STUDIES WITH PLASMINOGEN ACTIVATORS

by

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Thesis submitted for the degree of

Doctor of Philosophy, University of Glasgow.

Volume 1.

Amended

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The thesis begins with a brief historical review of the development of ideas in fibrinolysis, and an account of current concepts of the components and functions of the plasminogen-plasmin system. There follows an account of the laboratory methods used in the thesis, including the euglobulin lysis and fibrin plate tests for plasminogen activator, plasminogen and fibrinogen assays, and as an index of the fibrinolytic coagulation defect, the thrombin clotting time. A description is given of a simple original method of preparing fibrinogen tagged with radioactive iodine, and of the electrophoretic and chromatographic properties of this tagged fibrinogen. Its use in a modified plasminogen-enriched radioactive clot/

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The findings suggest that in vivo thrombolytic agents might cause breakdown of thrombi into platelet emboli, which would represent an obvious hazard in the case of coronary and cerebral thrombosis. Thrombi made from hyperlipidaemic blood were markedly resistant to lysis, an observation supporting the view that hyperlipidaemia may predispose to thrombotic vascular occlusion.

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PREFACE.

The experiments presented in this thesis were carried out in the University Department of Medicine, Glasgow Royal Infirmary, in the laboratory of Professor A. S. Douglas. The work was performed while I was in turn a registrar, then an honorary senior registrar supported by an individual research grant from the Medical Research Council, and finally a lecturer in medicine. Colleagues who collaborated in the studies included Professor Douglas, Mr. W. H. Bain, Miss B. C. Bayley, Mr. M. K. Browne, Miss W. E. Clement, Miss S. B. Gale, Mr. W. Reid, Dr. B. M. Rifkind and Dr. F. Walker. Much of the data has already been published, or has been accepted for publication, as shown below:

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CONTENTS.

VOLUME 1.

CHAPTER 1.

INTRODUCTION.

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Thrombolytic therapy, which may be defined as the use of plasminogen activators or other fibrinolytic substances to induce the lysis of preformed thrombi in vivo, has obvious conceptual attractions in the management of thrombo-embolic vascular occlusion compared with conventional anticoagulant drugs; at best anticoagulants can only prevent extension of thrombosis and restoration of vascular patency depends on relatively/

relatively slow natural processes, whereas with thrombolytic therapy speedy removal of thrombi might be able to restore vascular patency quickly before distal tissue necrosis has taken place.

In view of the encouraging results which have been obtained with thrombolytic enzymes in animal and human experiments, and from individual well-studied patients with vascular occlusion, intensive efforts are needed to place this approach to treatment on a basis from which large-scale, well designed clinical trials can be carried out. With this object in view, a long-term programme of investigation of the problems involved in thrombolytic therapy is being carried out in the University Department of Medicine, Glasgow Royal Infirmary, and the studies presented in this thesis were performed as part of this programme.

The thesis begins with a brief historical review of the development of ideas in fibrinolysis and an account of current concepts of the components and functions of the plasminogen-plasmin system. There follows an account of/

of the appraisal and development of laboratory methods used in this thesis in the study of thrombolytic activity; this in turn is followed by chapters on the biochemical effects and advantages and disadvantages of a variety of plasminogen activators. Finally, a report is given of certain factors, particularly platelet and lipid content, which influence the patterns of response of artificial thrombi to plasminogen activator in vitro, and which may have a bearing on the thrombolytic process in vivo.

Plan of the thesis.

The thesis is presented in two volumes; volume 1 contains the text and references, and volume 2, figures and tables. The tables are in two parts; those in appendix 2 are those to which reference is made in the text, and those in appendix 3 are detailed tables from which, as indicated in the legends, the figures were compiled.

CHAPTER 2.

THE PLASMINOGEN-PLASMIN SYSTEM.

Historical Introduction.

It has been known for more than a century that under certain circumstances clots in shed blood may undergo spontaneous lysis, but it is only in the past 10 or 15 years that detailed knowledge of the enzyme system responsible for such fibrinolytic activity, the plasminogen-plasmin system has accumulated.

The first observations on the solution of fibrin clots appear to have been made in the middle of the 19th century by Denis (1838) and Zimmerman (1846) who found that fibrin from human and bovine blood dissolved under certain circumstances. Dastre (1893) suggested that this phenomenon was not solution in the true sense but was due to digestion of the fibrin, a process for which he suggested the term 'fibrinolysis'. Denys and de Marbaix (1889) showed that proteolytic activity could be found in serum after treatment with chloroform. Delezenne and Pozerski (1903) observed that/

that chloroform-induced proteolytic activity disappeared if untreated serum was added and suggested that the action of chloroform was to remove or destroy inhibitors of proteolytic activity. Hedin (1904) showed the presence of a proteolytic enzyme in the globulin fraction of human serum which itself had no demonstrable proteolytic activity; the albumin fraction obtained from serum on ammonium sulphate fractionation inhibited the proteolytic activity of the globulins. In 1946 Christensen confirmed the effect of chloroform on the inhibitors of proteolysis and presented evidence that chloroform activation of serum is an autocatalytic activation of a proenzyme.

A major advance in understanding of plasma fibrinolytic activity, from which most subsequent knowledge has arisen, was the observation of Tillett and Garner (1933) that the culture filtrate of certain strains of haemolytic streptococci contained a factor which caused rapid lysis of fibrin from human plasma. Milstone (1941) showed that purified human fibrin was resistant to the streptococcal product, but if a small amount of the euglobulin fraction of human serum was added,

lysis/

lysis ensued rapidly. Kaplan (1944) and Christensen (1945) showed that the streptococcal factor and the euglobulin fraction of serum when mixed together gave rise to a proteolytic enzyme; this was produced by conversion to an active form of a normally inert enzyme precursor in the globulin fraction. A new and now generally accepted nomenclature was proposed by Christensen and MacLeod (1945); the streptococcal factor was named streptokinase, the inert globulin, plasminogen and the active enzyme to which it is converted, plasmin; the enzyme system as a whole is named the plasminogen-plasmin or fibrinolytic enzyme system.

Components and Functions of the Plasminogen-Plasmin System.

Though of considerable complexity, the plasminogen-plasmin system has four main components: plasminogen, plasmin, activators and inhibitors.

Plasminogen, a plasma globulin inactive in its native state, is converted by activators to plasmin, a proteolytic enzyme which/

which under suitable circumstances digests fibrin to give soluble products (figure 1).

It is not known where in the body plasminogen is synthesised. Using a fluorescent antibody technique, Banhart and Riddle (1961) have produced evidence to suggest that eosinophils may transport and possibly also synthesise plasminogen. Pure preparations of plasminogen have not yet been produced and accordingly characterisation is inadequate. Plasminogen is a beta globulin and is concentrated in Cohn fraction III (Cohn et al., 1946). Two molecular weights have been suggested - 143,000 (Shulman et al., 1958) and 83,000 (Davies and Engelert, 1960). Plasminogen is stable in dilute mineral acid and advantage is taken of this property in the most commonly used method of preparation, that of Kline (1953), which uses Cohn fraction III as starting material. Such acid-fractionated plasminogen is very poorly soluble at neutral pH. Also starting with Cohn fraction III, Alkjaersig (1964) using DEAE chromatography with lysine or epsilon aminocaproic acid (EACA) as stabilisers and solubilisers, has prepared a preparation which is freely soluble/

soluble at pH 7. Similar preparations have been made by among others, Derechin et al. (1962), Wallen and Bergstrom (1960) and Cole and Mertz (1961), and a preparation of this type is now commercially available (A. B. Kabi, Stockholm).

Activation of Plasminogen. Activation of plasminogen, that is its conversion to plasmin, is a proteolytic phenomenon. Alkjaersig et al. (1958) found that the process is analogous to the formation of other proteolytic enzymes from their inactive precursors (e.g. trypsin, pepsin, thrombin). Three factors are common to the activation process (i) the activation is itself an enzymatic reaction; (ii) it is irreversible; and (iii) it involves the splitting of a limited number of peptide bonds. Plasminogen activators possess the property of splitting lysine and arginine esters and such esters act as competitive inhibitors of plasminogen activation; it would therefore appear probable that lysine and arginine bonds are split during the activation process. Alkjaersig et al. (1958) also studied the release of trichloracetic acid (TCA) soluble material after spontaneous activation of plasminogen in glycerol, and after activation with/

with streptokinase and urokinase (see below). After streptokinase and urokinase activation, TCA soluble material accounted for about 16 per cent of the protein in the original plasminogen preparation; after spontaneous activation the figure was about 25 per cent. Shulman et al. (1958) observed a decrease in molecular weight after activation of plasminogen which was in accord with above data.

At the present time, little is known about the interactions of activators and plasminogen but as the proteins involved become available in more purified forms, their precise inter-relationships at a molecular level will no doubt become better understood.

Activators of Plasminogen.

Plasminogen activation can be brought about in four main ways:

(1) By substances which activate plasminogen directly, e.g. urokinase, tissue activator.

(2) By substances which convert a normally inert proactivator to/

to activator, e.g. streptokinase, tissue lysokinase. Proactivator has only been demonstrated as an activity and not as a molecular identity; it may be plasminogen itself (see chapter 5), and the need to postulate proactivator activity has probably arisen because of differences in species specificity of activator (Macfarlane,1964).

(3) By procedures, e.g. dilution or addition of chloroform, which can bring about activation in vitro.

(4) By administration of substances, not themselves plasminogen activators, which can bring about the release of activator in vivo e.g. nicotinic acid, bacterial pyrogen.

Tissue Activator. Plasminogen activator activity can be extracted from almost all body tissues except liver and placenta (Albrechtsen, 1957). High concentrations are found in the prostate, uterus, thyroid, lungs, ovary, adrenals and lymph nodes. Red cells also contain a plasminogen activator (Künzer and Haberhausen, 1963).

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A tissue activator, which can easily be extracted from pig heart with 2 Molar potassium thiocyanate, has been shown to produce activation of plasminogen by a slow, probably stoichiometric reaction (Abe and Astrup, 1960). Bachman and Sherry (1964) have also extracted and partially purified and characterised a pig heart activator.

Astrup (1956) believes that tissues contain lysokinases which like streptokinase, require proactivator to produce plasminogen activation.

Lack (1964) has found considerable amounts of tissue activator in the lysosomal fraction of tissue homogenates. He suggests that anoxia may be an important cause of release of this activator from lysosomes, and he also speculates that release of lyosomal activator may be a cause of the excessive plasma fibrinolytic activity found following local vasoconstriction, ischaemia, anoxia, anaphylaxis and sudden death.

Todd (1964) has presented evidence to suggest that fibrinolytic/

fibrinolytic activity in the tissues is concentrated round blood vessels, particularly veins.

Plasma activator. Plasminogen activator activity present in trace quantities in normal plasma, is found in increased amounts after exercise, emotional stress, adrenaline and nicotinic acid injection, electric shock and administration of bacterial pyrogen (Sawyer et al., 1960a). The low levels of plasminogen activator present in shed blood are probably responsible for the clot lysis activity seen in the dilute clot lysis technique of Fearnley and Tweed (1953). Caution must be observed in assuming from the demonstration of fibrinolytic activity in plasma samples in vitro that the same activity was present in vivo; the trauma of venepuncture or manipulation after the withdrawal, may have initiated the activity found in the test-tube.

Fearnley et al. (1952) found that in vitro plasma activator is labile at room temperature, less labile at 4°C. and apparently stabilised by fibrin formation. Fletcher et al. (1964a) have shown/

shown that plasma activator activity induced in vivo by nicotinic acid injection has a half-life in the circulation of about 15 minutes.

Source of plasma activator. This is uncertain. Kwaan et al. (1957) have shown that plasma activator levels rise in isolated venous segments in response to paravenous injection of serotonin, acetyl choline and adrenaline. This response, and the appearance of activator in the venous blood of ischaemic limbs, is blocked by atropine, and Kwaan and his associates suggest that a cholinergic effector mechanism may be involved. Though they did not identify the effector cells, they concluded that circulation through the vasa vasorum has a significant role in the production of fibrinolytic activity within veins. It is an attractive hypothesis that local activator release from the vessel wall may be a response to intravascular thrombosis, with serotonin from aggregating platelets as the trigger (Kwaan et al., 1958).

Support for the view that vein walls are in part at least the source of plasma activator has been provided by Todd (1958) who, using a histochemical technique, found that plasminogen activator/

activator was concentrated round veins, venules and pulmonary arteries, and appeared to be closely related to their endothelium. Warren (1964), using a similar histochemical method, found evidence of plasminogen activator round both arteries and veins; the activity appeared to arise from the endothelium, and from vasa vasorum.

Chakrabarti et al. (1963) also found in experiments on unobstructed veins exposed at operation, that trauma to the vein wall resulted in a release of fibrinolytic activity into the lumen; non-fibrinolytic plasma introduced into the lumen also rapidly developed considerable fibrinolytic activity. The authors considered that their results were best explained by the release of plasminogen activator from the vein walls.

Urokinase. Urokinase, the physiological plasminogen activator present in normal urine, is considered in chapter 6.

Bacterial Activators. Streptokinase is considered in chapter 5. Staphylococci have also been shown to produce an exotoxin named staphylokinase/

staphylokinase which is a plasminogen activator (Gerheim et al., 1948).

Autocatalytic Activation. Alkjaersig et al. (1958) have demonstrated that if the reaction is stabilised in 50 per cent glycerol, plasmin itself can produce activation of activator-free plasminogen in a first-order reaction. There is evidence, already cited, that the plasmin so produced may differ from that arising from streptokinase and urokinase activation. As has already been discussed, chloroform probably produces plasminogen activation in vitro by destruction of inhibitors, thus permitting autocatalytic activation to occur.

Trypsin can also activate plasminogen, the activation kinetics being those of a first-order enzymatic reaction (Alkjaersig et al., 1958).

Hageman Factor. There is evidence to suggest that activation of Hageman factor (factor XII) may promote fibrinolysis (Niewiarowski and Prou-Wartelle, 1959; Iatridis and Ferguson, 1962).

Non-Enzymatic Agents. By a mechanism which is not understood/

understood, tolbutamide and chlorpropamide (Fearnley et al., 1960), testosterone and nandrolone (Fearnley and Chakrabarti, 1962), phenformin (Fearnley and Chakrabarti, 1964) and corticosteroids (Chakrabarti et al., 1964) all increase plasma fibrinolytic activity in vivo, though all are fibrinolytically inert in vitro.

When injected intravenously nicotinic acid causes a sharp but not sustained rise in plasma activator levels, an effect which is shared with other vaso-active substances and which is discussed in chapter 8. Bacterial pyrogen when given intravenously also causes an intense but transient rise in plasma fibrinolytic activity; resistance to the effect develops rapidly (Meneghini, 1949).

Von Kaulla (1962) has studied in vitro a large number of compounds, many of which are urea or urethane derivatives, and others are hydrotropic, e.g. 2,4-dimethyl benzene sulphonate and p-iodobenzoic acid, sodium salt, which when incubated in the presence of human plasma produce plasminogen activation. Von Kaulla has suggested that, like chloroform, they may do so/

so by destroying an inhibitory mechanism.

Activator in Secretions. Plasminogen activator activity is present in milk, (Astrup and Sterndorff, 1953), tears (Storm, 1955), saliva (Albrechtsen and Thaysen, 1955) and seminal fluid (von Kaulla and Shettles, 1953).

Plasmin.

Plasmin is the proteolytic enzyme which evolves from plasminogen by the activation procedures already discussed. As already mentioned, different methods of activation may produce a variety of molecular species of plasmin. Plasmin can digest many proteins including fibrinogen (figure 2) and fibrin. Other substrates include glucagon, ACTH and growth hormone (Mirsky et al., 1959), factor V (Alagille and Soulier, 1956), antihaemophilic globulin (Lewis et al., 1949) and certain components of complement (Pillemer et al., 1952). In vitro, plasmin has equal affinity for fibrinogen as for fibrin (Ratnoff, 1953). Plasmin may also be involved in the production of physiologically active polypeptides, e.g. bradykinin (Eisen, 1964).

Inhibitors of the Plasminogen-Plasmin System.

Inhibitors/

Inhibitors of the plasminogen-plasmin system are of two main types; those which inhibit plasminogen activation (antiactivators) and those which inhibit formed plasmin (antiplasmins).

Plasma Antiactivator. The evidence to support the existence of antiactivator in plasma is suggestive but not conclusive, as the methods used have not fully distinguished between antiplasmin and antiactivator activity (Lewis and Ferguson, 1951; Mullertz, 1957; Jacobssen, 1955; Nilsson et al., 1961a; Paraskevas et al., 1962). Further evidence to suggest the presence of antiactivator activity in plasma is presented in chapter 6.

Other Antiactivators. A variety of aliphatic amino compounds including lysine and ornithine (Mullertz, 1954) are competitive inhibitors of plasminogen activation, the most potent being EACA (Alkjaersig et al., 1959a; Ablondi et al., 1959). Other antiactivators include p-aminomethylbenzoic acid (Lohmann et al., 1964) and aminomethyl cyclohexane carboxylic acid (Okamoto and Okamoto, 1962), the antifibrinolytically active isomer/

isomer of which is about 10 times more potent than EACA as an antiactivator (Dubber et al., 1964).

Trasylol, a polypeptide with a molecular weight of 6,200, commercially prepared from bovine lung, and marketed as a trypsin inhibitor, also has antiactivator properties (Steichele and Herschlein, 1961).

Plasma Antiplasmin. Plasma and serum exert a substantial inhibitory action on plasmin. Norman and Hill (1958) have shown that there are at least two antiplasmins in serum. One in the alpha-2 globulin fraction reacts quickly as a competitive inhibitor of plasmin. The other, in the alpha-1 fraction reacts more slowly but firmly with plasmin to produce an inactive complex. Platelets also show antiplasmin activity (Johnson and Schneider, 1953).

Fletcher (1960) reports that there is in plasma about 60 per cent more antiplasmin activity than plasminogen, i.e. than potential plasmin, but according to Norman (1960) there is about 30 times more antiplasmin than plasminogen. The discrepancy probably arises because of the different assay methods/

methods used by the two authors: antiplasmin estimations vary greatly under differing experimental conditions. In any event there is agreement that antiplasmin levels considerably exceed potential plasmin levels.

Other Antiplasmins. Numerous substances have been shown to possess antiplasmin activity including EACA (Alkjaersig et al., 1959a), aminomethyl cyclohexane carboxylic acid (Dubber et al., 1965a), Trasylol (Steichele and Herschlein, 1961), basic amino acids (Mullertz, 1954), soya-bean trypsin inhibitor (Christensen and MacLeod, 1945), heavy metals (Kowalski and Latallo, 1956) and toxic phosphorus compounds (Mounter and Shipley, 1958).

The Antifibrinolytic Activity of Lipids. It would seem probable that in some circumstances lipids exert an inhibitory effect on fibrinolysis; the evidence, which is conflicting and confused, has recently been reviewed by Howell (1964).

Fibrinolysis in vivo - The Sherry Hypothesis.

Sherry and his associates (Sherry et al., 1959a; Fletcher et al., 1959; Alkjaersig et al., 1959b; Sherry et al., 1959b) have/

have proposed a dual-phase concept of plasminogen activation which is now widely accepted as the probable mechanism by which in vivo the relatively non-specific proteolytic enzyme plasmin is largely restricted to a single highly specific action, that of fibrin lysis. The suggested mechanism is schematically illustrated in figure 3. According to this hypothesis, plasminogen in a system including both plasma and clot exists, in a physical sense, in a dual phase, plasminogen in the plasma constituting the soluble phase and, in the clot, the gel phase. As a consequence of this physical distribution, the results of plasminogen activation in the two phases are entirely different. Plasminogen activation in the soluble or plasma phase, provided that it is not unduly rapid, produces no appreciable effect on susceptible substrates in the plasma, for plasma antiplasmin rapidly inhibits plasmin as it is formed (figure 3, left hand section). However rapid plasma plasminogen activation may by a temporary overwhelming of the antiplasmin mechanism permit the appearance of free plasmin in the circulation with digestion of susceptible substrates including fibrinogen and other coagulation factors, and hence produce a haemorrhagic state/

state (see below). Plasminogen activation in the clot or gel phase, where effective antiplasmin levels are said to be relatively low (Sawyer et al., 1961), produces a different result (figure 3, right hand section); in this circumstance, because of the intimate spatial relationship of plasminogen and fibrin, fibrin lysis or thrombolysis results.

The Sherry hypothesis is supported by the in vitro observations that using the plasminogen enriched radioactive clot system (see chapter 4) lysis is readily produced by activator in the surrounding medium, lytic activity being a function of clot plasminogen content. Moreover such clots are resistant to lysis by plasmin in the surrounding medium (Alkjaersig et al., 1959b). Sherry et al. (1959a) have suggested that the main function of plasma plasminogen is to endow any clots which may form with the means to mediate their subsequent lysis when activator diffuses into them. Plasminogen is known to have a strong affinity for fibrinogen and fibrin (Blömback and Blömback, 1956) and indeed it is difficult without

specific/

specific solubilising agents for plasminogen to prepare plasminogen-free fibrinogen (Mosesson, 1962). Activator is also absorbed on fibrin in the process of clotting, and Gross (1963) has shown, using an autoradiographic technique, that isotopically labelled streptokinase can penetrate deeply into thrombus within a short time of contact.

According to the Sherry hypothesis, the ideal approach to therapeutic thrombolysis would be so to elevate plasma activator levels as to produce rapid activation of intrinsic thrombus plasminogen, with soluble-phase plasminogen activation proceeding at a rate within the capacity of the plasma antiplasmin mechanism to keep pace with plasmin production; this ideal can probably never be realised.

Systemic Hyperplasminaemic States.

The consequence of excessively rapid plasma plasminogen activation is temporary overwhelming of the antiplasmin mechanism with the appearance of free plasmin in the circulation - hyperplasminaemia. A major component of the haemostatic defect which occurs following hyperplasminaemia is the presence in the circulation of products of proteolysis of fibrinogen by plasmin./

plasmin. Normally in the final stage of blood coagulation thrombin, a highly specific proteolytic enzyme, converts fibrinogen to fibrin monomer by splitting off specific peptides (Scheraga and Laskowski, 1957). After polymerisation of fibrin monomer, the final visible clot is formed in a gelation step; in the presence of factor XIII the final clot is stable unless exposed to proteolytic enzymes. Sherry and his associates (Fletcher et al., 1962a; Alkjaersig et al., 1962; Bang et al., 1962) have shown that in the presence of fibrinogen breakdown products, clot formation is delayed and defective, due to a defect in fibrin polymer formation. On naked-eye examination, clots formed in the presence of fibrinogen breakdown products are loose and friable, and their abnormal structure has also been demonstrated with the electron microscope. The abnormality is due to incorporation with fibrin monomer as it polymerises of breakdown products of fibrinogen proteolysis by plasmin; the breakdown products lack the correct structure to form a sound polymer. The main breakdown product responsible for the defect has been identified and/

and characterised. Niewiarowski and Latallo (1957), Niewiarowski and Kowalski (1958) and Triantaphyllopoulos (1958) have observed an anti-thrombin effect of the products of fibrinogen proteolysis; Alkjaersig et al. (1962) who originally challenged this view, are now said to accept that under certain circumstances fibrinogen breakdown products may show anti-thrombin properties (Hirsch, 1965).

Other factors in the genesis of the haemostatic failure of hyperplasminaemia are digestion of factor V and antihaemophilic globulin, and accelerated lysis of such clots as do form.

Causes of Hyperplasminaemic States. Hyperplasminaemic states may occur when plasma activator levels are sufficiently high to cause plasma plasminogen activation at a rate in excess of the capacity of the antiplasmin mechanism to neutralise the plasmin so formed. Such high plasma activator levels may arise when tissues rich in activator have been handled or damaged, e.g. during surgical operation, especially cardiac surgery with an extracorporeal circulation (von Kaulla and Swan, 1958; Gans et al., 1962). Hyperplasminaemia may also complicate obstetric accidents/

accidents, e.g. amniotic fluid embolism, accidental haemorrhage, intrauterine death with a retained dead foetus (Schneider, 1959). Proteolytic states have also been reported in prostatic carcinoma, especially if metastases are present (Tagnon et al., 1952), pancreatic neoplasm (Ratnoff, 1952), leukaemia (Mikata et al., 1959), and cirrhosis of the liver (Grossi et al., 1961). The genesis of naturally occurring hyperplasminaemic states is probably often extremely complex. It may be in some cases at least that the initial event is release into the circulation of thromboplastic substances, with intravascular clotting, and that hyperplasminaemia ensues as a homeostatic response which 'overshoots' (Fletcher et al., 1962b; Sharp, 1964).

As will be seen later in this thesis, some degree of hyperplasminaemia is probably inevitable in patients in whom a thrombolytic state has been induced for therapeutic purposes.

Fibrinolysis and the Haemostatic Mechanism.

It is attractive to speculate that the plasminogen-plasmin system, which when fully activated in vivo by streptokinase is sufficiently potent to produce total digestion of circulating fibrinogen/

fibrinogen (chapter 5), may under physiological circumstances be in dynamic equilibrium with the coagulation system to maintain an intact patent vascular tree. This hypothesis, whose proponents have included Copley (1957), Astrup (1956) and Fearnley (1961) would suggest that the coagulation system is constantly active laying down a thin layer of fibrin on the endothelium to seal any deficiencies which may occur, and that in order to maintain vascular patency the plasminogen-plasmin system is also constantly active to remove such fibrin deposits after they have served their haemostatic function. It has also been proposed (Mole, 1948; Biggs and Macfarlane, 1962a; Nilsson et al., 1961a; Fletcher et al., 1962b) that a reduction in plasma fibrinolytic activity might permit accumulation of fibrin on the endothelium, thus forming the basis of an atheromatous plaque (Duguid, 1949).

Though there can be no doubt as to the ability of the coagulation system to produce fibrin in vivo, the physiological role of the plasminogen-plasmin system is not so firmly established. Evidence to support the view that it is constantly active/

active in vivo includes the demonstration of fibrinolytic activity in plasma obtained from normal subjects under physiological circumstances (Fearnley and Tweed, 1953; Sawyer et al., 1960a). Indirect support for a role for the plasminogen-plasmin system in vivo can be found in the observations that in general plasma fibrinolytic activity appears to be somewhat reduced in patients suffering from diseases associated with thrombosis (Hume, 1958; Lackner and Mersky, 1960; Nestel, 1959). Further, urokinase excretion is reduced in diseases in which thrombotic episodes are common, e.g. carcinomatosis, though it is increased after myocardial infarction, a conflicting observation (Smyrniotis et al., 1959).

On the other hand if a dynamic equilibrium between clotting and lysis does obtain, then a change in one of the components of the equilibrium state, for example the impaired coagulation in certain haemorrhagic disorders might be expected to lead to a compensating diminution in the other component and hence to a decrease in plasma fibrinolytic activity. However,

fibrinolytic/

fibrinolytic activity in patients with haemophilia, Christmas disease and von Willebrand's disease has been reported as normal (Kamel et al., 1963). Moreover, 131_I labelled fibrinogen survival is normal in patients with haemophilia (Rausen et al., 1961), and prolonged administration of EACA to a group of haemophiliacs did not result in an increase in plasma fibrinogen levels (Gordon et al., 1965).

Until more conclusive observations have been made, the concept of a dynamic equilibrium between clotting and lysis must remain an attractive but unproven hypothesis; at the present time all that can be said with certainty is that each system can interact with the activity of the other at many levels, e.g. both systems may be activated by activation of Hageman factor; platelets have antiplasmin activity; fibrinogen and factors V, VIII and prothrombin are digested by plasmin; fibrinogen breakdown products, which may have an anti-thrombin effect, inhibit fibrin polymerisation.

Thrombolytic Therapy.

There is now good evidence that intravascular administration of plasminogen activators to increase levels of plasma thrombolytic/

thrombolytic activity can bring about the lysis of preformed thrombi in man and experimental animals. Johnson and Tillett (1952) found that thrombi produced in the marginal ear veins of rabbits could be lysed by a systemic infusion of streptokinase, and Sherry et al. (1954) showed that in dogs streptokinase and a variety of proteolytic enzymes could lyse peripheral arterial thrombi. Similar observations in relation to urokinase have been made by Tsapogas and Flute (1964). The key observations in man are those of Johnson and McCarty (1959) who reported that it was possible to lyse artificially produced thrombi in the forearm veins of human volunteers by systemic infusion of streptokinase; in control subjects not given streptokinase the veins went on to fibrosis. The effectiveness of thrombolytic therapy in man has also been demonstrated in individual well-studied cases of peripheral vascular occlusion treated by plasminogen activator infusion (Verstreate et al., 1963; McNicol et al., 1963).

In view of the conceptual attractions of thrombolytic therapy, and the initial clinical successes, intensive efforts are needed to extend knowledge of the biochemical effects of plasminogen/

plasminogen activators and to improve methods used in their study, in order to place this approach to treatment on a routine basis so that large well designed clinical trials may be carried out to assess its general applicability in the treatment of thrombo-embolic occlusive vascular disease.

The majority of the investigations presented in this thesis were carried out during therapeutic administration of plasminogen activators. A few of the studies were primarily pharmacological and were performed to elucidate certain of the biochemical effects of thrombolytic activity in vivo, and the remainder were concerned with methodology, or with the response of thrombi in vitro to plasminogen activators.

CHAPTER 3.

LABORATORY METHODS.

It is the purpose of this chapter to give an account of the more important laboratory methods used in this thesis.

In the study of the effects of plasminogen activators it is necessary to have assay systems for plasminogen activator activity in plasma and other fluids; the methods which are available are discussed below. Test systems must also be available to measure the coagulation defect produced by plasmin activity; in most cases the thrombin clotting time or the one-stage prothrombin time are suitable for this purpose, though in some circumstances a whole blood clotting time, recalcification time or assay of specific coagulation factors may also be helpful. Finally, to give understanding of the mechanisms of production of fibrinolytic states and the associated coagulation defect, plasminogen and fibrinogen must be assayed.

Activator Assays. No specific assay system for activator in biological fluids is available. The fibrin plate test and the ^{131}I labelled clot system measure the ability of the plasma or other fluid/

fluid under test to lyse preformed fibrin, a property which is largely dependent on the activator content of the fluid under test, though both systems are partially sensitive to plasmin in the fluid being assayed. The radioactive clot system, which is described in detail in chapter 4, is relatively insensitive, and is somewhat complex. The fibrin plate test, in which sensitivity can be increased by the use of resuspended euglobulin precipitates in place of native plasma, requires overnight incubation and so is not suitable for concurrent control of thrombolytic therapy. The euglobulin lysis technique is primarily a measure of plasma activator content, but it also depends on plasminogen levels and probably also on fibrinogen levels. As discussed in chapter 5, when plasminogen is low, euglobulin lysis times may be long even in the presence of increased concentrations of activator.

In a group of 40 patients with renal disease, euglobulin lysis results correlated well with those of the fibrin plate test (figure 4), but there was no correlation between euglobulin lysis activity and plasminogen levels (figure 5) or fibrinogen levels (figure 6).

Description of Methods./

Description of Methods.

Fibrin Plate Test. In the fibrin plate test, a fibrinogen solution, rich in plasminogen, is clotted with thrombin in a Petri dish. If a small amount of a solution containing activator is placed on the surface of the plate, the plate plasminogen is converted to plasmin and after incubation the holes made in the plate where the fibrin is lysed are measured. Areas of lysis are usually expressed in square millimetres as the product of two perpendicular diameters of the area of lysis. Areas of lysis are not linearily related to activator concentration and where the results are to be subjected to statistical analysis a standard curve should be run with each batch of plates, activity in the test sample being read off the curve (Smyrniotis et al., 1959).

Such a fibrin plate is primarily sensitive to activator but it is also susceptible to digestion by plasmin. Response to activator can be eliminated by heating the plate to destroy plasminogen (80°C. for 45 minutes; Lassen (1952)). Because of partial denaturation of the fibrin, response to plasmin in a heated plate is about 50 per cent of that found in a comparable unheated/

unheated plate (Alkjaersig et al., 1959a).

Assay technique. The method used in this thesis was slightly modified from that of Nilsson and Olow (1962). The fibrinogen was bovine fibrinogen, Blömback fraction 1 - 0 (Blömback and Blömback, 1956) kindly supplied by Dr. I. N. Grondhal, Stockholm. 2.5 ml. of 0.1 per cent bovine fibrinogen in tris buffer, 0.15 Molar, pH 7.8 were clotted with 0.5 ml. thrombin, 50 N.I.H. units/ml. (Parke Davis thrombin topical), in a perspex dish, internal diameter 11.5 cms. After application of samples (30µl. of plasma or resuspended euglobulin precipitate), the plates were incubated for 18-20 hours at 37°C. Resuspended euglobulin precipitates were prepared by adding 0.1 ml. plasma to 1.9 ml. 0.014 per cent acetic acid, pH 5.4; the mixture was kept at 4°C. for 10 minutes and then centrifuged at 500 g. for 10 minutes. The precipitate was dissolved in 0.1 ml. citrate saline (3.8 per cent sodium citrate in 0.9 per cent sodium chloride solution). Resuspended euglobulin precipitates produced larger areas of lysis than the native plasma from which they were prepared.

Samples were kept at 4°C. until assayed and were applied to/

to the plates as soon as possible after withdrawal.

Euglobulin Lysis Activity. In this technique the euglobulin fraction of plasma, which includes fibrinogen, plasminogen and plasminogen activator, is precipitated at pH 5.4 and low ionic strength. The supernatant which contains antiplasmin, is discarded. Accordingly lysis times of clots made from the euglobulin precipitate are much shorter than those found with native plasma.

The technique used was that of Nilsson and Olow (1962). To 9.5 ml. of 0.014 per cent acetic acid, pH 5.4, was added 0.5 ml. plasma. The mixture was kept at 4°C. for 10 minutes and then centrifuged at 4°C. at 500 g. for 10 minutes. The supernatant was discarded and the precipitate dissolved in 0.5 ml. of 0.1 Molar barbitone buffer, pH 7.3 (sodium diethyl barbitone, 11.75 gm; HCl, 0.1 N, 430 ml; Na Cl, 14.67 gm; distilled water to 1000 ml.). After clotting with thrombin, 0.5 ml. of a 2 N.I.H. unit/ml. solution, the clot was incubated at 37°C. and the time necessary for complete clot lysis was recorded.

Sherry and Alkjaersig (1957) have shown that in fibrinolytic assays, activity is proportional to the reciprocal of the lysis time/

time and accordingly a logarithmic plot of lysis time against units of activity shows a linear relationship. Such a plot can be used to express observed lysis times in terms of arbitrary units of activity, as suggested by Sherry et al. (1959b). Such units can be used for statistical purposes in analysis of data. In the present studies, a lysis time of 300 minutes was assigned 1 unit of activity. Observed lysis times can be converted to units by dividing the lysis time in minutes into 300.

Radioactive Clot Assay. The method is described in chapter 4.

Thrombin Clotting Time. The thrombin clotting time was used in this thesis primarily as an index of defective fibrin polymerisation, but it is also prolonged by reduced fibrinogen concentrations (figure 7).

The method used was that of Fletcher et al. (1959). To 0.1 ml. plasma was added 0.3 ml. of the "thrombin titration mixture" of Seegers and Smith (1942). This was made up fresh on each occasion with 6 ml. saline, 1 ml. tris buffer, 0.1 Molar, pH 7.5, 2 ml. 0.7 per cent calcium chloride and 2 ml. 15 per cent acacia solution. The clotting time of the plasma with "thrombin titration/

titration mixture" was estimated at 37°C. after addition of 0.1 ml. thrombin, 10 N.I.H. units/ml.

One-Stage Prothrombin Times were estimated as described by Douglas (1962).

Plasminogen Assay. The method used was the caseinolytic assay of Remmert and Cohen (1949) as modified by Alkjaersig et al. (1959b). Antiplasmin is first destroyed by incubating the plasma with acid. The acid is neutralised with alkali and buffer, and streptokinase is then added to convert the plasminogen to plasmin. The plasmin so produced is assayed by a caseinolytic technique, the amount of tyrosine released from the casein being a measure of the amount of plasmin present. The casein solution used was prepared by boiling for 20 minutes 25 gm. casein (L. Light and Co. Ltd., Colnbrook) in 500 ml. phosphate buffer, 0.1 Molar, pH 7.6 (see below). The solution was filtered while hot and after cooling, the pH was readjusted to 7.6. The solution was then dialysed overnight against a large volume of constantly stirred phosphate buffer.

Assay technique. To 0.5 ml. plasma was added 0.5 ml. 1/6 N. hydrochloric acid. After standing for 15 minutes at room temperature/

temperature to destroy antiplasmin, 0.5 ml. 1/6 N. sodium hydroxide was added, followed by 1.0 ml. phosphate buffer, 0.1 Molar, pH 7.6, 0.5 ml. streptokinase solution 2,000 units/ml. (Varidase Lederle) and 2.0 ml. 5 per cent casein solution. After addition of casein and thorough mixing, the assay mixture was incubated at 37°C. for 62 minutes. At 2 minutes and 62 minutes, 2 ml. aliquots were taken and to each was added 2 ml. 10 per cent trichloracetic acid. After centrifugation (500 g. for 10 minutes) 1 ml. of the supernatant was added to 5 ml. 0.5 N. sodium hydroxide and 1.5 ml. 5 per cent trichloracetic acid, followed by 1.5 ml. dilute (1:2) Folin Ciocalteu reagent. After standing for 15 minutes for colour development, the optical density of the 62 minute sample was read at 650 mμ with the 2 minute sample as blank. Tyrosine release was read off a standard curve; 1 casein unit equals 180 μg. tyrosine released in 1 hour.

Fibrinogen Assay. The method described is that of Ratnoff and Menzie (1951) as modified by Alkjaersig (1960). In this assay fibrinogen is clotted with thrombin; the fibrin so formed is hydrolysed/

hydrolysed with sodium hydroxide and the tyrosine released is estimated colorimetrically. A constant proportion of tyrosine in the fibrinogen molecule is assumed (1 : 11.7).

Assay technique. In a 15 ml. test tube were placed "0.2 ml." glass beads (diameter 0.15 mm.), 6.0 ml. saline, 0.1 ml. thrombin solution, 100 N.I.H. units/ml., 0.2 ml. 2.5 per cent calcium chloride and 0.2 ml. plasma. The tube was shaken but not inverted and the fibrin was caught up on the glass beads. After standing for 1 hour at 4°C. the tube was shaken again and centrifuged for 10 minutes at 500 g. The glass beads and adherent fibrin were washed 3 times with saline. After final centrifugation and decantation of the washing fluid, 0.4 ml. 10 per cent sodium hydroxide solution was added and the tube boiled in a water bath for 20 minutes. After cooling to room temperature 0.6 ml. 5 per cent trichloracetic acid, 2 ml. 0.5 N. sodium hydroxide and 0.6 ml. dilute (1:2) Folin Ciocalteu reagent were added. After standing for 15 minutes for colour development, optical density at 650 mμ was read against a reagent blank. Readings were converted to fibrinogen concentration, mg/100 ml., from a standard tyrosine curve.

Collection of blood samples. In all the studies reported in this thesis/

thesis, citrated blood was used; 9 volumes of blood collected by clean venepuncture were mixed with 1 volume of 3.8 per cent sodium citrate in a graduated centrifuge tube. Plasma was obtained by centrifugation at 500 g. for 10 minutes, and in expressing results of plasma assays, no correction has been made for dilution by citrate.

Standard phosphate buffer. The standard phosphate buffer used in this thesis was a buffer of 0.1 Molar, pH 7.6, made up as follows: $Na\,H_2\,PO_4 \cdot 2H_2O$, 2.028 gm.; $Na_2\,H\,PO_4 \cdot 2H_2O$, 14.964 gm.; 9.0 gm. Na Cl; water to 1000 ml.

CHAPTER 4.

PREPARATION AND PROPERTIES OF ^{131}I TAGGED FIBRINOGEN, AND ITS USE AS A SUBSTRATE IN A THROMBOLYTIC ASSAY SYSTEM.

This chapter describes a method of preparing fibrinogen tagged with radioactive iodine, some of the properties of this tagged fibrinogen, and its use in the assay of thrombolytic activity. The method of fibrinogen-tagging, devised in collaboration with Miss W. E. Clement (Clement and McNicol, 1959) has subsequently been modified as described by McNicol and Douglas (1964).

Materials.

Radioactive Iodine (^{131}I) was obtained from the Radiochemical Centre, Amersham, as radioactive sodium iodide, in 0.1 ml. dilute sodium thiosulphate solution. At various times the quantity of radioactivity used varied from 1 to 6 mc.

Fibrinogen was prepared from expired bank plasma by a phosphate buffer method (Biggs and Macfarlane, 1962b).

Thrombin./

Thrombin. Parke Davis thrombin topical.

Plasminogen. The plasminogen used to make radioactive clots, prepared by the method of Kline (1953), was kindly supplied by Miss M. Mackay. It was insoluble at neutral pH.

Plasmin was prepared by spontaneous activation of plasminogen in glycerol according to the method of Alkjaersig et al. (1958). The plasminogen for this purpose, soluble at neutral pH, was supplied by A. B. Kabi.

Ion Exchange Resin. Amberlite IRA 400 Cl.

Streptokinase. A highly purified preparation supplied by A. B. Kabi.

Urokinase. Leo urokinase was used (Leo Laboratories, Copenhagen).

Methods.

Buffer for electrophoresis. Barbitone buffer 0.1 Molar, pH 8.6 (Flynn and de Mayo, 1951).

Paper electrophoresis was carried out in a horizontal electrophoresis tank on Whatman No. 1 paper for 16 hours at 130 volts. Strips were stained for protein with bromophenol blue.

Cellulose acetate electrophoresis was carried out using oxoid cellulose/

cellulose acetate strips which were run for 2 hours at 150 volts in a Shandon horizontal electrophoresis tank. After staining with Ponceau S, strips were scanned in a recording densitometer (Chromoscan, Joyce Loebl and Co., Gateshead on Tyne).

Chromatography was carried out by ascending chromatography with butanol acetic acid as solvent. Strips were stained for amino acids with diazotized sulphanilic acid and 10 per cent potassium carbonate, and with 1 per cent palladium chloride for iodide. Monoiodotyrosine, diiodotyrosine and potassium iodide were run as markers.

Scanning for radioactivity. Electrophoresis strips and chromatograms were scanned for radioactivity by passing them between two Geiger-Muller tubes linked through a rate meter to a recording milliammeter.

Autoradiography. Kodak 'Kodirex' film was used.

Tyrosine assay was carried out using Folin Ciocalteu's reagent, optical density at 650 mμ being recorded and tyrosine content of unknown/

unknown solutions being read off a standard tyrosine curve.

Preparation of Radioactive Iodine Tagged Fibrinogen. To 6 mc. of radioactive sodium iodide in 0.1 ml. dilute sodium thiosulphate solution were added 0.025 ml. of a normal solution of stable iodine in methanol, 0.025 ml. 0.01 N. sulphuric acid and 5 ml. of human fibrinogen in citrate saline, concentration 4 mg./ml. After 5 minutes incubation at room temperature, the fibrinogen was passed through an ion-exchange resin column with 5 ml. 0.1 Molar phosphate buffer, pH 7.6. After addition of heparin (0.1 ml. of a 1 mg./ml. solution), the fibrinogen was stored in small aliquots at -20°C.

Properties of ^{131}I Tagged Fibrinogen.

The quality of the ^{131}I tagged fibrinogen produced is dependent on the quality of the starting fibrinogen. Figure 8 shows a paper electrophoresis strip of a tagged fibrinogen preparation, clottable radioactivity 94 per cent, with a control strip of normal serum run simultaneously. The figure also shows a scan for radioactivity, and it will be seen that all the radioactivity was with the fibrinogen. The hazard of a poor starting/

starting fibrinogen is illustrated in figure 9, which shows an electrophoresis strip of tagged fibrinogen prepared from a fibrinogen solution which was contaminated with gamma globulin; only 58 per cent of the radioactivity was thrombin-clottable. In the studies presented in this thesis using radioactive iodine tagged fibrinogen, thrombin-clottable radioactivity varied from 89 - 94 per cent, and trichloracetic acid radioactivity was always greater than 97 per cent.

Figure 10 shows a cellulose acetate electrophoresis strip of a normal plasma to which a trace of tagged fibrinogen had been added. Above the electrophoresis strip is shown a densitometer tracing from an optical scan of the stained strip, and below is shown an autoradiogram with radioactivity well localised to the fibrinogen peak.

Figure 11 shows a chromatogram of the tagged fibrinogen scanned for radioactivity which was all at the origin; no iodide was washed out. Chromatography was repeated after trypsin hydrolysis; figure 12 shows radioactivity, 63 per cent of which was opposite /

opposite mon- and di-iodotyrosine markers.

Discussion.

The method of tagging fibrinogen with radioactive iodine which has been described in this chapter was quickly and easily carried out. If the initial fibrinogen was of good quality, thrombin-clottable radioactivity was up to 94 per cent, with at least 97 per cent precipitable by trichloracetic acid. The specificity of the tag was demonstrated by electrophoresis and by chromatography. Chromatography after hydrolysis suggests that tagging was largely due to iodination of tyrosines.

The next section of this chapter presents the use of tagged fibrinogen in the plasminogen enriched clot assay system for plasminogen activator.

Activator assay with Plasminogen enriched ^{131}I labelled Clots.

The method described, slightly modified from that of Alkjaersig et al. (1959b) is dependent on the release of radioactivity from plasminogen-enriched ^{131}I tagged fibrin clots when incubated in the presence of a plasminogen activator. Plasminogen enrichment is intended to ensure that, after washing to reduce blank/

blank radioactivity, the clots retain sensitivity to activator. Expired bank plasma trace labelled with ^{131}I tagged fibrinogen prepared as described above, and enriched with plasminogen, is clotted with thrombin round stainless steel wire spirals. After washing, the clots are incubated at 37°C. with the test plasma. Radioactivity released from the clot in a standard time is a measure of activator concentration in the test plasma.

Method.

To 0.5 ml. of pooled bank plasma trace labelled with ^{131}I tagged fibrinogen so that 1 mg. of fibrinogen gave approximately 2500 counts, was added 0.1 ml. plasminogen, 6 casein units/ml; this plasminogen being insoluble at neutral pH was not eluted during washing. The plasma was clotted round a stainless steel wire spiral in a 75 x 8 mm. test tube by addition of 0.1 ml. thrombin, 10 N.I.H. units/ml. The clots were incubated at 37°C. for 30 minutes to ensure complete clotting. Still on the wire spirals, the clots were removed from the test tubes and washed at 37°C. in phosphate buffer, 0.1 Molar, pH 7.6., to reduce blank radioactivity and remove serum with its high antiplasmin/

antiplasmin content. Washing was always carried out for at least 1 hour and sometimes overnight. For any one experiment, the washing period for all the clots was constant.

Activator Assay.

The activator assay was carried out as follows. The test solution (0.5 ml.) was incubated with a preformed clot for 30 minutes. The clot was then removed on its wire spiral and residual radioactivity counted in a well-type scintillation counter with a pulse-height analyser. Results were recorded after subtraction of (i) background and (ii) mean counts from two control clots incubated with saline. Results were sometimes expressed in terms of counts, and sometimes, knowing the specific radioactivity of the fibrin, in terms of micrograms of fibrin lysed per millilitre of test solution per hour.

Reproducibility of the assay system, and correlation of radioactivity release with tyrosine release. Forty-two radioactive clots were prepared as described above, and were incubated with a solution of streptokinase in saline, 50 units/ml. Seven clots were incubated for 10 minutes, 7 for 20 minutes, 7 for 30 minutes, 7 for/

for 40 minutes, 7 for 50 minutes and 7 for 60 minutes, and radioactivity release was counted. Results are as shown in figure 13. The coefficient of variation is 14.5 per cent (calculated by Dr. R. A. Robb).

In this experiment tyrosine release from the radioactive clots was also estimated. Figure 14 shows the excellent correlation found between tyrosine release and radioactivity release ($r = +0.954$, $p < 0.001$).

Effect of plasminogen content. As shown in figure 15, susceptibility to lysis by streptokinase and urokinase was dependent on clot plasminogen content.

Response to Exogenous Plasmin of plasminogen enriched tagged clots. The response of the tagged clots to lysis by exogenous plasmin was also studied. An activator free plasmin solution, 7 casein units/ml., was compared in the radioactive clot assay system with varying concentrations of urokinase. The plasmin solution was assayed in quadruplicate and gave results of 183, 194, 216, 229 (mean = 206) μg. fibrin lysed/ml. test solution/hour. The results with a urokinase dilution curve are shown in figure 16. Interpolating into the dilution curve gives a thrombolytic potency for/

for the plasmin solution similar to that of a 10 unit/ml. urokinase solution; i.e. the assay system was relatively more sensitive to activator than to plasmin, 7 units/ml. of plasmin being about twice the maximum plasmin activity which can be induced by activation of plasma plasminogen, whereas 10 units/ml. of urokinase represents a relatively low activator level. From the dilution curve it will be seen that quantitative aspects of the assay system were not satisfactory with concentrations of urokinase below 20 units/ml.

Discussion.

The plasminogen enriched radioactive iodine labelled clot has definite advantages as a substrate for assay of thrombolytic activity. It measures the property which thrombolytic therapy is designed to induce in plasma, that is the ability to lyse preformed human fibrin. A result is obtained relatively quickly, the system is more sensitive to activator than to plasmin (see also chapter 7) and there is good correlation between radioactivity release and tyrosine release. On the other hand the method requires relatively complex apparatus, quantitative aspects are somewhat/

somewhat disappointing (coefficient of variation 14.5 per cent), and the method is poor with low levels of activity.

CHAPTER 5.

IN VIVO STUDIES WITH STREPTOKINASE.

Streptokinase was the first activator of the fibrinolytic enzyme system to become available for investigative purposes in man and it is still the only fibrinolytic agent commercially available in this country for intravascular administration. It is a bacterial protein with a molecular weight of about 50,000, which is produced by certain strains of streptococci: in recent years most streptokinase preparations have been produced by streptococci of type C (H 46 A) (von Kaulla, 1963a). The toxicity of early preparations of streptokinase prevented their intravascular administration but Fletcher and Johnson (1957) described a method of purification which made possible commercial production of streptokinase preparations suitable for intravascular administration, which if given with corticosteroids are largely devoid of toxicity.

There is marked species variability in response to streptokinase. Human plasminogen is readily activated, the mechanism/

mechanism of activation being discussed below, but rabbit and dog plasminogens are more resistant to activation by streptokinase and bovine plasminogen is virtually totally resistant (Cliffton and Downie, 1950).

The only known biochemical effect of streptokinase is to bring about the conversion of plasminogen to plasmin. A two-phase reaction is involved. Streptokinase first reacts rapidly and stoichiometrically with a proactivator in plasma (Troll and Sherry, 1955) to form activator which brings about the enzymatic conversion of plasminogen to plasmin by splitting off nitrogen containing material (Alkjaersig et al., 1958). There is controversy as to the nature of proactivator, some (e.g. de Renzo, 1960) believing it to be plasminogen itself, while others interpret the evidence as suggesting that plasminogen and proactivator are separate entities (e.g. Lassen, 1960).

Streptokinase shares with other streptococcal proteins the ability to act as an antigen in man and antibodies to streptokinase are distributed through the population, varying very widely in concentration; in streptokinase therapy enough streptokinase must be given to bind circulating antibody and other/

other non-specific inhibitors before the specific effect on the fibrinolytic enzyme system is seen (e.g. Fletcher et al., 1958). These authors have shown that the streptokinase-antibody complex is cleared rapidly from the circulation at two rates; a very fast rate (50 per cent plasma clearance time - 18 minutes) and a somewhat slower rate (50 per cent plasma clearance time - 83 minutes). Surprisingly enough immediate deleterious effects due to the immunological consequences of streptokinase infusion have not been reported, possibly because of the rapidity with which the streptokinase-antibody complex is removed from the circulation.

In this chapter an account is given of the biochemical effects of streptokinase given intravascularly to six patients with thrombo-embolic occlusive vascular disease.

Materials and Methods.

Streptokinase. A highly purified preparation, suitable for intravascular administration, was provided by A. B. Kabi. Vials of streptokinase were reconstituted with saline immediately before use. All patients except number 6 were given streptokinase by/

by intravenous infusion using a constant rate infusion pump (B. Braun, Melsungen, West Germany) delivering a concentrated streptokinase solution into a freely running intravenous drip; patient 6 was given streptokinase intra-arterially.

Streptokinase sensitivity test. This test was designed to find a concentration of streptokinase which would neutralise circulating antibody and other inhibitors and leave over enough free streptokinase to produce a plasma-clot lysis time of about 20 minutes (Johnson et al., 1957). To 0.2 ml. aliquots of the plasma in a series of test tubes was added respectively 2, 4, 6, 8, 16, 24, 32, 48 and 80 microlitres of streptokinase solution, 500 units/ml. After clotting with thrombin, 0.1 ml. of a 20 N.I.H. units/ml. solution, the tubes were incubated at 37°C. and the times for complete clot lysis measured. For each patient, plasma volume was estimated from body weight (39 ml./kg.) and an initial dose of streptokinase calculated on the basis of plasma volume and the concentration of streptokinase, estimated as described above, which produced a lysis time of 15 - 20 minutes.

Recalcification times were measured by the method of Biggs and Macfarlane/

Macfarlane (1962c).

Antihaemophilic-globulin assays and Christmas factor assays were carried out by Professor A. S. Douglas by a one-stage method (Douglas, 1965) which depends on the clotting time of recalcified plasma from patients with severe haemophilia (or Christmas disease) which has been "contact activated" by kaolin. Cephalin, as prepared by Bell and Alton (1954) was added to the substrate system.

Factor V was assayed by Professor Douglas by the technique described by Douglas and Biggs (1953) but the readings were taken only 5 and 6 minutes after the start of the incubation. Results are expressed as a percentage of the pre-infusion value.

Prothrombin assays were carried out using tosyl arginine methyl ester (TAMe) by the technique of Glueck and Sherry (1954).

Radioactive clot activator assays were carried out as described in chapter 4.

Fibrin plate tests were carried out as described in chapter 3, using bovine fibrinogen. No "proactivator", apart from that present in the test plasmas, was added.

Other assays were carried out as described in chapter 3.

Results./

Results.

The biochemical effects of streptokinase administration to 6 patients are described. In each case an initial fibrinolysis inducing dose of streptokinase was given calculated as described above from the streptokinase sensitivity test. Details of estimated plasma volumes, streptokinase concentrations and initial dosages are shown in table 1.

Patient 1. Results are shown in figure 17 and table 2. After infusion of an initial dose of 155,000 units over 20 minutes, administration was continued with 45,000 units over the succeeding 40 minutes. The initial dose produced no activity in the radioactive clot test, and the changes recorded in plasminogen and fibrinogen are within the limits of experimental error. At the end of the infusion there was marked activity in the radioactive clot test, and whole clot lysis and euglobulin lysis were much accelerated. There was a fall in plasminogen to 0.9 units/ml., with a level of 1.2 units 3 hours after the infusion and 3.2 units at 24 hours. Fibrinogen fell from 250 mg./100 ml. to 140 mg./100 ml. and had not fully returned to its pre-infusion level at 24 hours. The thrombin/

thrombin clotting time, 10 seconds before streptokinase was given, was 19 seconds at the end of the infusion and 16 seconds 3 hours later. At 24 hours it was again 10 seconds.

Patient 2. Results are shown in figure 18. The initial dose in this patient was large - 750,000 units. When 500,000 units had been given, no evidence of fibrinolytic activity was seen, but when the full initial dose had been given, high levels of circulating activator were found and plasminogen was depressed to near zero. The initial dose of streptokinase was followed by infusion of 50,000 units/hour for 5 hours. During this period activator levels remained high and plasminogen depressed. Fibrinogen levels slowly fell and at the same time the thrombin clotting time increased from 12 to 19 seconds. Four hours after the end of the infusion, plasminogen, fibrinogen and thrombin times were still abnormal, but had all returned to normal the next day.

Patient 3. An initial dose of 80,000 units was followed by 80,000 units/hour for 5 hours: results are shown in figure 19. The initial dose produced a slight increase in activator activity in the radioactive clot test, and minor changes in plasminogen and fibrinogen. Prothrombin time, recalcification time, prothrombin, factor/

factor V, antihaemophilic globulin and Christmas factor were unaffected. As the infusion continued, activator levels quickly rose and plasminogen fell to zero where it remained throughout the infusion; plasminogen had not returned to pre-infusion levels 23 hours after the infusion ended. Early in the infusion fibrinogen fell to zero, but thereafter it climbed to about 100 mg./100 ml. where it remained while streptokinase was being given.

The prothrombin time, 14.5 seconds before infusion, increased to 60 seconds, and the recalcification time, 205 seconds before infusion, to over 800 seconds. Prothrombin fell to 35 per cent of its pre-infusion level, factor V to 25 per cent of the pre-infusion level and antihaemophilic globulin to zero. Christmas factor was unaffected.

Patient 4. An initial dose of 460,000 units was given over the first hour, followed by 50,000 units/hour for a further two hours (figure 20). In this patient the initial dose produced a rise in activator assay in the radioactive clot system and a fall in plasminogen from 3.0 to 1.5 units/ml; there was also a fall in fibrinogen from 244 to 151 mg./100 ml. The initial dose produced a slight rise in prothrombin time and thrombin time, and/

and a fall in antihaemophilic globulin, prothrombin and factor V assays. During the following 2 hours there was a further fall in plasminogen, fibrinogen, antihaemophilic globulin, prothrombin and factor V. An hour after the end of the infusion no plasminogen was detectable. At this point also fibrinogen levels were most depressed and the greatest abnormality was seen in the thrombin clotting time and the prothrombin time. Euglobulin lysis time, 360 minutes before infusion, fell to 13 minutes after the initial dose had been given, had risen to 240 minutes at the end of the infusion, presumably because of plasminogen depletion. As in patient 3, antihaemophilic globulin, factor V and prothrombin were reduced but Christmas factor was unaffected.

Patient 5. After an initial dose of 250,000 units given over 1 hour, therapy was continued for a further 21¼ hours with an infusion of 75,000 units of streptokinase/hour. Results are shown in figure 21, where it will be seen that there was a short accidental break in the infusion at 16 hours, due to blood clot in the infusion tubing. This clot, resulting from back flow of blood during a change of tubing, persisted unlysed in the 1000 unit/ml. solution of streptokinase which was being given to the patient, failure of lysis being presumably/

presumably due to plasminogen depletion. In the radioactive clot assay system, large amounts of thrombolytic activity were present in the plasma throughout the infusion, a finding confirmed in the fibrin plate test. Euglobulin lysis on the other hand was not accelerated and euglobulin lysis times were in excess of 3 days for the greater part of the infusion. During the break in infusion at 16 hours when a little plasminogen was present in the plasma, slight euglobulin lysis activity was seen, though at other times (e.g. 4.5 and 5.5 hours) when a little plasminogen was also assayed, euglobulin lysis was grossly prolonged. Fibrinogen fell to 125 mg./100 ml. at 8 hours and remained at about that level for the duration of the infusion and for 16 hours after it: 40 hours after the end of infusion, fibrinogen had risen to 238 mg./100 ml. Thrombin clotting time climbed slowly during the infusion and reached a maximum value, 8 seconds above the pre-infusion level, at the time of the break in infusion; thereafter it fell slowly and had returned to pre-infusion levels at 16 hours after the end of infusion.

Patient 6. Streptokinase was given by intra-arterial perfusion using a constant-rate infusion pump. An initial dose of 350,000 units/

units was given over 5½ hours followed by 12,500 units/hour for the remaining 20½ hours (figure 22). Thrombolytic activity in venous blood as demonstrated in the euglobulin lysis time and the fibrin plate test, was induced by the initial dose and was maintained for the duration of the perfusion. Plasminogen was rapidly depleted and was zero at 16 and 26 hours: it had not returned to the pre-infusion value 30 hours after the end of infusion. Fibrinogen fell from a pre-infusion level of 276 mg./100 ml. to 112 mg./100 ml. at 8 hours; thereafter it slowly increased, being 148 mg./100 ml. at 26 hours. The thrombin clotting time showed the same pattern, the maximum abnormality being at 8 hours, with thereafter gradual improvement.

Discussion.

Since Tillet and his associates (1955) first administered streptokinase intravenously to man, it has been given to animals and man by numerous investigators, and from its use has emerged much of our current knowledge of the fibrinolytic enzyme system. As streptokinase is still the only fibrinolytic activator commercially available/

available for intravascular administration, detailed knowledge of its effects is clearly important.

The studies reported in this chapter confirm that by intravascular administration of appropriate amounts of streptokinase intense thrombolytic activity can be imparted to the plasma. In all the patients, the ability of the plasma to dissolve preformed fibrin clots was investigated and found to be present either in the radioactive clot test or the fibrin plate test.

The present experiments also confirm the observation first made by Johnson et al. (1957) that in plasma the specific biochemical effect of streptokinase as an activator of plasminogen is only seen when enough streptokinase has been administered to neutralise circulating antibody and other inhibitors. In patient 2 for example the initial dose (i.e. the amount of streptokinase to neutralise circulating antibody and then induce a brisk lytic state) was calculated as 750,000 units; 500,000 units produced a barely detectable effect. In patient 3 on the other hand the total dose of streptokinase given was 480,000 units and this served not only to neutralise antibody but to maintain for 5 hours intense fibrinolytic/

fibrinolytic activity with a major coagulation defect. It is clear that administration of a suitable initial dose, individually calculated for each patient, is an essential preliminary to rational use of streptokinase.

The coagulation defect. As has already been discussed in chapter 2, the coagulation defect induced by streptokinase administration has been studied in detail by Sherry and his associates, who have shown that a major component is defective fibrin polymerisation caused by the digestion of circulating fibrinogen by free plasmin (Alkjaersig et al., 1962; Bang et al., 1962; Fletcher et al., 1962a). Fibrinogen breakdown products become incorporated with fibrin monomer as it polymerises and as a result fibrin polymerisation and clot structure are strikingly defective. Free plasmin appears in the circulation (hyperplasminaemia) when after the infusion of large amounts of activator, sudden massive plasmin production temporarily overwhelms the physiological antiplasmin mechanism. The defect in fibrin polymerisation is reflected in the prolongation of the thrombin clotting time seen in all the present/

present patients and also contributes to the prolongation of the prothrombin time and the recalcification time. The thrombin clotting time as illustrated in figure 7, is also prolonged by hypofibrinogenaemia per se.

Other factors are involved in the genesis of the coagulation defect. Factor V is particularly susceptible to digestion by plasmin (Lewis et al., 1949; Alagille and Soulier, 1956; Fletcher et al., 1959; Donaldson, 1960; Nilsson and Olow, 1962) and reduced levels of antihaemophilic globulin in the presence of increased fibrinolytic activity have also been reported (Lewis et al., 1949; Alagille and Soulier, 1956; Niewiarowski and Latallo, 1957; Donaldson, 1960). Low levels of Christmas factor have been found less often (Alagille and Soulier, 1956; Donaldson, 1960). In the present patients fibrinogen was depleted in all and depletion of prothrombin, factor V and antihaemophilic globulin was found in all the patients in whom these factors were assayed. The factor V assays may have been affected by the presence of breakdown products which may have exaggerated the fall in factor V levels/

levels seen, but the fall in prothrombin was almost certainly real as a synthetic substrate (TAMe) was used for its assay, there being no dependence for an end-point on clot formation which might be interfered with by fibrinogen breakdown products. The reduced levels of antihaemophilic globulin assayed were also probably real, as in the same plasma samples, and employing an identical technique, (apart from the use of plasma from a patient with Christmas disease instead of a patient with haemophilia,) Christmas factor assayed at 100 per cent.

Duration of the lytic state and coagulation defect after cessation of therapy. Activator levels in the plasma were found to decline very rapidly after the infusion of streptokinase was stopped. For example, in patients 1, 3 and 4, activator levels were high in samples taken off just before the infusions were stopped, but 1 hour later, virtually no activator was found. This observation is in agreement with the very short half-life (15 minutes) of nicotinic-acid induced plasma activator found by Fletcher et al. (1964a).

The/

The fibrinogen breakdown products responsible for defective fibrin polymerisation have a half-life in the circulation of about 9 hours (Fletcher et al., 1962a), and this is reflected in the duration of elevation of the thrombin time or recalcification time found in the present series of patients after the cessation of streptokinase therapy. For example in patient 1, the thrombin time was still abnormal 3 hours after the end of infusion but was normal at 24 hours: in patient 2 the thrombin time was abnormal $4\frac{1}{2}$ hours after the end of infusion but was normal at 24 hours; and in patient 3 recalcification time was abnormal 7 hours after the end of infusion, but was normal 16 hours after the infusion stopped.

The return to normal of plasminogen, fibrinogen, prothrombin, factor V and antihaemophilic globulin presumably occurs as these factors are synthesised and released into the circulation. In patient 1, neither plasminogen nor fibrinogen had returned to pre-infusion levels at 24 hours; and in patient 6, both plasminogen and fibrinogen were essentially normal at 30 hours after the end of the streptokinase infusion. In each of the remaining patients in the final sample studied plasminogen was still/

still depleted but fibrinogen was normal, suggesting a slower rate of synthesis of plasminogen than fibrinogen. In patients 3 and 4 antihaemophilic globulin, prothrombin and factor V returned to pre-infusion levels more rapidly than did fibrinogen.

Streptokinase Dosage Schedules.

There is general agreement as to the need for initiation of streptokinase therapy with an individually calculated antibody neutralising dose. It would also seem clear that thereafter in order to restore vascular patency as rapidly as possible the plasma surrounding a thrombus in vivo should have imparted to it as high a level of thrombolytic activity as is compatible with a tolerable coagulation defect. Opinions differ as to how this objective can best be achieved. The manufacturers of the streptokinase preparation used in these studies advise that after administration of an initial dose, treatment should be continued with one third to one half of this initial dose given each hour. It would seem probable that a dosage schedule of this sort will with some patients result in overdosage; for example in patient 2, where the initial dose was 750,000 units, 250,000 units/hour would, according to the manufacturers suggestion be indicated for/

for maintaining therapy, whereas in fact 50,000 units/hour maintained a brisk fibrinolytic state.

The theoretical effects of three other possible dosage schedules are illustrated schematically in figure 23: low dosage therapy, intermediate and high dosage. In the hypothetical low dosage regimen (figure 23, left hand section), an antibody neutralising dose is followed by an infusion rate which produces only a slight fall in plasminogen, and a negligible coagulation defect: plasma thrombolytic activity is also low. McNicol et al. (1963) have suggested in patients with arterial obstruction the use of a low dosage schedule of this type, given by intra-arterial perfusion, with a catheter tip near the thrombus, in the hope that small amounts of streptokinase which may produce a negligible systemic fibrinolytic state, might induce intense thrombolytic activity round the catheter tip at the site of vascular occlusion. However, Hume (1964) stated as a result of experiments in dogs, that local perfusion is no more effective in producing thrombolysis in arteries than the same dosage of streptokinase given intravenously.

With/

With intermediate dosage schedules (figure 23, centre section), plasminogen is partially but not completely depleted, and a constant state of hyperplasminaemia is produced together with a serious and sustained coagulation defect. Johnson and McCarty (1959) found that this biochemical state, with both streptokinase and plasmin in the circulation, was highly effective in producing the lysis of artificial thrombi induced in the forearm veins of human volunteers. Patients 2 and 4 show findings approximating to those anticipated in this hypothetical state, though in both patients plasminogen levels tended to drift down during the infusion, and as the plasminogen levels fell, the coagulation defect became more marked, presumably because of increasing plasmin production.

The third schedule of streptokinase administration, a high dosage or "plasminogen depletion" regimen, is also illustrated schematically in figure 23 (right hand section), and Verstreate and his associates (1964) have suggested that this is the most satisfactory method of streptokinase administration. According to this scheme of treatment, after giving an initial antibody neutralising dose of streptokinase, therapy is maintained with large/

large doses of streptokinase designed to bring about rapidly and thereafter to maintain complete, or almost complete plasminogen depletion. It is suggested that there will be an initial transient phase of hyperplasminaemia, and in consequence a coagulation defect as plasminogen is falling; but when complete plasminogen depletion has been achieved, as newly synthesised plasminogen is released into the circulation it is converted to plasmin, the rate of plasmin production being within the capacity of the antiplasmin mechanism to bring about almost immediate neutralisation. Accordingly the deleterious effects of the initial transient hyperplasminaemia will pass off as the infusion continues.

Patients 3 and 6 illustrate that plasminogen depletion is attended in practice by the sequence of events postulated above. In both patients plasminogen levels fell to zero early in the infusion and remained at zero levels for the remainder of the infusion. Fibrinogen levels were lowest, and the coagulation defect most marked at the beginning of the infusion, but thereafter a progressive improvement in both fibrinogen levels and the coagulation defect was observed.

Hazards of plasminogen depletion. Johnson and McCarty (1959) have/

have pointed out that when plasminogen depletion has been induced, fibrin which forms will lack plasminogen and hence will be resistant to subsequent lysis. This circumstance is illustrated in patient 5, in whom the infusion tubing became obstructed by unlysable clot. Johnson and McCarty (1959) have also suggested that in their human volunteers clot lysis is more readily produced with intermediate dosage of streptokinase when both activator and plasmin are present in the circulation than with high dosage streptokinase when only activator is present.

The evidence suggests that when total plasminogen depletion has been produced, conventional anticoagulants should be given as soon as streptokinase administration stops to prevent laying down of unlysable fibrin in the blood vessels during the relatively long period of plasminogen regeneration.

Patient 5 also illustrates that in the presence of plasminogen depletion the euglobulin lysis time is not a satisfactory index of plasma thrombolytic activity. In this patient euglobulin lysis times were prolonged despite high levels of activator in the circulation; euglobulin lysis activity was only seen when there was/

was a short accidental break in streptokinase infusion and though activator levels fell, a little plasminogen was detected in the plasma (figure 21).

Conclusions.

Intravascular administration of streptokinase in appropriate dosage readily produces and maintains an intense thrombolytic state in the plasma. A degree of hyperplasminaemia appears to be an inevitable concomitant of intense plasma thrombolytic activity, with in consequence a coagulation defect due in part to defective fibrin polymerisation resulting from the presence in the circulation of products of proteolysis of fibrinogen by plasmin, and in part to digestion of coagulation factors. Various possible streptokinase dosage schedules have been discussed, but the most satisfactory method of streptokinase administration to induce a maximum thrombolytic effect with minimum hazard to the patient, remains to be established by further clinical and laboratory investigation.

CHAPTER 6.

IN VITRO AND IN VIVO STUDIES OF A PREPARATION OF UROKINASE.

It has been known for many years that urine can digest blood clots (Sahli, 1885), an action due to its content of a plasminogen activator named urokinase (Williams, 1951; Astrup and Sterndorff, 1952; Sobel et al., 1952). Urokinase, itself a proteolytic enzyme, activates plasminogen by first order kinetics, probably by splitting lysine and/or arginine bonds (Kjeldgaard and Ploug, 1957; Alkjaersig et al., 1958). It is a colourless protein with a high degree of stability over a wide pH range (Ploug and Kjeldgaard, 1957).

Bjerrehous (1952) has shown that urokinase concentrations are similar in bladder urine and urine obtained from the renal pelvis. Celander and Guest (1960) demonstrated that there are at least two urinary proteins with urokinase activity and von Kaulla and Swan (1958) and Smyrniotis et al. (1959) have produced evidence to suggest that urokinase may represent in part at least excreted plasma plasminogen activator. There is also evidence to support the/

the concept that it has a physiological role in maintaining the patency of the urinary tract by promoting the lysis of fibrinous deposits (McNicol et al., 1961).

Because of its human origin, it has been hoped that urokinase might have many advantages, including freedom from toxicity and antigenicity, as a therapeutic fibrinolytic substance. Interest in the use of urokinase was stimulated by its isolation and purification by Ploug and Kjeldgaard (1957). There are reports in the literature of the use of urokinase activated plasmin (e.g. Sokal et al., 1958; Sussman and Fitch, 1958). Hansen et al. (1961) also report on the infusion of urokinase to 22 subjects, though they present no data on the biochemical effects induced, and Fletcher et al. (1964b) report in an abstract on the administration of high doses of urokinase to a group of 34 patients.

The present chapter gives an account of the study in vitro and in vivo of a commercial preparation of urokinase.

Materials and Methods.

Urokinase. A purified preparation, isolated from the urine of normal males was made available by Leo Laboratories. The preparation,/

preparation, supplied as sterile yellowish-white pellets, was freely soluble in 0.9 per cent sodium chloride. The manufacturers state that potency in arbitrary units (Ploug and Kjeldgaard, 1957) varies from batch to batch from 5000 to 12000 units/mg. The materials used in this study had a potency of 7100 units/mg. Calcium content of a 10,000 unit/ml. solution was less than 0.2 mEq./litre (assay carried out by Miss M. Gray).

Recalcification times were estimated as described by Biggs and Macfarlane (1962c). In some of the experiments the plasma was low-spun, and in others where it was high spun, cephalin (Bell and Alton, 1954) was added to the system in place of saline. In all cases EACA was present in a concentration of 10^{-2} Molar to inhibit lysis.

Thromboplastin generation tests were carried out as described by Biggs and Douglas (1953) with the following modifications. The volumes of plasma, serum, platelet syspension, Molar/40 calcium chloride and urokinase (10,000 units/ml. solution) were 0.3 ml. In each case saline was added to give a constant volume of 1.5 ml. and EACA was present in a concentration of 10^{-2} Molar to/

to inhibit lysis.

Antihaemophilic globulin, Christmas factor and factor V assays were carried out as described in chapter 5.

Electrophoresis. Oxoid cellulose acetate strips were used for electrophoresis. Strips were run for two hours at 150 volts in a Shandon horizontal electrophoresis tank with the barbitone buffer of Flynn and de Mayo,(1951). After staining, strips were scanned in a Chromoscan recording densitometer.

Esterolytic assays, using tosyl arginine methyl ester (TAMe) were carried out by the method of Troll et al. (1954). Where serum was used in the assays it was dialysed with constant stirring overnight at 4°C. against a large volume of 0.9 per cent saline. Optimum urokinase concentration was found to be 200 units/ml. and incubation was for 30 minutes.

Urokinase sensitivity tests. To 0.2 ml. of citrated plasma were added, using a micro-burette, 80µl. of urokinase solution, 500 units/ml., and thrombin,0.1 ml. of a 20 N.I.H. unit/ml. solution. The plasma was incubated at 37°C. and the time for complete clot lysis measured.

Thrombin. Parke Davis thrombin topical.

Plasminogen./

Plasminogen. A preparation of human plasminogen, soluble at neutral pH, was made available by A. B. Kabi.

Radioactive clot assays were carried out as described in chapter 4.

Other assays were carried out as described in chapter 3.

Results of In Vitro Studies.

Fibrinolytic activity in purified systems.

In purified systems, the urokinase preparation was found to be a potent activator of plasminogen.

(i) Fibrin plate test. In a concentration of 10 units/ml. the urokinase preparation tested in triplicate gave zones of lysis of 400, 440 and 380 sq. mm. No lysis was seen with the same concentration in another plate containing EACA at a concentration of 5×10^{-3} Molar, a concentration adequate to inhibit plasminogen activation but not to inhibit plasmin (Alkjaersig et al., 1959a).

(ii) Radioactive clot assay. Tested in triplicate in the radioactive clot assay, a urokinase solution, 10 units/ml. gave 162, 181 and 147 μg. fibrin lysed/ml. test solution/hour.

Esterolytic activity. As has been reported by Sherry et al. (1959a) urokinase/

urokinase possesses esterolytic activity against TAMe. The present preparation demonstrated this, 1 mg. of the urokinase preparation producing lysis of 161 $\pm$ 23 μM TAMe in 30 minutes (Table 2).

Natural Inhibitors of Urokinase.

It was observed that in systems containing serum or plasma, also in vivo (see below), the activity of urokinase was much less than that seen with similar amounts of urokinase in purified systems.

Fibrin Plate test. Figure 24 shows the effects of plasma in inhibiting urokinase in the fibrin plate test. Mean values and standard deviations from observations on 18 plasmas are shown. Complete inhibition of 1 unit of urokinase was observed when 40 μl. or more of plasma was added to the urokinase preparation before assay.

Radioactive clot systems. Similar results were found in the radioactive clot system where in each of 25 experiments inhibition was complete with 50 μl. of plasma per unit of urokinase; with 10 μl. of plasma per unit of urokinase, inhibition was 22 $\pm$ 10 per cent/

cent (Table 3).

Electrophoresis studies. To identify the protein fraction responsible for this inhibitory effect, electrophoresis of 10 µl. of serum was carried out on cellulose acetate. After electrophoresis the wet cellulose acetate paper was divided longitudinally, that is, at right angles to the origin. One half of the paper was stained with Ponceau S; the other half was cut, parallel with the origin, into strips 0.5 cm. wide. Ten µl. of urokinase solution, 20 units/ml. was evenly applied as a streak along the long axis of each strip, which was then placed, urokinase side down, on a fibrin plate. After incubation for 18 hours at 37°C. the strips were lifted from the surface of the plate and the area of lysis associated with each strip was measured. Using as control the area of lysis produced by urokinase which had been applied to a strip cut well away from the serum pattern, inhibition of urokinase activity produced by each serum band was measured. Results are shown in figure 25, where it can be seen that inhibitory activity is largely concentrated in the alpha-1 and alpha-2 globulin bands.

Caseinolytic assays. As the action of urokinase in the fibrin plate and/

and radioactive clot assays is mediated through its ability to convert plasminogen to plasmin, and as it is the plasmin so produced which brings about the lysis observed in the assay systems, the inhibitory effects seen may be due in part at least to the antiplasmin content of the plasma or serum. In order to demonstrate an "antiurokinase" as opposed to an antiplasmin effect in serum, the following experiment was carried out using a caseinolytic assay. Urokinase (0.25 ml. of a 25 unit/ml. solution) was incubated at 37°C. for one hour with 0.5 ml. plasminogen solution, 7 casein units/ml. After addition of 0.5 ml. of serum the caseinolytic activity induced was measured. The assay was also carried out adding the serum first and incubating the serum at 37°C. for one hour with the urokinase before adding the plasminogen solution. Twenty-five sera were examined in this way. Results are shown in figure 26, where it will be seen that in 20 of the 25 experiments, caseinolytic activity was greater when plasminogen was added first than when serum was added first. Mean caseinolytic activity (expressed as optical density readings) when serum was added first was 0.16 ± 0.04, and when plasminogen was added first

was/

was 0.18 ± 0.05. The difference is highly significant ($t = 3.905$, $p<0.001$).

Esterolytic system. Serum was also found to inhibit the esterolytic activity of urokinase on TAMe. The results are shown in table 2. Mean esterolytic activity of 1 mg. urokinase (7100 units) in 8 experiments was 161 ± 23 μM. TAMe lysed in 30 minutes. When serum, 20 μl./unit of urokinase, was added, mean esterolytic activity per mg. urokinase was in 8 experiments 111 ± 57 μM. TAMe. The difference is signficant ($t = 2.292$, $p<0.05$).

Thromboplastic or Coagulative Activity of Urokinase.

Since human urine is known to contain thromboplastic activity, it was decided to study the coagulative properties of the urokinase preparation. Urokinase in increasing concentrations was substituted for saline in a recalcification time assay, using fresh normal plasma as substrate and incorporating EACA at a concentration of 10^{-2} Molar to inhibit lysis. A progressive reduction in the recalcification time, linearly related to urokinase concentration in a semilogarithmic plot, was found and is shown in figure 27.

Substituted/

Substituted for brain extract in the one-stage prothrombin time, it was found that the urokinase preparation in a concentration of 10,000 units/ml. had a similar potency to a 1:750 dilution of the brain extract in use in the laboratory at that time.

The coagulative property of the urokinase was readily demonstrated in plasma collected and clotted in silicone tubes. In such an experiment the addition of urokinase at a concentration of 1500 units/ml. reduced the recalcification time from 450 to 175 seconds. In an experiment using Hageman plasma reductions in recalcification times were very readily observed when small concentrations of the urokinase preparation were present in the system. The figures obtained are shown in table 4, where it can be seen that urokinase in a concentration of 2 units/ml. shortened the recalcification time from 825 to 585 seconds.

Table 5 shows the effect of urokinase in the thromboplastin generation test in partially correcting for antihaemophilic globulin deficiency, Christmas factor deficiency and platelet deficiency. Urokinase was found to substitute partially for Christmas factor and antihaemophilic globulin. In the test for substitution/

substition of urokinase for platelets no correction was seen when the plasma or serum was tested at the standard dilution, but when plasma and serum were further diluted 1:4 slight correction of the abnormality resulting from platelet lack was seen.

Electrophoretic Studies. To attempt separation of fibrinolytic from coagulative activity the urokinase preparation was studied after electrophoresis. Acetate buffer, pH 4.5, 0.1 Molar, was found to give the best separation. After electrophoresis of 20 µl. of urokinase solution, 10,000 units/ml., on cellulose acetate, each paper was divided, at right angles to the origin, into three equal portions. One portion was stained with nigrosin, the other two were cut parallel with the origin into strips 1 cm. wide. Each strip from the second portion of the paper was applied to the surface of a fibrin plate and the extent of lysis after overnight incubation was assessed. To each strip from the third portion of the paper was added, in a 12 x 75 mm. test tube, 0.2 ml. of saline. Each strip was pounded with a glass rod for several minutes; the strips did not disintegrate. Thirty minutes later the ability of 0.1 ml. of the eluting fluid to shorten the recalcification/

recalcification time of normal plasma was measured. Results are shown in figure 28. In this buffer system urokinase separated into at least 4 components. The fibrinolytic activity was largely associated with slow moving components, whereas the coagulative property was more uniformly distributed.

RESULTS OF IN VIVO STUDIES.

The results of intravenous infusion of the urokinase preparation in 4 adult subjects will be described.

Subject 1. 40,000 units of urokinase were infused over 1 hour. Results are shown in figure 29 and table 6. At the end of the infusion the radioactive clot test, the fibrin plate test and the euglobulin lysis test all showed evidence of activator in the circulation. There was a slight fall in plasminogen, but no significant changes occurred in prothrombin time, thrombin time, fibrinogen, recalcification time, antihaemophilic globulin and Christmas factor assays.

Subject 2 was given 40,000 units/hour for two hours. The effects of the infusion are presented in figure 30 and table 7. At the end of 1 hour activator activity was seen in the radioactive clot assay and euglobulin lysis was accelerated; no activity/

activity was seen in the fibrin plate test. At two hours there was a 50 per cent fall in plasminogen, and the radioactive clot assay showed increased activity; lytic activity was also seen in the fibrin plate test. There were no significant changes in thrombin time, prothrombin time and fibrinogen levels. In an AHG assay clotting times fell from 171 seconds before infusion to 160 seconds at the end of infusion. Clotting time in the Christmas factor assay fell from 149 seconds before infusion to 105 seconds at the end of infusion.

Subject 3 was given 80,000 units in 1 hour. The effects of the infusion are presented in figure 31 and table 8. The infusion produced activity in the fibrin plate test and acceleration of euglobulin lysis. There were no significant changes in thrombin time, prothrombin time, plasminogen or fibrinogen. Recalcification time (cephalin added) fell from 82 to 27 seconds. The changes in AHG assay, Christmas factor assay and factor V assay are set out in table 8. In AHG and Christmas assays, apparent AHG and Christmas activities were increased - that is, clotting times were shortened. There was also a sharp fall in factor V activity.

Subject 4/

Subject 4 had an infusion of 60,000 units over 30 minutes (figure 32 and table 9). The radioactive clot assay, the fibrin plate test and the euglobulin lysis time all showed a marked increase in activator activity. Peak activity in the radioactive clot assay was 970 counts/100 seconds. A urokinase solution 20 units/ml. gave 6800 counts/100 seconds; 20 units/ml. is approximately the concentration which should have been induced in the plasma by rapid infusion of 60,000 units.

Urokinase Sensitivity Tests. These were carried out before and again 10 days after infusion. Results are shown in table 10, where it can be seen that urokinase sensitivity had not been significantly altered by the infusions.

Antigenicity. Another subject was given an intravenous infusion of 30,000 units urokinase. One week later a challenging dose of 1 mg. was injected intravenously and one further week later intradermal injection of 10 μg. produced no skin reaction. Serum obtained at the time of the intradermal test was examined by Dr. Robert Goudie for precipitating antibody, using double diffusion in an agar-gel plate. The serum was used undiluted and the antigen, urokinase, was tested at 1 and 0.1 mg./ml.

No/

No precipitins were found after seven days diffusion at 20°C.

Discussion.

In vitro testing of the urokinase preparation demonstrated that it was an effective activator of the plasminogen-plasmin system. In the fibrin plate test no activity was seen in the presence of EACA at a concentration of 5×10^{-3} Molar which inhibits plasminogen activation (Alkjaersig et al., 1959a). Esterolytic activity against a synthetic substrate was shown by the present preparation, 1 mg. of which lysed 161 ± 22 μM. TAMe in 30 minutes.

The inhibitory action of plasma and serum on urokinase, previously reported by Hansen et al. (1961) and Paraskevas et al. (1962) can readily be demonstrated in the fibrin plate test and the radioactive clot test. As all activator assays at present available measure the plasmin produced by plasminogen activation, inhibition can be due either to antiplasmin activity or to antiactivator activity; and the inhibitory effect here reported appears to be largely localised in the alpha-1 and alpha-2 globulin fractions to which Norman and Hill (1958) have demonstrated/

demonstrated that the antiplasmins belong. The caseinolytic assays in which with sera from most subjects, less casein is digested when urokinase and serum are incubated together before addition of further purified plasminogen than when serum is added after plasminogen, suggest that the inhibitory effect is due, in part at least, to direct inhibition of plasminogen activation. The finding in the esterolytic assays that some sera inhibit the action of urokinase on TAMe reinforces the view that some sera contain antiactivator activity. However as sera also contain plasminogen and antiplasmin, the results must be regarded only as strongly suggestive of the existence of antiurokinase activity in plasma or serum.

Thromboplastic or Coagulative Activity.

As shown in figure 28, the preparation is not a homogeneous protein; there can be no doubt from the experiments that the preparation can be separated into components with much lytic activity and others with less or none. There is suggestive but not final evidence that the coagulative activity may be separated from the lytic activity.

Whole normal urine is known to contain thromboplastic activity/

activity (Tocantins and Lindquist, 1947). Though the thromboplastic activity seen in vitro in the recalcification time and the thromboplastin generation test with a solution of 10,000 units/ml. is not potent (equivalent to a 1:750 solution of brain extract in the prothrombin time), definite evidence of coagulative activity was seen in vivo in subjects 2 and 3 where plasma concentrations of urokinase can have been at most 25 units/ml. In these subjects infusion of urokinase was followed by shortening of recalcification time and an apparent rise in AHG and Christmas factor levels, probably by distortion of the assay system by the non-specific coagulative activity of the urokinase. The fall in factor V activity in subject 3 may perhpas be most readily explained on the basis of factor V consumption due to some measure of intravascular coagulation.

Iatridis et al. (1960) found that a urokinase preparation had a 'Hageman-like' effect. In terms of current knowledge there would seem to be two possible explanations of the coagulative phenomena observed in the experiments presented in this chapter - either inadequate removal of the thromboplastic component of urine or a Hageman-like effect of the urokinase molecule/

molecule itself. Since there can be no doubt that the preparation used in these experiments was not a homogeneous protein, it might well be possible to separate the preparation into its several components on a large scale; the fractions obtained could then be tested for coagulative activity and lytic activity to determine whether a product can be obtained which has lytic activity but no coagulative properties.

It is perhaps surprising that with the dosage used in the present experiments in vivo it was possible to demonstrate a coagulative effect. The generation of thrombin from prothrombin is autocatalytic, and therefore prothrombin conversion, once triggered by the effect demonstrated in vitro, may have been perpetuated by the autocatalytic reaction in vivo.

Fibrinolytic Effects in vivo.

In the urokinase sensitivity tests it was found that in vitro a concentration of 200 units/ml. of plasma gave a mean lysis time of 12 minutes. In fibrinolytic therapy with streptokinase it is usually thought desirable to induce in the plasma a brisk fibrinolytic state and initial doses of streptokinase are calculated to produce plasma clot lysis times of 15 to 20 minutes/

minutes (see chapter 5). On the basis of the urokinase sensitivity tests, and assuming a plasma volume of 3 litres, a dose of about 600,000 units would be necessary to produce such activity. Hansen et al. (1961) found that at least 250,000 units of urokinase had to be administered to produce whole-plasma clot lysis times of less than three hours and Fletcher et al. (1964b) gave 1800$\pm$ 400 units/lb. body weight/hour of another commercial preparation (i.e. about 250,000 units/hour) to induce brisk plasma fibrinolytic activity. It is not therefore surprising that in the present study only slight or moderate fibrinolytic activity, substantially less than that induced in healthy subjects by strenuous exercise (table 21) was seen after infusion of 40 - 80,000 units of urokinase. In view of the coagulative activity seen in vivo in subjects 2 and 3 there must be some doubt about the wisdom of administering doses of the preparation studied adequate to produce brisk fibrinolytic activity. It may be however that with larger doses of urokinase any fibrin deposited would be so rapidly lysed, that no untoward clinical effects will be produced. This view is supported by the report of Fletcher et al. (1964b), using

large/

large doses of another commercial preparation of urokinase which is also coagulative in vitro (Dubber et al., 1965b), that no significant evidence of coagulative activity in vivo was found. Further work is necessary to produce good clinical and laboratory evidence on this important point.

CHAPTER 7.

EXPERIMENTS WITH "PLASMIN" PREPARATIONS.

For some years there has been controversy as to whether the best approach to thrombolytic therapy is with plasminogen activator or with preformed plasmin, but there now appears to be general agreement that the use of activators is to be preferred. There are superficial attractions in the use of plasmin, the enzyme which actually brings about the lysis of fibrin, but for reasons to be presented and discussed in this chapter, plasminogen activators are not only more effective in bringing about the lysis of preformed fibrin but also are less liable to produce a coagulation defect. Experiments with Actase, a commercial "plasmin" preparation which will be shown to consist largely of streptokinase, are also described.

Materials and Methods.

Activator-free plasmin was prepared by the method of Alkjaersig et al. (1958) by spontaneous activation in glycerol of a preparation of human plasminogen soluble at neutral pH (supplied by A. B. Kabi).

The/

The preparation was dialysed overnight before use.

Actase, a commercial preparation described by the manufacturers as human plasmin, was supplied by Ortho Pharmaceutical Ltd.

Other materials and methods are described in chapters 3, 4, 5 and 6.

Results.

Comparison of Streptokinase, Urokinase and Plasmin in Fibrinolytic and Coagulation Systems.

Table 11 shows the results of an experiment in which mixtures of normal plasma with saline, streptokinase, urokinase and plasmin were assessed for fibrinolytic activity and defective coagulation. It will be seen that in assays where the thrombolytic activity was measured against preformed fibrin, i.e. the fibrin plate test and the radioactive clot test, plasmin was no more effective than saline, but caused major defects in the one-stage prothrombin time and thrombin clotting time. Streptokinase and urokinase on the other hand imparted to the plasma marked lytic activity against preformed fibrin without causing a coagulation defect. Euglobulin lysis activity was less intense with plasmin than with the activators.

In Vitro Studies with Actase./

In Vitro Studies with Actase.

(i) Caseinolytic activity of Actase. One vial of Actase was reconstituted with 10 ml. distilled water and 0.5 ml. aliquots were assayed in the caseinolytic system for plasminogen (chapter 3). The assays were carried out in triplicate: identical results were obtained with (1.3, 1.3 and 1.2 units/ml.) and without (1.2, 1.3, 1.4 units/ml.) streptokinase.

(ii) Activity in the Fibrin Plate Test. Results of assay of Actase reconstituted as described in the preceding paragraph, are set out in table 12, which also shows results from a dilution curve of a standard streptokinase solution, 10 units/ml., with activator-free plasmin, 1.3 units/ml., added to simulate the plasmin content of Actase. The negligible lytic effect of the activator-free plasmin alone is also shown. In this system the streptokinase content of a vial of Actase appeared to be about 65,000 units.

Actase Infusions.

Two subjects received Actase by intravenous infusion. Subject 1 had a calculated plasma volume of 2880 ml. An 18 minute/

minute lysis time in the streptokinase sensitivity test was given by a concentration of 40 units/ml., giving a total initial "fibrinolysis-inducing" dose of 115,200 units of streptokinase. Results of infusion of the contents of 1 vial of Actase over 1 hour are shown in figure 33, where it will be seen that there was a trivial increase in plasma activator levels and a slight drop in plasma plasminogen following infusion. Fibrinogen levels and thrombin clotting times were unchanged (table 13.)

Subject 2 had a calculated plasma volume of 3000 ml. A 20 minute lysis time in the streptokinase sensitivity test was given by a concentration of 200 units/ml., indicating a total initial "fibrinolysis-inducing" dose of streptokinase of 600,000 units. Results of infusion of the contents of 2 vials of Actase over 1 hour are shown in table 14, where it will be seen that there was a slight increase in plasma activator levels as measured by the euglobulin lysis time and radioactive clot test, and there was a slight drop in plasma plasminogen levels. There were no changes in the fibrin plate test, fibrinogen levels or thrombin clotting time.

Discussion.

In the controversy as to whether the best approach to thrombolytic/

thrombolytic therapy is with activator or plasmin, evidence to support the use of activator has been provided largely by the work of Sherry and his associates (see reviews by Sherry and Fletcher, 1960, and Sawyer et al., 1961). Sherry's group (Alkjaersig et al., 1959b) first showed that preformed thrombi are relatively resistant to plasmin in the surrounding medium, but are sensitive to activator. The results presented in table 11 confirm that streptokinase and urokinase are much more effective than activator-free plasmin in imparting thrombolytic activity to human plasma. Under the specific conditions of this particular in vitro experiment, the activators induced plasma thrombolytic activity without the serious coagulation defect produced by a thrombolytically inert concentration of plasmin, which was however able to reduce plasma fibrinogen levels and accelerate euglobulin lysis activity. The disparity in assay of plasma-plasmin mixtures between the euglobulin lysis results on the one hand and the fibrin plate tests and radioactive clot tests on the other, presumably rests on the incorporation of plasmin within the clot and in close proximity to fibrin in the euglobulin tests, but its presence in the surrounding medium/

medium only and not incorporated with the fibrin, in the other tests. This experiment supports the concept that lysis results more readily from activation of intrinsic clot plasminogen than from surface action of plasmin. Furthermore, as pointed out by Alkjaersig et al. (1959b), if such a mechanism were operative, the rate of lysis of thrombus would not only be a function of the concentration of activator surrounding the thrombus, but also as shown by Alkjaersig et al. (1959b) and supported by the observations displayed in figure 15, a function of plasminogen concentration within the clot.

On the basis of the postulated physiological mechanism for fibrinolysis (chapter 2) a small amount of plasmin will on infusion be neutralised by antiplasmin, and it might be expected that to produce with plasmin a thrombolytic effect in the plasma surrounding a thrombus, sufficient plasmin must be infused to neutralise circulating antiplasmin; that is, it will be necessary to induce a hyperplasminaemic state with all its deleterious consequences. Arguments in favour of plasmin as a therapeutic weapon have been put by, among others, Cliffton (1957), Moser (1959) Ambrus and his group (Sokal et al., 1958) and Amris and his associates (Amris and Amris, 1963; Amris et al., 1963).

The/

The Ambrus group (Ambrus and Markus, 1960) claims that the plasmin-antiplasmin complex formed on infusion of plasmin is readily dissociable and constitutes a reservoir of plasmin which may be released at the site of a thrombus, but no good evidence has been produced to support the relevance of this concept to thrombolytic therapy in man. Most claims of clinical benefit with plasmin therapy relate to the use of Actase (e.g. Moser, 1959) which as discussed below, owes the greater part of its activity to its streptokinase content, and Johnson (1964) has found that activator-free plasmin, when infused in doses sufficient to produce a serious coagulation defect, is ineffective in producing in vivo in man the lysis of experimental thrombi which are readily lysed with streptokinase and urokinase.

The preparation of Actase used in the experiments described in the present chapter had a plasmin content of about 13 units/vial, and a streptokinase content of about 65,000 units/vial. In the fibrin plate test, its activity appeared to be almost entirely due to its content of streptokinase, since an activator-free plasmin preparation tested in the same concentration as the plasmin content of/

of Actase solution produced negligible lysis. The total content of plasmin present in the Actase vial might have been expected to have been produced from about 7 ml. of normal plasma, and to have been neutralised by the antiplasmin content of 3 or 4 ml. of normal plasma; accordingly for practical purposes Actase should be regarded as a streptokinase preparation.

The dosage of Actase suggested by the manufacturers was one or two vials infused over 1 hour on any one day. Subject 1, who was given 1 vial of Actase, had an initial dose level for streptokinase calculated at 115,200 units, significantly in excess of the streptokinase content of the 1 vial of Actase which he was given, and subject 2 had an initial dose level for streptokinase calculated at 600,000, much in excess of the streptokinase content of the two vials of Actase. As might have been anticipated in the circumstances, trivial and barely detectable levels of fibrinolytic activity were found in the plasmas of these two subjects after Actase infusion. Fletcher et al. (1960) examined an Actase preparation available in the United States at that time, and found a much lower concentration of streptokinase, about 6500 units/vial than/

than that in the preparation used in the present studies. These authors found that infusion of the American preparation, also in the manufacturers' recommended dosage, did not produce any fibrinolytic response.

Despite extensive publication of clinical results obtained with Actase, in which it was regarded as a plasmin preparation, (e.g. Symposium, Angiology, 1959; Moser, 1958; Sussman and Fitch, 1958) it is clear that Actase must be regarded for all practical purposes as a preparation of streptokinase and that all the problems associated with streptokinase, especially the need for initial dosage calculation, are present with Actase.

CHAPTER 8.

IN VIVO STUDIES WITH COMPLAMIN, A NICOTINIC ACID - THEOPHYLLINE ESTER, AS A FIBRINOLYTIC AGENT.

Though the feasibility of thrombolytic therapy with fibrinolytic enzymes has been demonstrated (see chapter 2 for a brief review of the literature), the expense involved in the preparation of suitable enzymes and the difficulties in laboratory control of enzyme therapy have stimulated a search for non-enzymatic agents which when administered might stimulate production or release of fibrinolytic enzymes in the body. One such substance is nicotinic acid, which, fibrinolytically inert in vitro, was discovered by Meneghini and Piccinini (1958) and Weiner et al. (1958) to have the property of inducing intense but transient fibrinolytic activity when injected intravenously in man. Because of the short duration of the fibrinolytic response and the rapid development of resistance, attempts have been made to provide modified preparations of nicotinic acid with enhanced activity, and it is the purpose/

purpose of this chapter to describe studies with one such preparation - 3-(methyloxyethylamino)-2-oxypropyl theophylline nicotinate - Complamin.

Nicotinic acid owes its fibrinolytic activity to its ability to increase plasma activator levels (Sawyer et al., 1960a) though the mechanism by which it does so is not known. In the present assessment of Complamin as a fibrinolytic agent, factors studied after its administration included plasma fibrinolytic activity; plasminogen and fibrinogen levels; and thrombin clotting time and one-stage prothrombin time.

Materials and methods.

Complamin was made available by Edinburgh Pharmaceutical Industries, as ampoules containing 300 mg. in 2 ml., and 150 mg. tablets. The minimum dosage of the intravenous preparation suggested by the manufacturers is 300 mg. t.i.d; and of the tablets 150 mg. t.i.d.

The laboratory methods used are described in chapters 3 and 4.

Results.

Single intravenous injections. Seven subjects (healthy colleagues) were/

were given a single intravenous injection of Complamin, 1 ml. of solution (i.e. 150 mg.) diluted in 10 ml. of saline, being injected over 60 seconds. The response in euglobulin lysis activity is shown in figure 34 where it will be seen that 5 minutes after injection there was a rise in euglobulin lysis activity from a mean pre-infusion level of 1.2 units to a mean level of 7.9 units. Euglobulin lysis activity was almost back to normal at 30 minutes. There was a slight drop in mean fibrinogen and plasminogen levels (tables 15 and 16) but there were no changes in thrombin clotting times or prothrombin times. The response of the same subjects on another day to exercise (5 minutes skipping with a heavy rubber tube) is also shown in figure 34, where a similar pattern of euglobulin lysis activity is seen. The peak rise is somewhat less than that seen with Complamin but the general pattern of rise and fall of activity is closely parallel. With exercise, there were no changes in plasminogen or fibrinogen levels and thrombin and prothrombin times (tables 17 and 18).

Repeated intravenous injections. The response in a healthy colleague to three intravenous injections of 150 mg. Complamin given at hourly intervals is shown in figure 35, where it will be seen that there/

there was a progressive reduction in euglobulin lysis activity induced by the second and third injections. Another healthy subject was given a daily intravenous injection of Complamin for five days. The euglobulin lysis response is shown in figure 36, where again a progressive reduction in response was seen.

Intravenous Infusions. An intravenous infusion of Complamin was given to two subjects with very similar results. The effects of the infusion in one of the subjects are shown in figure 37. Over the first 30 minutes 150 mg. was infused; over the next 30 minutes 300 mg; and over the last 30 minutes 600 mg. As detected by the radioactive clot method, euglobulin lysis and fibrin plate test, infusion produced a marked, but transient increase in plasma thrombolytic activity, which fell off rapidly despite progressive doubling of the infusion rate. There were no significant changes in plasminogen, fibrinogen, thrombin time or prothrombin time. The data for the second subject are presented in table 19. In a third subject, 150 mg. of Complamin infused over 1 hour produced a rise in euglobulin lysis activity from 0.8 units before infusion to 1.6 units after 30 minutes and 1.2 units at the end of the infusion. One hour later the euglobulin lysis activity was 1.1 units.

Oral Administration/

Oral Administration.

Complamin tablets were given to 4 normal adult male subjects. One subject was given 2 tablets only, the others were given 2 tablets on one day and 4 tablets several days later. The results are shown in table 20, where it will be seen that with 2 tablets there was a minor increase in euglobulin lysis activity in subjects 1 and 3, but no change in subjects 2 and 4. With four tablets, subject 2 showed no change. Euglobulin lysis activity doubled in subject 4 and showed a significant increase in subject 3. With 2 tablets, where a rise in euglobulin lysis activity was seen, it was noted in the 30-minute samples but had subsided at 60 minutes. With 4 tablets the effect seemed to be somewhat longer lasting, being noted in both subjects 2 and 4 at 120 minutes.

Complamin and Exercise.

In order to study the inter-relationships between the fibrinolytic responses to Complamin and to exercise, two healthy subjects participated in the following experiments, each experiment being carried out separated from the others by an interval of several days.

(a) 5 minutes exercise, followed in 30 minutes by 5 minutes exercise

(b)/

(b) 5 minutes exercise, followed in 30 minutes by 150 mg. Complamin intravenously.

(c) 150 mg. Complamin intravenously, followed in 30 minutes by 150 mg. Complamin intravenously.

(d) 150 mg. Complamin intravenously, followed in 30 minutes by 5 minutes exercise.

Response of euglobulin lysis activity is shown for experiments (a) and (b) in figure 38 and for experiments (c) and (d) in figure 39.

It will be seen that for both subjects, if exercise was followed by exercise, or Complamin by Complamin, the second stimulus provoked a much reduced euglobulin lysis response, whereas if exercise was followed by Complamin, or Complamin by exercise, a full response was observed. It may be that the reduced response on the second occasion to repeated exercise is due to fatigue and a less vigorous performance of the exercise.

Discussion.

The results presented in this chapter demonstrate that Complamin/

Complamin, like nicotinic acid, when given rapidly intravenously, produces a marked but transient rise in plasma fibrinolytic activity, comparable in duration (not more than 30 minutes) and intensity to that produced by five minutes strenuous exercise. The fibrinolytic response falls off rapidly with injections repeated at hourly intervals and at 24-hour intervals. The response was also transient when Complamin was given by intravenous infusion, contrary to the observations of Amery et al. (1962) who found a response for an hour or more when Complamin was given in this way. Given by mouth, Complamin either, in some subjects, failed to produce a fibrinolytic response or, in other subjects, produced a trivial effect.

The mechanism by which Complamin produces increased levels of fibrinolytic activity in the circulation is not clear, though it is shared by some other vaso-active drugs, e.g. adrenaline and pitressin (von Kaulla, 1963b). Histamine, however, which produces vaso-dilation, does not cause increased plasma fibrinolytic activity (Weiner et al., 1959), and there is no correlation between the intensity of the flush produced by Complamin and the degree of fibrinolytic activity induced. Kwaan et al. (1958) have/

have shown that fibrinolytic principles can be released from the vessel wall in response to paravenous injection of adrenaline, serotonin and acetyl choline in the same and in the opposite arm, and it may be that a neural pathway, stimulated by Complamin and other substances, can bring about plasminogen activator release into the circulation.

The short duration of the fibrinolytic response to Complamin is due in part to the rapid clearance of fibrinolytic activator from the circulation by the liver (Fletcher et al., 1964a) and also to the rapid development of a refractory state to the drug. The experiments in which Complamin given after exercise, or exercise taken after Complamin, produce a full fibrinolytic response, suggest that the failure to produce a repeated or sustained response to Complamin is not due to exhaustion of the body's fibrinolytic resources, but to resistance to the action of the drug. The experiments suggest that it may be possible to find a drug to produce a sustained release of plasminogen activator into the circulation.

The failure in the present experiments to see significant changes in plasminogen, fibrinogen, thrombin clotting time and one-stage prothrombin time is probably due to the short duration of/

of the increase in activator levels produced in healthy subjects by Complamin injection. Fletcher et al. (1964a), who gave nicotinic acid by intravenous injection to patients with cirrhosis of the liver, in whom there is impaired clearance of plasminogen activator from the circulation, and hence a persistence of high levels of activator for much longer than in normal subjects, found evidence of hyperplasminaemia including a drop in plasminogen and fibrinogen values and a rise in the thrombin clotting time and in one-stage prothrombin time.

Conclusions. In thrombolytic therapy with streptokinase, where intense fibrinolytic activity (e.g. activity at least as great as the maximum response seen to Complamin in any subject) can readily be achieved and maintained, therapy may need to be continued for 48 hours or more (e.g. Johnson and McCarty, 1959; Verstreate et al., 1963; McNicol et al., 1963) to remove thrombus and restore vascular patency. It would therefore appear most improbable that therapy with Complamin would be of benefit in the treatment of acute thromboembolic occlusive vascular disease. On the other hand, as the present studies demonstrate, Complamin clearly does not exploit the full fibrinolytic resources of the body and a search for further/

further modifications of nicotinic acid or other pharmacological substances which would produce a sustained fibrinolytic response, would seem worthwhile. In particular, a non-toxic substance active when given by mouth, might by long term administration to increase physiological fibrinolytic activity, have an important role in the prevention of vascular thrombosis.

CHAPTER 9.

PLASMINOGEN-PLASMIN SYSTEM ACTIVITY IN SURGICAL OPERATIONS.

Fibrinolytic activity in the blood arising in association with surgical operations was first noted by Macfarlane (1937) and has since been frequently studied. The increased fibrinolytic activity may be partly a physiological non-specific response to stress, and anxiety with adrenalin release may also play a part (Latner, 1947). Other possible factors include division of adhesions and handling of lung, with release of tissue activator into the circulation (Marchal et al., 1960), reduced pH and hypoxaemia (von Kaulla and Swan, 1958) and the effects of anaesthesia (von Kaulla, 1947).

Sudden death is often followed by intense fibrinolytic activity (Berg, 1950) and Schneck and von Kaulla (1961) have observed increased plasma fibrinolytic activity associated with syncope or collapse during procedures such as pneumoencephalography; it may be that hypotension and cardiac arrest predispose to the development/

development of pathological plasminogen-plasmin system activity.

In view of the obvious potential use of tissue or other endogenous activators as therapeutic agents, an account is given in the present chapter of the activity of the fibrinolytic enzyme system in 10 patients before, during and after a major abdominal operation, and the biochemical features found in two patients with a severe haemorrhagic state associated with increased plasma fibrinolytic activity are also described.

Materials and methods.

Whole clot lysis times were estimated as described by McNicol and Douglas (1964).

Whole blood clotting times were determined by the method of Lee and White (1913).

Other methods were as described in chapters 3 and 4.

Results.

1. Fibrinolytic Activity in a Group of Patients Undergoing Excision of the Rectum. In a group of 10 patients in whom combined abdomino-perineal excision of the rectum was performed (surgeon - Mr. M. K. Browne), blood samples were obtained immediately before/

before the administration of the anaesthetic, during the operation at a time when much tissue dissection was taking place, and again as soon as the operation had been completed. In no case was there a clinical haemostatic defect. For each sample, a fibrin plate test, euglobulin lysis time, plasminogen assay, fibrinogen assay and thrombin clotting time were carried out. Results are shown in figure 40, where it will be seen that during surgery there was a marked increase in activator activity as measured in the fibrin plate test and the euglobulin lysis time; pre-operative levels had largely been regained in the samples obtained after surgery. There were also minor falls in plasminogen and fibrinogen levels, and a slight rise in the thrombin time.

2. Individual Patients with a Fibrinolytic Coagulation Defect during Surgery.

Patient 11. During excision of the rectum in a 30 year old woman with ulcerative colitis (surgeon - Mr. A. A. Bonar), oozing of blood was noted to be excessive and bleeding from the perineal wound continued after operation despite repacking. Seven hours after surgery the patient had a cardiac arrest. Resuscitation was by open heart massage and bleeding in the chest and from the perineal wound/

wound increased despite fibrinogen infusion (figure 41). At this time the blood was apparently incoagulable, but clotting occurred with thrombin in 93 seconds. Whole clot lysis time was 30 minutes. Plasminogen was assayed as zero and fibrinogen 40 mg./100 ml. The fibrinolytic inhibitor epsilon aminocaproic acid (EACA) was given intravenously as shown in figure 41; after a loading dose of 5 gm., 1 gm./hour was given for 5 hours, then 0.5 gm./hour for a further 5 hours. Transfusion requirements from the beginning of operation until EACA was given were 30 pints. Within 5 minutes of giving EACA bleeding ceased and did not recur, and the patient's course thereafter was uneventful. The progressive improvement in the laboratory tests can be seen in figure 41.

Patient 12. Immediately after spleno-renal anastomosis and splenectomy for haematemesis due to hepatic cirrhosis and portal hypertension (surgeon - Professor W. A. Mackey) this patient, a 52 year old woman, was found to have a haemorrhagic state with incoagulable blood. Laboratory study at this time (figure 42) showed increased thrombolytic activity in the fibrin plate test but euglobulin lysis was normal, presumably because of plasminogen depletion which was also found. The whole blood clotting/

clotting time was infinite. After treatment with EACA (10 gm. i.v.) the haemorrhagic state was corrected almost immediately. The improvement in the laboratory findings can be seen in figure 42.

Discussion.

The findings presented in figure 40 confirm that increased plasminogen-plasmin system activity occurs during a major surgical operation. In the present series of patients, studied in association with abdomino-perineal excision of the rectum, a procedure involving much tissue trauma, increased levels of plasminogen activator in the circulation were associated with a slight fall in mean plasminogen and fibrinogen levels and a trivial coagulation defect; this had corrected itself by the end of operation. As none of the patients had a clinically manifest defect of haemostasis, it would seem probable that the fibrinolytic activity seen was a physiological response to the stresses of operation, pre-operative anxiety and the administration of an anaesthetic, though the release of activator from damaged tissues may also have contributed.

In/

In patient 11 on the other hand, in association with the same operation there developed a profound and clearly pathological fibrinolytic state, with a life-threatening haemostatic defect. Although plasminogen was assayed as zero in the caseinolytic technique, a little plasminogen was presumably present to give the very rapid whole clot lysis time of 30 minutes. As plasma fibrinogen was only 40 mg./100 ml., trace quantities of plasminogen could presumably bring about its rapid lysis in the presence of large amounts of activator. Alternatively, the rapid clot lysis may have been due to free plasmin which had become bound to antiplasmin before the assay was carried out. The striking clinical response to EACA therapy confirms the role of pathological fibrinolytic activity in the genesis of the haemorrhagic state.

In patient 12, also with a haemorrhagic state associated with increased fibrinolytic activity and rapidly controlled by EACA therapy, large amounts of activator were found in the fibrin plate test, but because of the low plasminogen level, with a relatively normal fibrinogen, euglobulin lysis activity was not accelerated.

Table 21 summarises biochemical data from other chapters in/

in this thesis relating to selected subjects in whom fibrinolytic activity was induced in vivo by streptokinase, urokinase, Complamin or exercise, together with some of the findings from the present chapter. It will be seen that urokinase, Complamin, exercise, and surgery in most subjects, can produce increased fibrinolytic activity without significant abnormalities in plasminogen, fibrinogen or thrombin time, whereas operation in some patients, and streptokinase probably always produce fibrinolytic activity associated with reduction in plasminogen and fibrinogen and a significant coagulation defect. The duration of the rise in plasma activator levels may be a factor in the differing responses seen; e.g. the urokinase infusion was only for 30 minutes, and the fibrinolytic responses to Complamin and exercise were transient. The duration of the fibrinolytic response in the 10 subjects shown in figure 40 was not determined. On the other hand, Sawyer et al. (1960b), comparing the fibrinolytic with the fibrinogenolytic properties of activators, have shown that for a given rate of fibrinolysis, a lesser fall of plasma plasminogen and less fibrinogenolysis is produced by urokinase than by streptokinase, and there may be important differences of this type between the action/

action of plasma activator and tissue activator. In any event, it is clear from patients 11 and 12 that endogenous activator can produce fibrinolytic activity associated with a severe haemorrhagic state, and that purified tissue activator cannot be expected, if made available for therapeutic use, to be free from risk of haemostatic failure.

CHAPTER 10.

THROMBOLYSIS STUDIED IN AN ARTIFICIAL CIRCULATION, WITH THROMBI PREPARED IN VITRO IN A CHANDLER'S TUBE.

In the investigation of the plasminogen-plasmin system, the human substrates most commonly employed have been whole blood clots, plasma clots and euglobulin clots and while from these systems has emerged most of the present knowledge of fibrinolysis, it is clear that such test tube clots are very different in structure and composition from the pathological thrombi which are the in vivo target of thrombolytic agents.

The structural differences between clot and thrombus have recently been reviewed by Poole (1964a), who pointed out that a clot has a uniform structure, with the formed elements distributed at random in the fibrin network, whereas a thrombus consists of two types of material; white thrombus, consisting largely of agglutinated platelets, and red thrombus, consisting of fibrin and red blood cells. Chandler (1958) demonstrated that it was possible to produce artificial thrombi in vitro by making blood/

blood flow round and round a closed circular loop of plastic tubing; when solidification occurs in this system, a thrombus is formed with a platelet head and a fibrin tail (figure 43); histologically Chandler thrombi very closely resemble pathological thrombi (figure 44).

The object of thrombolytic therapy is to increase plasma thrombolytic activity by infusion of plasminogen activators and so to accelerate the physiological process of thrombolysis. It is the purpose of this chapter to describe experiments in which factors affecting the process of thrombolysis in vitro were studied using Chandler-type thrombi as substrate, in the belief that the results obtained may have more relevance to the in vivo situation than experiments with blood clots. Thrombi, trace labelled with radioactive iodine-tagged fibrin, were made in a Chandler's tube and transferred to an artificial circulation where they were perfused with streptokinase; patterns of radioactivity distribution during perfusion were studied.

Materials and methods.

Streptokinase. A highly purified preparation was supplied by A. B. Kabi./

Kabi.

Urokinase. Leo urokinase was used.

Non-esterified fatty acids were estimated by the method of Dole (1956). Values above 100 μ.eq./litre were regarded as elevated.

Triglycerides were measured by a modification of the method of van Handel and Zilversmit (1957). Values above 200 mg./100 ml. were regarded as abnormal.

Cholesterol was measured by the method of Zlatkis et al. (1953). The upper limit of normal was accepted as 275 mg./100 ml. Lipid assays were performed by Dr. B. M. Rifkind.

Preparation of artificial thrombi. Artificial thrombi were made by a modification of the method of Chandler (1958). Whole blood was used, 20 ml. being collected with a plastic syringe and immediately transferred to a 'Chandler's tube' made with 27.5 inches of transparent vinyl plastic tubing, internal diameter 12.5 mm. (Portland Plastics, Ltd., Kent). At the same time a trace of ^{131}I tagged fibrinogen was added (usually about 0.1 ml. to give 1000 counts/second) and the tubing, made into a continuous loop with a nylon adaptor, was placed on the turntable of a blood cell suspension mixer revolving at 28.5 r.p.m. The tube was left to revolve for one hour at room temperature when the resulting thrombus/

thrombus was decanted and washed three times with saline. The radioactivity of the thrombus was then counted and it was transferred to the perfusion circulation.

Perfusion circulation. The perfusion circulation (figure 45), devised by Mr. W. H. Bain, was made up with 'Tygon' plastic tubing, internal diameter 0.25 inch, with a length of narrow tubing, internal diameter 0.0625 inch as a collateral bypassing the site of insertion of the thrombus. A sigmamotor pump (Sigmamotor Pump Co., Middleport, N.Y.) was used at a flow rate of 140 ml./minute, giving a mean upstream pressure of 80 mm. of mercury. The perfusion fluid was 0.9 per cent saline, the total volume being 200 ml., and perfusion was carried out at a temperature of about 28°C. Thrombi were inserted into the circulation through a break in the tubing to sit on a stainless steel gauze basket (mesh size 120). Until lysis had taken place, occlusion by the thrombus of the main channel of the circulation was usually almost or quite complete and most of the flow was deviated through the collateral circulation, but as lysis occurred, the flow through the main channel progressively increased.

Other methods /

Other methods used are described in chapters 3 and 4.

Results.

In each case the thrombus was perfused for 24 hours and 1 ml. samples were taken from the circulation at 0, 4, 8, 12, 20 and 24 hours. After centrifugation for 10 minutes at 1000 g., radioactivity in 0.5 ml. of supernatant was counted. At the conclusion of the experiment, the wire gauze basket with any thrombus still remaining in it was cut out and radioactivity counted. The perfusing fluid was filtered and non-filterable radioactivity counted. Radioactivity in solution, at the site of thrombus insertion and non-filterable (i.e. in fragments) was expressed in each case after correction for radioactivity decay, as a percentage of initial thrombus radioactivity.

'Normal' Thrombi. Thrombi were made from seven healthy colleagues, aged 21 - 42 years, who all had normal fibrinogen and plasminogen assays (table 22). Figure 46 shows the white head and fibrin tail of a thrombus prepared in this way from a patient with hyperlipidaemia (patient 3a, table 27), and figure 47 illustrates the striking differences between histological structure of head and tail in a thrombus prepared from a normal subject.

Autoradiography/

Autoradiography of the platelet head was carried out and while radioactivity was associated with platelet clumps, resolution was not adequate to determine whether the radioactivity was present in the spaces between individual platelets (histological preparations made and reported by Dr. F. Walker).

The pattern of release of radioactivity into solution during perfusion with streptokinase is shown in figure 48, and table 23 shows mean values and standard deviations for percentage radioactivity in solution, in fragments and at the site of insertion after perfusion for 24 hours. It will be seen that the rate of release of radioactivity had fallen off after 12 hours and that only a mean of 62 per cent of the radioactivity was in solution at 24 hours, when rather more than 25 per cent was present as non-filterable fragments. A proportion of the fragments appeared to be fibrin, but the majority (figure 49) were yellowish-white millet seed sized granules the histological appearances of which, shown in figures 50 and 51, are compatible with an origin from platelet aggregates. Tables 23 and 24 and figure 48 also show that in the absence of streptokinase in the perfusing fluid, thrombi showed less than 10 per cent lysis in 24 hours.

Effect of EACA. This is shown in figure 52 and table 23 where it

will/

will be seen than when EACA was present in the circulation in a concentration 10^{-3} Molar, the clots were as stable as when no streptokinase was present in the circulation, there being only 7 - 8 per cent soluble radioactivity after perfusion for 24 hours.

Effect of Plasminogen Enrichment. Plasminogen enriched thrombi were made from two normal subjects with plasma plasminogen levels of 2.4 and 2.6 casein units/ml. by adding to the Chandler's tube with the blood and ^{131}I tagged fibrinogen, respectively 44 and 48 units of plasminogen in saline, to give plasma plasminogen levels about three times normal. Results of perfusion with streptokinase of the two plasminogen enriched thrombi are shown in tables 23 and 24,and figure 53 shows the results from one of the experiments. Plasminogen enrichment resulted in very rapid and almost complete lysis. In both experiments, after only 4 hours perfusion with streptokinase, over 90 per cent of the radioactivity was in solution and only 4 and 7 per cent respectively was present in fragments in the circulation.

Effect of Platelet Count on Susceptibility to Lysis. Thrombi were made/

made from two patients with a low platelet count (patient (a), refractory anaemia, platelet count 12,500/c.mm., and patient (b), idiopathic thrombocytopenic purpura, platelet count 37,500/c.mm.) and from two patients with thrombocythaemia (patient (c), platelet count 1,250,000/c.mm. and patient (d), platelet count 1,150,000/c.mm.). Results of perfusion with streptokinase are shown in tables 24 and 26 and for patients (a) and (c) patterns of release of soluble radioactivity are shown in figure 53. With the thrombocytopenic thrombi, lysis was much more complete than with 'normal' thrombi, and less radioactivity was present in fragments. For both patients (a) and (b), the percentage of radioactivity in solution after perfusion for 24 hours was more than 2 standard deviations above the mean value for the 'normal' thrombi, and the percentage of radioactivity in fragments was more than 2 standard deviations below the mean of the normals. Figure 54 shows the histological appearance of the head of the thrombus prepared on another occasion from patient (a) at a time when the platelet count was 22,000/c.mm. The appearances resemble those of a simple blood clot and are in striking contrast to those seen in figure 47 which illustrates the appearance of a 'normal'/

'normal' platelet head, the head of the thrombocytopenic thrombus lacking the normal coral-like platelet aggregates and having a much more loose, open structure.

Both the thrombocythaemic thrombi were found to be resistant to lysis. The percentage of radioactivity in solution at 24 hours was more than 2 standard deviations below the mean 'normal' value, and the percentage radioactivity in fragments was more than 2 standard deviations above mean 'normal' values.

Hyperlipidaemic Thrombi. Thrombi were made from 4 patients with elevated blood lipid levels. Details of the lipid abnormalities present and of conventional fibrinolytic studies, are shown in table 27. Patient 1, a man aged 30 years, had idiopathic hypercholesterolaemia. Patient 2, a man aged 56 years, also had idiopathic hypercholesterolaemia with xanthomatosis. Patient 3, a girl of 15 years, had severe diabetes mellitus and Hashimoto's thyroiditis. When sample 3a was taken, she was in ketoacidosis. Sample 3b was taken 14 days later when her diabetic state was under control and non-esterified fatty acid, cholesterol and triglyceride levels had fallen substantially, though cholesterol and triglyceride levels were still above normal. The diagnosis of Hashimoto's thyroiditis was/

was only confirmed after sample 3b had been taken, and the patient was not receiving thyroxine at the time of study. Patient 4, a man of 22 years, had a nephrotic syndrome. It will be noted (table 27) that all four patients had normal plasminogen assays. Patients 2 and 3 also had euglobulin lysis times within normal, but patients 1 and 4 had a marked reduction in euglobulin lysis activity. All four patients, including the two with euglobulin lysis times within the normal range, yielded thrombi which were much more resistant to lysis than those prepared from normal subjects (figure 55 and table 28). The percentage of soluble radioactivity at 24 hours with all the hyperlipidaemic thrombi was more than 3 standard deviations below the mean value obtained with 'normal' thrombi.

Stability of Streptokinase.

During perfusion, there was a definite fall in plasminogen activator activity in the perfusing fluid. For example, in one experiment (carried out by Dr. B. Sweet) in which the perfusing fluid was assayed for activator activity in a clot lysis system incorporating purified fibrinogen and plasminogen, lysis times rose from 3 minutes with fluid removed at the beginning of the perfusion/

perfusion, to 9 minutes with fluid removed after 24 hours, indicating a marked loss of streptokinase activity in the perfusion fluid. However, failure to obtain complete lysis of the Chandler thrombi cannot be attributed to this decline in the lytic activity of the perfusing fluid, for as shown in table 29, addition to the perfusing fluid of further streptokinase, 200 units/ml., or urokinase 200 units/ml. after 24 hours perfusion produced no increment in soluble radioactivity.

Discussion.

The data presented indicate that perfusion with streptokinase of artificial thrombi with a histological structure similar to that of pathological thrombi, results in substantial but not complete thrombolysis. About two-thirds of the radioactivity of thrombi trace-labelled with ^{131}I fibrin was found in solution after about 24 hours in the artificial circulation, and only 10 per cent of the radioactivity remained at the site of insertion of thrombi into the circulation. About 30 per cent of the radioactivity was found in the circulation after perfusion as particulate matter, many of the fragments having a histological structure suggesting their composition/

composition to be from platelet aggregates. Support for the contention that the unlysed particulate radioactivity in the circulation was associated with platelet aggregates is obtained from the experiment with blood from patients with low platelet counts in which lysis was much more complete and little radioactivity was present in fragments, and blood from patients with high platelet counts in which lysis was much less complete and much more radioactivity was present in fragments.

The nature of the substance which is responsible for maintaining platelet aggregation is unknown (Poole, 1946b), but the experiments described in this chapter can be interpreted as suggesting that fibrin may contribute to the maintenance of platelet aggregation. With the plasminogen enriched thrombi, lysis was rapid and almost complete. Little radioactivity was present as particles in the circulation. Platelets are known to contain significant amounts of antiplasmin (Johnson and Schneider, 1953), which may neutralise plasmin and protect fibrin in the immediate vicinity, and plasminogen enrichment of thrombi may provide enough plasmin to swamp the antiplasmin mechanism and make available free plasmin to lyse the fibrin holding platelets aggregated, hence the/

the small amount of radioactivity present in particles after streptokinase perfusion of plasminogen enriched thrombi.

In view of the structural resemblance of the thrombi used in these experiments to pathological thrombi, it would appear possible that a similar pattern of thrombolysis with release of platelet-rich particles into the circulation, may pertain during thrombolysis in vivo. While release of platelet emboli may not be significantly deleterious in the treatment of peripheral vascular occlusion, such a phenomenon would be associated with obvious and important hazards in the cerebral circulation. This potential danger lends weight to the present reluctance, based on the risk of converting ischaemic to haemorrhagic infarcts, to use thrombolytic agents in the treatment of cerebral thrombosis or cerebral embolism.

Nilsson et al. (1961b) have suggested that EACA, a potent competitive inhibitor of plasminogen activation, can be given with streptokinase in thrombolytic therapy, with elimination of plasma proteolytic activity, but unimpaired thrombolytic activity. The results of the experiments with EACA presented here would suggest that it is highly probable that EACA if given with streptokinase would inhibit/

inhibit thrombolytic activity in vivo.

The utility of this approach to the study of thrombolysis is also illustrated by the experiments with hyperlipidaemic blood. As might be expected from the present confusion as to the role of lipids as inhibitors of fibrinolysis (Howell, 1964), in two of the hyperlipidaemic patients plasma fibrinolytic activity as assessed by the euglobulin lysis method was normal and in two patients was much reduced, a finding which may possibly be related in part to elevated fibrinogen levels. However blood from all four yielded thrombi which were resistant to streptokinase and it is possible that the present technique can highlight abnormalities in response to lytic agents which are poorly or inconsistently reflected in other test systems.

Conclusions.

The technique described in this chapter of studying factors affecting thrombolysis, by perfusing with streptokinase in an artificial circulation thrombi with a histological structure closely resembling that of pathological thrombi, produced in vitro in a Chandler's tube, may have more relevance to in vivo response to thrombolytic agents than in vitro experiments with blood clots. The/

The results presented suggest that when thrombi are exposed to streptokinase the fibrin component may be largely dissolved, but platelet emboli, possibly cemented by fibrin protected from lysis by platelet antiplasmin content, may be released into the circulation. The general applicability of the technique in the study of thrombolytic mechanisms has also been illustrated by its use to demonstrate the inhibitory effects of circulating EACA and of hyperlipidaemia on thrombolytic activity.

CHAPTER 11.

SUMMARY AND CONCLUSIONS.

Over the past ten years, with the demonstration of the feasibility of treatment of thrombo-embolic occlusive vascular disease with fibrinolytic enzymes, there has been intensive study of the components and functions of the fibrinolytic enzyme, or plasminogen-plasmin system. The studies reported in this thesis were carried out in an effort to increase understanding of the plasminogen-plasmin system, and of the consequences of plasminogen activation, with a view if possible to the eventual routine use of fibrinolytic enzymes in the treatment of patients with vascular occlusion.

The Plasminogen-Plasmin System.

The plasminogen-plasmin system may have a function in vivo complementary to the coagulation system in maintaining an intact patent vascular tree. According to this hypothesis, the coagulation system seals with a haemostatic fibrin plug any deficiencies which may appear in the vascular endothelium and the/

the fibrinolytic mechanism removes such fibrin deposits after they have served their haemostatic function and endothelial repair has occurred.

The main components of the plasminogen-plasmin system are plasminogen, plasmin, activators and inhibitors. Plasminogen, a normally inert plasma globulin, is converted by activators to plasmin, a proteolytic enzyme which can digest many proteins including fibrin, fibrinogen, factor V, prothrombin and antihaemophilic globulin. Activators of plasminogen include tissue activators; a plasma activator; a urinary activator named urokinase which may represent in part at least excreted plasma activator; bacterial activator, e.g. streptokinase which is produced by certain strains of haemolytic streptococci; and substances, fibrinolytically inert in vitro, which when injected intravenously produce an increase in plasma activator levels, e.g. bacterial pyrogen and nicotinic acid.

According to Sherry et al. (1959a) under physiological conditions inhibitory mechanisms restrict plasmin to digestion of fibrin. In plasma there is more antiplasmin than plasminogen, that is than potential plasmin, and if plasminogen activation takes place/

place relatively slowly, then as plasmin is produced, it is neutralised by antiplasmin, and so plasma proteins are protected from digestion. In thrombi on the other hand, where plasminogen concentrations are relatively high and effective antiplasmin concentrations are said to be relatively low, activator diffusing into thrombus activates plasminogen in close proximity to the fibrin which is digested - the process of thrombolysis.

The necessity for the antiplasmin mechanism, which in vivo normally confers substrate specificity on plasmin and protects plasma proteins from digestion can be appreciated if the effects of free plasmin in the circulation - hyperplasminaemia - are considered. Hyperplasminaemia results in a grave coagulation defect, due in part to depletion of coagulation factors - for example, fibrinogen, factor V, antihaemophilic globulin - and in part to defective fibrin polymerisation resulting from the presence in the circulation of polypeptides arising from the digestion of fibrinogen by plasmin; these interfere with the normal conversion of fibrin monomer to fibrin polymer. Accelerated lysis of such clots as do form increases the haemostatic defect. Full plasmin-antiplasmin union takes place relatively slowly and

a/

a temporary overwhelming of the antiplasmin mechanism may occur when the sudden arrival of large amounts of plasminogen activator brings about quick and massive conversion of plasminogen to plasmin, with the appearance of free plasmin in the circulation. Large amounts of plasminogen activator may appear in the circulation in spontaneous pathological fibrinolytic states, and in thrombolytic therapy.

Owing to the defect in fibrin polymerisation, the whole blood clotting time is prolonged, as are the thrombin clotting time and the one-stage prothrombin time. Plasminogen, fibrinogen, factor V, prothrombin and antihaemophilic globulin assays show low values.

It is believed that physiological plasma fibrinolytic activity may be due to trace quantities of activator in the plasma and it is now generally accepted that the object of fibrinolytic or thrombolytic therapy is to increase plasma activator levels and so accelerate the process of thrombolysis. The ideal is to produce rapid digestion of thrombi without significant hyperplasminaemia; in practice this ideal is probably impossible to achieve.

Studies with Plasminogen Activators.

Human/

Human and animal experiments in the literature have suggested that activators of the plasminogen-plasmin system may be successfully used in the treatment of thrombo-embolic vascular occlusion, and the work presented in this thesis was carried out as part of a long-term clinical and laboratory programme of investigation of the problems involved in thrombolytic therapy.

After two chapters devoted to methods, including an original method of preparing fibrinogen tagged with radioactive iodine, an account is given of in vitro and in vivo studies with a variety of plasminogen activators - streptokinase, urokinase, a commercial plasmin preparation which owes the greater part of its thrombolytic activity to its content of streptokinase, and a nicotinic acid compound. In view of the possible use of tissue or other endogenous activators in therapy, an account is given of biochemical findings in patients with increased levels of plasminogen activator in the plasma during surgical operations, which may perhaps be due to release into the circulation of tissue activator. Finally an account is given of some factors, particularly platelet and lipid content, affecting the in vitro response/

response to streptokinase of artificial thrombi.

Laboratory methods. The methods are not original, though some have been modified in minor ways for the present studies. Methods described include assays for plasminogen activator activity, plasminogen, fibrinogen, and the hyperplasminaemic coagulation defect. Activator assay techniques described include the euglobulin lysis method, which is simple and speedy, but can give misleading results if plasma plasminogen levels are low, and the fibrin plate test, which measures plasma thrombolytic activity, but takes many hours to carry out. Evidence is presented that in a large group of tests, there was good correlation between results with euglobulin lysis and with the fibrin plate test. An account is then given of a caseinolytic technique for plasminogen assay, and a standard method of fibrinogen assay. There follows a description of the thrombin clotting time, which was used as a combined index of defective fibrin polymerisation and hypofibrinogenaemia.

Fibrinogen tagged with radioactive iodine. The next chapter (chapter 4) gives an account of a simple original method of preparing fibrinogen tagged with radioactive iodine. The method was quick and/

and easy to perform, and produced a tagged fibrinogen in which thrombin clottable radioactivity varied from 89 to 96 per cent, trichloracetic acid precipitable radioactivity always being greater than 97 per cent. The electrophoretic and chromatographic properties of this tagged fibrinogen are described, and an account is given of its use in a modified version of the plasminogen-enriched ^{131}I fibrin labelled clot system of activator assay originally described by Alkjaersig et al. (1959b). Though a good correlation was demonstrated between radioactivity release and tyrosine release, quantitative aspects of the method were somewhat disappointing (coefficient of variation, 14.5 per cent). Though the clots were primarily sensitive to activator, they also showed some response to plasmin in the test solution; they appeared to measure the property which thrombolytic therapy is intended to impart to the plasma, that is the ability to dissolve preformed human fibrin.

Streptokinase. The thesis then goes on (chapter 5) to give an account of the biochemical effect of intravascular administration of a highly purified preparation of streptokinase to 6 subjects. Streptokinase is the only plasminogen activator commercially available/

available at the present time in this country for intravascular administration. It is a streptococcal antigen and antibodies to streptokinase are distributed through the population, varying widely in concentration. Accordingly each subject was given an initial dose of streptokinase, individually calculated from an in vitro sensitivity test, to neutralise circulating antibody to streptokinase and leave over enough free streptokinase to induce a brisk thrombolytic state in the plasma. Streptokinase administration was continued thereafter for a period varying in different subjects from 40 minutes to 26 hours, the dosage rate of the continuing infusion being from 12,500 to 80,000 units per hour. In all cases marked plasminogen activator activity was imparted to the plasma by streptokinase administration. The necessity for individual initial dosage calculation is illustrated by the findings in two of the subjects; in one, an initial dose of 500,000 units produced a barely detectable effect, whereas in the other a total dose of 480,000 units produced and maintained for five hours high levels of thrombolytic activity in the circulation. In association with the thrombolytic state, all the patients showed evidence of hyperplasminaemia, with low plasminogen and fibrinogen levels,/

levels, and prolongation of the thrombin clotting time and one-stage prothrombin time. In the patients in whom the assays were carried out, prothrombin, factor V and antihaemophilic globulin were also reduced. After streptokinase administration ceased, thrombolytic activity in the plasma declined very rapidly; in the patients in whom samples were taken one hour later, virtually no activity was found. On the other hand, the duration of the streptokinase induced coagulation defect was many hours.

Several different approaches to the problem of choice of dosage schedule of streptokinase are discussed. There is suggestive but by no means complete evidence that the most practical, though possibly not the most effective, method of streptokinase administration may be by the 'plasminogen depletion' technique, in which after administration of a suitable initial antibody neutralising dose, therapy is maintained with large amounts of streptokinase designed to produce rapidly and thereafter to maintain total, or almost total, plasminogen depletion. In this circumstance the coagulation defect is most marked near the onset

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of the infusion but thereafter gradually improves, plasmin being produced only at the rate made possible by the appearance of new plasminogen in the circulation as it is synthesised; the rate of plasmin production in this circumstance appears to be within the capacity of the antiplasmin mechanism to bring about rapid neutralisation. Much more work is needed to determine the best method of streptokinase administration in routine clinical circumstances.

Urokinase. Chapter 6 gives a report of in vitro and in vivo studies with a commercial preparation of urokinase, the physiological plasminogen activator present in normal urine. The preparation was found to be a potent activator of plasminogen in purified systems, and also to possess esterolytic activity against tosyl arginine methyl ester. Serum was found to inhibit the plasminogen activator and the esterolytic activity of the urokinase, the inhibitory effect being located in the alpha-1 and alpha-2 globulin fractions of the serum, to which the antiplasmins belong. However in a caseinolytic assay system, with purified plasminogen as substrate, serum appeared to exert a distinct antiactivator, as opposed to an antiplasmin, effect.

Since/

Since human urine is known to contain thromboplastic activity, the urokinase preparation was examined for coagulative properties. It was found to shorten the recalcification time of normal and Hageman factor deficient plasma, to substitute for brain extract in the one-stage prothrombin time, and to substitute partially for antihaemophilic globulin, Christmas factor and platelets in the thromboplastin generation test. On electrophoresis on cellulose acetate the urokinase preparation separated into at least four components and it appeared to be possible by electrophoresis to achieve a partial separation of plasminogen activator from the coagulative activity.

The urokinase preparation was given by intravenous infusion to four patients; in each slight thrombolytic activity was produced in the plasma, and in two of the subjects, definite laboratory but not clinical evidence of coagulative activity in vivo was found. Urokinase is known to have 'Hageman-like' effects, which may be due to an intrinsic action of the urokinase molecule, or to inadequate removal of the thromboplastic component of the urine. Although it is possible that in vivo at a clinical level the coagulative properties detected in the present preparation may be overshadowed by/

by the fibrinolytic effects of the preparation, which may possibly be devoid of hazard of producing intravascular clotting, it would seem worth while for the manufacturers to attempt to produce a preparation free from coagulative properties.

In one patient in whom antibodies to urokinase were looked for after an infusion and a subsequent intradermal challenging dose, there was no evidence of antigenicity, and in all four patients the response to urokinase in vitro was the same before and 10 days after the infusions, again suggesting that urokinase is not antigenic.

Activator or plasmin? There has been much controversy in the literature as to whether activator or plasmin offers the best approach to thrombolytic therapy, but the case for the use of activator seems to have been established. Evidence to support the use of activator includes the observation already cited that preformed thrombi are relatively resistant to plasmin in the surrounding medium, but are sensitive to activator. Further, on the basis of the postulated physiological mechanism for thrombolysis, a small amount of plasmin will on infusion be neutralised by antiplasmin. To produce with plasmin a fibrinolytic effect in the plasma surrounding a thrombus, sufficient plasmin must/

must be infused to neutralise circulating antiplasmin; that is, it will be necessary to induce a hyperplasminaemic state with all its deleterious consequences. Those who argue in favour of plasmin as a therpeutic weapon claim that the plasmin-antiplasmin complex formed on infusion of plasmin is readily dissociable and constitutes a reservoir of plasmin which may be released at the site of a thrombus, but they have produced no good evidence to support the relevance of this concept to thrombolytic therapy in man (Ambrus and Markus, 1960).

Studies with plasmin preparation. Chapter 7 begins with a description of in vitro experiments in which a comparison is made of streptokinase, urokinase and activator-free plasmin as regards thrombolytic activity and ability to produce a coagulation defect. When mixed with plasma the activators endowed it with marked thrombolytic activity but in the concentrations used, a coagulation defect was not produced, whereas plasmin, when mixed with plasma in a concentration which did not cause detectable thrombolytic activity, produced a striking coagulation defect. In vivo and in vitro studies were also made with Actase, a commercial "plasmin" preparation, which/

which was shown to owe the greater part of its thrombolytic activity to its content of streptokinase. When infused intravenously in the manufacturer's recommended dosage to two subjects with streptokinase antibody levels in excess of the streptokinase content of the Actase administered, negligible plasma thrombolytic activity was produced. Clearly commercial "plasmin" preparations of this type must be used with the same criteria for individual dosage calculation as apply to streptokinase.

Complamin, a nicotinic acid-theophylline ester, was selected for study (chapter 8) as representative of a group of substances, themselves fibrinolytically inert, which when injected intravenously induce increased levels of plasma plasminogen activator activity; the mechanism responsible for the activator release is not known. Evidence is presented that, as with its parent substance nicotinic acid, the plasminogen activator activity induced in vivo by intravenous injection of Complamin is intense but transient; a refractory state soon develops in which the response to further Complamin injections is lost. The response to a single Complamin injection was comparable in duration and degree to that observed

after/

after 5 minutes strenuous exercise in the same subjects. Some subjects showed trivial increases in plasminogen activator activity after taking Complamin by mouth.

When two Complamin injections were given at an interval of 30 minutes, there was a much reduced response to the second injection; similarly when exercise was taken twice at an interval of 30 minutes, there was a diminished response to the exercise on the second occasion. However, when exercise was followed by Complamin, or Complamin by exercise, a full plasminogen activator response to the second stimulus in each case, as well as to the first was seen. The findings suggest that the failure of response to repeated injections of Complamin was due to resistance to the drug and not to exhaustion of the body's reserve of plasminogen activator; accordingly it might be possible to find a pharmacological agent to produce a sustained rise in plasminogen activator levels in the plasma.

Fibrinolytic activity during surgical operation. In view of the obvious possibility that endogenous plasminogen activator extracted from the tissues or the plasma might be of therapeutic use, the activity/

activity of the plasminogen-plasmin system in patients undergoing surgical operations was studied (chapter 9); one of the causes of the increased fibrinolytic activity found in such patients is thought to be release of tissue activator into the circulation. Ten patients were studied before, during and after excision of the rectum, a procedure in which there is much tissue trauma. Although in no patient was there a clinical haemostatic defect, each showed a marked increase in plasma activator levels accompanied by a slight fall in plasminogen and fibrinogen assays and a rise in the thrombin clotting time. Two patients with a serious haemostatic defect associated with increased plasminogen-plasmin system activity were investigated. In each, increased levels of plasminogen activator in the circulation were accompanied by depletion of plasminogen and fibrinogen, and a severe coagulation defect. It is clear from the findings that endogenous plasminogen activator, perhaps of tissue origin, can produce plasma fibrinolytic activity associated with severe hyperplasminaemia, and that purified endogenous activator cannot be expected, if made available for therapeutic use, to be free from risk of inducing haemostatic/

haemostatic failure.

Studies with artificial thrombi. The concluding chapter in the thesis (chapter 10) describes in vitro experiments using artificial thrombi in which a number of factors affecting the response of such thrombi to perfusion with plasminogen activator were studied. Thrombi, trace-labelled with ^{131}I tagged fibrin, were prepared by allowing whole blood, mixed with a small amount of ^{131}I tagged fibrinogen, to clot in a closed loop of rotating plastic tubing. Chandler (1958) showed that in such circumstances thrombi were produced which closely resembled pathological in vivo thrombi, with a white platelet rich head and a red tail with fibrin, red cells and white cells. Radioactive thrombi made in this way were transferred after washing to a perfusion circulation comprising a pump, plastic tubing and a reservoir, where for 24 hours they were perfused with a solution of streptokinase in saline. The thrombi were inserted into a wire basket in one branch of the perfusion circulation; the site of occlusion thus produced was by-passed by a length of narrower tubing simulating a collateral vessel in vivo. Patterns of radioactivity release during 24 hours perfusion with streptokinase, 200 units/ml.,/

200 units/ml., were studied.

With thrombi produced from normal blood, after perfusion for 24 hours, about 60 per cent of the total radioactivity was in solution, 10 per cent was still attached to the basket into which the thrombus had been inserted, and 30 per cent was in fragments in the circulation. The fragments had a histological appearance suggesting their origin to be largely from platelet aggregates. In the absence of streptokinase, or with the fibrinolytic inhibitor epsilon aminocaproic acid in the circulation, the thrombi were more than 90 per cent stable for 24 hours.

Thrombi made from blood which had been artificially enriched with plasminogen were also perfused with streptokinase; lysis was very rapid and almost complete. When thrombi were made from thrombocytopenic blood, lysis was also more rapid and complete than with thrombi from normal blood, and thrombi from thrombocythaemic blood showed increased resistance to lysis compared with normal. The findings suggest that when thrombi are subjected to lysis, the fibrin component dissolves but the platelet rich head breaks up into macroscopic platelet clumps. The experiments/

experiments with plasminogen-enriched thrombi suggest that the platelets in these clumps may be held together by fibrin which is protected from lysis by the antiplasmin content of the platelets. In thrombolytic therapy, formation of such lysis-resistant platelet emboli would represent an important hazard in the cerebral circulation. Thrombi were also made from 4 patients with hyperlipidaemia, two of whom had normal euglobulin lysis times and two had prolonged lysis times. The thrombi from all four were resistant to lysis in the artificial circulation and it may be that this technique can highlight abnormalities which are poorly or inconsistently reflected in other methods of assessing substrate responsiveness to the activity of the plasminogen-plasmin system.

Conclusion. Despite intensive investigative efforts in many laboratories over the past 10 years, the goal of routine thrombolytic therapy with plasminogen activators has not been achieved. Outstanding problems include the lack of suitable plasminogen activators, and the side-effects of those which are available; lack of suitable simple assay systems for control of therapy; uncertainty as to the best approach to selection of dosage schedules and/

and control of therapy; and ignorance of the effects of plasminogen activators on pathological thrombi in vivo. In the work presented in this thesis, a variety of plasminogen activators was used to investigate some of these problems in vitro and in vivo. While there is still much to be learned before plasminogen activators can be used as a routine in the treatment of thrombo-embolic vascular occlusion, the problems are a challenge to the clinical investigator, and from the attempts made to solve them should emerge more understanding of the components and functions in health and disease of the fibrinolytic enzyme system.

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STUDIES WITH PLASMINOGEN ACTIVATORS

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Volume 2.

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VOLUME 2.

APPENDIX 1.

FIGURES.

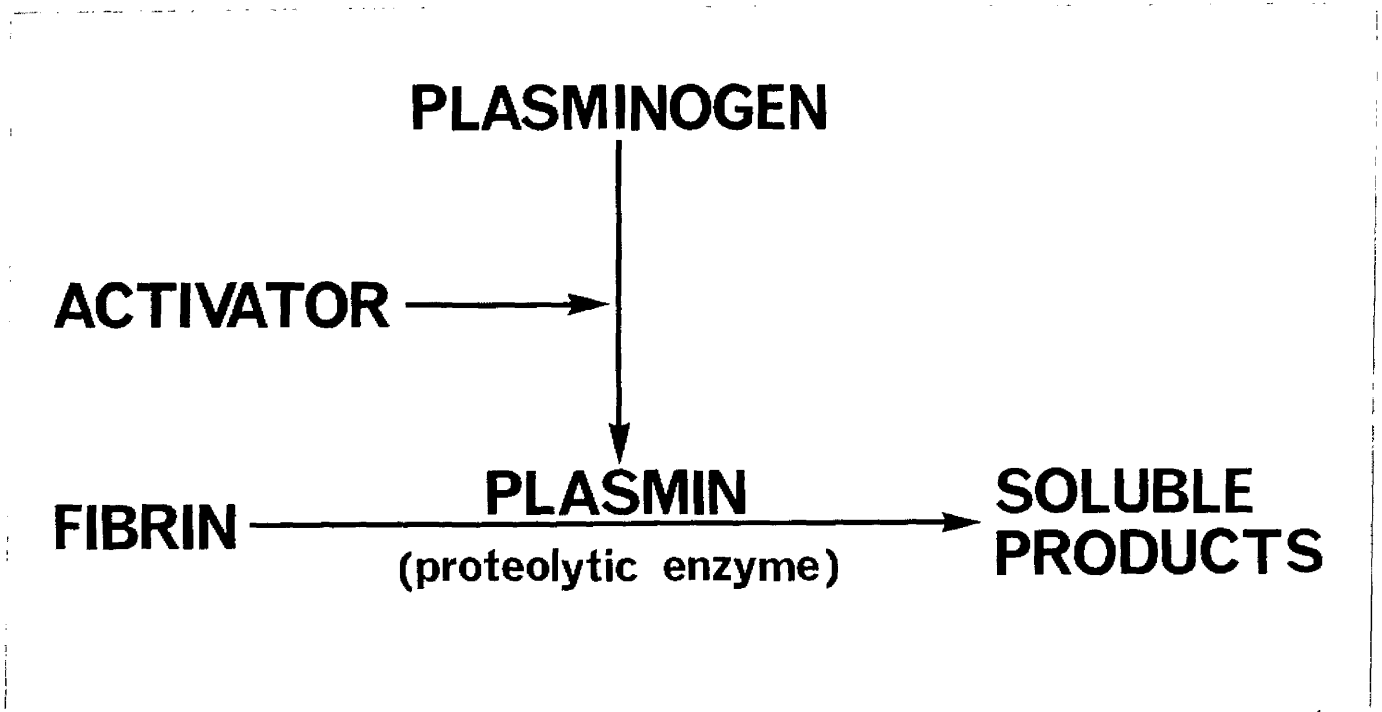


Figure 1 shows the basic components of the plasminogen-plasmin or fibrinolytic enzyme system.

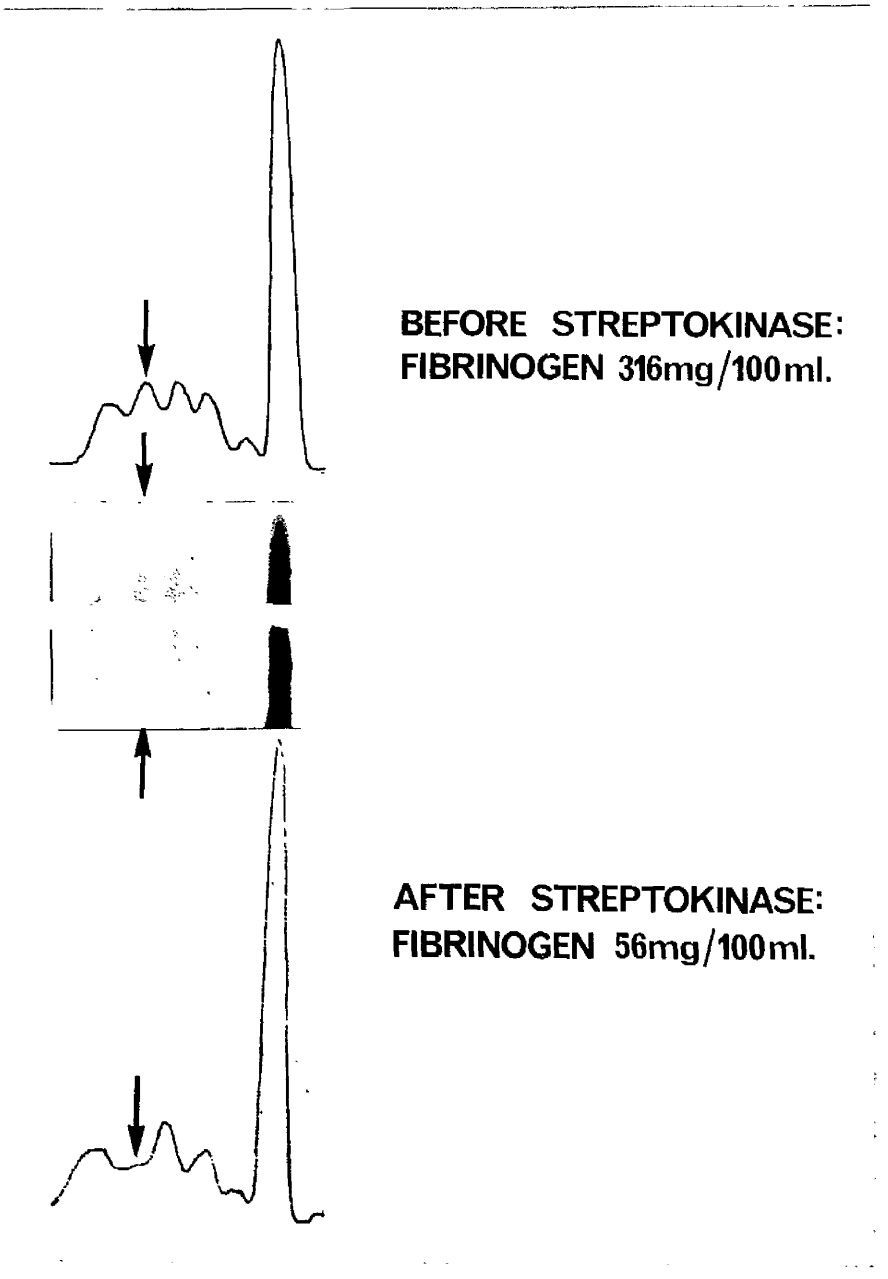


Figure 2 shows in the upper portion an electrophresis strip of a normal plasma and in the lower portion an electrophoresis strip of the same plasma after addition of streptokinase, 100 units/ml. As can be seen from the strips, and the densitometer tracings made from them, the plasmin produced by the streptokinase has resulted in very substantial digestion of fibrinogen. The cellulose acetate electrophoresis strips were run, stained and scanned as described in chapter 4.

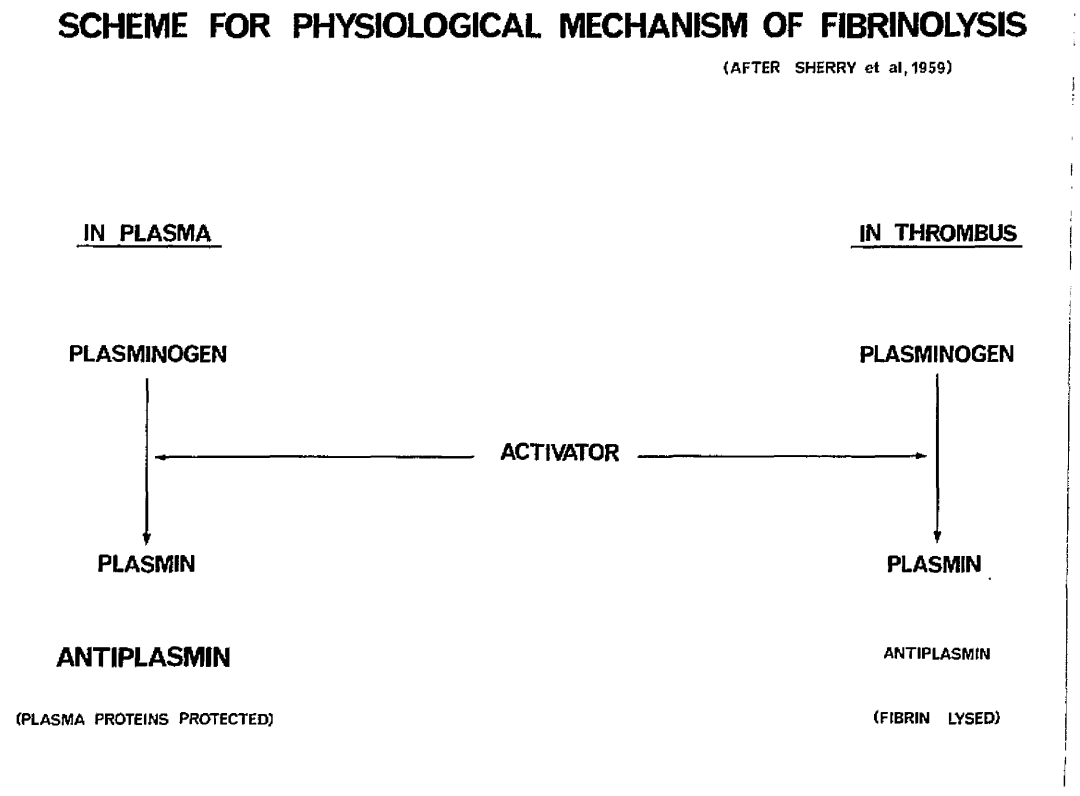


Figure 3 shows the physiological dual-phase mechanism of plasminogen activation in vivo postulated by Sherry et al. (1959a).

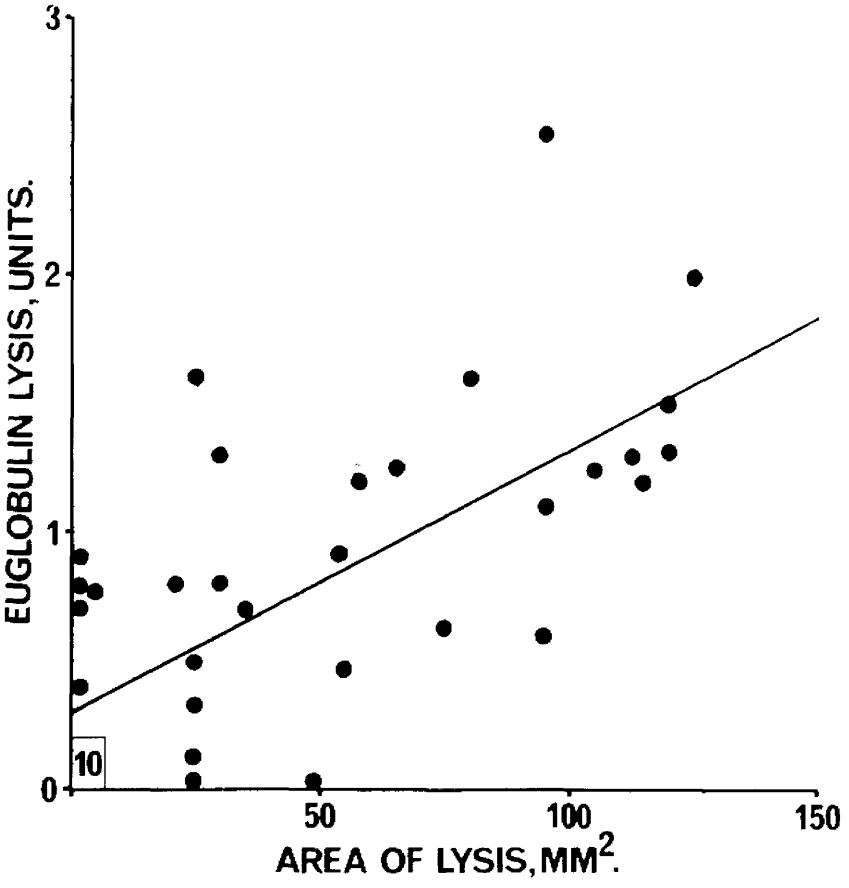


Figure 4. Correlation between euglobulin lysis activity and fibrin plate tests in 40 patients with renal disease ($r = +0.564$, $p < 0.001$; $y = 0.32 + 0.01\ x$). The figure is prepared from table 30.

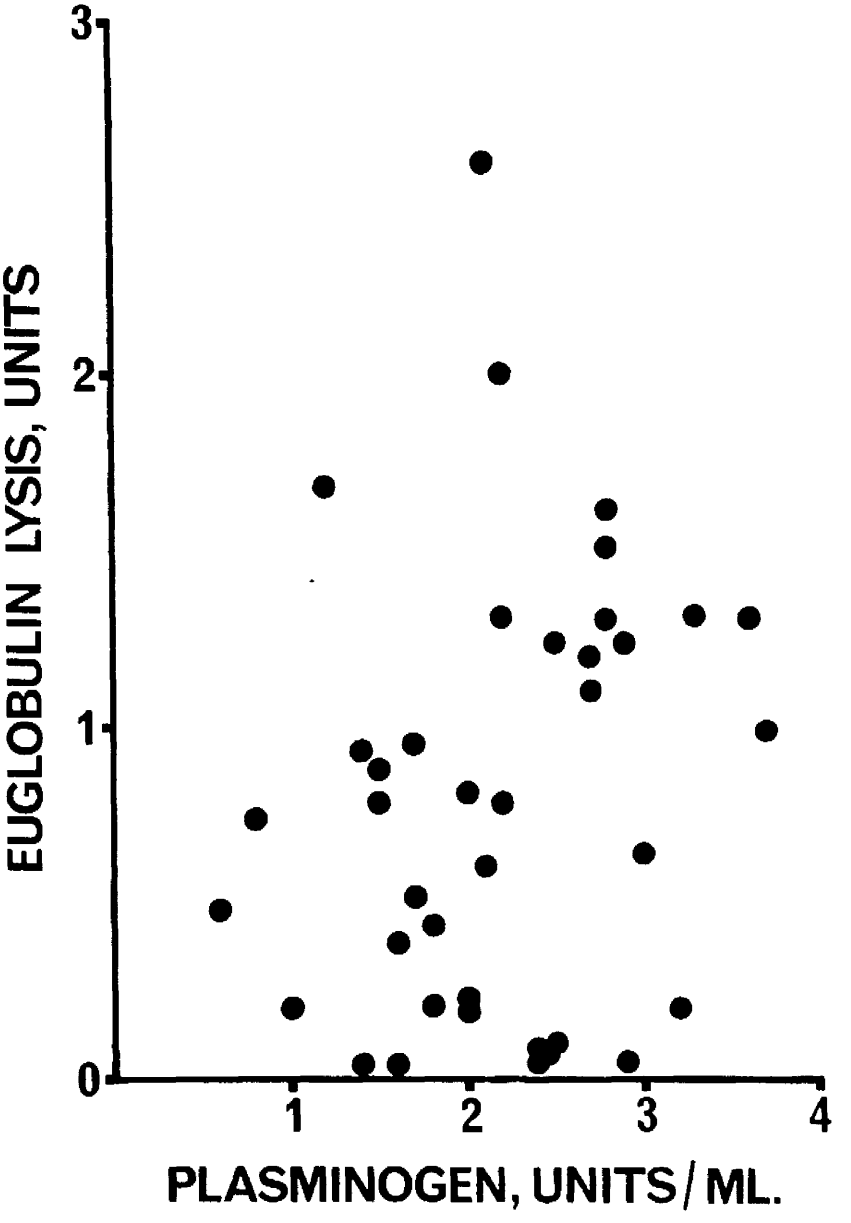


Figure 5 shows lack of correlation between euglobulin lysis activity and plasminogen levels in 40 patients with renal disease ($r = +0.160$, $p > 0.1$). The figure is prepared from table 30.

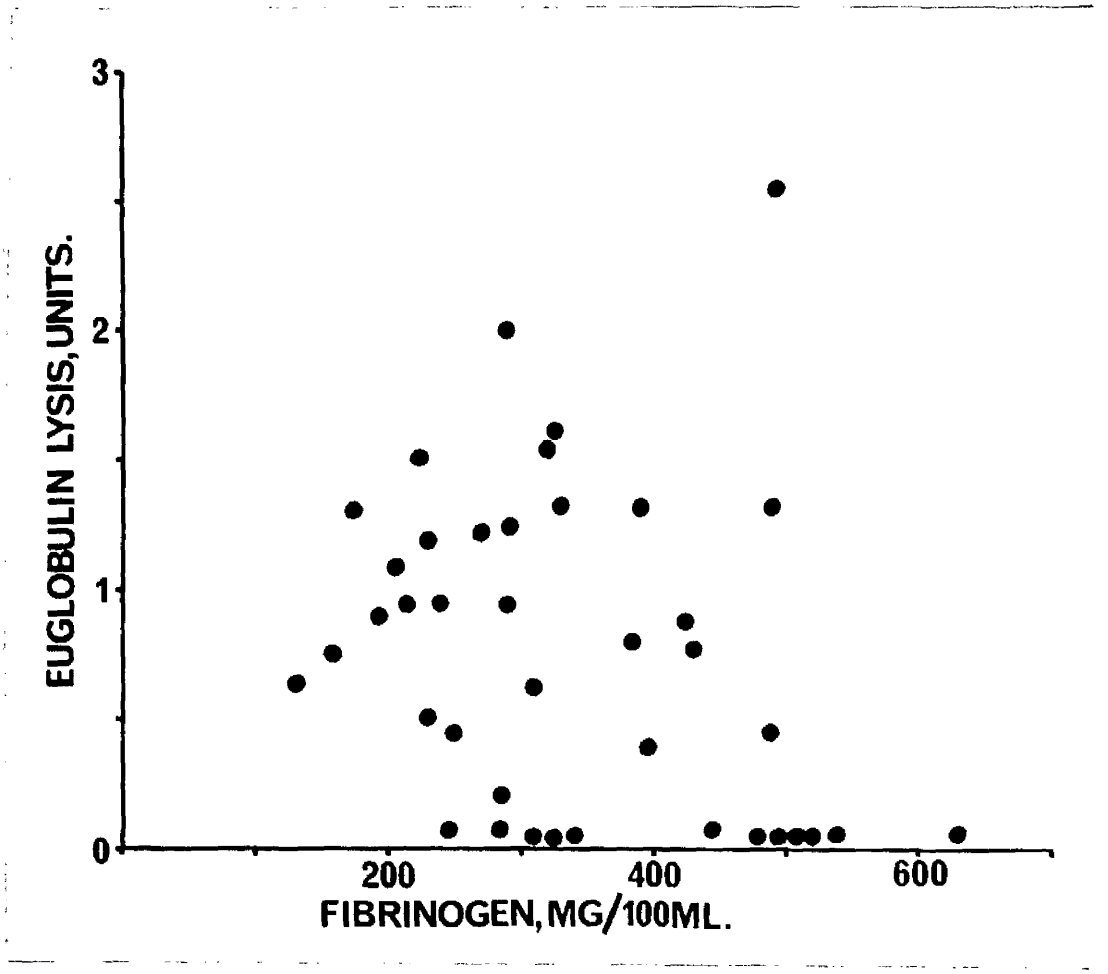


Figure 6 shows lack of correlation between euglobulin lysis activity and fibrinogen levels in 40 patients with renal disease ($r = -0.232$, $p > 0.1$). The figure is prepared from table 30.

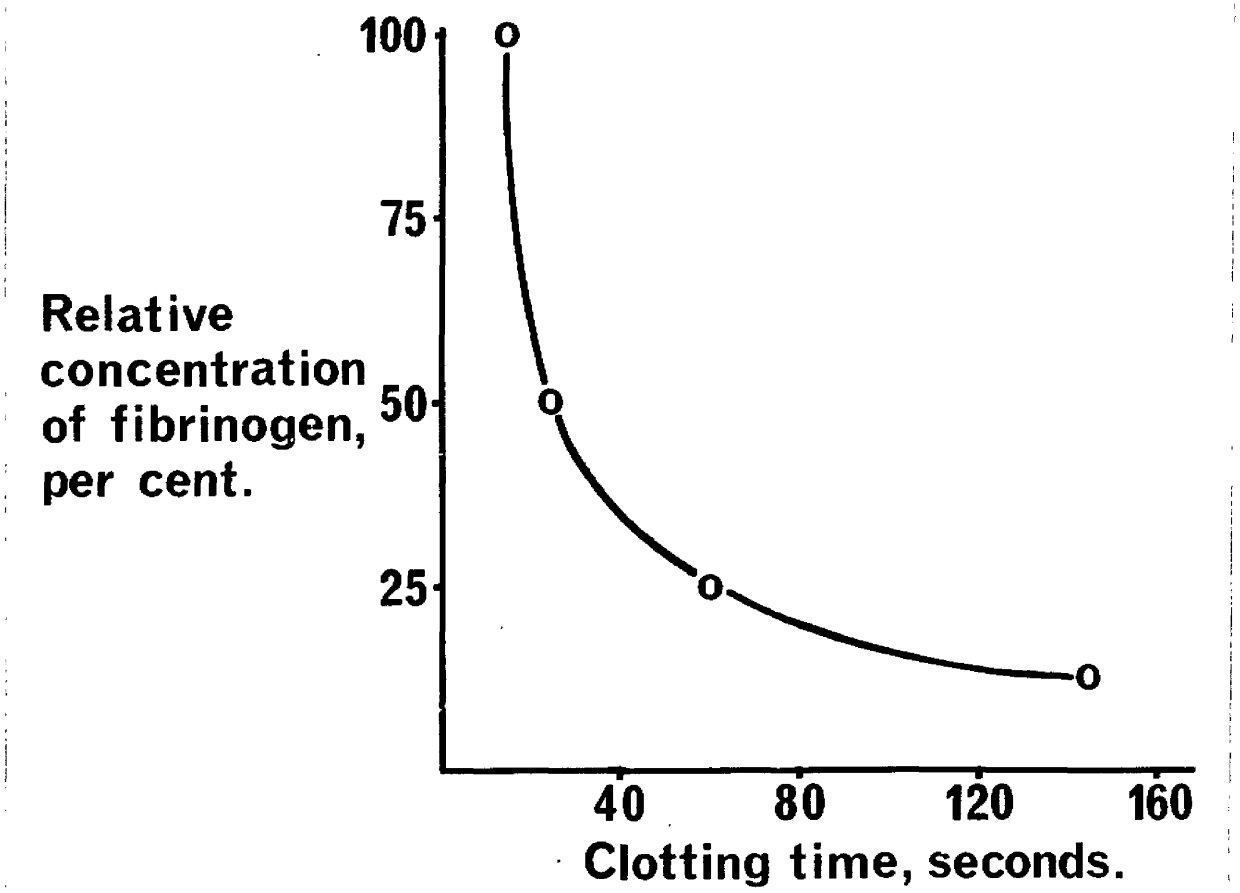


Figure 7 shows the relationship, in a purified system, between fibrinogen levels and thrombin clotting times. The figure is prepared from table 31.

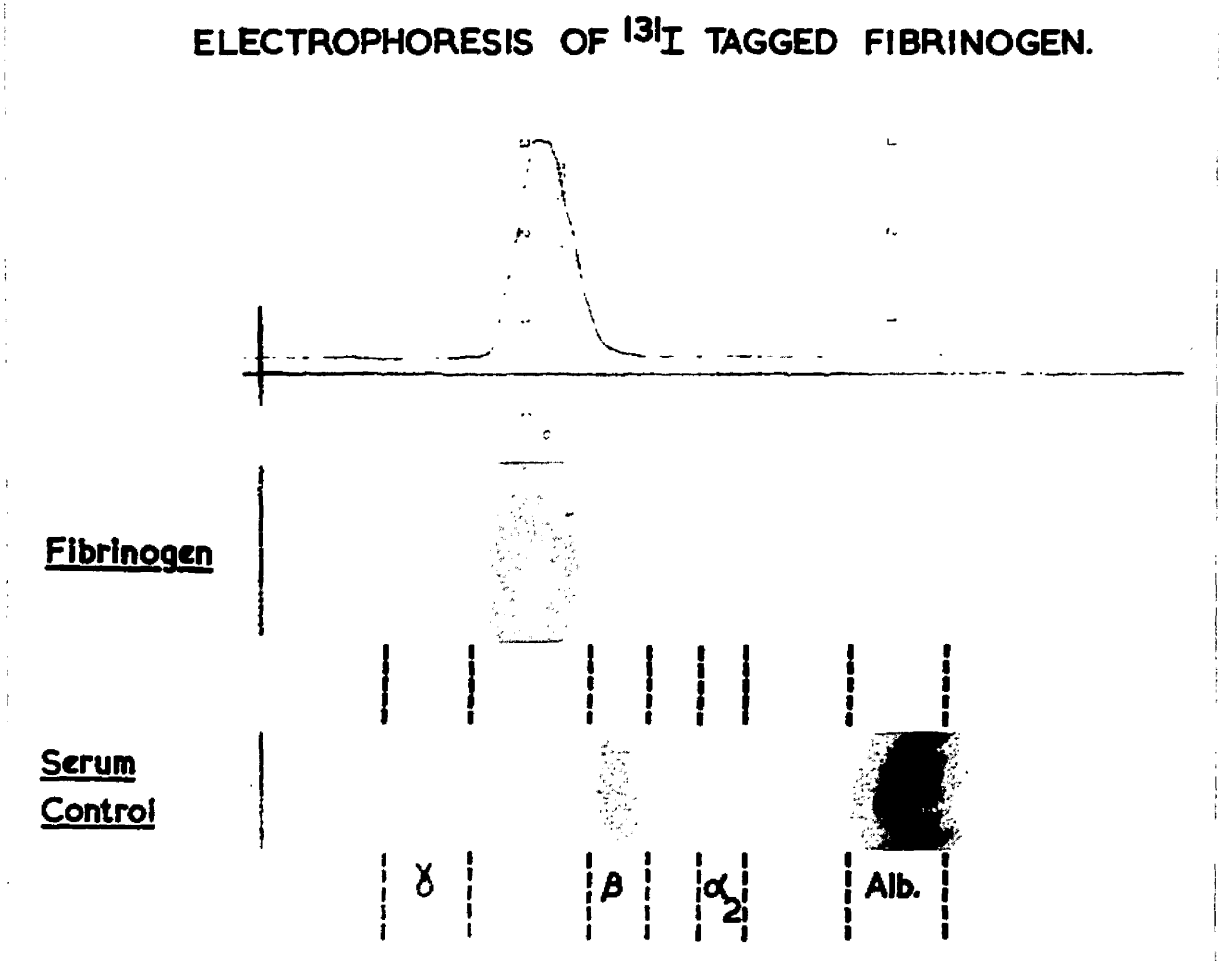


Figure 8. Paper electrophoresis strip of fibrinogen tagged with radioactive iodine. A control strip of normal serum run simultaneously is shown below, and a recording of the radioactivity in the fibrinogen above, the plasminogen strip.

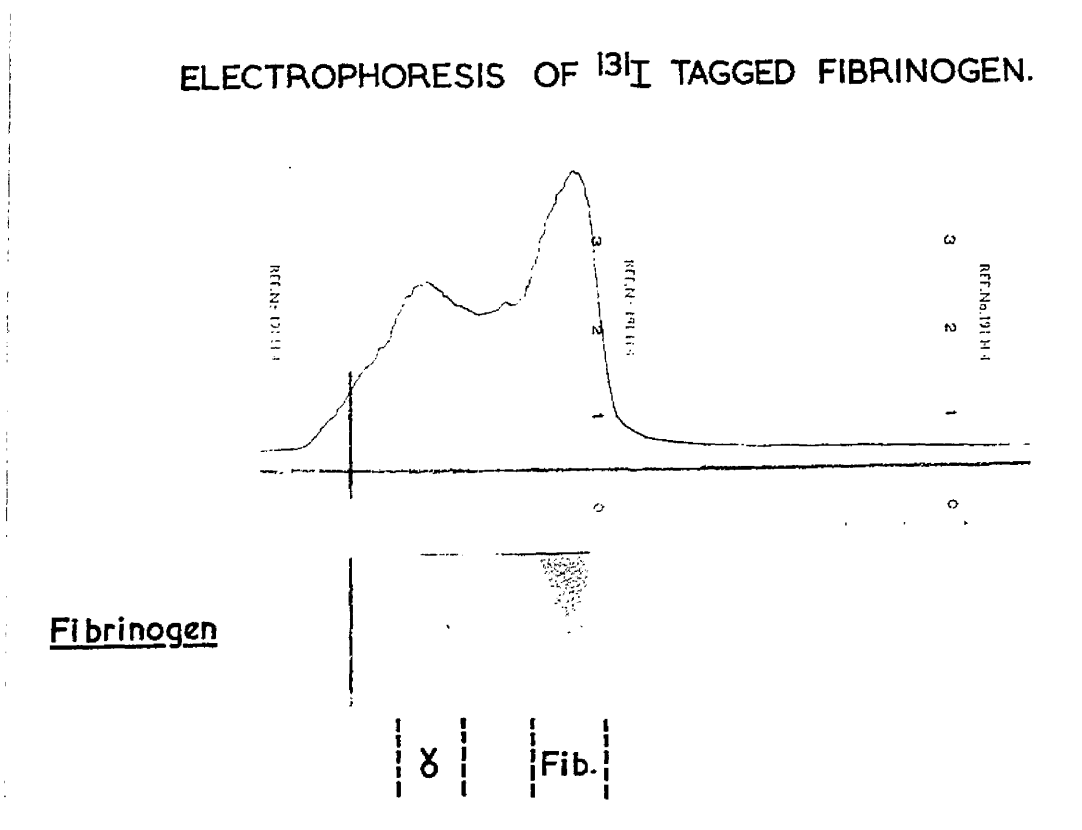


Figure 9. Electrophoresis strip of tagged fibrinogen contaminated with tagged gamma globulin. A recording of radioactivity is shown above the fibrinogen strip and a control strip of normal serum run simultaneously below it.

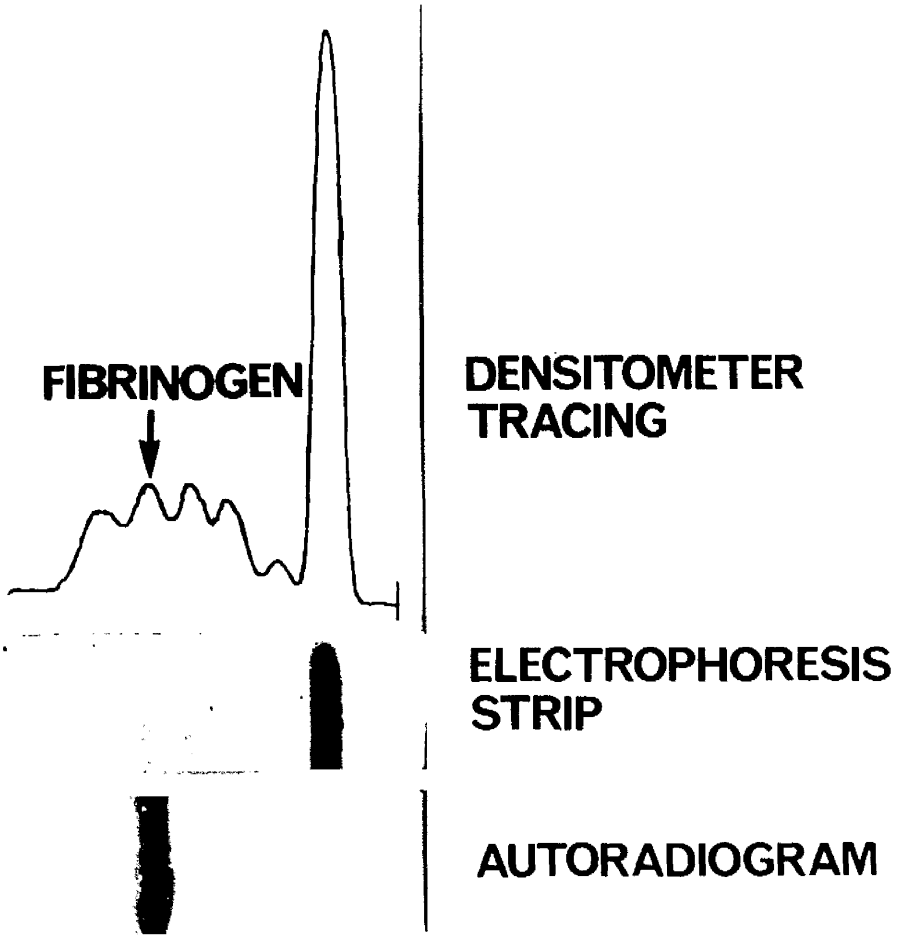


Figure 10 shows a cellulose acetate electrophoresis strip of a normal plasma to which a trace of fibrinogen tagged with radioactive iodine had been added. Also shown are an optical scan of the stained strip and an autoradiogram prepared from the strip.

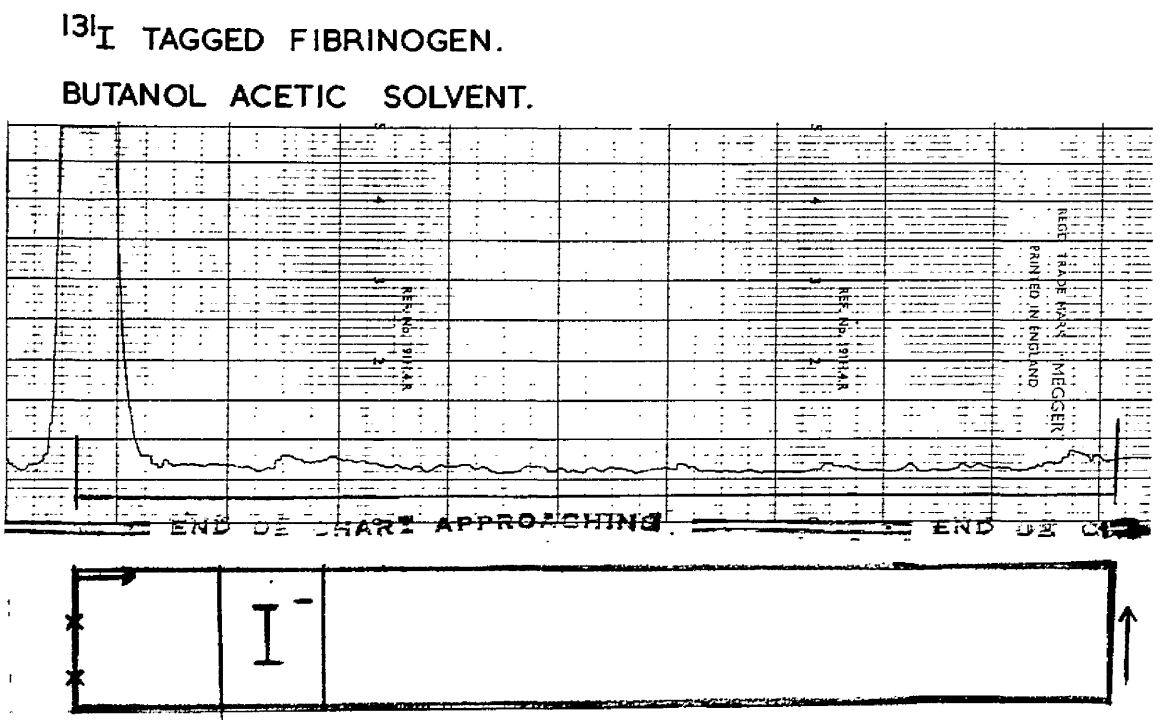


Figure 11. Chromatogram of tagged fibrinogen with a recording of radioactivity above the chromatography strip.

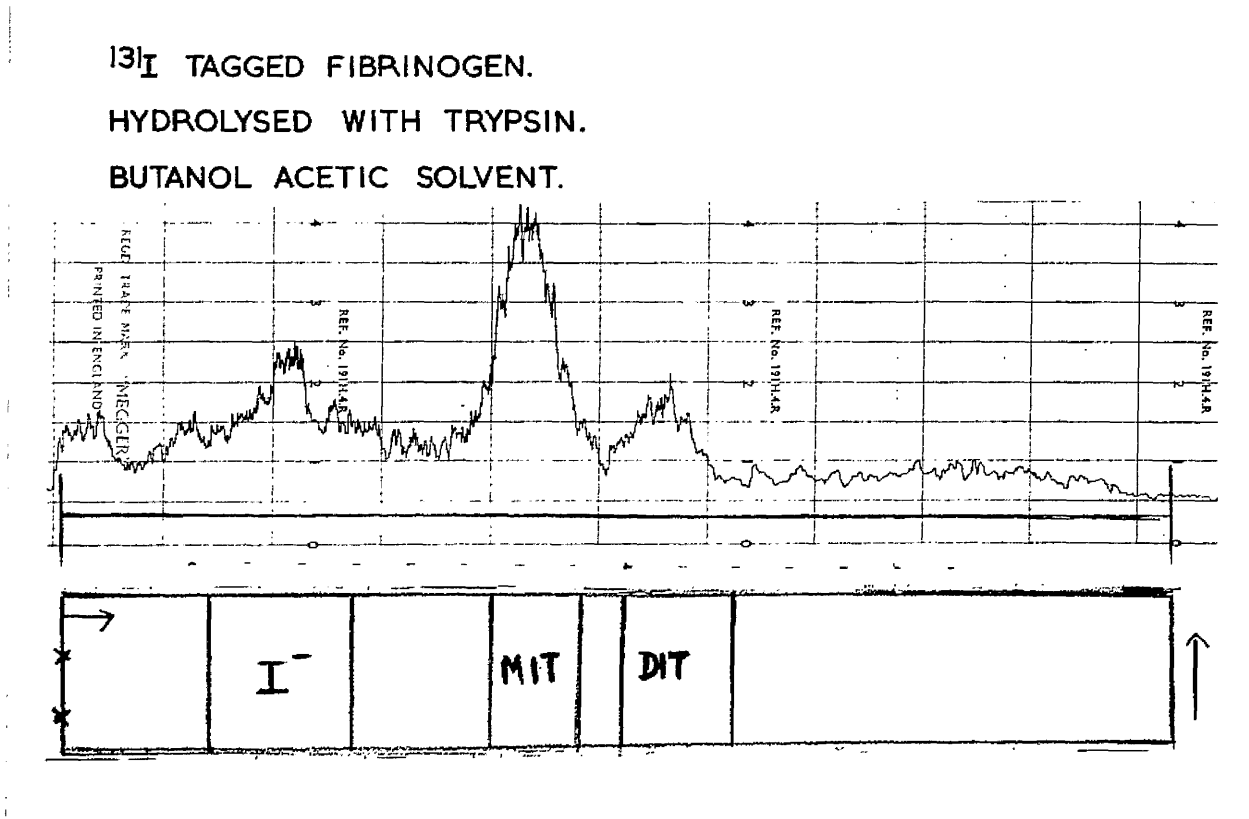


Figure 12. Chromatogram of tagged fibrinogen after hydrolysis with trypsin. A recording of the radioactivity is shown above the chromatography strip.

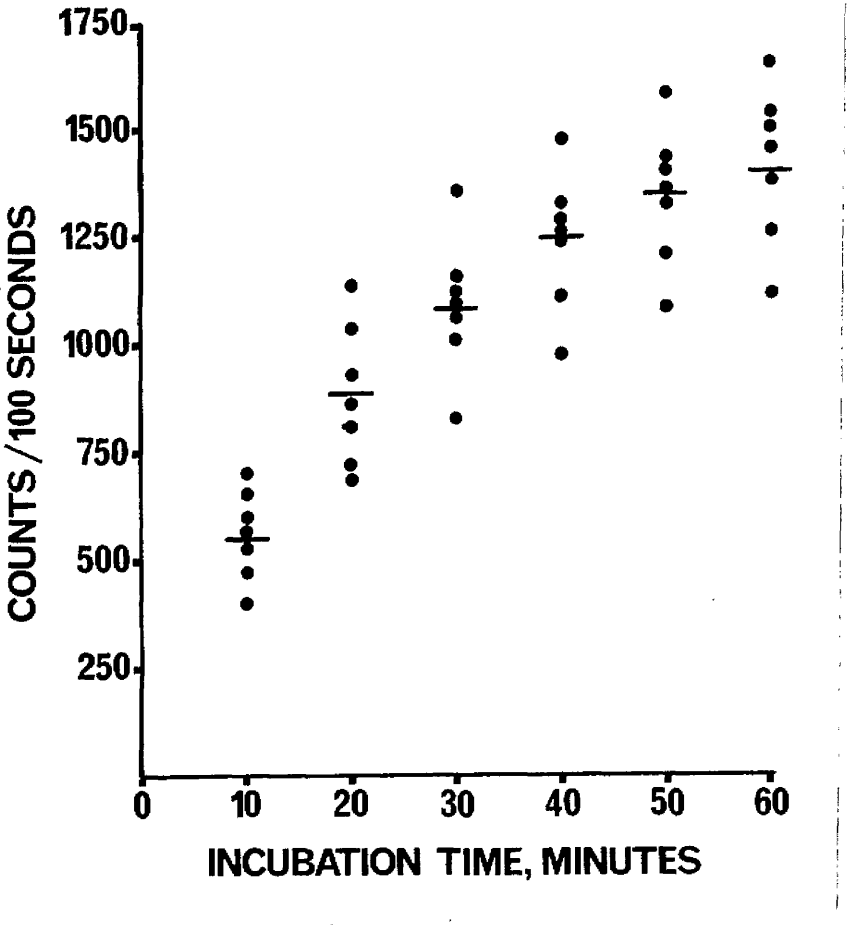


Figure 13. Relationship between duration of incubation with streptokinase, 50 units/ml., and release of radioactivity in the plasminogen enriched radioactive iodine tagged clot system. The figure is prepared from table 32.

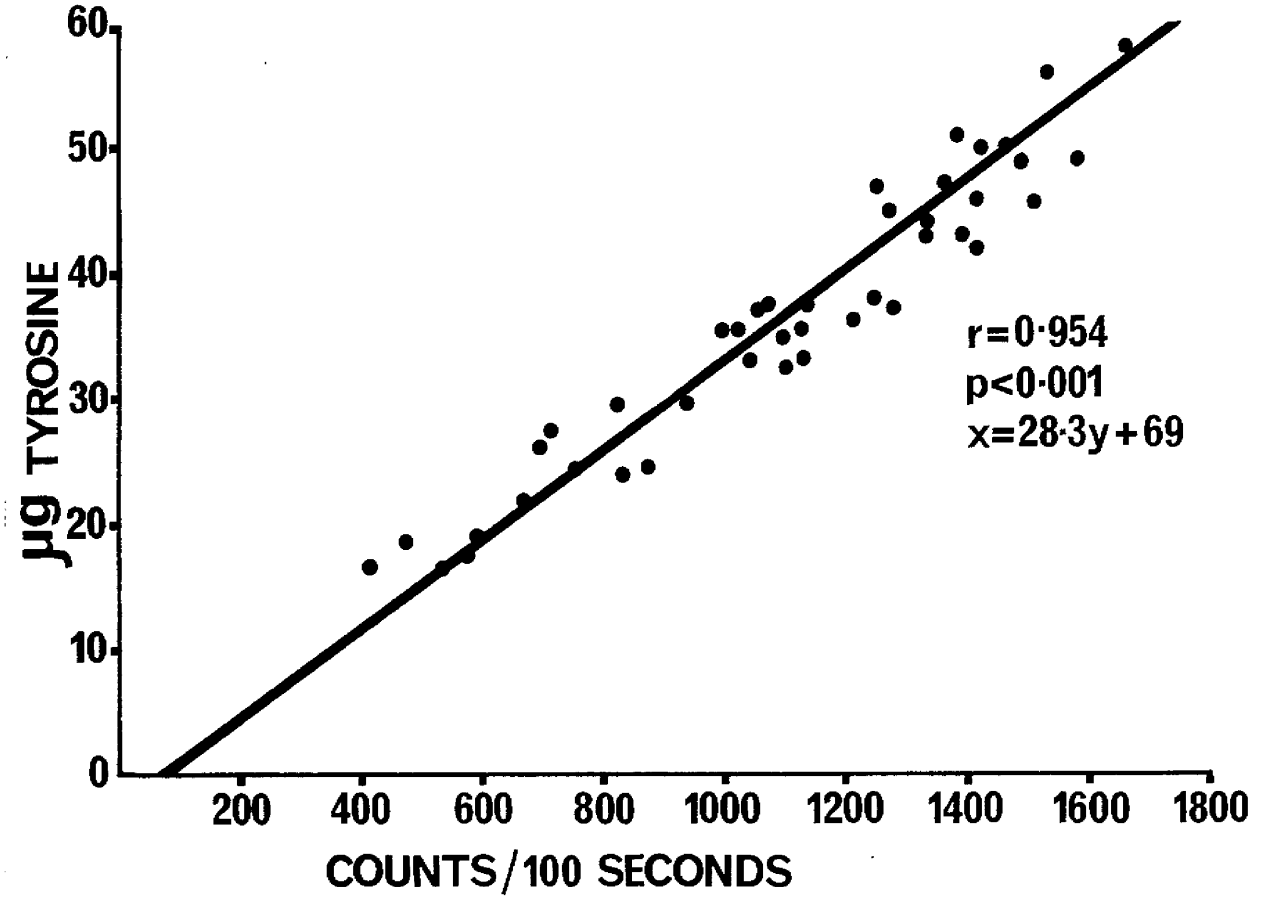


Figure 14 shows the relationship between radioactivity release and tyrosine release in the plasminogen enriched radioactive clot system. The figure is prepared from table 33.

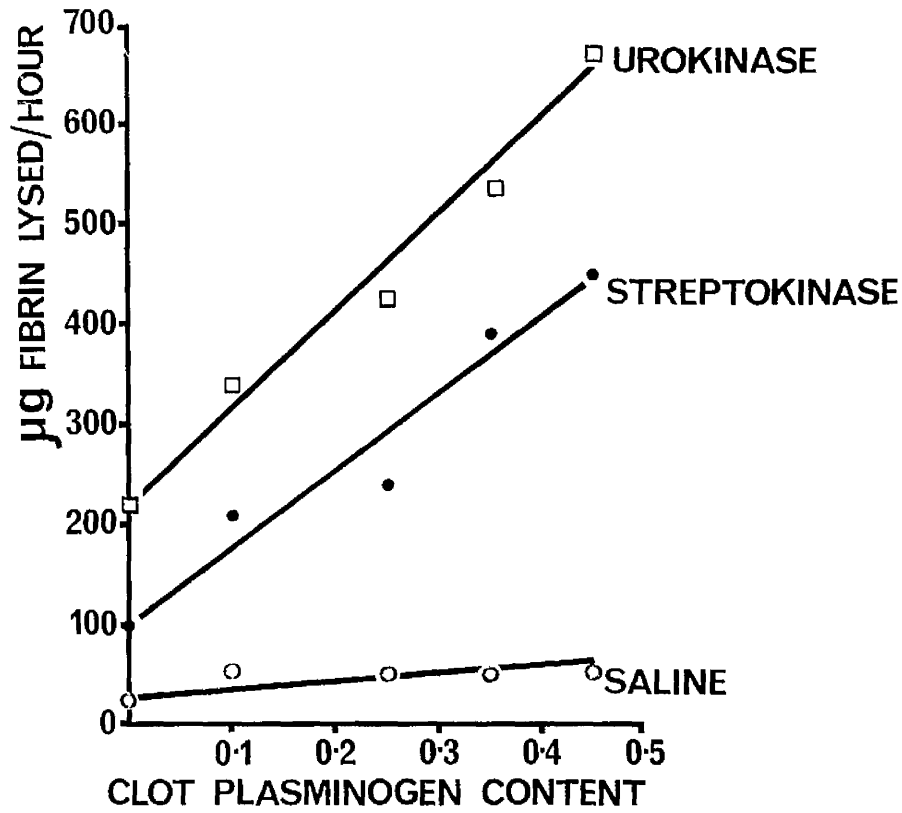


Figure 15. The effect of increasing concentrations of plasminogen on susceptibility to lysis in the plasminogen enriched radioactive clot system. The figure is prepared from table 34.

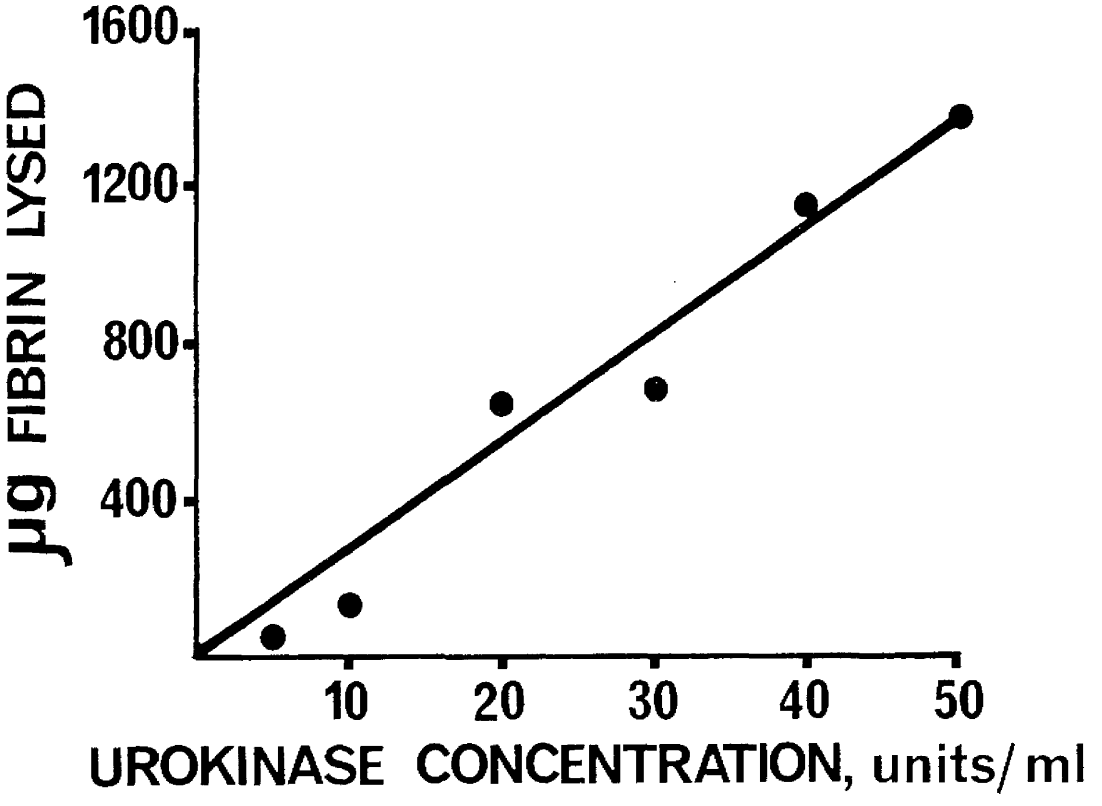


Figure 16 shows the relationship between urokinase concentrations and lysis of plasminogen enriched radioactive clots. The figure is prepared from table 35.

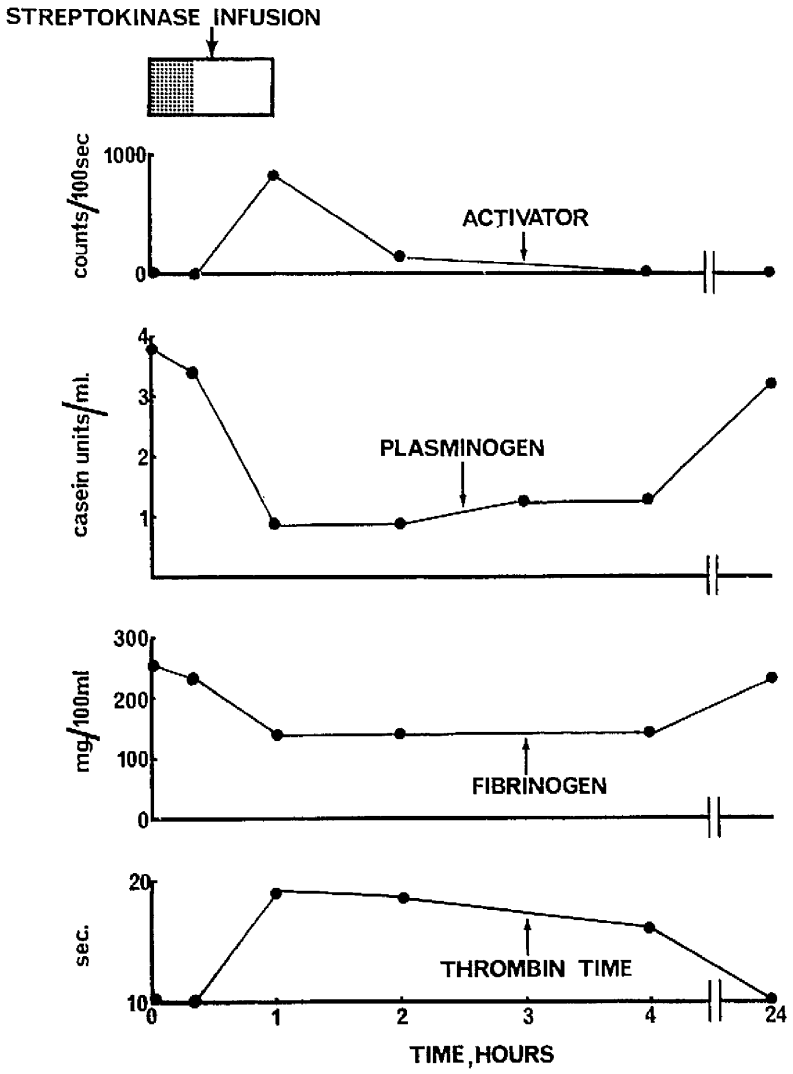


Figure 17. Effects of streptokinase infusion in patient 1, chapter 5. An initial dose of 155,000 units was given in the period shown with hatching and the infusion was continued with a further 45,000 units. The figure is prepared from table 36.

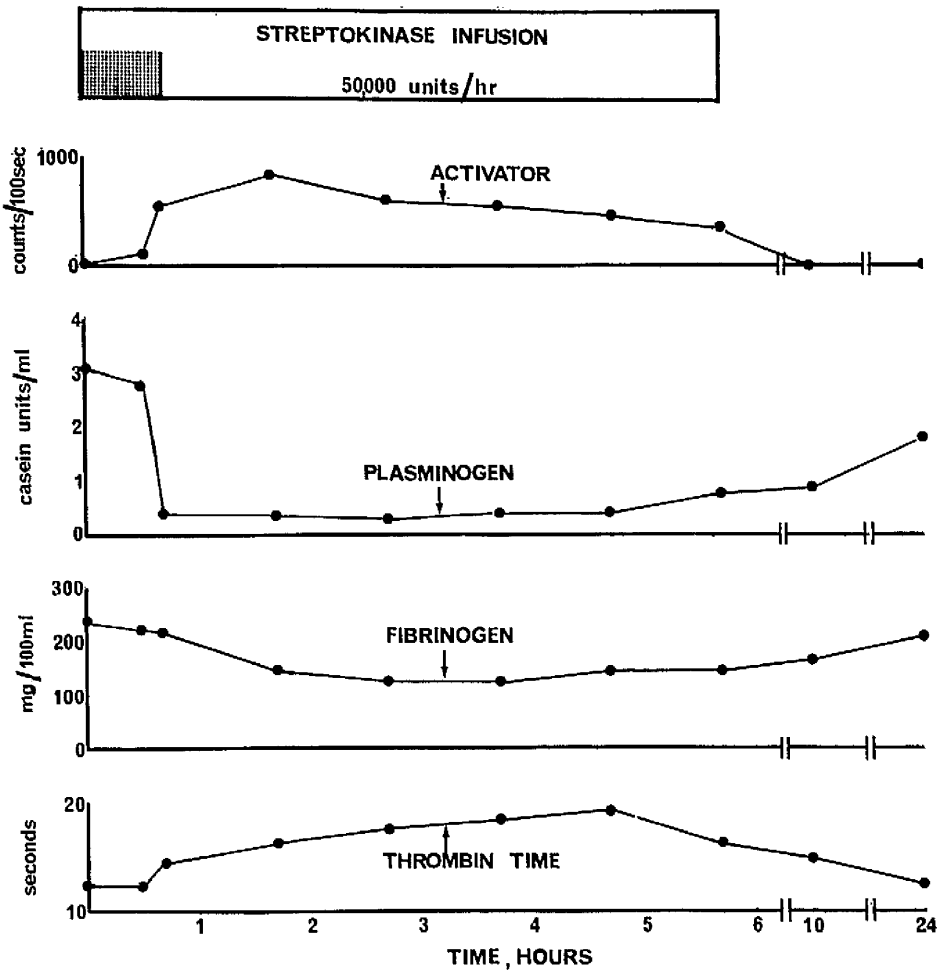


Figure 18. Effects of streptokinase infusion in patient 2, chapter 5. An initial dose of 750,000 units was given in the period shown with hatching. The figure is prepared from table 37.

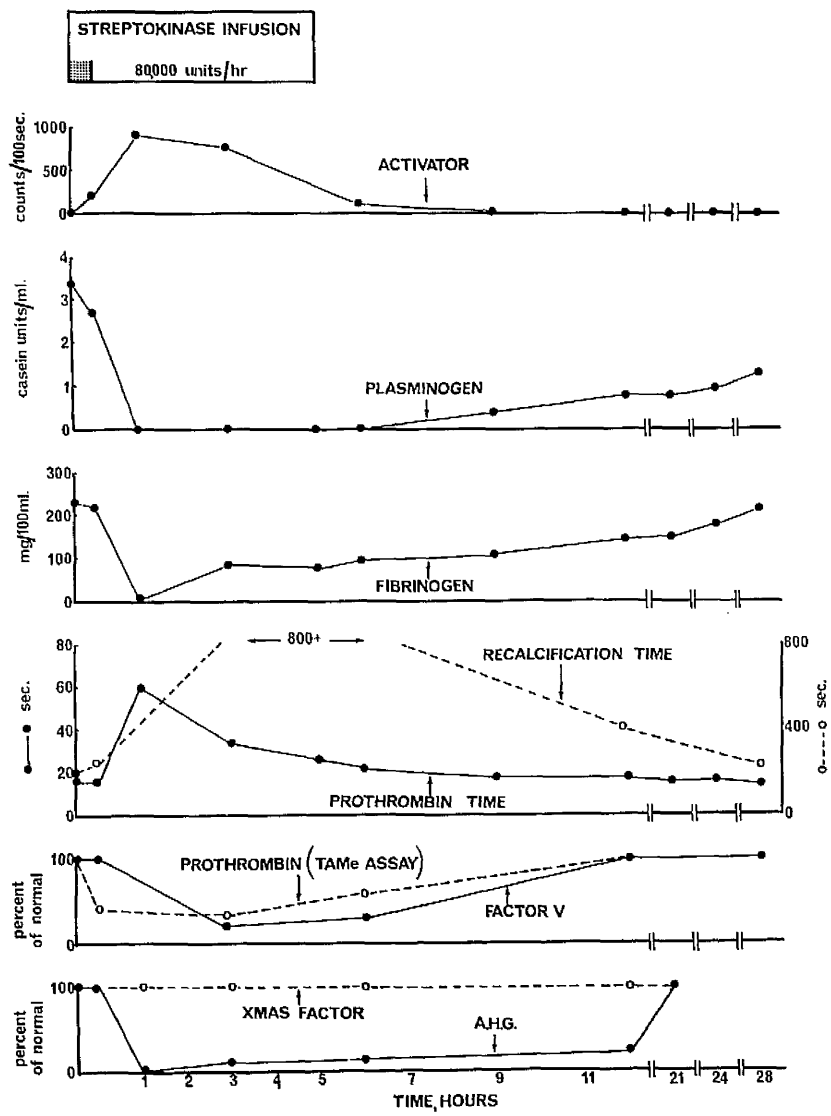


Figure 19. Effects of streptokinase infusion in patient 3, chapter 5. An initial dose of 80,000 units was given in the period shown with hatching. The figure is prepared from table 38.

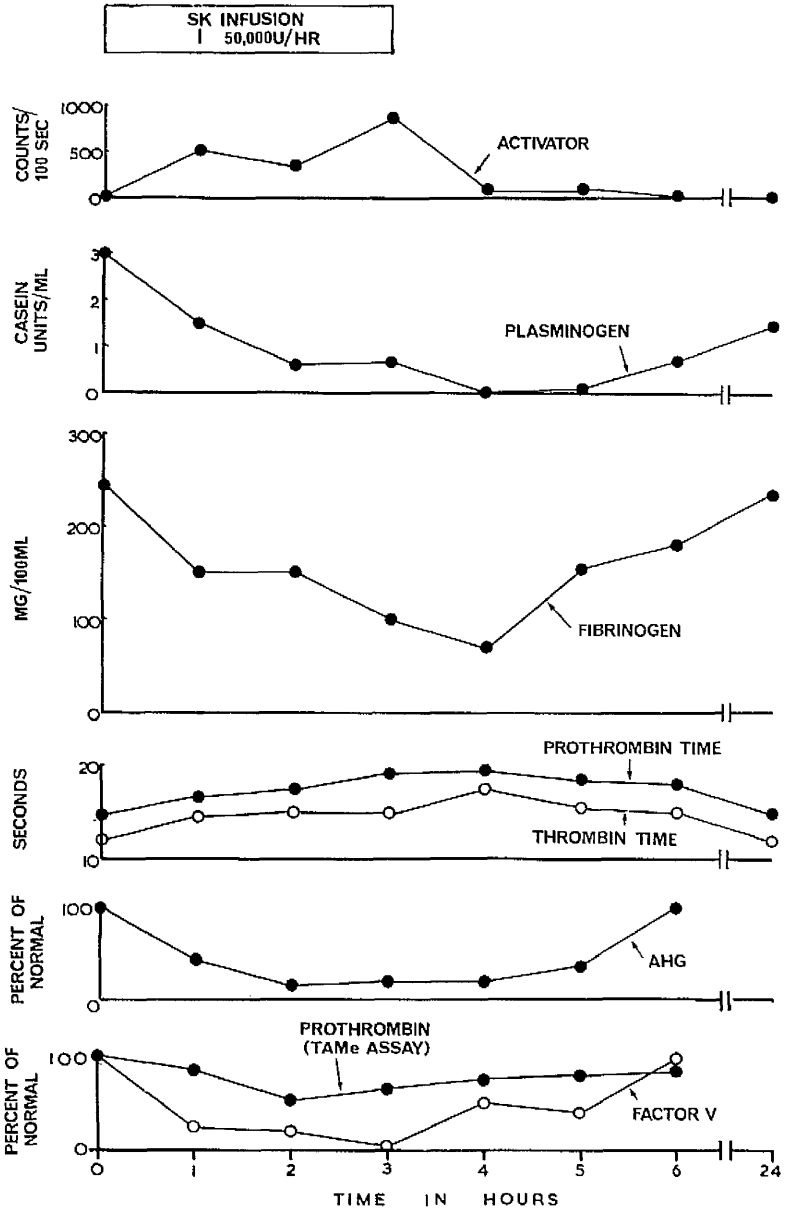


Figure 20. Effects of streptokinase infusion in patient 4, chapter 5. An initial dose of 460,000 units was given over the first hour. The figure is prepared from table 39.

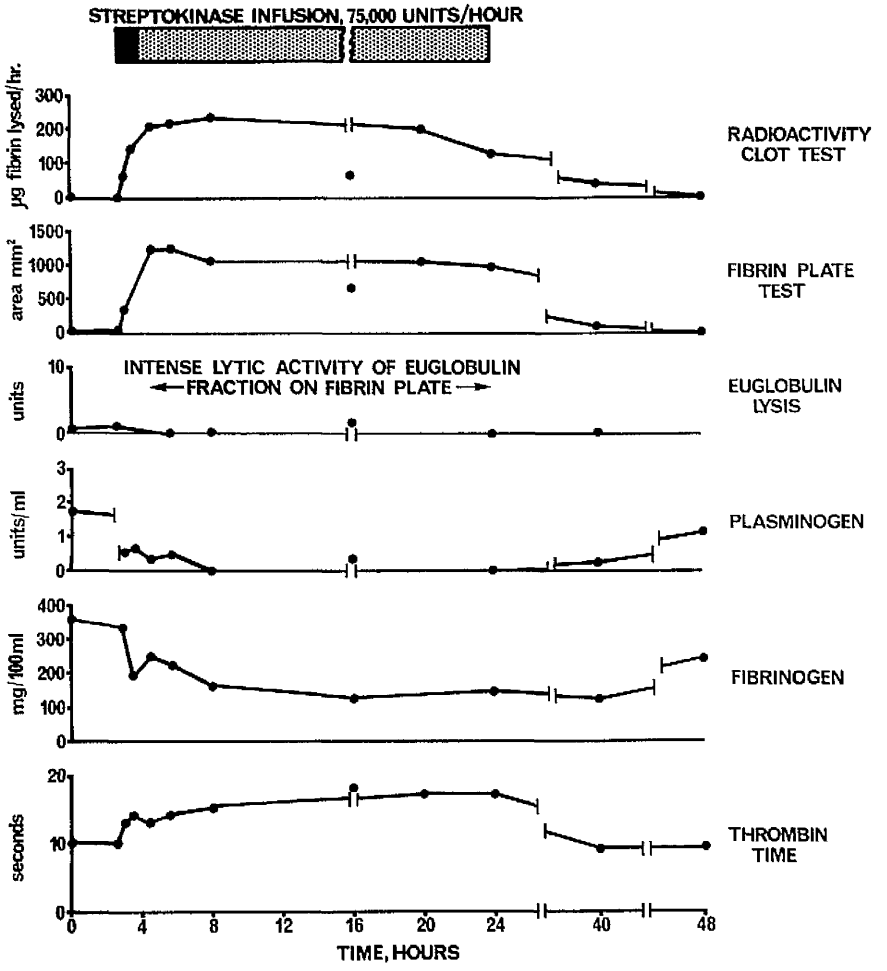


Figure 21. Effects of streptokinase infusion in patient 5, chapter 5. An initial dose of 250,000 units was given in the period shown in black. The figure is prepared from table 40.

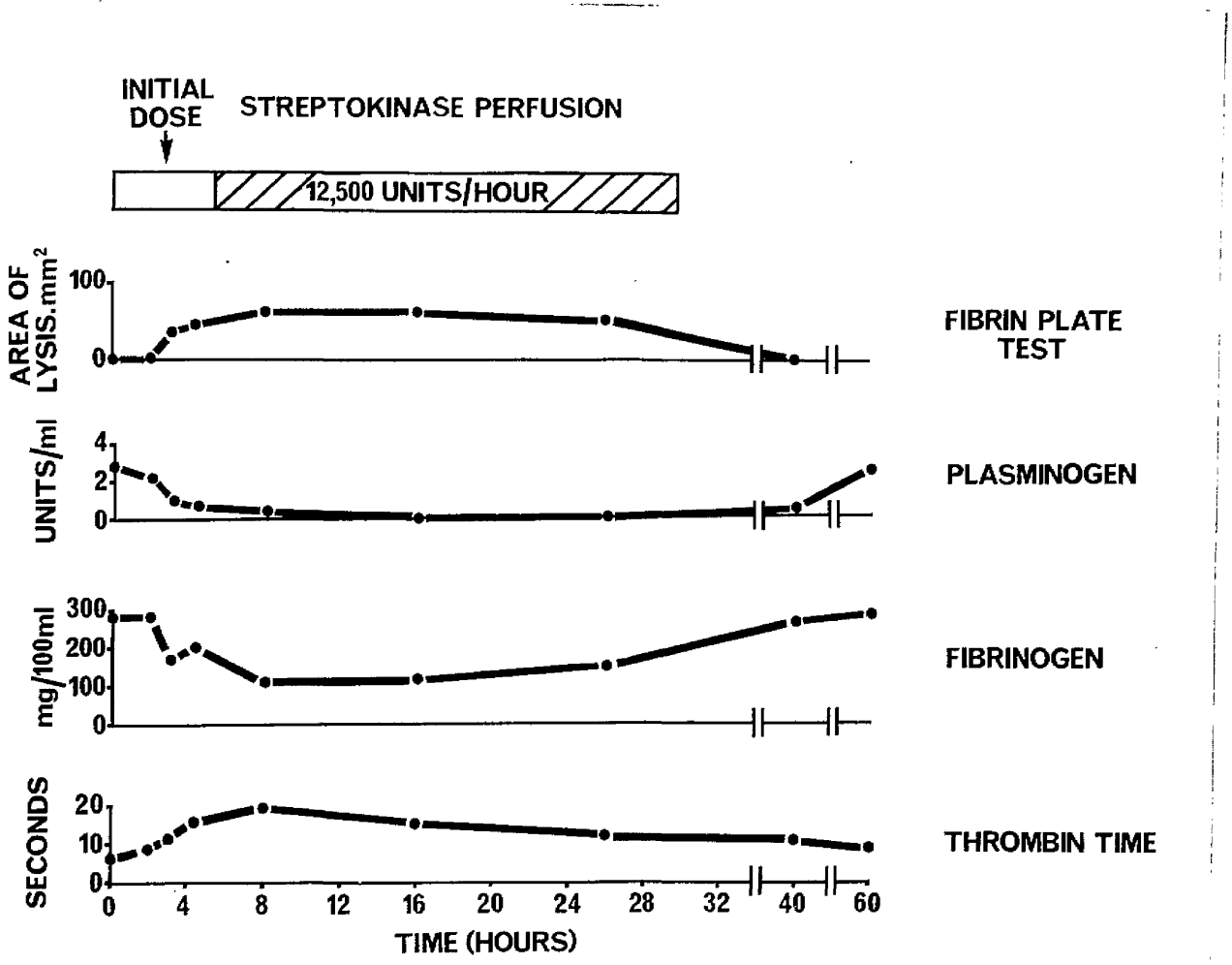


Figure 22. Effects of intra-arterial perfusion with streptokinase in patient 6, chapter 5. The initial dose was 350,000 units. The figure is prepared from table 41.

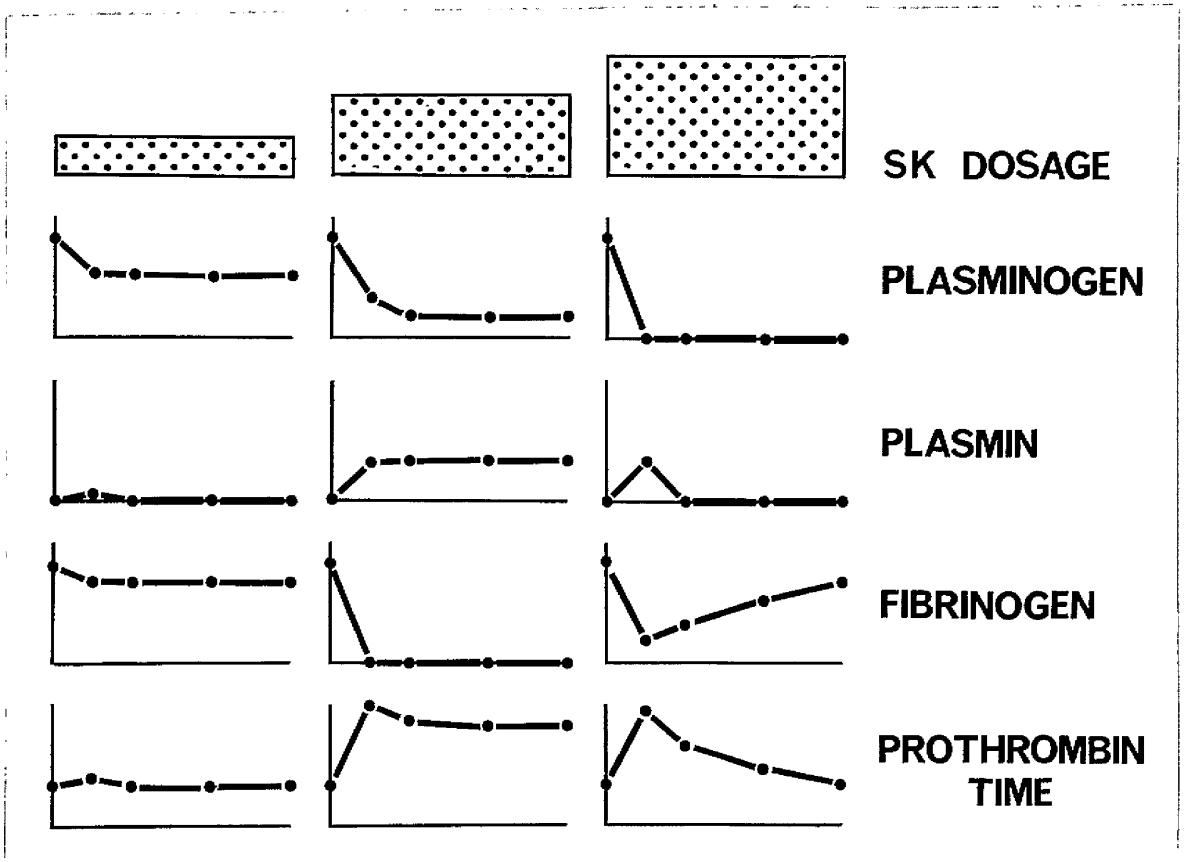


Figure 23 shows three possible approaches to choice of streptokinase infusion rate: low dosage, intermediate dosage and high dosage. In each case an initial antibody neutralising dose is assumed to have been administered first.

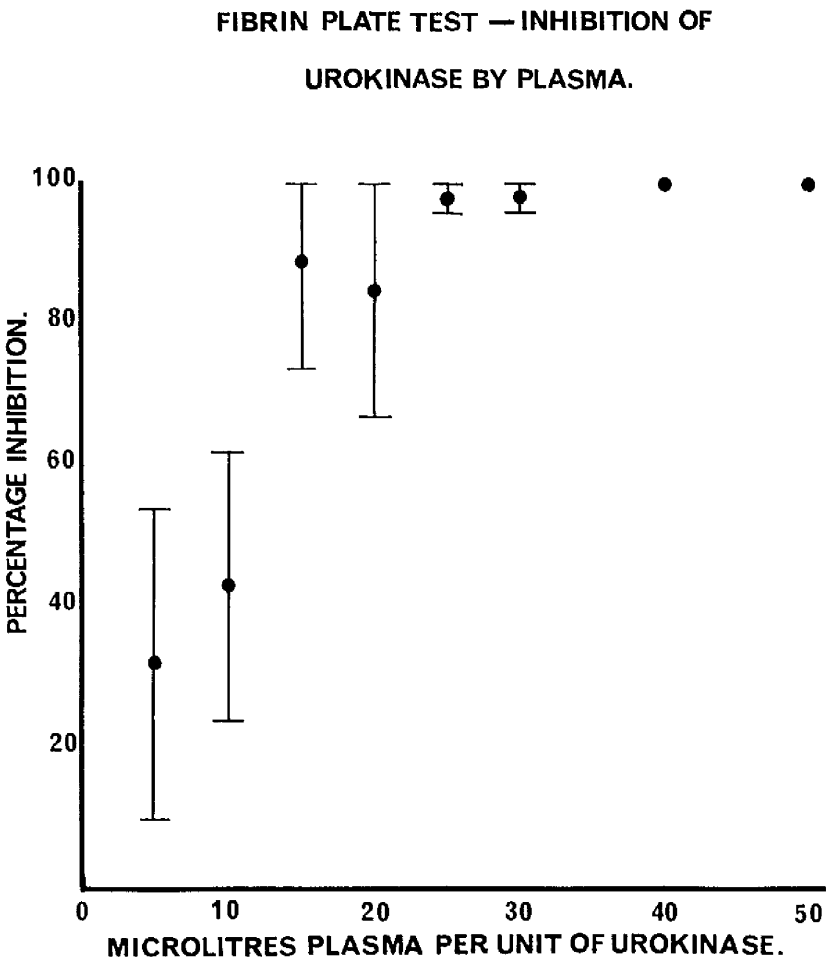


Figure 24. Effect of plasma in inhibiting urokinase in the fibrin plate test. Mean values and standard deviations from observations on 18 plasmas are shown. The figure is prepared from table 42.

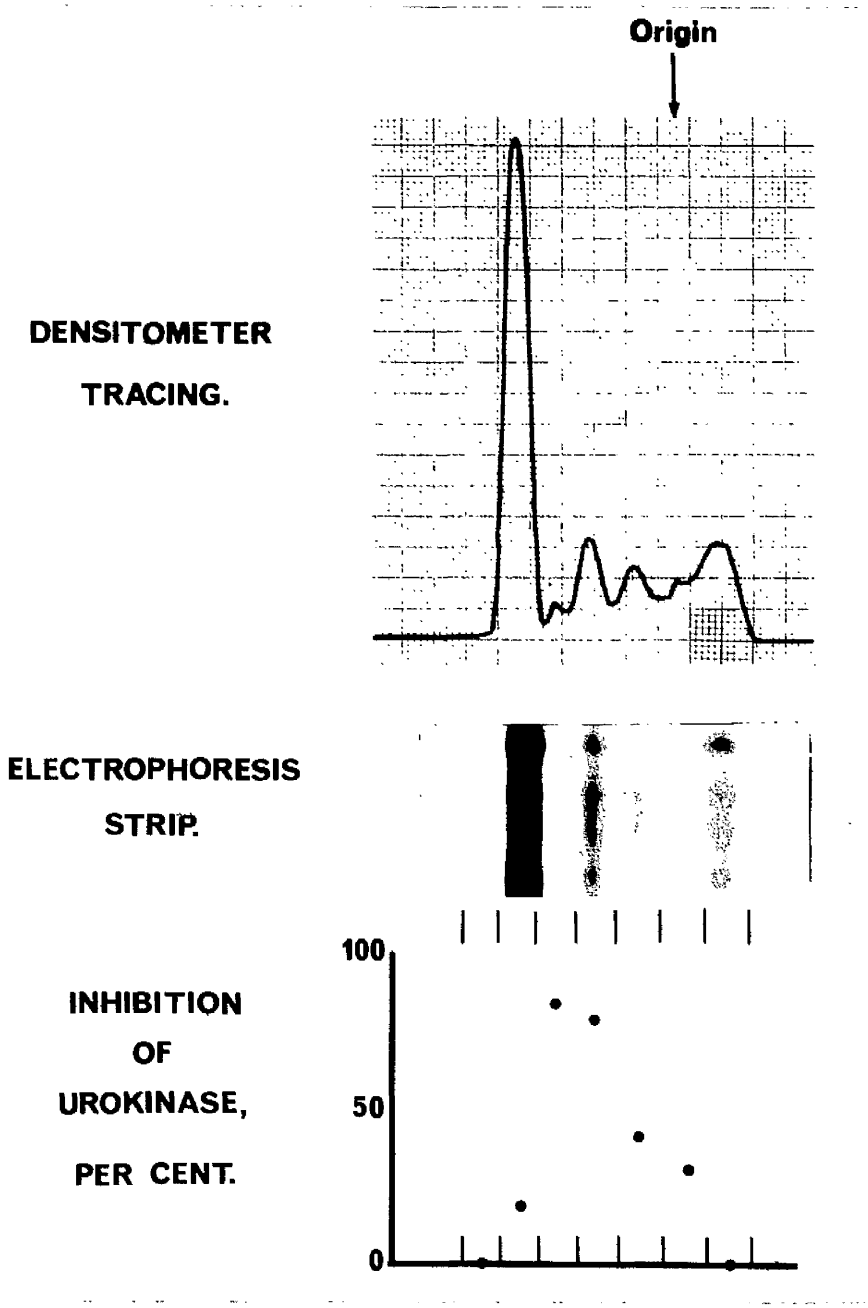


Figure 25. Effect of various serum fractions, obtained by electrophoresis on cellulose acetate, in inhibiting urokinase in the fibrin plate test.

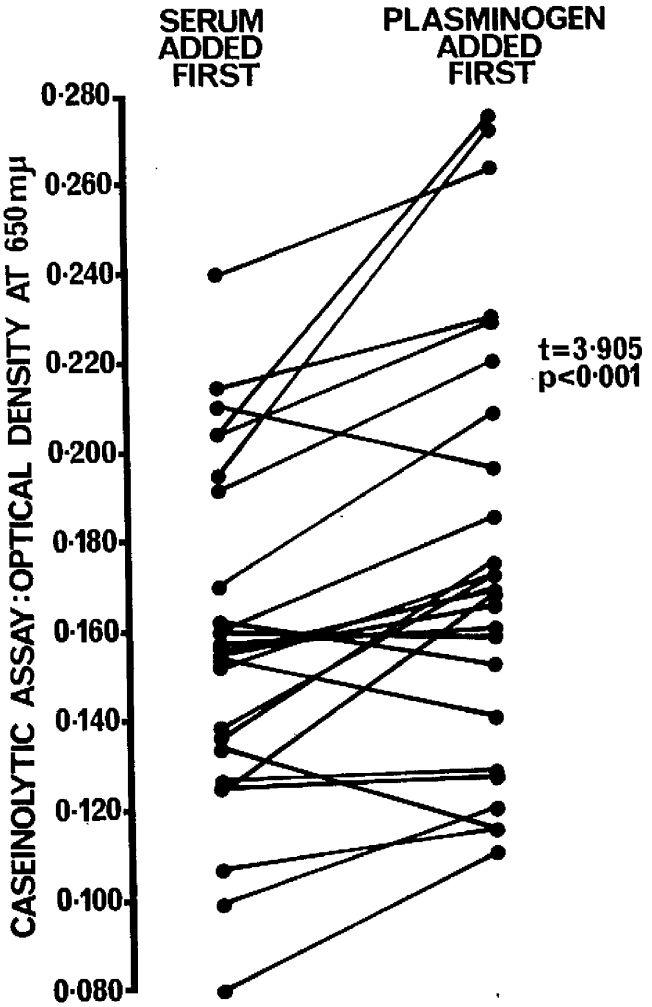


Figure 26. Caseinolytic activity produced by urokinase activation of purified plasminogen, with serum added in one series of experiments before the plasminogen and in the other experiments after the plasminogen. The figure is prepared from table 43.

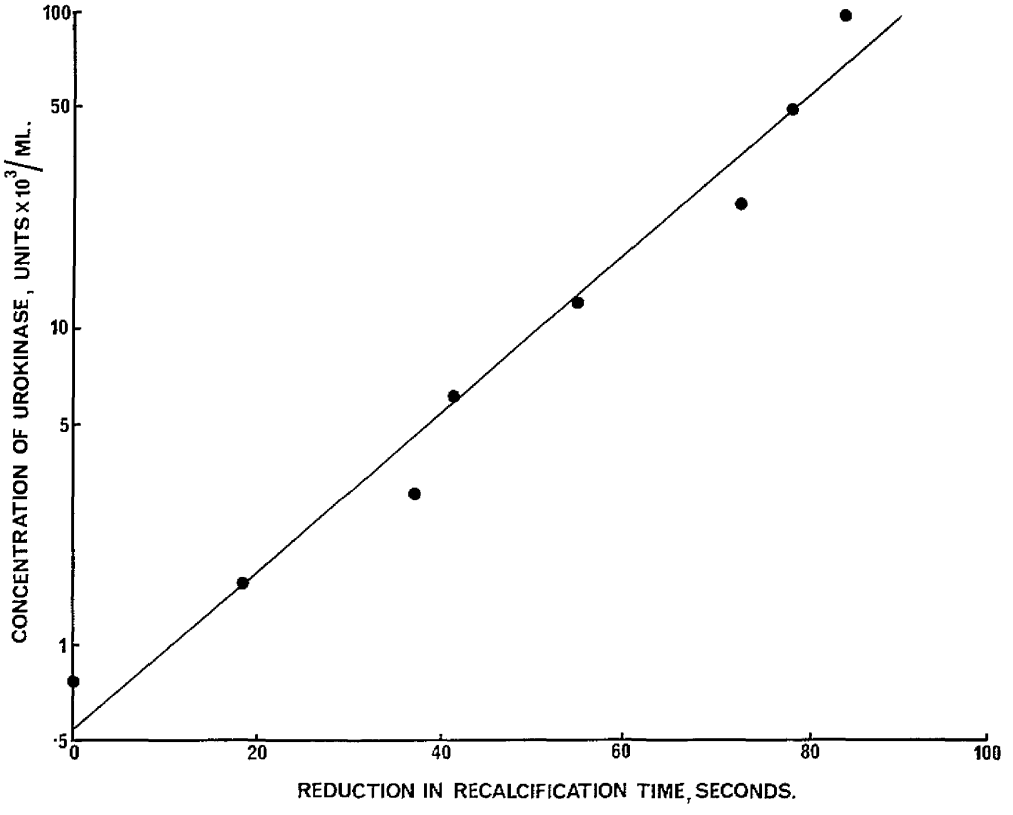


Figure 27. Reduction in clotting time with increasing amounts of urokinase added to normal plasma before recalcification. The figure is prepared from table 4.

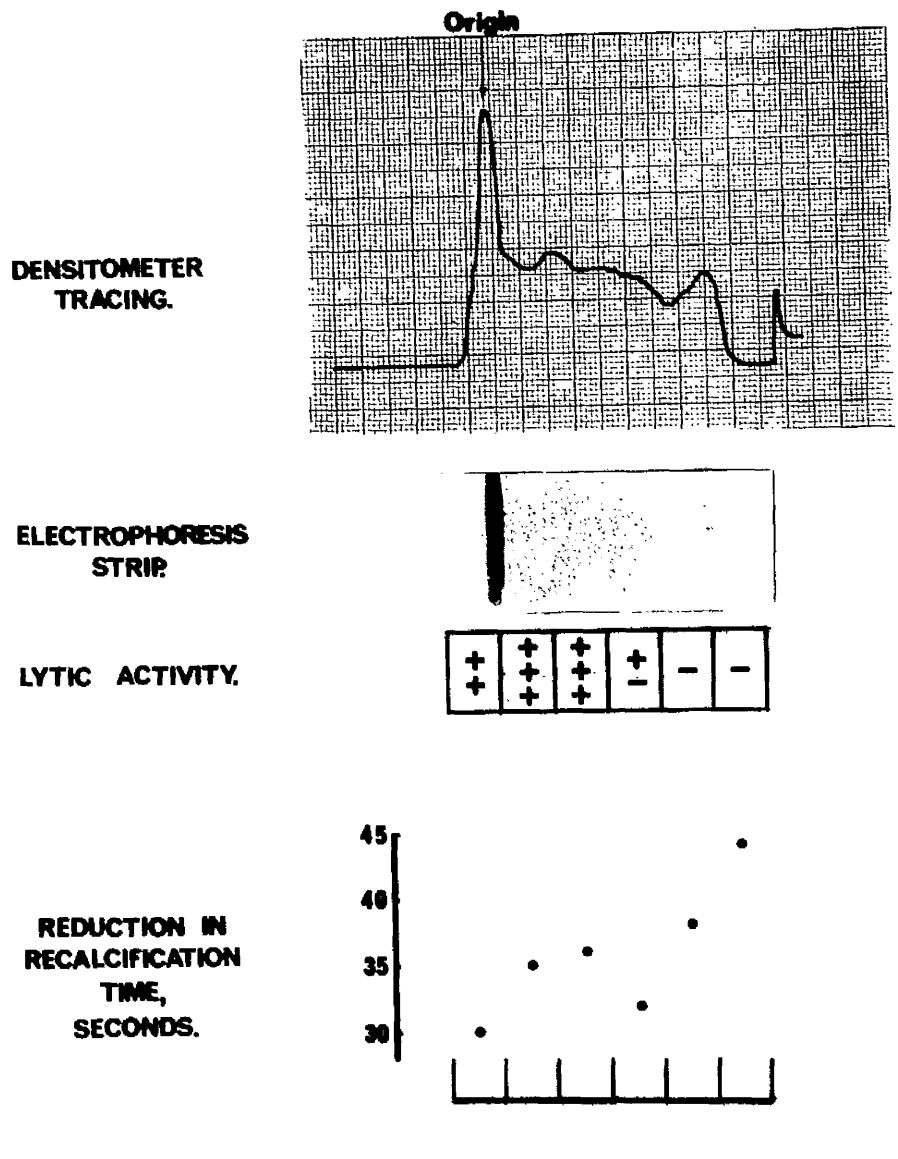


Figure 28 shows separation of the urokinase preparation by electrophoresis on cellulose acetate into at least 4 components in the buffer system used (acetate buffer 0.1 Molar, pH 4.5). These components have differing levels of fibrinolytic activity and may have differing coagulative properties.

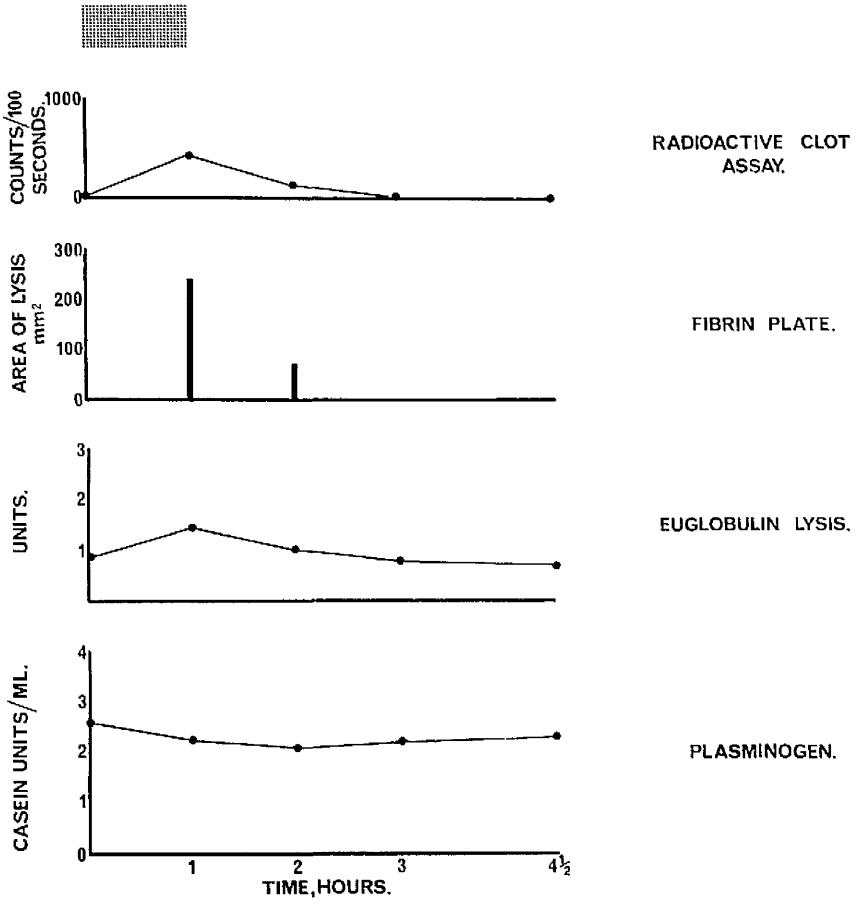


Figure 29. Effect in subject 1, chapter 6, of infusion of 40,000 units of urokinase over 1 hour, shown with hatching. The figure is prepared from table 6.

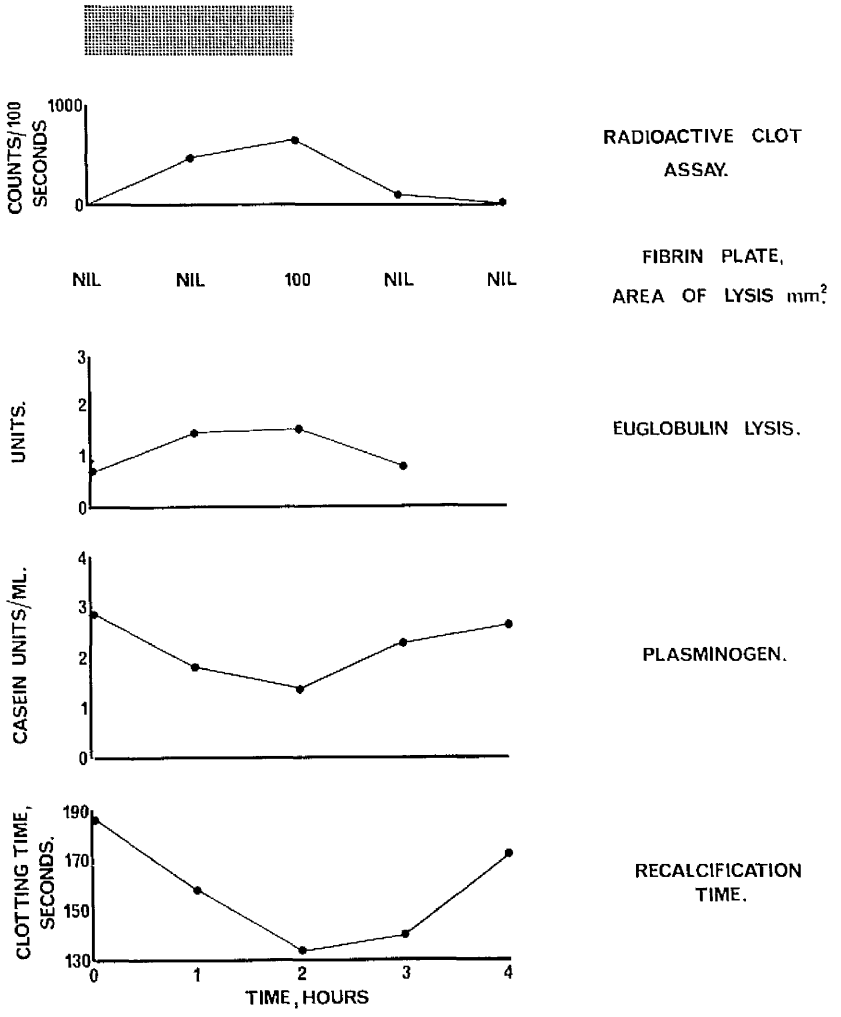


Figure 30. Effect in subject 2, chapter 6, of infusion of 80,000 units of urokinase over two hours, shown with hatching. The figure is prepared from table 7.

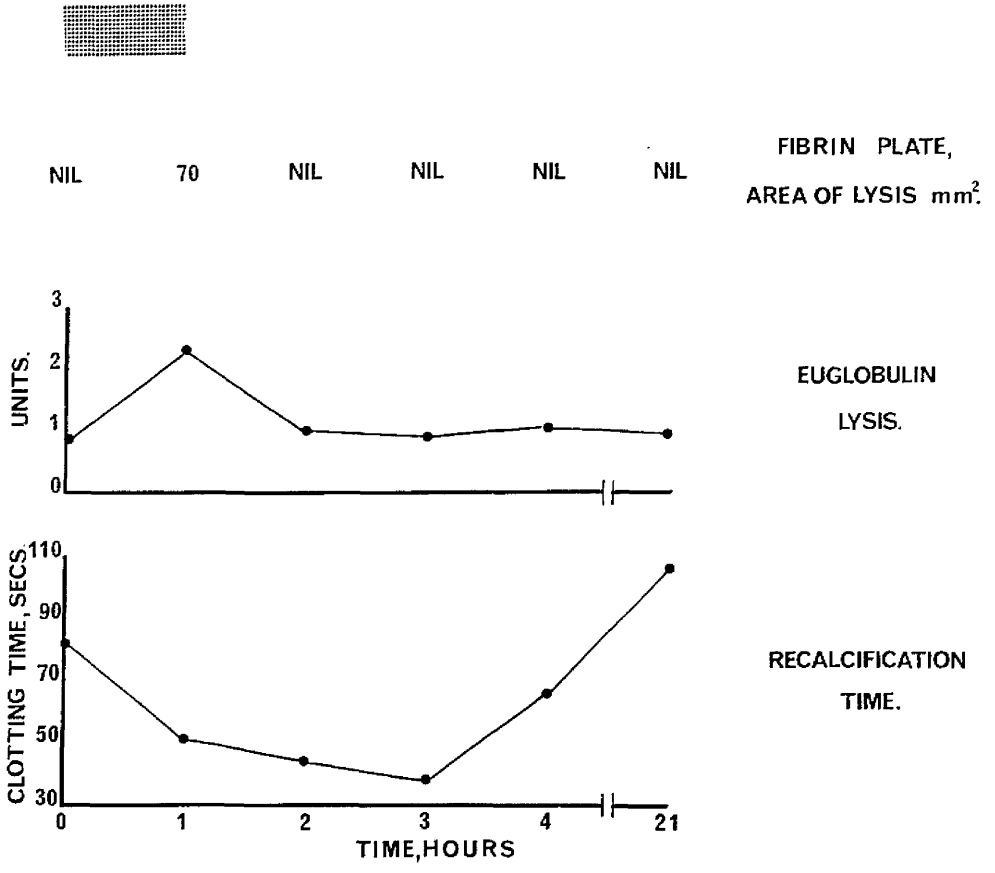


Figure 31. Effect in subject 3, chapter 6, of infusion of 80,000 units of urokinase over 1 hour, shown with hatching. The figure is prepared from table 8.

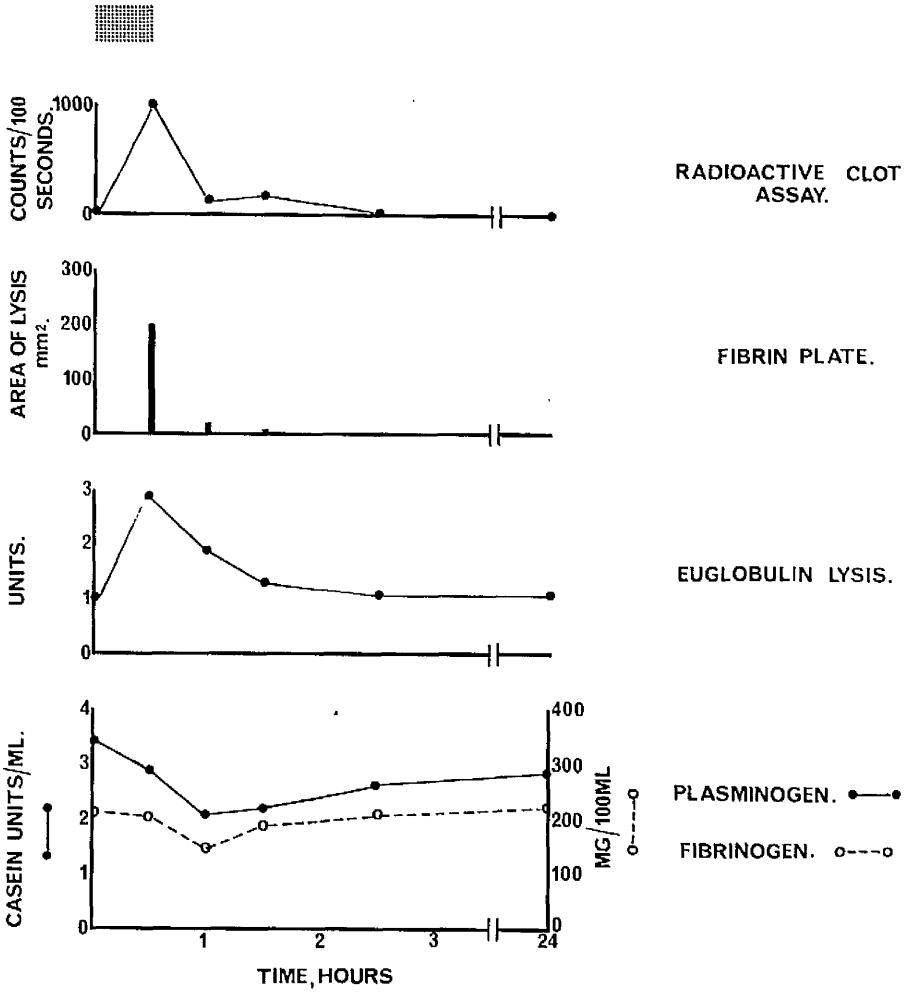


Figure 32. Effect in subject 4, chapter 6, of infusion of 60,000 units of urokinase over 30 minutes, shown with hatching. The figure is prepared from table 9.

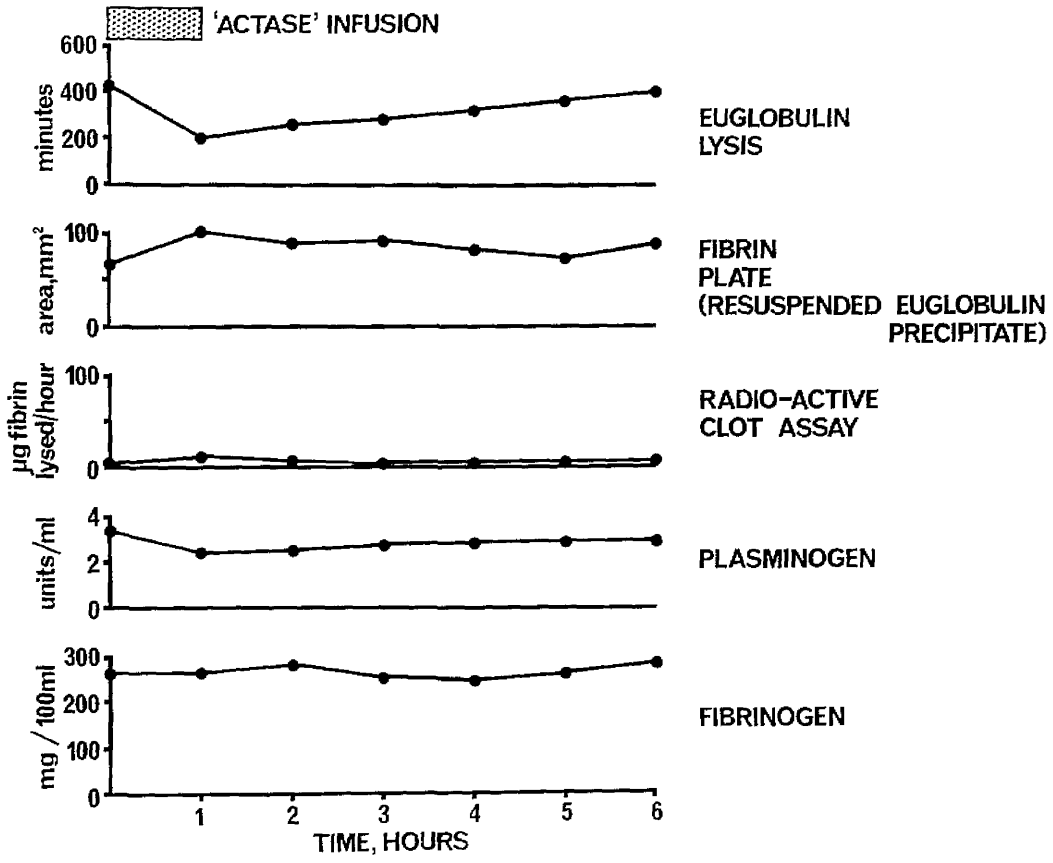


Figure 33 shows the effect in subject 1, chapter 7, of infusion of 1 vial of Actase. The figure is prepared from table 13.

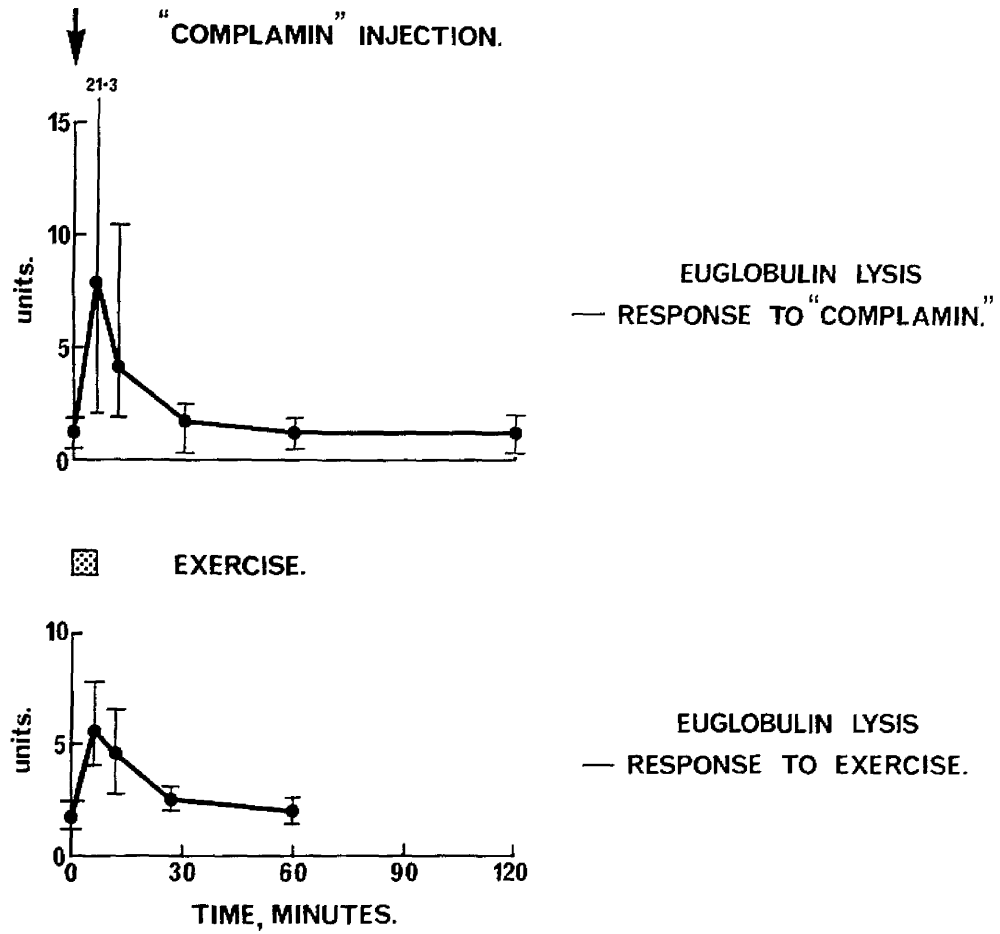


Figure 34 shows in the upper portion the response in euglobulin lysis activity to intravenous injection of 150 mg. Complamin in 7 healthy subjects. Mean values and ranges are shown. The lower half of the figure illustrates the effect of 5 minutes strenuous exercise on euglobulin lysis activity in the same subjects. The figure is prepared from tables 45 and 46.

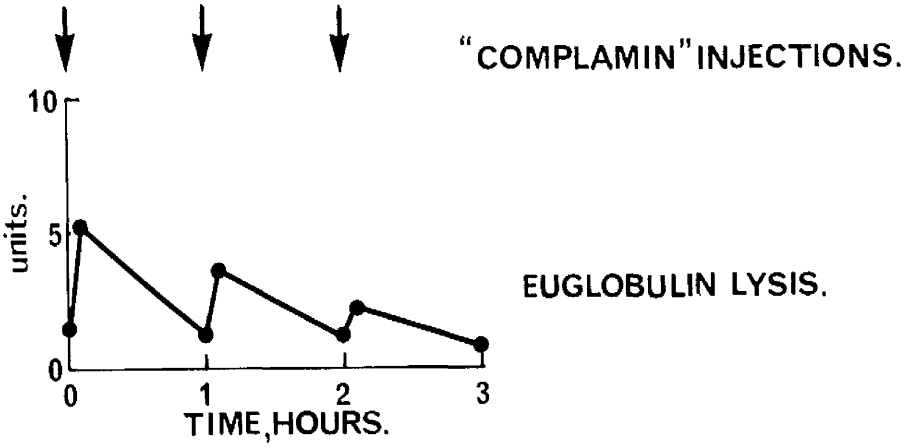


Figure 35 shows the effect on euglobulin lysis activity of three intravenous injections of 150 mg. Complamin given to a healthy subject at hourly intervals. The figure is prepared from table 47.

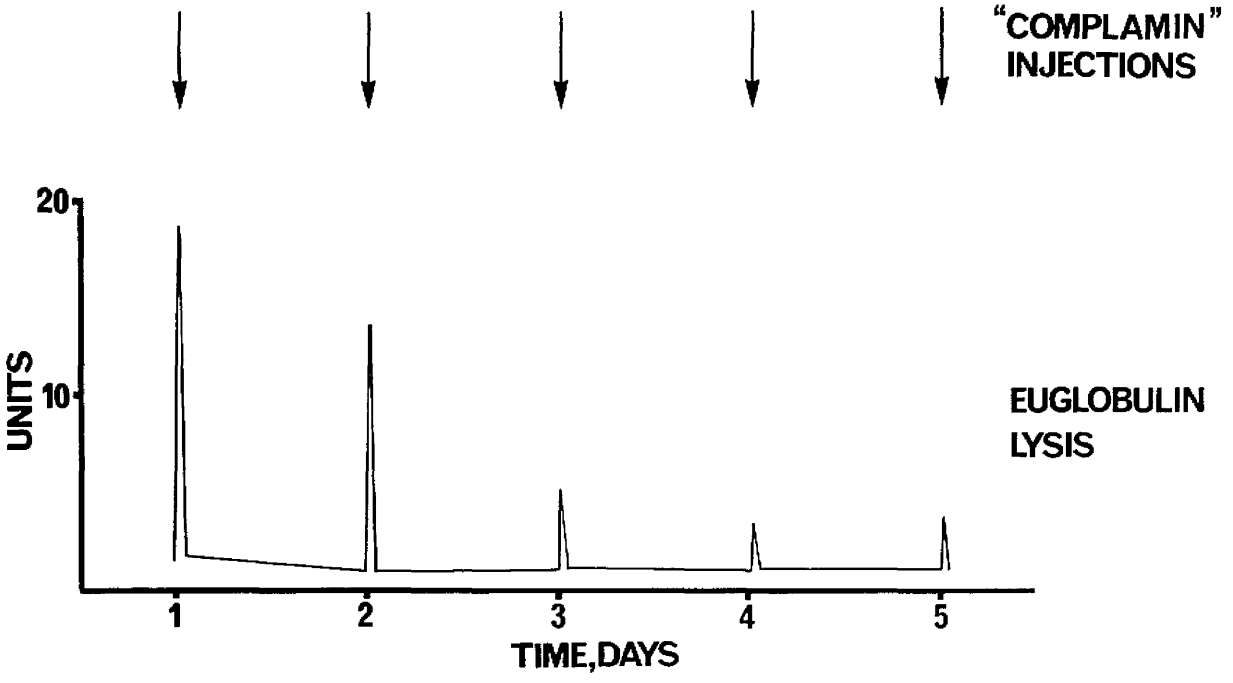
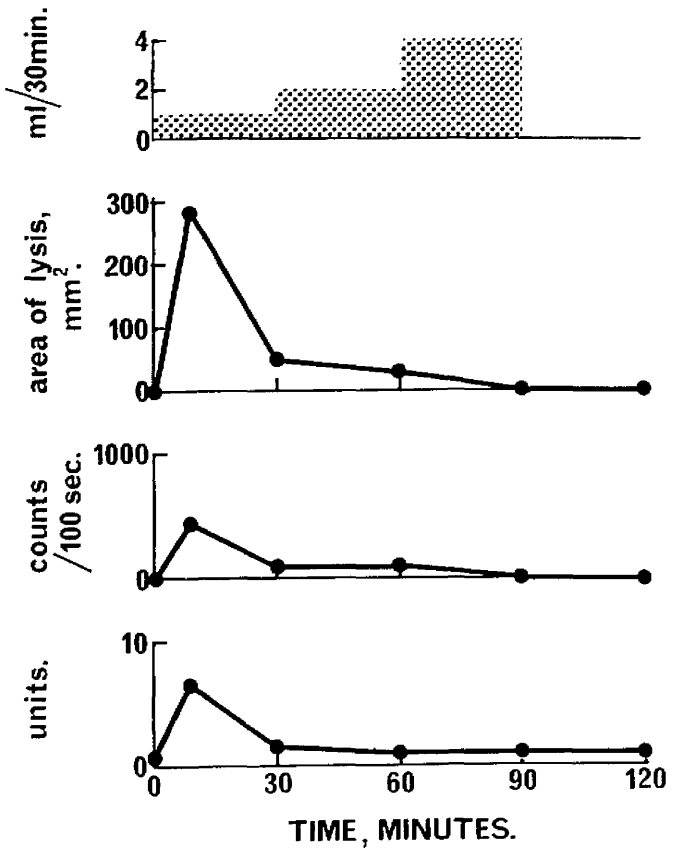


Figure 36 shows the effect on euglobulin lysis activity of a daily intravenous injection of 150 mg. Complamin for 5 days. The figure is prepared from table 48.



"COMPLAMIN" INFUSION RATE.

FIBRIN PLATE TEST.

RADIOACTIVE CLOT ASSAY.

EUGLOBULIN LYSIS.

Table 37 shows the effect of intravenous infusion of Complamin at a progressively increased rate (1 ml. = 150 mg.). The figure is prepared from table 19.

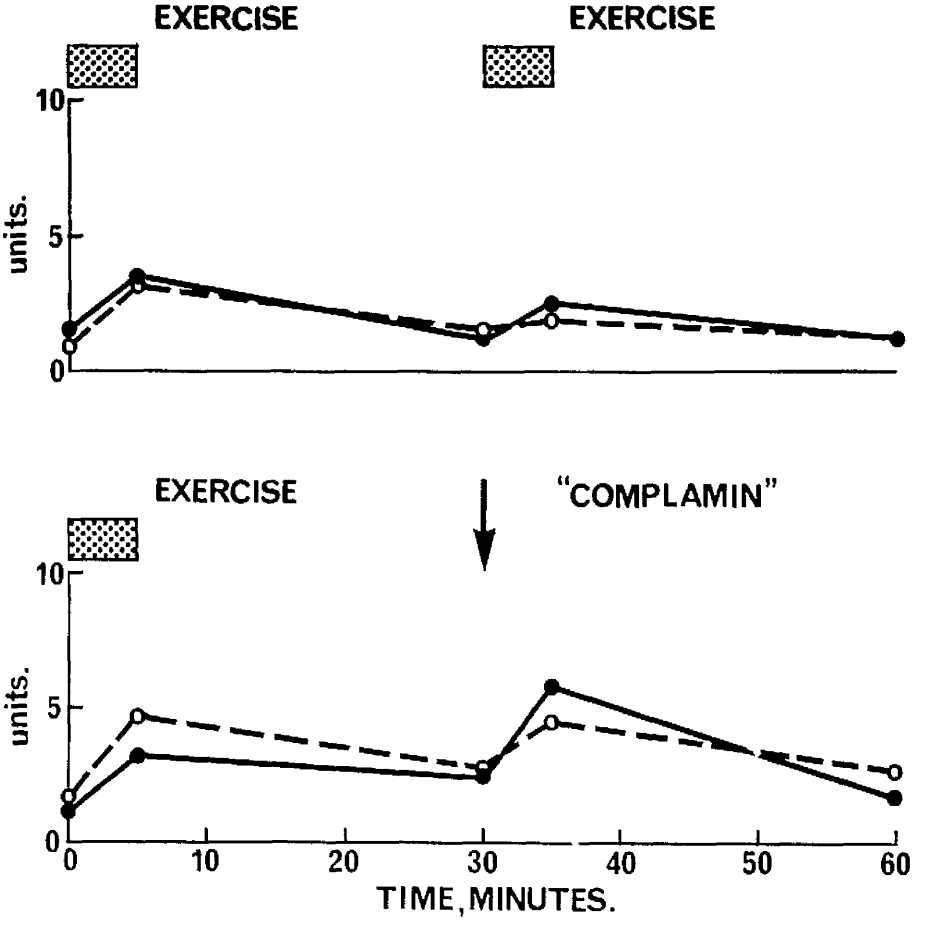


Figure 38 shows in two subjects, the effect on euglobulin lysis activity of 5 minutes exercise followed at 30 minutes by a further 5 minutes exercise (upper part of diagram) and on another occasion the effect of 5 minutes exercise followed at 30 minutes by 150 mg. Complamin intravenously (lower part of diagram.) The figure is prepared from table 49.

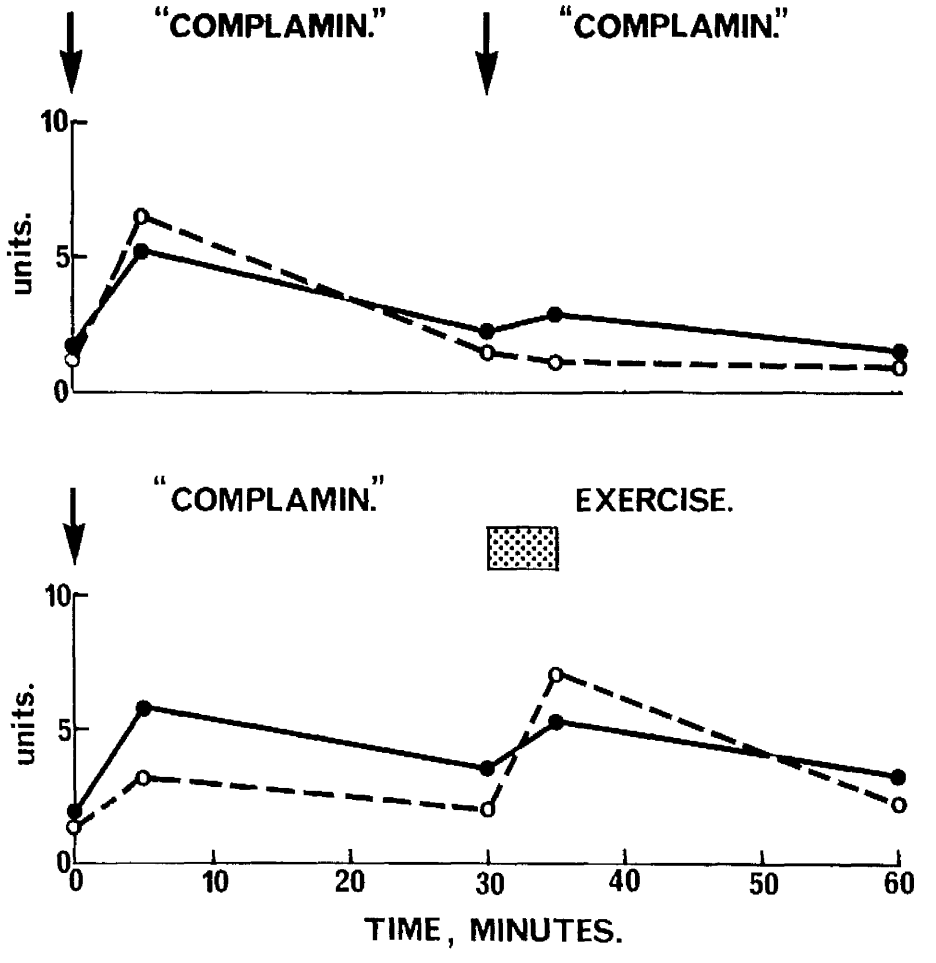


Figure 39 shows, in the same two subjects as figure 38, the effect on euglobulin lysis activity of 150 mg. Complamin injected intravenously followed in 30 minutes by a second intravenous injection of 150 mg. Complamin (upper part of diagram) and on another occasion the effect of 150 mg. Complamin injected intravenously followed in 30 minutes by 5 minutes exercise (lower part of diagram).

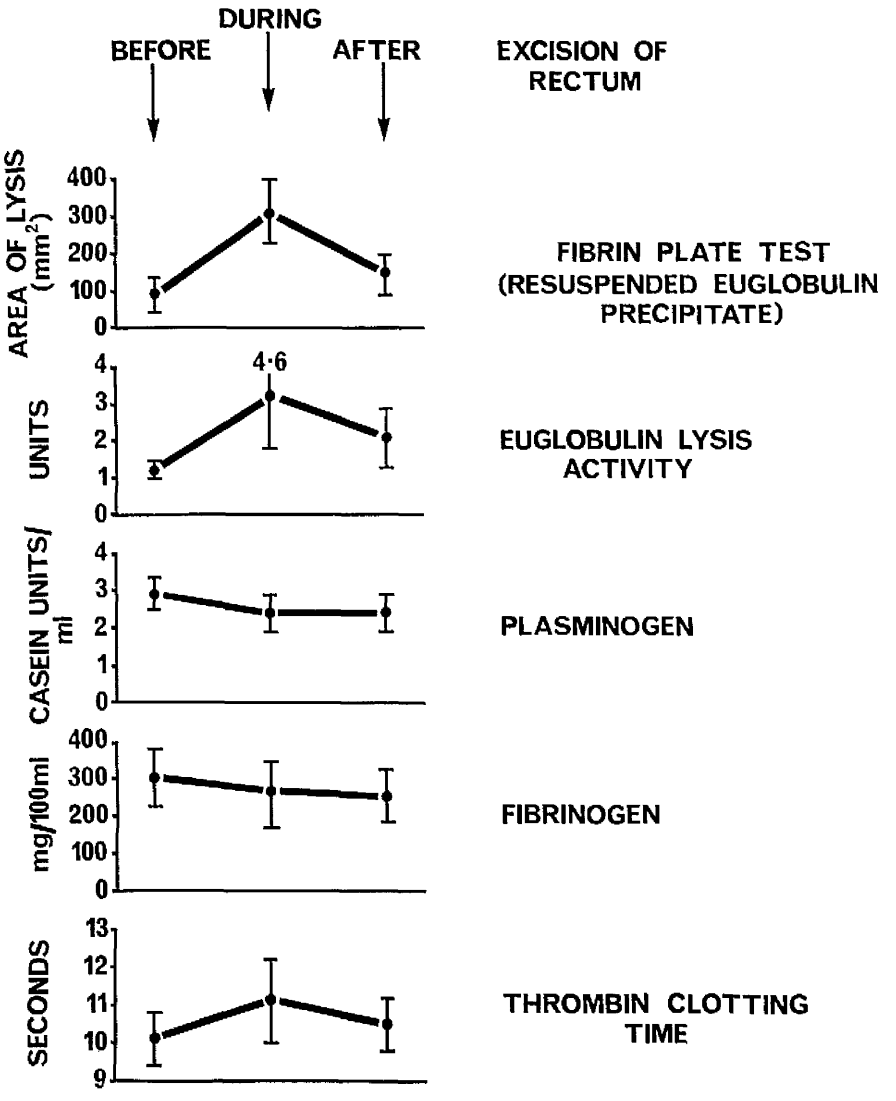


Figure 40 shows the effect on the plasminogen-plasmin system of excision of the rectum in 10 patients. Mean values and standard deviations are shown. The figure is prepared from tables 50 - 54.

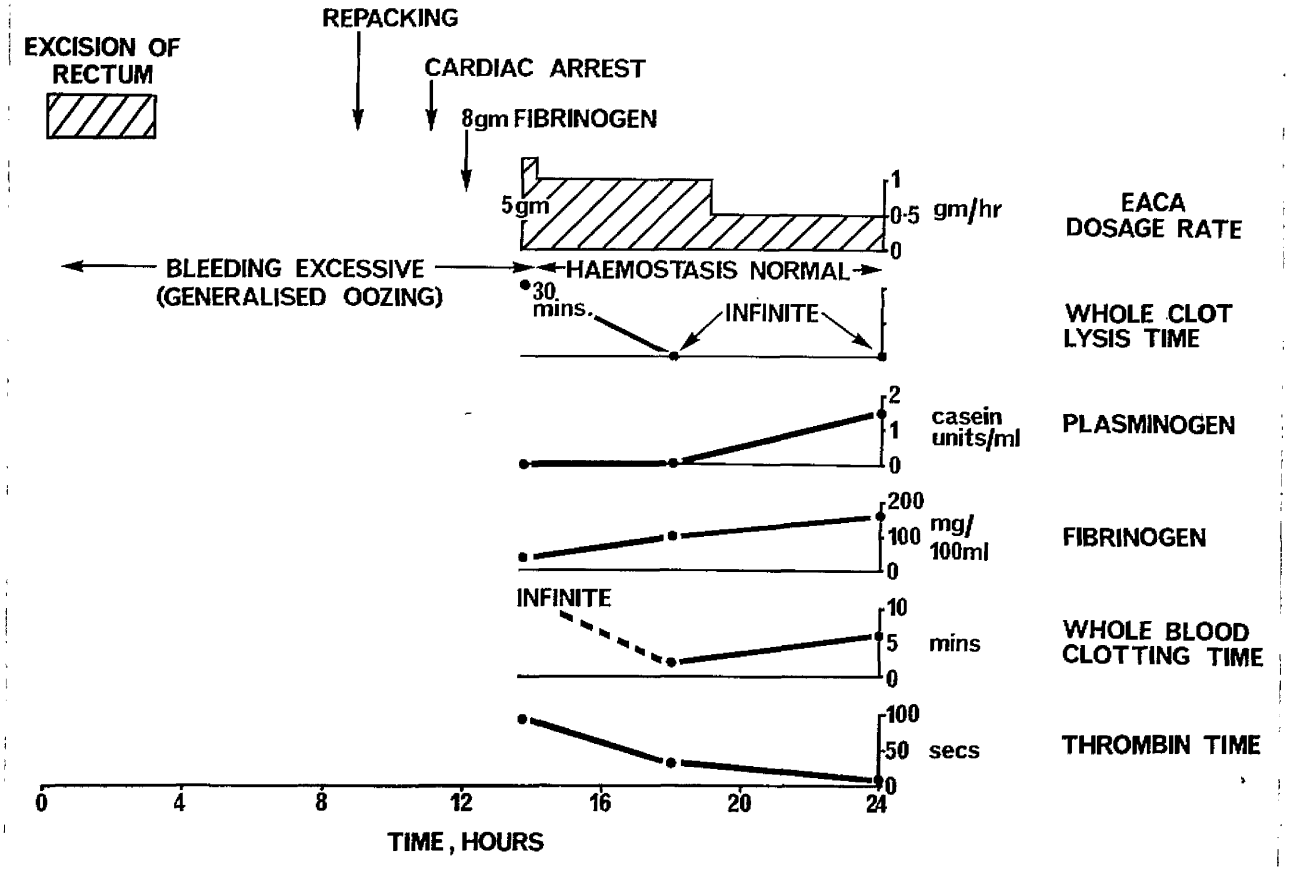


Figure 41 shows the course of a pathological "fibrinolytic" state arising during excision of the rectum (patient 11, chapter 9). The figure is prepared from table 55.

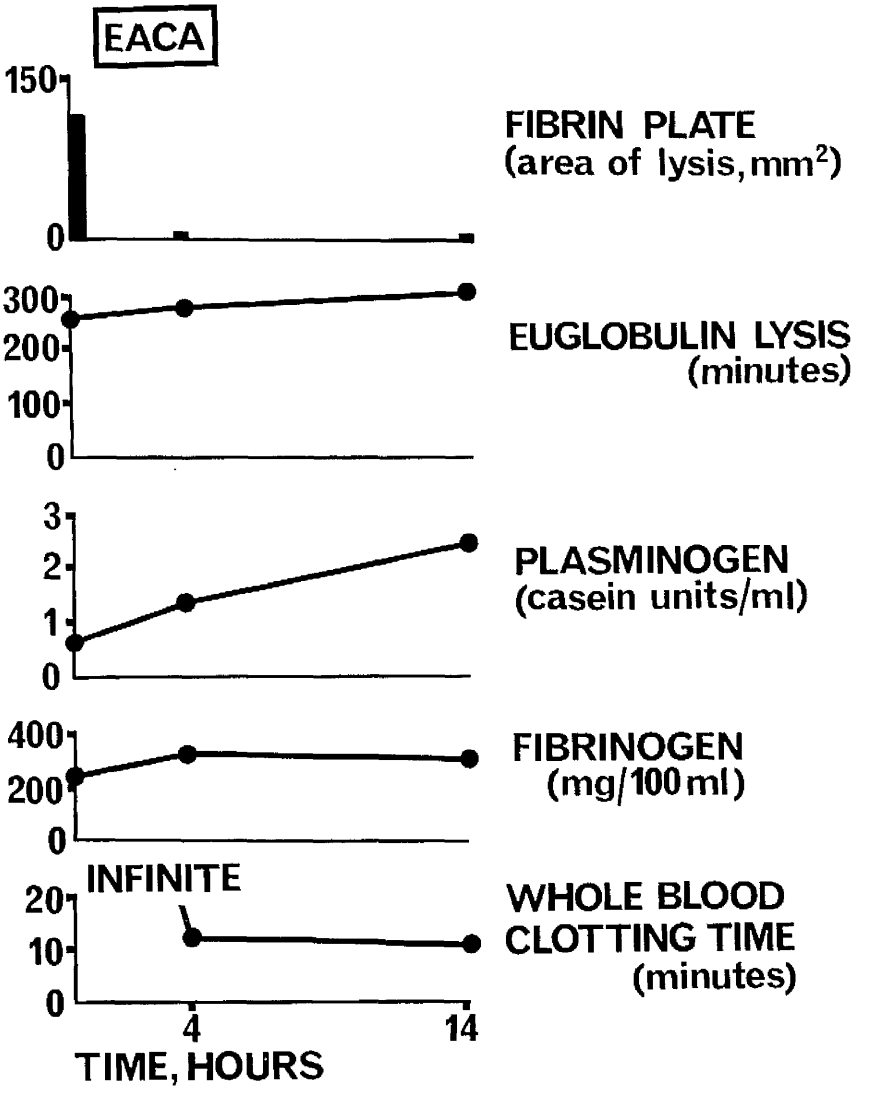


Figure 42 shows the course of a pathological "fibrinolytic" state arising after spleno-renal anastomosis and splenectomy for haematemesis due to hepatic cirrhosis and portal hypertension (patient 12, chapter 9). Ten gm. EACA were given over 4 hours. The figure is prepared from table 56.

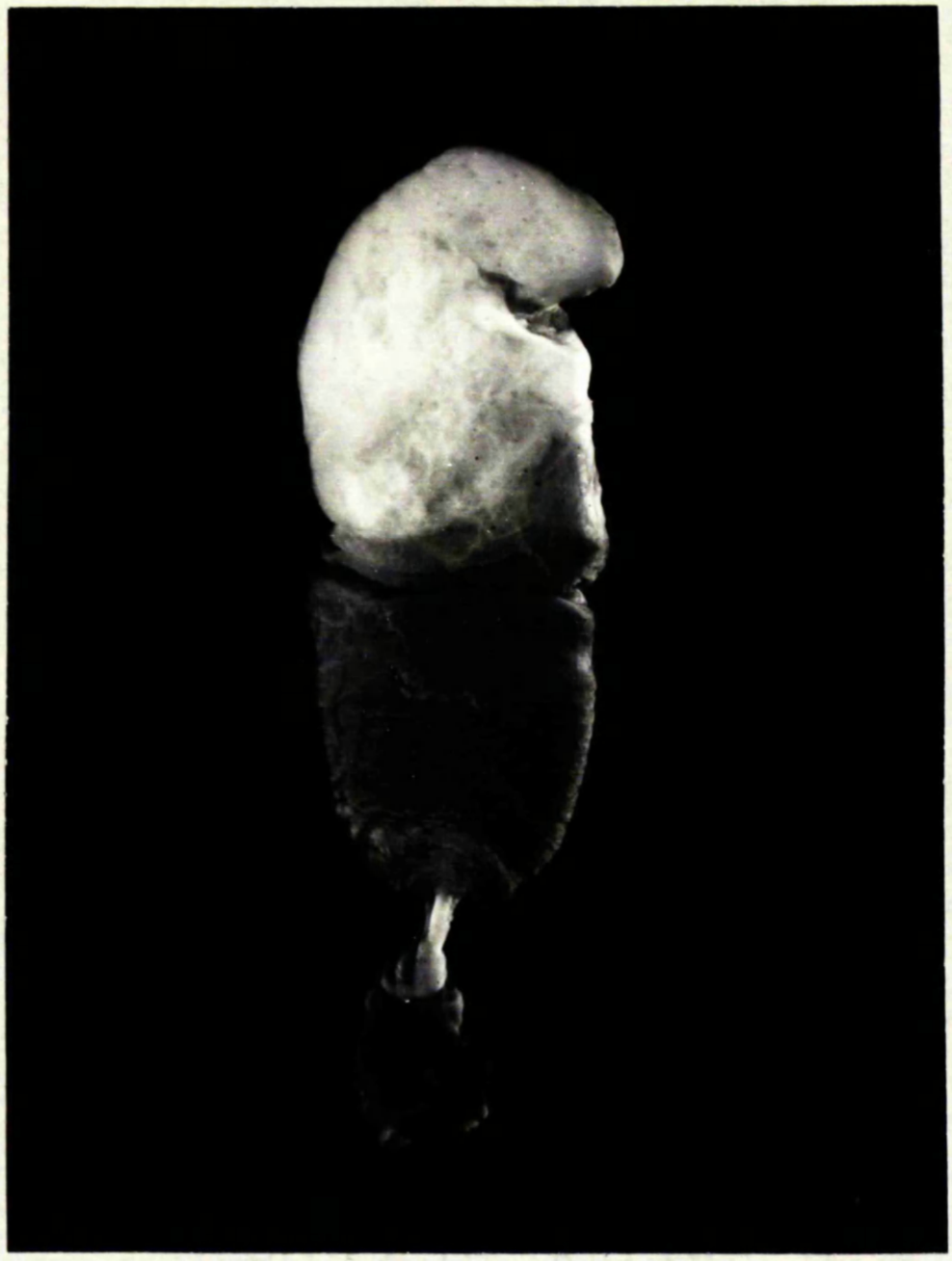


Figure 43 shows an artificial thrombus (length about 2 cm.) made in a Chandler's tube by recalcification of platelet-rich plasma. The white head and the fibrin tail can be seen.

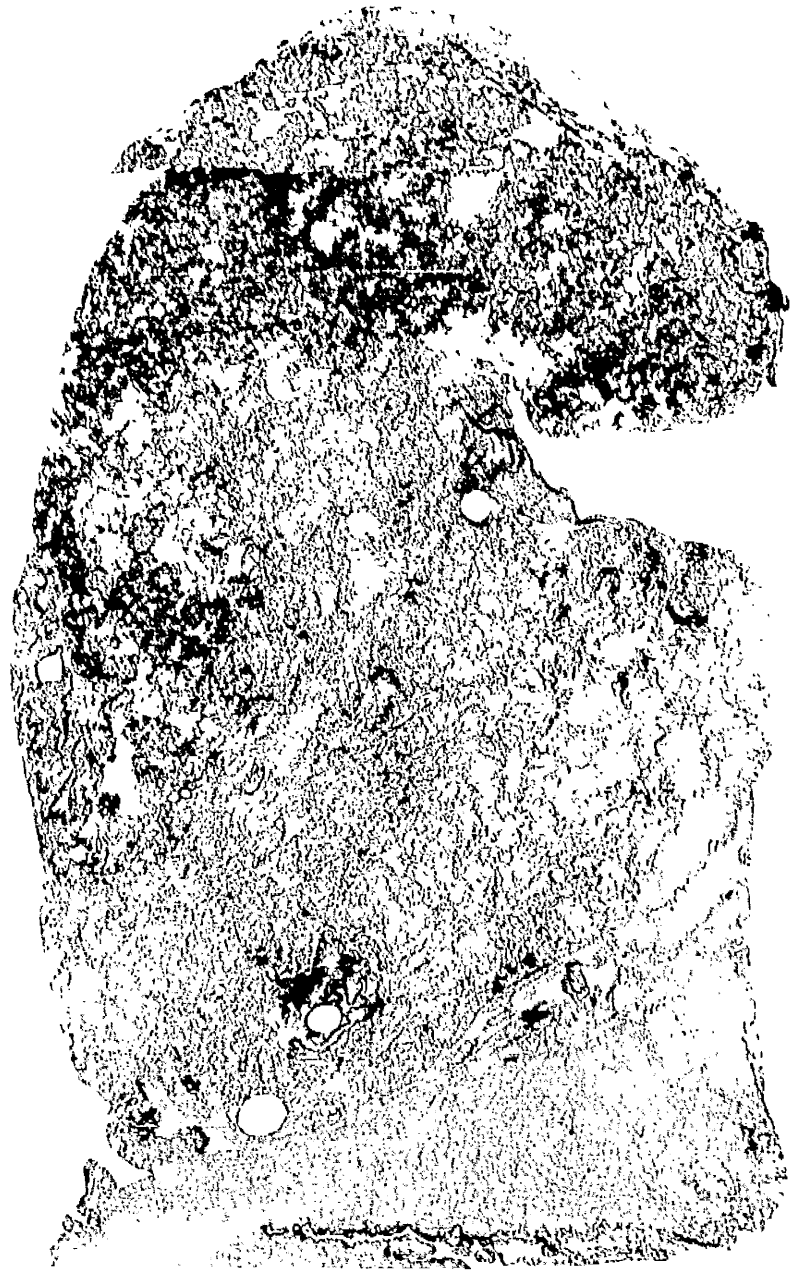


Figure 44 shows a longitudinal section through the thrombus illustrated in figure 43. The contrast between the organised coral-like platelet head and the fibrin tail can be seen. Original magnification x 10. Stain - Picro-Mallory. (Photomicrograph prepared by Dr. F. Walker).

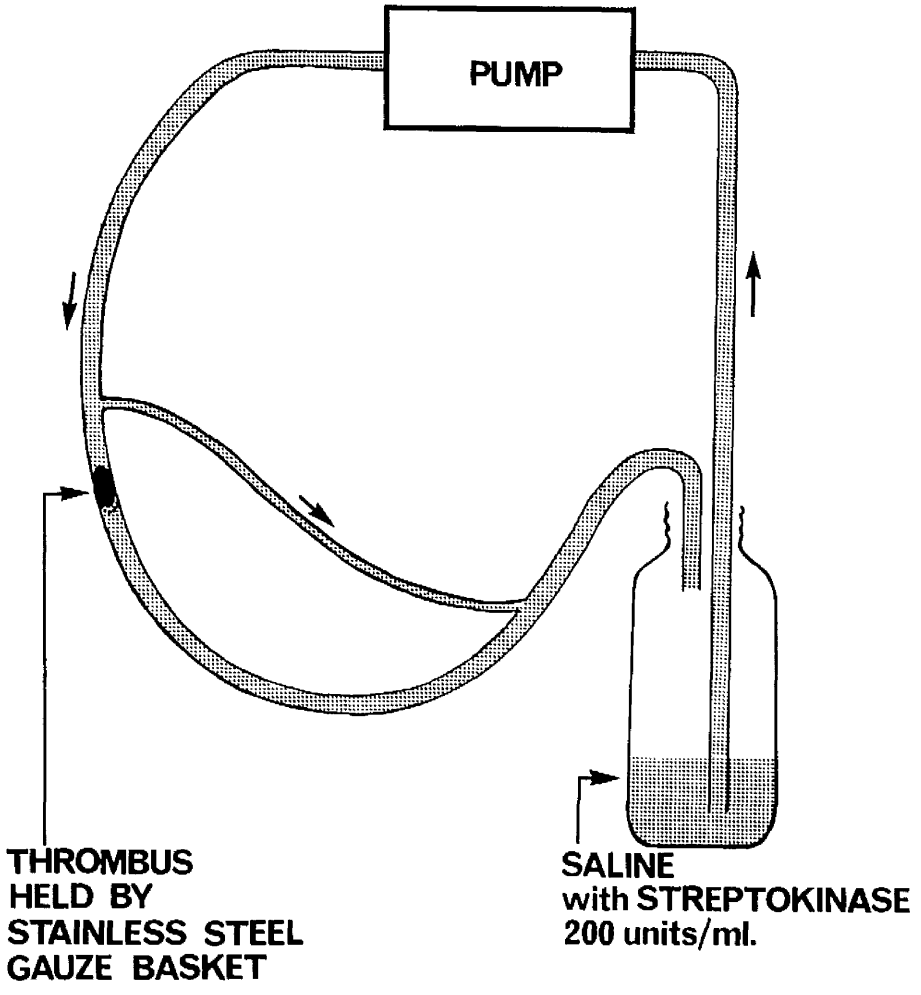


Figure 45 is a diagramatic representation of the circulation used for perfusion of thrombi prepared in the Chandler's tube.

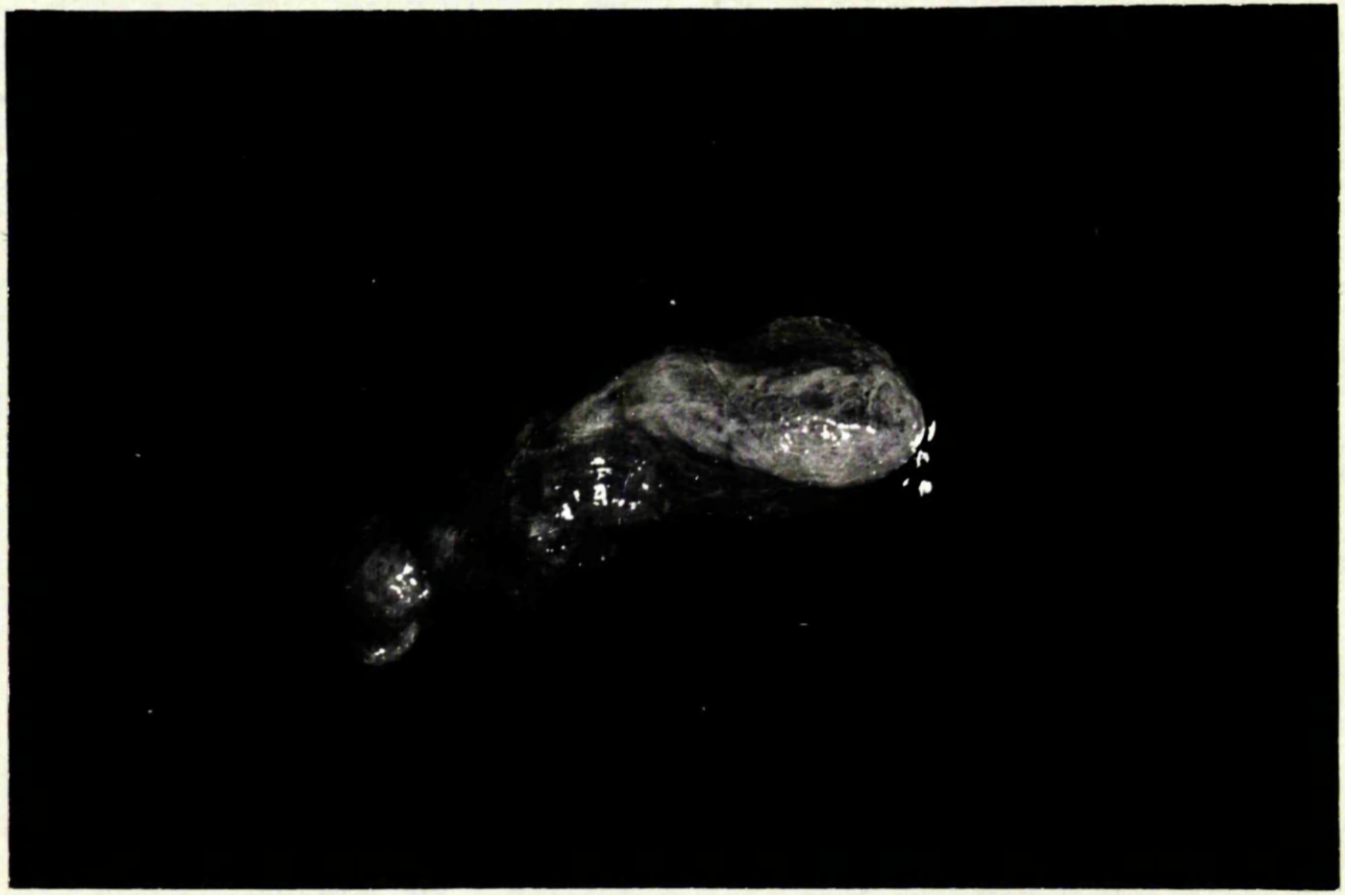


Figure 46 shows a whole blood thrombus (length about 2 cm.) prepared in a Chandler's tube from a patient with hyperlipidaemia (patient 3a, table 27). The white head and the fibrin tail can be seen, though the differentiation is less clear than in the plasma thrombus shown in figure 43.

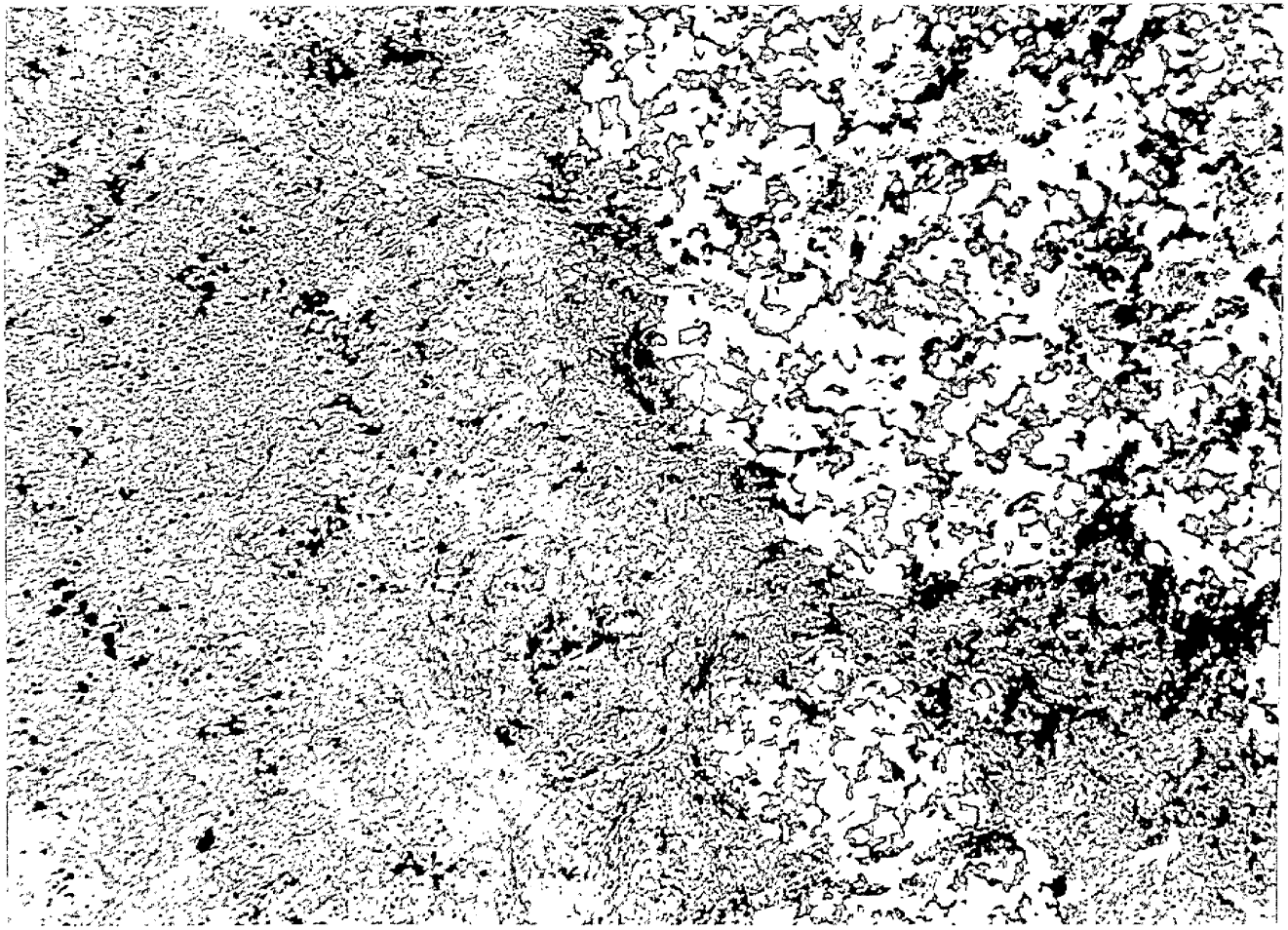


Figure 47. Histological appearances of the junction between the platelet-rich head on the right, and the fibrin tail, on the left, of a thrombus prepared in the Chandler's tube from normal blood. Original magnification x 60. Stain - Picro-Mallory. (Photomicrograph prepared by Dr. F. Walker).

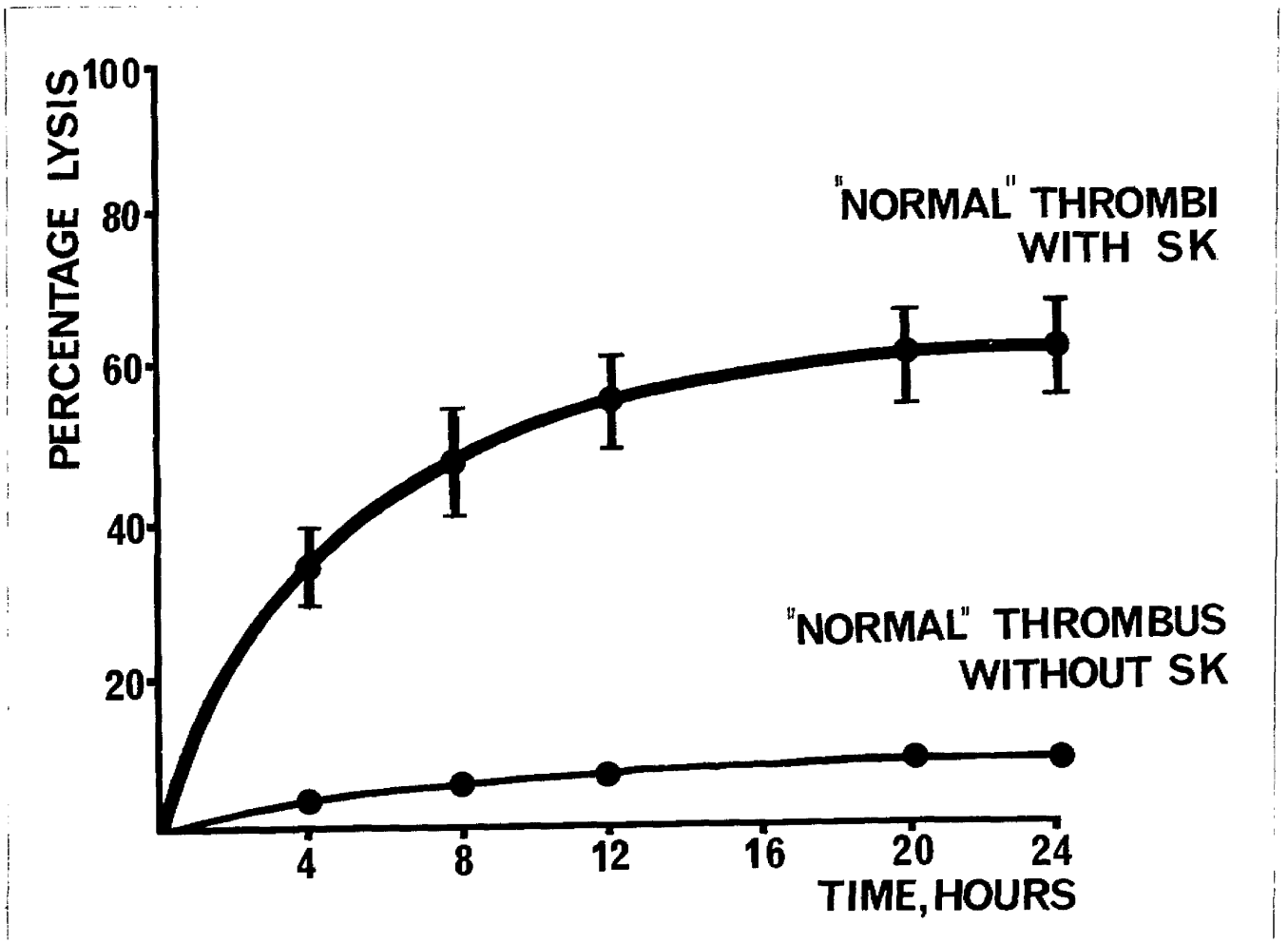


Figure 48. Patterns of radioactivity release during perfusion with streptokinase of Chandler thrombi prepared from 7 normal subjects. Mean values and standard deviations are shown. The figure also shows the stability of a 'normal' thrombus perfused with saline alone. The figure is prepared from tables 24 and 29.

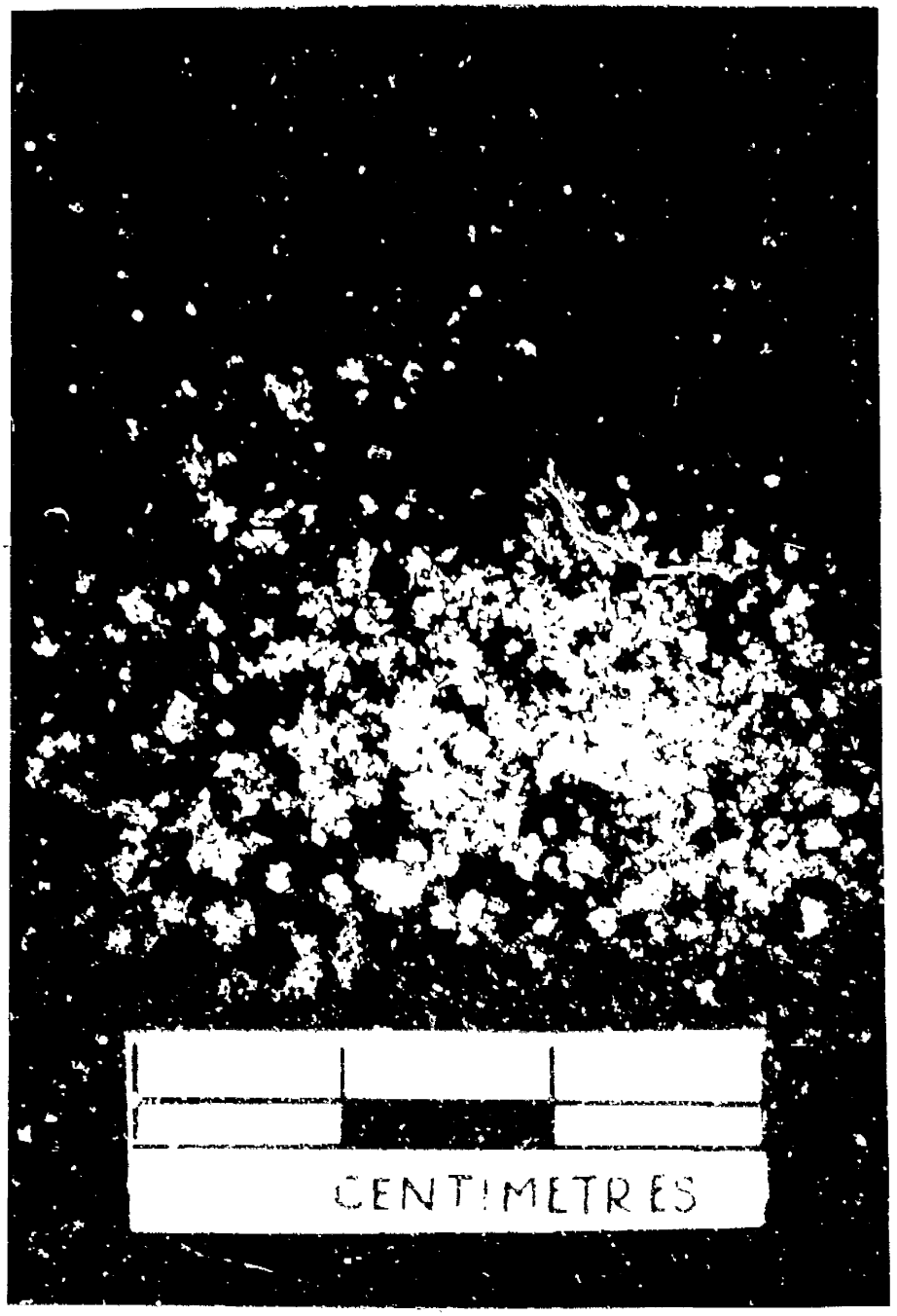


Figure 49. Naked eye appearance of fragments released into the artificial circulation after 24 hours perfusion with streptokinase of a "normal" thrombus.

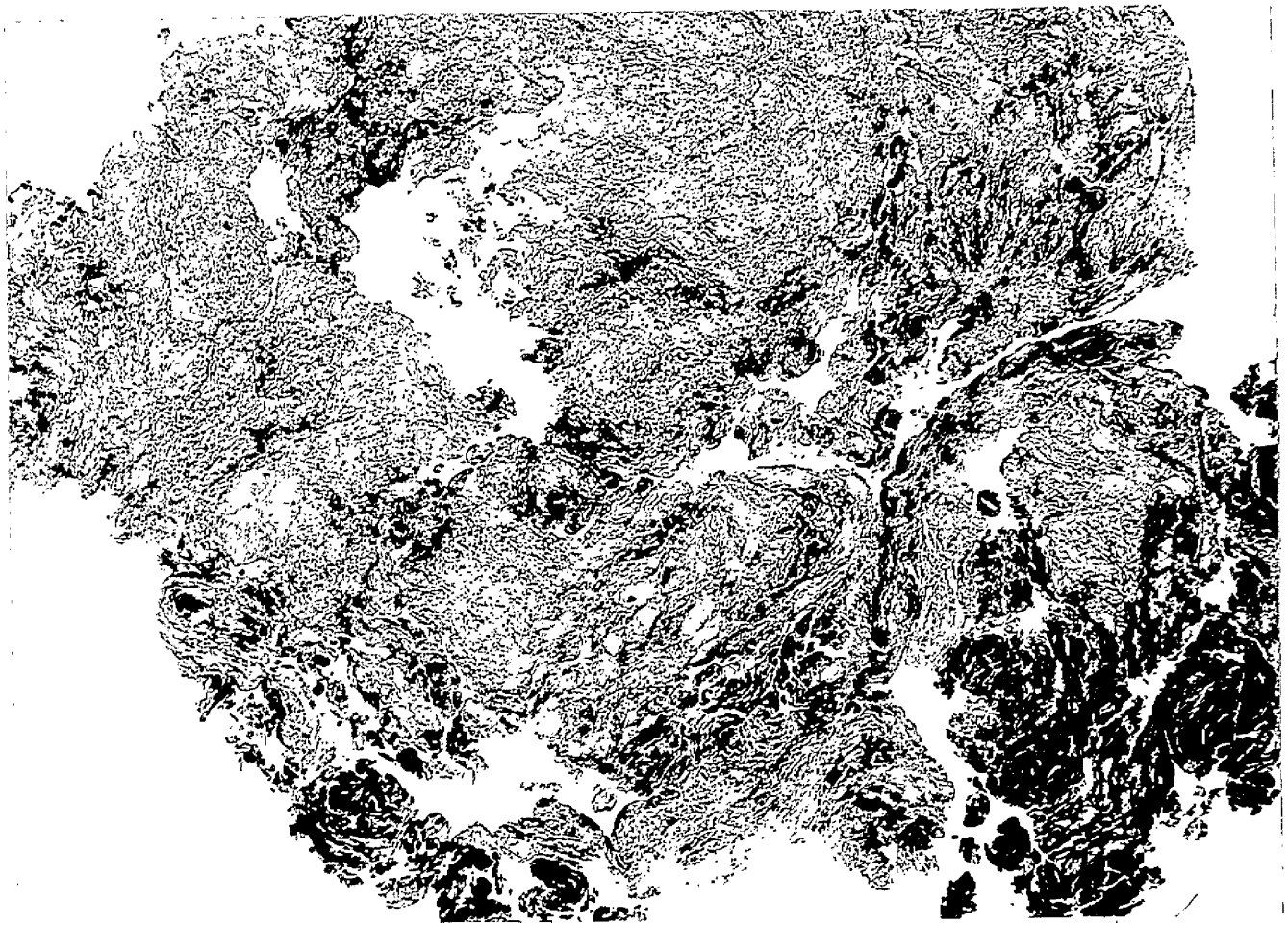


Figure 50. Histological appearance of large fragment found in the artificial circulation after perfusion of "normal" thrombus with streptokinase. Original magnification x 60. Stain - Picro-Mallory. (Photomicrograph prepared by Dr. F. Walker).

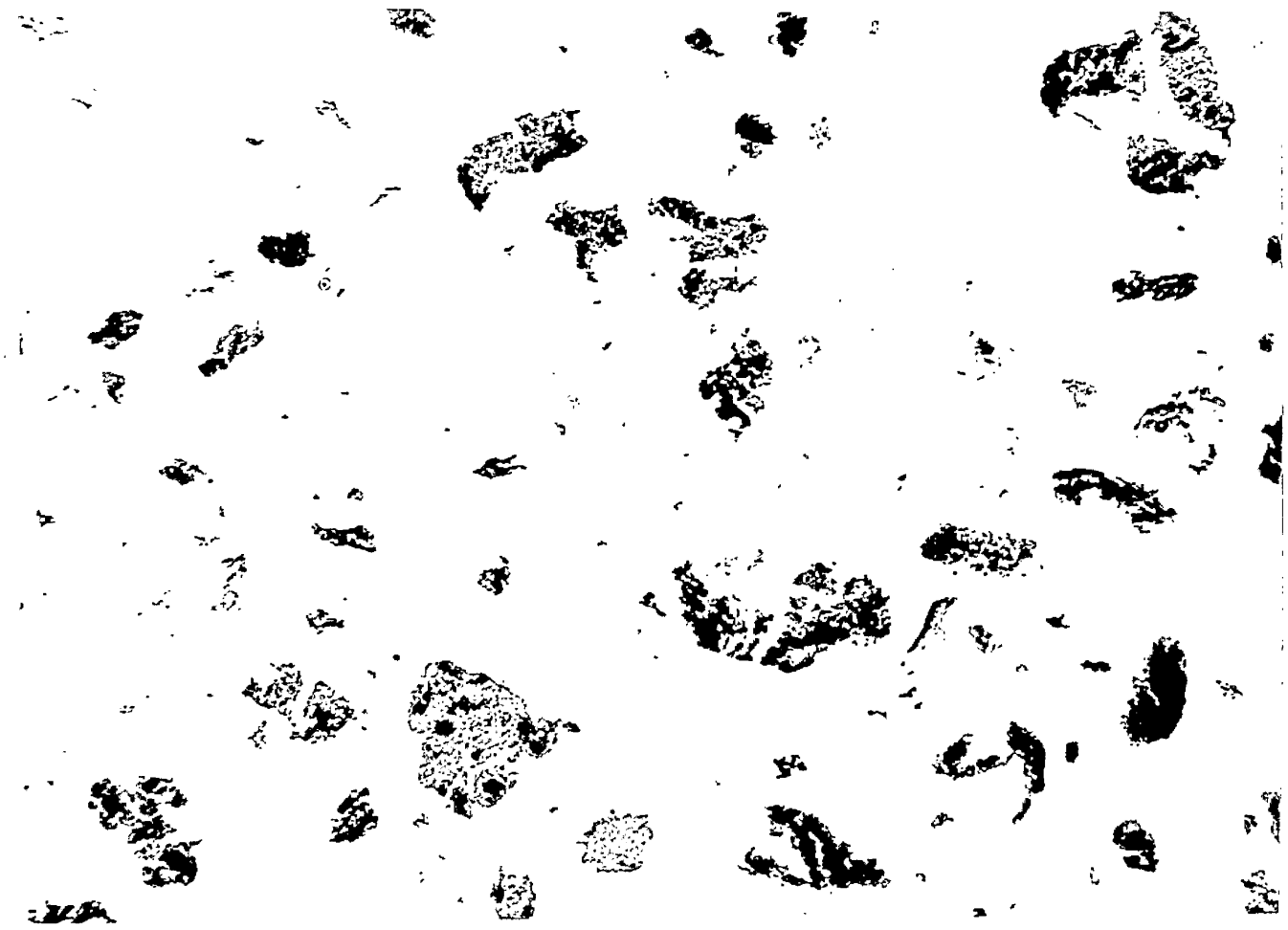


Figure 51. Histological appearance of small fragments found in artificial circulation after perfusion of "normal" thrombus with streptokinase. Original magnification x 60. Stain - Picro-Mallory. (Photomicrograph prepared by Dr. F. Walker).

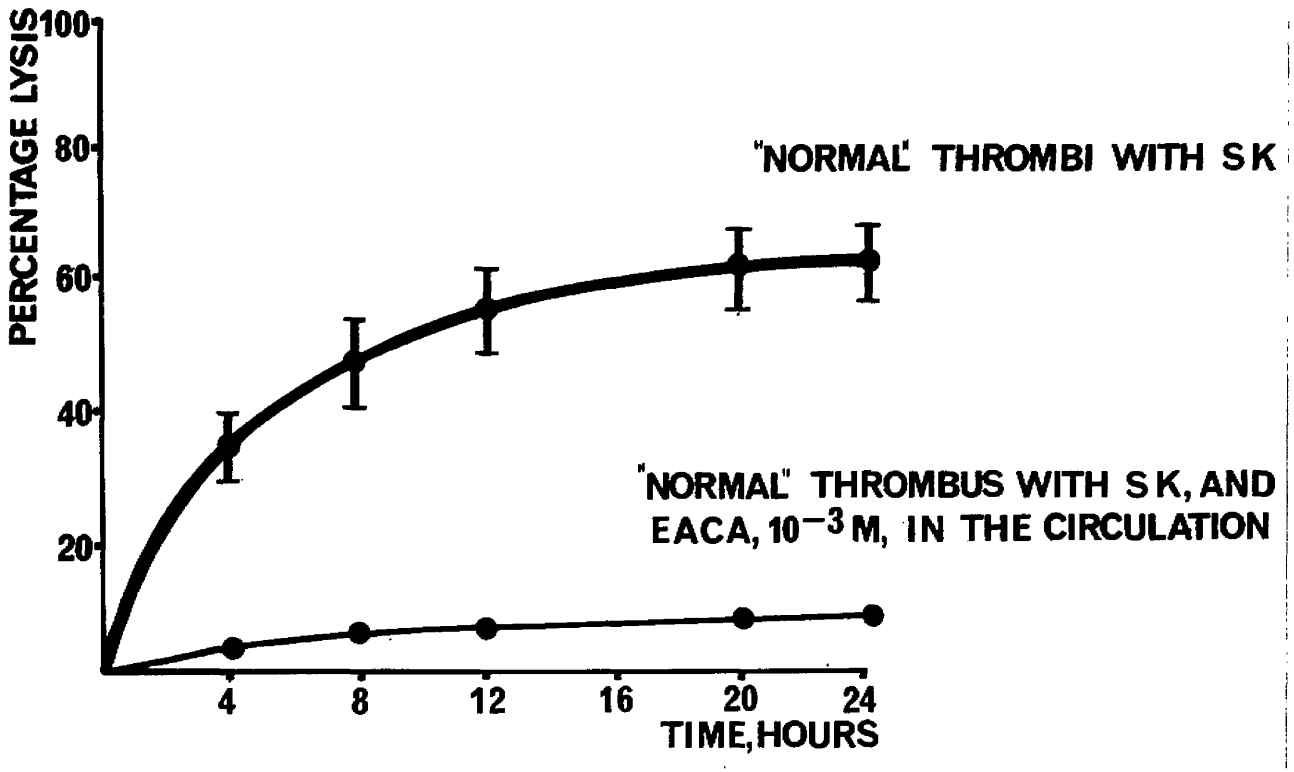


Figure 52 shows the effect of EACA at a concentration of 10^{-3} Molar in inhibitory lysis by streptokinase of artificial thrombus. The figure is prepared from tables 24 and 29.

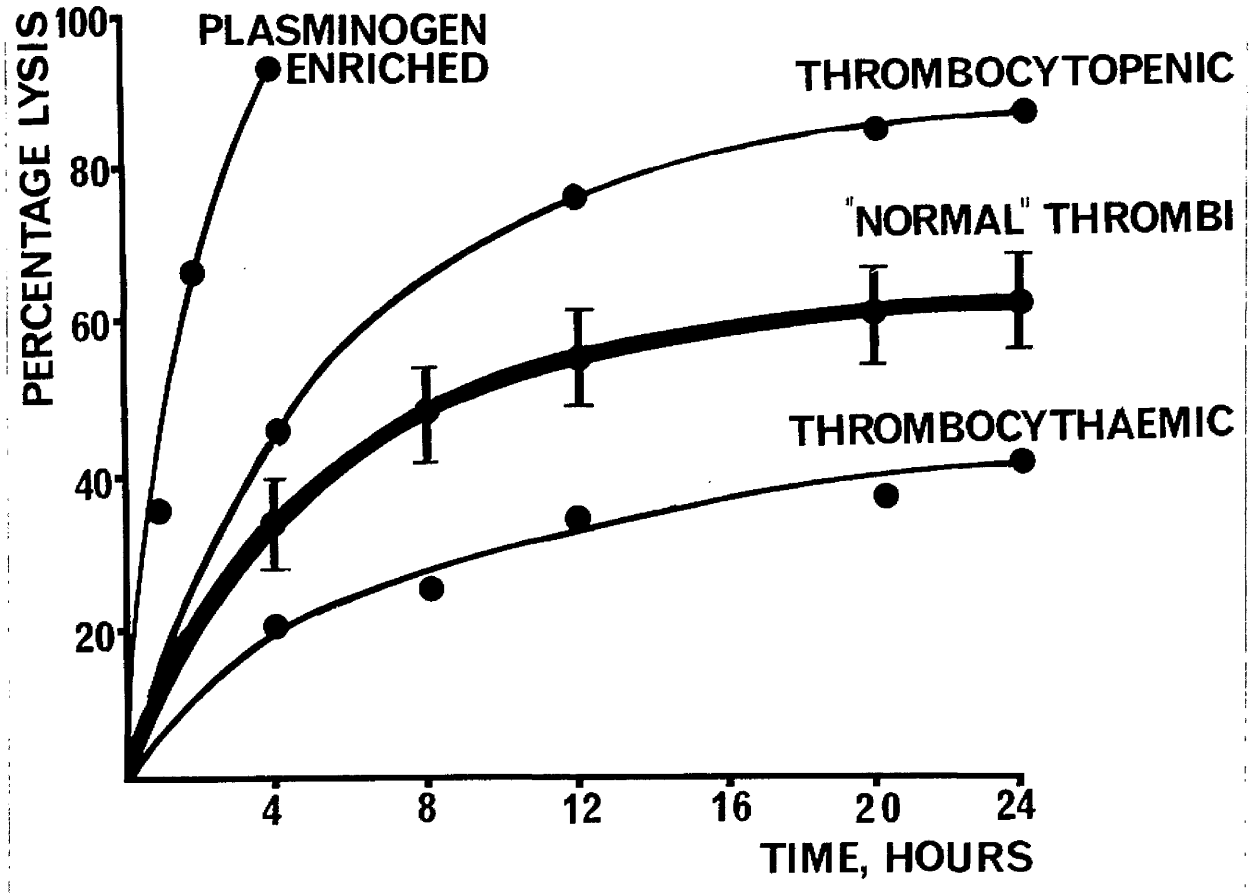


Figure 53 shows the effect on patterns of thrombolysis during perfusion with streptokinase of high and low platelet counts in the blood from which the thrombi were prepared. The effect of enrichment of thrombus with plasminogen is also shown. The figure is prepared from tables 24, 25 and 29.

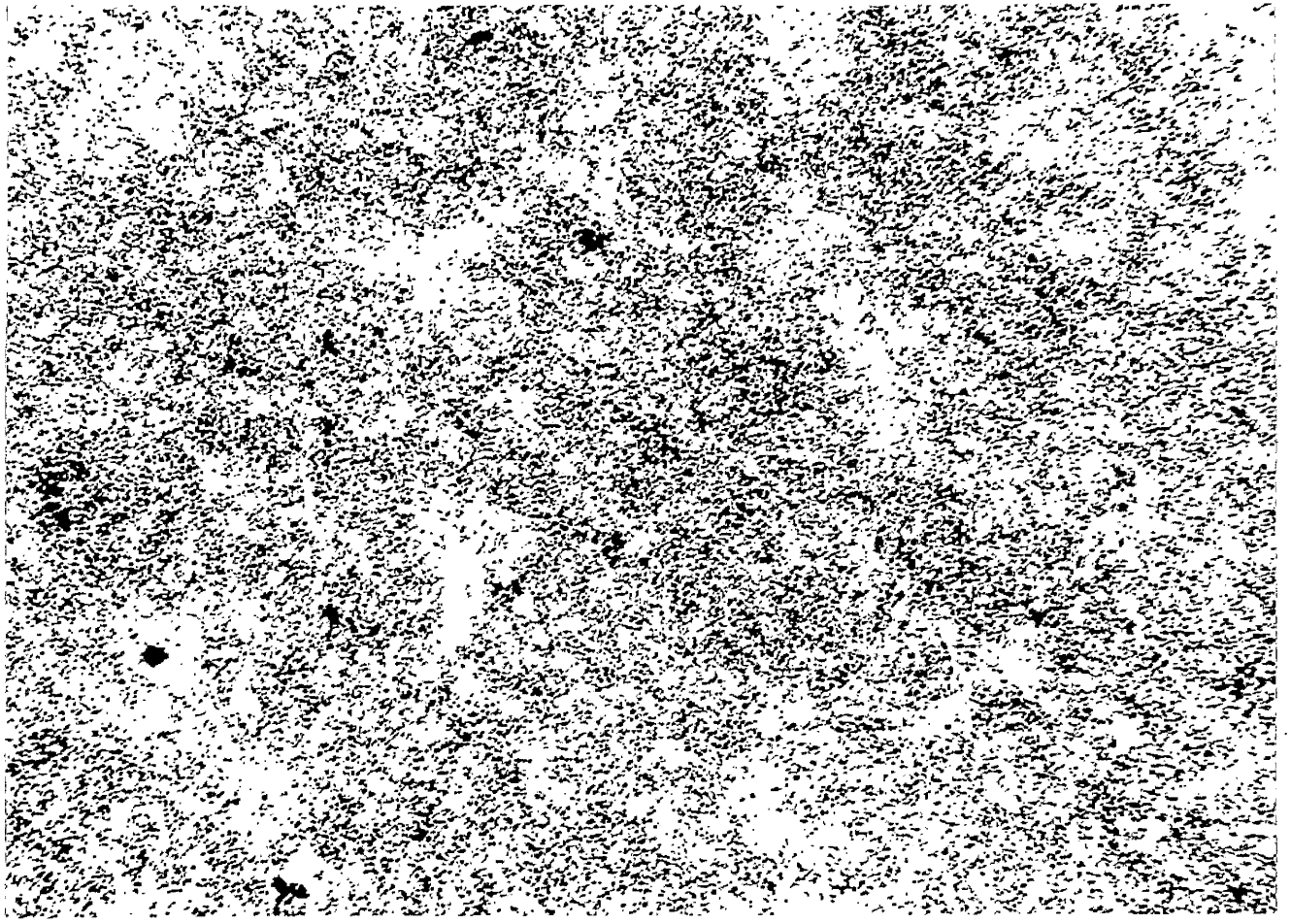


Figure 54 shows the histological appearance of the head of a thrombus made in the Chandler's tube from thrombocytopenic blood. Original magnification x 60. Stain - Martius scarlet blue. (Photomicrograph prepared by Dr. F. Walker).

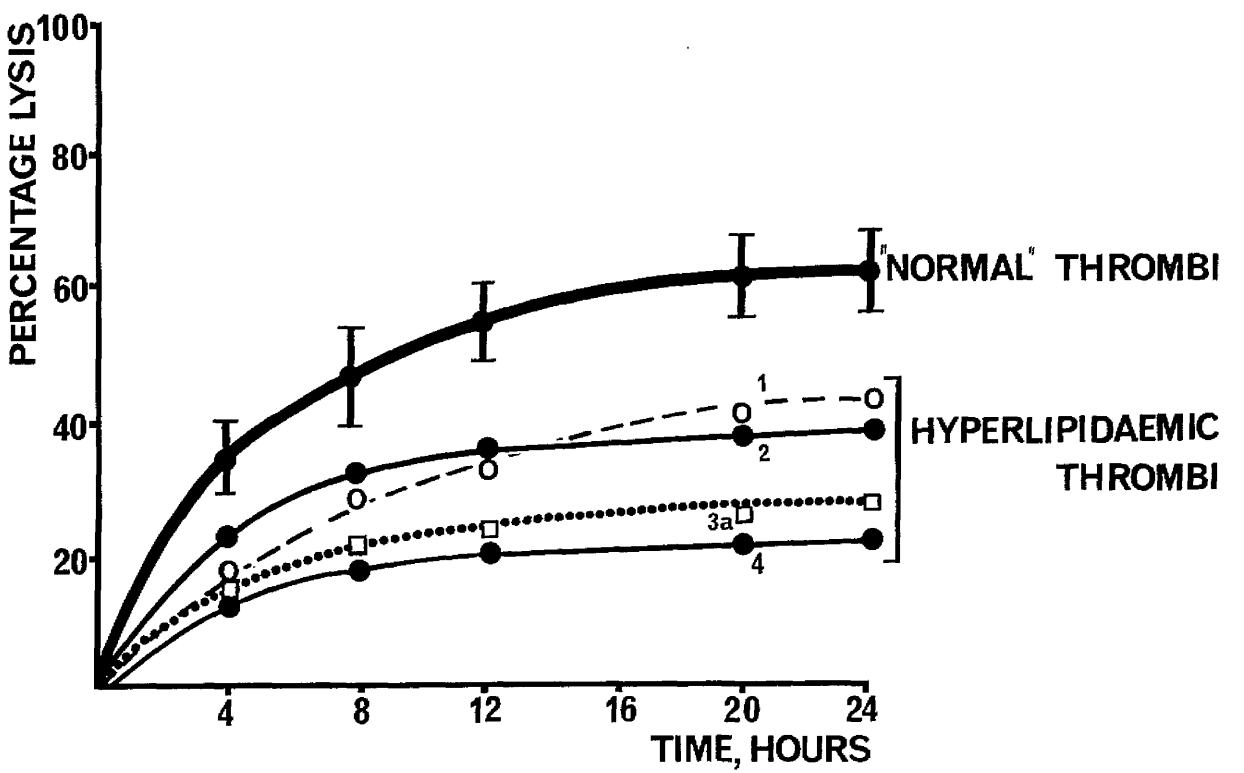


Figure 55. Patterns of radioactivity release during perfusion with streptokinase of thrombi made from hyperlipidaemic blood (table 27). The figure is prepared from tables 24 and 29.

APPENDIX 2.

MAIN TABLES.

Table 1. Streptokinase infusions: details of initial doses.

Patient Number	Estimated plasma volume, ml.	Streptokinase concentration, units/ml.	Initial Streptokinase dosage, units
1	3120	50	155,000
2	3620	240	750,000
3	2280	36	80,000
4	2290	200	460,000
5	3100	80	250,000
6	2028	200	350,000

Shown are details of estimated plasma volumes, streptokinase concentrations in the sensitivity test giving a lysis time of 15-20 minutes, and initial doses administered, for the six patients given intravascular streptokinase.

Table 2. Hydrolysis of TAMe by urokinase.

μM TAMe lysed in 30 minutes	
Without serum	With serum
160	155
160	75
210	20
155	95
145	160
165	56
160	170
132	160
Mean ± SD	160.9 ± 22.6 114.4 ± 57.4

Shown are μM TAMe lysed in 30 minutes by 1 mg. urokinase, and the effect of different sera in inhibiting this activity. Mean values and standard deviations are also shown.

Table 3. Inhibitory action of plasma on urokinase in the radioactive clot system

Percentage inhibition of urokinase activity
24
32
10
6
28
26
34
12
19
27
22
29
20
30
31
16
1
21
36
23
Mean ± SD 22.3 ± 9.6

Shown is the inhibitory effect on urokinase of plasma from 20 different subjects, tested in the radioactive clot system. In each case 0.5 ml. urokinase, 20 units/ml, was assayed after addition of 0.1 ml. plasma, and activity compared with that produced by urokinase to which saline had been added.

Table 4. Coagulative activity of urokinase with normal and Hageman-factor deficient plasma.

Urokinase concentration, (units) in test system	Recalcification time, seconds Normal Plasma	Hageman plasma
0	246	825
2	-	585
5	-	540
10	-	461
20	-	335
45	-	315
90	-	250
185	-	220
375	-	150
750	248	138
1500	228	130
3000	210	-
6250	205	-
12500	192	-
25000	173	-
50000	168	-
100000	163	-

Shown is the effect of urokinase on the recalcification times of fresh normal plasma and Hageman-factor deficient plasma. In each case a saline control is also shown. Tests were carried out in the presence of EACA at a concentration of 10^{-2} Molar to inhibit lysis. The data for the normal plasma are graphically displayed in figure 27.

Table 5. The effect of urokinase in the thromboplastin generation test

		Thromboplastin generation test Clotting times (seconds)					
Incubation time, mins.		1	2	3	4	5	6
Normal plasma. Normal serum. Platelets		30	10.5	10.5	11	11	11
Normal plasma. Christmas serum Platelets	Without UK	171	141	139	140	117	84
	With UK	115	100	96	80	70	54
Haemophilic plasma. Normal serum Platelets	Without UK	35	29	31	31	30.5	31
	With UK	74	42	45	26	26.5	26
Normal plasma and serum (diluted 1:4) Platelets		113	80	46	27	21	17
Normal plasma and serum (diluted 1:4) No platelets	Without UK	158	149	141	140	127	101
	With UK	112	106	100	92	92	88

UK = Urokinase

Effect of urokinase (10,000 units/ml) in the thromboplastin generation test in partially correcting defects due to anti-haemophilic globulin, Christmas factor and platelet deficiency. The tests were carried out in the presence of EACA at a concentration of 10^{-2} Molar.

Table 6. Biochemical effects of urokinase infusion.

	Time of Sampling (hours)				
	0	1	2	3	4½
Activator assay (counts/100 sec)	0	462	139	0	0
Fibrin plate test (mm^2)	0	240	64	0	0
Euglobulin lysis (units)	0.83	1.53	1.07	0.81	0.76
Plasminogen (units/ml)	2.6	2.2	2.1	2.2	2.3
Fibrinogen (mg/100ml)	270	262	268	271	275
Thrombin time (secs.)	10.5	10.5	9.5	9.5	10.5
Prothrombin time (secs.)	16.5	16	16.5	15.5	15.5
Recalcification time (secs.)*	134	135	131	128	148
AHG assay (secs.)	172	143	168	173	174
Christmas factor assay (secs.)	112	119	104	106	126

Effect in subject 1, Chapter 6, of infusion of 40,000 units of urokinase over 1 hour. The data are displayed in part in figure 29.

* Cephalin added

Table 7. Biochemical effects of urokinase infusion.

	Time of sampling (hours) 0	1	2	3	4
Activator Assay (counts/100 sec.)	0	466	671	112	0
Fibrin plate test (mm^2)	0	0	100	0	0
Euglobulin lysis (units)	0.72	1.45	1.52	0.84	-
Plasminogen (units/ml)	2.9	1.8	1.4	2.3	2.6
Fibrinogen (mg/100ml)	240	235	220	198	210
Prothrombin time (secs.)	16.5	16.0	16.0	15.5	15.5
Recalcification time (secs.)	187	158	133	137	172
AHG assay (secs.)	171	176	160	174	172
Christmas factor assay (secs.)	149	147	105	106	115

Effect in subject 2, Chapter 6, of infusion of 80,000 units of urokinase over 2 hours. The data are displayed in part in figure 30.

Table 8. Biochemical effects of urokinase infusion

	Time of sampling (hours)					
	0	1	2	3	4	21
Fibrin plate test (mm^2)	0	70	0	0	0	0
Euglobulin lysis (units)	0.96	2.5	1.06	0.99	1.09	0.99
Plasminogen (units/ml)	2.6	2.5	2.5	2.3	2.4	2.5
Fibrinogen (mg/100ml)	167	172	158	168	172	181
Recalcification time (secs) *	82	52	44	37	66	108
Thrombin time (secs)	10½	11	11	11	11	11
Factor V assay (per cent)	100	55	45	18	100	100
AHG assay (secs)	126	85	86	87	119	111
Christmas factor assay (secs)	115	66	56	62	106	110

Effect in subject 3, Chapter 6, of infusion of 80,000 units of urokinase over 1 hour. The data are displayed in part in figure 31.

* Cephalin added.

Table 9. Biochemical effects of urokinase infusion.

	Time of sampling (hours)					
	0	½	1	1½	2½	24
Activator assay (counts/100 sec)	0	970	168	209	36	0
Fibrin plate test (mm^2)	0	210) 192)	24	9	0	0
Euglobulin lysis (units)	1.03	2.82	1.92	1.33	1.18	1.05
Plasminogen (units/ml)	3.4	2.9	2.1	2.2	2.6	2.8
Fibrinogen (mg/100ml)	218	208	154	196	214	220
Prothrombin time (secs)	16.0	16.5	15.5	15.5	15	15.5
AHG assay (secs)	178	173	178	180	182	179
Christmas factor assay (secs)	156	152	155	160	155	151

Effect in subject 4, Chapter 6, of infusion of 80,000 units urokinase over 30 minutes. The data are displayed in part in figure 32.

Table 10. Urokinase sensitivity tests before and 10 days after urokinase infusion

Subject Number	Lysis times in urokinase sensitivity tests	
	Before infusion	10 days after infusion
1	12 mins. 15 secs.	10 mins. 45 secs.
2	14 mins. 25 secs.	12 mins. 10 secs.
3	10 mins. 30 secs.	11 mins. 30 secs.
4	9 mins. 40 secs.	8 mins. 55 secs.

Shown for the 4 subjects (Chapter 6) given urokinase infusions are the urokinase sensitivity tests before and 10 days after infusion.

Table 11. Streptokinase, urokinase and plasmin compared in fibrinolytic and coagulation tests.

Plasma +	Radioactive clot assay (ug. fibrin lysed/ hour)	Fibrin plate test(percentage activity in terms of standard urokinase)	Euglobulin lysis time (mins.)	Fibrinogen (mg./100 ml.)	Prothrombin time (secs.)	Thrombin time (secs.)
Saline	45	0	276	228	26,27	23,23.5
Streptokinase	399	80	17	196	25,26	23,23
Urokinase	333	50	26	208	25,26	25,26
Plasmin	48	1	65	122	54,58	38,40

The effect of 1 ml. of saline, streptokinase (500 units/ml) urokinase (100 units/ml.) and plasmin (14.0 casein units/ml.) added to 1 ml. of normal plasma. Activity in the fibrin plate test is expressed as a percentage of the activity of a 10 units/ml. urokinase solution, from which a dilution curve was prepared (table 44).

Table 12. Fibrin plate tests with streptokinase and Actase.

Test substance		Area of lysis, mm^2
Streptokinase solution,	100%	440
10 units/ml., with human	50%	380
plasminogen, 1.3 units/ml	25%	288
	12.5%	180
	6.25%	110
Actase solution	1:1,500	360
	1:3,000	256
Activator-free plasmin, 1.3 units/ml		20

Shown are experiments designed to assess the streptokinase content of a vial of Actase. Zones of lysis with serial dilutions of a streptokinase solution, 10 units/ml, to which 1.3 units/ml of human plasminogen had been added to simulate the plasmin content of Actase, are shown together with zones of lysis with two dilutions of an Actase solution made by dissolving the contents of 1 vial in 10 ml saline. Also shown is the negligible lysis produced by plasmin on its own. By interpolation into the dilution curve, the 1:1,500 dilution of Actase appears to have a streptokinase concentration of 4.2 units/ml and the 1:3,000 dilution 2.25 units/ml giving a streptokinase concentration per vial of 63,000 and 67,500 units respectively.

Table 13. Biochemical effects of Actase infusion

Time (hours)	Euglobulin lysis (mins.)	Fibrin plate (mm^2)	Radioactive clot test (μg fibrin lysed/hour)	Plasminogen (units/ml)	Fibrinogen (mg/100ml)	Thrombin time (secs.)
0	425	64	5	3.4	266	10.5, 10
1	200	100	9	2.4	265	10.5, 10.5
2	250	88	6	2.5	283	10.5, 10.5
3	290	90	3	2.7	250	10.5, 10
4	330	80	4	2.7	238	10, 10
5	370	72	5	2.9	260	10.5, 10.5
6	400	88	5	2.8	278	10.5, 10

The effect of intravenous infusion of 1 vial actase in the first hour of study in subject 1, Chapter 7. Resuspended euglobulin precipitates were used in the fibrin plate tests. The results are shown in part in figure 33.

Table 14. Biochemical effects of Actase infusion

Time (hours)	Euglobulin lysis (mins.)	Fibrin plate (mm^2)	Radioactive clot test (μg fibrin lysed/hour)	Plasminogen (units/ml)	Fibrinogen (mg/100ml)	Thrombin time (secs.)
0	412	36	6	3.8	650	9.5, 10
1	340	35	12	3.2	668	10, 10
2	360	20	8	3.4	606	9.5, 9.5
4	390	28	9	3.5	618	9.5, 10

The effect of intravenous infusion of 2 vials actase in subject 2, Chapter 7. Actase was infused at a constant rate in the first hour of study. Resuspended euglobulin precipitates were used in the fibrin plate tests.

Table 15. Plasminogen assays after Complamin injection.

Subject Number	Plasminogen assay, units/ml at varying times (mins.) after injection					
	0	5	10	30	60	120
1	2.8	2.3	3.0	2.7	3.2	2.8
2	3.0	2.8	2.2	2.3	2.6	3.0
3	2.8	2.2	2.2	2.4	-	2.4
4	2.9	2.3	2.3	2.3	2.4	2.7
5	1.9	2.1	2.0	2.0	2.2	1.9
6	3.2	3.6	3.4	3.2	3.3	3.1
7	2.7	2.5	2.9	2.8	2.7	2.6
Mean $\pm$ S.D.	2.8 $\pm$ 0.4	2.5 $\pm$ 0.5	2.6 $\pm$ 0.5	2.5 $\pm$ 0.4	2.7 $\pm$ 0.5	2.6 $\pm$ 0.4

Plasminogen levels in 7 healthy subjects before and at varying times after intravenous injection of 150 mg Complamin. Mean values and standard deviation are also shown. The fall in plasminogen levels from the zero sample to the 5 minute sample is not significant ($t = 1.871$, $0.4 < p > 0.5$).

Table 16. Fibrinogen assays after Complamin injection.

Subject Number	Fibrinogen assay, (mg/100 ml) at varying times (mins.) after injection					
	0	5	10	30	60	120
1	240	227	213	226	248	246
2	207	205	203	206	196	206
3	250	256	240	255	260	252
4	228	196	220	244	214	244
5	267	194	200	205	229	263
6	348	356	372	321	345	301
7	287	294	276	269	292	298
Mean ± S.D.	261 ± 48	244 ± 60	246 ± 61	247 ± 39	255 ± 51	259 ± 33

Fibrinogen levels in 7 healthy subjects before and at varying times after intravenous injection of 150 mg. Complamin. Mean values and standard deviations are also shown. The fall in fibrinogen levels from the zero sample to the 5 minute sample is not significant ($t = 0.582$, $0.5 < p > 0.6$).

Table 17. Effect of exercise on plasminogen levels.

Subject Number	Plasminogen assay, units/ml, at varying times (mins) in relation to exercise				
	Before	0	5	20	55
1	2.8	2.6	2.9	2.7	3.0
2	3.0	3.1	2.9	3.0	3.2
3	3.1	3.0	3.2	2.8	2.9
4	3.0	3.1	3.1	3.2	2.8
5	2.1	2.3	2.2	-	2.2
6	3.3	2.9	3.0	3.2	3.1
7	2.6	2.3	2.4	2.7	2.5
Mean $\pm$ S.D.	2.8 $\pm$ 0.4	2.8 $\pm$ 0.4	2.8 $\pm$ 0.4	2.9 $\pm$ 0.2	2.8 $\pm$ 0.4

Plasminogen levels in 7 healthy subjects before and at varying times after 5 minutes exercise. Mean values and standard deviations are also shown

Table 18. Effect of exercise on fibrinogen levels.

Subject Number	Fibrinogen assay, mg/100ml, at varying times (mins.) in relation to exercise				
	Before	0	5	20	55
1	242	220	231	250	245
2	204	221	207	209	198
3	247	229	251	256	266
4	211	207	220	205	199
5	245	256	251	-	240
6	330	340	321	332	315
7	296	285	292	317	305
Mean ± S.D.	254 ± 38	251 ± 47	253 ± 44	262 ± 26	253 ± 46

Fibrinogen levels in 7 healthy subjects before and at varying times after 5 minutes exercise. Mean values and standard deviations are also shown.

Table 19. Fibrinolytic effect of Complamin infusion.

Time (mins.)	Subject Number	Fibrin plate test (mm^2)	Radioactive clot test (counts/100 secs)	Euglobulin lysis activity (units)
0	1	0	0	0.9
	2	0	0	1.7
10	1	289	479	6.7
	2	380	552	8.1
30	1	42	103	1.9
	2	36	52	2.3
60	1	30	97	1.1
	2	25	17	2.1
90	1	0	0	1.0
	2	0	0	1.9
120	1	0	0	1.0
	2	0	0	1.8

Shown are the effects on fibrinolytic assays of intravenous infusion of Complamin in two subjects. In each case, for the first 30 mins., 150 mg was infused, 300 mg for the second 30 mins., and 600 mg for the third 30 min. period. The data for patient 1 are presented graphically in figure 37. For both subjects, variations in one-stage prothrombin time and thrombin clotting times at no point were greater than 1 second.

Table 20. The effect on euglobulin lysis activity of Complamin taken by mouth.

Subject Number	Number of tablets	Euglobulin lysis (units) at varying times (minutes) after ingestion					
		0	10	30	60	120	240
1	2	2.0	-	2.5	2.0	1.9	2.1
2	2	1.1	1.2	1.0	0.9	1.1	1.2
3	2	1.0	0.9	1.3	1.0	1.2	1.1
4	2	1.5	1.4	1.6	1.5	1.4	1.4
2	4	1.4	1.3	1.5	1.4	1.2	1.3
3	4	1.1	-	1.4	1.5	1.9	1.3
4	4	1.3	1.4	2.6	2.5	1.5	1.4

Table 21. Comparison of the effects of a variety of plasminogen activators.

	Euglobulin lysis activity (units)	Plasminogen (units/ml.)	Fibrinogen (mg./100 ml.)	Rise in thrombin time (secs.)
Streptokinase (table 41)	6.0	1.0	173	5
Urokinase (table 9)	2.8	2.9	208	0
Complamin (tables 15,16,45)	7.9	2.5	244	0
Exercise (tables 17,18,46)	5.6	2.8	251	0
Surgical operation: mean of 10 patients (tables 50-54)	3.2	2.4	264	1.0
Surgical operation (table 55)	10*	0	45	87

Shown are euglobulin lysis activity, plasminogen and fibrinogen levels and the rise in thrombin clotting time from pre-treatment or normal levels, in subjects with fibrinolytic activity induced by a variety of circumstances.

* Whole clot lysis time, expressed in the same units.

Table 22. Plasminogen and fibrinogen levels in normal subjects from whom Chandler's thrombi were prepared.

Subject number	Plasminogen (units/ml.)	Fibrinogen (mg./100 ml.)
1	2.9	324
2	2.1	296
3	2.4	310
4	2.6	293
5	2.3	225
6	2.7	270
7	2.5	286

Table 23. Lysis of thrombi in the artificial circulation.

Type of thrombus		Percentage of total radioactivity		
		In solution	In fragments	At site of insertion
Normals (7 subjects)		62 ± 6	28 ± 5	10 ± 2.5
Normal, without SK	a	8	16	76
	b	11	6	83
Normal, plasminogen enriched	a	93	7	0
	b	95	4	1
Normal, with EACA	a	8	10	82
	b	7	12	81

Shown are patterns of distribution of radioactivity after perfusion in the artificial circulation of thrombi prepared in the Chandler's tube. For the normal thrombi, mean values and standard deviations are shown, calculated from the data presented in table 57. The data are displayed in part in figures 48, 52 and 53.

Table 24. Lysis of thrombi in the artificial circulation.

Type of thrombus		Percentage of total radioactivity in solution at varying times (hours) after start of perfusion.				
		4	8	12	20	24
Normal, no SK	a	4	5	6	8	8
	b	5	6	7	9	11
Normal, with EACA	a	3	4	6	7	8
	b	2	3	5	7	7
Thrombocytopenic	a	45	–	76	83	87
	b	42	58	66	73	76
Thrombocythaemic	c	20	25	33	37	41
	d	22	29	35	39	45
Hyperlipidaemic	1	17	30	33	41	43
	2	22	32	35	37	38
	3 a	14	21	23	25	27
	3 b	15	24	27	31	33
	4	12	17	19	21	22

Shown are the percentages of total radioactivity in solution at varying times after the start of perfusion with streptokinase under a variety of experimental conditions. The data are displayed in part in figures 48, 52, 53 and 55.

Table 25. Lysis of thrombi in the artificial circulation.

Experiment number	Percentage of total radioactivity in solution at varying times (hours) after start of perfusion.			
	1	2	3	4
1	36	66	-	93
2	42	72	88	95

Shown are the percentages of total radioactivity in solution at varying times after the start of perfusion with streptokinase of plasminogen enriched thrombi. The data are presented in part in figure 53.

Table 26. Lysis of thrombi in the artificial circulation.

Type of thrombus		Percentage of total radioactivity		
		In solution	In fragments	At site of insertion
Thrombocytopenic	a	87	12	1
	b	76	17	7
Thrombocythaemic	c	41	47	12
	d	45	39	16

Shown are patterns of distribution of radioactivity after perfusion in the artificial circulation of thrombi, prepared in the Chandler's tube, from two patients with a low platelet count and from two with a high platelet count. Results from patients a and c are shown in figure 53.

Table 27. Hyperlipidaemic patients: fibrinolytic and lipid assays.

Patient number	Fibrinogen (mg./100 ml.)	Plasminogen (units/ml.)	Euglobulin lysis (mins.)	N.E.F.A. (μ.eq./litre)	Cholesterol (mg./100 ml.)	Triglyceride (mg./100 ml.)
1	464	2.1	1440	389	424	93
2	290	3.6	225	555	394	105
3 a	646	3.8	310	1995	749	1000
b	392	2.4	170	426	645	284
4	562	2.3	1440	518	928	500

Shown are fibrinogen and plasminogen levels, euglobulin lysis activity and serum lipid assays, for 4 patients with hyperlipidaemia. Pattern of lysis of thrombi prepared from these patients are shown in figure 55 and table 27. Patient 3, with diabetes mellitus, was studied during an episode of ketoacidosis (3a) and also 14 days later (3b) when the diabetic state was well controlled with insulin.

Table 28. Lysis of thrombi in the artificial circulation.

Type of thrombus			Percentage of total radioactivity		
			In solution	In fragments	At site of insertion
Hyper-lipidaemic patients	1		43	9	48
	2		38	20	42
	3	a	27	21	52
		b	33	25	42
	4		22	17	61

Shown are patterns of distribution of radioactivity, after perfusion with streptokinase in the artificial circulation of thrombi, prepared as described in the text, from 4 patients with raised serum lipid levels (see tables 24 and 27). Patient 3 was studied on two occasions.

Table 29. Lysis of thrombi in the artificial circulation.

Subject number	Percentage of total radioactivity in solution at varying times (hours) after the start of perfusion.				
	4	8	12	20	24
1	36	51	58	62	64
2	30	42	50	56	58
3	32	47	56	65	67
4	43	57	63	67	69 (70)
5	38	52	60	65	67
6	27	40	47	52	53
7	31	43	47	54	56 (55)
Mean ± S.D.	34 ± 5.5	47 ± 6.3	54 ± 5.9	60 ± 6.1	62 ± 6.3

Shown for thrombi from 7 normal subjects are percentages of total radioactivity in solution at varying times after start of perfusion with streptokinase. The data are graphically displayed in figure 48. For subject 4, the figure in brackets in the 24 hour column relates to the findings at 30 hours in this subject, urokinase, 200 units/ml. having been added to the perfusion fluid at 24 hours; and for subject 7 the figure in brackets refers to the 30 hour findings, further streptokinase, 200 units/ml. having been added to the perfusion fluid at 24 hours.

APPENDIX 3.

SUPPORTING TABLES.

Table 30. Euglobulin lysis activity and associated factors in patients with renal disease.

Patient Number	Euglobulin lysis activity (units)	Fibrin plate test (area of lysis mm^2)	Fibrinogen (mg./100 ml.)	Plasminogen (units/ml.)
1	< 0.2	0	254	-
2	0.47	54	260	0.6
3	0.44	0	487	1.8
4	0.38	23	395	1.6
5	0.62	75	307	2.1
6	1.10	90	204	2.7
7	0.63	90	143	3.0
8	2.60	90	495	2.1
9	1.62	83	315	2.8
10	1.30	30	490	3.6
11	1.33	142	387	3.3
12	< 0.2	0	307	1.6
13	< 0.2	0	347	2.9
14	0.79	3	382	1.5
15	1.36	115	173	2.2
16	1.36	105	330	2.8
17	1.50	144	226	2.8
18	0.20	0	283	3.2
19	0.73	0	145	0.8
20	1.23	53	270	2.9
21	1.20	115	230	2.7
22	< 0.2	0	517	2.4
23	0.97	0	275	3.7
24	< 0.2	0	442	2.4
25	0.87	28	190	1.5
26	0.77	33	430	2.2
27	0.94	52	238	1.7
28	< 0.2	0	632	2.4
29	0.23	0	280	2.0
30	2.0	126	240	2.2
31	1.23	65	285	2.5
32	0.93	0	212	1.4
33	0.21	0	493	1.0
34	1.67	25	325	1.2
35	< 0.2	25	458	1.4
36	0.22	0	510	1.8
37	0.23	49	325	2.0
38	0.51	25	227	1.7
39	0.82	24	418	2.0
40	< 0.2	25	535	2.5

Shown for each of 40 patients with a variety of acute and chronic renal diseases is the relationship between euglobulin lysis activity, fibrin plate test, and fibrinogen and plasminogen levels. The data are graphically displayed in figures 4,5 and 6.

Table 31. Relationship between fibrinogen levels and thrombin clotting time.

Clotting time (secs.)	Relative concentration of fibrinogen (per cent)
14	100
33	50
50	25
143	12.5

Shown is the relationship between thrombin clotting time and fibrinogen concentration using a purified fibrinogen solution. The fibrinogen concentration of the undiluted solution was 262 mg./100 ml. The data are graphically displayed in figure 7.

Table 32. Relationship between duration of incubation and release of radioactivity in the radioactive clot test.

Counts/100 seconds after varying incubation times (mins.)					
10	20	30	40	50	60
527	821	1092	978	1582	1509
597	946	1359	1276	1202	1455
471	1043	1076	1327	1072	1106
573	1136	819	1252	1423	1263
706	693	1105	1111	1331	1531
662	870	1010	1486	1404	1646
407	774	1160	1232	1368	1392
Mean 563	890	1089	1237	1340	1415
S.D. ± 105	± 164	± 162	± 161	± 164	± 181

Shown are patterns of radioactivity release in the plasminogen enriched radioactive clot assay after incubation with 0.5 ml. streptokinase solution, 50 units/ml. The data are displayed graphically in figure 13.

Table 33. The correlation between radioactivity release and tyrosine release in the radioactive clot assay system.

Tyrosine content (ug.)	Counts/ 100 sec.	Tyrosine content (ug.)	Counts/ 100 sec.
16.8	407	35.6	978
18.5	471	37.5	1111
18.9	597	38.4	1232
21.2	662	43.0	1327
27.5	706	49.1	1486
16.4	527	36.8	1072
17.3	573	36.4	1202
25.8	693	43.7	1331
24.5	724	50.1	1423
29.7	946	49.2	1582
33.7	1043	46.7	1252
33.4	1136	42.0	1404
24.8	870	51.1	1386
24.1	821	46.0	1405
29.8	819	32.5	1106
35.6	1010	45.3	1263
37.4	1076	43.2	1392
35.6	1105	49.6	1455
47.3	1359	57.9	1646
31.2	1160	45.6	1509
34.9	1092	56.3	1531

The results are displayed graphically in figure 14.

Table 34. Relationship between plasminogen content and lysis of radioactive clots.

Volume of plasminogen added (ml.)	Radioactive clot test: µg. fibrin lysed/hour with:		
	Saline	Streptokinase 500 u./ml.	Urokinase 500 u./ml.
0	27	100	215
0.1	54	203	339
0.25	52	240	427
0.35	51	395	534
0.45	56	450	676

The effect in the radioactive clot test of increasing concentrations of plasminogen in the clot. The plasminogen used was a Kline type preparation, concentration 8.0 units/ml. The data are graphically displayed in figure 15.

Table 35. Relationship between urokinase concentration and lysis of radioactive clots.

Urokinase concentration (units/ml.)	µg. fibrin lysed/ml. test solution/hour	
	Duplicate assays.	Mean value.
5	32 84	60
10	124 172	148
20	608 720	664
30	648 740	696
40	1084 1236	1160
50	1376 1488	1432

Shown is the effect of increasing quantities of urokinase in the plasminogen enriched radioactive iodine labelled clot system. In the same experiment plasmin, concentration 7 units/ml., produced lysis of a mean of 212 ug. fibrin/hour (232 and 192 ug.). The data are graphically displayed in figure 16.

Table 36. Biochemical effects of streptokinase infusion.

	Time of sampling					
	0	20 mins.	60 mins.	2 hrs.	4 hrs.	24 hrs.
Activator assay (counts/100 secs.)	0	0	422	26	0	0
Whole clot lysis time (hours)	> 48	> 48	18 mins.	14	> 48	> 48
Euglobulin lysis time (mins.)	480	390	12	136	280	355
Plasminogen (units/ml.)	3.8	3.4	0.9	1.0	1.2	3.2
Fibrinogen (mg./100 ml.)	250	238	140	160	155	210
Thrombin time (secs.)	10	10	19	18	16	10

Shown are the biochemical changes induced in patient 1 by streptokinase infusion. An initial dose, 155,000 units, was given over the first 20 minutes and 45,000 units over the succeeding 40 minutes. The data are graphically presented in figure 17.

Table 37. Biochemical effects of streptokinase infusion.

Time (hours)	Radioactive clot assay (counts/100 secs.)	Plasminogen (units/ml.)	Fibrinogen (mg./100 ml.)	Thrombin time (secs.)
0	0	3.1	240	12.5
0.5	102	2.7	222	12.5
0.75	554	0.4	218	14.5
1.75	812	0.4	149	16.5
2.75	609	0.3	131	17.5
3.75	547	0.4	128	18.5
4.75	475	0.4	143	19.5
5.75	353	0.7	148	16.5
10	0	0.8	166	15.0
24	0	1.8	210	12.5

Shown is the effect of streptokinase infusion in patient 2. An initial dose of 750,000 units was given over the first hour, followed by 50,000 units/hour for a further 5 hours. The data are presented graphically in figure 18.

Table 38. Biochemical effects of streptokinase infusion.

Time (hrs.)	Activator Assay (counts/ 100 secs.)	Plasminogen (units/ml.)	Fibrinogen (mg./100 ml.)	Prothrombin time (secs.)	Recalcification time (secs.)	Prothrombin assay (per cent)	Factor V assay (per cent)	AHG assay (per cent)
0	0	3.4	231	14.5	205	100	100	100
20 mins.	198	2.7	219	15	265	38	100	100
1	871	0	0	60	-	-	-	0
3	784	0	84	34	>800	35	25	11
5	-	0	77	26	>800	-	-	-
6	103	0	96	22	>800	57	28	15
8	0	0.37	108	18	-	-	-	-
12	0	0.78	147	18	420	100	100	22
21	0	0.8	150	16	-	-	-	100
24	0	1.0	178	16	-	-	100	-
28	0	1.23	214	14	249	100	100	-

Shown is the effect of streptokinase infusion in patient 3. An initial dose of 80,000 units was given followed by 80,000 units/hour for a further 5 hours. Prothrombin, Factor V and AHG are expressed as percentages in terms of the pre-infusion level. The data are graphically displayed in figure 19.

Table 39. Biochemical effects of streptokinase infusion.

Time (hours)	Radioactive clot assay (counts/100 secs.)	Euglobulin lysis time (mins.)	Plasminogen (units/ml.)	Fibrinogen (mg./100 ml.)	Prothrombin time (secs.)	Thrombin time (secs.)	AHG (per cent)	Prothrombin (per cent)	Factor (per cent
0	0	360	3.0	244	14.5	12	100	100	100
1	497	13	1.5	151	16.5	14.5	47	89	25
2	348	28	0.6	150	17.5	15	16	57	20
3	852	240	0.7	98	19	15	19	66	8
4	73	-	0	72	19.5	17.5	19	73	50
5	68	-	0.1	154	18.5	15.5	36	80	40
6	0	-	0.7	180	18	15	100	83	100
24	0	320	1.5	233	15	12	-	-	-

Shown is the effect of streptokinase infusion in patient 4. An initial dose of 460,000 units was given over the first hour, followed by 50,000 units/hour for a further two hours. AHG, prothrombin and factor V are expressed as percentages in terms of the pre-infusion level. The data are also graphically displayed in figure 20.

Table 40. Biochemical effects of streptokinase infusion.

Time	Radioactive clot assay (µg. fibrin lysed/hour)	Fibrin plate test (area of lysis mm^2)	Euglobulin precipitate on fibrin plate (area of lysis mm^2)	Euglobulin lysis (units)	Plasminogen (units/ml.)	Fibrinogen (mg./100 ml.)	Thrombin time (secs.)
0	0	16	20	0.75	1.7	357	10
2.75	0	30	42	1.1	-	-	10
3.25	65	324	-	-	0.5	330	13
3.75	142	-	-	-	0.6	192	14
4.5	206	1216	> 1500	0(>3 days)	0.3	250	13
5.5	211	1221	>1500	0(>3 days)	0.36	223	14
8	237	1074	> 1500	0(>3 days)	0	160	15
16	66	648	>1500	1.6	0.3	125	18
20	202	1074	>1500	-	-	-	17
24	133	961	>1500	0(>3 days)	0	140	17
40	41	96	120	0(>3 days)	0.2	120	9
64	0	0	0	-	1.16	238	9.5

Shown is the result of infusion of streptokinase in patient 5. An initial dose of 250,000 units was given over 1 hour from the 2 hrs. 45 mins. point. Treatment was thereafter continued to 24 hours with 75,000 units/hour. The data are graphically presented in figure 21.

Table 41. Biochemical effects of streptokinase given intra-arterially.

Time (hrs.)	Euglobulin lysis (units)	Fibrin plate test (mm^2)	Plasminogen (units/ml.)	Fibrinogen (mg./100 ml.)	Thrombin time (secs.)
0	1.0	0	2.8	276	6
2	1.0	0	2.2	278	9
3	6.0	70	1.0	173	11
4½	8.6	96	0.7	199	16
8	10	110	0.4	112	19
16	11.5	110	0	121	15
26	10	100	0	148	12
40	1.8	0	0.4	260	11.5
60	1.1	-	1.3	280	9.5

Shown is the result of intra-arterial perfusion with streptokinase in patient 6. The assays were carried out in venous blood. An initial dose of 350,000 units was given over 5½ hours followed by 12,500 units/hour for the following 24½ hours. The data are graphically displayed in figure 22.

Table 42. Inhibitory effect of plasma on urokinase in the fibrin plate test.

Plasma number	Percentage inhibition of urokinase activity with the following volumes of plasma per unit of urokinase.					
	5 µl.	10 µl.	15 µl.	20 µl.	25 µl.	30 µl.
1	63	71	97	100	100	100
2	62	69	98	100	100	100
3	55	64	98	100	100	100
4	25	50	92	80	100	100
5	51	63	96	100	100	100
6	6	20	94	96	100	100
7	59	62	97	100	100	100
8	26	48	92	100	100	100
9	17	24	86	52	88	98
10	0	16	68	64	92	99
11	2	17	76	68	96	99
12	17	41	93	82	100	100
13	46	51	93	100	100	100
14	10	26	88	76	100	100
15	18	37	92	100	100	100
16	32	42	93	84	100	100
17	45	52	94	91	100	100
18	0	13	49	45	76	93
Mean ± S.D.	29 ± 22	43 ± 20	89 ± 15	85 ± 18	99 ± 3	99 ± 3

Shown for 18 normal plasmas is the percentage inhibition of urokinase activity in the fibrin plate test produced by varying proportions of plasma and urokinase. With more than 30 ul./unit of urokinase, 100 per cent inhibition was always seen. Percentage inhibition was calculated by reference to 3 urokinase dilution curves. The data are graphically presented in figure 24.

Table 43. Inhibitory action of serum on urokinase in a caseinolytic assay system.

Plasma number	Optical density readings in caseinolytic assay	
	Serum added before plasminogen	Serum added after plasminogen
1	0.136	0.175
2	0.160	0.185
3	0.170	0.208
4	0.155	0.165
5	0.157	0.160
6	0.154	0.140
7	0.195	0.272
8	0.240	0.263
9	0.191	0.220
10	0.204	0.275
11	0.215	0.230
12	0.210	0.196
13	0.204	0.229
14	0.152	0.172
15	0.138	0.172
16	0.125	0.127
17	0.107	0.115
18	0.162	0.152
19	0.125	0.167
20	0.133	0.115
21	0.080	0.110
22	0.126	0.128
23	0.156	0.168
24	0.099	0.120
25	0.160	0.158
Mean $\pm$ S.D.	0.158 ± 0.043	0.176 ± 0.048

Table 44. Comparison of mixtures of plasma with streptokinase, urokinase and plasmin in the fibrin plate test.

Test substance		Area of lysis (mm^2)
Urokinase solution 10 units/ml.	100%	460
	50%	380
	25%	272
	12.5%	180
	6.25%	100
Plasma + saline		0
Plasma + streptokinase		432
Plasma + urokinase		378
Plasma + plasmin		16

Shown are areas of lysis in the fibrin plate test with a dilution curve of a standard urokinase solution, 10 units/ml., and the areas of lysis found with mixtures of 1 ml. plasma and 1 ml. of respectively saline, streptokinase (500 units/ml.), urokinase (100 units/ml.) and plasmin (14 units/ml.). From the data shown the percentage activities of the various plasma mixtures in terms of the standard urokinase solution were calculated and are shown in Table 11.

Table 45. Euglobulin lysis activity following Complamin injection.

Subject number	Euglobulin lysis activity (units) at varying times (mins.) after Complamin injection.					
	0	5	10	30	60	120
1	0.8	21.3	2.2	0.8	0.8	0.9
2	2.3	5.3	4.9	2.5	2.2	2.1
3	1.2	2.6	2.6	1.8	2.0	1.4
4	1.4	2.2	1.9	1.6	1.3	1.2
5	0.3	4.3	3.3	1.2	0.3	0.3
6	1.1	3.9	3.6	3.2	1.8	1.3
7	1.0	15.8	10.3	1.0	0.9	1.1
Mean	1.2	7.9	4.1	1.7	1.3	1.2

Shown is euglobulin lysis activity in 7 healthy subjects before and at varying intervals after intravenous injection of 150 mg. Complamin. Mean values are also shown. The data are graphically displayed in figure 34. In no subject did the one-stage prothrombin time or the thrombin clotting time change from control values by more than 1 second.

Table 46. Euglobulin lysis activity following exercise.

Subject number.	Euglobulin lysis activity (units) at varying times (mins.) in relation to exercise.				
	Before	0	5	20	55
1	1.31	7.24	6.47	2.52	2.08
2	1.99	4.54	4.30	2.31	1.96
3	1.86	5.81	4.45	2.46	2.10
4	1.85	7.89	5.81	3.13	2.11
5	1.43	5.86	5.13	2.61	2.12
6	1.82	4.01	3.35	2.43	2.03
7	1.58	3.95	2.76	2.02	1.46
Mean	1.69	5.61	4.61	2.5	1.98

Shown is euglobulin lysis activity in 7 healthy subjects before and at varying intervals after 5 minutes strenuous exercise. Mean values are also shown. The data are graphically displayed in figure 34. In no subject did the one-stage prothrombin time or the thrombin clotting time change from control values by more than 1 second.

Table 47. Effect of repeated Complamin injections

Time of sampling (mins.)	Euglobulin lysis activity (units)
0	1.7
5	5.5
60	1.4
65	3.2
120	1.2
125	2.6

Shown is the effect on euglobulin lysis activity in a normal subject of 3 intravenous injections of 150 mg. Complamin given at hourly intervals (i.e. after the zero, 60 and 120 minute samples had been withdrawn). The data are shown graphically in figure 35.

Table 46. Effect of repeated Complamin injections.

Time after Complamin injection (mins.)	Euglobulin lysis (units) on successive days.				
	1	2	3	4	5
0	1.2	0.9	1.1	0.9	1.0
5	18.8	13.6	5.5	3.6	4.3
10	15.0	7.5	2.5	1.6	1.1
20	3.6	1.0	1.1	1.2	1.0
30	1.7	1.1	1.0	1.1	0.9

Euglobulin lysis activity induced by intravenous injection of 150 mg. Complamin on 5 successive days. The data are graphically displayed in figure 36.

Table 49. Euglobulin lysis activity: experiments with exercise and Complamin.

Time (mins.)	Sample number	Subject number	Experiment 1	Experiment 2	Experiment 3	Experiment 4
0	1	1	0.97	1.98	1.15	1.16
		2	1.56	1.84	1.75	1.61
5	2	1	3.16	4.54	6.41	3.09
		2	3.36	3.06	5.20	5.88
30	3	1	1.36	2.83	1.76	2.21
		2	1.62	2.65	2.25	3.61
35	4	1	1.94	4.56	1.46	7.28
		2	2.40	5.88	2.81	5.36
60	5	1	1.28	2.72	1.15	2.04
		2	1.18	1.39	1.52	3.33

Euglobulin lysis activity in experiments with two healthy subjects after exercise and Complamin (150 mg. I.V.). In experiment 1, 5 mimutes exercise was taken from 0-5 mins. and 30-35 mins. In experiment 2, exercise was taken from 0-5 mins. and Complamin was given at 30 mins. In experiment 3, Complamin was given at 0 and 30 mins. and in experiment 4, Complamin was given at 0 mins. and exercise taken from 30-35 mins. The results of experiments 1 and 2 are shown in figure 38, and of experiments 3 and 4 in figure 39.

Table 50. Effect of surgery: fibrin plate test.

Patient number	Fibrin plate test (area of lysis mm^2)		
	Before operation	during operation	after operation
1	30	289	130
2	24	340	96
3	120	320	84
4	49	210	96
5	96	256	192
6	144	196	156
7	150	396	192
8	88	270	100
9	132	460	280
10	100	392	120
Mean ± S.D.	93 ± 46	313 ± 85	145 ± 61

Areas of lysis in the fibrin plate test (resuspended euglobulin precipitate) from 10 patients studied before, during and after excision of the rectum. Mean values and standard deviations are also shown. The data are graphically displayed in figure 40.

Table 51. Effect of surgery: euglobulin lysis activity.

Patient number	Euglobulin lysis activity (units).		
	Before operation	during operation	after operation
1	1.4	2.7	1.7
2	1.3	3.6	2.0
3	1.4	2.6	1.4
4	1.3	1.5	1.3
5	1.0	2.5	1.9
6	0.9	1.8	1.5
7	1.1	4.8	2.9
8	1.1	2.7	2.1
9	1.0	6.1	3.8
10	1.5	3.5	2.5
Mean ± S.D.	1.2 ± 0.2	3.2 ± 1.4	2.1 ± 0.8

Euglobulin lysis activity for 10 patients studied before, during and after excision of the rectum. Mean values and standard deviations are also shown. The data are graphically displayed in figure 40.

Table 52. Effect of surgery: plasminogen assays.

Patient number	Plasma plasminogen (units/ml.)		
	Before operation	during operation	after operation
1	2.7	2.6	2.4
2	3.2	3.0	2.9
3	2.4	1.8	1.9
4	3.6	2.8	2.9
5	2.2	2.0	2.1
6	2.8	2.4	2.6
7	3.1	2.2	2.4
8	3.3	2.3	2.0
9	2.8	1.7	1.6
10	3.0	3.1	2.9
Mean $\pm$ S.D.	2.9 $\pm$ 0.4	2.4 $\pm$ 0.5	2.4 $\pm$ 0.5

Plasma plasminogen levels from 10 patients studied before, during and after excision of rectum. Mean values and standard deviations are also shown. The data are graphically displayed in figure 40.

Table 53. Effect of surgery: fibrinogen assays.

Patient number	Plasma fibrinogen (mg./100 ml.)		
	Before operation	during operation	after operation
1	270	235	220
2	310	260	250
3	246	250	280
4	462	470	430
5	320	280	250
6	315	250	276
7	362	275	220
8	285	215	196
9	246	190	210
10	248	210	196
Mean $\pm$ S.D.	306 $\pm$ 67	264 $\pm$ 78	253 $\pm$ 69

Plasma fibrinogen levels from 10 patients studied before, during and after excision of rectum. Mean values and standard deviations are also shown. The data are graphically displayed in figure 40.

Table 54. Effect of surgery: thrombin clotting time.

Patient number	Thrombin clotting time (secs.)		
	Before operation	during operation	after operation
1	10.5	11.0	10.5
2	10.0	11.5	10.0
3	9.0	11.0	9.5
4	9.5	10.5	10.0
5	10.5	10.0	10.0
6	10.0	10.0	11.0
7	10.5	12.0	11.0
8	11.5	10.5	12.0
9	9.5	10.5	10.0
10	10.0	13.5	10.5
Mean ± S.D.	10.1 ± 0.7	11.1 ± 1.1	10.5 ± 0.7

Thrombin clotting times from 10 patients studied before, during and after excision of the rectum. Mean values and standard deviations are also shown. The data are displayed graphically in figure 40.

Table 55. Hyperplasminaemic state complicating surgical operation.

	Before EACA	4 hours after EACA	10 hours after EACA
Whole clot lysis time	30 mins.	>24 hrs.	>24 hrs.
Plasminogen (units/ml.)	Nil	Nil	1.6
Fibrinogen (mg./100 ml.)	45	110	165
Whole blood clotting time (mins.)	Infinite	2	5
Thrombin clotting time (secs.)	97	17	11

Coagulation and fibrinolytic studies in a patient with a hyperplasminaemic state after excision of rectum. The effect of EACA treatment is shown. The data are graphically presented in figure 41.

Table 56. Hyperplasminaemic state complicating surgical operation.

	Time of sampling (hours)		
	0	4	14
Fibrin plate test (area of lysis mm^2)	115	9	8
Euglobulin lysis (mins.)	255	272	298
Plasminogen (units/ml.)	0.6	1.3	2.2
Fibrinogen (mg./100 ml.)	218	305	296
Whole blood clotting time (mins.)	Infinite	12	10

Coagulation and fibrinolytic studies in a patient with a hyperplasminaemic state treated with EACA (10 gms. I.V. given over 4 hours). The data are graphically displayed in figure 42.

Table 57. Lysis of thrombi in the artificial circulation.

Subject number	Percentage of total radioactivity	
	In fragments	At site of insertion
1	31	7
2	32	10
3	26	7
4	21	10
5	22	11
6	33	14
7	32	12
Mean ± S.D.	28 ± 5	10 ± 2.5

Distribution of total radioactivity with thrombi from normal subjects after 24 hours perfusion with streptokinase. Mean values and standard deviations are also shown, and are presented in table 23.