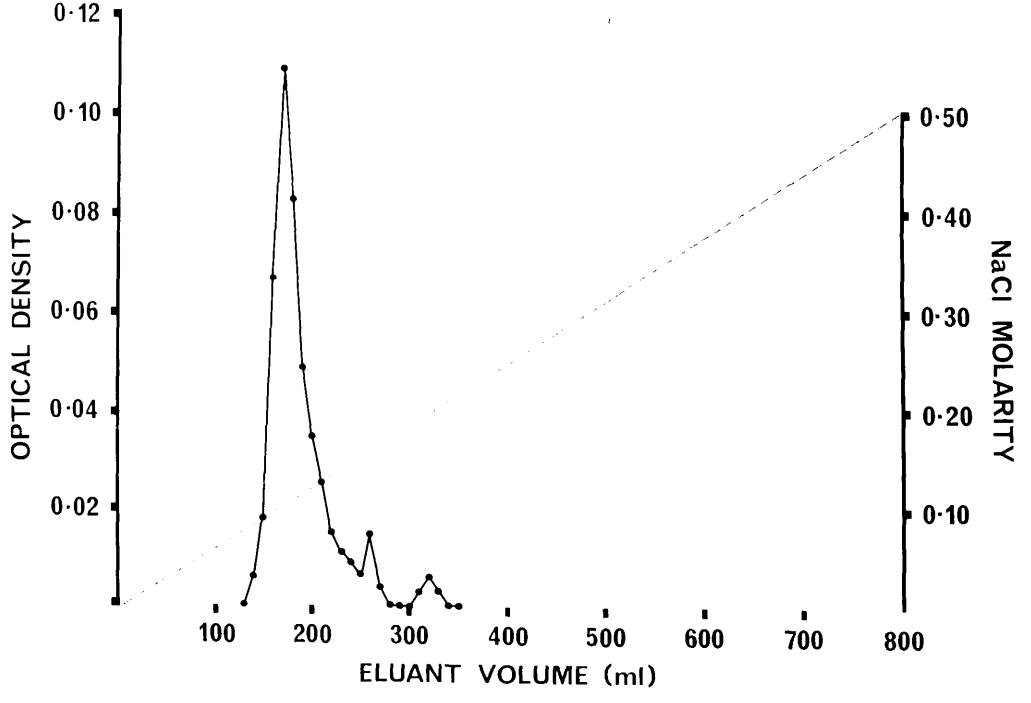

Figure 45 illustrates graphically the elution pattern obtained from the fractionation of Kabi human fibrinogen on a DEAE cellulose column. Elution was carried out with a linear concentration gradient of 0.1 M sodium carbonate buffer, pH 8.9 to 0.1 M sodium carbonate buffer pH 8.9 plus 0.5 M sodium chloride.

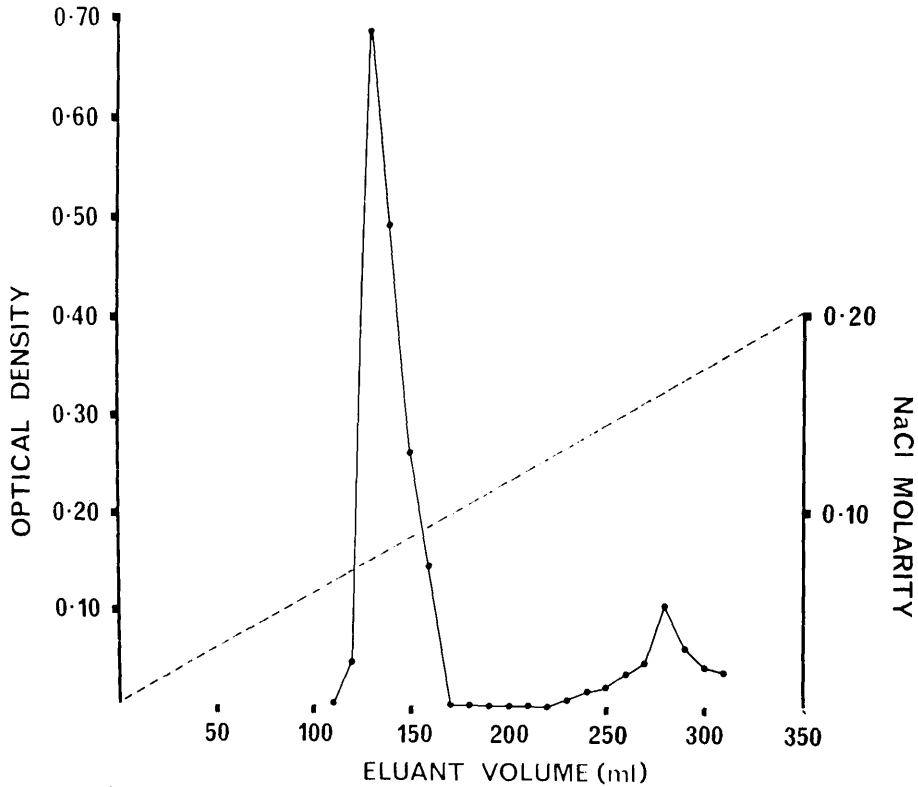

Figure 46 shows graphically the elution pattern obtained from the fractionation of purified plasminogen on a DEAE cellulose column. Elution was performed with a linear concentration gradient of 0.1 M carbonate buffer, pH 8.9 to 0.1 M carbonate buffer, pH 8.9 plus 0.2 M sodium chloride.

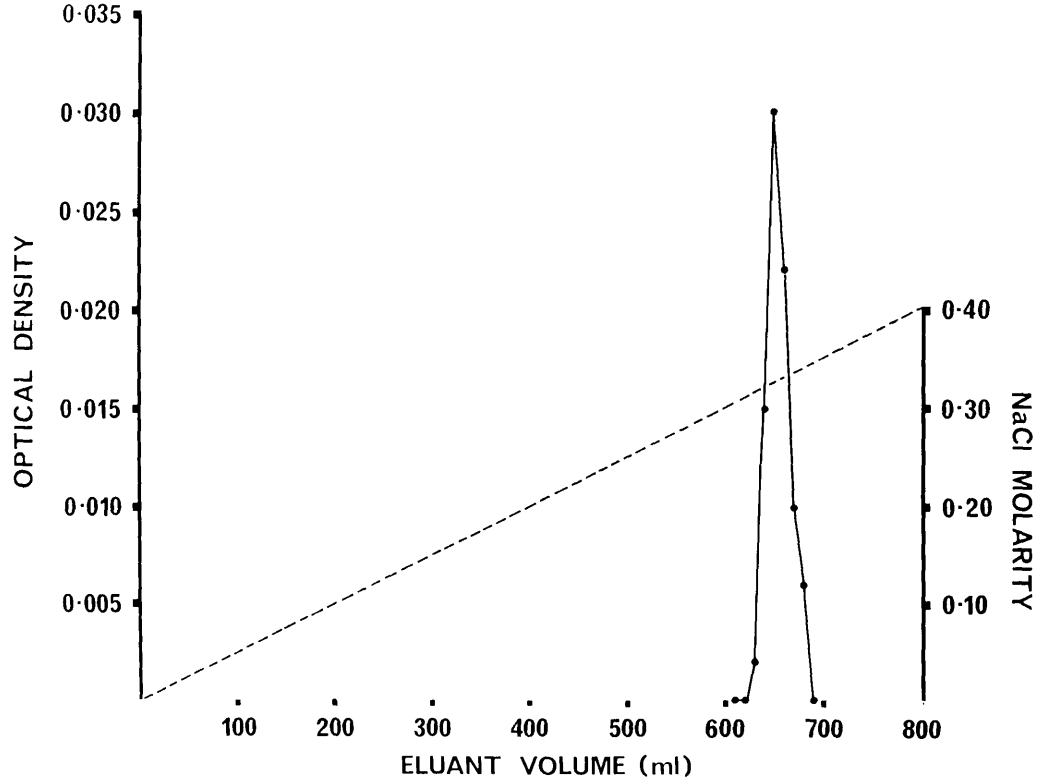

Figure 47 illustrates graphically the elution pattern obtained from the fractionation of a mixture of equal parts of trypsin and soybean trypsin inhibitor on a DEAE cellulose column. Elution was achieved with a linear concentration gradient of 0.1 M carbonate buffer, pH 8.9 to 0.1 M carbonate buffer, pH 8.9 plus 0.4 M sodium chloride.

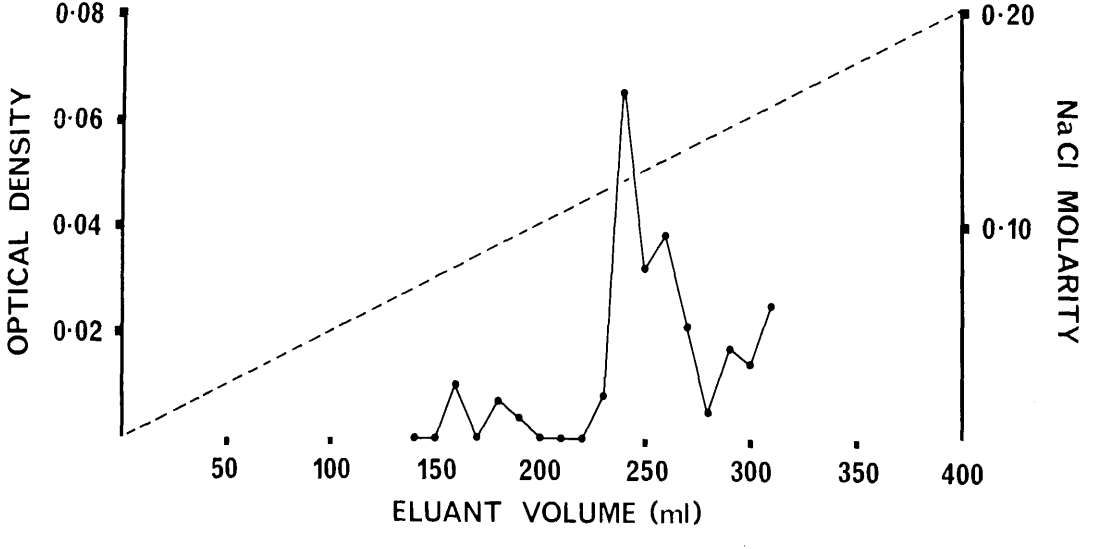

Figure 48 shows graphically the elution pattern obtained from the fractionation of urokinase on a DEAE cellulose column. Elution was performed with a linear concentration gradient of 0.1 M carbonate buffer, pH 8.9 to 0.1 M carbonate buffer, pH 8.9 plus 0.2 M sodium chloride.

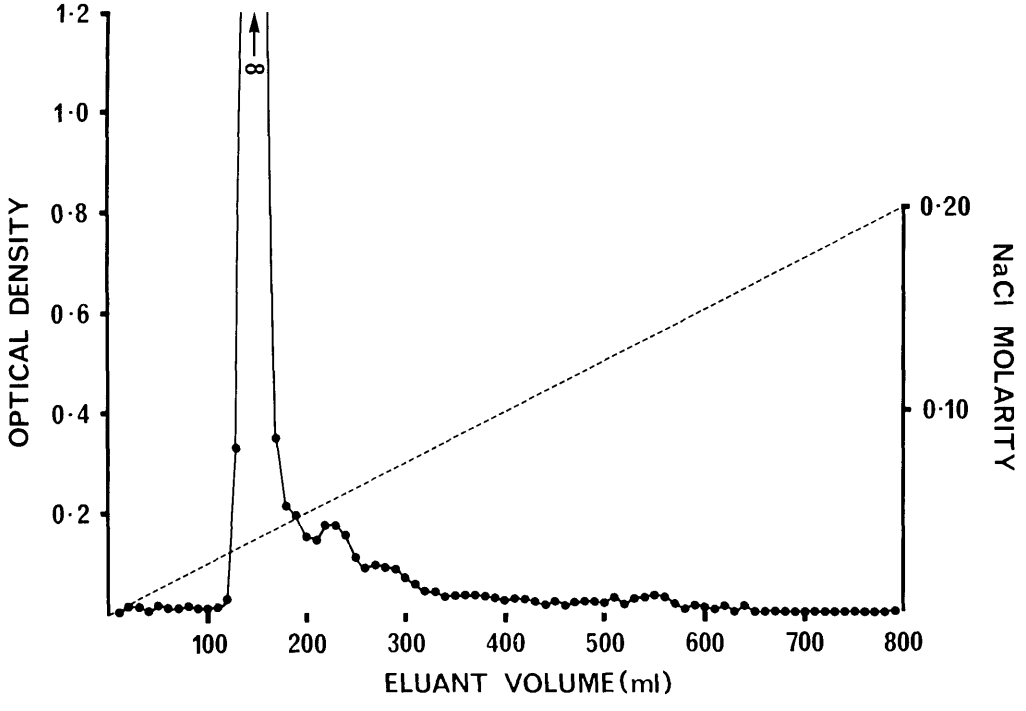

Figure 49 illustrates graphically the elution pattern obtained from the fractionation, on a DEAE cellulose column, of the fibrinogen degradation product 'a', prepared by incubating, at 37°C for 10 minutes, a mixture of 200 mg fibrinogen and 0.667 mg trypsin. Fractionation was achieved using a linear concentration gradient of 0.1 M carbonate buffer, pH 8.9 to 0.1 M carbonate buffer, pH 8.9 plus 0.2 M sodium chloride.

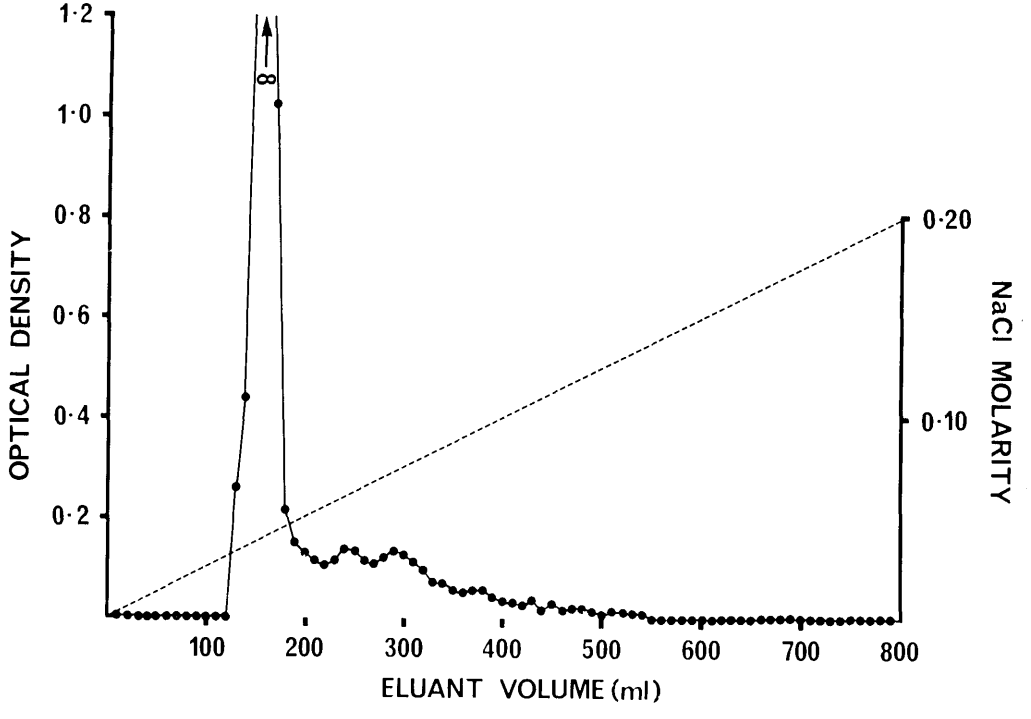

Figure 50 shows graphically the elution pattern obtained from the fractionation, on a DEAE cellulose column, of the fibrinogen degradation product 'b', prepared by incubating, at 37°C for 10 minutes, a mixture of 200 mg fibrinogen and 6.67 mg trypsin. Fractionation was performed with a linear concentration gradient of 0.1 M carbonate buffer, pH 8.9 to 0.1 M carbonate buffer, pH 8.9 plus 0.2 M sodium chloride.

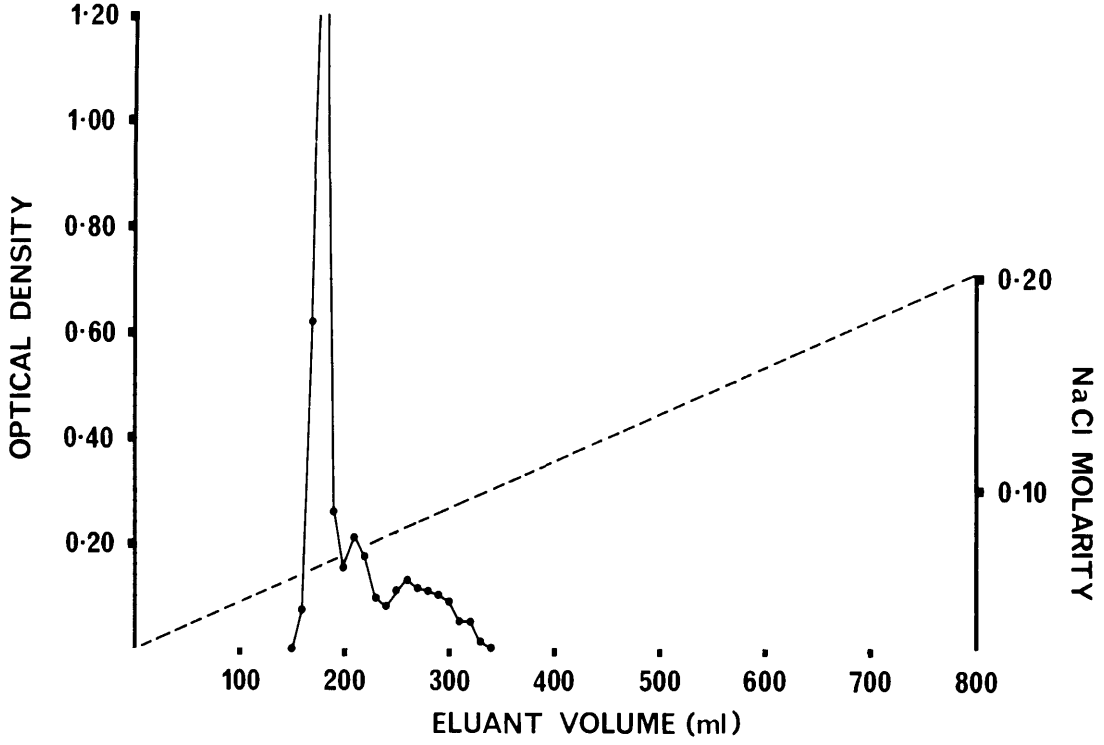

Figure 51 shows graphically a second elution pattern obtained from the fractionation of product 'b'. The conditions of preparation of the product and the elution of the product from the DEAE cellulose column are identical to those described in figure 50. The elution patterns illustrated in figures 50 and 51 are, within the limits of experimental error, identical.

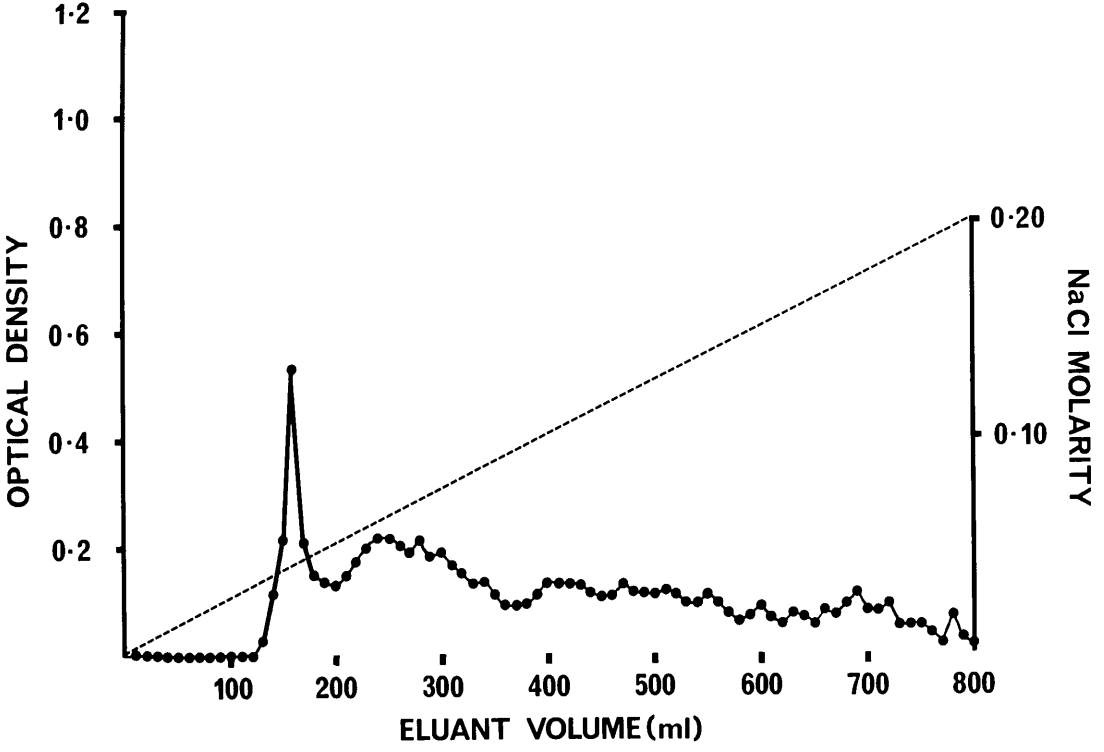

Figure 52 illustrates graphically the elution pattern obtained from the fractionation, on a DEAE cellulose column, of the fibrinogen degradation product 'c', prepared by incubating 200 mg fibrinogen and 6.67 mg trypsin at 37°C for 16 hours. Fractionation was achieved with a linear concentration gradient of 0.1 M carbonate buffer, pH 8.9 to 0.1 M carbonate buffer, pH 8.9 plus 0.2 M sodium chloride.

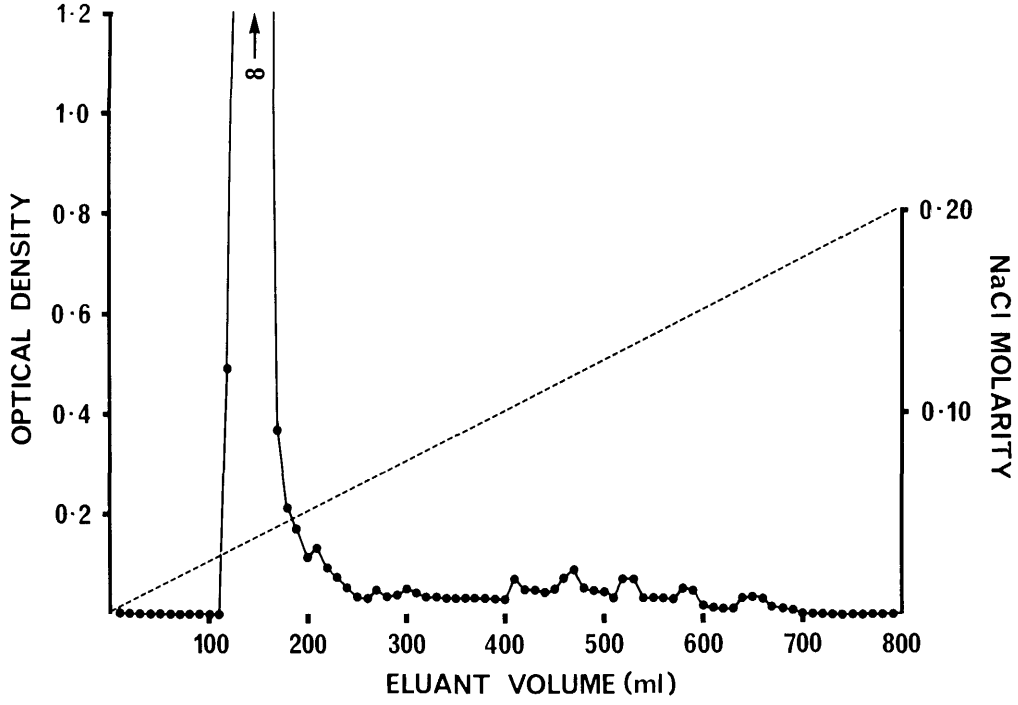

Figure 53 illustrates graphically the elution pattern obtained for the fractionation, on a DEAE cellulose column, of the fibrinogen degradation product 'd', prepared by incubating, at 37°C for 10 minutes a mixture of fibrinogen, plasminogen and urokinase. Fractionation was achieved by the use of a linear concentration gradient of 0.1 M carbonate buffer, pH 8.9 to 0.1 M carbonate buffer, pH 8.9 plus 0.2 M sodium chloride.

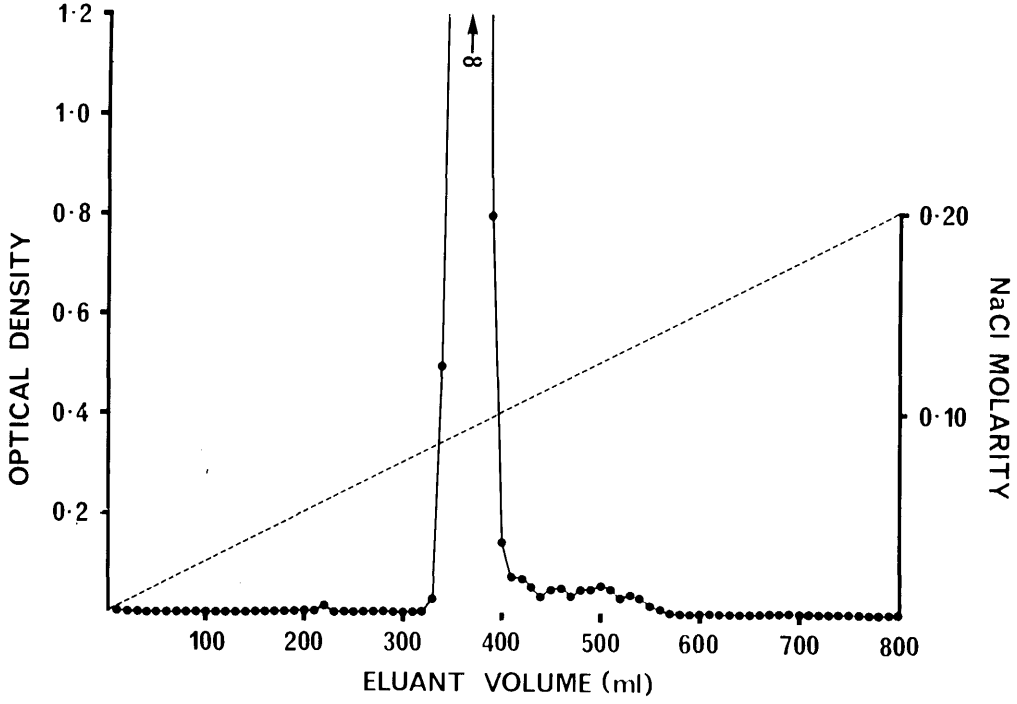

Figure 54 shows graphically the elution pattern obtained for the fractionation, on a DEAE cellulose column, of Kabi human fibrinogen. Elution was performed with a linear concentration gradient of 0.1 M carbonate buffer, pH 8.9 to 0.1 M carbonate buffer, pH 8.9 plus 0.2 M sodium chloride.

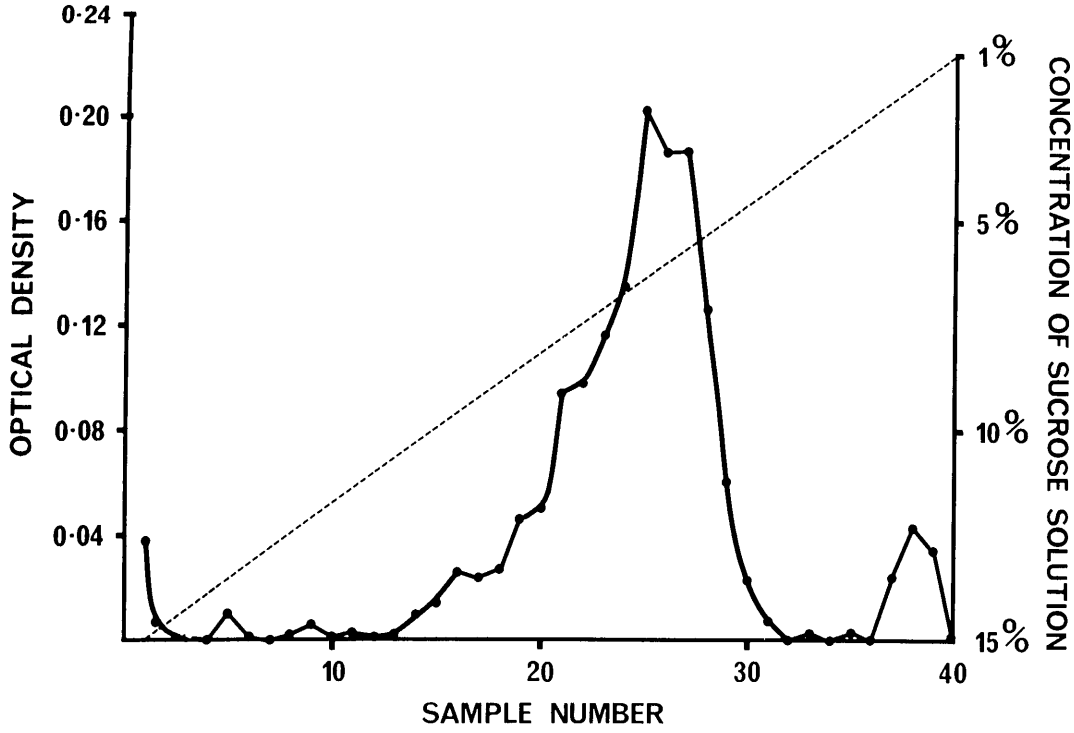

Figure 55 illustrates graphically the elution pattern obtained for the fractionation of Kabi human fibrinogen, under the influence of ultra-centrifugation, in a sucrose density gradient of 1 - 15% sucrose.

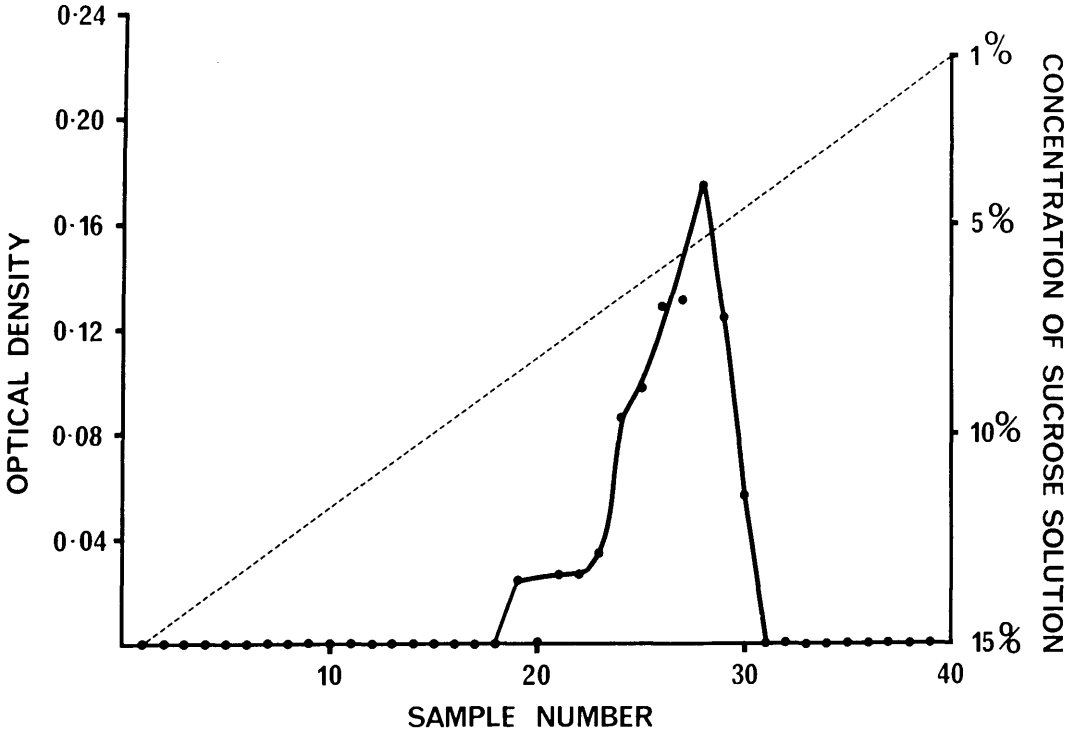

Figure 56 shows graphically the elution pattern obtained for the fractionation, in a sucrose density gradient of 1 - 15% sucrose, of 'purified' fibrinogen. The fibrinogen preparation was obtained from the main protein component of Kabi human fibrinogen fractionated on a DEAE cellulose column.

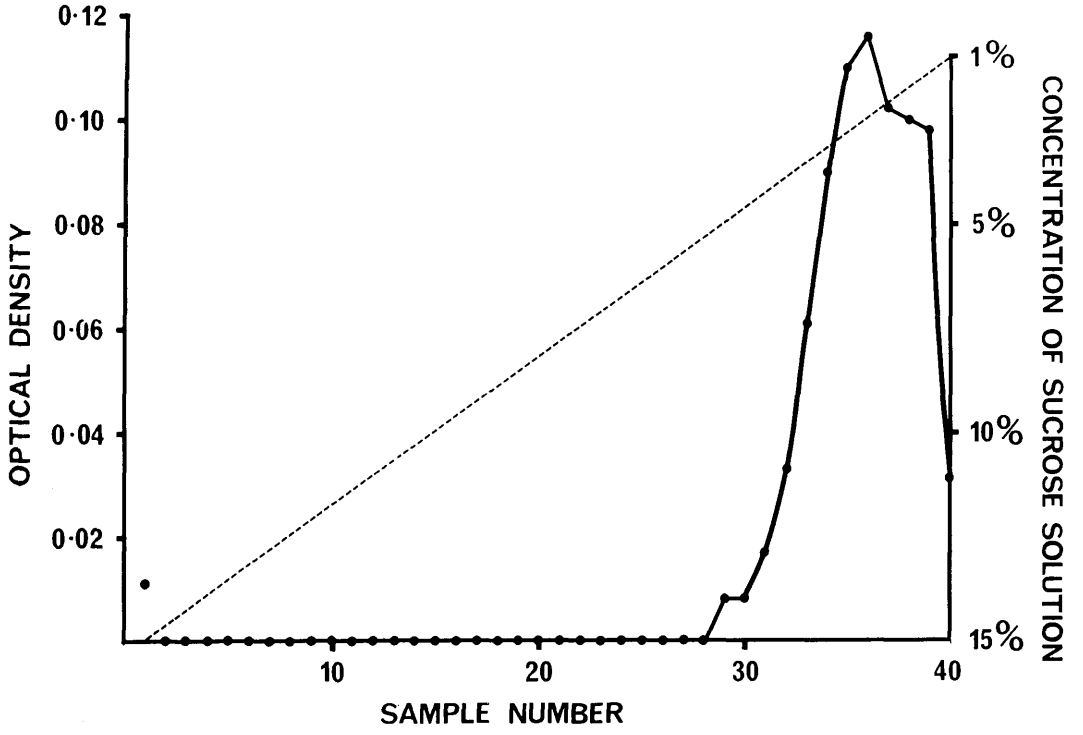

Figure 57 illustrates graphically the elution pattern obtained for the fractionation, by ultracentrifugation, of the enzyme ribonuclease (RNase) in a sucrose density gradient of 1 - 15% sucrose.

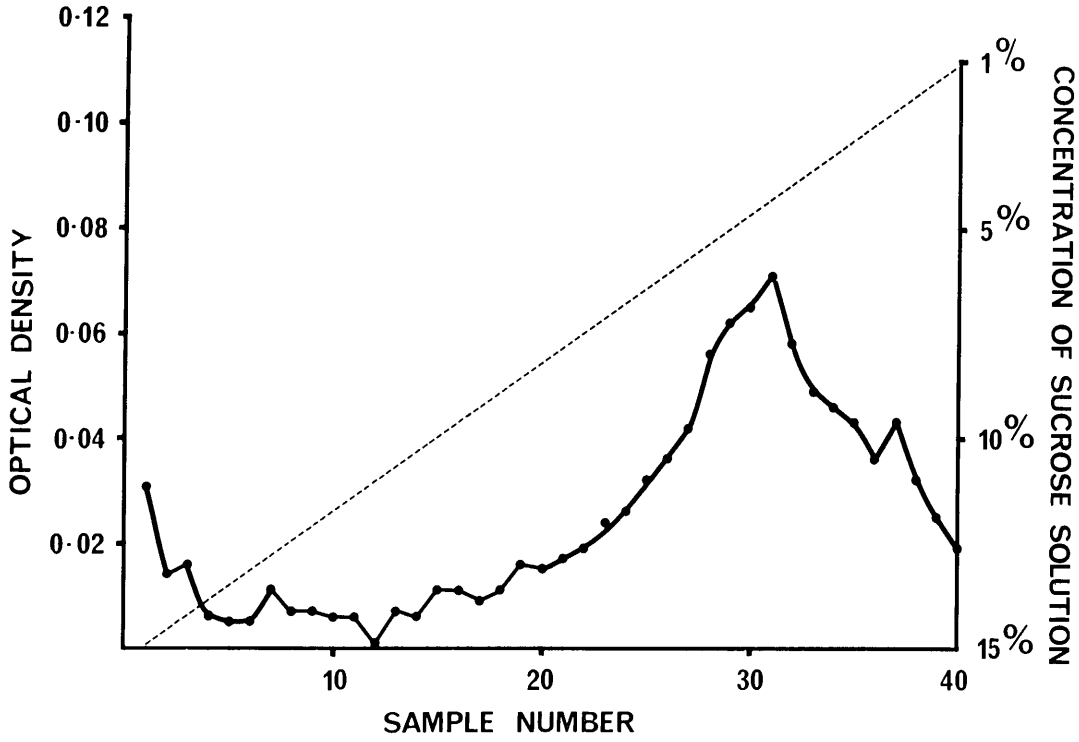

Figure 58 shows graphically the elution pattern obtained for the fractionation, by ultracentrifugation, of the fibrinogen degradation product 'a' in a sucrose density gradient of 1 - 15% sucrose.

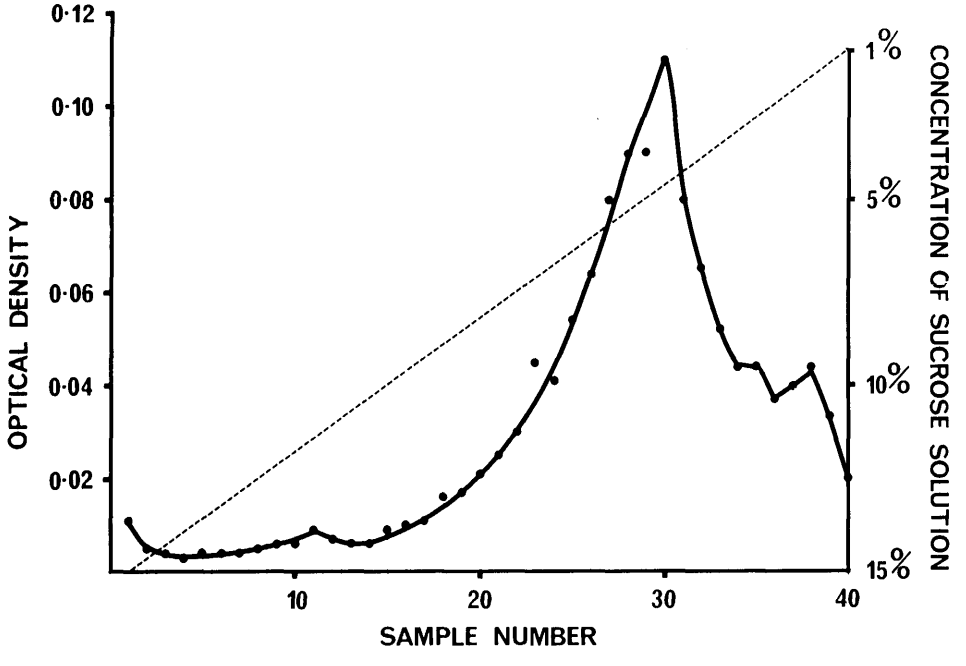

Figure 59 illustrates graphically the elution pattern obtained for the fractionation of the fibrinogen degradation product 'b', by ultra-centrifugation, in a sucrose density gradient of 1 - 15% sucrose.

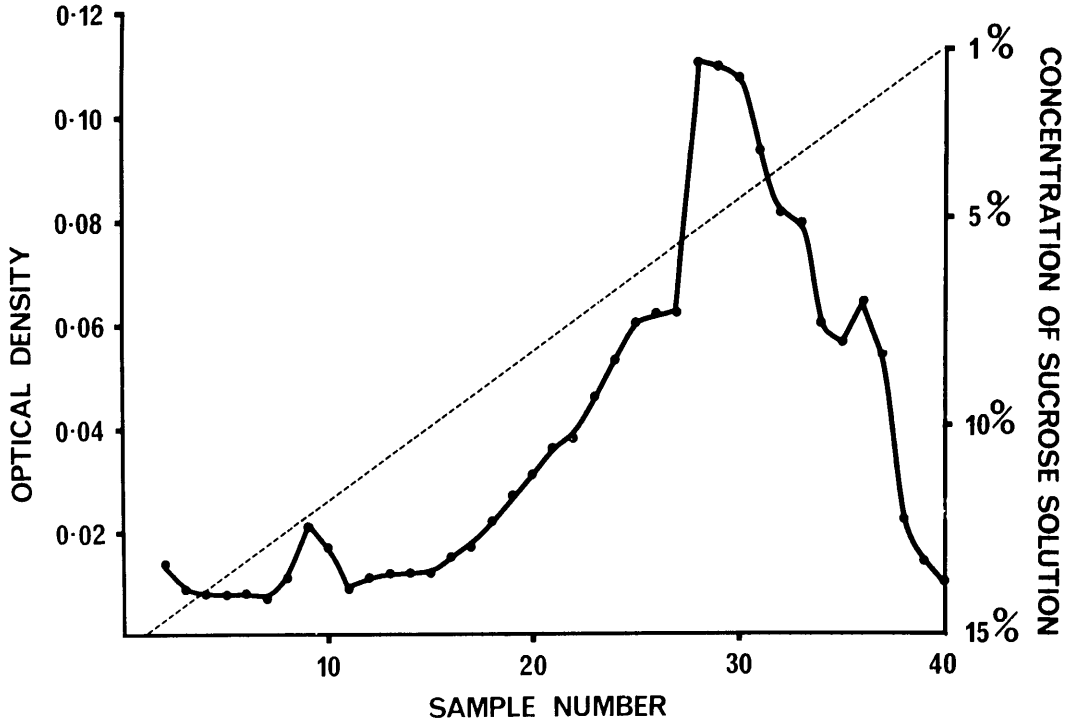

Figure 60 shows graphically the elution pattern obtained for the fractionation of the fibrinogen degradation product 'd', by ultracentrifugation, in a sucrose density gradient of 1 - 15% sucrose.

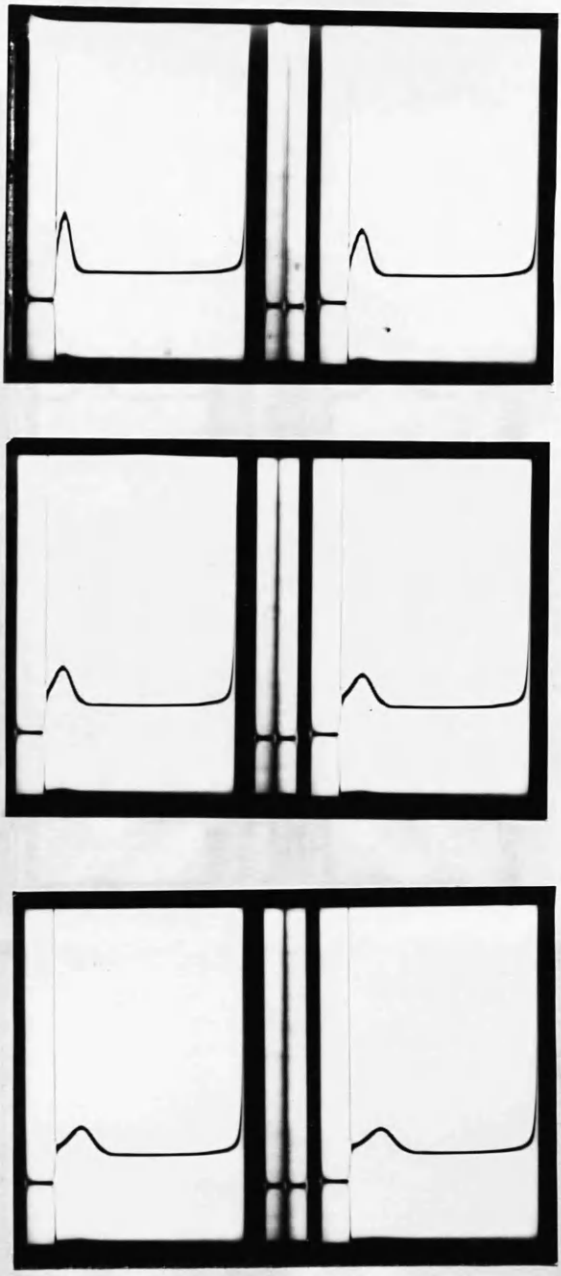

Figure 61 shows the ultracentrifugation patterns obtained with the Schliern optical system of a Spinco, Model E, Ultracentrifuge during the determination of the sedimentation coefficient of the fibrinogen degradation product 'b'. The patterns read from left to right and top to bottom and represent the patterns at the time intervals zero, 8, 16, 24, 32 and 64 minutes from the start of the run.

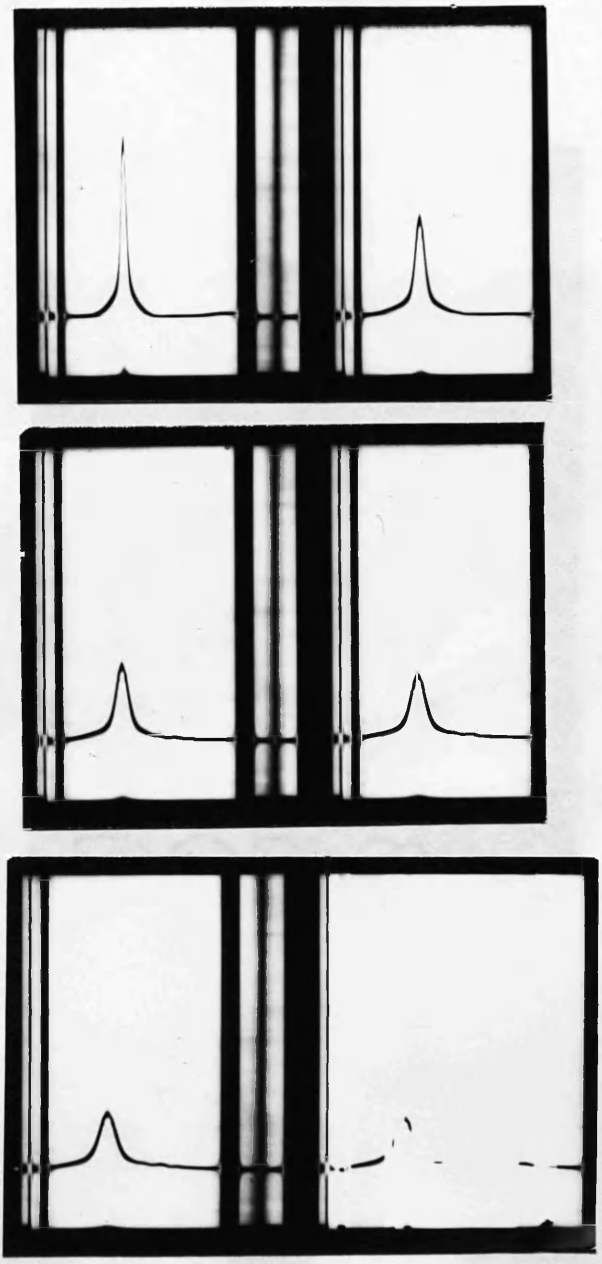

Figure 62 shows the ultracentrifugation patterns obtained with the Schliern optical system of a Spinco, Model E, Ultracentrifuge during the determination of the diffusion coefficient of the fibrinogen degradation product 'b'. The patterns read from left to right and from top to bottom and represent the patterns obtained at the time intervals 8, 16, 24, 32, 40 and 48 minutes from the start of the run.

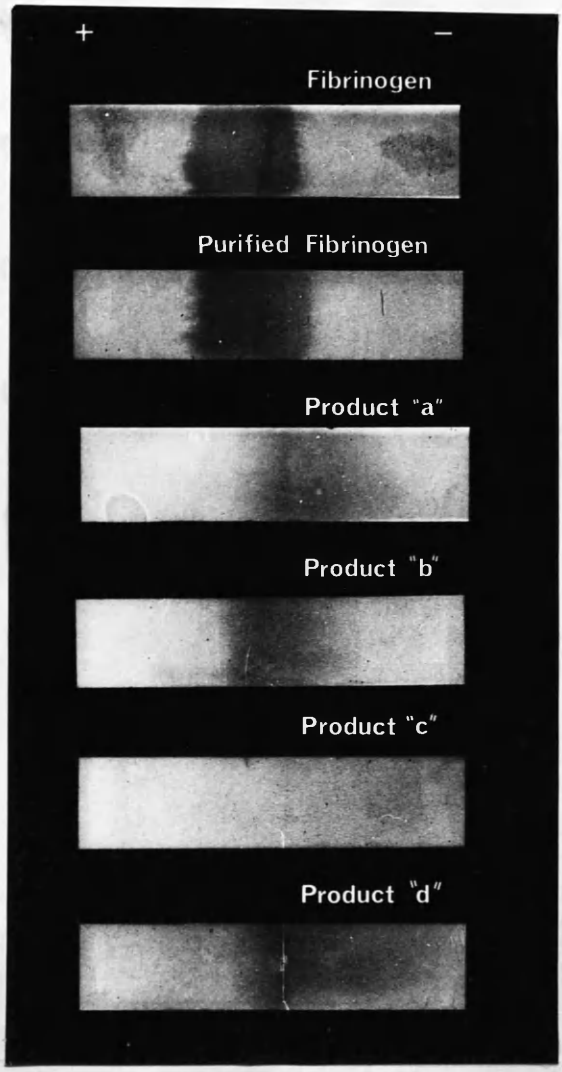

Figure 63 illustrates the electrophoretic patterns obtained with fibrinogen, before and after passage through a DEAE cellulose column, and the fibrinogen degradation products 'a', 'b', 'c' and 'd' as a result of horizontal paper electrophoresis in 0.1 M barbitone buffer, pH 8.6 for 17 hours at 200 volts. The electrophoretic strips were stained with Bromophenol blue.

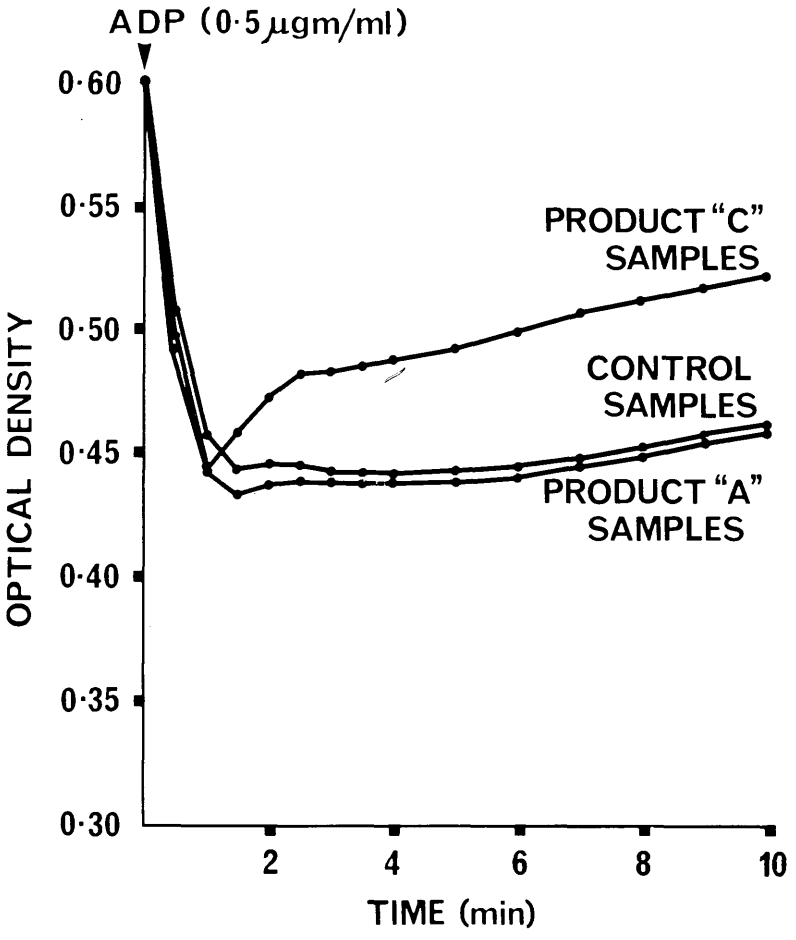

Figure 64 shows the effect of the fibrinogen degradation products 'a' and 'c' on ADP induced platelet aggregation. The concentration of both products was 100 µg/ml plasma. Product 'a' has no significant effect on platelet aggregation; the mean optical density readings of the control and test samples, 1 minute after the addition of ADP (0.5 µg/ml plasma), are 0.456 ± 0.090 and 0.442 ± 0.103 ($t = 1.172$, $0.20 < p < 0.30$). Product 'c' causes significant inhibition of platelet aggregation; the mean optical density readings of the control and test samples, 2 minutes after the addition of ADP, are 0.445 ± 0.121 and 0.473 ± 0.105 ($t = 3.233$, $0.01 < p < 0.02$). The optical density readings, at these time intervals, of the seven samples investigated are presented in tables 66 and 74.

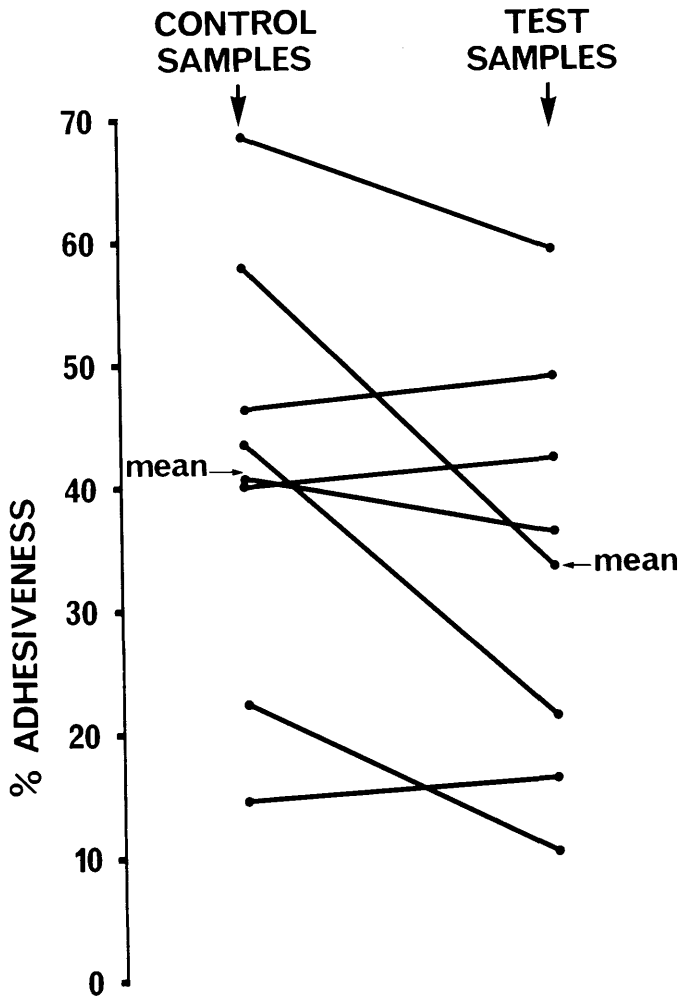

Figure 69 illustrates the effect of product 'a' (100 µg/ml blood) on platelet adhesiveness in citrated whole blood. There is no significant difference between the results of the control samples and the samples treated with product 'a'. The mean percentage platelet adhesiveness of the control samples is 41.9 ± 17.4 and of the test samples 33.5 ± 16.7 ($t = 2.149$, $0.05 < p < 0.10$). The figure is compiled from the data of table 67.

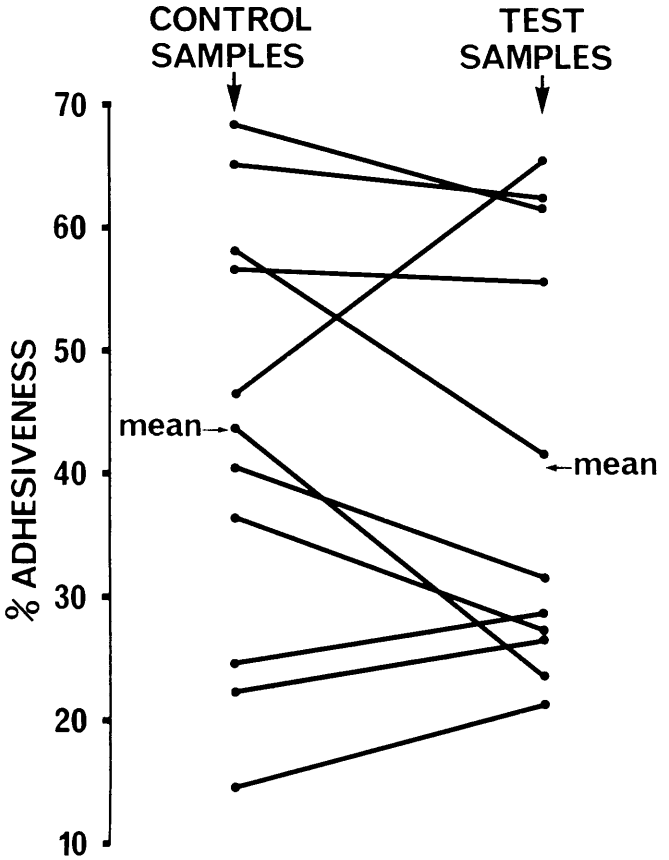

Figure 66 shows the effect of product 'a' (200 µg/ml blood) on platelet adhesiveness in citrated whole blood. Product 'a' has no significant effect on platelet adhesiveness; the mean percentage platelet adhesiveness of the control samples is 43.4 ± 17.8 and the test samples 40.6 ± 17.4 ($t = 0.826$, $0.40 < p < 0.50$). The data of table 68 was used to compile this figure.

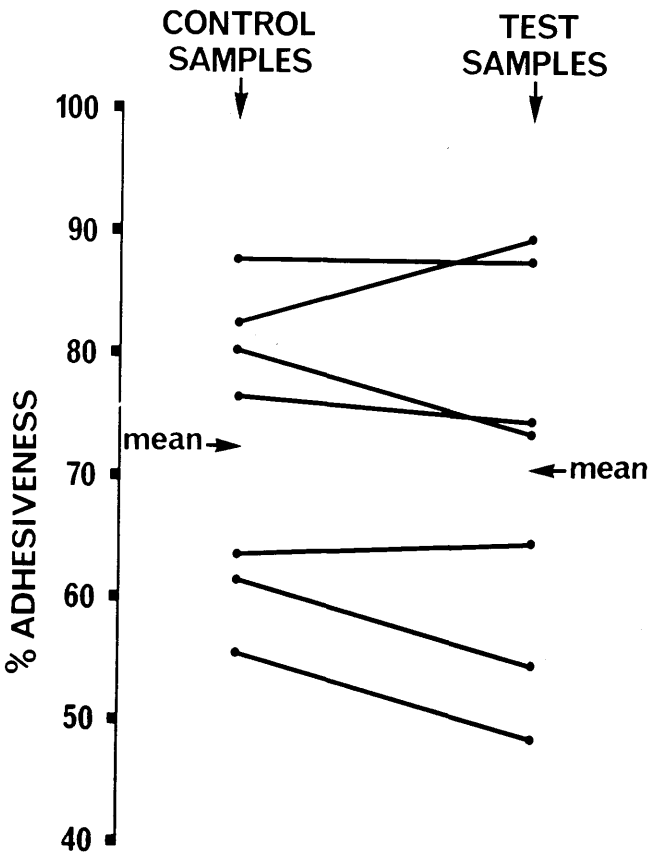

Figure 67 illustrates the effect of product 'a' (100 µg/ml blood) on platelet adhesiveness in citrated whole blood after the blood and product had been agitated together at 37°C for 10 minutes. There is no significant difference between the results of the control samples and the samples treated with product 'a'; the mean percentage platelet adhesiveness of the control samples is 72.5 ± 12.3 and of the test samples 70.4 ± 15.6 ($t = 1.084$, $0.30 < p < 0.40$). The figure was constructed from the data of table 69.

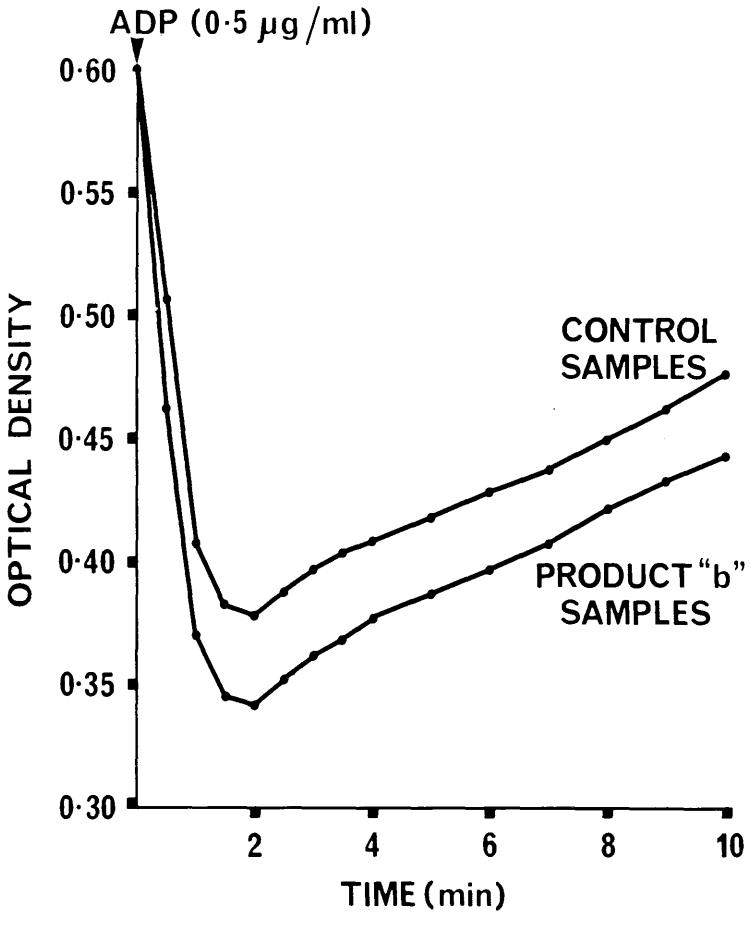

Figure 68 shows the effect of product 'b' (100 µg/ml plasma) on ADP induced platelet aggregation. Product 'b' causes significant enhancement of platelet aggregation; the mean optical density reading, 1½ minutes after the addition of ADP (0.5 µg/ml plasma), of the control samples is 0.378 ± 0.140 and of the test samples 0.341 ± 0.141 ($t = 3.253$, $0.01 < p < 0.02$). The optical density readings, at this time interval, of the seven plasma samples investigated are presented in table 70.

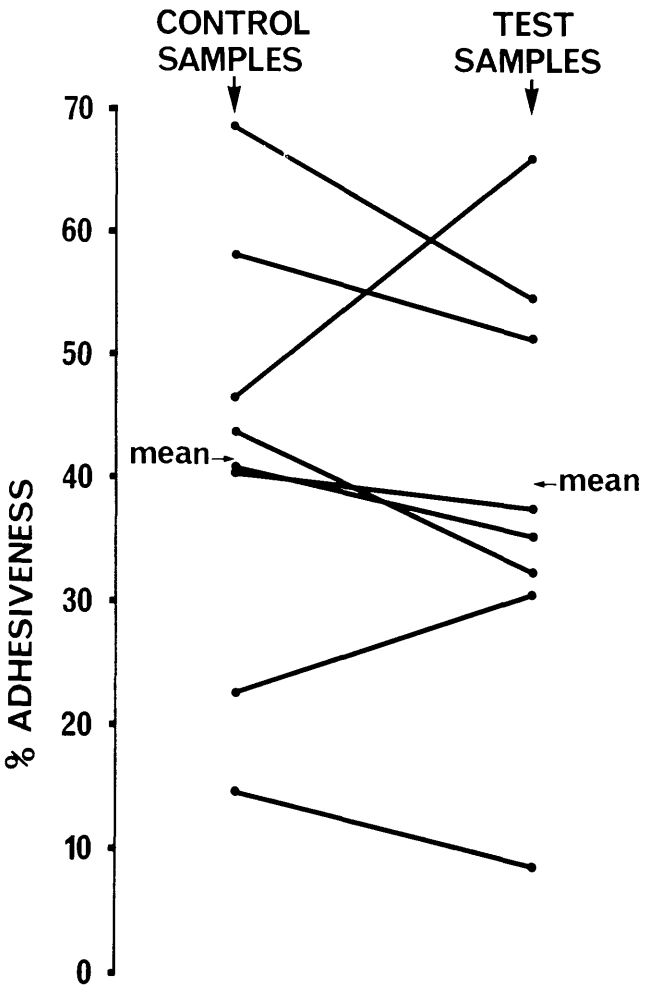

Figure 69 illustrates the effect of product 'b' (100 µg/ml blood) on platelet adhesiveness in citrated whole blood. Product 'b' has no significant effect on platelet adhesiveness; the mean percentage platelet adhesiveness of the control samples is 41.9 ± 17.4 and of the test samples 39.6 ± 17.6 ($t = 0.594$, $0.50 < p < 0.60$). The figure is compiled from the data of table 71.

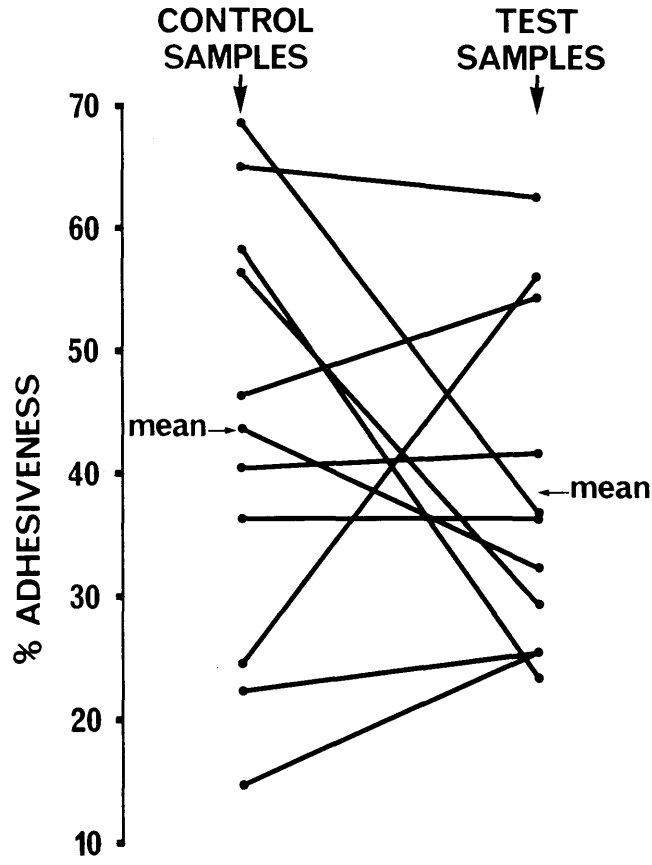

Figure 70 shows the effect of product 'b' (200 µg/ml blood) on platelet adhesiveness in citrated whole blood. There is no significant alteration in the platelet adhesiveness of samples treated with product 'b'; the mean percentage platelet adhesiveness of the control samples is 43.4 ± 17.8 and of the test samples 38.5 ± 15.7 ($t = 1.251$, $0.20 < p < 0.30$). The results of table 72 were used to construct this figure.

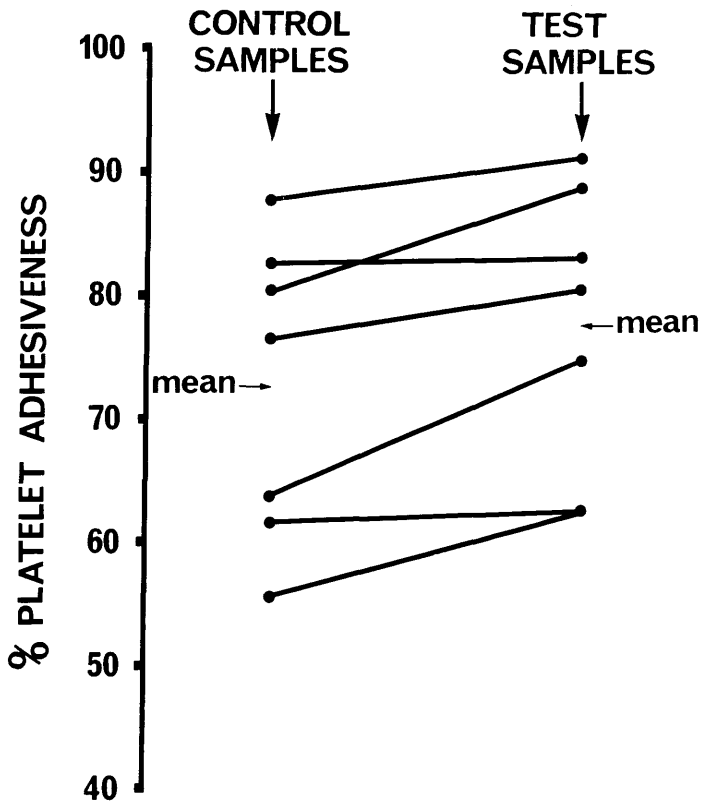

Figure 71 illustrates the effect of product 'b' (100 µg/ml blood) on platelet adhesiveness in citrated whole blood after the blood and product had been agitated together at 37°C for 10 minutes. Product 'b' causes a significant increase in platelet adhesiveness; the mean percentage platelet adhesiveness of the control samples is 72.5 ± 12.3 and of the test samples 77.6 ± 11.7 ($t = 3.456$, $0.01 < p < 0.02$). The figure is constructed from the data of table 73.

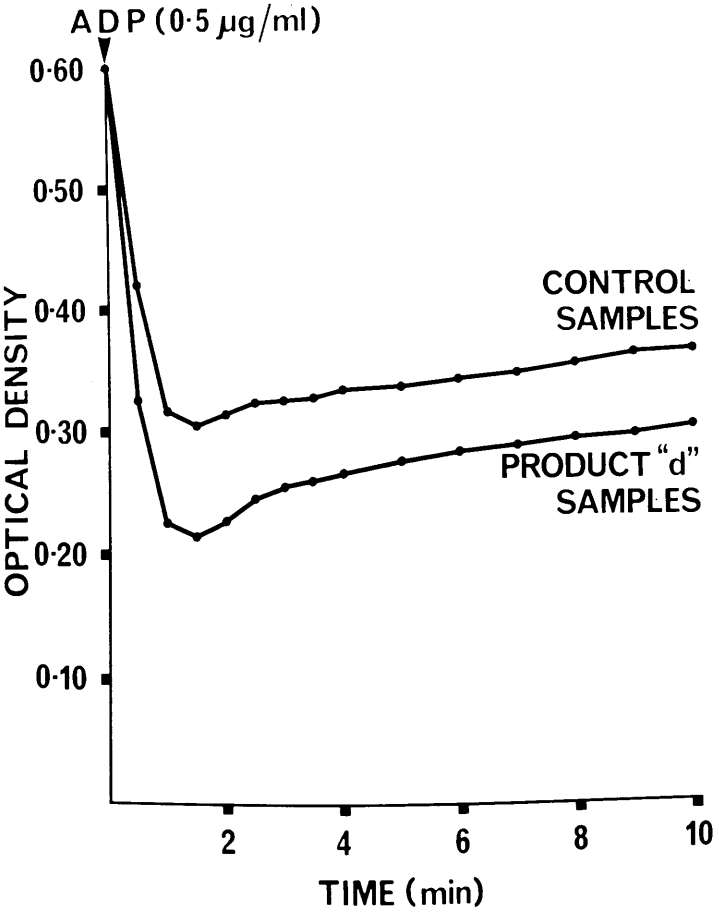

Figure 72 shows the effect of product 'd' (100 µg/ml plasma) on ADP induced platelet aggregation of platelet rich plasma. Product 'd' causes a significant enhancement of platelet aggregation; $1\frac{1}{2}$ minutes after the addition of ADP (0.5 µg/ml plasma), the mean optical density reading of the control samples is 0.305 ± 0.159 and of the test samples 0.216 ± 0.112 ($t = 2.415$, $0.025 < p < 0.050$). The optical density readings, at this time interval, of the ten plasma samples investigated are presented in table 75.

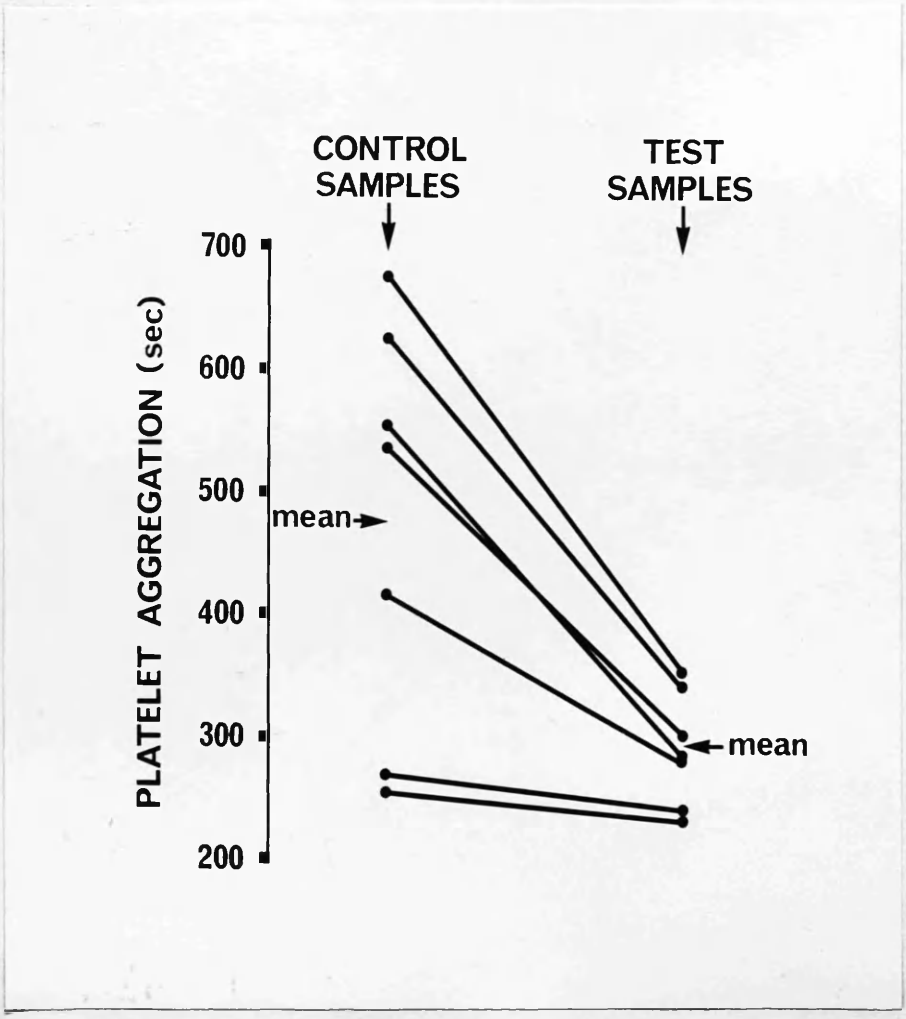

Figure 73 illustrates the effect of product 'd' (180 µg/ml plasma) on the rate of formation of platelet aggregates in the Chandler tube. The speed of platelet aggregation is accelerated in each of the seven plasma samples investigated. The mean time of platelet aggregation in the control loops is 477 ± 168 seconds and of the test loops 294 ± 45 seconds ($t = 3.874$, $0.005 < p < 0.010$). The figure is compiled from the data of table 76.

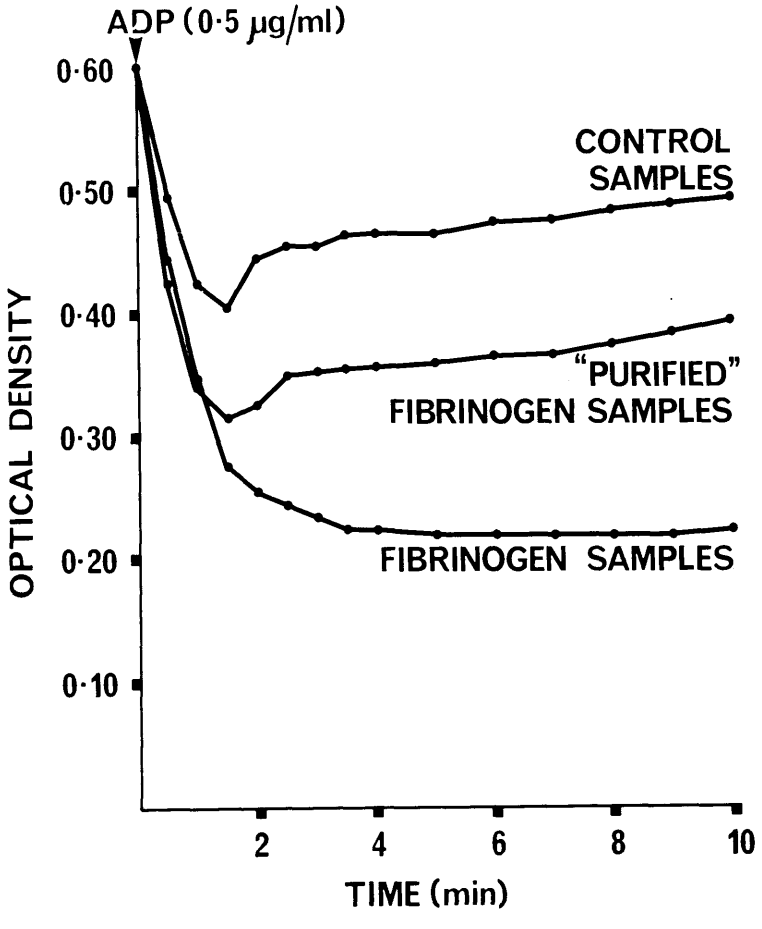

Figure 74 shows the effect of fibrinogen (100 µg/ml plasma) and 'purified' fibrinogen (100 µg/ml plasma) on ADP induced platelet aggregation of platelet rich plasma. Fibrinogen causes significant enhancement of aggregation but 'purified' fibrinogen has no effect on aggregation. 1½ minutes after the addition of ADP (0.5 µg/ml plasma) the mean optical density reading of the control samples is 0.406 ± 0.135, of the fibrinogen treated samples 0.275 ± 0.198 and of the 'purified' fibrinogen treated samples 0.312 ± 0.123 ($t = 3.126$, $0.020 < p < 0.025$ and $t = 2.361$, $0.05 < p < 0.10$ respectively). The optical density readings, at this time interval, of the seven plasma samples investigated are shown in tables 77 and 78.

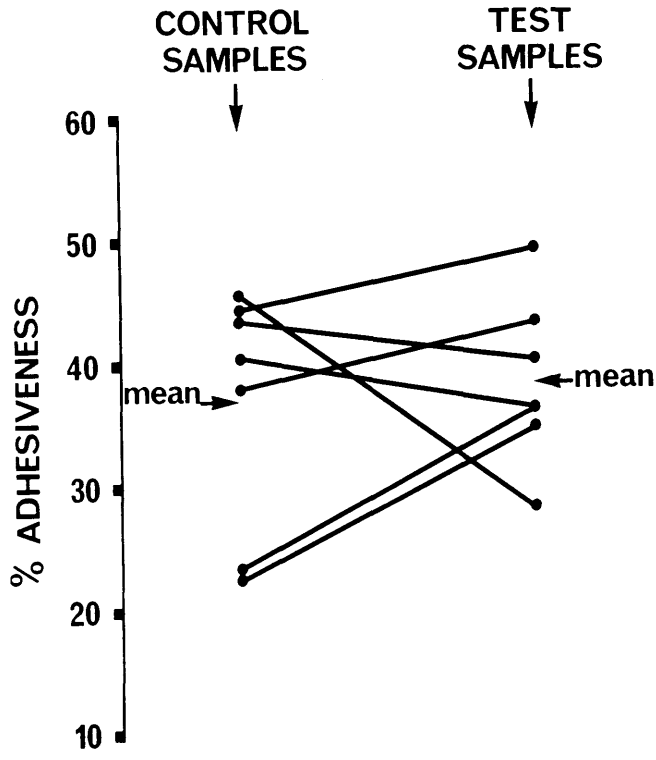

Figure 75 illustrates the effect of fibrinogen (100 µg/ml blood) on platelet adhesiveness in citrated whole blood. There is no significant difference between the results of the control and fibrinogen treated samples; the mean percentage adhesiveness of the control samples is 37.0 ± 9.9 and of the test samples 30.5 ± 6.8 ($t = 0.359$, $0.70 < p < 0.80$). The figure was constructed from the results of table 79.

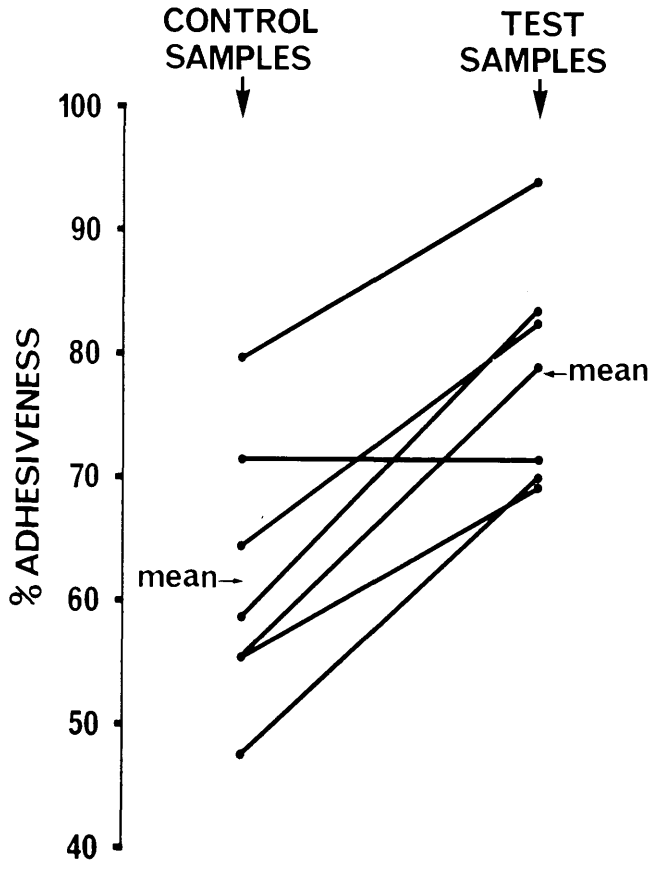

Figure 76 shows the effect of fibrinogen (100 µg/ml blood) on platelet adhesiveness in citrated whole blood after the blood and fibrinogen had been agitated together at 37°C for 10 minutes. Fibrinogen produces a significant increase in platelet adhesiveness; the mean percentage platelet adhesiveness of the control samples is 61.7 ± 11.0 and of the test samples 78.6 ± 9.0 ($t = 5.166$, $0.001 < p < 0.005$). The figure is compiled from the data of table 80.

APPENDIX 2

Tables 26 - 80.

Table 26 The effect of streptokinase on platelet aggregation

Subject No.	Time of platelet aggregation (seconds) Control	Test
1	475	362
2	391	235
3	526	369
4	370	279
5	546	445
6	268	229
7	328	234
8	252	170
9	322	242
10	476	268
11	246	217
Mean	382	276
S.D.	± 110	± 81

Table 26 shows the effect of streptokinase on the rate of formation of platelet aggregates in the Chandler tube. The concentration of streptokinase used was the concentration which produced maximum lytic activity as estimated for each plasma sample. The difference between the results of the control and test loops is statistically significant; $t = 6.602$, $p < 0.001$. The data is presented in figure 5.

Table 27 Effect of trypsin (10 µg/ml) on platelet aggregation

Subject No.	Time of platelet aggregation (seconds)	
	Control	Test
1	306	164
2	390	113
3	471	149
4	537	227
5	508	151
6	351	100
7	636	152
Mean	457	151
S.D.	± 115	± 41

Table 27 shows the effect of trypsin (10 µg/ml plasma) on platelet aggregation in the Chandler tube. The differences between the results in the control and test loops are statistically significant; $t = 7.764$, $p < 0.001$. These results have been used to compile figure 6.

Table 28 Effect of trypsin (1 mg/ml) on platelet aggregation

	Time of platelet aggregation (seconds)	
Subject No.	Control	Test
1	704	86
2	632	40
3	763	32
4	655	52
5	688	44
6	661	61
7	556	28
Mean	± 666	± 49
S.D.	± 64	± 20

Table 28 shows the effect of trypsin (1 mg/ml plasma) on platelet aggregation in the Chandler tube. The differences between the results in the speed of formation of platelet aggregates in the untreated and trypsin treated plasma samples are statistically significant; $t = 26.491$, $p < 0.001$. Figure 7 is compiled from these results.

Table 29 The effect of trypsin on platelet poor plasma

	Time of platelet aggregation (seconds)	
Subject No.	Control	Test
1	328	169
2	347	379
3	348	349
4	395	184
5	980	507
6	630	351
7	503	213
Mean	504	307
S.D.	± 236	± 124

Table 29 shows the effect of incubating trypsin (10 µg/ml plasma) with platelet poor plasma, before the addition of platelet rich plasma, on the rate of formation of platelet aggregates in the Chandler tube. The differences between the control and trypsin treated samples are statistically significant; $t = 2.970$, $0.020 < p < 0.025$. The data is illustrated in figure 8.

Table 30 Effect of trypsin on platelet rich plasma

	Time of platelet aggregation (seconds)	
Subject No.	Control	Test
1	530	135
2	546	434
3	573	117
4	395	140
5	322	99
6	553	250
7	315	283
Mean	462	208
S.D.	± 114	± 122

Table 30 shows the effect of incubating trypsin (10 µg/ml plasma) with platelet rich plasma, before the addition of platelet poor plasma, on platelet aggregation in the Chandler tube. The differences between the rate of formation of platelet aggregates in the control and trypsin treated plasma samples are statistically significant; $t = 4.500$, $0.001 < p < 0.005$. The results are also presented in figure 9.

Table 31 Effect of adenosine diphosphate (ADP) on platelet aggregation

	Time of platelet aggregation (seconds)	
Subject No.	Control	Test
1	576	567
2	776	690
3	508	476
4	697	584
5	629	507
6	998	844
7	657	586
8	499	459
9	692	632
10	517	489
Mean	655	583
S.D.	± 152	± 117

Table 31 shows the effect of ADP (0.5 µg/ml plasma) on the rate of formation of platelet aggregates in the Chandler tube. The difference between the results of the control and test loops is statistically significant; $t = 4.824$, $p < 0.001$. The data is also presented in figure 10.

Table 32 Effect of ADP on platelet aggregation on recalcified and non recalcified plasma

	Time of platelet aggregation (seconds)	
Subject No.	Control	Test
1	48	90
2	97	82
3	70	44
4	229	50
5	36	49
6	65	36
7	51	65
Mean	85	59
S.D.	± 66	± 20

Table 32 shows the effect of ADP (0.5 µg/ml plasma) on platelet aggregation in plasma recalcified with one-tenth plasma volume M/4 $CaCl_2$ and in plasma to which an equivalent volume of 0.9 per cent sodium chloride had been added. There is no significant difference between the speed of formation of platelet aggregates in the control and test loops; $t = 0.938$, $0.30 < p < 0.40$. This data was used to compile figure 11.

Table 33 Effect of adenosine on platelet aggregation

Subject No.	Time of platelet aggregation (seconds)	
	Control	Test
1	303	409
2	421	490
3	360	450
4	406	459
5	366	509
6	475	549
7	306	353
Mean	± 377	± 460
S.D.	± 62	± 65

Table 33 shows the effect of adenosine (100 µg per ml plasma) on platelet aggregation in the Chandler tube. The rate of formation of platelet aggregates is delayed in every plasma sample treated with adenosine. The mean time for platelet aggregation to occur in the control loop is significantly less than that for the adenosine treated loop ; $t = 6.606$, $p < 0.001$. The results are presented in figure 12.

Table 34 Effect of combined action of trypsin and ADP on platelet aggregation

Subject No.	Time of platelet aggregation (seconds)		
	Control	ADP	ADP plus trypsin
1	576	300	53
2	454	415	252
3	636	227	143
4	508	336	82
5	537	407	204
6	421	386	109
7	998	375	117
Mean	590	349	137
S.D.	± 194	± 67	± 70

Table 34 shows the effect of ADP (0.5 µg per ml plasma) and the combined action of ADP, at the same concentration, and trypsin (10 µg per ml plasma) on platelet aggregation in the Chandler tube. ADP alone produces significant acceleration of the rate of formation of platelet aggregates over that of the control loops; $t = 2.970$, $0.020 < p < 0.025$. The combined action of ADP and trypsin causes a significant increase in the rate of formation of platelet aggregation over the rate of the ADP treated plasma samples; $t = 8.194$, $p < 0.001$. These results have been used to compile figure 13.

Table 35 Effect of adenosine (100 µg per ml) on trypsin treated plasma

Subject No.	Time of platelet aggregation (seconds)	
	Control	Test
1	46	169
2	106	333
3	93	79
4	86	136
5	55	92
6	117	98
7	96	227
8	88	53
9	122	115
10	127	138
11	131	124
12	119	166
13	125	107
14	98	101
15	59	70
16	66	213
Mean	96	139
S.D.	± 28	± 71

Table 35 shows the effect of incubating adenosine (100 µg/ml plasma) with plasma treated with trypsin (10 µg/ml plasma) on platelet aggregation in the Chandler tube. The rate of formation of platelet aggregation is retarded in ten of the plasma samples investigated and the differences between the results of the control and test loops are statistically significant; $t = 2.284$, $0.025 < p < 0.050$. Figure 14 is compiled from these results.

Table 36 Effect of adenosine (200 µg per ml) on trypsin treated plasma

	Time of platelet aggregation (seconds)	
Subject No.	Control	Test
1	59	96
2	75	143
3	120	123
4	93	115
5	85	110
6	75	135
7	162	223
Mean	96	135
S.D.	± 35	± 42

Table 36 shows the effect of incubation of adenosine (200 µg per ml) with plasma treated with trypsin (10 µg per ml plasma) on platelet aggregation in the Chandler tube. The differences between the speed of aggregation in the control and test loops are significant; $t = 4.289$, $0.005 < p < 0.010$. The results are illustrated in figure 15.

Table 37 Effect of streptokinase on ADP induced platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.216	0.310
2	0.230	0.515
3	0.078	0.530
4	0.132	0.520
5	0.545	0.560
6	0.580	0.575
7	0.590	0.590
8	0.215	0.218
9	0.521	0.590
10	0.555	0.585
Mean	0.366	0.499
S.D.	± 0.208	± 0.129

Table 37 shows the effect of streptokinase on ADP induced platelet aggregation. The concentration of streptokinase was that concentration which produced maximum lytic activity as determined for each plasma sample. The optical density readings of the control and test samples, $4\frac{1}{2}$ minutes after the addition of ADP (0.5 µg/ml plasma), are shown. The differences between the results of the two samples are statistically significant; $t = 2.413$, $0.025 \; p \; 0.050$. The results are a detailed analysis of one of the points on the curves shown in figure 16.

Table 38 Effect of streptokinase on platelet aggregation in diluted plasma

Subject No.	Optical Density Readings	
	Control	Test
1	0.378	0.350
2	0.245	0.185
3	0.255	0.312
4	0.368	0.378
5	0.368	0.375
6	0.050	0.275
7	0.198	0.210
Mean	0.266	0.298
S.D.	± 0.119	± 0.077

Table 38 shows the effect of streptokinase (100 N.I.H. units/ml plasma) on ADP induced platelet aggregation in plasma diluted with 0.9 per cent sodium chloride in the ratio 1: 2.5. The optical density readings of the control and test samples, 3 minutes after the addition of ADP (1.0 μg per ml plasma), are shown. There is no significant difference between the results of the control and streptokinase treated samples; $t = 1.122$, $0.30 < p < 0.40$. These results were used to compile a section of figure 17.

Table 39 Effect of streptokinase on platelet aggregation in acid - alkali treated plasma

Subject No.	Optical Density Readings	
	Control	Test
1	0.537	0.318
2	0.580	0.370
3	0.368	0.430
4	0.580	0.520
5	0.579	0.600
6	0.431	0.460
7	0.580	0.549
8	0.541	0.572
9	0.522	0.090
10	0.369	0.362
11	0.310	0.350
12	0.560	0.445
13	0.550	0.472
14	0.585	0.530
15	0.501	0.489
16	0.592	0.498
17	0.291	0.251
Mean	0.499	0.430
S.D.	± 0.066	± 0.129

Table 39 shows the effect of streptokinase (2,000 N.I.H. units/ml plasma) on ADP induced platelet aggregation in which the antiplasmin has been destroyed by acid - alkali treatment. The optical density readings of the control and test samples, 10 minutes after the addition of ADP (0.5 µg/ml plasma), are shown. The differences between the results of the control and streptokinase treated samples are significant; $t = 2.574$, $0.020 < p < 0.025$. These results were used to compile a section of figure 18.

Table 40 Effect of streptokinase on nor-adrenaline induced platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0	0
2	0.457	0.492
3	0.251	0.240
4	0.515	0.520
5	0.600	0.600
6	0.459	0.500
7	0.558	0.248
8	0.560	0.520
9	0	0
10	0.314	0.385
Mean	0.371	0.351
S.D.	± 0.224	± 0.219

Table 40 shows the effect of streptokinase (100 N.I.H. units/ml plasma) on nor-adrenaline induced platelet aggregation. The optical density readings of the control and test plasma samples, 10 minutes after the addition of nor-adrenaline (1.0 µg/ml plasma), are shown. Streptokinase has no significant effect on platelet aggregation in this system; $t = 0.623$, $0.50 < p < 0.60$. These results were used in the determination of the mean values of the control and test plasma samples shown in figure 19.

Table 41 Effect of trypsin (10 µg/ml) on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.480	0.505
2	0.578	0.560
3	0.540	0.535
4	0.525	0.580
5	0.520	0.560
6	0.485	0.525
7	0.378	0.525
8	0.480	0.490
9	0.578	0.590
10	0.565	0.570
Mean	0.513	0.544
S.D.	± 0.061	± 0.033

Table 41 shows the effect of incubating trypsin (10 µg/ml plasma) with platelet rich plasma on ADP induced platelet aggregation. The optical density readings of the control and test plasma samples, 1 minute after the addition of ADP (0.5 µg/ml plasma), are shown. There is no significant difference between the results of the control and trypsin treated samples; $t = 2.119$, $0.05 < p < 0.10$. These results were used to produce part of figure 20.

Table 42 Effect of trypsin (100 µg/ml) on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.518	0.508
2	0.575	0.560
3	0.546	0.532
4	0.465	0.450
5	0.495	0.470
6	0.550	0.475
7	0.475	0.468
8	0.525	0.460
9	0.575	0.548
10	0.570	0.535
Mean	0.529	0.501
S.D.	± 0.041	± 0.041

Table 42 shows the effect of incubating platelet rich plasma with trypsin (100 µg/ml plasma) on ADP induced platelet aggregation. The optical density reading of the control and test samples, 1½ minutes after the addition of ADP (0.5 µg/ml plasma), are shown. The differences between the results of the control and trypsin treated samples are significant; $t = 3.925$, $0.001 < p < 0.005$. A section of figure 20 is compiled from these results.

Table 43 Effect of trypsin (1 mg/ml) on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.478	0.480
2	0.578	0
3	0.580	0.088
4	0.538	0.490
5	0.560	0.082
6	0.555	0.318
7	0.490	0.385
Mean	0.540	0.263
S.D.	± 0.041	± 0.204

Table 43 shows the effect of trypsin (1 mg/ml plasma) on platelet aggregation. The optical density readings of the control and test samples, 2 minutes after the addition of 0.9% sodium chloride to the control samples and trypsin to the test samples, are shown. The differences between the control and trypsin treated samples are significant; $t = 3.080$, $0.020 < p < 0.025$. These results were used in the compilation of a section of figure 21.

Table 44 Effect of trypsin on platelet aggregation in fibrinogen depleted plasma

Subject No.	Optical Density Readings	
	Control	Test
1	0.522	0.322
2	0.515	0.412
3	0.518	0.260
4	0.530	0.390
5	0.521	0.280
6	0.540	0.350
7	0.530	0.400
Mean	0.525	0.345
S.D.	± 0.009	± 0.060

Table 44 shows the effect of trypsin (1 mg/ml plasma) on platelet aggregation in plasma pretreated with streptokinase (1,000 N.I.H. units/ml plasma) at 37°C for 10 minutes. The optical density readings of the control and test plasma samples, 30 seconds after the addition of 0.9% sodium chloride to the control samples and trypsin to the test samples, are shown. The differences between the results of the control and trypsin treated samples are significant; $t = 6.485$, $p < 0.001$. A portion of figure 22 is compiled from these results.

Table 45 Effect of trypsin on platelet aggregation in diluted plasma

Subject No.	Optical Density Readings	
	Control	Test
1	0.364	0.386
2	0.235	0.220
3	0.234	0.350
4	0.355	0.322
5	0.360	0.360
6	0.040	0.092
7	0.192	0.180
Mean	0.254	0.273
S.D.	± 0.118	± 0.110

Table 45 shows the effect of trypsin (10 µg/ml plasma) on ADP induced platelet aggregation in plasma diluted with 0.9% sodium chloride in the ration 1: 2.5. The optical density readings of the control and test samples, 4 minutes after the addition of ADP (1.0 µg/ml plasma), are shown. The differences in the results of the control and trypsin treated samples are not significant; $t = 0.965$, $0.30 < p < 0.40$. This data was used to compile a section of figure 17.

Table 46 Effect of calcium chloride (0.25 m mole/ml) on the action of trypsin on platelet aggregation

	Optical Density Readings	
Subject No.	Control	Test
1	0.208	0.155
2	0.315	0.325
3	0.345	0.270
4	0.425	0.445
5	0.530	0.330
6	0.290	0.280
7	0.490	0.512
Mean	0.372	0.331
S.D.	± 0.115	± 0.118

Table 46 shows the effect of incubating trypsin (10 µg/ml plasma) with platelet rich plasma, to which calcium chloride (0.25 m mole/ml plasma) had been added, on ADP induced platelet aggregation. The optical density readings of the control and test plasma samples, 1 minute after the addition of ADP (0.5 µg/ml plasma), are shown. The differences between the readings in the control and trypsin treated samples are not significant; $t = 1.363$, $0.20 < p < 0.30$. These results were used to compile a section of figure 23.

Table 47 Effect of calcium chloride (1.0 m mole/ml) on the action of trypsin on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.080	0.142
2	0.440	0.535
3	0.558	0.539
4	0.450	0.532
5	0.100	0.412
6	0.491	0.520
7	0.480	0.439
Mean	0.371	0.446
S.D.	± 0.196	± 0.143

Table 47 shows the effect of incubation of trypsin (10 µg/ml plasma) with plasma, to which calcium chloride (1.0 m mole/ml plasma) had been added, on ADP induced aggregation. The optical density readings of the control and test samples, 3 minutes after the addition of ADP (0.5 µg/ml plasma), are shown. There is no significant difference between the results of the control and trypsin treated samples; $t = 1.690$, $0.10 < p < 0.20$. A portion of figure 24 was compiled from these results.

Table 48 Effect of trypsin on platelet poor plasma

Subject No.	Optical Density Readings	
	Control	Test
1	0.520	0.563
2	0.579	0.519
3	0.579	0.472
4	0.562	0.540
5	0.266	0.217
6	0.542	0.490
7	0.310	0.372
Mean	0.480	0.453
S.D.	± 0.133	± 0.121

Table 48 shows the effect of incubating platelet poor plasma with trypsin (100 µg/ml plasma), before the addition of platelet rich plasma, on ADP induced platelet aggregation. The optical density readings of the control and test samples, 10 minutes after the addition of ADP (0.5 µg/ml plasma), are shown. The differences between the results of the control and trypsin treated samples are not statistically significant; $t = 1.170$, $0.20 < p < 0.30$. These results were used to construct a section of figure 25.

Table 49 Effect of trypsin on platelet rich plasma

Subject No.	Optical Density Readings	
	Control	Test
1	0.560	0.541
2	0.330	0.339
3	0.560	0.540
4	0.550	0.450
5	0.562	0.518
6	0.346	0.319
7	0.480	0.432
Mean	0.484	0.448
S.D.	± 0.104	± 0.083

Table 49 shows the effect of incubating platelet rich plasma with trypsin (100 µg/ml plasma), before the addition of platelet poor plasma, on ADP induced platelet aggregation. The optical density readings of the control and test samples, 10 minutes after the addition of ADP (0.5 µg/ml plasma), are shown. There is a significant difference between the results of the control and trypsin treated plasma samples; $t = 2.768$, $0.025 < p < 0.050$. A part of figure 26 is compiled from these results.

Table 50 Effect of trypsin on washed platelets

Subject No.	Optical Density Readings	
	Control	Test
1	0.460	0.480
2	0.540	0.515
3	0.390	0.522
4	0.522	0.490
5	0.535	0.540
6	0.520	0.519
7	0.556	0.548
Mean	0.503	0.516
S.D.	± 0.058	± 0.025

Table 50 shows the effect of trypsin (10 µg/ml suspension) on ADP induced platelet aggregation of washed platelets suspended in 0.1 M tris buffer, pH 7.5. The optical density readings of the control and test samples, 10 minutes after the addition of ADP (0.5 µg/ml suspension), are shown. The differences between the results of the control and trypsin treated samples are not significant; $t = 0.291$, $0.70 < p < 0.80$. A section of figure 27 was compiled from these results.

Table 51 Effect of adenosine on trypsin treated plasma

	Optical Density Readings	
Subject No.	Control	Test
1	0.521	0.578
2	0.412	0.580
3	0.468	0.595
4	0.548	0.578
5	0.535	0.580
6	0.522	0.480
7	0.432	0.580
Mean	0.491	0.567
S.D.	± 0.054	± 0.039

Table 51 shows the effect of adenosine (100 µg/ml plasma) on ADP induced platelet aggregation of platelet rich plasma treated with trypsin (100 µg/ml plasma). The optical density readings of the control and test samples, one minute after the addition of ADP (0.5 µg/ml plasma) are shown. The differences between the results of the control and adenosine treated samples are significant; $t = 2.724$, $0.025 < p < 0.050$. These results were used to construct a portion of figure 31.

Table 52 Effect of adenosine (0.5 mg/ml) on trypsin treated plasma

Subject No.	Optical Density Readings	
	Control	Test
1	0.435	0.405
2	0	0.270
3	0.010	0.350
4	0.290	0.280
5	0	0.358
6	0.155	0.138
7	0.346	0.320
Mean	0.177	0.303
S.D.	± 0.182	± 0.086

Table 52 shows the effect of adenosine (0.5 mg/ml plasma) on platelet aggregation in platelet rich plasma to which trypsin (1 mg/ml plasma) had been added. Trypsin alone was added to the control samples and trypsin and adenosine were added simultaneously to the test samples. The optical density readings, 4 minutes after the additions were made to each sample, are shown. There is no significant difference between the results of the control samples and the samples to which adenosine had been added; $t = 1.818$, $0.10 < p < 0.20$. A section of figure 22 was compiled from these results.

Table 53 Effect of trypsin on nor-adrenaline induced platelet aggregation

	Optical Density Readings	
Subject No.	Control	Test
1	0.215	0
2	0.420	0.410
3	0.538	0.425
4	0.560	0.540
5	0.275	0.458
6	0.560	0.522
7	0.540	0.552
8	0.582	0.545
9	0.260	0.025
10	0.450	0.470
Mean	0.440	0.395
S.D.	±0.141	± 0.207

Table 53 shows the effect of trypsin (10 µg/ml plasma) on nor-adrenaline induced platelet aggregation. The optical density readings of the control and test samples, 2 minutes after the addition of nor-adrenaline (1.0 µg/ml plasma), are shown. The differences between the results of the control and trypsin treated samples are not significant; $t = 1.691$, $0.10 < p < 0.20$. These results were used to construct a portion of figure 32.

Table 54 Effect of urokinase on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.225	0.355
2	0	0.570
3	0.174	0.370
4	0.560	0.560
5	0.570	0.558
6	0.580	0.595
7	0.235	0.240
8	0.560	0.600
9	0.550	0.565
10	0.548	0.580
Mean	0.400	0.499
S.D.	± 0.264	± 0.128

Table 54 shows the effect of urokinase on ADP induced platelet aggregation. The concentration of urokinase was the concentration which produced maximum lytic activity as determined for each plasma sample. The optical density readings of the control and test samples, 3 minutes after the addition of ADP (0.5 µg/ml plasma), are shown. The differences between the results of the control and urokinase treated samples are statistically significant; $t = 3.134$, $0.01 < p < 0.02$. These results were used to compile a section of figure 33.

Table 55 Effect of chymotrypsin on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.252	0.226
2	0.253	0.179
3	0.200	0.312
4	0.400	0.422
5	0.520	0.466
6	0.302	0.455
7	0.620	0.582
Mean	0.364	0.377
S.D.	± 0.156	± 0.144

Table 55 shows the effect of chymotrypsin (100 µg/ml plasma) on ADP induced platelet aggregation. The optical density readings of the control and test samples, 5 minutes after the addition of ADP (0.5 µg/ml plasma), are shown. There is no significant difference between the results of the control and chymotrypsin treated samples; $t = 0.412$, $0.60 < p < 0.70$. A section of figure 34 is compiled from these results.

Table 56 Effect of ficin on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.472	0.389
2	0.485	0.388
3	0.252	0.238
4	0.253	0.158
5	0.200	0.272
6	0.400	0.445
7	0.520	0.480
8	0.302	0.434
9	0.602	0.620
10	0.225	0.256
11	0.490	0.315
12	0.096	0.024
Mean	0.360	0.335
S.D.	± 0.160	± 0.165

Table 56 shows the effect of ficin (10 µg/ml plasma) on ADP induced platelet aggregation. The optical density readings of the control and test samples, 5 minutes after the addition of ADP (0.5 µg/ml plasma) are shown. The differences between the results of the control and ficin treated samples are not significant; $t = 0.996$, $0.30 < p < 0.40$. The results were used to construct a section of figure 35.

Table 57 Effect of heparin (10 units/ml) on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.196	0.122
2	0.410	0.132
3	0.160	0.110
4	0	0
5	0.310	0.284
6	0	0
7	0.182	0.060
8	0.311	0.062
Mean	0.196	0.096
S.D.	± 0.146	± 0.091

Table 57 shows the effect of heparin (10 units/ml plasma) on ADP induced platelet aggregation. The optical density readings of the control and test samples, 4 minutes after the addition of ADP (0.5 μg/ml plasma) are shown. The differences between the results of the control and heparin treated samples are significant; $t = 2.594$, $0.025 < p < 0.020$. A section of figure 36 was compiled from these results.

Table 58 Effect of heparin (0.05 units/ml) on platelet aggregation

	Optical Density Readings	
Subject No.	Control	Test
1	0.210	0.192
2	0.558	0.540
3	0.502	0.512
4	0.582	0.488
5	0.581	0.512
6	0.480	0.280
7	0.590	0.522
8	0.522	0.493
9	0.588	0.409
10	0.190	0.202
11	0.530	0.410
12	0.240	0.212
13	0.524	0.488
14	0	0.005
Mean	0.436	0.376
S.D.	± 0.139	± 0.167

Table 58 shows the effect of heparin (0.05 units/ml plasma) on ADP induced platelet aggregation. The optical density readings of the control and test samples, 10 minutes after the addition of ADP (0.5 µg/ml plasma), are shown. The differences between the results of the control and heparin treated samples are significant; $t = 3.297$, $0.005 < p < 0.010$. A portion of figure 37 was compiled from these results.

Table 59 Effect of heparin (500 units/ml) on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.581	0.640
2	0.480	0.592
3	0.590	0.600
4	0.188	0.490
5	0.522	0.538
6	0.588	0.618
7	0.190	0.290
8	0.240	0.298
Mean	0.422	0.508
S.D.	± 0.184	± 0.140

Table 59 shows the effect of heparin (500 units/ml plasma) on ADP induced platelet aggregation. The optical density readings of the control and test samples, 10 minutes after the addition of ADP (0.5 ug/ml plasma), are shown. The differences between the results of the control and heparin treated samples are significant; $t = 2.563$, $0.025 < p < 0.050$. These results were used to compile a part of figure 38.

Table 60 Effect of heparin on washed platelets

Subject No.	Optical Density Readings	
	Control	Test
1	0.460	0.516
2	0.540	0.540
3	0.390	0.410
4	0.522	0.500
5	0.535	0.500
6	0.520	0.540
7	0.556	0.578
Mean	0.503	0.512
S.D.	± 0.058	± 0.053

Table 60 shows the effect of heparin (10 units/ml suspension) on ADP induced aggregation of washed platelets suspended in 0.1 M tris buffer, pH 7.5. The optical density readings of the control and test samples, 10 minutes after the addition of ADP (0.5 µg/ml suspension), are shown. There is no significant difference between the results of the control and heparin treated samples; $t = 0.756$, $0.40 < p < 0.50$. These results were used to compile a section of figure 27.

Table 61 Effect of streptokinase on platelet adhesiveness

Subject No.	Percentage platelet adhesiveness	
	Control	Test
1	40.5	57.8
2	26.3	7.1
3	60.1	14.9
4	90.1	0
5	84.2	22.8
6	36.3	13.3
7	26.3	20.1
8	19.7	13.0
Mean	47.9	21.3
S.D.	± 27.2	± 17.4

Table 61 shows the effect of streptokinase on platelet adhesiveness in citrated whole blood. The concentration of streptokinase was the concentration which produced maximum lytic activity as estimated for each sample. The difference between the results of the control and streptokinase treated samples is significant; $t = 2.402$, $0.025 < p < 0.050$. These results were used to compile figure 39.

Table 62 Effect of streptokinase on platelet adhesiveness

Subject No.	Percentage platelet adhesiveness	
	Control	Test
1	53.1	48.9
2	37.2	37.2
3	65.8	84.9
4	80.1	89.7
5	46.9	85.3
6	78.0	78.9
7	48.9	60.0
8	20.0	35.5
9	31.3	32.5
10	59.1	86.3
Mean	52.0	63.9
S.D.	± 19.5	± 23.7

Table 62 shows the effect of streptokinase on platelet adhesiveness of citrated whole blood after agitating the blood and streptokinase together at 37°C for 10 minutes. The concentration of streptokinase was the concentrations which produced maximum lytic activity as determined for each sample. The difference between the results of the control and test samples is significant; $t = 2.780$, $0.020 < p < 0.025$. Figure 40 is compiled from these results.

Table 63 Effect of trypsin on platelet adhesiveness

	Percentage platelet adhesiveness	
Subject No.	Control	Test
1	93.1	76.8
2	67.6	90.7
3	89.3	78.6
4	37.3	62.1
5	5.6	73.8
6	15.2	39.7
7	24.6	61.6
8	48.4	35.7
9	35.6	41.8
10	67.2	54.5
Mean	48.4	61.5
S.D.	± 30.1	± 18.6

Table 63 shows the effect of trypsin (10 µg/ml blood) on platelet adhesiveness in citrated whole blood. There is no significant difference between the results of the control and trypsin treated samples; $t = 1.513$, $0.10 < p < 0.20$. These results were used to construct figure 41.

Table 64 Effect of trypsin on platelet adhesiveness

Subject No.	Percentage platelet adhesiveness	
	Control	Test
1	70.0	78.0
2	80.4	78.7
3	49.4	62.2
4	58.4	79.7
5	43.4	72.7
6	52.5	56.2
7	57.0	62.2
8	60.2	67.6
Mean	58.9	69.7
S.D.	± 11.7	± 8.9

Table 64 shows the effect of trypsin (10 ug/ml blood) on platelet adhesiveness in citrated whole blood after the blood and trypsin had been agitated together at 37°C for 10 minutes. The difference between the results of the control and trypsin treated samples is significant; $t = 3.011$, $0.01 < p < 0.02$. The results were used to compile figure 42.

Table 65 Effect of trypsin on platelet adhesiveness

Subject No.	Percentage platelet adhesiveness	
	Control	Test
1	13.2	98.6
2	29.8	89.5
3	25.9	25.9
4	0	3.2
5	0	18.2
6	0	45.6
7	5.4	26.7
Mean	10.6	44.0
S.D.	± 12.7	± 36.6

Table 65 shows the effect of trypsin (10 µg/ml plasma) on platelet adhesiveness in platelet rich plasma. The differences between the results of the control and trypsin treated samples are significant; $t = 2.801$, $0.025 < p < 0.050$. The results are also presented in figure 43.

Table 66 Effect of product 'a' on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.480	0.480
2	0.310	0.272
3	0.535	0.532
4	0.380	0.405
5	0.470	0.460
6	0.576	0.576
7	0.442	0.370
Mean	0.456	0.442
S.D.	± 0.090	± 0.103

Shown is the effect of product 'a' (100 µg/ml plasma) on ADP induced platelet aggregation in platelet rich plasma. The optical density readings of the control and test samples 1 minute after the addition of ADP (0.5 µg/ml plasma) are shown. Product 'a' has no significant effect on platelet aggregation ($t = 1.172$, $0.20 < p < 0.30$). These results were used to construct a section of figure 64.

Table 67 Effect of product 'a' on platelet adhesiveness

	Percentage platelet adhesiveness	
Subject No.	Control	Test
1	40.5	36.5
2	58.1	33.7
3	46.4	49.0
4	68.8	59.1
5	43.9	21.2
6	22.3	10.3
7	40.3	42.1
8	15.0	16.3
Mean	41.9	33.5
S.D. ±	17.4 ±	16.7

Shown is the effect of product 'a' (100 µg/ml blood) on platelet adhesiveness in citrated whole blood. Product 'a' has no significant effect on platelet adhesiveness ($t = 2.149$, $0.05 < p < 0.10$). Figure 65 is compiled from these results.

Table 68 Effect of product 'a' (200 µg/ml) on platelet adhesiveness

Subject No.	Percentage platelet adhesiveness	
	Control	Test
1.	24.6	28.9
2	56.5	55.8
3	36.2	27.1
4	65.0	62.2
5	40.5	31.8
6	58.1	42.0
7	46.4	65.4
8	68.8	61.9
9	43.9	23.8
10	22.3	26.9
11	15.0	21.1
Mean	43.4	40.6
S.D.	± 17.8	± 17.4

Shown is the effect of product 'a' (200 µg/ml blood) on platelet adhesiveness in citrated whole blood. There is no significant difference between the results of the control samples and the samples treated with product 'a', ($t = 0.826$, $0.40 < p < 0.50$). These results were used to compile figure 66.

Table 69 Effect of product 'a' (100 µg/ml) on platelet adhesiveness

Subject No.	Percentage platelet adhesiveness	
	Control	Test
1	80.1	73.4
2	63.8	64.7
3	76.2	74.5
4	82.7	89.3
5	55.3	48.6
6	61.3	54.3
7	87.9	87.9
Mean	72.5	70.4
S.D.	± 12.3	± 15.6

Shown is the effect of product 'a' (100 µg/ml blood) on platelet adhesiveness in citrated whole blood after blood and product 'a' had been agitated together at 37°C for 10 minutes. The differences between the results of the control and product 'a' treated samples are not significant ($t = 1.084$, $0.30 < p < 0.40$). Figure 67 was compiled from these results.

Table 70 Effect of product 'b' on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.331	0.268
2	0.256	0.250
3	0.360	0.300
4	0.590	0.582
5	0.430	0.415
6	0.500	0.420
7	0.182	0.155
Mean	0.378	0.341
S.D.	± 0.140	± 0.141

Shown is the effect of product 'b' (100 µg/ml plasma) on ADP induced platelet aggregation of platelet rich plasma. The optical density readings of the control and test samples, $1\frac{1}{2}$ minutes after the addition of ADP (0.5 µg/ml plasma), are shown. Product 'b' causes significant enhancement of platelet aggregation; $t = 3.253$, $0.01 < p < 0.02$. These results were used to compile a section of figure 68.

Table 71 Effect of product 'b' on platelet adhesiveness

	Percentage platelet adhesiveness	
Subject No.	Control	Test
1	40.5	35.1
2	58.1	51.2
3	46.4	66.0
4	68.8	54.5
5	43.9	32.3
6	22.3	30.9
7	40.3	37.7
8	15.0	8.8
Mean	41.9	39.6
S.D.	± 17.4	± 17.6

Shown is the effect of product 'b' (100 µg/ml blood) on platelet adhesiveness in citrated whole blood. Product 'b' has no significant effect on platelet adhesiveness ($t = 0.594$, $0.50 < p < 0.60$). Figure 69 is constructed from these results.

Table 72 Effect of product 'b' (200 µg/ml) on platelet adhesiveness

Subject No.	Percentage platelet adhesiveness	
	Control	Test
1	24.6	56.1
2	56.5	29.2
3	36.2	36.7
4	65.0	62.9
5	40.5	41.9
6	58.1	23.4
7	46.4	54.2
8	68.8	36.4
9	43.9	32.3
10	22.3	25.1
11	15.0	25.2
Mean	43.4	38.5
S.D. ±	17.8	± 13.7

Shown is the effect of product 'b' (200 µg/ml blood) on platelet adhesiveness of citrated whole blood. Product 'b' has no significant effect on platelet adhesiveness ($t = 1.251$, $0.20 < p < 0.30$). These results were used to construct figure 70.

Table 73 Effect of product 'b' (100 µg/ml) on platelet adhesiveness

	Percentage platelet adhesiveness	
Subject No.	Control	Test
1	80.1	88.6
2	63.8	74.9
3	76.2	80.2
4	82.7	83.0
5	55.3	62.6
6	61.3	62.4
7	87.9	91.8
Mean	72.5	77.6
S.D.	± 12.3	± 11.7

Shown is the effect of product 'b' (100 µg/ml blood) on platelet adhesiveness in citrated whole blood after the blood and product 'b' had been agitated together at 37°C for 10 minutes. The differences between the results of the control and product 'b' treated samples are significant; $t = 3.456$, $0.01 < p < 0.02$. Figure 71 is compiled from these results.

Table 74 Effect of product 'c' on platelet aggregation

	Optical Density Readings	
Subject No.	Control	Test
1	0.430	0.482
2	0.252	0.285
3	0.559	0.570
4	0.326	0.390
5	0.445	0.470
6	0.561	0.560
7	0.542	0.555
Mean	0.445	0.473
S.D.	± 0.121	± 0.105

Shown is the effect of product 'c' (100 µg/ml plasma) on ADP induced aggregation in platelet rich plasma. The optical density readings of the control and test samples, 2 minutes after the addition of ADP (0.5 µg/ml plasma), are shown. Product 'c' results in significant inhibition of platelet aggregation ($t = 3.233$, $0.01 < p < 0.02$). A section of figure 64 was constructed from these results.

Table 75 Effect of product 'd' on platelet aggregation

	Optical Density Readings	
Subject No.	Control	Test
1	0.030	0
2	0.325	0
3	0.272	0
4	0.292	0.272
5	0.290	0.240
6	0.130	0.133
7	0.550	0.430
8	0.480	0.463
9	0.452	0.425
10	0.228	0.200
Mean	0.305	0.216
S.D.	± 0.159	± 0.112

Shown is the effect of product 'd' (100 µg/ml plasma) on ADP induced platelet aggregation in platelet rich plasma. The optical density readings of the control and test samples, $1\frac{1}{2}$ minutes after the addition of ADP (0.5 µg/ml plasma), are shown. Platelet aggregation is enhanced in the presence of product 'd' ($t = 2.415$, $0.025 < p < 0.050$). A section of figure 72 was compiled from these results.

Table 76 Effect of product 'd' on platelet aggregation in the Chandler tube

Subject No.	Time of platelet aggregation (seconds) Control	Test
1	533	303
2	418	286
3	270	242
4	556	289
5	255	237
6	678	356
7	629	342
Mean	477	294
S.D.	± 168	± 45

Shown is the effect of product 'd' (180 µg/ml plasma) on the rate of formation of platelet aggregates in the Chandler tube. The speed of platelet aggregation is accelerated in the samples treated with product 'd' ($t = 3.874$, $0.005 < p < 0.010$). Figure 73 is compiled from these results.

Table 77 Effect of fibrinogen on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.520	0.480
2	0.482	0.302
3	0.427	0.405
4	0.272	0
5	0.180	0.105
6	0.408	0.130
7	0.550	0.500
Mean	0.406	0.275
S.D.	± 0.135	± 0.198

Shown is the effect of fibrinogen (100 µg/ml plasma) on ADP induced platelet aggregation in platelet rich plasma. The optical density readings of the control and test samples, $1\frac{1}{2}$ minutes after the addition of ADP (0.5 µg/ml plasma), are shown. Fibrinogen produces significant enhancement of platelet aggregation ($t = 3.126$, $0.020 < p < 0.025$). A section of figure 74 is compiled from these results.

Table 78 Effect of 'purified' fibrinogen on platelet aggregation

Subject No.	Optical Density Readings	
	Control	Test
1	0.520	0.451
2	0.482	0.170
3	0.427	0.361
4	0.272	0.280
5	0.180	0.160
6	0.408	0.295
7	0.550	0.470
Mean	0.406	0.312
S.D.	± 0.135	± 0.123

Shown is the effect of 'purified' fibrinogen (100 µg/ml plasma) on ADP induced platelet aggregation in platelet rich plasma. The fibrinogen was 'purified' by passage through a DEAE cellulose column. The optical density readings of the control and test samples, $1\frac{1}{2}$ minutes after the addition of ADP (0.5 µg/ml plasma), are shown. The differences between the results of the control and test samples are not significant ($t = 2.361$, $0.05 < p < 0.10$). These results were used to compile a section of figure 74.

Table 79 Effect of fibrinogen on platelet adhesiveness

Subject No.	Percentage platelet adhesiveness	
	Control	Test
1	44.4	49.5
2	38.0	43.3
3	40.7	36.6
4	45.9	28.1
5	43.8	40.3
6	22.9	35.0
7	23.2	36.4
Mean	37.0	38.5
S.D.	± 9.9	± 6.8

Shown is the effect of fibrinogen (100 µg/ml blood) on platelet adhesiveness in citrated whole blood. The difference between the control and the fibrinogen treated samples is not significant ($t = 0.359$, $0.70 < p < 0.80$). Figure 75 was constructed from these results.

Table 80 Effect of fibrinogen (100 µg/ml) on platelet adhesiveness

Subject No.	Percentage platelet adhesiveness	
	Control	Test
1	71.3	71.3
2	58.8	83.6
3	47.5	70.1
4	64.3	82.7
5	79.8	94.0
6	55.2	69.4
7	55.2	79.0
Mean	61.7	78.6
S.D.	± 11.0	± 9.0

Shown is the effect of fibrinogen (100 µg/ml blood) on platelet adhesiveness in citrated whole blood after the blood and fibrinogen had been agitated together at 37°C for 10 minutes. The difference between the results of the control and fibrinogen treated samples is significant ($t = 5.166$, $0.001 < p < 0.005$). Figure 76 is compiled from these results.