PLASMINOGEN ACTIVATION AND ITS INHIBITION

by

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PREFACE

The work presented in this thesis was carried out in the laboratory of Professor A.S. Douglas, in the University Department of Medicine, Glasgow Royal Infirmary. While performing the work, I have held the post of honorary senior house officer, supported by a grant made for this appointment to Professor A.S. Douglas by the Medical Research Council. I was directed in the experimental work for this thesis by Professor E.M. McGirr, Professor A.S. Douglas and Dr. G.P. McNicol. Specific mention must be made of some portions of the work which I did not personally perform. Miss P. Wilson carried out column chromatography (chapter 6); Dr. B.M. Rifkind and Miss M. Gale performed lipid assays (chapter 7); and Dr. B. Sweet prepared rabbit anti-human fibrin serum (chapter 8). Some of the work described in the thesis has been published, as detailed below, and the remainder is being prepared for publication:-

Dubber, A.H.C., McNicol, G.P., Douglas, A.S., Melander, B., (1964), Some properties of the antifibrinolytically active isomer of amino-methylcyclohexane carboxylic acid, Lancet, 2, 1317.

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CHAPTER 1

INTRODUCTION

Interest in the components and functions of the fibrinolytic enzyme system or plasminogen-plasmin system has been stimulated largely by the availability of activators and inhibitors of this system, as preparations suitable for use in man. Administration of these substances as therapeutic agents in man has lead to more fundamental understanding of the physiological function of the fibrinolytic enzyme system and the biochemical abnormalities which accompany pathological activity of this system. This thesis is concerned with the study of certain aspects of plasminogen activation and its inhibition.

Acute and frequently life-threatening haemostatic defects may be precipitated in part or wholly by disordered activity of the fibrinolytic enzyme system and there is increasing evidence that inhibitors of the system may have value in the therapy of such states. Studies made for this thesis included investigation of two compounds which possess activity as fibrinolytic inhibitors (chapters 4 and 5).

A number of clinical trials and individual well-documented cases have served to establish the feasibility and usefulness of plasminogen activators in the treatment of thrombo-embolic vascular disease. Up to the present time, the most frequently employed

activator has been the bacterial protein, streptokinase. This activator has certain disadvantages, in particular its antigenicity. On the other hand, urokinase, the activator prepared from human urine, has presumed non-antigenicity. However, early preparations possessed marked thromboplastic activity. Recently a commercial preparation of urokinase has been made available for trial by clinical investigators. One of the chapters of this thesis describes studies made with this preparation with a view to defining any coagulative properties it might possess and includes investigation of its effect on plasminogen activation in vivo (chapter 6).

The physiological role of the fibrinolytic enzyme system is considered by many authorities to be the maintenance of a patent vasculature. One hypothesis concerning the development of atherosclerosis links plasma lipid levels with the coagulation system and the fibrinolytic enzyme system. The hypothesis suggests that high plasma lipid levels may accelerate clotting and inhibit lysis thus permitting the increased occurrence and persistence of intravascular fibrin. It is thought that such fibrin can become incorporated in the subendothelial layer of the arterial wall leading to the development of atherosclerotic plaques. The effect of fat

feeding on blood clotting, fibrinolysis and platelet aggregation was investigated as part of the work for this thesis (chapter 7).

In chapter 8 of this thesis, an account is presented of detailed investigation of the biochemical abnormalities occurring in the defibrination syndrome; the patients studied had a variety of associated clinical disorders. It is generally believed that abnormal fibrinolysis may contribute in part at least to the coagulation defect associated with some defibrination syndromes.

The thesis is presented in 2 volumes. The first contains the text and references and the second figures and tables. The text begins with an historical introduction to the fibrinolytic enzyme system and an account of current concepts of its components and functions. There follows a description of the methods used in the work for this thesis and thereafter the presentation and discussion of the investigative work mentioned above. The figures are numbered in order of their introduction in the text and the tables are arranged similarly.

CHAPTER 2

THE FIBRINOLYTIC ENZYME SYSTEM

Historical Introduction

Current extensive knowledge of components and functions of the fibrinolytic enzyme system has largely accumulated over the past two decades although the earliest observations of fibrinolysis appear to have been made nearly 200 years ago by Morgagni (1769) and Hunter (1794). In their writings is recorded the finding of post-mortem fluidity of blood in cases of sudden death. Half a century later, Denis (1838) noted that washed human fibrin clots would dissolve in alkaline solution. Plósz (1873) demonstrated that fibrin was rendered insoluble following repeated salt extraction and concluded that the substance responsible for dissolution had been eluted during the process. He suggested that the dissolving agent might be a tryptic enzyme. Dastre (1893) called the spontaneous dissolution of whole blood clots, fibrinolysis, and attributed the phenomenon to enzymes attached to fibrin.

Delezenne and Pozerski (1903) confirmed the earlier observations of Denys and de Marbaix (1889) that proteolytic activity appeared in serum mixed with chloroform and also found that this activity disappeared on addition of untreated serum. They concluded that the effect of chloroform might be to destroy proteolytic inhibitors.

Nolf (1904) reported that the incoagulability of blood observed after peptone injection was associated with a marked increase in fibrinolytic activity and suggested that the source of the substance producing fibrinolysis might be the vascular wall.

It will be seen that these early observations and hypotheses concerning the nature of fibrinolysis foreshadowed findings made after the lapse of many years.

A fundamental discovery in the field of fibrinolysis was made by Tillett and Garner (1933) who showed that rapid lysis of human plasma clots occurred if they were formed in the presence of culture filtrates of certain strains of haemolytic streptococci. They thought that such culture filtrates contained an enzyme with a direct proteolytic effect on fibrin. However, Milstone (1941) demonstrated that the effect of the streptococcal substance was dependent on a factor contained in the globulin fraction of human serum since pure human fibrin was resistant to lysis by the streptococcal substance alone but was rapidly lysed in its presence on addition of a small amount of human serum globulins. He named this accessory component contained in human serum globulins 'lytic factor'.

Kaplan (1944) thought that 'lytic factor' was the same agent as the proteolytic substance which appeared in serum on addition of chloroform.

Christensen (1945) and Christensen and MacLeod (1945) extended this observation and postulated that the streptococcal substance was a kinase which activated a globulin precursor of the fibrinolytic enzyme of human blood.

These workers also proposed the terminology now most widely used by workers in the field of fibrinolysis. They called the streptococcal substance, streptokinase. The globulin precursor of the active fibrinolytic enzyme was named plasminogen and the active enzyme, plasmin. The enzyme system responsible for the phenomenon of fibrinolysis was called the plasminogen-plasmin system or fibrinolytic enzyme system.

Components and Functions of the Fibrinolytic Enzyme System

There are four principal components of the fibrinolytic enzyme system: plasminogen, plasmin, activators and inhibitors (figure 1).

Plasminogen. Plasminogen is the circulating plasma precursor of the proteolytic enzyme plasmin. Kowalski et al. (1958) have suggested that plasminogen thought to be identical with plasma plasminogen, is present in various tissues; Christensen (1954) has demonstrated plasminogen in certain exudates and transudates and Lodi et al. (1961) its presence in lymph.

The site of plasminogen synthesis in the body is not definitely known although Barnhart and Riddle (1963) using a fluorescent antibody technique, have shown that eosinophils contain and may produce plasminogen.

Plasminogen is a beta globulin but since absolutely pure preparations have not yet been obtained, its characterisation remains inadequate. Shulman et al. (1958) and Singher (1960) have shown that the plasminogen molecule contains about 8 hexose groups suggesting that it is a glycoprotein. The former workers have found a molecular weight for plasminogen of 143,000. More recently Davies and Englert (1960) and Hagan et al. (1960) have reported it to be 83,000.

The most widely used methods of preparation of plasminogen have been evolved from that of Kline (1953). This method is based on two properties of plasminogen, its marked acid resistance (von Kaulla, 1949) and its concentration in Cohn fraction III (Christensen and Smith, 1950)which is used as starting material. Such acid-fractionated plasminogen is sparingly soluble and unstable at neutral pH. More recently, Alkjaersig (1964), has produced, using DEAE-cellulose chromatography, a stable plasminogen preparation of high specific activity which is readily soluble at neutral pH. Reports on similar

preparations have been made by Davies and Englert (1960), Hagan et al. (1960), Derechin (1962), Derechin et al. (1962), Wallén (1962 a, b), Hink and McDonald (1963), and Robbins and Summaria (1963). In this method basic amino acids, such as lysine or epsilon aminocaproic acid (EACA) are used as selective effluents or to enhance solubility and prevent co-precipitation of plasminogen during the purification prodedure. A commercial preparation of this type has been made available recently (A. B. Kabi, Stockholm).

Conversion of Plasminogen to Plasmin Troll and Sherry (1955) and Alkjaersig et al. (1958 a, b) have shown that the conversion of plasminogen to plasmin (plasminogen activation) is a first-order enzymatic step which involves the splitting of peptide bonds, probably lysine and/or arginine bonds since the esters of these amino acids are also split by plasminogen activators and they act as competitive inhibitors of plasminogen activation.

Activators and procedures which activate plasminogen to plasmin may be divided into four main groups. Certain activators, including urokinase and tissue activators, bring about direct conversion of plasminogen to plasmin. Some substances appear to convert an inert proactivator to activator. This group includes streptokinase and tissue

lysokinases. Proactivator has been postulated as an activity and has not yet been identified. It is thought that proactivator may be plasminogen itself (De Renzo, 1960). Verstraete (1966) has suggested that plasmin is the proactivator and that a streptokinase-plasmin complex possesses the property of plasminogen activation. Macfarlane (1964) has suggested that the hypothesis concerning proactivator arises out of differences in species specificity of activator. The third group of activating procedures includes administration of certain substances, among them adrenaline and nicotinic acid, not themselves plasminogen activators, which appear to trigger activator release into the blood stream. The fourth group comprises a variety of procedures which bring about plasminogen activation in vitro. These procedures include dilution of plasma, addition of chloroform to plasma and trypsin activation.

Tissue Activator Astrup and Permin (1947) demonstrated that fibrinolytic activity present in tissues is due to plasminogen activator activity and not to a fibrinolytic enzyme. Tissue activator may be prepared by potassium thiocyanate extraction of human or animal tissue (Astrup and Stage, 1952). Tissue activator is relatively stable especially at acid pH (Astrup and Sterndorff, 1956). Mullertz (1953) considers that tissues contain lysokinases which, like streptokinase, activate an inert proactivator. Abe and Astrup (1960) have shown that

conversion of plasminogen to plasmin by a thiocyanate activator extract of pig heart is a slow, possibly stoichiometric, reaction. Activator activity is present in most tissues. Uterus, adrenals, lymph nodes, prostate and lung are particularly rich in activator content while there is none in normal liver and placenta (Albrechsten, 1957). Using an autoradiographic technique, Todd (1959) has demonstrated the presence of activator in tissues closely related to the endothelium of veins and pulmonary arteries. Baron and Barnett (1960) have shown that plasminogen activator is produced by a continuous metabolic process and released into the supernatant by a variety of primary and continuous human cell cultures. Lack and Ali (1964) have produced evidence to suggest that tissue activator is concentrated in the lysosomal fraction of tissue homogenates. Lack (1964) suggests that local anoxia may influence the release of tissue activator and that this release may be responsible for the increased plasma fibrinolytic activity found after sudden death, in response to local ischaemia and after anaphylaxis.

Blood Activator Under physiological conditions, plasminogen activator activity can be detected in shed blood (Fearnley and Tweed, 1953). Plasminogen activator can be separated from plasma by precipitation of the euglobulins which also contain plasminogen, a quarter of the

fibrinogen but with only traces of fibrinolytic inhibitory activity (Kowalski et al., 1959). Flute (1960) isolated by electrophoresis a fraction of normal plasma which behaved as a labile plasminogen activator. Fearnley et al. (1952) showed that there was a rapid loss of fibrinolytic activator activity from blood samples stored at room temperature. The loss was less at 4°C. and the activity stabilised by fibrin formation. Flute (1963) considers that blood activator is a lysokinase.

Increase in physiological fibrinolytic activity may be brought about by, among others, exercise and adrenaline injection (Biggs et al., 1947; Ogston and Fullerton, 1961), electric shock (Fantl and Simon, 1948) and anaesthesia (von Kaulla, 1947). The concept of stress as a stimulant to increase physiological fibrinolytic activity is now generally accepted. Fletcher et al. (1964) have shown that the increased plasma activator activity induced in vivo by nicotinic acid injection has a half-life in the circulation of between 14 and 15 minutes.

The source of blood activator has not been established. Kwaan et al. (1957 a, b) found an increase in fibrinolytic activity in isolated venous segments following the paravenous injection of acetyl choline, adrenaline and serotonin and concluded that the activity was released

from vein walls by a cholinergic-effector mechanism since the effect was blocked by atropine. Kwaan et al. (1958 a,b) have put forward the hypothesis that local activator release from the vessel wall may be a response to intravascular thrombosis. They suggest that serotonin released from aggregating platelets may be the stimulus for its production. The work of Todd (1959), mentioned previously, lends support to the view that vein walls may be the source of activator appearing in blood. Chakrabarti et al. (1963) found that trauma to the wall of an unobstructed vein exposed at operation resulted in increased plasma activator activity upstream from the site of trauma. These workers also showed that non-fibrinolytic plasma introduced into the lumen of an isolated venous segment rapidly developed fibrinolytic activity. They considered that release of activator from the vein walls was a reasonable explanation for their results. More recently Warren (1964), using a histochemical technique similar to that of Todd (1959), found evidence of activator activity in close association with the endothelium of veins, venules and small arteries including the vasa vasorum.

Urokinase Urokinase is the physiological activator which has been isolated from human urine. Investigation of a preparation of urokinase was part of the work for this thesis and the properties of this activator

are discussed in chapter 6.

Activators in Secretions Plasminogen activator activity has been demonstrated in milk (Astrup and Sterndorff, 1953), seminal fluid (von Kaulla and Shettles, 1953), saliva (Albrechsten and Thaysen, 1955) and tears (Storm, 1955).

Bacterial Activators These include streptokinase and staphylokinase.

Streptokinase Streptokinase is present in culture filtrates of certain strains of haemolytic streptococci. Fletcher and Johnson (1957) described a purification process which made possible the intravascular administration of streptokinase preparations in man. Streptokinase is a bacterial protein of molecular weight about 50,000 and exhibits antigenic properties (Fletcher et al., 1958).

Cliffton and Downie (1950) demonstrated the marked species variability which occurs in response to streptokinase administration. Streptokinase readily brings about the activation of human plasminogen, is less effective with rabbit, dog, cat and monkey plasminogen and bovine and porcine plasminogen are almost totally resistant to it.

Streptokinase first acts stoichiometrically with blood proactivator (Troll and Sherry, 1955). The concept of proactivator has been mentioned earlier. This reaction is rapid and the activator so formed

converts plasminogen to plasmin by a first-order enzymatic reaction with release of nitrogen-containing moieties (Alkjaersig et al., 1958 b).

It has been used extensively as a thrombolytic agent in man (see later). Its effectiveness in this respect was established by Johnson and McCarty (1959). In a controlled trial, monitored by venography, they demonstrated the disappearance of artificially induced thrombi in the forearm veins of human volunteers, following systemic streptokinase infusion. Veins containing thrombi in control subjects not given streptokinase went on to fibrosis.

Antibodies to streptokinase are distributed widely in the population and depending on recent or remote streptococcal infection, their level varies considerably. A priming dose of streptokinase, which can be assayed, is required to neutralize antistreptokinase antibody before streptokinase can exert its plasminogen activating effect (Johnson et al., 1957).

Staphylokinase Staphylokinase is a staphylococcal exotoxin, and was shown to be a plasminogen activator (Gerheim and Ferguson, 1949). In certain respects it differs from streptokinase. Although it produces rapid activation of plasminogen at sufficiently high concentrations (Davidson, 1960), there is a limiting concentration above which it exerts

an antiplasmin-like effect (Sweet et al., 1965). It also appears to activate plasminogen directly in contrast to streptokinase. Lack and Towers (1962) have demonstrated that staphylokinase is antigenic. Because of the antiplasmin-like effect of currently available preparations and because in other respects it has no advantages compared with streptokinase, it seems unlikely that staphylokinase will have value as a thrombolytic agent.

Non-enzymatic Agents Adrenaline has already been mentioned as increasing spontaneous fibrinolytic activity on intravenous injection (Biggs et al., 1947). Another vaso-active substance, nicotinic acid, has been found to have a similar effect when administered systemically (Weiner et al., 1958). Both these substances produce intense but transient rises. In this group must be included bacterial pyrogen since, when administered by the intravenous route, it has an effect on plasma fibrinolytic activity closely resembling that of adrenaline and nicotinic acid (Meneghini, 1949).

Fearnley and his colleagues have recently investigated various drugs in a search for a substance which would enhance physiological fibrinolytic activity. Tolbutamide and chlorpropamide were found to increase plasma fibrinolytic activity in patients with atherosclerosis

(Fearnley et al., 1960) although the effect is temporary in many people, resistance developing after several weeks' treatment (Fearnley and Chakrabarti, 1964). Another oral antidiabetic agent, phenformin, has been found to give an increase in fibrinolytic activity without provoking resistance (Fearnley and Chakrabarti, 1964). Testosterone and androlone, given by frequent intramuscular injection, enhance fibrinolytic activity and a short-lived effect has been found using anabolic steroids, including clofibrate (Fearnley et al., 1963). The most potent effect has been demonstrated with corticosteroids (Chakrabarti et al., 1964). All these substances are fibrinolytically inert in vitro and the mechanism of their action in vivo has not been elucidated.

Spontaneous Activation Plasminogen dissolved in phosphate buffer, pH 7.6, and 50 per cent glycerol and maintained at 30°C undergoes virtually complete spontaneous activation, in a first-order reaction, in about 4 days. The reaction is thought to be autocatalytic since it is said to occur with activator-free preparations (Alkjaersig et al., 1958 a). Astrup (1960) has suggested that spontaneous activation is due to the presence of small amounts of activator which contaminate the preparation.

The mechanism of chloroform-induced activation is thought to be

destruction of inhibitors to fibrinolysis with subsequent autocatalytic activation (Christensen, 1946).

Von Kaulla (1962) has suggested that the in vitro plasminogen activation produced by many urea or urethane derivatives and hydrotropic compounds is due to a similar mechanism.

Trypsin Activation Purified plasminogen is activated by trypsin. If the reaction is stabilized in glycerol, the activation kinetics are those of a first-order enzymatic reaction (Alkjaersig et al., 1958 b). This mechanism has not been proved to occur in vivo.

Plasmin Plasmin is the proteolytic enzyme which is produced from its inactive precursor plasminogen by the activation procedures already discussed. Alkjaersig et al. (1958 a, b) studied the release of trichloracetic acid (TCA) soluble moieties after autocatalytic activation in glycerol and after streptokinase and urokinase activation. After the former, TCA soluble material represented 25 per cent of the original protein and after the latter, the figure was 16 per cent. These observations suggest that different activation procedures may produce differing molecular species of plasmin. The molecular weight of plasmin formed spontaneously from plasminogen has been suggested to be 107,000 (Shulman et al., 1960).

In vitro, plasmin has equal affinity for fibrinogen and fibrin (Ratnoff, 1953). Among other plasma proteins susceptible to digestion by plasmin are ACTH, glucagon, growth hormone (Mirsky et al.,1959), prothrombin (Alagille and Soulier, 1956), factor V (Greenwalt and Triantaphyllopoulos, 1954) antihaemophilic globulin (A.H.G.) (Lewis et al.,1949) and certain components of complement (Pillemer et al., 1953). Eisen (1964) has suggested that plasmin may be involved in the formation of plasma kinins, such as bradykinin, from their inactive alpha-2-globulin precursors.

Inhibitors of the Fibrinolytic Enzyme System There are two groups of inhibitors of the fibrinolytic enzyme system: those which inhibit the activation of plasminogen called antiactivators, and those which inhibit the proteolytic activity of formed plasmin, called antiplasmins.

Plasma Antiactivator The separate existence of a physiological antiactivator in plasma has not been established since assay systems at present available do not distinguish activator inhibition from anti-plasmin activity. There is some evidence to suggest that compounds which inhibit activator activity are present in the circulating blood (Lewis and Ferguson, 1951; Jacobsson, 1955; Mullertz, 1957; Paraskevas et al.,1962; McNicol at al.,1963 a).

Other Antiactivators A number of aliphatic amino compounds, of which the most potent is EACA, competitively inhibit plasminogen activation. These are reviewed in chapter 4 in which an account is given of aminomethyl cyclohexane carboxylic acid (AMCA), a more potent antiactivator than EACA. The investigation of AMCA formed part of the work for this thesis. A brief account is also given in chapter 4 of p-aminomethyl benzoic acid (PAMBA), which also has antiactivator properties.

Another compound, the polypeptide Trasylol, possesses antiactivator activity and its investigation is described in chapter 5 as part of the work for this thesis.

Plasma Antiplasmin The existence of two separate antiplasmins in plasma has been established by the work of Norman (1958) and Norman and Hill (1958). According to electrophoretic migration patterns, they have been designated alpha-1-globulin antiplasmin and alpha-2-globulin antiplasmin. The alpha-2-protein is heat-stable, acts rapidly and behaves as a competitive inhibitor of plasmin. The alpha-1-protein is heat-labile and reacts more slowly and non-dissociably. It is generally agreed that antiplasmin levels in plasma exceed potential plasmin levels. Fletcher (1960) has reported that plasma antiplasmin levels are 60 per

cent greater than potential plasmin levels, while Norman (1960) has suggested that antiplasmin levels are about 30 times greater.

Platelet Antiplasmin Johnson and Schneider (1953) have shown that platelets possess antiplasmin activity.

Other Antiplasmins There are numerous compounds with antiplasmin activity which are chiefly of theoretical interest. These include pancreatic trypsin inhibitor and soya bean trypsin inhibitor (Christensen and Macleod, 1945), quaternary amines (Astrup and Alkjaersig, 1951), lauryl amine (Astrup and Alkjaersig, 1952), basic amino acids (Mullertz, 1954), their esters (Troll et al., 1954), heavy metals (Kowalski and Latallo, 1956), heparin (von Kaulla and McDonald, 1958) and toluidine blue (Lombardo, 1958). Other antiplasmins include EACA and AMCA. These are discussed in chapter 4. Their utility as antiplasmins is restricted to in vitro work. Trasylol, the polypeptide proteolytic inhibitor, may be a therapeutically useful antiplasmin. It is discussed in chapter 5.

The Antifibrinolytic Effect of Lipids This effect was investigated as part of the work for this thesis and is discussed in chapter 7.

Physiological Fibrinolysis Sherry and his co-workers have postulated an hypothesis to account for the largely specific in vivo effect of the

fibrinolytic enzyme system - that of lysis of fibrin (Alkjaersig et al., 1959 a; Sherry et al., 1959 a, b; Fletcher et al., 1962 a). Figure 2 is a schematic representation of the hypothesis which has been called the dual phase concept of plasminogen activation. According to the concept, plasminogen, in a plasma-clot system, exists, in a physical sense in a soluble and a gel phase. Plasminogen in plasma constitutes the soluble phase and plasminogen in clot the gel phase. Because of this physical distribution of plasminogen, the biochemical effects of plasminogen activation in each phase differ. When soluble phase plasminogen is activated to plasmin, it is rapidly neutralized by plasma antiplasmin so that no appreciable proteolysis of plasma proteins, susceptible to digestion by plasmin, is detectable. It is only when plasma plasminogen is rapidly converted to plasmin and the antiplasmin mechanism is temporarily overwhelmed that systemic proteolysis with a haemorrhagic state occurs. Such hyperplasminaemic states are discussed below. In the gel phase, activator, adsorbed to fibrin during its formation, also diffuses into the clot where plasminogen is in intimate spatial relationship with fibrin and levels of antiplasmin are said to be low (Sawyer et al., 1961). Thus free plasmin is formed in close proximity to its substrate fibrin which is then lysed.

There is experimental evidence which, though not conclusive, lends support to this hypothesis. Fibrinogen and fibrin have strong affinities for plasminogen (Blomback and Blomback, 1956). The preparation of fibrinogen free from plasminogen is exceedingly difficult unless specific plasminogen-solubilizing agents are used (Bergstrom and Wallén, 1961). Fibrinous deposits or thrombi are found to contain considerable quantities of plasminogen. Clots rendered plasminogen-poor rapidly take up plasminogen when exposed to plasma. Plasminogen-enriched radioactive clots lyse rapidly when immersed in a medium containing activator and clot lysis rates are linearly related to the logarithm of either clot plasminogen concentration or activator concentration (Alkjaersig et al.,1959 a). Using isotopically-labelled streptokinase Gross, (1963) has reported that this activator rapidly and deeply penetrates thrombus in contact with it.

It is suggested by Sherry et al. (1959 a) that an important function of plasma plasminogen may be to endow fibrin with a mechanism which can promote its subsequent lysis.

Another hypothetical explanation of the mechanism involved in lysis of thrombus is that of Ambrus and Markus (1960). These authors believe that specificity of plasmin for fibrin is conferred by means of a

plasmin-antiplasmin complex, which may function as a reservoir of plasmin in the circulation. Circulating proteins are protected from proteolysis by plasmin when it is bound to antiplasmin. Plasmin becomes available when required at sites of intravascular fibrin formation.

The Relationship between the Fibrinolytic Enzyme System and the Coagulation System Astrup (1956) has suggested that in health, the fibrinolytic enzyme system may be in dynamic equilibrium with the coagulation system. He considers that the latter system is constantly laying down fibrin deposits to seal any sites of tissue damage in the vascular tree and that, in order to maintain vascular patency, the former system constantly removes such fibrin once the integrity of the vessel wall has been restored.

Duguid (1955) has argued that presisting mural deposits of fibrin can become incorporated in the arterial wall thus forming the basis of an atherosclerotic plaque.

Astrup extended Duguid's theory by suggesting that in diseased states accompanied by reduction in fibrinolytic activity, resolution of fibrin deposits could be delayed thus permitting the pathological process proposed by Duguid.

Evidence in favour of a dynamic equilibrium of clotting and lysis is conflicting. Fearnley and Tweed (1953) and Sawyer et al. (1960) have demonstrated fibrinolytic activity in plasma from normal subjects which supports the view that the fibrinolytic enzyme system is constantly active in vivo. A minor reduction in fibrinolytic activity has been described in certain diseased states associated with thrombosis. Hume (1958), Lackner and Merskey (1960) and James et al. (1961) have reported decreased fibrinolytic activity after cardiac infarction. Nestel (1959) has reported similar findings in patients with intermittent claudication; Franz et al. (1961) in post-operative patients and Fearnley et al. (1963) in patients suffering from diabetes mellitus. Smyrniotis et al. (1959) have found a decrease in urinary activator excretion in patients with cancer.

If the concept of a dynamic equilibrium were true, it might be expected that patients with haemorrhagic diatheses due to deficiencies of clotting factors would have diminished plasma fibrinolytic activity to compensate for their impaired coagulation. However, Kamel et al. (1963) have reported that fibrinolytic activity is normal in patients with haemophilia, Christmas disease and von Willebrand's disease and Rausen et al. (1961) have found that ^{131}I-labelled fibrinogen

survival is normal in haemophilic patients. In addition, Gordon et al. (1965) found that prolonged administration of the fibrinolytic inhibitor, EACA, to a group of haemophiliacs did not bring about an increase in plasma fibrinogen levels.

From these observations, it will be seen that Astrup's hypothesis remains unproven. There is accumulating evidence to suggest that the coagulation system and the fibrinolytic enzyme system have a complex inter-relationship. This evidence, reviewed in chapter 8 which is concerned with a number of defibrination syndromes of differing aetiologies, includes the findings that Hageman factor may activate both systems; platelets possess antiplasmin activity; prothrombin factor V, antihaemophilic globulin (A.H.G.) and fibrinogen are susceptible to digestion by plasmin and fibrinogen breakdown products may have an antithrombin effect and inhibit fibrin polymerisation.

Therapeutic Thrombolysis The earliest observations that administration of plasminogen activator might bring about lysis of *in vivo* thrombi were made by Johnson and Tillett (1952) who found that thrombi produced in the marginal ear veins of rabbits could by lysed by intravascular administration of streptokinase. In man, the work of Johnson and McCarty (1959) previously mentioned, established the value of systemic

streptokinase infusion in lysing artificially produced thrombi in the forearm veins of human volunteers. The feasibility and effectiveness of thrombolysis in man with streptokinase infusion has been established by clinical trials (Fletcher et al., 1959 a, b; Deutsch and Fischer, 1960) and in individual well-documented cases of peripheral vascular occlusion (Amery et al., 1963; McNicol et al., 1963 b; Verstraete et al., 1963). Compared with streptokinase which is antigenic, urokinase has obvious attractions as a thrombolytic agent and in chapter 6 of this thesis is described investigation of a commercial preparation of urokinase with a view to establishing its suitability for this purpose.

Hyperplasminaemic States Disordered fibrinolytic states with a resultant coagulation defect occur when large amounts of plasminogen activator are released into the circulation so that conversion of plasminogen to plasmin is rapid and complete. The plasmin-antiplasmin 'buffering'system is temporarily overwhelmed and free plasmin circulates - hyperplasminaemia. The ensuing coagulation defect is due in part to digestion of prothrombin, factor V and antihaemophilic globulin (A.H.G.); in part to defective fibrin polymerisation brought about by circulation of fibrinogen and fibrin breakdown products and in

part to accelerated lysis of such fibrin thrombi as do form (Alkjaersig et al.,1962; Bang et al.,1962; Fletcher et al.,1962 b).

A number of steps are involved in fibrin gel formation. Thrombin, a highly specific proteolytic enzyme (Blömback and Yamashina, 1958), splits off specific peptides from fibrinogen, thus converting it to fibrin monomer (Scheraga and Laskowski, 1957). The fibrin polymer is formed by condensation of a number of fibrin monomers and the visible fibrin clot is then formed in a gelation step. The clot is rendered insoluble by the enzymatic action of factor XIII, which has been postulated to bring about the formation of sulphydryl linkages between the polymers (Laki and Gladner, 1964). Bang et al. (1962) have shown, using the electron microscope, that clots formed in the presence of fibrinogen and fibrin breakdown products are loose and friable and have an abnormal 'frayed-rope' appearance. The abnormality is due to incorporation within the fibrin polymer of breakdown products which lack the correct configuration for a structurally sound polymer. An antithrombin effect of the products of fibrin and fibrinogen proteolysis has also been observed (Niewiarowski and Latallo, 1957; Triantaphyllopoulos, 1958).

Hyperplasminaemic states have been reported to occur when

tissues rich in activator have been handled especially during pulmonary surgery (Soulier et al., 1952) and surgery with extracorporeal circulation (von Kaulla and Swan, 1958); in association with prostatic carcinoma particularly with metastases (Tagnon et al., 1952) and in cirrhosis of the liver (Kwaan et al., 1956). Systemic proteolysis by plasmin may contribute to the coagulation defect which may complicate certain obstetric accidents, most commonly amniotic fluid embolism, intrauterine death and accidental haemorrhage (Schneider, 1959). As will be discussed in chapter 8 of this thesis, the genesis of defibrination syndromes may be in part due to hyperplasminaemia and it is possible that 'pure' hyperplasminaemic states are rare, with the exception of that which is probably inevitable in patients receiving thrombolytic therapy.

As has been mentioned, the work for this thesis has involved the investigation of two compounds which exhibit antifibrinolytic properties (AMCA and Trasylol), one of which also possesses anticoagulant and antitryptic activity. A commercial preparation of urokinase has been screened for thromboplastic activity. The effect of fat feeding on clotting, lysis and platelet aggregation has been examined and finally a number of patients with the defibrination syndrome have been studied in an attempt to elucidate further the problem of its diagnosis and therapy.

CHAPTER 3

MATERIALS AND METHODS

In this chapter is presented an account of the materials and standard fibrinolytic and coagulation methods used in the work for this thesis. A brief explanation of the basis for each method, and sources of error in interpretation of results, is also given. In certain instances, it was necessary to modify a number of the tests for particular experiments. Such modifications and methods devised or used for one particular aspect of the work in this thesis are set out in the methods section of the appropriate chapter.

Assay systems are described for plasminogen, plasminogen activators and for fibrinogen, which, as fibrin, is the physiological substrate for plasmin. Plasma fibrinolytic inhibitor activity was also measured. A method for assay of streptokinase antibody is given and the thrombin clotting time, widely used as a measure of the coagulation defect due to hyperplasminaemia, is described.

A brief account is given of the one-stage prothrombin time, the plasma recalcification time and the thromboplastin generation test. The assay systems used to determine levels of factor V, antihaemophilic globulin (A.H.G.), Christmas factor and Hageman factor are outlined. The method used to count platelets is given and details of the system

employed to assess speed of platelet aggregation are provided.

Materials

Streptokinase Streptokinase - Streptodornase Varidase (Lederle) was the preparation employed with the exception of one series of experiments, described in chapter 7, in which the preparation of streptokinase used was Kabikinase provided by A.B. Kabi, Stockholm. The stock solution of the Varidase preparation contained streptokinase, 10,000 N.I.H. units/ml. of 0.9 per cent saline. This was stored at 4°C. and diluted in 0.9 per cent saline as required. The Kabikinase preparation was highly purified streptokinase intended for intravascular administration and supplied as a powder in ampoules of 250,000 N.I.H. units. For the series of experiments in chapter 7, one ml. amounts containing 8,000 N.I.H. units were required. Accordingly, one ampoule was dissolved in 0.9 per cent saline to give a concentration of 8,000 N.I.H. units/ml. and this volume was stored at -20°C. in one ml. amounts and thawed immediately before use.

Urokinase Two preparations were used, one provided by Leo Laboratories Ltd., Copenhagen, and one by Abbott Laboratories, North Chicago, Illinois. The latter preparation is described in detail in chapter 6. Urokinase (Leo) was a purified preparation isolated from

human urine. It was a sterile yellowish white powder, freely soluble in 0.9 per cent saline. The potency of the preparation used in the work for this thesis was 5,000 Ploug units per mg. protein (Ploug and Kjeldgaard, 1957). The fibrinolytic and coagulative properties of this preparation have been described by McNicol et al. (1963 a).

Human Plasminogen This was a purified preparation, soluble at neutral pH, containing 0.3 casein units/mg. protein, supplied by A.B. Kabi. The casein unit of activity is defined below.

Human Plasmin This was prepared by the method of Alkjaersig et al. (1958 a), by spontaneous activation of Kabi human plasminogen. To each flask of human plasminogen was added 10 ml. of 50 per cent glycerol-saline. The spontaneous activation to plasmin at 37°C., was 98 per cent complete after 6 weeks. Completion was assessed by caseinolytic assay, described below, of the preparation with and without the addition of streptokinase, 2,000 units/ml. Without streptokinase activation, the preparation contained 6.1 casein units/ml. and with streptokinase activation, 6.2 casein units/ml.

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

concentration.

Trypsin This was bovine trypsin (L. Light and Co. Ltd., Colnbrook). The preparation was twice crystallized and salt-free.

Casein Solution This was prepared as described by McNicol and Douglas (1964a), by boiling 25 gm. casein (L. Light and Co. Ltd.) in 500 ml. phosphate buffer, 0.1 Molar, pH 7.6, for 20 minutes. While hot, the pH of the solution was adjusted to 7.6 and the solution filtered. It was then dialysed for 24 hours with constant stirring at 4°C. against 4 litres of phosphate buffer, 0.1 Molar, pH 7.6. The dialysate was changed once at 12 hours. The pH of the casein solution was checked at the end of dialysis and readjusted, if necessary, to 7.6. The solution was stored at 4°C.

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

Human Fibrinogen This was prepared from expired pooled bank plasma by a phosphate buffer method (Biggs and Macfarlane, 1962 a). The final concentration of fibrinogen was about 4 mg./ml. of citrate-saline solution.

Radioactive Iodine (^{131}I) This was obtained from the Radiochemical Centre, Amersham, as sodium iodide, in 0.1 ml. dilute sodium thiosulphate.

Radioactive Iodine-tagged Human Fibrinogen Human fibrinogen was labelled with radioactive iodine by a modification of the method of Clement and McNicol (1959), (McNicol and Douglas, 1964a). To one to two mc. radioactive iodine were added 0.025 ml., N. stable iodine in methanol; 0.025 ml., 0.01 N. H_2SO_4; 0.025 ml. heparin (25 units/ml.) and 5 ml. of human fibrinogen in citrate-saline. The mixture was allowed to incubate for 5 minutes at room temperature. It was then passed through a chloride-phase ion-exchange resin column, (Amberlite IRA 400 (Cl), B.D.H. Laboratory Chemicals Division, Poole) with 5 ml. phosphate buffer, 0.1 Molar, pH 7.6. The tagged fibrinogen was stored in small amounts at -20°C. Clottable radioactivity of this preparation varied from 89 to 94 per cent.

Acetyl-l-Lysine Methyl Ester (AcLMe) This was a preparation dried with phosphate buffer, pH 7.6, and NaCl (Cyclo Chemical Corporation, Los Angeles, Calif.). Twenty mg./ml. in distilled water gave a solution of pH 7.6 containing 0.016 Molar AcLMe in 0.066 Molar phosphate buffer and 0.6 per cent NaCl.

Chromotropic Acid Reagent In a 1,000 ml. volumetric flask containing 200 ml. cold distilled water were added 100 ml., 2 per cent chromotropic acid (4, 5-dihydroxy-2, 7-naphthalene disulphonic acid, disodium salt (Kodak Ltd., London)). This mixture was placed in an ice bath and 600 ml. cold concentrated H_2SO_4 added in 50 ml. aliquots, allowing 20 minutes between each addition. This was allowed to stand 16 hours at 4°C, brought up to room temperature, distilled water to 1,000 ml. added and then filtered through a medium-pore, sintered glass funnel. The solution was stored in the dark at 4°C and was discarded if it turned brown on standing.

Epsilon Aminocaproic Acid (EACA) This was a 25 per cent solution provided by Boots Pure Drug Co. Ltd., Nottingham.

Plasma Samples Plasma samples were obtained by mixing, without frothing, 9 volumes of whole blood, collected by clean venepuncture, with 1 volume of 3.8 per cent sodium citrate and the plasma separated by centrifugation (600 g. for 10 minutes at 4°C). Where plasma was used in assays, no correction of results was made for dilution by citrate.

Expired Pooled Bank Plasma used in the work for this thesis had a fibrinogen content of 220 mg./100 ml. and a plasminogen content of 2.2 casein units/ml.

Silicone Glassware Glassware was silicone-coated by the method described by Dacie (1956 a), using a 10 per cent (V/V) solution of silicone M 441 (I.C.I. Ltd., Manchester) in petroleum ether. The glassware was washed and re-treated for each experiment.

Methods

Plasminogen Assay The method used was the caseinolytic assay of Remmert and Cohen (1949), as modified by Alkjaersig et al. (1959 a). In this assay, plasma is incubated with acid to destroy antiplasmin. The acid is neutralized with alkali and buffer. Streptokinase is added to convert plasminogen to plasmin. Casein is employed as substrate for the plasmin so formed. The enzymatic action of plasmin on casein is stopped by precipitation of protein with trichloracetic acid (TCA). TCA-soluble moieties, including tyrosine, released by the proteolytic activity of plasmin on casein, are in the supernatant. The amount of tyrosine released from the casein is a measure of the amount of plasmin present.

To 0.5 ml. plasma was added 0.5 ml., 1/6 N. HCl. The plasma was incubated with the acid for 15 minutes at room temperature and then 0.5 ml., 1/6 N. NaOH was added followed by 1.0 ml. phosphate buffer, 0.1 Molar, pH 7.6. Streptokinase, 0.5 ml. of a

solution containing 2,000 units/ml., was next added. After addition of 2.0 ml., 5 per cent casein solution and thorough mixing, the digestion mixture was incubated at 37°C for 62 minutes. Aliquots, each of 2.0 ml., were removed from the digestion mixture at 2 minutes and 62 minutes and each added to 2 ml., 10 per cent TCA. The supernatant was obtained by centrifugation (1,000 g. for 10 minutes) and tyrosine measured by addition of 1.0 ml. of the supernatant to 5.0 ml., 0.5 N. NaOH; 1.5 ml., 5 per cent TCA and 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 in a spectrophotometer (SP500, Unicam Instruments, Cambridge) at 650 mμ with the 2 minute sample as blank. Tyrosine release was read off a standard curve. One casein unit of plasminogen released 180 μgm. tyrosine/hour.

Activator Assays No direct assay for activator in biological fluids is available. Five assay systems are described. The euglobulin lysis and dilute plasma clot lysis tests measure primarily plasma activator activity. The fibrin plate test and the radioactive iodine (^{131}I) labelled clot assay measure the ability of the test plasma or other biological fluid to lyse preformed fibrin. An assay system for urokinase,

employing the synthetic ester AcLMe, is also described. This assay system is useful as a biochemical standardization for concentrated solutions of pure urokinase.

Euglobulin Lysis Test In this test, the plasma euglobulin fraction is precipitated at pH 5.4 and low ionic strength. The precipitate contains plasminogen activator and plasminogen, a quarter of the plasma fibrinogen, but with only traces of the plasma antiplasmins which are chiefly in the supernatant (Kowalski et al., 1959). Because antiplasmin is not present in the clots made from the euglobulin precipitate, lysis times are much shorter than those found with clots made from native plasma. In the presence of normal concentrations of plasma plasminogen and probably fibrinogen, the lysis time is a measure of the plasma activator activity. Low plasminogen concentrations, even in the presence of high activator activity, lead to prolonged euglobulin lysis times because there is insufficient protential plasmin to digest the fibrin of the clot (McNicol et al., 1962, a) and there is evidence to suggest that an increased concentration of fibrinogen, which as fibrin is substrate for plasmin, may also lengthen the lysis time of the euglobulin clot (Bang et al., 1960).

The method used was that of Nilsson and Olow (1962). A

precipitate was formed by addition of 0.5 ml. plasma to 9.5 ml., 0.014 per cent acetic acid, pH 5.4. After standing for 10 minutes at 4°C, the precipitate was obtained by centrifugation (1,000g. for 10 minutes at 4°C). The supernatant was discarded and the precipitate resuspended in 0.5 ml. barbitone buffer, pH 7.3 (sodium diethyl barbitone, 11.75 gm.; HCl, 0.1N., 430 ml.; NaCl, 14.67 gm.; distilled water to 1,000 ml.). The resuspended precipitate was clotted with 0.5 ml. thrombin, 2 N.I.H. units/ml., and the time for lysis of the clot observed at 37°C. Results of euglobulin lysis tests may be expressed in minutes but where statistical analysis of results is necessary, in units of activity. Fibrinolytic activator activity is inversely proportional to lysis time (Sherry and Alkjaersig, 1957) and using this relationship in a double logarithmic plot, observed lysis times are expressed as units of activity, a lysis time of 300 minutes being arbitrarily assigned a value of one unit of activity. To convert lysis times to units, the lysis time in minutes is divided into 300.

Dilute Plasma Clot Lysis Test This is a dilution technique which depends on the fact that dilution favours fibrinolysis possibly by depressing inhibitory activity (Fearnley and Lackner, 1955).

The method used in this thesis was that of Fearnley et al. (1957),

modified to use plasma instead of whole blood. To 1.7 ml. phosphate buffer, pH 7.4, containing 0.1 ml. thrombin (50 N.I.H. units/ml. saline) was added 0.2 ml. plasma. The thrombin was added immediately before the plasma. The plasma was separated from citrated whole blood collected as described previously, the citrated whole blood having been kept on ice, centrifuged at 4°C and the plasma added to the buffer without delay. Clotting occurred within a minute and the tubes were then stored for 30 minutes at 4°C and transferred for 5 minutes to a waterbath at 37°C. At this time, brisk rotation of the tube between the palms of the hands freed the clot from the wall of the tube. The clot was then observed at 37°C until its complete disappearance which was the end-point. Lysis times in healthy subjects during the day were greater than 400 minutes.

Fibrin Plate Test In this test a solution of fibrinogen is clotted with thrombin in a perspex dish resembling a Petri dish. Test fluids are pipetted on to the fibrin surface. Plasminogen is always associated with the fibrinogen used for the test. If the test fluid contains activator, plasminogen will be converted to plasmin which then digests fibrin to produce holes in the fibrin film. The areas of lysis are not linearly related to activator concentration and where

statistical analysis of results is required, a standard curve must be run with each batch of test fluid, activity in each sample being read off the curve (Smyrniotis et al., 1959).

The method used in this thesis was a modification of that of Nilsson and Olow (1962). Bovine fibrinogen, Blomback fraction I - 0 (Blomback and Blomback, 1956), was used. To 23.7 ml. tris buffer, 0.15 Molar, pH 7.8, was added 0.7 ml., 0.1 per cent bovine fibrinogen. The fibrinogen solution was clotted with 0.6 ml. thrombin (50 N.I.H. units/ml.), in a perspex dish of internal diameter 11.5 cm. Samples of human plasma, resuspended euglobulin precipitate, other biological fluid or homogenized tissue were applied in 30 μ litre amounts to the surface of the fibrin. The plate was then incubated at 37^{o}C for 16 hours. Holes made in the plate where fibrin had been lysed were then measured and results expressed in square millimetres as the product of two perpendicular diameters of the area of lysis.

Resuspended euglobulin precipitates produced larger holes than the plasmas from which they were prepared because plasma antiplasmin is largely discarded with the supernatant (Kowalski et al., 1959).

The precipitate was prepared by addition of 0.1 ml. plasma to 1.9 ml., 0.014 per cent acetic acid, pH 5.4. After standing for 10

minutes at 4°C, the precipitate was obtained by centrifugation (1,000 g. for 10 minutes at 4°C) and resuspended in citrate-saline solution (one volume 3.8 per cent sodium citrate and 9 volumes 0.9 per cent saline).

Radioactive Clot Assay The method described is modified from that of Alkjaersig et al. (1959 a), (McNicol and Douglas, 1964a). The assay depends on the release of radioactivity from ^{131}I-tagged plasma clots, enriched with plasminogen, when incubated in the presence of a plasminogen activator. Plasminogen is added to clots during their formation to ensure that they remain sensitive to activator after they have been washed to remove blank radioactivity. The clots are formed round stainless steel wire spirals. After washing, the clots are incubated at 37°C with the test plasma. Radioactivity released into the test plasma from the clot in a standard time is a measure of activator concentration in the test plasma.

Expired pooled bank plasma was trace labelled with ^{131}I-tagged fibrinogen so that 10 ml. plasma gave approximately 2,500 counts in 10 seconds. To 0.5 ml. labelled plasma was added 0.1 ml. plasminogen, 6 casein units/ml. The plasminogen was insoluble at neutral pH and was therefore not eluted during washing. The plasma was clotted with 0.1 ml. thrombin, 10 N.I.H. units/ml., round the corkscrew end

of a stainless steel wire in a 75 x 8 mm. test tube and washed in phosphate buffer, 0.1 Molar, pH 7.6. The minimum washing time was 60 minutes. The test plasma, 0.5 ml., was incubated with a radioactive clot for 30 minutes. The clot was then removed on its wire spiral and radioactivity in the test solution counted. Results were recorded after subtraction of background radioactivity and the mean count from two saline control tubes in which clots had been incubating. Results were expressed either as counts or, after caluculation of the specific radioactivity of the fibrin as μgm. fibrin lysed / ml. of test solution / hour.

Assay of Urokinase with the Synthetic Ester, AcLMe This assay depends on the ability of urokinase to split AcLMe into acetyl-l-lysine and methyl alcohol. The amount of methyl alcohol liberated from the synthetic substrate is measured colorimetrically. The method is not sufficiently sensitive to measure urokinase in urine and is useful for biochemical standardization of concentrated solutions of pure urokinase.

The assay system was that of Sherry et al. (1964). To 3.0 ml. AcLMe, 20 mg./ml., at 37°C was added 0.3 ml. urokinase dissolved in phosphate buffer, 0.1 Molar, pH 7.6. The concentration of the urokinase solution was not less than 1,000 C.T.A. units/ml. The C.T.A.

unit is defined in chapter 6. At 1 minute, a one ml. aliquot was removed into 0.5 ml., 15 per cent TCA and again at 15 and 30 minutes. To 0.5 ml. of each of these specimens was added 0.1 ml., 2 per cent potassium permanganate ($KMnO_4$) and after one minute, 0.1 ml., 10 per cent Na_2SO_3 which had been freshly prepared. After addition of 4.0 ml. chromotropic acid reagent and thorough mixing, specimens were placed in a boiling water bath for 15 minutes. After a few minutes for cooling, colour intensity was read in a spectrophotometer (S.P. 500, Unicam Instruments) at 580 mμ against the one minute specimen as blank.

A standard curve was run with each test batch. The stock standard was a solution of methyl alcohol containing one μ mole/ml., made up in 0.066 Molar phosphate buffer containing 5 per cent TCA. The standards were treated from the point at which $KMnO_4$ was added. In the work for this thesis, 0.5 ml. of a solution containing one μ mole/ml. gave an optical density reading of 0.150. The results were calculated from the standard curve and expressed as μ moles of methyl alcohol liberated per ml. of enzyme solution per hour.

Fibrinogen Assay The method described is modified from that of Ratnoff and Menzie (1951) (McNicol and Douglas, 1964a). In this method plasma fibrinogen is clotted with an excess of thrombin and the fibrin

formed, after washing free from other plasma proteins, is hydrolysed by boiling with sodium hydroxide. The tyrosine released during hydrolysis is estimated colorimetrically. It is assumed that there is a constant proportion of tyrosine in the fibrinogen molecule (1.0:11.7).

Into a 15 ml. thick-walled glass test tube were placed "0.2 ml." glass beads, of diameter not greater than 0.15 mm.; 6.0 ml., 0.9 per cent saline; 0.2 ml., 2.5 per cent $CaCl_2$; 0.2 ml. plasma and finally 0.1 ml. thrombin, 100 N.I.H. units/ml. The tube was shaken without inversion and the fibrin harvested on the glass beads. After standing for 30 minutes at $4^{\circ}C$, any further fibrin formed was harvested on the glass beads by shaking the test tube without inversion. The tube was then centrifuged (1,000 g. for 10 minutes). The supernatant was discarded using a Pasteur pipette and the glass beads washed three times with 6.0 ml., 0.9 per cent saline, with centrifugation (1,000 g. for 10 minutes) after each washing and careful decantation of the saline. To the glass beads and adherent fibrin was then added 0.4 ml., 10 per cent NaOH and the tube boiled in a water bath for 20 minutes. The tube was sealed with tinfoil to prevent evaporation. After cooling, the tyrosine released was estimated by addition of 2.0 ml., 0.5 N. NaOH; 0.6 ml., 5 per cent TCA and 0.6 ml. dilute (1:2) Folin Ciocalteu reagent. After

standing for 10 minutes to permit maximum colour development, the optical density was read in a spectrophotometer (SP 500, Unicam Instruments) at 650 mμ. against a reagent blank. The chief sources of error in this assay are failure to wash the glass beads adequately, careless decantation of saline washings and adherence of fibrin to the walls of the test tube. Optical density readings were converted from a standard tyrosine curve to mg. fibrinogen/100 ml. plasma.

Assay for Fibrinolytic Inhibitor Activity: Urokinase Sensitivity Test

In this test, plasma is clotted in the presence of a standard amount of the plasminogen activator urokinase. The lysis times of such standard clots provide a comparative measure of overall fibrinolytic inhibitor levels. Urokinase is employed as activator in this system because of its presumed non-antigenicity and the relative uniformity of the fibrinolytic response which it produces (Fletcher et al., 1965). It is therefore assumed that inhibitory activity against urokinase is due to plasma antifibrinolytic activity and not to specific antibody to urokinase as may occur with the bacterial protein activators streptokinase and staphylokinase, antibodies to streptokinase and staphylokinase being present in very variable titres throughout the population. Accordingly, in any one patient, in whom plasminogen and

fibrinogen levels are constant, changes in lysis time in the urokinase sensitivity test will reflect variations in fibrinolytic inhibitor levels. The test is therefore useful in detecting any increase in fibrinolytic inhibition which may be imparted to plasma by administration of a fibrinolytic inhibitor. Similarly in a population with plasminogen and fibrinogen levels within the normal ranges, the scatter of lysis times is a measure of the scatter of inhibitor levels.

The test does not distinguish between plasma antiactivator activity and plasma antiplasmin activity though with the relatively high concentration of urokinase incorporated in the test system, variations in lysis times are more likely to be due to fluctuations in antiplasmin activity.

In this assay system, modified slightly from that of McNicol et al. (1963 a), to 0.2 ml. plasma was added 0.32 ml. urokinase containing 500 Ploug units/ml. The mixture was clotted with thrombin 0.1 ml., 20 N.I.H. units/ml. The lysis time of the plasma clot at 37°C was observed. Results may be expressed as minutes or for statistical analysis as units of activity, one unit being defined as the reciprocal of a lysis time of 10 minutes.

Streptokinase Sensitivity Test This test is designed to measure the

concentration of streptokinase which would neutralize plasma antibody to streptokinase and other inhibitors and leave sufficient free streptokinase to produce a plasma clot lysis time of about 20 minutes (Johnson et al., 1957).

To 0.2 ml. plasma in each of a series of test tubes, streptokinase, 500 N.I.H. units/ml., was added in volumes ranging from 2 to 160 μ litres as measured in a microburette (Micro-metric Instrument Co., Cleveland, Ohio). Each plasma/streptokinase mixture was clotted with thrombin, 0.1 ml. (20 N.I.H. units/ml.), and incubated at 37°C. The time for clot lysis to occur was observed. The required concentration of streptokinase was calculated on the basis of the plasma volume to which it would be added.

Thrombin Clotting Time This test is used as an index of defective fibrin polymerisation. However, evidence will be presented in chapter 8 that reduced concentration of fibrinogen per se may prolong the thrombin clotting time.

The method described is that of Fletcher et al. (1959 a). To 0.1 ml. plasma was added 0.3 ml. 'thrombin titration mix' (Seegers and Smith, 1942). Thrombin, 0.1 ml. (6 N.I.H. units/ml.) was added and the clotting time of this mixture recorded at 37°C. The

thrombin titration mix was freshly prepared immediately before use as follows:- 6 ml., 0.9 per cent saline; 2 ml., 0.7 per cent $CaCl_2$; 2 ml., 15 per cent acacia; 1 ml. tris buffer, 0.1 Molar, pH 7.5.

One-stage Prothrombin Time This test was carried out as described by Douglas (1962). A frozen saline extract of human brain was the brain extract used and the concentration of the 0.025 Molar $CaCl_2$ was checked by titration.

Plasma Recalcification Time This was measured by the method of Biggs and Macfarlane (1962 b).

Thromboplastin Generation Test This was the method of Biggs and Douglas (1953). In most of the experiments, the method was modified by using commercial platelet substitute (Bell and Alton, 1954), supplied by Stayne Laboratories, Ltd., High Wycombe, Bucks.

Factor V Assay This was the method of Douglas and Biggs (1953) with the modification that clotting times were recorded at 5 and 6 minutes after the start of incubation.

Antihaemophilic Globulin (A.H.G.) and Christmas Factor Assays These were carried out by a one-stage method (Douglas, 1965) which depends on the clotting time of plasma from patients with high-grade haemophilia or Christmas disease, which has been maximally "contact-

activated" by kaolin. Cephalin (Bell and Alton, 1954) was added to the substrate system.

Hageman Factor Assay This method (Douglas, 1963) depends on the recalcification time of the test plasma mixed with Hageman factor deficient plasma. A dilution curve was made with pooled normal plasma and clotting times of the test plasma converted to per cent Hageman factor from the curve. The system was maximally "contact-activated" with kaolin and cephalin (Bell and Alton, 1954) was added.

Platelet Count The method used was that described by Dacie (1956 b).

Platelet Aggregation This was measured by a modification of the Chandler's tube technique (Chandler, 1958). Chandler showed that when whole blood was rotated in a closed loop of plastic tubing, when it solidified, its histological structure closely resembled a pathological *in vivo* arterial thrombus. This technique has been modified to use platelet-rich plasma (Cunningham et al. 1965). When platelet-rich plasma is made to flow round a Chandler's tube, the first stage in formation of the thrombus is a "snow-storm" effect produced by platelet aggregation. These platelet clumps eventually coalesce to form the white head of the thrombus and after a further interval, a fibrin tail forms. Under rigidly standardised conditions, the time between re-

calcification of the plasma and the appearance of the "snow-storm" effect is measured.

The method used in this thesis was slightly modified from that of Cunningham et al. (1965). Nine volumes of whole blood were collected in a silicone centrifuge tube containing 1 volume, 3.8 per cent sodium citrate and mixed without frothing using a non-water-wettable seal to avoid contact activation of the blood during mixing. After centrifugation, (600 g. for 5 minutes at 4°C, 10 minutes after collection), 5 ml. of the platelet-rich plasma were transferred, using a silicone Pasteur pipette, to a Chandler's tube. The tube was made with transparent plastic vinyl tubing of length 70 cm. and internal diameter 1.25 cm. (Portland Plastics Ltd., Hythe, Kent). The tube contained 10 ml., 0.9 per cent saline. This mixture was recalcified with 0.5 ml., 0.25 Molar $CaCl_2$ and a stop-watch started. The tube was made a continuous loop with a nylon adaptor and rotated on the turntable of a blood-cell suspension mixer (Matburn Ltd., London), revolving at 28.5 r.p.m. in a glass-fronted incubator at 37°C. The time for the "snow-storm" appearance to occur after recalcification was noted and taken as the time for platelet aggregation.

CHAPTER 4

IN VITRO AND IN VIVO STUDIES OF AMINOMETHYL CYCLOHEXANE CARBOXYLIC ACID, A SYNTHETIC FIBRINOLYTIC INHIBITOR

The possible use of inhibitors of the fibrinolytic enzyme system for the treatment of haemostatic failure due to pathological fibrinolytic activity (McNicol and Douglas, 1964) has stimulated the search for potent inhibitors of fibrinolysis. In this chapter will be described the results of a laboratory and clinical appraisal of the synthetic amino acid inhibitor 1-(aminomethyl) cyclohexane-4-carboxylic acid (Mitsubishi Kasei Kogyo Company, 1961), the anti-fibrinolytic effect of which was discovered by Okamoto and Okamoto (1962).

A number of amino compounds have been shown to possess antifibrinolytic effects. In vitro, lysine and ornithine inhibit the activation of plasminogen (Mullertz, 1954) and arginine and lysine esters competitively inhibit the proteolytic activity of plasmin and the activation of plasminogen by urokinase, streptokinase and trypsin (Alkjaersig et al., 1958 b). The synthetic amino acid EACA, which differs from lysine in the absence of the alpha-amino group, was discovered by Okamoto and his associates (Mitsubishi Kasei Kogyo Kabushiki Kaisha Company, 1957) and was shown to be an effective

inhibitor of plasminogen activation by Ablondi et al. (1959) and Alkjaersig et al. (1959 b).

Sjoerdsma and Nilsson (1960) investigated a number of aliphatic amino compounds and found activity as inhibitors of plasminogen activation among those amino acids with a carbon chain length of 4-8 C atoms. The most potent was the 6-carbon compound, EACA. The terminal position of the amino group appears to be critical in that while EACA is a potent inhibitor, norleucine (alpha-aminocaproic acid) is inactive. Likewise the carboxyl group probably is important because the addition of an alpha-amino group to EACA (alpha-epsilon-aminocaproic acid or lysine) results in impaired activity.

The search for a more potent fibrinolytic inhibitor lead to the investigation of amino compounds with a cyclic structure. At the present time, the most potent of these are aminomethyl cyclohexane carboxylic acid and p-aminomethyl benzoic acid, (PAMBA), (Lohmann et al., 1964). Okamoto and Okamoto (1962) initially reported that aminomethyl cyclohexane carboxylic acid had 10 to 30 times the activity of EACA. Animal toxicity studies, including acute and chronic toxicity studies and investigation of the effect of long-term administration on conception and foetal development, were reported by Melander et al. (1965),

whose results indicated that the compound was considerably less toxic to administer than are the amounts of EACA in current use.

The use of EACA as a fibrinolytic inhibitor is now well-documented. At concentrations above 10^{-4} Molar, it competitively inhibits plasminogen activation and at concentrations above 10^{-2} Molar, it non-competitively inhibits the proteolytic activity of plasmin (Ablondi et al., 1959; Alkjaersig et al., 1959 b). Effective blood levels of EACA are maintained by a daily dose of the order of 15 to 20 gm. (McNicol et al., 1962 b). The decrease in toxicity and the alleged increase in potency of aminomethyl cyclohexane carboxylic acid compared with EACA would represent a considerable advance in the search for a more potent fibrinolytic inhibitor and for this reason, its investigation was undertaken. Melander et al. (1965) found that the synthetic pathways described in the chemical literature for aminomethyl cyclohexane carboxylic acid result in a mixture of isomers containing 10 to 25 per cent of one of these isomers which possesses the entire antifibrinolytic activity of the mixture. Groth and Hassel (1965) have shown that the trans-isomer is the active form.

In this account the following aspects of the compound have been studied: its mode of action as an inhibitor of the fibrinolytic enzyme

system; its effect on the trypsinogen-trypsin system and on the proteolytic activity of pepsin; its effect on the coagulation system; and an appraisal of its in vivo potency.

In many of the experiments, the effect of the compound was compared with that of EACA and in one in vitro experiment its potency relative to PAMBA was demonstrated.

Materials

Aminomethyl Cyclohexane Carboxylic Acid There were two preparations. A mixture of the isomers containing between 10 and 25 per cent of the antifibrinolytically active isomer is obtained by a synthetic procedure involving hydrogenation of aminomethyl benzoic acid. This mixture, which has been called AMCHA, was provided as a 10 per cent solution. The active isomer, separated from the mixture by chromatography, has been called AMCA and was also provided as a 10 per cent solution. The chemical formula of the compound together with that of EACA is shown in figure 3.

Trypsinogen and Enterokinase These were purified bovine preparations from L. Light and Co., Ltd.

Pepsin This was a three times crystallized preparation obtained from the Sigma Chemical Co., St. Louis, Mo.

Urea-denatured Haemoglobin This was a twice crystallized bovine preparation from the Sigma Chemical Co.

Methods

Caseinolytic Assay In the experiment carried out to measure inhibition by AMCHA and by EACA of caseinolytic activity of bank plasma, the final volume of the digestion mixture was increased to 6.0 ml. by addition of 1.0 ml. of varying concentrations of AMCHA or EACA. Saline was substituted for inhibitor in the control experiment.

In the kinetic studies, the method was modified by using plasminogen, plasmin, trypsinogen or trypsin instead of plasma. The acid/alkali treatment was omitted and the final volume of the digestion mixture was 5.0 ml., AMCHA or EACA occupying a volume of 1.0 ml. and saline replacing inhibitor in the control experiment.

The kinetics of inhibition by AMCA of plasminogen activation were investigated using a modification of the technique described by Alkjaersig et al. (1958 b). The digestion mixture was 0.5 ml. human plasminogen of varying concentrations, 0.5 ml. streptokinase, (50 N.I.H. units/ml.) and 0.2 ml. AMCA or saline in the control experiment. The activation reaction was allowed to proceed for one hour at 37^{o}C and the reaction stopped by addition of 2 ml., 0.25N.HCl

which lowers the pH of the digestion mixture to 2. At pH 2, plasmin is stable. AMCA was removed by dialysis at 4°C against 0.01 N.HCl. Streptokinase was denatured or inactivated by precipitation of plasmin with 1.0 Molar NaCl at pH 2 (Troll and Sherry, 1955). The precipitate was washed twice in 0.01 N.HCl containing 1.0 Molar NaCl, collected by centrifugation, dissolved in 3 ml. distilled water and plasmin then assayed by the caseinolytic technique.

Urokinase Sensitivity Test This was modified in order to examine the antifibrinolytic effect in vitro of AMCHA and AMCA compared with EACA. The final volume of the test mixture was increased to 0.82 ml. to accommodate inhibitor. In the control experiment, saline was substituted for inhibitor.

Estimation of Plasma Levels of AMCA This method was modified from that of Nilsson et al. (1960) and is based on the urokinase sensitivity test. To 0.2 ml. amounts of citrated plasma, withdrawn from the subject immediately before ingestion of AMCA, were added increasing concentrations of AMCA in 0.2 ml. volumes. A saline control was also prepared. After addition of 0.32 ml. urokinase (500 Ploug units/ml.) and clotting with 0.1 ml. thrombin (20 N.I.H. units/ml.), the lysis time at 37°C of each clot so formed was observed. A control

curve was prepared by plotting on the abscissa the Molar concentration of AMCA in each plasma volume against the logarithm of the corresponding lysis time in seconds on the ordinate. A linear relationship was found. The concentration of AMCA in plasma withdrawn from the subject at different times after ingestion of the drug was estimated by testing 0.2 ml. amounts of each plasma in this system with 0.2 ml. saline added to adjust the volume to that of the control curve system. The concentration of AMCA was derived by conversion of the clot lysis time in seconds to the corresponding logarithm and reading off the control curve the Molar concentration of AMCA which, added to the pre-ingestion plasma, gave the same logarithm.

Assay of Pepsin Activity The method used was described by McNicol (1964) modified from that of Northrop et al. (1948). The substrate used was urea-denatured bovine haemoglobin. In 80 ml., 0.05 Molar glycine solution were dissolved 2.5 gm., bovine haemoglobin. The pH was adjusted to 2 with about 20 ml., 0.2 N.HCl. The substrate was prepared immediately before use. Pepsin was used in a concentration of 0.005 mg./ml. To 4 ml. substrate was added 0.5 ml. inhibitor diluted in saline. A saline control was also prepared. After the

addition of 0.5 ml. pepsin solution, the assay mixture was incubated at 37°C for 62 minutes. Aliquots of 2.0 ml. were removed at 2 and 62 minutes and added to 2.0 ml., 10 per cent NaOH which arrests enzyme activity. After centrifugation (1,000 g. for 10 minutes), 1.0 ml. of the supernatant was added to 4.0 ml., 0.5 N.NaOH; 1.5 ml., 5 per cent TCA and 1.5 ml. dilute (1:2) Folin Ciocalteu reagent. After standing for 15 minutes for maximal colour development, the optical density of the 62 minute sample was read at 650 mμ. in a spectrophotometer (SP500, Unicam), against the 2 minute sample as blank.

Glycine was used as a buffer because addition of EACA or AMCHA to the acid-haemoglobin solution without glycine raised the pH out of the range in which pepsin is active, but with glycine, addition of EACA or AMCHA did not significantly alter the pH. The details of these effects are shown in table 1. In this experiment, 0.5 ml. of varying concentrations of AMCHA or EACA was added to 4.0 ml. acid-haemoglobin substrate. The concentrations of AMCHA and EACA shown in table 1 were calculated in the final volumes of 4.5 ml.

Thromboplastin Generation Test This was modified by increasing the final volume of the incubation mixture by 0.3 ml. The additional 0.3 ml.

volume was occupied by varying concentrations of AMCHA and in the control experiment, saline was substituted for AMCHA.

Other Materials and Methods were described in chapter 3.

Results

Inhibition of the Caseinolytic Activity of Bank Plasma by AMCHA This was measured in a caseinolytic assay system. Expired pooled bank plasma was the source of human plasminogen. The activator was streptokinase, 100 N.I.H. units/ml., a concentration which gave a lysis time of 7 minutes in the streptokinase sensitivity test with the plasma. Activation of plasma plasminogen by streptokinase was allowed to proceed for 30 minutes at 37°C. AMCHA was added in varying concentrations immediately before streptokinase or immediately after streptokinase, but prior to plasmin assay. The results of these experiments are shown in figure 4. It can be seen that inhibition by AMCHA of the caseinolytic activity of bank plasma was much more marked when AMCHA was added before activation than when it was added after activation.

The similar results observed when EACA was tested in this system are shown in figure 5.

Comparison of figures 4 and 5 shows that in this system, AMCHA

had twice the potency of EACA. For example, when added prior to streptokinase, 2×10^{-4} Molar AMCHA or 4×10^{-4} Molar EACA produced 20 per cent inhibition of caseinolytic activity and when added after streptokinase, 14 per cent inhibition was observed at a concentration of 3×10^{-3} Molar AMCHA or 6×10^{-3} Molar EACA.

Inhibition by AMCHA of the Proteolytic Activity of Plasmin:Kinetics of the Reaction In this experiment, casein was used as substrate for human plasmin. The concentration of casein in the digestion mixture was varied sevenfold, the highest concentration being 2 per cent. The concentration of plasmin was constantly 1.5 casein units/ml. of the digestion mixture and that of AMCHA 0.2 Molar.

The data are shown in figure 6 as double reciprocal plots (Lineweaver and Burk, 1934). These plots fulfil the Lineweaver-Burk criteria for uncompetitive inhibition by AMCHA of the proteolytic activity of plasmin.

Figure 7 shows the data obtained when EACA was incorporated in this experiment at a concentration of 0.4 Molar. The double reciprocal plots of figure 7 fulfil the Lineweaver-Burk criteria for non-competitive inhibition by EACA of the proteolytic activity of plasmin.

Inhibition by AMCHA of Plasminogen Activation by Streptokinase: Kinetics of the Reaction These were investigated in a caseinolytic assay system in which purified human plasminogen was the substrate for the activator streptokinase. The highest plasminogen concentration added was 23 casein units/ml., and the concentration was varied ten-fold. The concentration of streptokinase was 10 N.I.H. units/ml. of the digestion mixture. The results are illustrated in figure 8 as double reciprocal plots. AMCHA was incorporated at a constant concentration of 0.05 Molar immediately before activation of plasminogen with streptokinase. These plots fulfil the Lineweaver-Burk criteria for competitive inhibition by AMCHA of plasminogen activation by streptokinase.

At a sufficiently high concentration, the presence of AMCHA would interfere with the assay of plasmin formed in this experiment and hence the observed results would not represent the true kinetics of inhibition of plasminogen activation. Table 6 shows the results of caseinolytic assays in which varying concentrations of plasmin were assayed with AMCHA 0.05 Molar and without AMCHA. It can be seen that the proteolytic activity of plasmin, in a range of concentrations approximately the same as that which could be produced by activation

of the plasminogen concentrations used in the previous experiment, was not influenced by the presence of 0.05 Molar AMCHA.

The results of the corresponding experiments for EACA are shown in tables 5 and 6 and the double reciprocal plots of figure 9 illustrate that 0.1 Molar EACA competitively inhibits plasminogen activation by streptokinase. This result is qualitatively in agreement with that of Alkjaersig et al. (1959 b).

The kinetics of inhibition by AMCA of plasminogen activation by streptokinase were studied using the technique, modified from that of Alkjaersig et al. (1958 b). The highest concentration of plasminogen added was 23 casein units/ml. and the concentration was varied seven-fold. Activation of the plasminogen with streptokinase 40 N.I.H. units/ml. of the digestion mixture was allowed to proceed for 60 minutes at 37°C. The plasmin formed after this time was assayed after removal of AMCA and inactivation or denaturation of streptokinase. The results are shown as double reciprocal plots in figure 10. AMCA was incorporated at a constant concentration of 0.02 Molar. The plots fulfil the criteria for competitive inhibition by AMCA of plasminogen activation. The experiment also confirms the observations made with AMCHA.

Inhibition by AMCHA of the Proteolytic Activity of Trypsin: Kinetics of the Reaction These were studied by the technique already described

for plasmin, in a caseinolytic assay system. The range of concentration of casein, the substrate for trypsin, was varied sevenfold, the maximum concentration being 2 per cent. The concentration of trypsin was 0.08 mg./ml. of the digestion mixture and that of AMCHA, 0.2 Molar. The results are shown in figure 11 as Lineweaver-Burk plots. The plots demonstrate that AMCHA is a non-competitive inhibitor of the proteolytic activity of trypsin.

Figure 12 shows the results of this experiment with 0.4 Molar EACA as the inhibitor. The double reciprocal plots demonstrate the EACA inhibits trypsin in a non-competitive manner.

Inhibition by AMCHA of Trypsinogen Activation by Enterokinase; Kinetics of the Reaction The results of this experiment are shown in figure 13. A caseinolytic assay system was used. The final concentration in the digestion mixture of enterokinase was 1.25 mg./ml. and the concentration of trypsinogen was varied fiftyfold, the maximum concentration being 0.5 mg./ml. AMCHA was incorporated in a concentration of 0.05 Molar. The experiment was also carried out in the presence of 0.1 Molar EACA and the similar results obtained are shown in figure 14. The Lineweaver-Burk plots of figures 13 and 14 fulfil the criteria for competitive inhibition by AMCHA and by EACA of

trypsinogen activation by enterokinase.

The concentration of AMCHA and EACA used in these experiments did not inhibit the proteolytic activity of trypsin in concentrations ranging from 50 mg./100 ml. to 5 mg./100 ml. of the digestion mixture, as shown in table 10.

In all these experiments it will be seen that AMCHA had about twice the potency of EACA. It will be seen too, in the kinetic experiments, that the modes of action of AMCHA and of EACA were qualitatively similar.

The Effect of AMCHA on Pepsin Activity The results are shown in figure 15. Inhibition of pepsin began at an AMCHA concentration of 5×10^{-3} Molar and 80 per cent inhibition was seen at an AMCHA concentration of 5×10^{-2} Molar.

Comparison of the effect of AMCHA on pepsin activity was made with EACA and the results are shown in figure 16. Inhibition of pepsin by EACA began at concentrations greater than 2×10^{-2} Molar EACA and 80 per cent inhibition was observed with 6×10^{-2} Molar EACA.

The Antifibrinolytic Effect of AMCHA and AMCA In Vitro In the kinetic studies, casein was the substrate employed to assay plasmin because the use of the physiological substrate fibrin in such studies gives poor

reproducibility. In order to study the antifibrinolytic effect of AMCHA and AMCA compared with that of EACA in a system incorporating fibrin, varying concentrations of these compounds were added to bank plasma and the lysis times of these mixtures observed in the urokinase sensitivity test. The results are shown in figure 17. In this system, in terms of Molar concentration, AMCA the active isomer was about ten times more potent than AMCHA, the mixture of isomers, and about one hundred times more potent than EACA.

A similar experiment was carried out to compare the anti-fibrinolytic effect of AMCA with that of PAMBA. These results are illustrated in figure 18 where it will be seen that AMCA was approximately eighteen times more potent than PAMBA.

The Effect of AMCHA on the Coagulation System Because of the complex inter-relationship of blood coagulation with the fibrinolytic enzyme system, and to examine the possibility that AMCHA might produce a haemostatic effect by influencing thromboplastin generation or the thrombin-fibrinogen reaction, the effect of AMCHA on the thromboplastin generation test, the one-stage prothrombin time and the thrombin clotting time was studied.

Varying concentrations of AMCHA were added to fresh normal

plasma which was tested in the one-stage prothrombin time and thrombin clotting time. In the thromboplastin generation test, AMCHA was added to the incubation mixture. The results are shown in tables 14 and 15, where it will be seen that at concentrations of AMCHA greater than 2.5×10^{-2} Molar, the one-stage prothrombin time and the thrombin clotting time were prolonged and at a concentration of 2.5×10^{-2} Molar, there was slight impairment of thromboplastin generation.

In Vivo Experiments with AMCHA and AMCA

The antifibrinolytic effect of AMCHA and AMCA was investigated in healthy adult volunteers. In the first experiments, 2 gm. of AMCHA were ingested by the subject immediately after withdrawal of a control specimen of venous blood. Thereafter specimens were withdrawn at 15 and 30 minutes and 1, 2, 3, 5, 7, 12, and 24 hours following ingestion of AMCHA. These plasma samples were tested in the urokinase sensitivity test and the clot lysis time of each specimen was taken as a measure of the antifibrinolytic effect imparted to that plasma by the AMCHA ingested. The results are shown in figure 19 along with the results of a control experiment carried out on a different day. It can be seen that, following the ingestion of AMCHA, there was a demonstrable

antifibrinolytic effect at 15 minutes, a peak effect at 2 hours and thereafter a more gradual diminution of the effect over the succeeding 5 hours with return to the pre-ingestion lysis time between 7 and 12 hours. In the control experiment, there was negligible diurnal variation of lysis time in the urokinase sensitivity test.

All of the plasma samples were dialysed at 4°C with constant stirring for 36 hours against 0.1 Molar phosphate buffer, pH 7.6, with two changes of buffer. At the end of dialysis, there had been a 25 per cent volume increase of all specimens and each specimen had a lysis time of about 18 minutes in the urokinase sensitivity test. Undialysed aliquots of all samples were diluted 25 per cent with 0.1 Molar phosphate buffer and also tested in the urokinase sensitivity test. Dilution by 25 per cent of the pre-ingestion sample resulted in a urokinase sensitivity test of 18 minutes. The other, undialysed, diluted specimens also gave prolonged lysis times compared with their undiluted values. For example, the two hour specimen, that withdrawn at the peak of the inhibitory effect, had a lysis time of 42 minutes when diluted and 39.5 minutes undiluted. Dialysis had therefore eliminated the substance producing the increased antifibrinolytic effect. These results are shown in detail in table 16.

The same subject ingested on different days 2 gm. EACA and 1 gm. AMCHA. Specimens of venous blood were withdrawn at the same times as in the previous experiment and the plasma samples tested as before. The results are shown in figure 20. It can be seen that the effect of 2 gm. EACA and of 1 gm. AMCHA was closely similar, with the difference that there was possibly a significantly greater antifibrinolytic effect at 7 hours in the AMCHA experiment compared with the 7 hour value in the EACA experiment. In this experiment AMCHA had in vivo about twice the potency of EACA.

Dialysis of these specimens in the manner already described eliminated the differences in clot lysis times, as shown in table 18.

The active isomer, AMCA, was investigated similarly. Figure 21 shows the effect of ingestion of 200 mg. AMCA compared with the effect of 2 gm. EACA ingested by the same subject on a different day. With AMCA, there was demonstrable antifibrinolytic activity at 15 minutes in the urokinase sensitivity test, maximum inhibitory effect between 1 and 5 hours, and a slow fall of inhibitory effect to pre-ingestion lysis times between 12 and 24 hours. With EACA on the other hand, peak inhibitory activity was demonstrable at 2 hours and return to pre-ingestion values occurred between 5 and 7 hours.

Compared with EACA in this in vivo experiment, AMCA had a tenfold increase in potency over EACA.

Another subject ingested 200 mg. AMCA and 1 gm. AMCHA on different days. The results are shown in figure 22, where it will be seen that closely similar effects were observed in the urokinase sensitivity test following administration of each preparation. AMCA, in this experiment, therefore shows a fivefold increase in potency over AMCHA.

The results of ingestion of 200 mg. AMCA by 5 other subjects are shown in table 21. In these subjects, effective antifibrinolytic activity was demonstrable in the urokinase sensitivity test for about 12 hours after ingestion of the compound.

Effect of AMCA in the Treatment of a Pathological Haemostatic Defect AMCA, in a dose of 200 mg., was given to a patient with metastatic carcinoma of the breast which was associated with abnormal bleeding into the skin and from venepuncture sites. The abnormal bleeding was thought initially to be due, in part at least, to pathological fibrinolytic activity. The evidence for this will be discussed in chapter 8, where the patient's case history and investigation are described. One of the features of the investigation of this patient was accelerated lysis

of Fearnley-type dilute plasma clots. Figure 23 shows the effect of 200 mg. AMCA, administered orally to the patient, in inhibiting plasma fibrinolytic activity. In the same figure is shown the very similar pattern, in duration and extent, seen on another day when the patient was given 2 gm. of EACA by mouth. Observations on a control day, falling between the administration of AMCA and EACA, are also shown.

No side effects were noted by any of the subjects who took either AMCHA or AMCA.

Estimation of Plasma Levels of AMCA An estimate was made of the plasma levels of AMCA achieved in one patient following an oral dose of 200 mg. AMCA. Plasma samples were withdrawn from the subject immediately before ingestion of AMCA and at 15 and 30 minutes and 1, 2, 5, 8, 12, and 24 hours after ingestion. The concentrations of AMCA dissolved in the pre-ingestion plasma are shown in table 23 and the control curve in figure 24. The results of testing each of the post-ingestion samples in the same system, together with their Molar concentrations of AMCA, derived from the control curve, are also shown in table 23. It will be seen that an estimated peak concentration of 2.0×10^{-5} Molar AMCA was found 2 hours after ingestion of the compound.

Discussion

The results presented in this chapter reveal that the synthetic amino acid, aminomethyl cyclohexane carboxylic acid, inhibits the fibrinolytic enzyme at two points: it inhibits both plasminogen activation and the proteolytic acitivity of formed plasmin. In the caseinolytic assay system with bank plasma as the source of plasminogen, addition of AMCHA either before streptokinase or after streptokinase, will largely distinguish between the effect of the compound on plasminogen activation and its effect on the activity of formed plasmin. This experiment demonstrates that effective inhibition by AMCHA of the activation process was produced by considerably lower concentrations of AMCHA than the concentration required to produce effective inhibition of the activity of formed plasmin. Inhibition by AMCHA of plasminogen activation began at a concentration greater than 10^{-4} Molar and of the proteolytic activity of formed plasmin at concentrations greater than 2×10^{-3} Molar. At the higher range of concentrations of AMCHA added before streptokinase, the observed inhibition of caseinolytic activity must still be due primarily to inhibition of plasminogen activation, because the same concentrations added after activation did not significantly inhibit the

activity of formed plasmin.

In the same system, EACA had about one half the potency of AMCHA. Added prior to the activation process, 4×10^{-4} Molar EACA produced 20 per cent inhibition of caseinolytic activity, the degree of inhibition produced by 2×10^{-4} Molar AMCHA. Added after activation, 14 per cent inhibition of caseinolytic activity was produced by 6×10^{-4} Molar EACA compared with 3×10^{-4} Molar AMCHA.

Since effective inhibition of plasminogen activation can be achieved by lower concentrations than those required to inhibit the activity of formed plasmin, the aim of therapy with the compound would be to administer a dose with would result in plasma concentrations between 10^{-4} and 10^{-3} Molar AMCHA.

The kinetic studies reveal that AMCHA at a concentration of 0.2 Molar is an uncompetitive inhibitor of the proteolytic activity of plasmin and that at the lower concentration of 0.05 Molar it competitively inhibits plasminogen activation by streptokinase. One critisicm of the experiment in which the kinetics of inhibition of plasminogen activation were demonstrated is that the presence of AMCHA may inhibit plasmin formed during activation. Since the amount of plasminogen activated in the presence of the inhibitor is calculated by assay of plasmin formed from it, any inhibitory effect of AMCHA on plasmin would mean that the total amount of plasmin formed

during activation had not been measured. Hence the true kinetics of inhibition would not be represented by the Lineweaver-Burk plots of figure 8. In the experiment, already discussed, to measure the inhibitory effect of AMCHA upon the caseinolytic activity of bank plasma, it was seen that much higher concentrations of AMCHA were required to inhibit formed plasmin than to inhibit plasminogen activation. In addition in another experiment, the results of which are shown in table 6, 0.05 Molar AMCHA, the concentration used to demonstrate its kinetics of inhibition of plasminogen activation, did not affect the caseinolytic assay of a range of concentrations of plasmin equivalent to the potential plasmin which could be formed by activation of the highest plasminogen concentration added in the kinetics experiment, varied down to negligible amounts. It appears reasonable therefore to assume that the Lineweaver-Burk plots of figure 8 do reflect accurately the kinetics of inhibition by AMCHA of plasminogen activation by streptokinase. This effect, i.e. competitive inhibition of plasminogen activation, was confirmed with the active isomer AMCA in the system, where the plasmin formed was assayed after removal or denaturation of the activator and inhibitor. With EACA, non-competitive inhibition of the proteolytic activity of plasmin and competitive inhibition of

plasminogen activation by streptokinase were demonstrated at EACA concentrations of 0.4 and 0.1 Molar respectively. The latter finding, together with the results for EACA in the experiment to demonstrate inhibition of caseinolytic activity of bank plasma, was qualitatively in agreement with the findings of Ablondi et al. (1959) and Alkjaersig et al. (1959 b).

The effects of AMCHA on the trypsinogen-trypsin system were shown to be non-competitive inhibition of the proteolytic activity of trypsin with 0.2 Molar AMCHA and competitive inhibition by AMCHA of trypsinogen activation by enterokinase with 0.05 Molar AMCHA. As was found with the plasminogen-plasmin system, the concentration of AMCHA which would inhibit formed trypsin was fourfold that which would inhibit the activation of trypsinogen. Further, when a range of concentrations of trypsin, varied from the highest potential concentration of trypsin which could be formed in the experiment to investigate the kinetics of inhibition of trypsinogen activation, to very low concentrations, was assayed with and without 0.05 Molar AMCHA, the results were the same within the limits of experimental error. It is again a fair assumption that failure to remove AMCHA did not affect the observed kinetics of inhibition by AMCHA of trypsinogen activation.

Using twice the concentrations of EACA compared with AMCHA, similar effects of EACA on the trypsinogen-trypsin system were observed.

AMCHA inhibits pepsin activity, 70 per cent inhibition being observed at a concentration of 4×10^{-2} Molar compared with 57 per cent inhibition by the same Molar concentration of EACA.

The anticoagulant effect on the thromboplastin generation test, the one-stage test and the thrombin clotting time with concentrations of AMCHA in the plasma at or greater than 2.5×10^{-2} Molar, would not be important in vivo, since such concentrations are much higher than those required to inhibit plasminogen activation - the aim of therapy with this fibrinolytic inhibitor.

In the in vitro plasma clot system, which is activated by a standard amount of urokinase, the Molar potency of AMCHA was about sixfold that of EACA and AMCA was about one hundred times more potent than EACA.

The in vivo experiments revealed first that an antifibrinolytic effect demonstrable in the urokinase sensitivity test could be produced by oral administration of AMCHA and of AMCA. Using the urokinase sensitivity test, comparable antifibrinolytic effects were

detected after administration of 200 mg. AMCA, 1 gm. AMCHA or 2 gm. EACA.

It was also shown that an antifibrinolytic effect could be produced in a patient with a haemostatic defect due, in part at least, to a pathological fibrinolytic state (see chapter 8), and that twice the dose of EACA administered to this patient produced a closely similar effect.

The effect of AMCHA and AMCA was more prolonged than that of EACA, a finding possibly due to slower renal excretion of AMCA and AMCHA compared with EACA; Melander et al. (1964) found in animal studies that maximum urinary recovery of EACA after intravenous administration was 72 per cent, compared with 64 per cent for AMCHA.

The maximum estimated plasma level of AMCA in one patient after an oral dose of 200 mg. was 2×10^{-5} Molar. Andersson et al. (1965) using their closely similar technique, found levels of about 1.2×10^{-4} Molar in serum after a dose of 0.01 gm./kg. body weight, a concentration which effectively inhibited pathological fibrinolytic activity.

Conclusions

Fibrinolytic inhibitors described prior to the discovery of aminomethyl cyclohexane carboxylic acid, have been aliphatic amino acids with carbon chain lengths of 4 - 8 C atoms and the amino group in the terminal position, the most potent being EACA. The structural resemblance of aminomethyl cyclohexane carboxylic acid to EACA is obvious, though the former's structure may be more rigid. The rigidity may be responsible for its increase in potency over EACA as a fibrinolytic inhibitor enabling it to 'key-in' more precisely or consistently to the active site on the activator molecule.

Effective inhibition by AMCHA of plasminogen activation may be produced by much lower concentrations of AMCHA than those required to inhibit formed plasmin and the aim of therapy with AMCHA would therefore be to achieve the concentrations which would inhibit plasminogen activation and thus prevent the continuing formation of plasmin.

In vitro in the kinetic experiments and in the in vivo experiments, AMCHA had a twofold increase in potency over EACA and in the in vivo experiments AMCA had ten times the potency of EACA. This increase in potency renders the active isomer AMCA much more

attractive as a fibrinolytic inhibitor than EACA. A minimum of 10 gm. daily by mouth of EACA is required to maintain effective inhibitory levels. From the in vivo experiments described in this chapter and from the work described by Andersson et al. (1965) a reasonable dosage schedule of AMCA would appear to be a loading dose of about 400 to 600 mg. with a maintenance dose of 100 to 200 mg. every five hours. Like EACA, AMCA is rapidly excreted in the urine and therefore lower dosage should be required for the treatment of urinary tract haemostatic failure. A further attraction of AMCA as a fibrinolytic inhibitor is its lowered toxicity in animals compared with EACA.

The inhibitory effect of the compound on trypsinogen activation may have relevance in the future in the treatment of the proteolysis of acute pancreatitis.

The inhibitory effect of the compound on pepsin is probably without therapeutic value, because of the very high concentrations which would be required to produce effective inhibition of pepsin activity.

The effects produced on the thromboplastin generation test and the thrombin clotting time indicate that any haemostatic effect

produced by the compound in vivo is not exerted by virtue of its influence on either extrinsic or intrinsic thromboplastin generation or on the thrombin-fibrinogen reaction.

In the future, AMCA may come to replace EACA in the treatment of disordered fibrinolytic states.

CHAPTER 5

IN VITRO AND IN VIVO STUDIES WITH TRASYLOL

Trasylol is a polypeptide of molecular weight 6,200 which was originally isolated from bovine parotid gland (Frey et al., 1950). It has been shown to possess potent antitryptic activity in vitro (Moshal et al., 1963), and to prevent the development of experimentally induced pancreatitis in animals (McCutcheon, 1963, 1964; McHardy et al., 1963). Because of these properties, it has been used extensively in the treatment of acute pancreatic disease (see Skyring et al., 1965 for a review of the literature). It has also been recognised to possess inhibitory effects on the fibrinolytic enzyme system (Marx et al., 1959; Steichele and Herschlein, 1961; Beck et al., 1963) and more recently it has been shown to inhibit blood coagulation (Amris, 1966). On theoretical grounds, a drug possessing both antifibrinolytic and anticoagulant effects might have considerable value in the treatment of spontaneous pathological bleeding states associated with hypofibrinogenaemia. Such states may complicate major surgical procedures especially pulmonary surgery (Soulier et al., 1952) and surgery with extracorporeal circulation (von Kaulla and Swan, 1958); certain obstetric conditions (Schneider, 1959); metastatic carcinoma particularly of the prostate (Tagnon et al., 1952)

and hepatic cirrhosis (Kwann et al., 1956). The precise mechanism of production of these acute haemostatic defects, which may be life-threatening, has not been elucidated. Possible explanations include widespread intravascular coagulation, or systemic hyperplasminaemia or a combination of these two (Fletcher et al., 1962 b; Sharp, 1964). Emergency laboratory differentiation of clotting from lysis may be impossible thus rendering difficult a rational approach to therapy (i.e. heparin for primary intravascular coagulation or a fibrinolytic inhibitor, either EACA or AMCA, for primary fibrinolysis).

The experiments with Trasylol described in this chapter were undertaken to study further its effects on blood coagulation and on the fibrinolytic enzyme system, with a view to evaluating its possible use in the therapy of defibrination syndromes. A limited investigation was also made of its role as a trypsin inhibitor.

In the in vitro investigative work made with the drug, its mode of action as an inhibitor of the fibrinolytic enzyme system, its potency as a fibrinolytic inhibitor compared with that of AMCA and EACA, its effect on blood coagulation and its influence on platelet aggregation were studied.

Trasylol was administered to a number of normal subjects and

its effect on physiological fibrinolysis, blood coagulation and platelet aggregation assessed. It was also given to two patients with hypofibrinogenaemia and its effect on the coagulation disorders studied.

Finally, its mode of action as a trypsin inhibitor was investigated and a system was devised to detect changes in antitryptic activity in plasma following the intravenous administration of large doses of the drug.

Materials

Trasylol The compound is isolated commercially from bovine lung. It was made available in ampoules each containing 25,000 Kallikrein inactivator units in 5 ml. of solution suitable for intravenous injection. One unit was equivalent to the Kallikrein inactivator activity of 0.195 μgm. of the pure crystalline substance. In most of the work presented in this chapter, its concentration has been described in units since, in clinical use, it is prescribed in units. In one experiment, in which its in vitro potency was assessed relative to that of AMCA and EACA, its concentration is given in terms of Molarity and weight.

Methods

Inhibition by Trasylol of the Esterolytic Activity of Urokinase on AcLMe The kinetics of this reaction were studied using the method of

Sherry et al. (1964) which is described in chapter 3. This method was slightly modified by adjusting the volume of AcLMe to 2.5 ml. Trasylol was contained in a volume of 0.5 ml. and saline substituted for Trasylol in the control experiment.

Thromboplastin Generation Test The effect of Trasylol in vitro on thromboplastin generation was studied in two ways. It was added to the incubation mixture as described in chapter 4. In another experiment, it was added to the substrate plasma in varying concentration so that the substrate system was 0.1 ml. plasma and 0.1 ml. Trasylol. Saline was substituted for Trasylol in the control experiment.

Other Materials and Methods were described in chapters 3 and 4.

Results

In Vitro Experiments

Inhibition by Trasylol of the Caseinblytic Activity of Bank Plasma

This inhibitory effect was measured in a caseinolytic assay system in which expired pooled bank plasma activated by streptokinase was the source of human plasminogen. Streptokinase was used in a concentration of 100 N.I.H. units/ml. plasma. This concentration of streptokinase gave a lysis time of 5 minutes in the streptokinase sensitivity test with the plasma. Activation by streptokinase of plasma plasminogen

was allowed to proceed for 30 minutes at 37°C. A range of concentrations of Trasylol was added to the system either immediately before streptokinase or immediately after streptokinase and therefore 30 minutes before plasmin assay. The results of these experiments are shown in figures 25 and 26. It will be seen that inhibition by Trasylol of the caseinolytic acitivity of streptokinase-activated plasma was more effective when Trasylol was added after activation but prior to plasmin assay than when it was added prior to activation. In this system, inhibition of caseinolytic activity was virtually complete when Trasylol was present in a concentration of 10 units/ml. of the digestion mixture, corresponding to 200 units/ml. in whole plasma.

Inhibition by Trasylol of the Proteolytic Activity of Plasmin: Kinetics of the Reaction Casein was used as substrate for human plasmin in this experiment. The casein concentration was varied sevenfold, the maximum being 2 per cent in the digestion mixture. The concentration of plasmin was constantly 1.5 casein units/ml. of the digestion mixture. Three concentrations of Trasylol were tested; 5, 8 and 10 units/ml. of the digestion mixture. In the experiment in which Trasylol, 10 units/ml. of the digestion mixture was incorporated, there was virutally complete inhibition of the proteolytic activity of

plasmin. The results are illustrated as double reciprocal plots in figures 27 and 28 and it can be seen that the relationship between substrate concentration and reaction velocity became disorganised, inhibition being more effective at low substrate concentrations.

Accordingly, the experiment was modified to use a fourfold variation in casein concentration, the highest concentration being 2 per cent of the digestion mixture. The concentration in the digestion mixture of plasmin was as in the previous experiment and that of Trasylol 6 units/ml. The results of this experiment are shown in figure 29 as double reciprocal plots. The plots fulfil the Lineweaver-Burk criteria for non-competitive inhibition by Trasylol of the proteolytic activity of plasmin.

Inhibition by Trasylol of Plasminogen Activation by Streptokinase: Kinetics of the Reaction These kinetics were investigated in a caseinolytic assay system in which purified human plasminogen was the substrate for streptokinase. The highest concentration of plasminogen was added to the digestion mixture from a solution containing 23.3 casein units of plasminogen/ml. and this concentration was varied eightfold. Streptokinase (50 N.I.H. units/ml.) was the activator. Trasylol was incorporated in three concentrations; 2, 5 and 10 units/ml. of the digestion mixture. The results of these

experiments are shown as double reciprocal plots in figure 30. It will be seen that, as in the experiment in which the kinetics of inhibition of the proteolytic activity of plasmin were studied, the relationship between substrate concentration and reaction velocity was disorganised when a wide variation in substrate concentration was employed. Inhibition by Trasylol of plasminogen activation by streptokinase was more effective at low concentrations of plasminogen.

The experiment was repeated using a threefold variation in plasminogen concentration, the maximum being 23.3 casein units/ml. and incorporating Trasylol in a concentration of 5 units/ml. of the digestion mixture. The data are shown as double reciprocal plots in figure 31 where it can be seen that the Lineweaver-Burk criteria for competitive inhibition by Trasylol of plasminogen activation by streptokinase are fulfilled.

In the experiment in which inhibition by Trasylol of the caseinolytic activity of bank plasma was investigated, it was seen that the same concentration of Trasylol had a greater inhibitory effect when added to the assay system after activation of plasma plasminogen with streptokinase but prior to plasmin assay than when added prior to activation. In the above kinetic experiments, measurement of reaction

velocity is dependent on assay of plasmin formed after the reaction between Trasylol, streptokinase and plasminogen. The continuing presence of Trasylol in the digestion mixture might therefore depress the activity of the plasmin so formed and hence the observed data might not reflect accurately the true kinetics of the reaction.

The kinetic studies were therefore repeated using the method modified from that of Alkjaersig et al. (1958 b), in which the inhibitor is removed by dialysis and streptokinase denatured or inactivated before assay of plasmin. Plasminogen was added to the digestion mixture from a solution containing 18 casein units of plasminogen/ml. and the concentration varied threefold. The concentration of Trasylol was 17 units/ml. of the digestion mixture. The concentration of the streptokinase solution added to the digestion mixture was 50 N.I.H. units/ml. The results are shown as double reciprocal plots in figure 32. The plots fulfil the Lineweaver-Burk criteria for competitive inhibition and confirm the previous observations.

Inhibition by Trasylol of Plasminogen Activation by Urokinase: Kinetics of the Reaction These kinetics were studied in the caseinolytic assay system used for streptokinase. The maximum concentration of plasminogen was added to the digestion mixture from a solution containing

17.5 casein units of plasminogen/ml. This concentration was varied threefold. The concentration of the urokinase solution added to the digestion mixture was 100 C.T.A. units/ml. The concentration of Trasylol was 5 units/ml. of the digestion mixture. The results are illustrated in figure 33 as double reciprocal plots which fulfil the Lineweaver-Burk criteria for competitive inhibition by Trasylol of plasminogen activation by urokinase.

Inhibition by Trasylol of the Esterolytic Activity of Urokinase on AcLMe: Kinetics of the Reaction These kinetics were studied because the esterolytic activity of urokinase may represent a different property of the urokinase molecule from its role as an activator of plasminogen, since AcLMe is a competitive inhibitor of plasminogen activation by urokinase in addition to being a substrate for urokinase (Sherry et al., 1964). The maximum concentration of AcLMe used was 16.9 mg./ml. of the digestion mixture and its concentration was varied fivefold. Urokinase was added to the digestion mixture from a solution containing 2,000 C.T.A. units/ml. Trasylol was present in a constant concentration of 400 units/ml. of the digestion mixture. The observed data are shown as double reciprocal plots in figure 34. The effect, found in the previous kinetic experiments, of more

efficient inhibition by Trasylol at low substrate concentration can be seen.

The experiment was repeated using a threefold variation in substrate concentration from the maximum of 16.9 mg./ml. of the digestion mixture. The concentration of Trasylol was increased to 450 units/ml. of the digestion mixture. The results are shown as double reciprocal plots in figure 35. The plots illustrate that the inhibition by Trasylol of the esterolytic activity of urokinase is of the 'mixed' type - competitive and non-competitive.

The Antifibrinolytic Effect of Trasylol In Vitro This effect was investigated by addition of increasing concentrations of Trasylol to bank plasma and by observation of the lysis times of these mixtures in the urokinase sensitivity test. AMCA and EACA were investigated in the same manner so that a comparison of the antifibrinolytic effect of Trasylol could be made with the effect of these amino acid fibrinolytic inhibitors. The results are graphically presented in figure 36. It can be seen that in terms of Molar concentration, Trasylol was about one hundred times more potent than AMCA and one thousand times more potent than EACA. On a weight basis, Trasylol was about two and one-half times more potent than AMCA.

The Effect of Trasylol on the One-stage Prothrombin Time and the Thrombin Clotting Time Trasylol in varying concentrations was added to fresh normal plasma which was then tested in the one-stage prothrombin time and the thrombin clotting time. The data are shown in table 34 from which it will be seen that Trasylol had no effect on the one-stage prothrombin time or on the thrombin clotting time.

The Effect of Trasylol in the Thromboplastin Generation Test Trasylol was added in varying concentrations to the incubation mixture of a normal thromboplastin generation test. The results are shown in tables 35 and 36 and graphically displayed in part in figure 37. It can be seen that a defect in the thromboplastin generation test was detectable when Trasylol was present in a concentration of 100 units/ml. of the incubation mixture. The defect, prolongation of the substrate plasma clotting times, persisted up to 16 minutes' incubation. With concentrations of Trasylol less than 100 units/ml. of the incubation mixture, no abnormality of the thromboplastin generation test was demonstrable.

Trasylol was also added to the substrate plasma in the thromboplastin generation test. The results are given in table 37 and in part in figure 38. It will be seen that prolongation of the substrate

plasma clotting times was demonstrable with Trasylol present in a concentration of 50 units/ml. of substrate plasma. This defect was only demonstrable up to the fourth minute of incubation even with the maximum concentration of Trasylol tested and was therefore less striking than the defect due to the presence of Trasylol in the incubation mixture.

The Effect on Platelet Aggregation of Trasylol Added In Vitro to Plasma Trasylol was added to plasma from each of seven normal subjects in a concentration of 300 units/ml. of the plasma. The effect of addition of Trasylol to the plasma on platelet aggregation was assessed in the Chandler's tube system. The data are shown in table 38. Trasylol, at this concentration, significantly prolonged the time for platelet aggregation, the mean time for platelet aggregation without Trasylol being 577 $\pm$ 28 seconds and with Trasylol 939 $\pm$ 186 seconds ($t = 4.797$; $0.001<p<0.005$).

The experiment was repeated with Trasylol in a concentration of 40 units/ml. of plasma and plasma from each of ten normal subjects was used. The data are shown in table 39. Trasylol, at this concentration, significantly prolonged the time for platelet aggregation. The mean time for platelet aggregation without Trasylol was

439 ± 152 seconds and with Trasylol 572 ± 227 seconds ($t = 2.543$; $0.02<p<0.05$).

This last experiment was repeated after further modification. Platelet aggregation with Trasylol, 40 units/ml. plasma and without Trasylol was assessed using plasma from each of ten normal subjects. The control and test systems were prepared in the Chandler's tubes and both were incubated for 10 minutes at 37°C before recalcification. The data are shown in table 40. In this experiment, the mean time for platelet aggregation without Trasylol was 293 ± 69 seconds and with Trasylol was 312 ± 86 seconds. This difference is not significant ($t = 0.625$; $0.5<p<0.6$).

In Vivo Experiments

The antifibrinolytic and anticoagulant effects of Trasylol were investigated following intravenous administration of the compound to healthy adult subjects.

Table 41 summarises the findings in one subject during a 12 hour Trasylol infusion. The infusion rate was 10,000 units of Trasylol over 30 minutes; 3,000 units/hour for the succeeding two and one-half hours and 1,000 units/hour for a further nine hours. Blood samples were obtained immediately before the infusion, after 30 minutes, 3 hours

and 15 hours. This last specimen was taken 3 hours after the end of the infusion. It can be seen that during the infusion, plasminogen activator activity in the euglobulin clot lysis test, Fearnley dilute plasma clot lysis test, and the fibrin plate test decreased and that this decrease had persisted 3 hours after the infusion had ceased. Using the urokinase sensitivity test, the Trasylol infusion was found to have imparted increased antifibrinolytic activity to the plasma; this effect which was maximal 30 minutes after the start of the infusion had somewhat declined 3 hours after the start, but was still apparent 3 hours after the infusion had ceased. During the infusion, no effect was observed on the one-stage prothrombin time, thrombin clotting time, or thromboplastin generation test. The platelet count did not vary significantly during the infusion. These results are shown in part in figure 39.

Shown in table 42 are the effects of intravenous administration of Trasylol in another subject who received a single injection of 50,000 units of Trasylol followed by an infusion of 50,000 units given over 30 minutes. Blood samples were taken immediately before the infusion and after 40 minutes i.e., 10 minutes after the infusion ceased. It can be seen that this dosage schedule produced a defect in the thromboplastin

generation test - the substrate plasma clotting times being prolonged. There was also evidence of decreased fibrinolytic activator activity in the euglobulin lysis test and of increased fibrinolytic inhibitory levels in the urokinase sensitivity test. These results are shown in part in figure 40.

Table 43 shows data obtained from a patient who received a single intravenous injection of Trasylol containing 100,000 units. Blood samples were obtained immediately before the injection and at 30 minutes and 1, 2, 3, 5, 7 and 9 hours after the injection. A thromboplastin generation test, urokinase sensitivity test and platelet count were performed on each sample. It will be seen that a defect had appeared in the thromboplastin generation test at 30 minutes and had disappeared by 3 hours. In the urokinase sensitivity test, increased fibrinolytic inhibition was present at 30 minutes and had persisted at 9 hours. Pre-treatment levels had returned by 24 hours. Platelet counts did not vary significantly. These results are graphically displayed in part in figure 41.

Two subjects were given Trasylol infusions at a progressively increasing rate of infusion over 5 hours. A constant infusion pump fitted with an infusion-rate selector (B. Braun, Melsungen) was used

to deliver the Trasylol solution. The infusion rate was 10,000 units in 0.6 ml. solution during the first hour; 20,000 units contained in 1.2 ml. during the second hour; 50,000 units in 3.0 ml. during the third hour; 100,000 units in 6.0 ml. during the fourth hour and 200,000 units in 12.0 ml. during the fifth and final hour. Blood samples were taken immediately before the infusion, at the end of each hour and two hours after the infusion ceased. The effects of each infusion were assessed by testing each specimen in the thromboplastin generation test and the urokinase sensitivity test. Platelet counts were also carred out on each specimen. The data for these experiments are given in tables 44 and 45 and graphically displayed in figures 42 and 43. The effects of the infusions were closely similar in both subjects. There were demonstrable defects in the thromboplastin generation tests at the end of the first hour. The defects became progressively worse with increasing dosage of Trasylol and had persisted two hours after completion of each infusion. Increased fibrinolytic inhibitory levels in the urokinase sensitivity tests were not apparent until the end of the fourth hour when the dose of Trasylol had been 100,000 units in the preceding hour. This effect had disappeared two hours after completion of each infusion.

Platelet counts were not significantly affected in either subject.

Effect of Trasylol Injection on Platelet Aggregation Measured in the Chandler's Tube This was studied in seven patients each of whom received a single intravenous injection of Trasylol containing 100,000 units. Platelet aggregation was measured by the Chandler's tube technique using silicone plasma samples taken immediately before injection and 30 minutes after injection. The results are shown in table 46. The mean time for platelet aggregation before the Trasylol injection was 537 $\pm$ 75 seconds and after the Trasylol injection was 277 $\pm$ 139 seconds. The acceleration of platelet aggregation after the Trasylol injection is significant ($t = 4.976$; $0.001<p<0.005$).

The mean platelet count for the 7 subjects immediately before injection was 309 $\pm$ 72 thousands/mm^3 and 30 minutes after injection was 308 $\pm$ 72 thousands/mm^3. These counts are almost identical (table 47).

Thromboplastin generation tests were also performed for each subject immediately before injection and 30 minutes after injection. The data are shown in table 48. Each thromboplastin generation test was impaired after the Trasylol injection. At 6 minutes' incubation, the mean substrate plasma clotting time after the Trasylol injection was 15.6 $\pm$ 2.6 seconds. Compared with the mean value before Trasylol (12.1 $\pm$ 2.1 seconds), the prolongation after Trasylol is significant ($t = 4.208$; $0.005<p<0.01$).

When the times in seconds by which platelet aggregation was accelerated after the Trasylol injection were plotted against the corresponding prolongations in seconds of the substrate plasma clotting times at 6 minutes' incubation in the thromboplastin generation test, a positive and highly significant correlation was observed ($r = +0.934$; $p<0.001$). These results are displayed graphically in figure 44. When the individual times for platelet aggregation before and after the Trasylol injection were plotted against the corresponding substrate plasma clotting times at 6 minutes' incubation in the thromboplastin generation test, a significant negative correlation was found ($r = -0.689$, $0.005<p<0.01$). These results are graphically displayed in figure 45. No correlation was observed when the results obtained before the Trasylol injection or after it were handled separately.

Effect of Trasylol in the Treatment of Hypofibrinogenaemia Trasylol was administered to two patients both of whom had hypofibrinogenaemia associated with metastatic carcinoma. In both patients, there was abnormal bleeding into the skin and from venepuncture sites. In each case, there was evidence to suggest that the hypofibrinogenaemia was due to intravascular clotting secondary to metastatic carcinoma. The evidence for this is discussed fully in chapter 8, where these patients'

histories and investigation are described.

In each patient, in addition to fibrinogen depletion, there was depletion of factor V and antihaemophilic globulin (A.H.G.).

Table 49 shows the data from one of these patients who was treated initially with heparin given by intravenous infusion; 1,000 units/hour for 48 hours then 1,500 units/hour for 7 hours. During the heparin infusion, fibrinogen, factor V and antihaemophilic globulin (A.H.G.) levels rose progressively. The platelet count remained steady. Therapy was then discontinued for $17\frac{1}{2}$ hours. At this time, fibrinogen, factor V and antihaemophilic globulin (A.H.G.) levels had fallen to the values found before heparin was given. There was no change in the platelet count. Trasylol was given by intravenous infusion at the rate of 100,000 units/hour for 7 hours. After 7 hours, there had been no alteration in the fibrinogen level though a rise had occurred in the levels of factor V and antihaemophilic globulin (A.H.G.). However the platelet count had fallen sharply and accordingly, Trasylol therapy was stopped. Heparin infusion was restarted; 1,500 units/hour for $16\frac{1}{2}$ hours. At the end of this time the level of fibrinogen had risen and there had been a further increase in factor V and antihaemophilic globulin (A.H.G.). The data are displayed in part in figure 46.

The data for the second patient are shown in table 50 and in part in figure 47. Trasylol was administered to this patient at the rate of 100,000 units/hour for 9 hours. At the end of this time, there had been a rise in the levels of fibrinogen, factor V and antihaemophilic globulin (A.H.G.) and in the platelet count.

Investigation of the Antitryptic Activity of Trasylol

Inhibition by Trasylol of the Proteolytic Activity of Trypsin: Kinetics of the Reaction A caseinolytic assay system was used. The concentration of casein, the substrate for trypsin, was varied sevenfold, its maximum being 2 per cent of the digestion mixture. The concentration of trypsin was constantly 45 μgm./ml. of the digestion mixture and that of Trasylol 100 units/ml. The results are given in figure 48 as double reciprocal plots. The plots fulfil the Lineweaver-Burk criteria for non-competitive inhibition by Trasylol of the proteolytic activity of trypsin.

Addition of Trasylol and Trypsin to Plasma: Assay of the Antitryptic Effect of Trasylol In this experiment, a range of concentrations of trypsin was added to expired pooled bank plasma, with and without Trasylol 40 units/ml. plasma. Tryptic activity was measured in a caseinolytic assay system. The data are present in table 52. It will

be seen that in the plasma-trypsin digestion mixtures, no free trypsin activity was detected until the concentration of trypsin in the system was 50 μgm./ml. From concentrations of trypsin varying in different plasma samples from 50 to 200 μgm./ml., tryptic activity began to appear. From 200 μgm./ml. upwards, tryptic activity rose steadily. Addition of Trasylol, 40 units/ml. plasma to this system produced inhibition of tryptic activity, tryptic activity being at least 20 per cent less in the presence of Trasylol than without it.

Antitryptic Effect of Trasylol Injection To each of seven normal subjects was administered a single intravenous injection of Trasylol containing 100,000 units. Plasma samples were obtained from each subject immediately prior to the injection and 30 minutes afterwards. Trypsin was added to each plasma sample to give a concentration of 1,000 μgm./ml. plasma. The tryptic activity of each of these samples was assayed immediately in a caseinolytic system. The data are presented in table 53. Mean trypsin activity in the plasma samples taken prior to Trasylol injection was 0.159 ± 0.079 optical density units and after Trasylol injection was 0.130 ± 0.094 optical density units. The decrease in trypsin activity after the Trasylol injection was significant ($t = 3.069$, $0.02<p<0.05$).

Discussion

The results presented in this chaper show that the polypeptide Trasylol, commercially extracted from bovine lung, depresses activity of the fibrinolytic enzyme system by inhibition of both plasminogen activation and the proteolytic activity of formed plasmin. In the investigative work concerning AMCHA, its separate inhibitory effects on plasminogen-plasmin conversion and on the proteolytic activity of formed plasmin were distinguishable because inhibition of plasminogen activation began at a much lower concentration of AMCHA (10^{-4} Molar) than that required to depress the proteolytic activity of formed plasmin (2×10^{-3} Molar). These properties of AMCHA were demonstrated by its addition to a caseinolytic assay system with bank plasma either before activation of the plasma plasminogen with streptokinase or after activation but prior to plasmin assay (figure 4). The effect of Trasylol on the caseinolytic activity of bank plasma differed from that of AMCHA. When Trasylol was added to bank plasma after activation of plasma plasminogen with streptokinase but prior to plasmin assay, a greater degree of inhibition of caseinolytic activity was observed than when the same concentration of Trasylol was added prior to activation (figures 25 and 26). From these results, it was

concluded that Trasylol effectively inhibited the proteolytic activity of formed plasmin but where Trasylol had been added prior to activation with streptokinase, the observed inhibition of caseinolysis might be due to inhibition of the plasmin formed after plasminogen-plasmin conversion rather than to any inhibitory affect on the activation of plasminogen. That Trasylol possessed an inhibitory effect on the activation of plasminogen was shown in the kinetic experiment in which the plasmin formed in a digestion mixture containing streptokinase, plasminogen and Trasylol was assayed in a caseinolytic system after removal of Trasylol by dialysis and inactivation or denaturation of streptokinase. In this system, therefore, the observed decrease in caseinolytic activity, compared with activity in the control experiment without Trasylol, was due to depression by Trasylol of plasminogen activation and not to the inhibitory effect of Trasylol on formed plasmin.

The kinetic studies with Trasylol show that its inhibitory effects on the plasminogen-plasmin system are neither wholly competitive nor non-competitive. In these studies, when a wide variation in substrate concentration was used, with a range down to seven or eightfold from dilution of the maximum concentration, a sudden sharp increase in inhibition was observed at low substrate concentrations. When these experiments were modified to use three to fivefold variation in the

substrate concentrations, the results obtained were capable of interpretation according to the Lineweaver-Burk criteria. In these modified systems, the inhibition by Trasylol of plasminogen activation by streptokinase and by urokinase was of the competitive type, that of the proteolytic activity of formed plasmin of the non-competivie type and that of the esterolytic activity of urokinase on AcLMe of the mixed competitive and non-competitive type. Competitive inhibition was confirmed by the experiment, already discussed, in which Trasylol was removed by dialysis and streptokinase denatured or inactivated; the plasmin previously formed by activation of plasminogen in the presence of Trasylol was then assayed. The findings of competitive inhibition by Trasylol of plasminogen activation by streptokinase and urokinase are in agreement with the results reported by Steichele and Herschlein (1961). These authors reported that the inhibition by Trasylol of the proteolytic activity of plasmin was also competitive, a result contrary to the observations reported in this paper. One explanation for the conflicting findings may be that the assay systems differed, Steichele and Herschlein employing a fibrin plate tehhnique and variation of the concentration of plasmin and not of its substrate, fibrin.

One hypothesis to account for the sharp increase in the inhibitory effect of Trasylol at low substrate concentrations is that the molecule may be capable of combining with the substrate alone as well as either competing with the substrate for the enzyme binding site (competitive inhibition) or combining with the enzyme-substrate complex (non-competitive inhibition). An inhibitory effect due to the formation of a complex of Trasylol and the substrate would only be detected where the concentration of Trasylol approached that of the substrate i.e., at low substrate concentrations, and would be very difficult to detect at high substrate concentrations. Trasylol may be capable of acting in this way since it is a polypeptide and may therefore possess a number of different active sites with affinities for many compounds.

The inhibition by Trasylol of plasminogen activation by urokinase differs in type from its inhibition of the esterolytic activity of urokinase on AcLMe, the former being competitive and the latter 'mixed' competitive and non-competitive. This difference suggests that the urokinase molecule may possess different binding sites for these two activities.

In the <u>in vitro</u> plasma clot system containing a standard amount

of urokinase, the Molar potency of Trasylol as a fibrinolytic inhibitor was about one hundred times that of AMCA and one thousand times that of EACA. Weight for weight, Trasylol was about two and one-half times more potent than AMCA. As has already been suggested, the Trasylol molecule, by virtue of its greater size and complexity compared with AMCA and EACA, may possess more active sites to account for its greater potency as a fibrinolytic inhibitor.

In the coagulation studies, it was seen that Trasylol had no effect in vitro on the one-stage prothrombin time or on the thrombin-fibrinogen reaction as assessed in the thrombin clotting time, findings in agreement with the observations of Amris, (1966.)

In vitro addition of Trasylol to the incubation mixture of the thromboplastin generation test produced lengthening of the substrate plasma clotting times. The abnormality was not observed until Trasylol was present in a concentration of 100 units/ml. of the incubation mixture. Trasylol in vitro in the substrate plasmas produced a similar abnormality which was observed at a Trasylol concentration of 40 units/ml. of plasma. These results may be due to an action of Trasylol impairing thromboplastin generation; alternatively Trasylol

may inhibit the conversion of prothrombin to thrombin or it may act in both ways. Amris (1966) has reported that Trasylol in vitro impairs thrombin generation and Nordstrom et al. (1965) have reported that in vivo in dogs, Trasylol protects against the effects of intravenous administration of formed thromboplastin. These observations support the view that Trasylol prevents prothrombin conversion to thrombin by formed thromboplastin. Blömback et al. (1966), using partially purified clotting systems, found inhibition of the reaction between anti-haemophilic globulin (A.H.G.) and the "serum components" of intrinsic thromboplastin in the presence of Trasylol, observations supporting the view that Trasylol inhibits thromboplastin generation. In the experiments reported in this thesis, it can be seen that the more striking abnormality was produced when Trasylol was present in the incubation mixture of the thromboplastin generation test than when present in the substrate plasma. It may be that the presence of Trasylol in the incubation mixture with its subsequent transfer to the substrate plasmas produced the more striking abnormality because of a summation of inhibitory effects on thromboplastin generation and on prothrombin conversion.

The effect of Trasylol in vitro on platelet aggregation was

examined at two concentrations. At 300 units/ml. of plasma, Trasylol produced a highly significant prolongation of platelet aggregation. The effect of Trasylol, 40 units/ml. of plasma, was then investigated since this was considered to be a concentration which could be achieved in vivo by intravenous administration of between 100,000 and 200,000 units of Trasylol. This concentration also prolonged platelet aggregation significantly though the effect was less striking than with 300 units/ml. However, when the effect of Trasylol, 40 units/ml. of plasma, was assessed after 10 minutes incubation of the plasma with Trasylol, no significant effect on platelet aggregation was observed. Amris (1966) has suggested that Trasylol may become bound to undialysable components in plasma, and one possible explanation of the loss of effect of Trasylol on platelet aggregation after incubation might be alteration of the physico-chemical properties of Trasylol during incubation.

In the in vivo studies in which Trasylol was administered to normal subjects, both anticoagulant and antifibrinolytic effects were imparted to plasma. The latter effect was demonstrable as an increase in overall plasma fibrinolytic inhibition in the urokinase sensitivity test and the former as a defect in the thromboplastin generation test. Though these

effects were observed to appear at very variable dosage levels of Trasylol in the different subjects. At least 100,000 units/hour seemed to be necessary to produce consistently significant levels of both anti-fibrinolytic and anticoagulant activity in any subject. The effects disappeared rapidly after withdrawal of Trapylol. Accordingly, it would seem reasonable to advise the use of high doses of Trasylol to initiate therapy, with lower maintenance doses thereafter. A dosage of 100,000 units over one hour or 100,000 units initially as a single intravenous injection with 50,000 units/hour thereafter seems appropriate, a recommendation in agreement with the findings of Amris (1966).

Thirty minutes after a single intravenous injection of 100,000 units of Trasylol, acceleration of platelet aggregation was observed, a finding in apparent discord with the in vitro observations already cited that Trasylol inhibits platelet aggregation. The discrepancy may be due in part at least to inactivation of the aggregation-inhibiting properties of Trasylol in the interval between its administration and plasma sampling; a different result might have been found if samples had been taken say 2 or 3 minutes after Trasylol injection. The acceleration of platelet aggregation at 30 minutes may also be due to

a homeostatic response to the injection of an anticoagulant. In spite of the acceleration of platelet aggregation, seen 30 minutes after Trasylol in the normal subjects, there was no significant variation in platelet counts during or after administration of Trasylol.

The correlations observed between times for platelet aggregation and clotting times in the corresponding thromboplastin generation tests suggest that in these in vitro studies the speed of platelet aggregation increased pari passu with defective thromboplastin generation, evidence in support of the hypothesis that the effect of Trasylol on platelet aggregation may be a homeostatic response to its anticoagulant effect.

In the two patients with defibrination, there was evidence of increase in some of the depleted coagulation factors during administration of Trasylol. In one patient, however, heparin infusion produced a rise in fibrinogen without effect on the platelet count whereas Trasylol infusion was associated with a fall in the platelet count.

In the studies with trypsin, Trasylol was shown to inhibit the proteolytic activity of this enzyme in a non-competitive manner. In vitro in plasma Trasylol was observed to have antitryptic activity. In vivo a single intravenous injection of Trasylol containing 100,000

units was shown to produce a small but significant increase in anti-tryptic activity. This dose is much larger than thos apparently used by Skyring et al. (1965) who considered that Trasylol in a double-blind trial had no effect in the treatment of acute pancreatitis. The evidence presented in this paper suggests that a dose of the order of at least 100,000 units/hour would be required to produce inhibition of trypsin in plasma. These findings are in agreement with the report of Goldberg and Roy (1965).

Conclusions

Trasylol is a competitive inhibitor of plasminogen activation by streptokinase and urokinase. The proteolytic activity of plasmin and trypsin is inhibited non-competitively and the esterolytic activity of urokinase is inhibited in a mixed competitive and non-competitive manner. These inhibitory effects are observed at high substrate concentrations. At low substrate concentrations, the inhibitory effects of Trasylol are relatively increased. Trasylol is considerably more effective in vitro as an inhibitor of fibrinolytic activity than AMCA or EACA: and in contrast to these amino acid inhibitors, Trasylol is more effective as an inhibitor of formed plasmin than of plasminogen activation.

Trasylol possesses anticoagulant effects probably due to

inhibition of prothrombin conversion and of intrinsic thromboplastin generation.

In vivo in high dosage it imparts antifibrinolytic, anticoagulant and antitryptic effects to plasma. It may accelerate platelet aggregation in vivo.

In one patient with hypofibrinogenaemia, its administration was associated with a fall in the platelet count. In another patient there was evidence of a rise in fibrinogen, factor V, antihaemophilic globulin (A.H.G.), and in the platelet count.

An initial dosage of 100,000 units as a single injection or as an infusion given over one hour followed by maintenance dosage of 50,000 units/hour seems appropriate in the treatment of pathological fibrinolysis. For the therapy of intravascular coagulation, higher dosage may be required, possibly 100,000 units/hour. This latter dosage would also be required in the treatment of systemic proteolysis due to trypsin.

CHAPTER 6

IN VITRO AND IN VIVO STUDIES OF A PREPARATION OF UROKINASE

The physiological plasminogen activator present in human urine, called urokinase by Sobel et al. (1952), was first isolated in a relatively pure state by Ploug and Kjeldgaard (1957). Activation of plasminogen by urokinase has been shown to be an enzymatic process proceeding with first-order kinetics probably by the splitting of arginine and/or lysine bonds (Kjeldgaard and Ploug, 1957; Alkjaersig et al., 1958 b). Purified urokinase is a colourless protein which is stable over the pH range 1 to 10 (Ploug and Kjeldgaard, 1957). Using DEAE-cellulose which adsorbs urokinase Celander and Guest (1960) found two elution peaks in urine with urokinase activity and these workers have suggested that urine may contain more than one plasminogen activator. There is some evidence that at least a proportion of urinary urokinase may represent excreted plasma plasminogen activator (von Kaulla and Riggenbach, 1958; Smyrniotis et al., 1959). McNicol at al. (1961 b) have suggested that urokinase may have a physiological role in maintaining patency of the urinary tract by promoting lysis of fibrinous deposits.

There has been interest for some years in the possible use of

fibrinolytic enzymes for the treatment of thrombo-embolic vascular disease. The enzyme which has been most widely employed for this purpose is the bacterial protein, streptokinase. As has been discussed, the use of streptokinase has certain disadvantages: it is difficult to prepare pyrogen-free; it is antigenic and plasma inhibitor levels to it vary widely according to the patient's exposure to streptococcal infection.

Because of its human origin, presumed non-antigenicity and the predictable fibrinolytic response which it produces (Fletcher et al., 1965), urokinase appears more attractive as an agent for thrombolytic therapy. However, the preparation of pure urokinase has posed considerable difficulty. Normal urine has been known for many years to possess thromboplastic activity (Tocantins and Lindquist, 1947) and Caldwell et al. (1963) have prepared and partially purified urinary thromboplastic material. One commercial preparation of urokinase has been shown to possess thromboplastic properties (McNicol et al., 1963 a). More recently, other commercial preparations have become available and it is the purpose of this chapter to given an account of investigations, in vitro and in vivo, of one of these preparations with particular reference to its effects on the coagulation system. Its

ability to activate plasminogen in vivo has also been studied.

Materials

Urokinase A purified preparation, isolated from the urine of normal males, was made available by Abbott Laboratories, North Chicago, Illinois. The preparation was supplied in two forms. One was the form routinely prepared for intravascular administration. It was a sterile, white, lyophilized powder. Its potency, stated by the manufacturer, was 40,000 C.T.A. units/mg. of protein. This preparation contained sodium chloride, human serum albumin and disodium versenate. A solution of one mg. of protein dissolved in one ml. sterile distilled water was stated to contain 0.9 per cent NaCl, 0.5 per cent albumin and 0.1 per cent disodium versenate. Before assessment of the effects of this preparation, in vitro, on the coagulation system, disodium versenate was removed by dialysis with constant stirring for 24 hours against 0.9 per cent NaCl at 4^{o}C, the dialysate being changed once at 12 hours. The second form of urokinase preparation was supplied free from sodium chloride, albumin and disodium versenate. It was a white powder stated to contain 35,000 C.T.A. units/mg. of protein. Both preparations were freely soluble in distilled water or 0.9 per cent NaCl. The calcium content of 20,000 C.T.A. units/ml. solutions were less than 1.5 m.eq./litre.

Another batch of urokinase produced by Abbott Laboratories, in the form prepared for intravascular administration, was investigated in vitro. This batch of urokinase had been subjected to an additional purification step during preparation compared with the batch of urokinase used for most of the investigative work to be described. Its activity was stated as 40,000 C.T.A. units/mg. protein. It was also dialysed before it was tested in vitro..

A C.T.A. unit refers to the standard urokinase unit recently adopted by the Committee on Thrombolytic Agents, of the National Heart Institute. The unit is based on the activity of a standard urokinase preparation measurable by fibrinolytic assays. A conversion factor worked out in several independent laboratories indicates that one C.T.A. unit, in terms of fibrinolytic assay, is equivalent to 0.7 Ploug units.

Human Fibrinogen The human fibrinogen employed for preparation of fibrinogen breakdown products, described below, was lyophilized human fibrinogen (A.B. Kabi, grade L). This was stated to be a virtually electrophoretically uniform preparation with about 95 per cent coagulability.

Methods

One-stage Prothrombin Time This test was modified by substitution of a 10,000 C.T.A. units/ml. solution of urokinase for a 1:500 dilution of the human brain extract in use at that time in the laboratory. EACA, 0.1 ml., was added to the system to give a final concentration of 10^{-2} Molar to inhibit lysis.

Recalcification Time Fresh normal plasma collected in glass or silicone was used. Varying concentrations of urokinase were substituted for saline in this test. EACA, 0.1 ml., was added to the system as in the one-stage prothrombin time. In one experiment, Hageman factor deficient plasma was used instead of fresh normal plasma.

Thromboplastin Generation Test This was modified by adjusting the final volume of the incubation mixture to 1.5 ml. The volumes of adsorbed plasma, serum, platelet suspension or platelet substitute and 0.025 Molar $CaCl_2$ were each 0.3 ml. EACA, 0.1 ml., was added to the system to give a final concentration of 10^{-2} Molar. The remaining 0.2 ml. volume contained varying concentrations of urokinase. Saline was substituted for urokinase in the control experiment.

Antihaemophilic Globulin (A.H.G.) and Christmas Factor Assays The effect of urokinase in these assay systems was investigated by substituting a 20,000 C.T.A. units/ml. solution of urokinase in each assay for fresh normal plasma obtained by pooling 10 normal plasmas. The systems containing urokinase were made up and re-calcified simultaneously with a control dilution curve prepared from the pooled fresh normal plasma and EACA, 0.1 ml., was present in the assay in a final concentration of 10^{-2} Molar to inhibit lysis.

Coagulation Assays made during the in vivo studies were performed in the presence of EACA in a concentration of 10^{-2} Molar to inhibit lysis.

Electrophoresis of Urokinase This was the method of McNicol et al. (1963 a). Oxoid cellulose acetate strips (Oxo Ltd., London) were used for electrophoresis. Strips were run for 2 hours at 150 volts in a horizontal electrophoresis tank (Shandon Scientific Co. Ltd., London). Acetate buffer, pH 4.5, 0.1 Molar, was the buffer system. The form of urokinase, free from albumin and disodium versenate was used and compared with urokinase (Leo). Twenty µlitres of each urokinase solution containing approximately 10,000 C.T.A. units/ml. were applied to strips for electrophoresis. After completion of electrophoresis, each strip was divided into three equal

portions at right angles to the origin. One portion was stained overnight with 0.001 per cent nigrosin in 2.0 per cent acetic acid. The other two were cut into strips one cm. wide, parallel to the origin. The strips from one of these portions were applied to the surface of a fibrin plate and lysis associated with each strip assessed after incubation of the plate at 37°C for 16 hours. Each strip from the third portion was eluted with 0.2 ml., 0.9 per cent NaCl, in a 12 x 75 mm. test tube, by pounding the strip with a glass rod for 5 minutes. The strips remained intact. After 30 minutes, 0.1 ml. of the eluate was tested in the recalcification time system described above. The stained portions were scanned in a recording and integrating densitometer (Chromoscan; Joyce, Loebl and Co., Ltd., Gateshead-on-Tyne).

Preparation of Fibrinogen Degradation Products This was the method of Nussenzweig et al. (1961). Four ml. human fibrinogen (50 mg./ml.) were incubated for 10 minutes at 37°C with 1.0 ml. human plasminogen (17.5 casein units/ml.) and 0.1 ml. urokinase (40,000 C.T.A. units/ml.). At the end of incubation, this mixture was layered on a DEAE-cellulose column of length 37.5 cm. and internal diameter 2.5 cm. The buffer system for the column was

Na_2CO_3, 0.1 Molar, pH 8.9 and the column flow rate 84 ml./hour. Elution was achieved during 6 hours using a linear concentration gradient beginning with the buffer alone and rising to the buffer with 0.2 Molar NaCl. The column was kept at 4°C.

The column effluent was concentrated with Sephadex, G-25 (Pharmacia, Uppsala, Sweden) using Seitz filters. Protein in the effluent was measured by the method of Lowry et al. (1951).

Column chromatography was carried out by Miss Patricia Wilson.

Other Materials and Methods were described in chapter 3.

Results

In Vitro Studies

Effect of Urokinase Compared with Human Brain Extract Solutions of both dialysed and pure urokinase, each containing 10,000 C.T.A. units/ml., were found to shorten the recalcification time of fresh normal plasma by the same amount as a 1:500 dilution of the human brain extract in use in the laboratory at that time. These results are shown in table 54.

Effect of Urokinase on the Recalcification Time of Silicone Plasma The effect of increasing concentrations of dialysed and

pure urokinase on the recalcification time of silicone plasma is shown in table 55. Compared with saline controls, there was a progressive reduction in the plasma recalcification time with increasing concentration of urokinase. These reductions in recalcification time were linearly related to urokinase concentration in a semilogarithmic plot as illustrated in figures 49 and 50. It can be seen that both dialysed and pure urokinase had closely similar effects. By extrapolation of both plots, the zero coagulant value found for each form of urokinase was 100 C.T.A. units/ml., i.e. 100 C.T.A. units/ml. was the maximum concentration added to the system which had no coagulant effect.

Effect of Dialysed Urokinase on the Recalcification Time of Hageman Factor Deficient Plasma The effect of increasing concentrations of dialysed urokinase on the recalcification time of Hageman factor deficient plasma is shown in table 56. Again, a progressive reduction in recalcification time linearly related to urokinase concentration in a semilogarithmic plot was observed (figure 51). The zero coagulant activity of the urokinase preparation in this experiment was also found by extrapolation to be 100 C.T.A. units/ml.

Effect of Two Batches of Dialysed Urokinase on the Recalcification Time of Fresh Normal Plasma In this experiment, the effect of the batch of urokinase, subjected to an additional purification procedure during its preparation, was compared with that of the batch employed in the other investigative work for this paper. The effect of increasing concentrations of each of these preparations on the recalcification time of fresh normal plasma is shown in table 57. It can be seen that both preparations had closely similar coagulant properties.

Effect of Urokinase Preparations in the Thromboplastin Generation Test Table 58 shows the effect of 4,000 C.T.A. units of both dialysed and pure urokinase in the thromboplastin generation test in partially correcting for deficiency of antihaemophilic globulin (A.H.G.) and Christmas factor. Both the dialysed and pure form of urokinase had closely similar effects.

Table 59 shows the effect of 6,000 C.T.A. units of both forms of urokinase in partially correcting the defect in the thromboplastin generation test due to absence of platelets. When the thromboplastin generation test was carried out with the standard dilutions of adsorbed plasma and serum but with a platelet

preparation diluted 1:3, no defect was produced. However, when the adsorbed plasma and serum were each diluted a further 1:4, a defect was found which was partially corrected by addition of 4,000 C.T.A. units of either form of urokinase. The effects of both forms were closely similar in these experiments.

Table 60 shows the partial correction produced by dialysed urokinase, 6,000 C.T.A. units, in the thromboplastin generation test in the absence of either adsorbed plasma or serum.

Effect of Urokinase in Antihaemophilic Globulin (A.H.G.) and Christmas Factor Assays Solutions containing 20,000 C.T.A. units of both dialysed and pure urokinase were found to produce the same correction as pooled normal plasma diluted to contain 2 per cent antihaemophilic globulin (A.H.G.) or Christmas Factor in the respective assays. The results are shown in detail in table 61.

Electrophoretic Studies with the Pure Form of Urokinase (Abbott) Compared with Urokinase (Leo). These results are illustrated in figure 52. It will be seen that in the acetate buffer, 0.1 Molar, pH 4.5, urokinase (Leo) separated into at least 4 components. Fibrinolytic activity was largely associated with slow moving components, whereas the coagulative property was more uniformly

distributed. The pure form of urokinase (Abbott), on the other hand, was electrophoretically uniform in the acetate buffer system and it was not possible to separate fibrinolytic and coagulative properties, both of which were demonstrable.

Column Chromatography for Preparation of Fibrinogen Degradation Products Separate runs were made on the DEAE-cellulose column with fibrinogen, plasminogen and urokinase. Each was recovered in the column effluent at a concentration of about 0.1 Molar NaCl.

With the 10 minute digestion of urokinase, plasminogen and fibrinogen, a single distinct protein peak was recovered in the effluent at a concentration of 0.035 Molar NaCl (figure 53). The protein recovery in the effluent concentrate was 3 mg./ml. This protein was electrophoretically different in charge from the human fibrinogen and its molecular weight estimated by a sucrose density gradient technique was 215,000 compared with an estimated molecular weight by the same method for the original fibrinogen of 340,000. Electrophoresis and sucrose density measurements were carried out by Miss Patricia Wilson. The protein was assumed to be an early fibrinogen degradation product.

Effect of Fibrinogen Degradation Products on In Vitro Platelet Aggregation The effect of these degradation products on platelet aggregation was tested by addition of 0.3 ml. of the degradation concentrate to each of 7 fresh normal plasmas in the Chandler's tube system. The volume of 0.9 per cent NaCl was adjusted to 9.7 ml. to accommodate the degradation concentrate in the test system. This concentration of degradation products (180 μgm./ml. plasma) significantly enhanced platelet aggregation, the time for platelet aggregation being 294 ± 14 seconds, compared with the control of 477 ± 168 seconds ($t = 3.874$, $0.005 < p < 0.01$). These results are shown in table 62.

In Vivo Studies

Urokinase Infusions The urokinase preparation was administered by intravenous infusion to two subjects.

In the first subject, the initial dose was calculated from the lysis time in a urokinase sensitivity test modified to use 100 μlitres of urokinase (500 C.T.A. units/ml.) in 0.2 ml. of the subject's plasma. This concentration of urokinase (250 C.T.A. units/ml. of the subject's plasma) gave a lysis time of 15 minutes in the test system. The initial dose was then calculated from the subject's weight (60 kg.) multiplied by his plasma volume per kg. (approximately

40 ml.) and the previously obtained urokinase concentration (250 C.T.A. units/ml. plasma). This calculated initial dose of 600,000 C.T.A. units was administered over 30 minutes followed by 100,000 C.T.A. units/hour for 12½ hours, 200,000 C.T.A. units in the succeeding hour and 400,000 C.T.A. units during the last hour of the infusion.

Fibrinolytic assays carried out during the infusion and after its conclusion are shown in table 63. Some of the results are graphically displayed in figure 54. It will be seen that after the initial dose of 600,000 C.T.A. units in the first half hour, there was evidence of marked activator activity in the radio-active clot assay, and fibrin plate test. Negligible activator activity was detected during the infusion of 100,000 C.T.A. units/hour while 200,000 C.T.A. units/hour produced a moderate increase in activity and marked activator activity was associated with the infusion of 400,000 C.T.A. units/hour.

The levels of plasminogen and fibrinogen fell during the infusion and the thrombin clotting time was prolonged. One and a half hours after completion of the infusion, there was evidence of persisting brisk activator activity. No activator activity was detectable 3 hours after the infusion was completed.

Coagulation assays were carried out during and after this infusion and the results are shown in table 64 and in part in figure 55. At the end of the first-half hour of the infusion, it can be seen that the recalcification time was accelerated and there was shortening of the recalcification time in a Hageman factor assay. The level of factor V had fallen while there were shortened clotting times in antihaemophilic globulin (A.H.G.) and Christmas factor assays. During the remainder of the infusion, the level of antihaemophilic globulin (A.H.G.) fell.

The second subject was given 500,000 C.T.A. units of urokinase/hour for 24 hours. The results of fibrinolytic tests are shown in table 65 and in part in figure 56. It can be seen that during the infusion, high levels of activator activity were detectable in the euglobulin lysis test and the fibrin plate test and there was striking prolongation of the thrombin clotting time. The plasminogen level fell steadily to zero at 12 hours and fibrinogen had fallen to 115 mg./100 ml. at 24 hours.

Coagulation assays in the same subject are shown in table 66 and in part in figure 57. At the end of the first hour of the infusion, the silicone plasma recalcification time was shortened. During the

infusion, the levels of factor V, antihaemophilic globulin (A.H.G.) and Christmas factor fell.

Table 67 shows control curves for the coagulation assays performed during the two infusions. EACA was present in the control system in a concentration of 10^{-2} Molar.

Effect of Urokinase Injection on In Vitro Platelet Aggregation This was studied in 7 normal subjects. To each subject was given, as a single intravenous injection, 80,000 C.T.A. units of urokinase. Immediately before and 10 minutes after the injection, blood samples were withdrawn for platelet counts, euglobulin lysis tests and to measure times for platelet aggregation. The results are shown in tables 68, 69 and 70.

After the urokinase injection, the mean time for platelet aggregation (412 ± 112 seconds) was significantly less than the mean time for platelet aggregation before the injection (506 ± 74 seconds) ($t = 2.800$, $0.02 < p < 0.05$) (table 68).

In each subject, euglobulin lysis activity was increased after the injection, mean activity being 1.61 ± 0.55 units compared with mean activity pre-injection (0.92 ± 0.30 units). This increase in activity after the urokinase injection is significant ($t = 5.145$, $0.001 < p < 0.005$) (table 69).

The mean platelet count after the injection (268.4 ± 85.5 thousands/mm^3) was lower than the mean platelet count before the injection (279.6 ± 86.0 thousands/mm^3). This fall in the mean platelet count is significant ($t = 2.575$, $0.02 < p < 0.05$) (table 70).

Discussion

The results presented in this chapter show that the urokinase preparation in both forms possesses coagulative activity. This activity was readily demonstrable in vitro. Though not potent (a solution of urokinase containing 10,000 C.T.A. units/ml. was equivalent to a 1:500 dilution of human brain extract in the one-stage prothrombin time), the activity was very non-specific. In the thromboplastin generation test, addition of between 4,000 and 6,000 C.T.A. units of urokinase partially corrected for defects due to deficiency of antihaemophilic globulin (A.H.G.), Christmas factor or platelets and 6,000 C.T.A. units produced slight correction compared with saline when substituted in the test for adsorbed plasma or serum. A 20,000 C.T.A. units/ml. solution was equivalent to plasma containing 2 per cent antihaemophilic globulin (A.H.G.) or Christmas factor in the respective assay systems. Substituted for saline in a system to measure the recalcification time of fresh normal

silicone plasma, a solution of urokinase containing 200 C.T.A. units/ml. shortened the clotting time by about 30 seconds. By extrapolation of the straight line graphs obtained in these experiments, the zero coagulant value for the preparation was found to be 100 C.T.A. units/ml. This same coagulant value was also found in the system with Hageman factor deficient plasma as substrate (figure 51). The findings were closely similar for both dialysed and pure forms of urokinase. Coagulant activity of the dialysed urokinase could not therefore be attributed to its content of human serum albumin. The zero coagulant value of 100 C.T.A. units/ml. was a finding in agreement with the results reported by Alkjaersig et al. (1965).

Urokinase (Leo) was investigated by McNicol et al. (1963 a) who reported it to have coagulant activity at a concentration of 2 Rbug units/ml. plasma in a recalcification time system with Hageman factor deficient plasma as substrate. Urokinase (Abbott) appears therefore to be considerably less coagulant than urokinase (Leo).

This difference was further reflected in the electrophoretic studies of urokinase (Leo) and urokinase (Abbott). Urokinase (Leo) was not a homogeneous preparation, 4 components at least being demonstrable. Its fibrinolytic activity was largely found with slow-

moving components while coagulant activity was more uniformly distributed. Urokinase (Abbott) appeared to be a uniform preparation and it was not possible to separate its fibrinolytic and coagulant properties.

Iatridis et al. (1960) have reported investigation of another preparation of urokinase which they found to possess a 'Hageman-like' effect. The electrophoretic finding with urokinase (Abbott) is suggestive, though not conclusive, evidence that its coagulant activity may be due to a 'Hageman-like' property of the urokinase molecule rather than to contamination of the preparation with thromboplastic moieties. Further evidence in support of this suggestion is provided by the findings with the batch of urokinase which had undergone an additional purification step in its preparation. This preparation of urokinase had a closely similar coagulant effect to the earlier preparation of urokinase when both were tested simultaneously in the same recalcification time system. However, Alkjaersig et al. (1965) consider the more purified batch to be considerably less coagulant than the earlier batch and found by a different method that it possessed a zero coagulant value of 350 units/ml.

From the two in vivo infusions of urokinase, it was seen that

400,000 C.T.A. units of urokinase/hour were required to produce brisk plasma fibrinolytic activity. Fletcher et al. (1965) have shown ^{131}I-clot lysis to be maintained at the 20 per cent level (i.e. the per cent lysis at 30 minutes of a ^{131}I-clot), by the infusion of about 4,000 C.T.A. units of urokinase/kg. of body weight/hour. The weight of the first subject described in this paper was about 80 kg. and of the second, 75 kg. so that the suggested requirement of 400,000 C.T.A. units/hour is in agreement with the work of Fletcher et al. (1965).

During both infusions, plasminogen activation and fibrinogenolysis were evidenced by a progressive fall in the level of plasminogen and of fibrinogen.

In the first subject, after the administration of 600,000 C.T.A. units there was evidence of coagulative activity in vivo; acceleration of the recalcification time and apparent increase in the levels of Hageman factor, antihaemophilic globulin (A.H.G.) and Christmas factor. These apparent rises may have been due to distortion of the assay systems by urokinase. The observed decrease in factor V activity may perhaps be explained in part by its consumption due to in vivo clotting.

In the second subject, there was also evidence of coagulative

activity, there being an acceleration of the recalcification time and a fall in factor V activity in the end of the first hour.

Acceleration of platelet aggregation on intravenous injection of urokinase is a finding not previously reported. In the experiment described in this paper, the effect was found with 80,000 C.T.A. units of urokinase. Assuming, for the 7 subjects, an average plasma volume of 3 litres, and that 10 minutes after the injection, all of the administered urokinase remained within the vascular compartment, then the effect was produced by at most, 25 to 30 C.T.A. units of urokinase/ml. of plasma. Further, it was accompanied by a slight but significant fall in the mean platelet count for the 7 subjects and by a significant increase in euglobulin lysis activity. An attempt to provide an explanation for this enhancement of platelet aggregation was made by column chromatography of a 10 minute digest of urokinase, plasminogen and fibrinogen. In this experiment, a single distinct protein peak was eluted at a Molarity of sodium chloride well separated from those previously established for urokinase, plasminogen and fibrinogen alone. This protein was assumed to be an early fibrinogen degradation product from its electrophoretic property and estimated molecular weight and when added to the Chandler's tube system,

significantly enhanced platelet aggregation. This effect is contrary to the findings of Kowalski et al. (1964) and Hirsch et al. (1965). However, the early degradation product which these latter workers investigated had a lower molecular weight than the product tested in the experiments described in this chapter. Using the same degradation concentrate, enhanced platelet aggregation was also found in an adenosine diphosphate system (Wilson, 1966).

The findings reported here suggest that the in vivo effect on platelet aggregation may be due to circulating early breakdown products of fibrinogen following the enhanced fibrinolytic activity found with administration of urokinase.

Conclusions

The evidence presented in this chapter shows that, though less thromboplastic than urokinase (Leo), urokinase (Abbott) both in vitro and in vivo, has coagulant activity. Further, evidence has been presented that urokinase accelerates platelet aggregation. While it is likely that the intense fibrinolytic activity which may be produced by intravenous administration of urokinase would largely cancel out thromboplastic activity in vivo, the thromboplastic or coagulative properties could predispose to thrombosis or perhaps more particularly to rethrombosis during therapy with urokinase and it would clearly be desirable to obtain a preparation free from such coagulative properties.

CHAPTER 7

THE EFFECT OF A HIGH FAT MEAL ON FIBRINOLYSIS, BLOOD COAGULATION AND PLATELET AGGREGATION

In this chapter is presented an account of the investigation of the influence of alimentary lipaemia on fibrinolysis, blood clotting and platelet aggregation. There has been interest in this effect because of its possible relationship to the established association of high plasma lipoprotein levels and ischaemic heart disease (Bronte-Stewart, 1958; Albrink and Man, 1959; Dawber et al., 1962; Keys et al., 1963).

It has been suggested that, under physiological conditions, continuing patency of the vascular tree may depend on a homeostatic equilibrium between the coagulation system and the fibrinolytic enzyme system, the former constantly laying down thrombi containing fibrin to seal any defects of the vascular endothelium and the latter constantly removing such fibrinous deposits once they have served their haemostatic function (Astrup, 1956).

The work of Duguid and others has shown that if intravascular thrombus persists, it can become incorporated in the subendothelial layer of the arterial wall leading to the formation of an atherosclerotic

plaque. Vascular occlusion overlying such a site may follow (Duguid, 1955).

If it is believed that coagulation and fibrinolysis are continuing complementary processes within the vascular tree, then any factor which accelerates clotting or inhibits fibrinolysis may permit the deposition and persistence of intravascular thrombi. One hypothesis for the relationship between high plasma lipoprotein levels, thrombosis and atherosclerosis suggests that high plasma lipoprotein levels may move the postulated equilibrium of clotting and lysis towards thrombosis by inhibition of fibrinolysis or by accelerating clotting. Poole (1958) has reviewed the work concerning the effect of alimentary lipaemia on blood coagulation. The most consistent effect has been demonstrated using Russell's viper venom. In the presence of this lipid-free coagulant, the plasma clotting time is shortened during the period between three and six hours following a fatty meal.

The effect of alimentary lipaemia on the fibrinolytic enzyme system is less well established. Most observers have found an inhibitory effect, but an appreciable number of workers have failed to detect any influence. One explanation for these conflicting reports

may be the differing experimental design and techniques which have been used (Howell, 1964).

Alimentary lipaemia has also been reported to accelerate platelet aggregation (Philp and Payling Wright, 1965); there is much evidence that platelet aggregation is an important early phase of thrombus formation (Mustard et al., 1964).

The object of the studies to be reported in this chapter was to examine the effect of alimentary lipaemia on the fibrinolytic enzyme system, platelet aggregation and 'Stypven' time, comparing the observations made after a high fat meal with those after a low fat meal. The fibrinolytic enzyme system was studied, using a variety of techniques to measure its several components. In particular, the lysis of Chandler thrombi by exogenous activator in an artificial circulation was investigated because it was thought that this technique might be more physiological than tests involving observation of the lysis of whole blood or plasma clots. An attempt was also made to correlate the degree of elevation of plasma lipid components following fat feeding with any observed changes in fibrinolytic or coagulant activity.

Materials

Russell's Viper Venom This was 'Stypven', Burroughs Wellcome and Co., London. One ampoule was dissolved in 0.9 per cent saline to give a 1:10,000 dilution of venom.

Other Materials were described in chapter 3.

Methods

Ten healthy adults, nine males and one female, whose ages ranged between 26 and 36 years, were investigated on two occasions, once after a low fat breakfast, the control situation, and once after the same breakfast to which had been added 270 gm. of double cream. Details of the breakfasts are provided in table 71. Before taking a breakfast, each subject had fasted and refrained from smoking from the previous evening. After each breakfast, a further 3 hours' fast was observed. At this time, subjects rested for 10 minutes before venous blood samples were withdrawn.

Blood Samples With minimal occlusion of the proximal circulation, 60 ml. of venous blood were obtained in plastic syringes, after a clean venepuncture using a needle of gauge number 19. The blood was allocated was follows:- 15 ml. for preparation of the Chandler thrombus were delivered into a plain silicone centrifuge tube; 27 ml.

to assess speed of platelet aggregation, for fibrinolytic tests and 'Stypven' time were mixed in 9 ml. amounts in three silicone centrifuge tubes each containing one ml., 3.8 per cent sodium citrate; 2.5 ml. for the platelet count were mixed in a sequestrene container (Stayne Laboratories, Ltd., High Wycombe); 10 ml. for assay of free fatty acids were mixed in a lithium heparin bottle (Stayne Laboratories Ltd.,) and the remainder collected as serum for cholesterol, phospholipid and triglyceride assays.

The silicone specimens were stored on ice. The citrated silicone specimens were centrifuged 10 minutes after collection (600g. for 5 minutes at 4°C).

Preparation of Artificial Thrombi and Estimation of Their Streptokinase-induced Lysis in the Chandler's Tube Chandler (1958) has shown that when whole blood is rotated in a closed loop of plastic tubing, when it solidifies, a thrombus is formed with a platelet head and a fibrin tail. Such thrombi were prepared for each subject after each type of meal by a modification of the method of Chandler (McNicol et al., 1965). Figure 58 shows a Chandler thrombus. Histologically these thrombi closely resemble pathological in vivo arterial thrombi (figure 59).

Fifteen ml. whole blood, collected in silicone and stored on ice, were transferred 5 minutes after collection to a Chandler's tube made with transparent vinyl plastic tubing of length 70 cm. and internal diameter 1.23 cm. (Portland Plastics Ltd., Hythe). At the same time a small quantity of radioactive iodine-tagged fibrinogen was added (about 0.1 ml. to give approximately 1,000 counts/second). The tubing was made a continuous loop with a nylon adaptor and the loop rotated on the turntable of a blood-cell suspension mixer (Matburn Ltd., London) revolving at 28.5 revs./minute. The loop was rotated for one hour at 37°C and the resulting thrombus decanted and washed three times with 10 ml., 0.9 per cent saline. Each washing consisted of four inversions of the test tube containing the saline and thrombus. After washing, the thrombus was blotted dry gently and weighed. Radioactivity of the thrombus was determined in a well-type scintillation counter and the thrombus transferred to a Chandler's tube containing 39 ml., 0.9 per cent saline to which had been added one ml. of streptokinase, 8,000 N.I.H. units/ml., giving a final concentration of streptokinase of 200 N.I.H. units/ml. The thrombus was perfused with streptokinase for 24 hours by rotation of the tube in an incubator at 37°C. At the conclusion of the

experiment, radioactivity of the thrombus was determined and, after correction for decay, was expressed as a percentage of the initial radioactivity of the thrombus. Per cent reduction in radioactivity was calculated from this. McNicol (1965) has shown that there is a good correlation between radioactivity release and tyrosine release from plasma clots immersed in a medium containing plasminogen activator. Per cent reduction in radioactivity was therefore used as a measure of lysis of the thrombus. At this stage, the thrombus was again blotted dry and weighed and the per cent weight loss after 24 hours perfusion calculated.

Russell's Viper Venom ('Stypven') Clotting Time This was a modification of the method of Maclagan et al. (1958). The plasma used was that collected in silicone and centrifuged at 600g. for 5 minutes at 4°C. It was further centrifuged (2,000g. for 15 minutes at 4°C) to render it platelet-poor. To 0.1 ml. of this plasma at 37°C was added simultaneously 0.1 ml. 'Stypven' (1:10,000) and 0.1 ml. 0.025 Molar $CaCl_2$ and the clotting time recorded.

Preparation of Plasma Thrombus This was the thrombus formed in the experiment to assess speed of platelet aggregation. The thrombus was removed from the Chandler's tube after rotation for

one hour at 37°C and its triglyceride content assayed.

Serum Cholesterol This was measured by a modification of the method of Zlatkis et al. (1953) (Henly, 1957).

Serum Triglycerides These were measured by the method of van Handel and Zilversmit (1957).

Serum Phospholipids These were measured by the method of Bartlett (1959).

Plasma Free Fatty Acids These were measured by the method of Dole et al. (1956) modified to use Nile Blue as the indicator.

Thrombus Lipid This was extracted with chloroform-methanol in the preparation of 2:1 and triglyceride content of the extract measured by the method of van Handel and Zilversmit (1957).

Thin Layer Chromatography The method of Robinson and Philips (1963) was used.

Dr. B.M. Rifkind and Miss M. Gale kindly carried out the lipid assays.

Other Methods were described in chapter 3.

Results

Plasminogen Assays The mean plasma plasminogen level for the 10 subjects after the high fat breakfast (2.344 $\pm$ 0.218 casein units/ml.)

did not differ significantly from the mean level after the control breakfast (2.375 ± 0.299 casein units/ml.) ($t = 0.375$, $0.7<p<0.8$). These results are shown in table 72.

Fibrinogen Assays The mean plasma fibrinogen level for the 10 subjects after the high fat breakfast (252.5 ± 94.0 mg./100 ml.) was almost identical with the mean value after the control breakfast (253.0 ± 97.0 mg./100 ml.) ($t + 0.098$, $0.90<p<0.95$). Table 73 shows these results.

Euglobulin Lysis Tests Results of euglobulin lysis tests for the ten subjects after both types of breakfast are shown in table 74. Mean euglobulin lysis activity after the high fat breakfast (1.10 ± 0.51 units) is significantly less than mean activity after the control breakfast (1.72 ± 0.88 units) ($t = 3.573$, $0.005<p<0.01$).

Urokinase Sensitivity Tests Table 75 shows the results of urokinase sensitivity tests for the 10 subjects after each type of breakfast. It will be seen that in this test there was a significant mean increase in resistance to lysis by urokinase of plasma clots formed after the high fat breakfast (0.85 ± 0.38 units) compared with the findings after the control breakfast (1.05 ± 0.59 units) ($t = 3.087$, $0.01<p<0.02$).

Lysis of Chandler Thrombi by Streptokinase in an Artificial Circulation Table 76 shows the results for the 10 subjects of per cent reduction in radioactivity from the thrombi formed after each type of breakfast. Mean per cent radioactivity lost after the high fat breakfast (35.1 ± 12.2 per cent) is significantly decreased compared with the control value (48.6 ± 15.7 per cent) ($t = 3.024$, $0.01<p<0.02$).

Table 77 shows the results of per cent weight loss of thrombi formed after each type of breakfast for 8 subjects. Mean per cent weight loss after the high fat breakfast (46.8 ± 14.3 per cent) is significantly less than mean per cent weight loss after the control breakfast (56.5 ± 12.3 per cent) ($t = 4.054$, $0.001<p<0.005$).

Russell's Viper Venom ('Stypven') Clotting Times A consistent acceleration of the 'Stypven' clotting time was found in each subject after the high fat breakfast, compared with the findings after the low fat breakfast. The observations are recorded in table 78. The mean 'Stypven' clotting time after the high fat breakfast was 15.6 ± 4.9 seconds, and after the low fat breakfast 22.9 ± 5.2 seconds. The difference is highly significant ($t = 12.726$, $p<0.001$).

Platelet Aggregation These results are shown in table 79. The mean

value for the 10 subjects for platelet aggregation in the Chandler's tube after the low fat breakfast was 521 ± 87 seconds and after the high fat breakfast was 603 ± 68 seconds. The prolongation of platelet aggregation after the high fat breakfast is significant ($t = 4.263$, $0.005<p<0.01$).

Platelet Counts The mean platelet count for the 10 subjects following the control breakfast was 219.7 ± 51.6 thousands/mm^3, and after the high fat breakfast was 232.4 ± 50.2 thousands/mm^3. The difference between these means is not significant ($t = 1.178$, $0.2<p<0.3$). The results are recorded in table 80.

Plasma Lipid Levels After each type of breakfast, the levels of serum triglycerides, serum phospholipids, plasma free fatty acids and serum cholesterol were measured.

Mean serum triglyceride level after the high fat breakfast (164 ± 82 mg./100 ml.) was significantly greater than control findings (81 ± 34 mg./100 ml.) ($t = 4.427$, $0.001<p<0.005$). These results are shown in table 81.

Mean serum phospholipid level after fat feeding (244 ± 38 mg./100 ml.) was significantly higher than the mean level after the low fat breakfast (212 ± 24 mg./100 ml.) ($t = 3.596$, $0.005<p<0.01$).

Table 82 shows these results.

After the high fat breakfast mean plasma free fatty acid level was 952 ± 177 μe/litre and compared with the control findings (561 ± 180 μe./litre) this rise after the high fat breakfast is highly significant ($t = 5.213$, $p<0.001$). Table 83 summarises these results.

Mean serum cholesterol level for the ten subjects after the high fat breakfast (210 ± 41 mg./100 ml.) did not differ significantly from the mean value after the control breakfast (223 ± 43 mg./100 ml.) ($t = 1.417$, $0.10<p<0.20$). These results are shown in table 84.

Thin Layer Chromatography of the Double Cream This was carried out to identify the lipid constituents of the cream. The chromatograms, which were run with appropriate lipid markers, are shown in figures 60 and 61. It will be seen that the constituents of the cream were chiefly triglycerides with some diglycerides, a trace of cholesterol and no phospholipid.

Plasma Thrombus Triglyceride Content Mean thrombus triglyceride content for the 10 subjects after the high fat breakfast (0.29 ± 0.22 mg./100 ml.) was greater than the mean control thrombus triglyceride content (0.15 ± 0.13 mg./100 ml.), but the difference is not formally significant ($t = 2.109$, $0.05<p<0.1$) (table 85).

Discussion

The results presented in this chapter indicate that during alimentary lipaemia, induced by ingestion of 95 gm. of fat in the form of 270 gm. double cream, there was both a significant decrease in spontaneous plasma clot lysis activity (euglobulin lysis test) and increased resistance of plasma clots to lysis by urokinase. Possibly most important, an increased resistance of preformed Chandler thrombi to lysis by extrinsic activator (streptokinase) in an artificial circulation was also demonstrated.

Circumstances which have been reported to influence spontaneous fibrinolysis such as diurnal variation (Fearnley et al., 1957), exercise (Biggs et al., 1947) and circulatory occlusion before venesection (Kwaan et al., 1958b) were eliminated as far as possible by the experimental design.

The observed decrease in physiological fibrinolysis after the high fat breakfast was not due to decrease in clot or thrombus plasminogen content or to increase in concentration of fibrin, since the plasma levels of plasminogen and fibrinogen were closely similar after each type of breakfast.

The finding of decreased euglobulin lysis activity during

alimentary lipaemia was contrary to the results obtained in normal subjects by other workers (see Howell, 1964 for a review of the literature). However, Gajewski (1961) did observe decreased activity in this test in a group of patients with severe atherosclerosis, following ingestion of saturated fat. One explanation for these differing results in normal subjects may be that previous observers have studied the euglobulin lysis test following meals containing 40 to 60 gm. saturated fat, whereas the ten subjects described in this chapter were investigated during the lipaemia induced by about twice this amount. The decreased activity which Gajewski observed in his atherosclerotic group, who were fed 40 to 60 gm. saturated fat, may be explained by the delay in "clearing" of lipaemia which is reported to occur in these subjects (Mitchell and Bronte-Stewart, 1959). Higher lipid levels may therefore have been induced in Gajewski's group due to fat feeding compared with findings in normal subjects. Pappenhagen et al. (1963) examined the effect of chylomicra and β-lipoprotein on euglobulin clot lysis, and reported prolongation of euglobulin lysis times with either fraction whether added to plasma before euglobulin precipitation or to the euglobulin suspension before clotting, a finding suggesting that entry of chylomicra or β-lipoprotein

into the clot may be a factor in the production of decreased euglobulin lysis activity. That the bulk of plasma lipid components is present in the euglobulin clot has been demonstrated by Rifkind (1965) using thin layer chromatography of lipid extracts of euglobulin clot, supernatant and native plasma. Traces, only, of the plasma lipid components were identified in the supernatant, whereas lipid components in the clot were quantitatively similar to those of the native plasma.

Riding and Ellis (1964) reported that purified β-lipoprotein possesses significant antiplasmin activity in vitro in a plasminogen assay system with casein as the substrate for plasmin. The increase in overall fibrinolytic inhibitory levels demonstrated in the urokinase sensitivity tests reported in this chaper might therefore be due to an antiplasmin effect of β-lipoprotein in the clot. The urokinase sensitivity test does not distinguish antiactivator and antiplasmin activity, so that an increase in antiactivator activity during lipaemia is a further possible explanation of the results.

The decreased thrombolysis found in the Chandler thrombi experiments may have greater significance than observation of lysis of whole blood or plasma clots, since the test system, namely

arterial-like thrombi subjected to lysis in an artificial circulation, uses a more physiological substrate than test tube clots. The decrease in lysis of thrombi prepared from lipaemic blood as compared with those formed from non-lipaemic blood is in agreement with the observation of Bang and Cliffton (1960), who induced thrombi in dogs during alimentary lipaemia and demonstrated decreased thrombolysis in vivo by streptokinase compared with findings in both fasting dogs and dogs fed a low-fat meal. These workers also showed, using a histochemical technique, that thrombus formed during lipaemia contained large amounts of fat in intimate contact with the fibrin mesh while thrombus formed in the fasting state contained no fat. They suggested that resistance to lysis of "lipaemic" thrombi might be due to a lipid coating of the fibrin strands which protects them from proteolysis. Bang (1964) provided further evidence for this hypothesis; using electron microscopy, he showed that during the conversion of fibrinogen to fibrin, lipoprotein forms a layer on the surface of fibrin fibrils.

It is usually assumed that changes in fibrinolysis or coagulation following fat feeding are due to the associated lipaemia. However, although there were significant rises in triglyceride,

phospholipid and free fatty acid levels following the fat breakfast in all ten subjects, no correlation was obtained between the actual levels or changes in the levels of any of these lipid components and the changes in fibrinolytic activity.

On the other hand, Russell's viper venom ('Stypven') clotting times were closely related to serum triglyceride levels. When the pairs of results (clotting times after the control breakfast and after the high fat breakfast) were plotted against their corresponding serum triglyceride levels expressed as logarithms, a highly significant inverse correlation was found ($r = -0.879$, $p<0.001$; $y = 2.71 - 0.0358x$). Similar correlations were found for the results following the control breakfast ($r = -0.823$, $0.005<p<0.01$; $y = 2.52 - 0.0285x$) and following the high fat breakfast ($r = -0.872$, $p<0.001$; $y = 2.82 - 0.0285x$). The correlation for the combined 'fat-free' and 'after fat' results is illustrated in figure 62. It is of interest that plasma clots from lipaemic blood tended to have a higher triglyceride content than control clots, although the differences were not significant.

No correlation was found between the levels of the other lipids measured and 'Stypven' times.

The correlation between 'Stypven' times and serum triglycerides suggests that the acceleration of 'Stypven' times during alimentary lipaemia may be mediated through the entry of triglyceride into the circulation.. The correlation noted between 'Stypven' times and triglyceride levels in the subjects after the control breakfast suggests that endogenously-derived, circulating triglyceride may also be important in influencing the results of the 'Stypven' time. These results are in agreement with the report of James et al. (1961) that there is a significant negative correlation ($r = -0.65$) between 'Stypven' time and lipaemia measured as plasma turbidity which is largely due to triglyceride-rich chylomicra. It is usually assumed that phospholipid or one of its subfractions is the lipid active in coagulation (see Biggs and Macfarlane, 1962 d, for a review of the literature). Failure to find any correlation between 'Stypven' times and plasma lipid components other than triglycerides does not preclude a correlation between 'Stypven' times and particular lipid subfractions, e.g. phosphatidyl ethanolamine (Billimoria et al., 1965).

The proglongation of platelet aggregation noted after the high fat breakfast differs from observations made by Philp and Payling Wright (1965), who reported increased adhesiveness of platelets

during lipaemia compared with findings in the fasting state. One explanation for these contrary observations may be that the method used in this thesis to assess speed of platelet aggregation differed from that employed by Philp and Payling Wright. These authors also reported that the platelet-count was in every instance lower during lipaemia than in the fasting state. In the experiments reported in this chapter, platelet-counts after the two types of breakfast (low fat and high fat) were not significantly different; accordingly relative thrombocytopenia is unlikely to have been a factor in the prolongation of platelet aggregation which was observed.

One hypothesis to account for prolongation of platelet aggregation during lipaemia is that chylomicra may interfere with platelet collisions. If this were valid, it might be anticipated that there would be a correlation between triglyceride levels and platelet aggregation: no such correlation was found.

Current knowledge suggests that formation of platelet micro-thrombi may be an important early step in the formation of macroscopic thrombus in the blood vessels and possibly therefore, diminished speed of platelet aggregation might oppose the other 'thrombosis-promoting' effects of the lipaemia i.e. decreased fibrinolysis and accelerated clotting.

Conclusions

The effect of a high fat meal was investigated in ten normal subjects, compared with findings after a low fat meal. The aim of the investigations was to examine the hypothesis that the possible equilibrium of fibrinolysis and coagulation may be displaced towards thrombosis by raised blood lipid levels.

Decreased fibrinolytic activity was found after the fat meal in the euglobulin lysis test and in the urokinase sensitivity test. Artificial thrombi also showed increased resistance to lysis by streptokinase - a result thought to be more significant in terms of the in vivo effect of lipaemia on spontaneous fibrinolytic activity.

The acceleration of 'Stypven' times during lipaemia originally reported by Fullerton et al. (1953) was confirmed. A highly significant inverse correlation was demonstrated between 'Stypven' times and triglyceride levels expressed as logarithms.

Apart from the finding of prolongation of platelet aggregation, the other effects of a fat meal appear to move the hypothetical equilibrium of clotting and lysis towards thrombosis.

CHAPTER 8

STUDIES OF SIX DEFIBRINATION SYNDROMES

Defibrination or acquired hypofibrinogenaemia is now widely recognised to be associated with a severe and frequently life-threatening haemorrhagic diathesis. The pathogenesis of the condition has not been adequately elucidated in spite of intense interest in the problem over the past 15 years. Three commonly accepted possible causes of the condition are local release of tissue thromboplastin, local release of fibrinolytic activator or primary release of thromboplastin with a secondary fibrinolytic response which "overshoots" (Schneider, 1959; Fletcher et al., 1962 a; Sharp, 1964).

Spontaneous pathological bleeding associated with hypofibrinogenaemia may complicate major surgical procedures especially pulmonary surgery (Soulier et al., 1962) and surgery with extracorporeal circulation (von Kaulla and Swan, 1958); certain obstetric accidents particularly abruptic placentae, amniotic fluid embolism and intrauterine death (Schneider, 1959); metastatic carcinoma, most commonly of the prostate (Tagnon et al., 1962); and hepatic cirrhosis (Kwaan et al., 1956). Administration of streptokinase and urokinase for thrombolysis must also be included

(Fletcher et al., 1962 a).

Sharp (1964) has suggested that, with only occasional exceptions, pathological fibrinolysis is associated with an episode of intravascular fibrin formation, the lytic system being activated to combat coagulation. There is accumulating evidence to suggest that such a complex inter-relationship may exist between the coagulation and fibrinolytic enzyme systems. Both systems may be activated by activation of Hageman factor (Niewiarowski and Prou-Wartelle, 1959; Iatridis and Ferguson, 1961). Platelets possess antiplasmin activity (Johnson and Schneider, 1953; Stefanini and Murphy, 1956; Sherry and Alkjaersig, 1957) and proactivator properties (Holemans and Gross, 1961). Explosive intravascular fibrin formation and pathological fibrinolysis may be associated with depletion of the same coagulation components namely fibrinogen (Schneider, 1959; Fletcher et al., 1962 a), prothrombin and factor V (Greenwalt and Triantaphyllopoulos, 1954; Alagille and Soulier, 1956) and antihaemophilic globulin (A.H.G.) (Lewis et al., 1949; Sharp et al., 1958). Fibrinogen breakdown products inhibit fibrin polymerisation (Alkjaersig et al., 1962; Fletcher et al., 1962 b) and may have an antithrombin effect (Niewiarowski and Latallo, 1957;

Triantaphyllopoulos, 1958).

When hypofibrinogenaemia occurs, it may be difficult or impossible to determine whether the main cause is clotting or lysis and hence to adopt a rational approach to therapy.

It is the purpose of this chapter to describe six patients in whom the defibrination syndrome was diagnosed. In two there was prolonged retention of a dead foetus; in three metastatic carcinoma and one arose in the course of thrombolytic therapy.

A section of the chapter is devoted to description of three *in vitro* experiments which were attempts to illustrate possible errors in interpretation of certain fibrinolytic tests which might be performed in the investigation of defibrination syndromes.

Materials and Methods

Fibrinogen The preparations used were Kabi human fibrinogen described in chapter 6 and plasminogen-poor human fibrinogen prepared by the method of Kekwick et al. (1955).

Prothrombin Assay This was the method of Glueck et al. (1954) in which tosyl arginine methylester (TAMe) is substrate.

Assay of Factors VII and X These were assayed together by a method which depends on shortening of the one-stage prothrombin time of coumarin plasma (Douglas, 1955).

Factor X Assay This was the one-stage method of Bachmann et al. (1958) in which oxalated ox plasma, Seitz-filtered to remove factor X, is substrate. The method was modified slightly by using cephalin prepared as described by Bell and Alton (1954).

Demonstration of Fibrin Degradation Products in Serum The method used was that of Ferreira and Murat (1963). This was an agar-gel immunodiffusion technique. Fibrin antiserum was prepared by Dr. B. Sweet by immunizing rabbits with a suspension of fibrin (prepared from Kabi human fibrinogen) in aluminium hydroxide. The fibrin suspension was injected twice at an interval of 14 days. Antiserum was adsorbed before use with aged human serum, overnight at $4^{o}C$.

Other Materials and Methods were described in chapter 3.

Case 1. This patient, who was 30 years old, was in the 33rd week of her 5th pregnancy. She was Rhesus negative. She was known to have developed anti-Rhesus antibodies during her 3rd pregnancy which terminated in the delivery of a stillborn foetus with hydrops foetalis. During her fourth pregnancy, labour had been induced because of a rising anti-Rhesus antibody titre. No abnormal bleeding had occurred during these pregnancies. In her 5th pregnancy, intrauterine death was suspected to have taken place between 24

and 26 weeks and admission to hospital was arranged to confirm the diagnosis and to induce labour. The parient reported the presence of a blood-stained vaginal discharge during the three days preceding admission. Foetal death was confirmed radiologically. A Fibrindex test (Ortho Pharmaceuticals Ltd., Bucks.) demonstrated hypofibrinogenaemia and subsequent biochemical estimation of fibrinogen gave a result of 82 mg./100 ml. One pint of triple strength plasma was administered (equivalent to approximately 3.3 gm. fibrinogen). On the following day, persistent oozing of blood was noted from venepuncture sites. Other investigations were carried out at this time in an effort to elucidate the aetiology of her hypofibrinogenaemia. The results are shown in table 86 (day 0). There was depletion of prothrombin, factor V, antihaemophilic globulin (A.H.G.), factor X and plasminogen. The low plasminogen level and normal platelet count were thought to favour a diagnosis of pathological fibrinolysis as the aetiology of her hypofibrinogenaemia. Accordingly, therapy with the fibrinolytic inhibitor, EACA, was commenced, the initial dose being 4 gm., followed by 1 gm. four-hourly. Table 86 also shows the results of investigations carried out on the 4th day of therapy (day 4) and post-delivery (days 6 and 8).

Figure 63 graphically displays some of the more striking results of table 86. It will be seen that therapy with EACA, which was discontinued 12 hours post-delivery (day 5), was associated with restoration of the moderate plasminogen depletion to a normal level but with failure to correct depletion of affected coagulation components. In particular, plasma fibrinogen remained at less than 100 mg./100 ml.

On the 4th day of EACA therapy, medical induction of labour was commenced. Twenty hours later, with the onset of uterine contractions there was brisk vaginal bleeding. During the subsequent three hours until delivery of a stillborn macerated foetus was accomplished, blood loss amounted to about 2.5 litres. The patient, severely shocked during this period, required transfusion and was given whole blood (2 litres) and fibrinogen (12 gm.). On the day following delivery (day 6), the fibrinogen level and the fibrinolytic assays were within the normal range. The depleted coagulation components were partially restored while the platelet count had fallen to 85,000/mm^3., possibly as a consequence of acute blood loss and replacement transfusion.

Case 2. This patient, who was 32 years old, was gravida 3. She was Rhesus negative and her husband was known to be homozygous for the Rhesus factor. She had had, previously, two uncomplicated

pregnancies. In the present pregnancy, anti-Rhesus antibodies had been detected at 16 weeks. In the 21st week, she developed pre-eclamptic toxaemia (peripheral oedema was present and her diastolic blood pressure was 95mm. Hg). She was treated with bed rest and sedation and had recovered by the 23rd week. At the 25th week, the anti-Rhesus titre (indirect antiglobulin technique) had risen dramatically to 1:1024. Intrauterine death was suspected at that time and confirmed at the 26th week. She was admitted early in the 32nd week for induction of labour and at the time of admission, a Fibrindex test (Ortho Pharmaceuticals Ltd.,) demonstrated hypo-fibrinogenaemia. On the following day (day 0, table 87) clottable plasma fibrinogen was 218 mg./100 ml. and the plasminogen level was normal as was the platelet count. There was evidence of a coagulation defect:- factor V, antihaemophilic globulin (A.H.G.) and factor X levels were low and the one-stage prothrombin time and thrombin clotting time were prolonged. In view of the normal platelet count, a diagnosis of intravascular clotting was thought to be unlikely and since there was a striking prolongation of the thrombin clotting time, the diagnosis of pathological fibrinolysis was entertained. Accordingly, therapy with EACA was administered in the same dosage as in case I. Table 87 also shows assay values for the second day of

therapy (day 2) and the immediate post-delivery day (day 5). Figure 64 displays in part some of the results of table 87. EACA was discontinued 12 hours post-delivery. On the fourth day of therapy (day 4), spontaneous labour ensued and delivery of a stillborn macerated foetus was accomplished. Blood loss associated with delivery did not exceed 200 ml. and no replacement therapy was required. On the post-delivery day, all of the coagulation and fibrinolytic assays were normal with the exception of the level of factor V though this had risen to 30 per cent from its pre-delivery level of 10 per cent.

Case 3. This female patient, who was 56 years old, had a history of carcinoma of the breast of 8 years' duration for which the initial treatment had been radiotherapy. In 1963, she presented with metastases in the left supraclavicular fossa and was treated with steroids and androgens. In February 1964, she developed obstructive jaundice which resolved rapidly after cessation of the hormone therapy. Shortly thereafter, she was noted to have a recurrence of the breast tumour associated with a fixed, stony hard mass in the left supra-clavicular fossa. In April 1964, she underwent a simple mastectomy followed by radiotherapy. She developed a persistent wound infection

and ultimately required plastic surgery. In August 1964, she was reviewed following the plastic repair and generalised capillary oozing was noted from the still unhealed wound edges. She volunteered the information that she had noted spontaneous bruising of her limbs for some months prior. Shortly thereafter, she required emergency admission to hospital because of the sudden appearance of a large fluctuant haematoma of her right lower abdomen and upper part of right thigh. Coagulation and fibrinolytic investigations at the time of admission are shown in table 88. The low fibrinogen level established defibrination while the findings of a prolonged thrombin clotting time accelerated euglobulin clot lysis time, low plasminogen level and normal platelet count were thought to favour a diagnosis of pathological fibrinolysis rather than intravascular clotting. It was decided to follow the levels of plasminogen and fibrinogen during therapy with EACA (days 2 to 7, figure 65). On day 4, the fibrinogen level was 170 mg./100 ml., and this appeared to be a response to therapy with EACA. However, on day 7, the fibrinogen level was again less than 100 mg./100 ml., so that its level on day 4 could not be considered to have occurred as a response to therapy. On day 7, it was noted that the platelet count had fallen and the possibility

of intravascular clotting was considered.

In view of failure of therapy with a fibrinolytic inhibitor, it was felt that a trial of heparin administered by continuous intravenous infusion was justifiable and that if it should correct the defect, this information would be helpful in handling any subsequent haemorrhagic episode. Heparin (1,500 units/hour) was administered for 12 hours. Subsequent assay of the pre-infusion fibrinogen level was 180 mg./100 ml., and though, after the infusion, the level was 240 mg./100 ml., this rise could not, with certainty, be attributed to heparin. Thereafter, the policy adopted was to follow the fibrinogen level without therapy and to reconsider the use of heparin should the level again fall below 100 mg./100 ml. During this period of observation, the patient developed small skin nodules surrounded by subcutaneous haemorrhage. At this time, fibrin degradation products were demonstrated in the patient's serum (figure 66). Because of this finding, it was decided to observe the response to further therapy with fibrinolytic inhibitors (days 30 to 59, figures 23 and 65). During this therapy, transfusion (whole blood, 3 litres; packed cells from whole blood, 1 litre) was required to maintain the patient's haemoglobin. Death occurred on day 60 and post-mortem findings were of marked widespread bruising

of skin; numerous small metastatic tumours in lungs, mediastinal lymph nodes, ribs, spine, skull and long bones; tumour infiltration of the left pectoralis major muscle and extensive tumour replacement of both adrenal glands. There was no evidence of intravascular occlusion or fibrin formation and no fibrin thrombi were present in the kidneys.

Case 4. This female patient, who was 38 years old, had a five months history of a continuous blood-stained vaginal discharge. Biopsy of the cervix uteri confirmed the clinical diagnosis of carcinoma of the cervix uteri. There was evidence of local metastases. Shortly after the diagnosis was confirmed, there was a sudden increase in vaginal bleeding and it was noted that the blood did not clot. Whole blood (2.5 litres) was transfused and radiotherapy to the pelvis including radium implants commenced. Half of the radiation dose had been administered with no decrease in blood loss when the patient developed a massive haematoma in the left thigh at the site of an intramuscular injection, with gross purpuric haemorrhage into the skin distal to the site. At the same time, large haematomata with distal purpura were noted in association with venepuncture sites. Investigations carried out at this time are shown in table 49. There was hypofibrinogenaemia

in association with plasminogen depletion and a relatively low platelet count. In the light of these findings, this patient was thought to have intravascular clotting with secondary fibrinolysis. Accordingly, a heparin infusion was administered continuously for 54 hours. Results of assays carried out during therapy are graphically displayed in figure 46. Rises occurred in the levels of fibrinogen, factor V, antihaemophilic globulin (A.H.G.) and plasminogen and the platelet count was steady. After withdrawal of therapy for 18 hours, these assays had returned to pre-infusion levels. As discussed in chapter 5, Trasylol (100,000 K.I.E. units/hour) was then infused for 7 hours. At this time, although the levels of factor V and antihaemophilic globulin (A.H.G.) had risen, the fibrinogen level was unchanged and the platelet count had fallen. In view of this last feature, discussed in chapter 5, Trasylol was withdrawn and heparin infusion (1,500 units/hour) recommenced. After a further 16 hours, fibrinogen concentration had increased though the platelet count remained low. Towards the end of the first heparin infusion, although vaginal bleeding did not diminish, the blood clotted and oozing of blood from venepuncture sites had ceased.

Case 5. This male patient, who was 71 years old, had a three months history of cough and haemoptysis. He sought medical attention because of bleeding from gums and bruising of the skin overlying the jaw. Examination revealed a number of subcutaneous nodules with surrounding haemorrhage; cervical lymphadenopathy; thrombosis of the left external jugular vein; hepatomegaly and congestive cardiac failure. Haemostatic investigations are shown in table 50. Hypofibrinogenaemia, plasminogen depletion, thrombocytopenia and depletion of factor V and antihaemophilic globulin (A.H.G.) were found. A clinical diagnosis of bronchogenic carcinoma with metastases was made and defibrination was considered to be due to intravascular clotting with secondary fibrinolysis. Trasylol (100,000 units/hour) was administered for 9 hours and the effect is illustrated in figure 47 where it will be seen that at the conclusion of this therapy, there had been rises in fibrinogen, plasminogen, factor V, antihaemophilic globulin (A.H.G.) and in the platelet count. Before the Trasylol infusion was commenced, the left external jugular vein thrombosis had disappeared clinically. The patient did not survive and post-mortem findings were of a bronchogenic carcinoma with metastases in liver, spleen, heart, duodenum, kidney and skin.

There were many areas where intravascular fibrin formation was found in close association with tumour, particularly in lungs, heart and duodenum. A major portal venous radical was thrombosed and there was centrilobular necrosis of the liver. Homogenates of some of these tissues were tested on fibrin plates and marked fibrinolytic activity was found in assocaition with kidney; moderate activity with heart; minimal activity with lung and none with liver or tumour.

Case 6. This male patient, who was 35 years old, had mitral stenosis with atrial fibrillation. He had required surgical removal of an embolus lodged in the left popliteal artery. Twenty-four hours following the embolectomy, thrombosis was demonstrable radiologically at the site of the earlier occlusion. The fresh thrombosis was treated by administration of the plasminogen activator, urokinase. The infusion was given via an arterial cannula the tip of which was lodged immediately proximal to the occlusion. Urokinase was administered using a constant infusion pump (B. Braun, Melsungen, West Germany). The initial infusion dose of urokinase was calculated from the patient's urokinase sensitivity test modified to use 160 µlitres of urokinase (500 Ploug units/ml.). This concentration of urokinase

(400 Ploug units/ml. of the patient's plasma) gave a lysis time of 20 minutes and therefore would be a sufficient concentration to neutralize any antiurokinase effect of the plasma leaving enough free urokinase to produce brisk fibrinolytic activator activity in the plasma (McNicol et al., 1963 a). The total 'priming' dose of urokinase (approximately 850,000 units) was calculated from the patient's weight (54 kg.) multiplied by his plasma volume (approximately 40 ml./kg.) and the previously estimated concentration of urokinase (400 Ploug units/ml. plasma). The 'priming' dose was administered over $2\frac{1}{2}$ hours, followed by 20,000 Ploug units/hour for 2 hours and 40,00 Ploug units/hour for $17\frac{1}{2}$ hours. Due to mechanical failure of the pump, there was a break in the infusion at 22 hours for $2\frac{1}{2}$ hours. At $24\frac{1}{2}$ hours, the infusion was restarted at a rate of 80,000 Ploug units/hour for a further $23\frac{1}{2}$ hours at which time there was radiological evidence of patency of the artery. Heparin (10,000 units) was given thereafter every 6 hours for 36 hours and at the same time an oral anticoagulant was started. These measures were instituted to prevent rethrombosis.

The results of fibrinolytic assays are shown in table 89 and displayed in part in figure 67. It will be seen that the 'priming' dose

of urokinase produced brisk fibrinolytic activator activity in the radioactive clot assay and euglobulin lysis test. During the administration of 20,000 Ploug units/hour, activity fell to zero but increased with 40,000 units. It had again fallen to zero one half-hour after the break in the infusion. After restarting with 80,000 Ploug units/hour activity was again brisk. During the infusion, there was considerable plasminogen depletion and progressive defibrination, the fibrinogen falling to 100 mg./100 ml. at 41½ hours.

Shown also in table 89 are the results of antihaemophilic globulin (A.H.G.) assay. It can be seen that there was a progressive fall in the level of antihaemophilic globulin (A.H.G.) during the infusion.

In Vitro Experiments

Relationship between Fibrinogen Concentration and Thrombin Clotting Time In this experiment, varying concentrations of human fibrinogen dissolved in 0.9 per cent NaCl were substituted for plasma in the thrombin clotting time. The results are shown in table 90. It can be seen that the thrombin clotting time was prolonged by reduced fibrinogen concentrations.

Relationship between Fibrinogen Concentration and Lysis Time In this experiment, 0.4 ml. of varying concentrations of plasminogen-poor human fibrinogen was added to 0.1 ml. human plasminogen (2.3 casein

units/ml.) and 0.05 ml. urokinase (20 Ploug units/ml.) and the mixture clotted with 0.1 ml. thrombin (20 N.I.H. units/ml.). The lysis times of these mixtures were observed at 37°C. The results are shown in table 91. It can be seen that clot lysis was accelerated by reduced fibrinogen concentrations.

Plasminogen Levels in Plasma and Serum Shown in table 92 are the results of assays of plasminogen levels in 11 normal plasmas and their corresponding sera. The sera were allowed to incubate at 37°C for 24 hours to ensure adequate clotting before separating the sera by centrifugation (1,000 g. for 10 minutes at 4°C) and assay of plasminogen. The mean plasminogen level in plasma was 3.7 $\pm$ 0.7 casein units/ml. and in serum was 3.8 $\pm$ 0.7 casein units/ml. This difference is not significant ($t = 1.862$, $0.05<p<0.10$).

Discussion

Numerous laboratory tests of the coagulation and fibrinolytic enzyme systems are available which might be carried out in patients with defibrination syndromes in order to try to determine the cause and type of their haemostatic defect. However, as will be discussed, no clear pattern of laboratory results emerges to differentiate from one another intravascular clotting, pathological fibrinolysis or both

states occurring together. Fibrinogen depletion is, of course, common to all three causes of the defibrination syndrome. When fibrin and/or fibrinogen are degraded by the proteolytic activity of plasmin, the degradation products interfere with fibrin polymerisation and prolongation of the thrombin clotting time is usually considered to be an index of the severity of defective fibrin polymerisation during hyperplasminaemia, (Alkjaersig et al., 1962; Fletcher et al., 1962 b). However, low levels of fibrinogen per se may prolong the thrombin clotting time as shown in table 90. Accordingly, lengthening of the thrombin clotting time may occur in hypofibrinogenaemia due to pure intravascular clotting. Accelerated lysis of whole blood and euglobulin clots is normally accepted as evidence of increased fibrinolytic activator activity in the blood from which they were formed. There are, however, two situations in which lysis times of such clots might be interpreted erroneously. Firstly, low fibrinogen concentration in the clots may result in accelerated lysis times in the absence of increased activator activity as shown in table 91. Secondly, in pathological fibrinolytic states, it would appear likely that activator release into the circulation may be a transient phenomenon bringing about rapid hyperplasminaemia. Since activator has a half-life in the

circulation of about 15 minutes (Fletcher et al., 1964), in less than 30 minutes after the release, there may be plasminogen depletion and a fall in fibrinogen without evidence of increased activator activity. In these circumstances, whole blood and euglobulin clot lysis times may be abnormally prolonged due to a combination of plasminogen depletion and absence of increased activator activity and the only finding diagnostic of a recent hyperplasminaemic state would be a low plasminogen level since plasminogen levels are probably unaltered or only minimally reduced in uncomplicated fibrin formation (table 92). Estimation of the plasminogen level would, therefore, appear to be the most useful diagnostic fibrinolytic test in the investigation of defibrination syndromes. Unfortunately, assay of plasminogen is a time consuming procedure and the information which it may provide is not immediately available in an emergency situation.

As was mentioned previously, prothrombin, factor V and anti-haemophilic globulin (A.H.G.) are consumed during clotting and there is evidence that these clotting factors are susceptible to digestion by plasmin though it must be remembered that assay systems for these coagulation components depend on fibrin clot formation as their endpoint

and this may be distorted by defective fibrin polymerisation. For this reason, the one-stage prothrombin time may also be invalidated. Hougie et al. (1957) have reported the presence of factor X in the same concentration in both plasma and serum while Biggs and Macfarlane (1963d) have found it to be consumed in vitro when plasma is clotted in the presence of tissue extract. It is conceivable, therefore, that where there is release of tissue thromboplastin into the circulation, bringing about intravascular fibrin formation, the level of factor X may fall. It is generally accepted that platelets are unaffected during hyperplasminaemia. Possibly they are protected by their antiplasmin activity. On the other hand, a fall in the platelet count invariably accompanies intravascular fibrin formation (Sharp et al., 1958). The platelet count, therefore, emerges as the most useful of the coagulation tests in the investigation of defibrination syndromes.

Case 1. This patient's defibrination syndrome was considered to be due to pathological fibrinolytic activity because of plasminogen depletion and the normal platelet count. It was thought that absence of increased activator activity could be explained by transient, intermittent release of tissue activator into the circulation as discussed earlier.

Although plasminogen depletion was corrected during administration of the fibrinolytic inhibitor, EACA, levels of the depleted coagulation components remained unchanged and their restoration to normal or near normal levels appeared to be a consequence of delivery.

Following failure of response to the fibrinolytic inhibitor it was necessary to reassess the diagnosis. A retrospective diagnosis was made in this patient of intravascular clotting and plasminogen depletion was the evidence of secondary fibrinolysis. One explanation for the normal platelet count might be that intravascular coagulation had ceased some time before investigation and rapid regeneration of platelets in the marrow had restored their circulating numbers to normal. The low level of factor X might best be explained by its consumption during clotting in the presence of tissue thromboplastin.

Case 2. In this patient, a diagnosis of pathological fibrinolysis was considered because of the striking abnormality of the thrombin clotting time. This abnormality was accepted as evidence of defective fibrin polymerisation due to the presence of fibrinogen degradation products in the circulation. The main degradation

fragment has a half-life in the circulation of 9.5 hours (Fletcher et al., 1962 b; Alkjaersig et al., 1962). The fibrinogen level within the normal range would not exclude a degree of fibrinogenolysis and because of the relatively long 50 per cent plasma clearance time of the main degradation fragment, it was considered that hyperplasminaemia and fibrinogenolysis might have occurred some hours previously so that a degree of plasminogen regeneration could have occurred, sufficient to restore the plasminogen level to within the range of normal.

As in case 1, therapy with EACA failed to correct or prevent deterioration of the patient's haemostatic defect. Indeed, it may have 'unmasked' intravascular clotting since the fibrinogen level fell to less than 100 mg./100 ml. and thrombocytopenia developed during therapy. These subsequent findings and the failure of response to the fibrinolytic inhibitor supported a retrospective diagnosis of intravascular clotting. The abnormal thrombin clotting time was probably due to a degree of secondary fibrinolysis since the initial level of fibrinogen per se was unlikely to be responsible for such a striking prolongation of the clotting time. The low level of factor X might be explained as in case 1; it may have been consumed during

clotting in the presence of tissue thromboplastin.

Case 3. Pathological fibrinolysis was diagnosed initially in this patient because of accelerated euglobulin clot lysis, prolongation of the thrombin clotting time, plasminogen depletion and the normal platelet count. However, as can be seen in figure 65, fluctuations in the levels of plasminogen, fibrinogen, thrombin clotting time and platelet count appear to have occurred as a consequence of the natural history of the patient's disease and not to medication with fibrinolytic inhibitors. Accelerated clot lysis and prolongation of the thrombin clotting time may have been due to reduced fibrinogen concentration (tables 90 and 91). The finding of fibrin breakdown products in this patient's serum was accepted at that time as strong presumptive evidence of pathological fibrinolysis as the primary cause of this patient's defibrination. However, Merskey et al. (1965) have demonstrated the presence of considerable concentrations of degradation products in defibrination states due to intravascular clotting associated with a variety of diseases.

Although heparin was administered to this patient, its trial cannot be considered sufficiently adequate to provide comment on its therapeutic or diagnostic value in this case.

In retrospect, in spite of absence of evidence at post-mortem and because of the lack of response to therapy with fibrinolytic inhibitors, defibrination in this patient would appear to have been due to intravascular clotting with secondary fibrinolysis. Failure to find any evidence of clotting at post-mortem could be explained by the occurrence of lysis either ante-mortem or post-mortem.

Case 4. In this patient, therapy with heparin on two occasions produced significant rises in fibrinogen, the depleted clotting factors and in plasminogen. The reasons for administration of a Trasylol infusion and the response to this have been discussed in chapter 5. The positive response to both heparin infusions confirmed the value of the treatment and supported the diagnosis of intravascular clotting as the primary cause of the patient's defibrination. The initial low plasminogen level was evidence of secondary fibrinolysis.

Case 5. In this patient, the diagnosis of intravascular clotting was confirmed at post-mortem. Evidence for associated fibrinolysis was the low plasminogen level and the clinical disappearance of external jugular vein thrombosis. It is of interest that tumour homogenate from this patient showed no activator activity on a fibrin plate. This suggests that pathological fibrinolysis in this patient secondary to

intravascular clotting was due to a 'homeostatic' protective response of the fibrinolytic enzyme system and not to coincidental release of activator from the diseased tissue.

Case 6. This case has been included as an example of a defibrination syndrome due to a fibrinolytic state. The case illustrates the difficulty which may arise in situations in which activator release and hyperplasminaemia have occurred some time before biochemical investigation. In figure 67, it will be seen that 17 hours after the end of the infusion, there was no activator activity detectable in the radioactive clot assay or in the euglobulin lysis test and at that time the low level of plasminogen was the only feature to suggest a recent brisk fibrinolytic state. In the assay system for antihaemophilic globulin (A.H.G.), consumption of this clotting factor was demonstrated. EACA was added in the assay system in a concentration of 10^{-2} Molar to inhibit lysis.

Conclusions

It can be seen that considerable difficulty may arise in interpretation of results of coagulation and fibrinolytic tests, and hence in establishing the aetiology, intravascular clotting or pathological fibrinolysis or a combination of these two, in patients in whom the

defibrination syndrome is diagnosed.

Of the numerous laboratory tests of coagulation and fibrinolysis which may be performed, a platelet count and assay of plasminogen appear to be the most useful in investigation of defibrination syndromes, thrombocytopenia occurring in defibrination states due to intravascular clotting and plasminogen depletion where there is pathological fibrinolysis. From the evidence presented in this chapter, it can be seen that thrombocytopenia may not be consistently found in individual patients with the defibrination syndrome due to primary intravascular clotting. Possibly because release of tissue thromboplastin may occur intermittently, intravascular coagulation may have ceased by the time investigation is undertaken and rapid regeneration of platelets in the marrow may have restored circulating platelets to normal. Further, because assay of plasminogen is a lengthy procedure, its level is not immediately available in an emergency situation.

Sharp (1964) has suggested that apart from iatrogenic defibrination arising in the course of thrombolytic therapy, defibrination is probably always due to a combination of both intravascular clotting and fibrinolysis. In the six cases described in this chapter, apart from the defibrination syndrome which occurred during thrombolytic therapy

with urokinase, there was evidence in the other five of both clotting and lysis.

Where the defibrination syndrome arises and it is impossible to establish either intravascular clotting or pathological fibrinolysis as the aetiology, it would seem wise to treat such cases expectantly with transfusion of whole blood and if necessary fibrinogen and by administration of steroids as suggested by Fletcher et al. (1962 b). Administration of specific therapy, heparin or a fibrinolytic inhibitor, should be restricted to situations which are of such seriousness as to justify the risk of a therapeutic trial.

CHAPTER 9

SUMMARY AND CONCLUSIONS

The availability of fibrinolytic activators and inhibitors as preparations suitable for use in man has stimulated intensive study of the components and functions of the fibrinolytic enzyme system. The work for this thesis was concerned with the investigation of certain aspects of plasminogen activation and its inhibition.

The Fibrinolytic Enzyme System

The principal components of this complex system are plasminogen, plasmin, activators and inhibitors. Plasminogen is a circulating plasma globulin which is the inactive precursor of the proteolytic enzyme plasmin. Conversion of plasminogen to plasmin may be brought about by a variety of activators, among them blood and tissue activators; urokinase, the activator present in human urine and streptokinase, the activator produced by certain strains of haemolytic streptococci. Plasmin is capable of digesting a number of plasma proteins including fibrinogen, fibrin, prothrombin, factor V and antihaemophilic globulin (A.H.G.). In vivo, the proteolytic activity of plasmin is normally restricted to fibrin. Sherry et al. (1959 a) have proposed an hypothesis to account for the in vivo specificity of plasmin for fibrin. According to the concept, the level of antiplasmin

in plasma greatly exceeds the level of plasminogen, i.e. potential plasmin. Thus, when plasma plasminogen is slowly converted to plasmin, it is rapidly neutralized by plasma antiplasmin so that circulating plasma proteins susceptible to digestion by plasmin are protected. Conversely in thrombi, levels of plasminogen are said to be high relative to the effective concentration of antiplasmin which is considered to be low. In this situation therefore, free plasmin may be formed in close proximity to its substrate, fibrin, which is lysed. Sherry and his associates have suggested that one function of the fibrinolytic enzyme system is to provide any thrombi which may form with a mechanism which can promote their subsequent lysis.

Astrup (1956) has suggested that, under physiological circumstances, a dynamic equilibrium may exist between the coagulation and fibrinolytic enzyme systems, the former depositing fibrin to seal any deficiencies which may appear in the vascular endothelium and the latter removing such fibrin once it has served its haemostatic function and integrity of the endothelium has been restored. In this way, patency of the vasculature may be maintained.

It is considered that disordered fibrinolytic activity may contribute in part at least to a proportion of coagulation defects which

may complicate major surgery; certain obstetric accidents; hepatic cirrhosis and metastatic carcinoma. Pathological fibrinolytic activity may occur in such situations if large quantities of plasminogen activator are released into the circulation so that conversion of plasminogen to plasmin is rapid. The production of large amounts of plasmin temporarily overwhelms the antiplasmin 'buffering' mechanism and free plasmin circulates - hyperplasminaemia. A coagulation defect ensues which is due in part to digestion of prothrombin, factor V and antihaemophilic globulin (A.H.G.); in part to digestion of fibrinogen and fibrin, the degradation products of which inhibit fibrin polymerisation and in part to accelerated lysis of such fibrin thrombi as do form.

There is evidence to suggest that administration of fibrinolytic inhibitors may have value in the management of coagulation defects arising as a consequence of disordered fibrinolytic activity.

Plasminogen Activation and its Inhibition

The studies made for this thesis included assessment of two compounds, aminomethyl cyclohexane carboxylic acid and Trasylol, known to possess inhibitory activity against the fibrinolytic enzyme system. Trasylol was also known to possess anticoagulant and anti-tryptic activities and these properties were further investigated. A

preparation of the plasminogen activator, urokinase, was investigated in order to define any coagulant activity it might possess and the importance of such activity during intravenous infusion of the preparation for the purpose of increasing plasma activator activity. A study of the possible inhibitory effect on fibrinolysis and coagulation occurring during alimentary lipaemia was undertaken and finally detailed investigation was carried out of a number of defibrination syndromes since it is considered that pathological activity of the fibrinolytic enzyme system may contribute in part at least to the coagulation defect of some of these syndromes.

Materials and Methods The account given in chapter 3 contains a description of the materials and standard fibrinolytic and coagulation methods used in the work for this thesis. Many of the methods were adapted for particular aspects of the work and these modifications have been described in the relevant chapters. The standard methods for which details are provided include assays for plasminogen, plasminogen activator activity and fibrinogen. Fibrinolytic inhibitor activity was measured in the urokinase sensitivity test which is a plasma clot lysis system activated by a standard amount of urokinase. Also described is the thrombin clotting time which may be an index

of defective fibrin polymerisation or of fibrinogen depletion. Among the standard coagulation tests used were the one-stage prothrombin time, the plasma recalcification time, the thromboplastin generation test and assays for factor V, antihaemophilic globulin (A.H.G.), Christmas factor and Hageman factor. Finally a method for measuring platelet aggregation is given.

Aminomethyl Cyclohexane Carboxylic Acid In chapter 4, an account is given of the in vitro and in vivo investigation of a synthetic amino compound with a cyclic structure, aminomethyl cyclohexane carboxylic acid. This compound has been reported to possess anti-fibrinolytic activity and to be more potent than EACA, the aliphatic amino acid the use of which as fibrinolytic inhibitor has been most fully documented up to the present time. The results of animal toxicity studies had also indicated that the compound was less toxic to administer than EACA. Because of the reports of increased potency and decreased toxicity compared with EACA, investigation of the compound was undertaken. Two preparations of the compound were studied. One was a mixture of the trans and cis isomers (AMCHA) and the other a solution of the trans isomer (AMCA) which had been shown to possess all of the antifibrinolytic activity of the mixture.

In vitro, in streptokinase-activated plasma, AMCHA was found to inhibit plasminogen activation at concentrations above 10^{-4} Molar and the proteolytic activity of plasmin at concentrations above 2.0×10^{-2} Molar. In the same system, EACA had one-half the potency of AMCHA. The inhibition by both AMCHA and AMCA of plasminogen activation was shown to be competitive in type while AMCHA inhibited the proteolytic activity of plasmin non-competitively.

The effects of AMCHA on the trypsinogen-trypsin system were competitive inhibition of trypsinogen activation by enterokinase and non-competitive inhibition of trypsin activity. It was also found that, at very high concentrations, AMCHA inhibited pepsin activity.

Added in vitro in the urokinase sensitivity test, it was demonstrated that AMCA and AMCHA had antifibrinolytic effects, AMCA being 10 times more potent than AMCHA and 100 times more potent than EACA. Another cyclic compound, p-aminomethyl benzoic acid (PAMBA) was compared with AMCA in this in vitro system and it was found that AMCA had an eighteenfold increase in antifibrinolytic potency compared with PAMBA.

In vitro, at a concentration of 2.5×10^{-2} Molar, AMCHA produced slight impairment of thromboplastin generation and at higher

concentrations the compound prolonged the one-stage prothrombin time and the thrombin clotting time.

In the in vivo experiments, it was found that oral administration of AMCA or AMCHA produced an increase in antifibrinolytic activity demonstrable in the urokinase sensitivity test. In vivo, AMCA had a fivefold increase in potency compared with AMCHA and a tenfold increase in potency compared with EACA. The antifibrinolytic effects of AMCA and AMCHA were slightly prolonged in vivo compared with EACA.

AMCA in a dose of 200 mg. was found to inhibit pathological fibrinolytic activity in a patient with hypofibrinogenaemia associated with accelerated lysis of Fearnley-type dilute plasma clots.

An indirect measure of levels of AMCA in plasma was made in one subject who ingested 200 mg. The highest concentration of AMCA found in plasma was estimated to be 2×10^{-5} Molar. This level was found in plasma withdrawn 2 hours after ingestion of AMCA.

It was concluded that AMCHA and AMCA were more potent fibrinolytic inhibitors than EACA. The in vivo antifibrinolytic effect demonstrated in the urokinase sensitivity test was considered to be due to competitive inhibition by AMCA or AMCHA of plasminogen activation

since no attempt was made to achieve the higher concentrations required to inhibit plasmin. The observed antifibrinolytic effects were not due to the influence of AMCA or AMCHA on blood clotting since the higher concentrations which impaired clotting were not achieved *in vivo*.

A suggested dosage schedule for AMCA, based on the investigative work described in chapter 4, is a loading dose of 400 to 600 mg. followed by 100 to 200 mg. every 5 hours. Such doses should be adequate to inhibit pathological fibrinolytic activity by preventing plasminogen activation and thus the formation of plasmin.

Because of the inhibitory effect on the trypsinogen-trypsin system, AMCA may have value in the treatment of the systemic proteolysis due to trypsin which is believed to accompany acute pancreatitis.

The effect of AMCHA on pepsin is probably without therapeutic relevance in view of the high concentration of the inhibitor which would be required *in vivo* to produce an effective inhibition of pepsin activity.

The increased potency and decreased toxicity of AMCA compared with EACA render AMCA the more attractive fibrinolytic inhibitor and it

may, in the future, come to replace EACA in the treatment of pathological fibrinolytic states.

Trasylol In chapter 5 is described the *in vitro* and *in vivo* investigation of the polypeptide Trasylol, which is known to possess antifibrinolytic activity. Recently, it has been shown to have anticoagulant activity in addition to its antifibrinolytic properties. It was thought that, on theoretical grounds, a drug possessing both anticoagulant and antifibrinolytic activity might have value in the therapy of spontaneous bleeding states associated with hypofibrinogenaemia since possible explanations of the aetiology of such states are that they are due to widespread intravascular clotting or to hyperplasminaemia or to a combination of these two. The further investigation of Trasylol was undertaken with a view to assessing its place in the treatment of such states.

The preparation of Trasylol used in the investigative work for this thesis was the form, made available for intravascular administration, which is commercially prepared from bovine lung.

Trasylol was found to be an effective inhibitor of plasminogen activation and of the proteolytic activity of formed plasmin. In contrast to the amino acid inhibitors AMCA and EACA, it was a more effective

inhibitor of the proteolytic activity of plasmin than of plasminogen activation. In purified systems, using a limited variation in substrate concentration i.e. low substrate concentrations were not employed, it was seen that Trasylol was a competitive inhibitor of plasminogen activation by either streptokinase or urokinase; a non-competitive inhibitor of the proteolytic activity of plasmin and a mixed competitive and non-competitive inhibitor of the action of urokinase on AcLMe. In the same purified systems, employing a wide range of substrate concentration, the inhibitory effects of Trasylol were found to be relatively increased at low concentrations of the substrate. In vitro, in the urokinase sensitivity test, Trasylol was considerably more potent as an inhibitor of fibrinolytic activity than AMCA and EACA.

The anticoagulant properites of Trasylol were found to be due either to an inhibitory effect on the conversion of prothrombin to thrombin or to inhibition of blood thromboplastin generation or to a combination of these two effects.

In the in vivo experiments, it was seen that in high dosage, at least 50,000 units/hour, Trasylol imparted antifibrinolytic and anticoagulant effects to plasma. Evidence was also presented that

the drug migh enhance platelet aggregation in vivo. This effect was observed 30 minutes after a single intravenous injection of 100,000 units.

Trasylol was administered as a continuous intravenous infusion at the rate of 100,000 units/hour to two patients with hypofibrinogenaemia due to intravascular clotting. In one of these patients, after an infusion of 9 hours, the levels of fibrinogen, factor V and antihaemophilic globulin (A.H.G.) and the platelet count had risen. In the other patient at the end of 7 hours, there had been no change in the level of fibrinogen though the levels of factor V and antihaemophilic globulin (A.H.G.) had risen. The platelet count had fallen - an effect which might have been predicted from the earlier observations of enhanced platelet aggregation after Trasylol injection.

Its effect on trypsin activity was studied in vitro. Trasylol was found to be a non-competitive inhibitor of trypsin and to impart antitryptic activity to plasma when added in vitro.

In vivo, 30 minutes after a single intravenous injection of 100,000 units of Trasylol, increased antitryptic activity was detectable in plasma.

From the in vitro and in vivo studies of Trasylol described in this thesis, a dosage rate of 100,000 units either as a single intravenous injection or administered as an infusion over one hour followed by an infusion of 50,000 units/hour as maintenance therapy seems appropriate in the treatment of systemic proteolysis due to plasmin. Higher dosage, 100,000 units/hour may be required for the treatment of intravascular clotting or systemic proteolysis due to trypsin. The evidence presented was considered sufficiently encouraging to warrant further investigation of the effect of Trasylol in defibrination states.

Urokinase The thesis continues with an account (chapter 6) of in vitro and in vivo studies with a commercial preparation of urokinase, the physiological plasminogen activator present in human urine. Since human urine is known to contain thromboplastic material, studies with the preparation were made with a view to defining any coagulative properties it might possess and the effect of such properties during its intravenous administration in man with the aim of increasing plasma fibrinolytic activator activity.

The preparation was made available in two forms. One was the form provided for intravascular administration and contained human serum albumin and disodium versenate. Disodium versenate

was first removed by dialysis before this form was examined for coagulative properties. The second form of the urokinase preparation was free from albumin and disodium versenate.

Both forms were found to have non-specific coagulative properties; they shortened the recalcification times of silicone normal plasma; substituted for human brain in the one-stage prothrombin time; and corrected partially for defects due to antihaemophilic globulin (A.H.G.), Christmas factor and platelet deficiency in the thromboplastin generation test.

On electrophoresis on cellulose acetate, the preparation of urokinase appeared to be homogeneous and it was not possible to separate fibrinolytic and coagulant activity.

The urokinase preparation was given by continuous intravenous infusion to two subjects; in both subjects brisk thrombolytic activity was produced. In the first subject 600,000 C.T.A. units was administered in 30 minutes and produced intense fibrinolytic activity. Subsequently, infusion of 100,000 C.T.A. units/hour produced negligible activity while 200,000 and 400,000 C.T.A. units/hour produced moderately intense and brisk activity respectively. In the second subject, the administration of 500,000 C.T.A. units/hour was

associated with brisk fibrinolytic activity. In both subjects, there was progressive fibrinogen and plasminogen depletion and there was also definite laboratory but not clinical evidence of coagulative activity.

In a group of 7 subjects who were each given a single injection of 80,000 C.T.A. units of urokinase, 10 minutes after the injection, there was evidence of enhanced platelet aggregation in a Chandler's tube system, associated with a fall in the platelet count and increase in euglobulin lysis activity. Evidence was presented that the accelerated platelet aggregation might have been due to an effect of early fibrinogen degradation products on platelet aggregation.

Urokinase is known to possess 'Hageman-like' effects, which may be due to an intrinsic property of the urokinase molecule or to contamination with thromboplastic components of urine. Since it was not possible to separate the urokinase preparation into components with coagulative or fibrinolytic activity, it was thought that this evidence was suggestive though by no means conclusive evidence to support the former hypothesis. Further evidence in support of this hypothesis was provided by investigation of a batch of the same commercial preparation of urokinase which had been

further purified compared with the original batch. Both the more purified batch and the original batch were found to possess closely similar coagulant effects demonstrable as shortening of the recalcification time of fresh normal plasma.

Although it is probable at a clinical level that fibrinolytic activity may cancel out coagulative activity, the coagulative activity might predispose to thrombosis, more particularly to rethrombosis during therapy, and it would be desirable, if possible, to obtain a preparation free from such coagulant activity.

The Effect of Alimentary Lipaemia on Fibrinolysis, Blood Coagulation and Platelet Aggregation The object of the study reported in chapter 7 was to investigate the effect of alimentary lipaemia on the fibrinolytic enzyme system, Russell's viper venom ('Stypven') time and platelet aggregation, comparing findings after a high fat breakfast with those after a low fat breakfast. It has been suggested that raised plasma lipoprotein levels may move the possible equilibrium of clotting and lysis towards thrombosis by accelerating clotting or by inhibition of spontaneous fibrinolysis. If the deposition and persistence of intravascular fibrin is encouraged in this way by

raised plasma lipoprotein levels, this would afford one explanation for their well-established association with the development of ischaemic heart disease since Duguid (1955) has shown that persisting intravascular thrombus may be incorporated in the arterial wall leading to the formation of an atherosclerotic plaque.

In the study reported in chapter 7, 10 normal subjects were investigated on two occasions, once after a low fat breakfast and once after the same breakfast to which had been added 95 gm. of fat in the form of double cream. The effect of both types of breakfast on the fibrinolytic enzyme system was studied, using a variety of techniques to measure its several components. In particular, the lysis of Chandler thrombi by streptokinase in an artificial circulation was studied since this technique was thought to be more relevant to the in vivo effect of lipaemia on the fibrinolytic enzyme system than study of the lysis of whole blood or plasma clots. Platelet aggregation after both types of breakfast was studied; there is considerable evidence that platelet aggregation in vivo is an important early phase of thrombus formation. After both types of breakfast, lipid levels were measured and an attempt was made to correlate changes in their levels with any observed changes in

fibrinolytic and coagulant activity.

The results presented in chapter 7 indicate that during alimentary lipaemia, induced by ingestion of 95 gm. of fat, there was both a significant decrease in spontaneous plasma clot lysis activity (euglobulin lysis test) and increased resistance of plasma clots to lysis by urokinase. Possibly most important, there was an increased resistance of Chandler thrombi to lysis by streptokinase in an artificial circulation. Because plasma levels of plasminogen and fibrinogen were closely similar after each type of breakfast, it was concluded that the observed decrease in physiological fibrinolysis during alimentary lipaemia was not due to decrease in clot or thrombus plasminogen content or to increase in their concentration of fibrin.

After the high fat breakfast, 'Stypven' times were accelerated compared with findings after the low fat breakfast.

Inhibition of platelet aggregation was observed after the high fat breakfast. Platelet counts after the two types of breakfast did not differ significantly; accordingly relative thrombocytopenia was not responsible for the prolongation of platelet aggregation which was observed.

It is usually assumed that changes in fibrinolysis or coagulation following fat feeding are due to the associated lipaemia. However, although there were significant rises in triglyceride, phospholipid and free fatty acid levels following the high fat breakfast in every subject, no correlation was obtained between the actual levels or changes in the levels of any of these lipid components and the changes in fibrinolytic activity.

On the other hand, there was a close correlation between 'Stypven' times and triglyceride levels. A highly significant, negative correlation was found when 'Stypven' times after the two types of breakfast were plotted against their corresponding serum triglyceride levels expressed as logarithms. Similar correlations were found for the results following the low fat breakfast alone and following the high fat breakfast alone.

No correlations were found between 'Stypven' times and the levels of the other lipids which were measured.

It was considered that these correlations suggested that entry of triglyceride into the circulation during alimentary lipaemia might be important in bringing about acceleration of 'Stypven' times and that endogenously-derived, circulating triglyceride might also be important in influencing the results of the 'Stypven' time.

It was concluded that apart from the observed prolongation of platelet aggregation, the other effects of a fat meal appear to move the possible equilibrium of clotting and lysis towards thrombosis and that diminished speed of platelet aggregation might oppose the 'thrombosis-promoting' effects of lipaemia i.e. decreased fibrinolysis and accelerated clotting.

Studies of Six Defibrination Syndromes. The detailed investigation of six patients in whom the defibrination syndrome was diagnosed is described in chapter 8. In two patients, there was prolonged retention of a dead foetus; in three metastatic carcinoma and one arose in the course of thrombolytic therapy.

The reason for undertaking the detailed investigation of these patients was to illustrate the difficulty which may arise in interpreting the results of such investigations and in determining whether the main cause of defibrination is intravascular clotting or pathological fibrinolysis.

In the cases 1 and 2 in which defibrination was associated with prolonged retention of a dead foetus, there was depletion of prothrombin, factor V, antihaemophilic globulin (A.H.G.) and factor X. In both cases, the platelet count was normal. In case 1, there was plasminogen

depletion and in case 2, marked prolongation of the thrombin clotting time. The low plasminogen level in case 1 and the long thrombin clotting time in case 2 were thought to be evidence in favour of pathological fibrinolytic activity and accordingly the fibrinolytic inhibitor EACA was administered to these patients. In both cases, EACA failed to correct the coagulation defects and restoration to normal appeared to be a consequence of delivery. Because of failure of response to EACA, it was thought that defibrination in these patients had been due to primary intravascular clotting, plasminogen depletion in case 1 and the very long thrombin clotting time in case 2 being evidence for secondary pathological fibrinolysis. An explanation to account for the normal platelet counts was that the release of tissue thromboplastin into the circulation might be an intermittent phenomenon. Intravascular clotting might therefore have ceased some time before investigation and rapid regeneration of platelets in the marrow might have restored to normal the number of circulating platelets. The depletion of factor X was explained by its possible consumption during clotting in the presence of tissue thromboplastin since this has been shown to occur <u>in vitro</u>.

In case 3, a patient with defibrination associated with metastatic carcinoma of the breast, the initial investigation showed accelerated lysis of euglobulin clots, plasminogen depletion and prolongation of the thrombin clotting time. The platelet count was normal. This evidence was interpreted as indicating pathological fibrinolysis as the primary cause of defibrination. However, prolonged therapy with EACA was not associated with a rise in the fibrinogen level. Subsequent investigation revealed intermittent thrombocytopenia and that the levels of fibrinogen and plasminogen and the platelet count appeared to fluctuate as a consequence of the natural history of the patient's disease and not the medication with EACA. Although heparin was administered to this patient, its trial was inadequate and no conclusion could be drawn as to its possible value in the therapy of this patient. It was concluded that intravascular clotting was the primary cause of defibrination in this patient and that plasminogen depletion was evidence of secondary pathological fibrinolysis. An explanation for the accelerated euglobulin clot lysis in the absence of increased plasminogen activator activity in the fibrin plate test was provided by the in vitro experiment in which lysis of plasma clots, containing a standard amount of plasminogen and urokinase, was accelerated by reduced concentrations of fibrinogen. It was also shown in vitro that the thrombin clotting time was prolonged by reduced fibrinogen concentration per se. Although at post-mortem,

there was no evidence in this patient of intravascular fibrin formation, it was thought that this could be explained by lysis either ante-mortem or post-mortem.

In cases 4 and 5, defibrination was associated with depletion of factor V, antihaemophilic globulin (A.H.G.) and plasminogen and thrombocytopenia. Because of both plasminogen depletion and thrombocytopenia, in each case the diagnosis made was intravascular clotting with secondary fibrinolysis. In case 4, during a heparin infusion, a rise occurred in fibrinogen, factor V and antihaemophilic globulin (A.H.G.) and the platelet count remained steady. After withdrawal of heparin, their levels fell to those found before treatment. An infusion of Trasylol was administered but did not produce a rise in the fibrinogen level while a fall in the platelet count occurred. A second heparin infusion was administered and again rises occurred in the levels of fibrinogen, factor V and antihaemophilic globulin (A.H.G.). The response to both heparin infusions was good evidence in favour of its therapeutic value in this patient and supported the diagnosis of intravascular clotting as the primary cause of the defibrination syndrome.

In case 5, a Trasylol infusion produced rises in the levels of

fibrinogen, factor V, antihaemophilic globulin (A.H.G.) and in the platelet count. At post-mortem, intravascular fibrin formation was found in close association with tumour thus confirming the clinical diagnosis.

Case 6 was included because it was a defibrination syndrome due to a pure fibrinolytic state induced by administration of a urokinase infusion. It also illustrated the value of plasminogen assay in the investigation of acute haemostatic defects. Seventeen hours after the end of the infusion, plasminogen depletion was the only feature to suggest a recent brisk fibrinolytic state.

It was concluded that assay of plasminogen and a platelet count were the most useful tests in the investigation of defibrination syndromes, but that the former is a time consuming assay the result of which may not be available in an emergency and the latter may not always be found since if intravascular clotting has ceased temporarily at the time of investigation, rapid marrow regeneration of platelets may have restored their level in the circulation to normal. It may therefore be impossible to determine the main cause of defibrination, either intravascular clotting or pathological fibrinolysis or a combination of these two. It appears wise therefore to treat such

cases with transfusion of blood and possibly fibrinogen and administration of hydrocortisone and to reserve specific therapy, either a fibrinolytic inhibitor or heparin, for life-threatening situations where conservative measures have failed.

Conclusion

Availability of fibrinolytic activators and inhibitors as preparations suitable for administration to man has stimulated interest in the components and functions of the fibrinolytic enzyme system in health and disease and lead to a search for preparations with greater potency and fewer side effects than those currently available.

Part of the work for this thesis was concerned with the investigation of two substances, aminomethyl cyclohexane carboxylic acid and Trasylol, which were found to be more potent inhibitors of the fibrinolytic enzyme system than EACA, the currently available fibrinolytic inhibitor.

Another portion of the work involved investigation of a preparation of urokinase, the plasminogen activator present in human urine. Urokinase, unlike streptokinase which is the most commonly employed fibrinolytic activator, is non-antigenic but earlier preparations

have been shown to possess thromboplastic activity. The preparation investigated in the work for this thesis was found to be less thromboplastic than earlier preparations.

Finally, two aspects of the function of fibrinolysis in disease were studied. The effect of high plasma lipid levels in spontaneous fibrinolysis was examined and found to be inhibitory and the role of pathological fibrinolysis in the development of defibrination syndromes was studied.

There remains much to be learned before the clinical uses of fibrinolytic activators and inhibitors are clearly defined and the role of the fibrinolytic enzyme system in health and disease is precisely understood.

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PLASMINOGEN ACTIVATION AND ITS INHIBITION

by

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Volume 2

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FIGURES

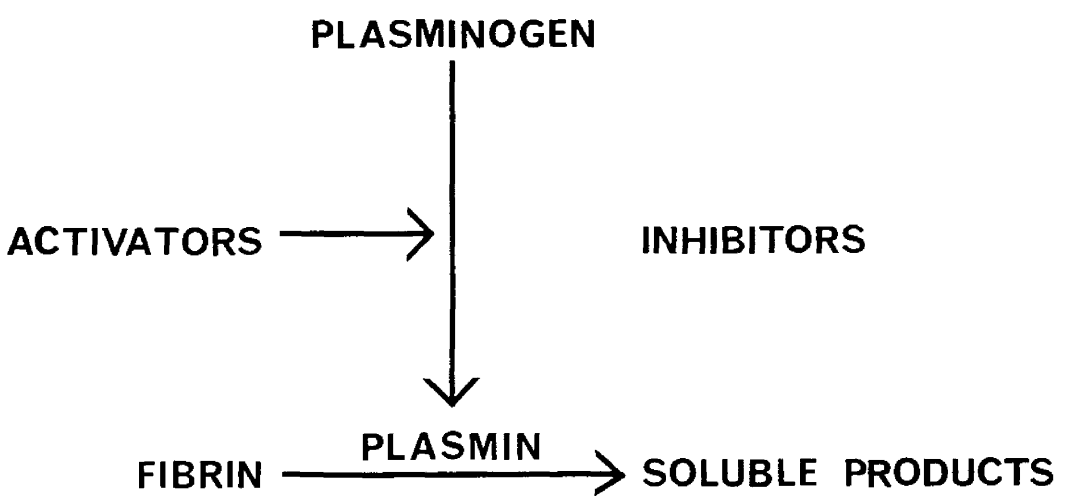


Figure 1 shows the principal components of the fibrinolytic enzyme system.

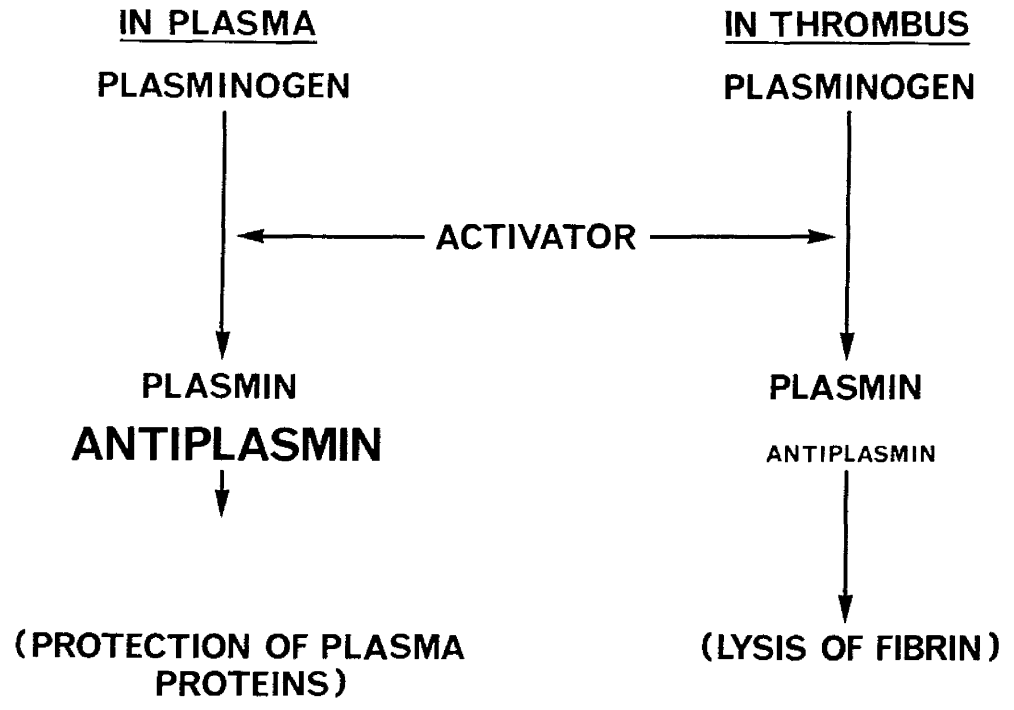
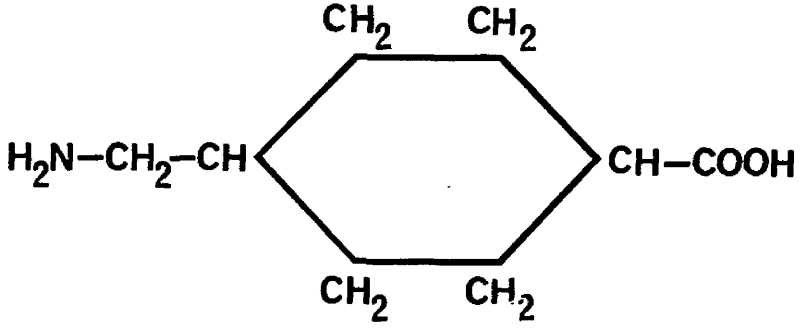


Figure 2 illustrates schematically the dual-phase concept of physiological plasminogen activation *in vivo* postulated by Sherry et al (1959a).

AMCHA



(AMINO–METHYL–CYCLOHEXANE CARBOXYLIC ACID)

EACA

$H_2N-CH_2.CH_2.CH_2.CH_2.CH_2.COOH$

(EPSILON–AMINO CAPROIC ACID)

Figure 3 shows the structural formulae of aminomethyl cyclohexane carboxylic acid (AMCHA) and epsilon aminocaproic acid (EACA).

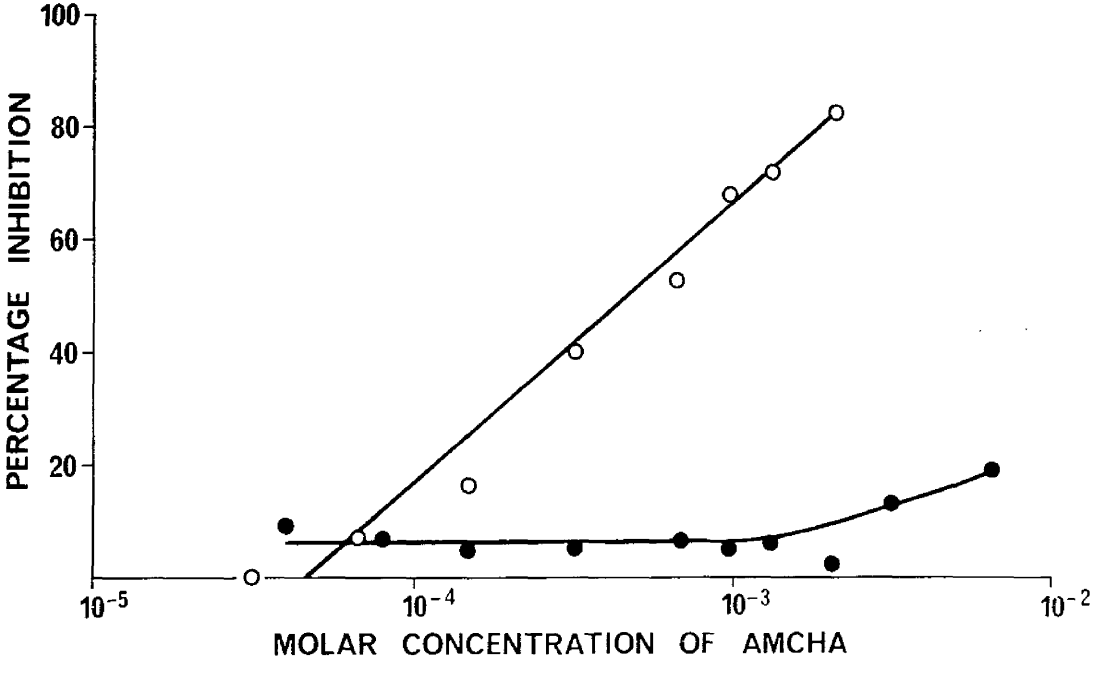


Figure 4 shows the effect in a caseinolytic assay system of AMCHA added to bank plasma before (o) and after (•) streptokinase. The horizontal scale is logarithmic (cycles x 3). The figure is compiled from the data of table 2.

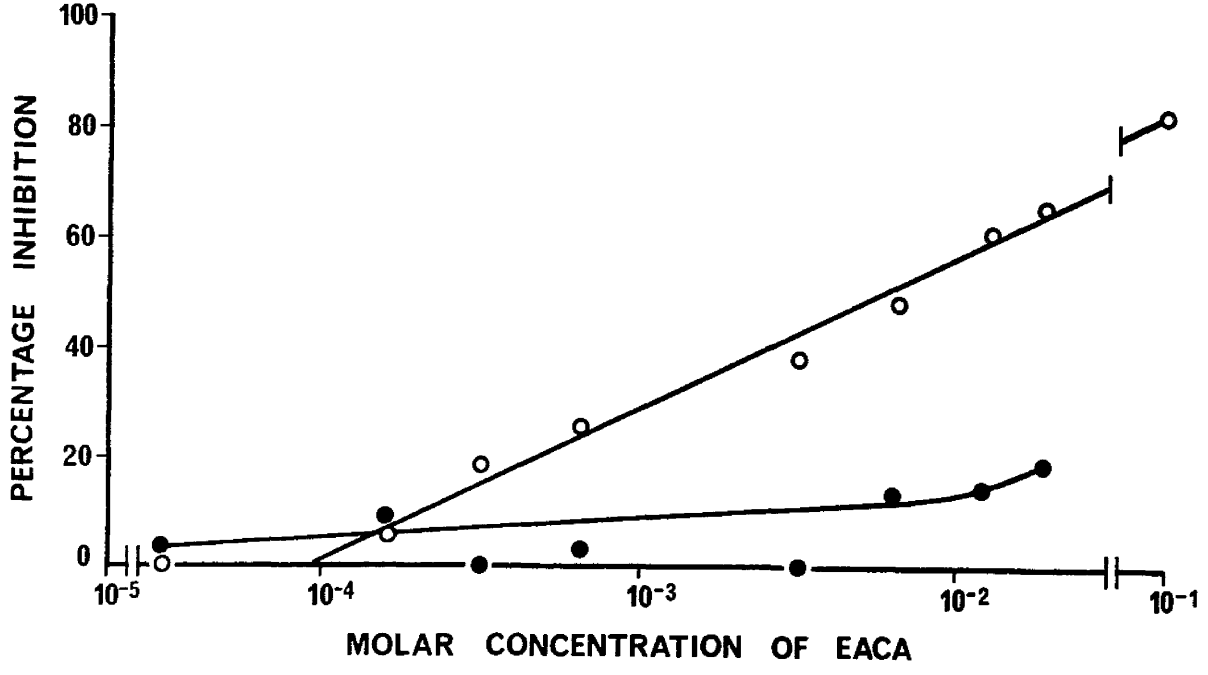


Figure 5 shows the effect in a caseinolytic assay system of EACA added to bank plasma before (o) and after (•) streptokinase. The horizontal scale is logarithmic (cycles x 4) The figure is compiled from the data of table 3.

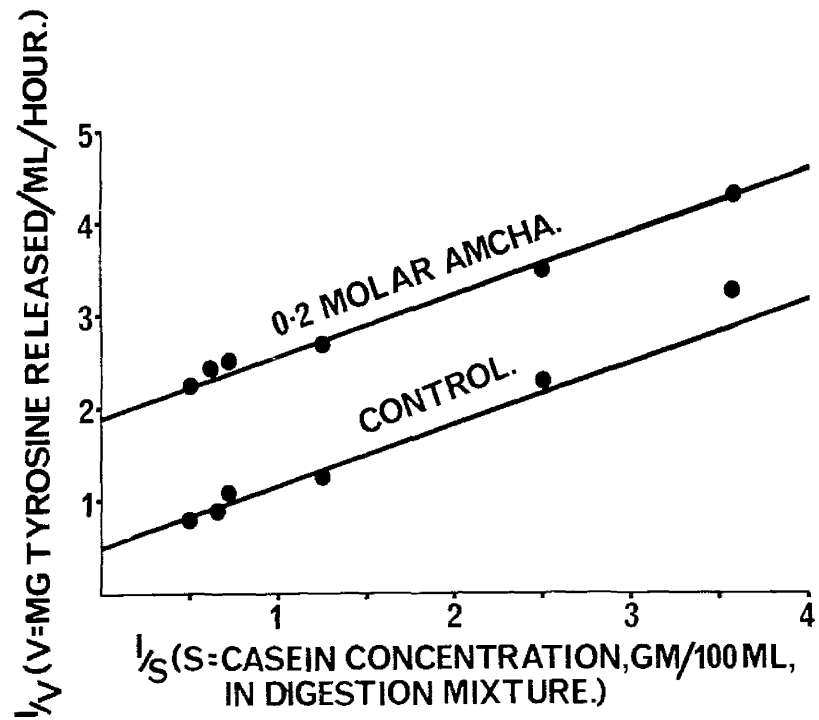


Figure 6 shows Lineweaver-Burk plots demonstrating that AMCHA is an uncompetitive inhibitor of plasmin. The plots are constructed from the data of table 4.

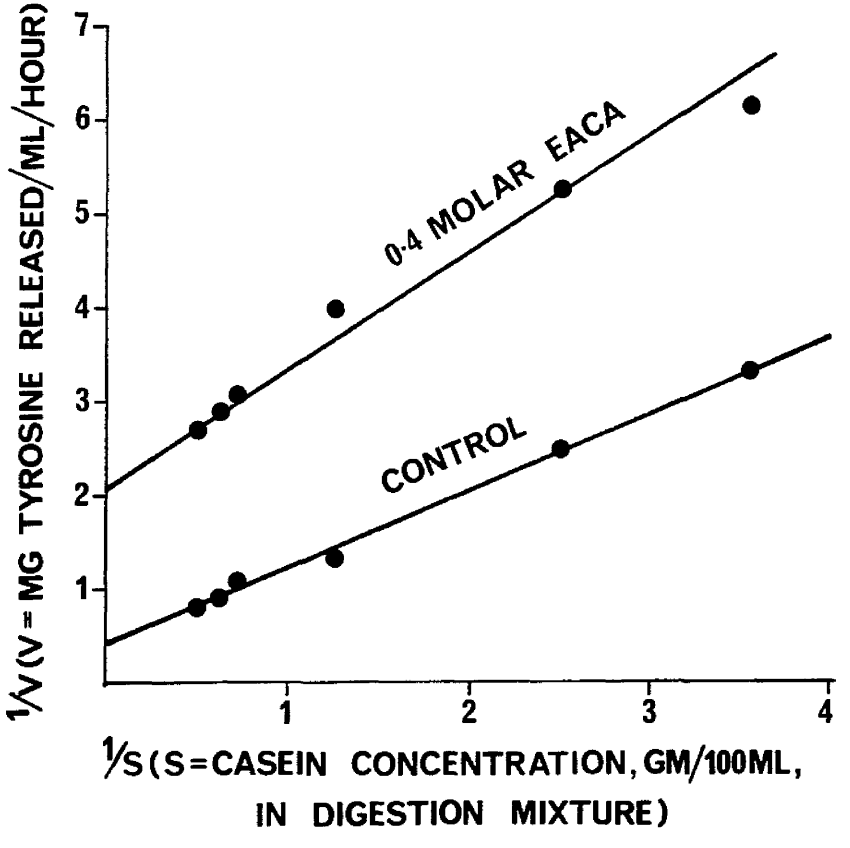


Figure 7 shows Lineweaver-Burk plots demonstrating that EACA is a non-competitive inhibitor of plasmin. The plots are constructed from the data of table 4.

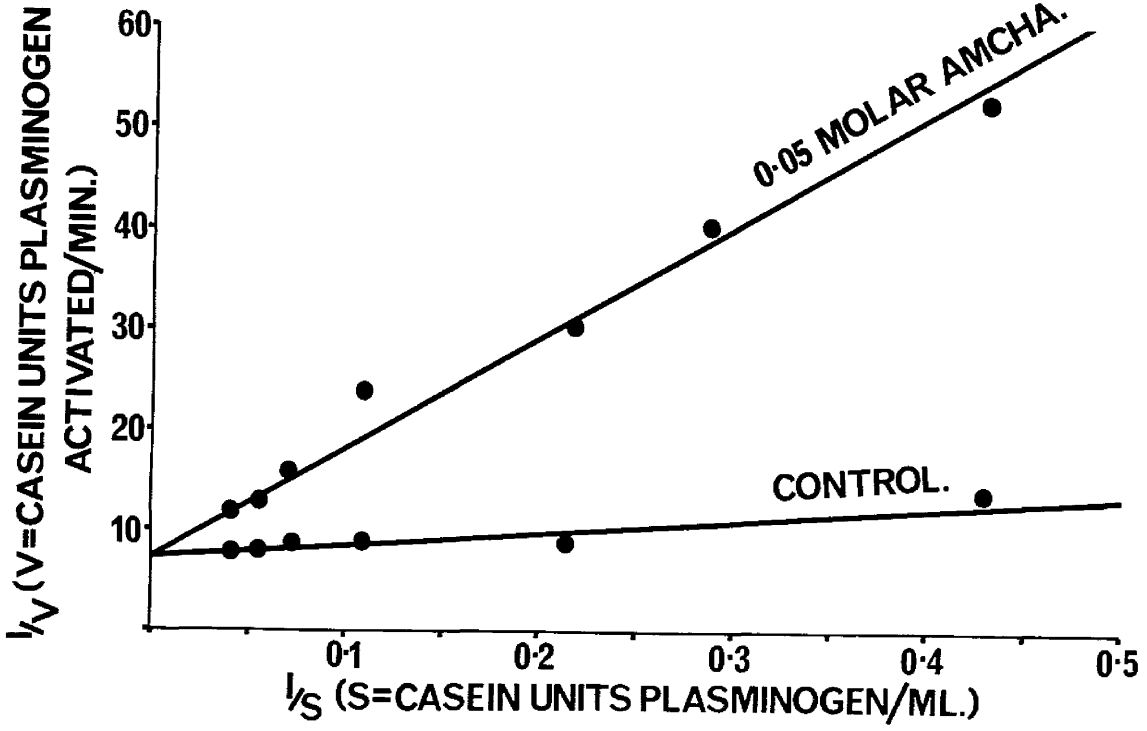


Figure 8 shows Lineweaver-Burk plots demonstrating that AMCHA is a competitive inhibitor of plasminogen activation. The plots are constructed from the data of table 5.

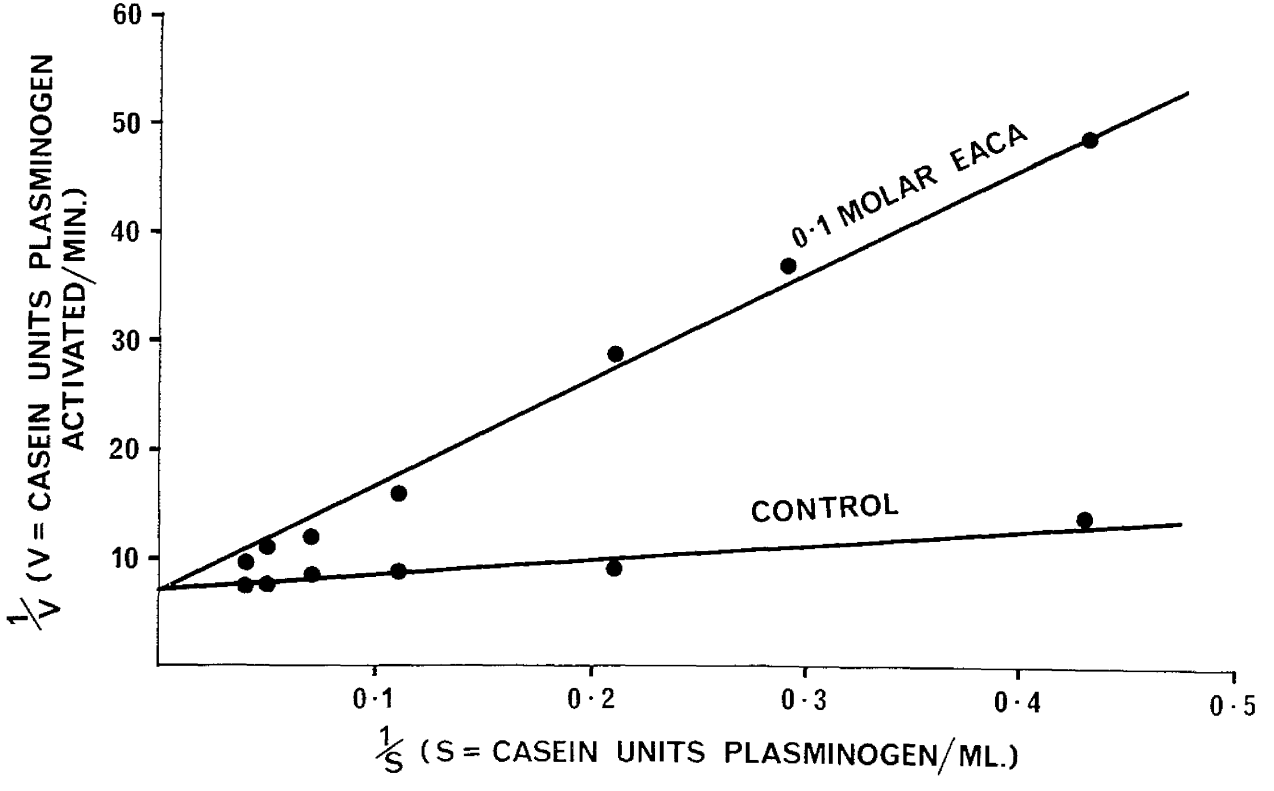


Figure 9 shows Lineweaver-Burk plots demonstrating that EACA is a competitive inhibitor of plasminogen activation. The plots are constructed from the data of table 5.

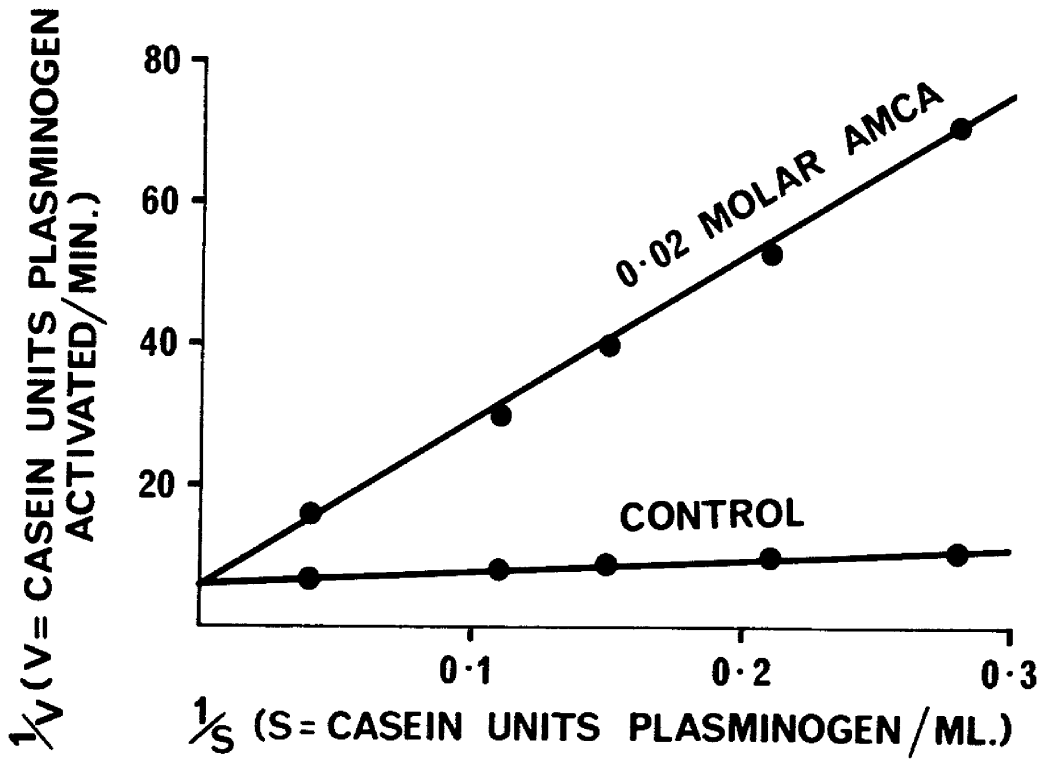


Figure 10 shows Lineweaver-Burk plots demonstrating that AMCHA is a competitive inhibitor of plasminogen activation. The plots are constructed from the data of table 7.

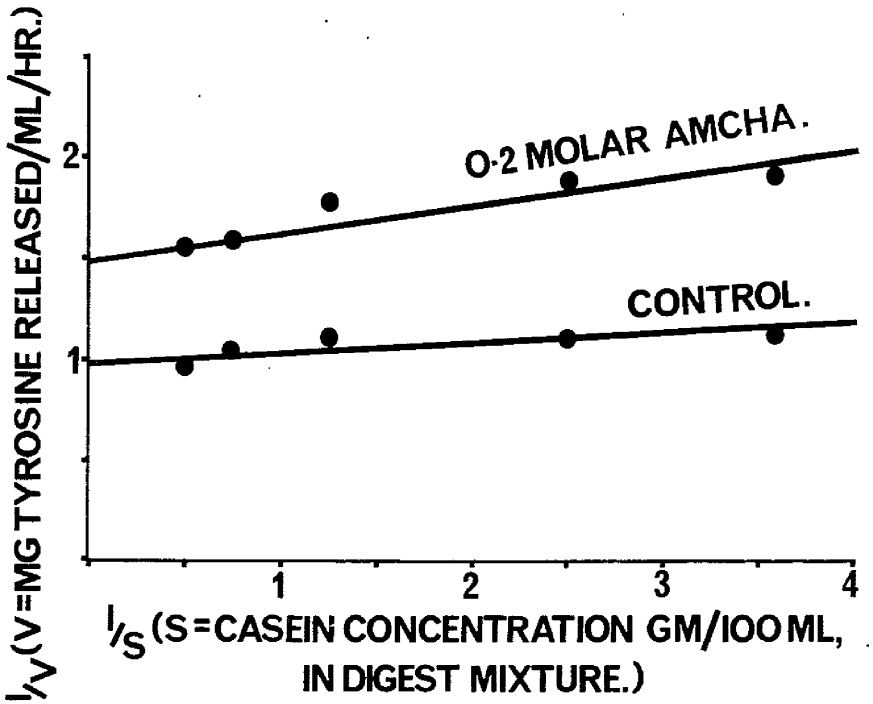


Figure 11 shows Lineweaver-Burk plots demonstrating that AMCHA is a non-competitive inhibitor of trypsin. The plots are constructed from the data of table 8.

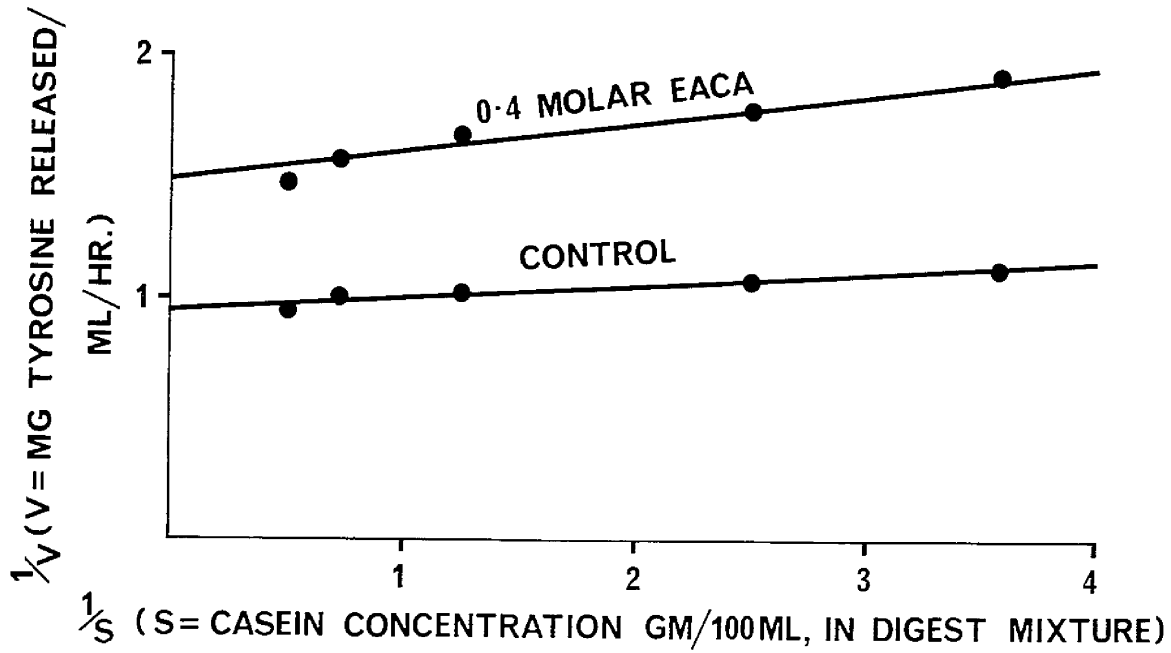


Figure 12 shows Lineweaver-Burk plots demonstrating that EACA is a non-competitive inhibitor of trypsin. The plots are constructed from the data of table 8.

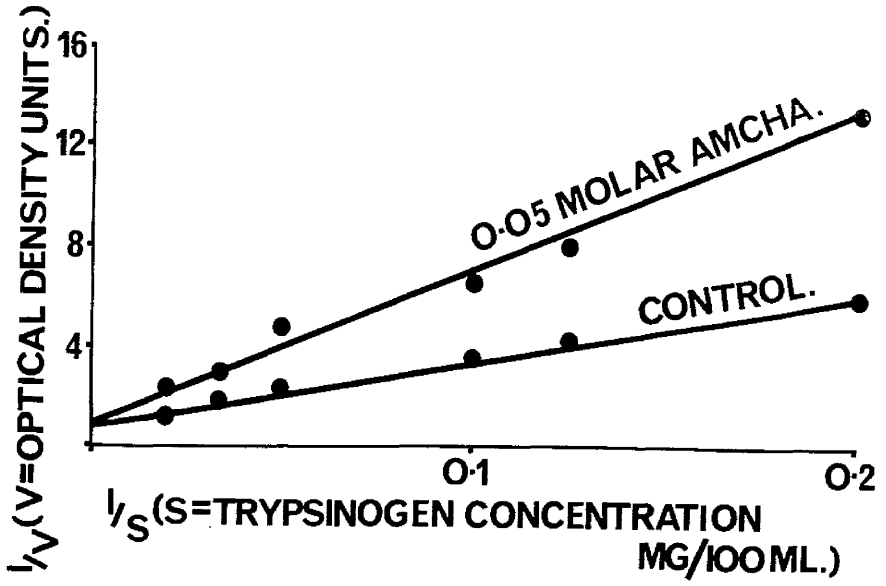


Figure 13 shows Lineweaver-Burk plots demonstrating that AMCHA is a competitive inhibitor of trypsinogen activation. The plots are constructed from the data of table 9.

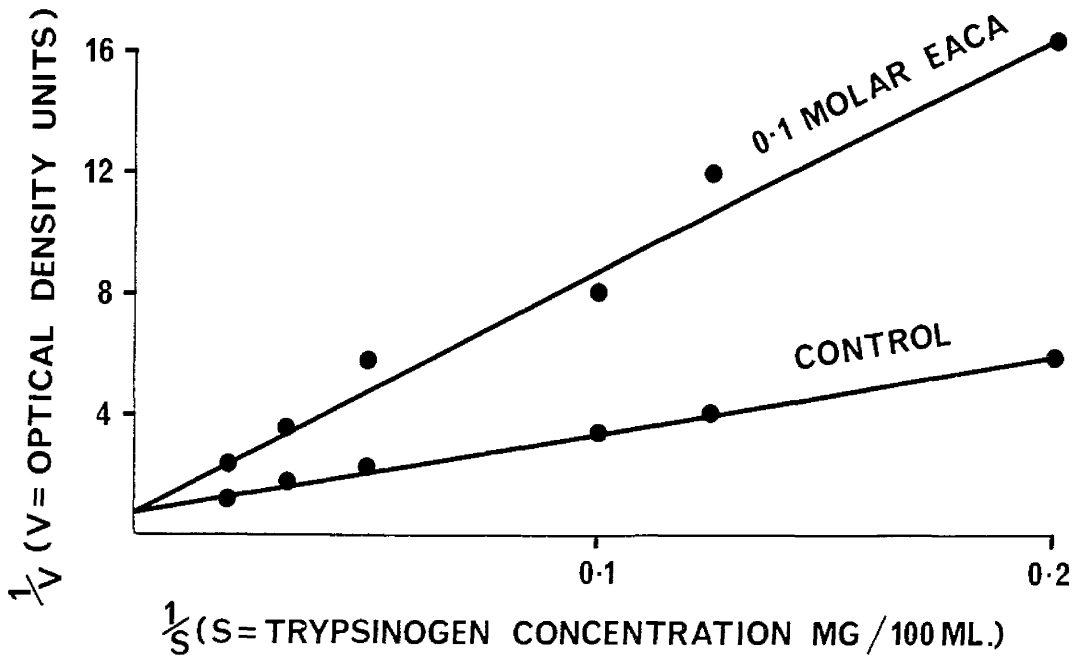


Figure 14 shows Lineweaver-Burk plots demonstrating that EACA is a competitive inhibitor of trypsinogen activation. The plots are constructed from the data of table 9.

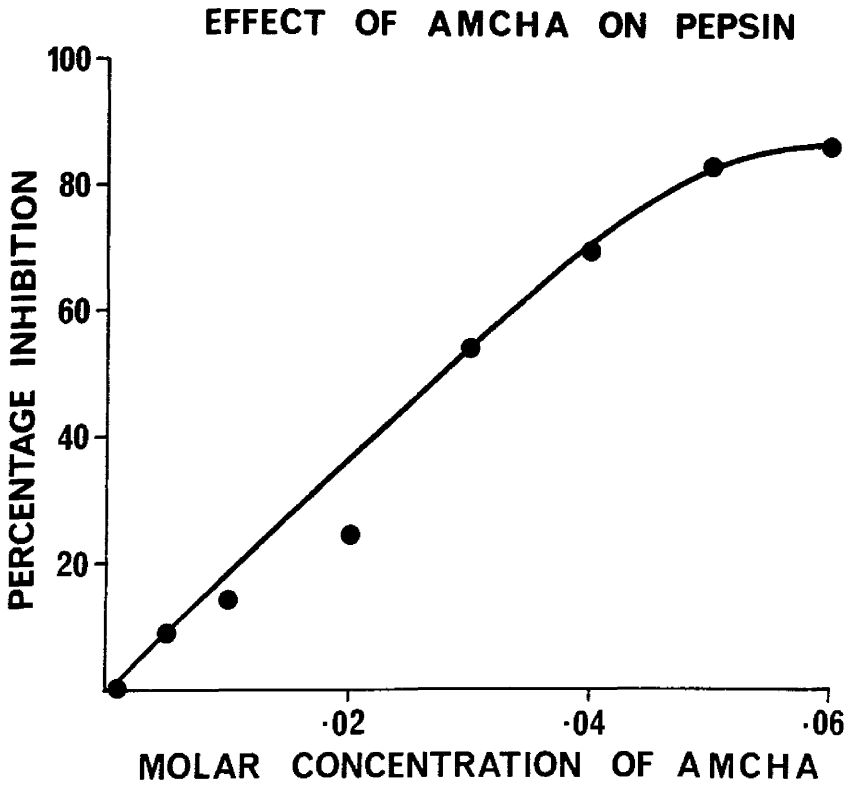


Figure 15 shows the inhibitory effect of AMCHA on pepsin. The figure is prepared from the data of table 11.

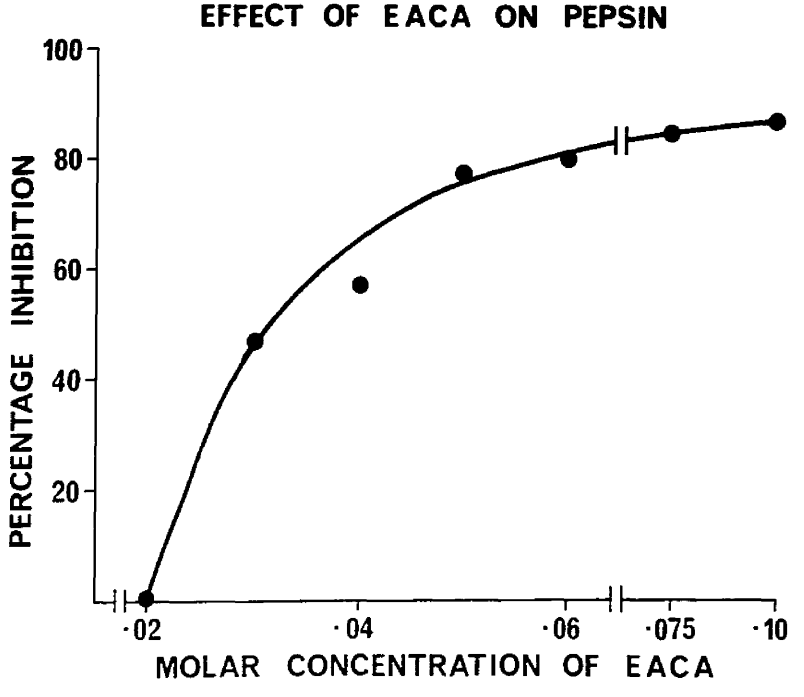


Figure 16 shows the inhibitory effect of EACA on pepsin. The figure is prepared from the data of table 11.

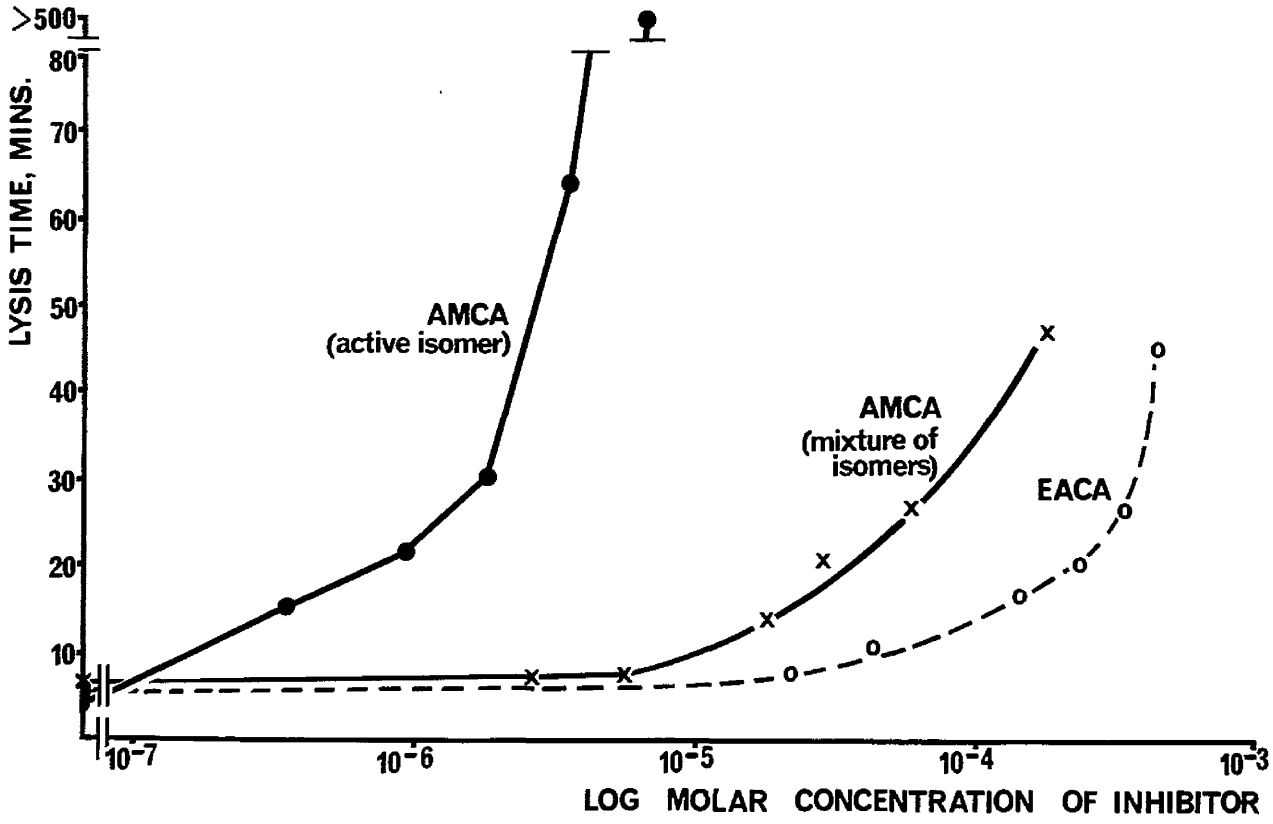


Figure 17 shows a comparison of the antifibrinolytic effect of the active isomer (AMCA), of the naturally occurring mixture of isomers (AMCHA), and of EACA in an in vitro system - the urokinase sensitivity test with bank plasma as substrate. The horizontal scale is in four logarithmic sycles. The figure is prepared from the data of table 12.

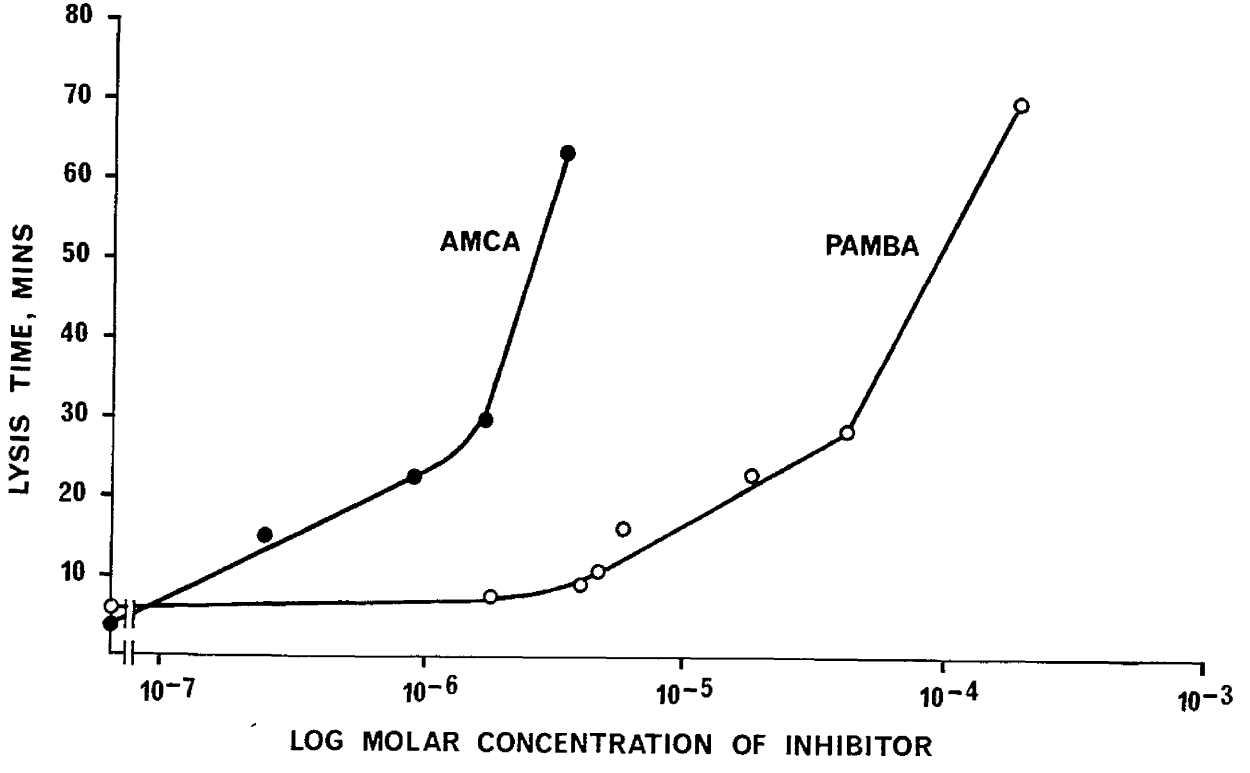


Figure 18 shows a comparison of the antifibrinolytic effect of AMCA and of PAMBA in an <u>in vitro</u> system - the urokinase sensitivity test with bank plasma as substrate. The horizontal scale is in four logarithmic cycles. The figure is prepared from the data of table 13.

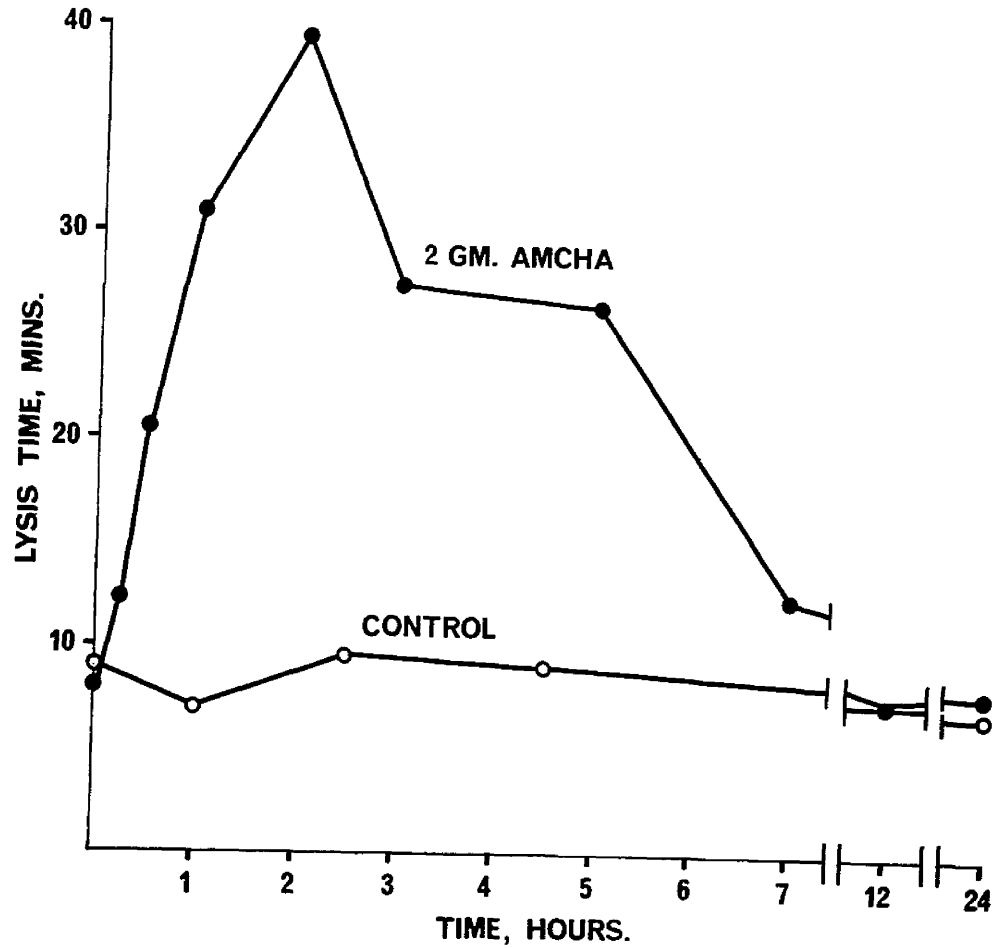


Figure 19 shows the effect over a 24 hour period on the urokinase sensitivity test of a normal subject, of ingestion of 2 gm. AMCHA. The figure is prepared from some of the data of table 16.

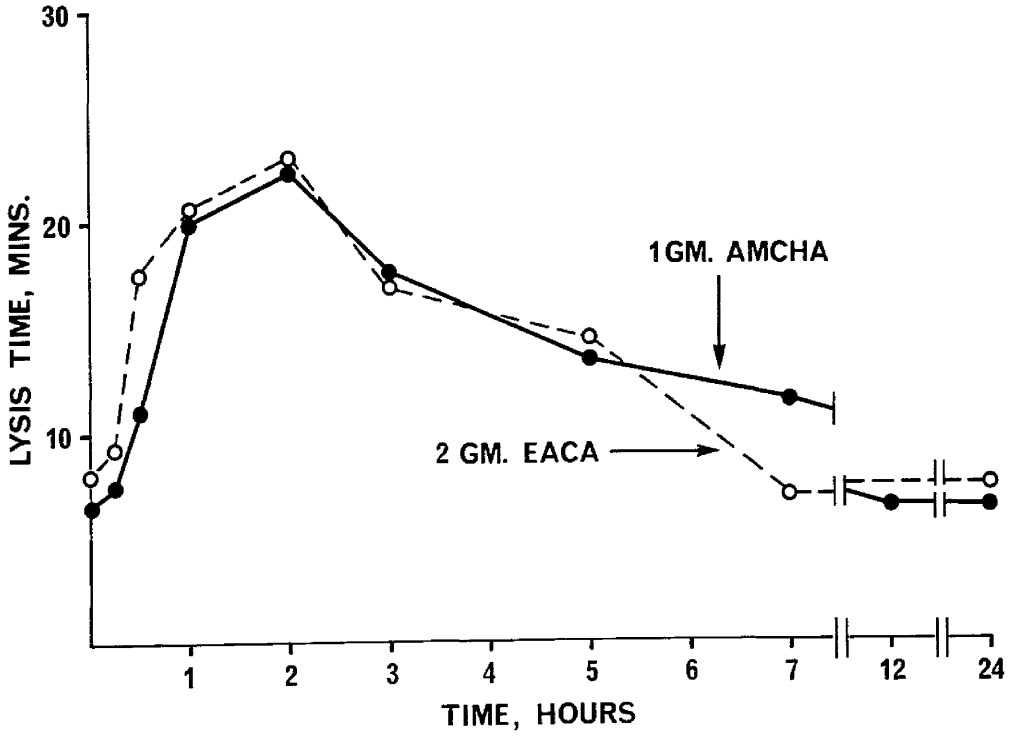


Figure 20 shows a comparison of the inhibitory effect of 1 gm AMCHA and 2 gm EACA, on the urokinase sensitivity test of a normal subject who ingested each compound on different days. The figure is prepared from the data of table 17.

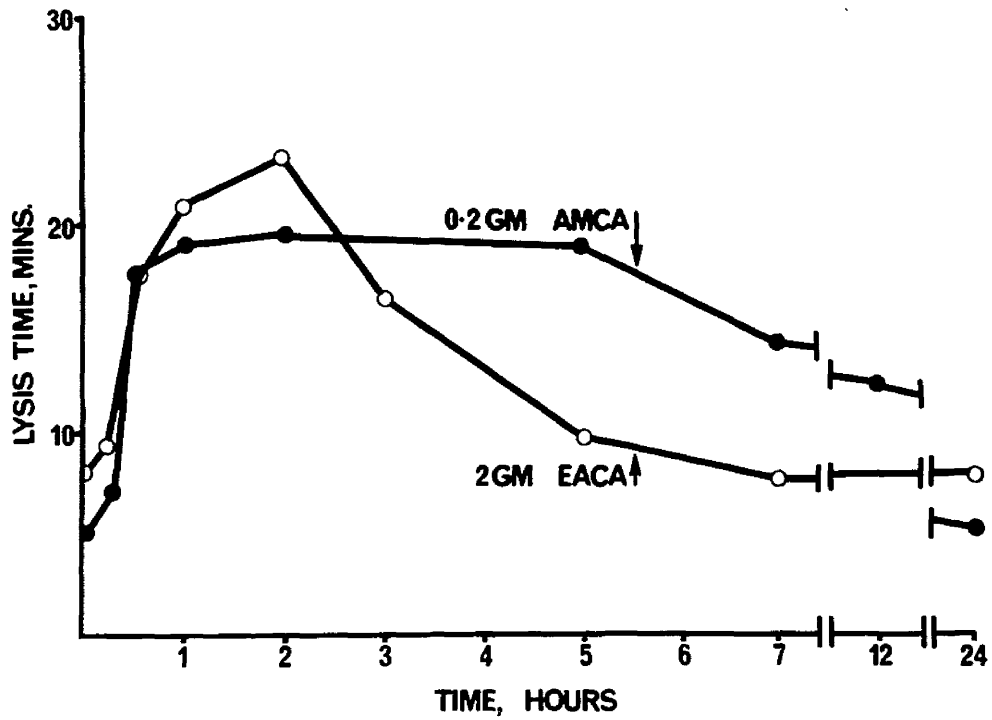


Figure 21 shows a comparison of the inhibitory effect of 200 mg AMCA and 2 gm EACA, on the urokinase sensitivity test of a normal subject who ingested each compound on different days. The figure is prepared from the data of table 19.

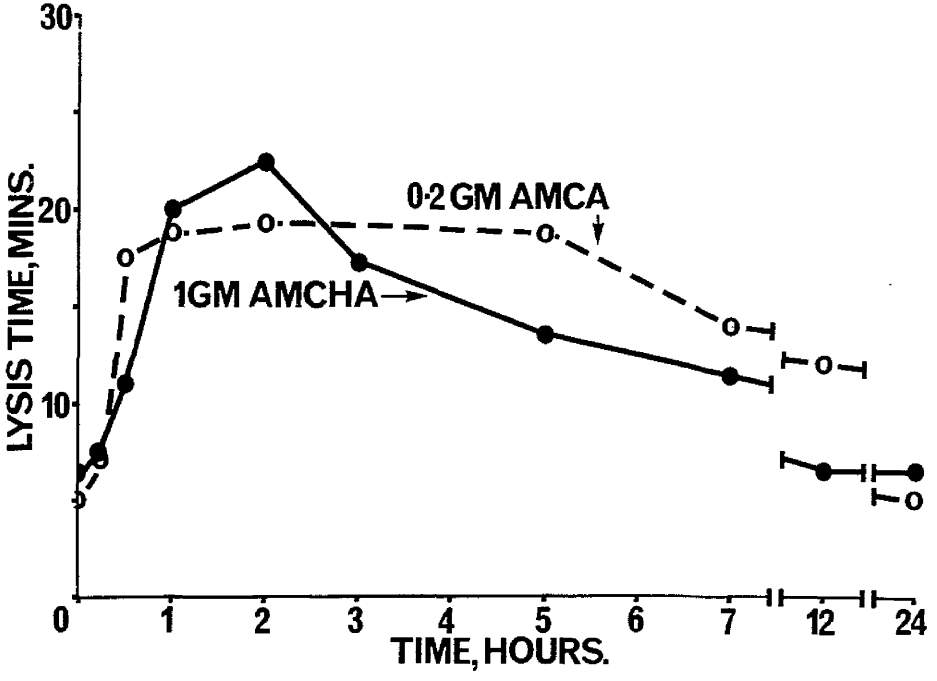


Figure 22 shows a comparison of the inhibitory effect of 200 mg AMCA and 1 gm AMCHA, on the urokinase sensitivity test of a normal subject who ingested each preparation on different days. The figure is prepared from the data of table 20.

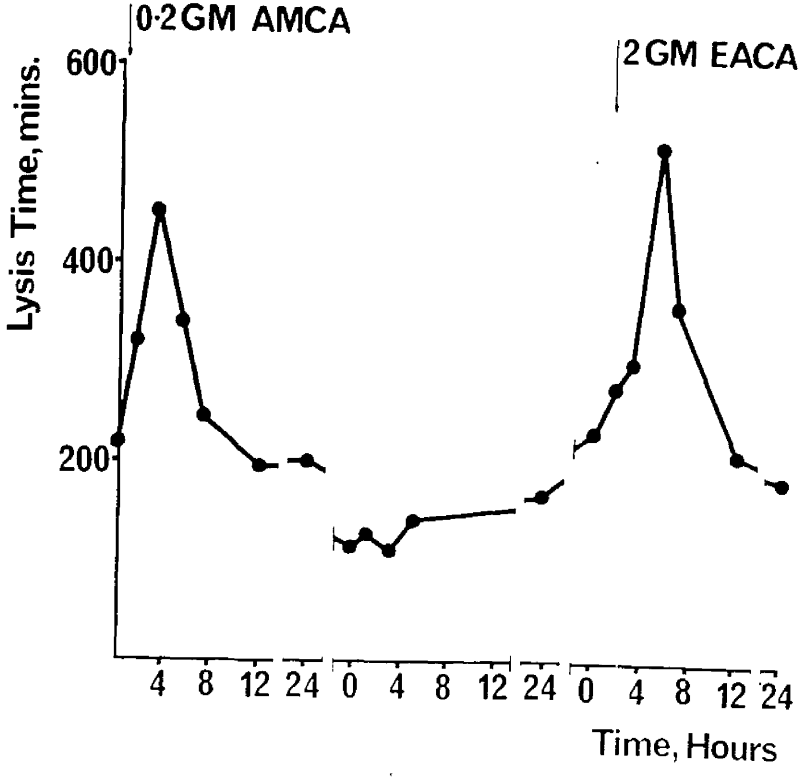


Figure 23 shows a comparison, in the Fearnley dilute plasma-clot lysis test, of the antifibrinolytic effect of 200 mg AMCA and 2 gm EACA. Each compound was ingested on different days by a patient in whom there was evidence of pathological fibrinolysis. The findings on an intervening day when no fibrinolytic inhibitor was given are also shown. The figure is prepared from the data of table 22.

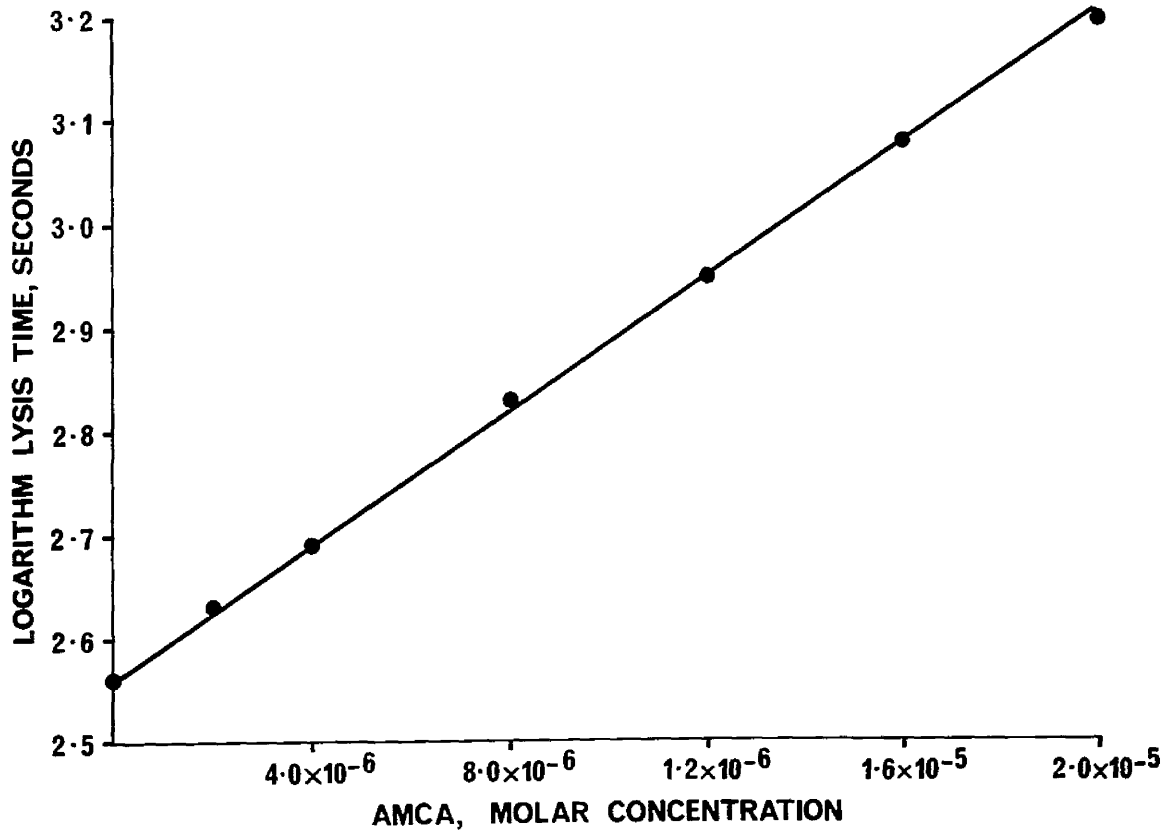


Figure 24 shows the control curve from which were derived plasma levels of AMCA in one subject who ingested 200 mg AMCA. The curve is prepared from the data of table 23.

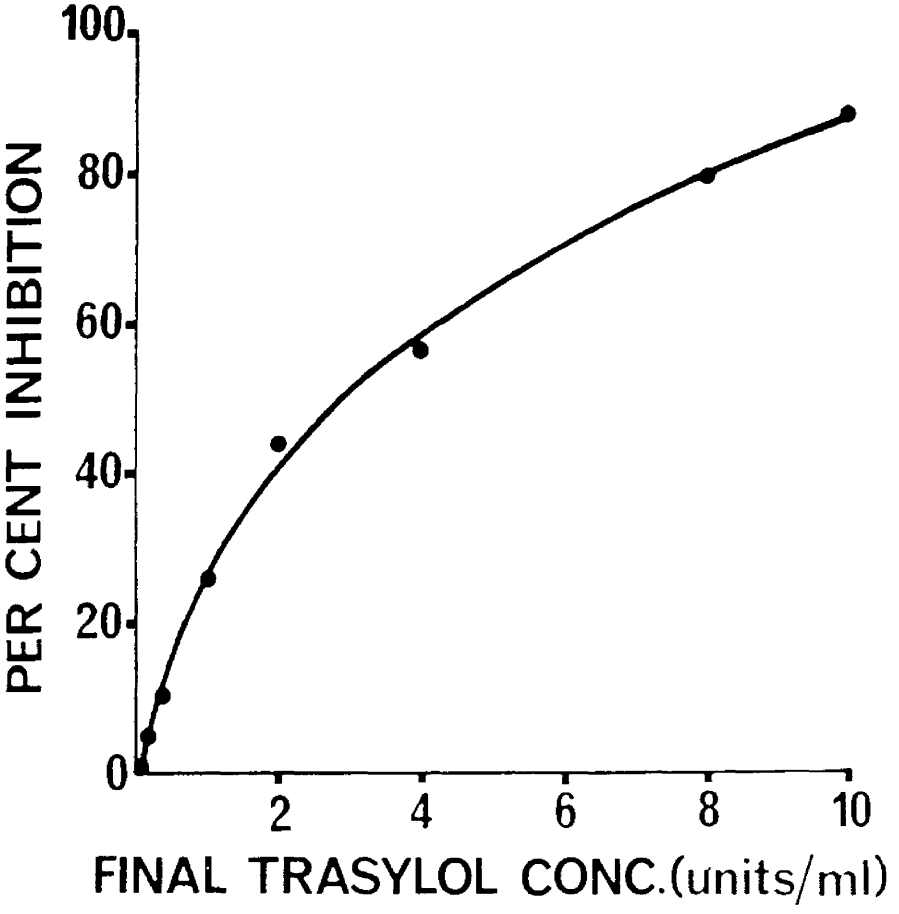


Figure 25 shows the effect in a caseinolytic assay system of Trasylol added to bank plasma before streptokinase-activation. The figure is constructed from the data of table 24.

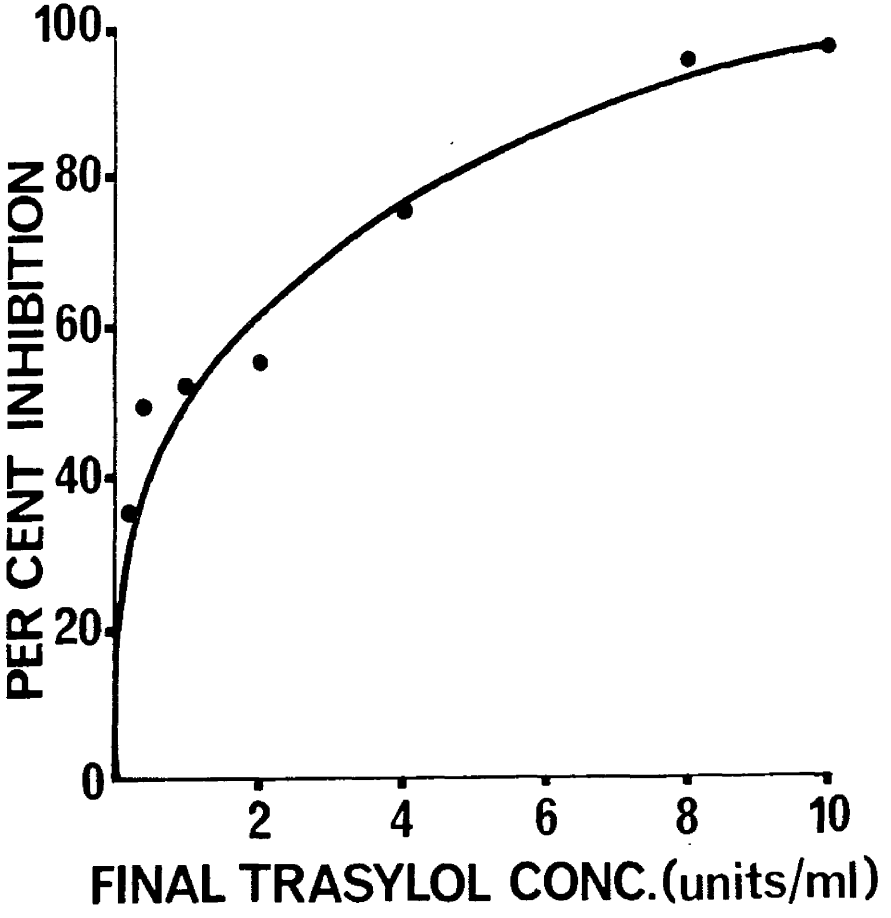


Figure 26 shows the effect in a caseinolytic assay system of Trasylol added to bank plasma after streptokinase-activation. The figure is constructed from the data of table 24.

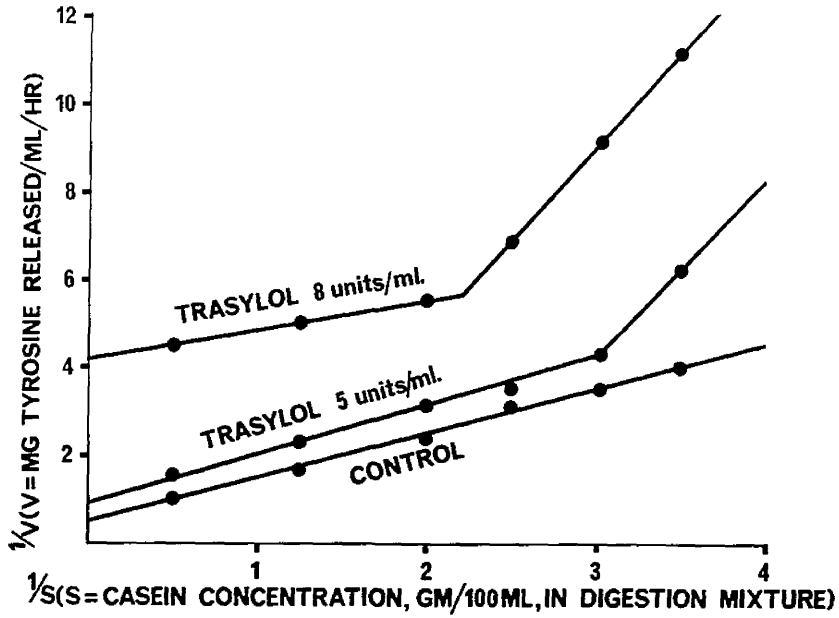


Figure 27 shows Lineweaver-Burk plots demonstrating the inhibitory effect of Trasylol on the proteolytic activity of plasmin using a sevenfold variation in substrate concentration. The figure is constructed from the data of table 25.

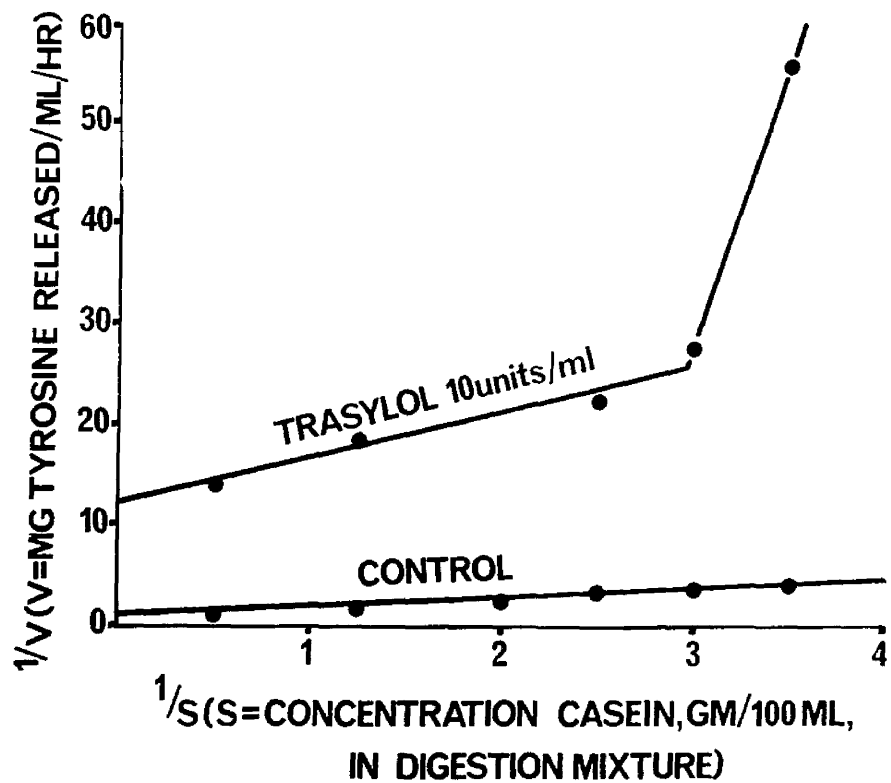


Figure 28 shows Lineweaver-Burk plots demonstrating the inhibitory effect of Trasylol on the proteolytic activity of plasmin using a sevenfold variation in substrate concentration. The figure is constructed from the data of table 25.

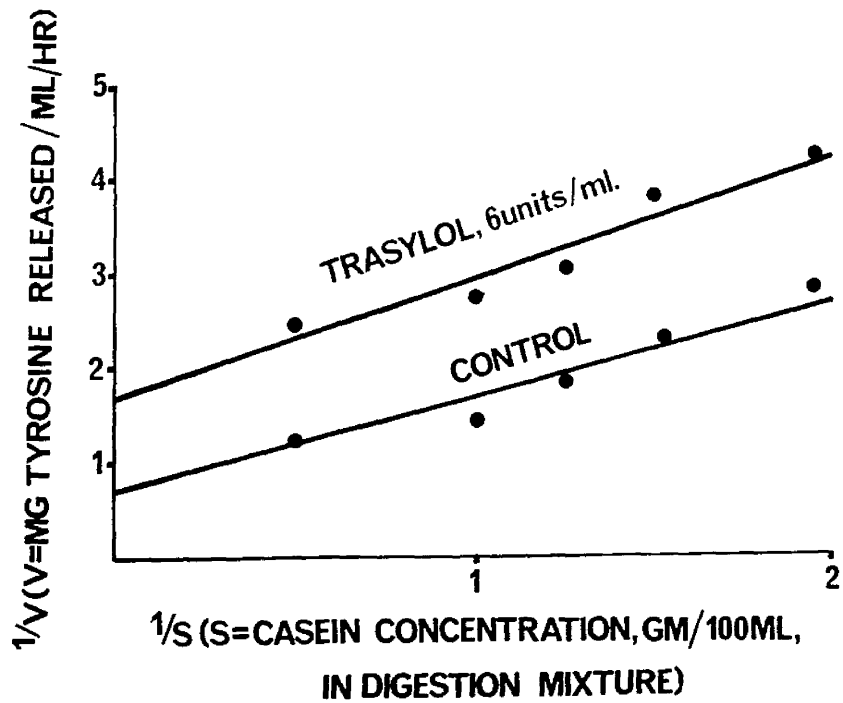


Figure 29 shows Lineweaver-Burk plots demonstrating that Trasylol is a non-competitive inhibitor of the proteolytic activity of plasmin. A fourfold variation in substrate concentration was used. The figure is constructed from the data of table 25.

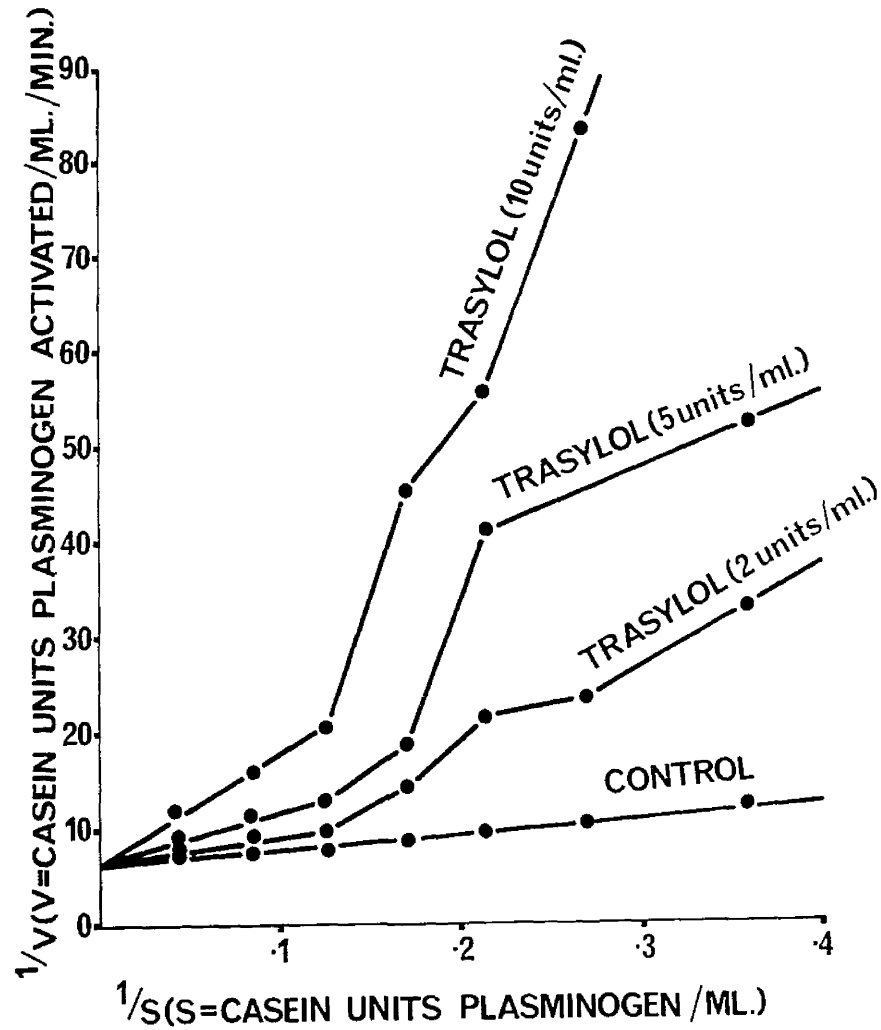


Figure 30 shows Lineweaver-Burk plots demonstrating the inhibitory effect of Trasylol on plasminogen activation by streptokinase using an eightfold variation in substrate concentration. The figure is constructed from the data of table 27.

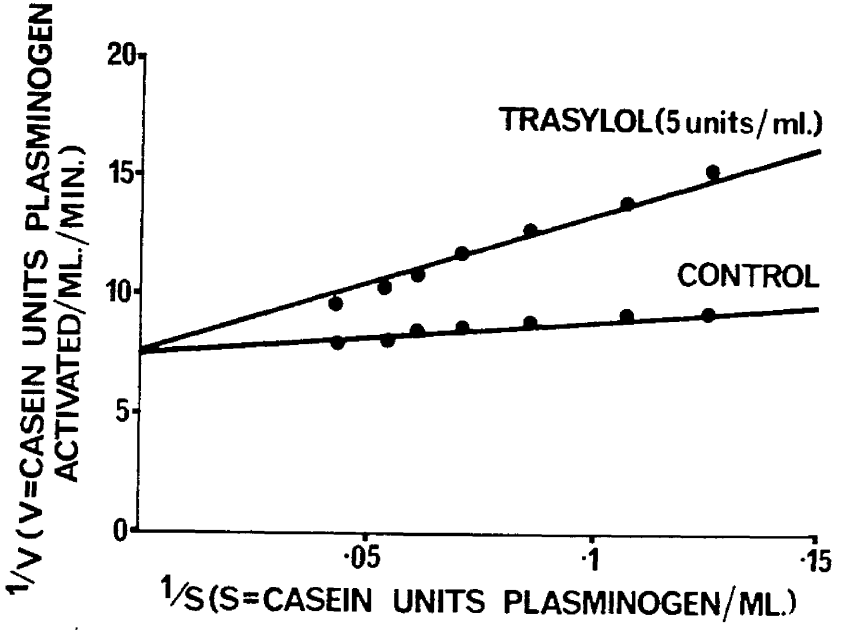


Figure 31 shows Lineweaver-Burk plots demonstrating that Trasylol is a competitive inhibitor of plasminogen activation by streptokinase. A threefold variation in substrate concentration was used. The figure is constructed from the data of table 28.

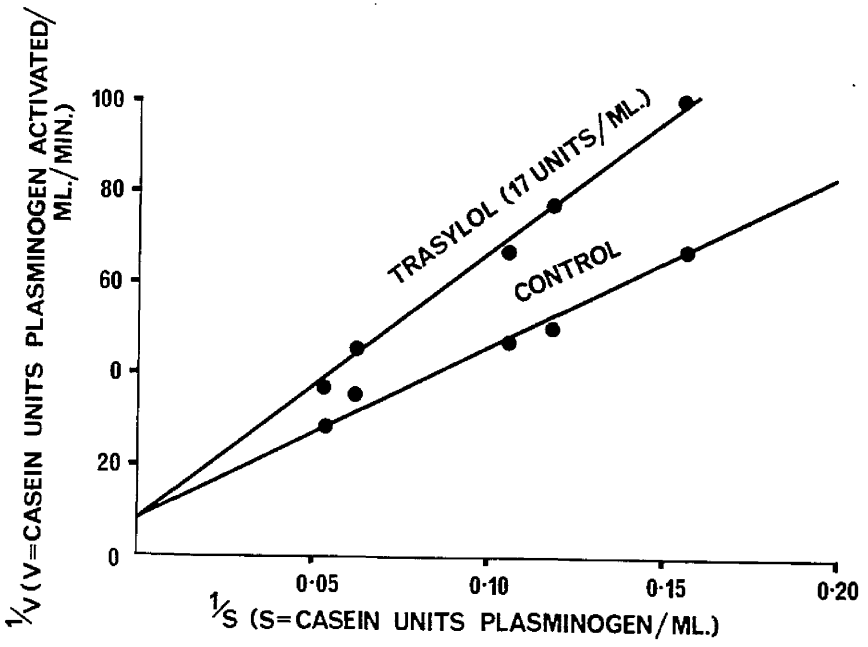


Figure 32 shows Lineweaver-Burk plots demonstrating that Trasylol is a competitive inhibitor of plasminogen activation by streptokinase. A three-fold variation in substrate concentration was used. In this experiment, Trasylol was removed by dialysis and streptokinase denatured or inactivated; the plasmin previously formed by activation of plasminogen in the presence of Trasylol was then assayed. The figure is constructed from the data of table 29.

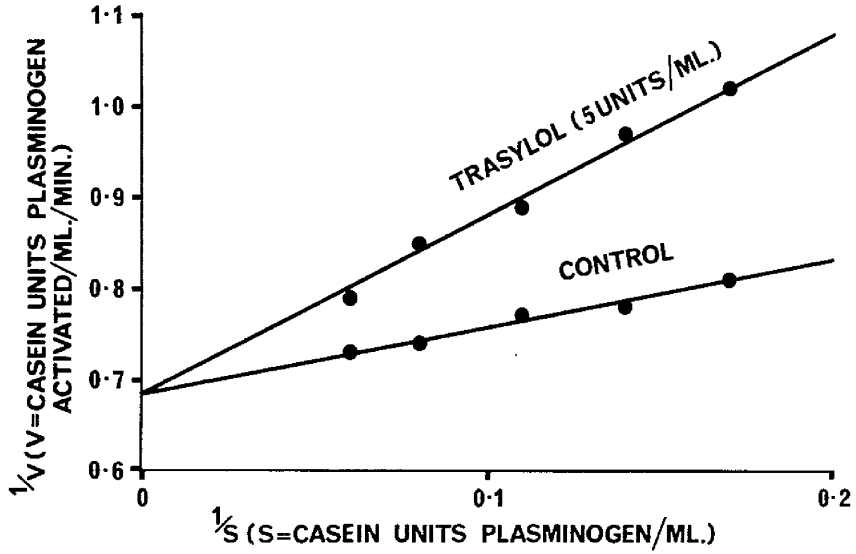


Figure 33 shows Lineweaver-Burk plots demonstrating that Trasylol is a competitive inhibitor of plasminogen activation by urokinase. A three-fold variation in substrate concentration was used. The figure is constructed from the data of table 30.

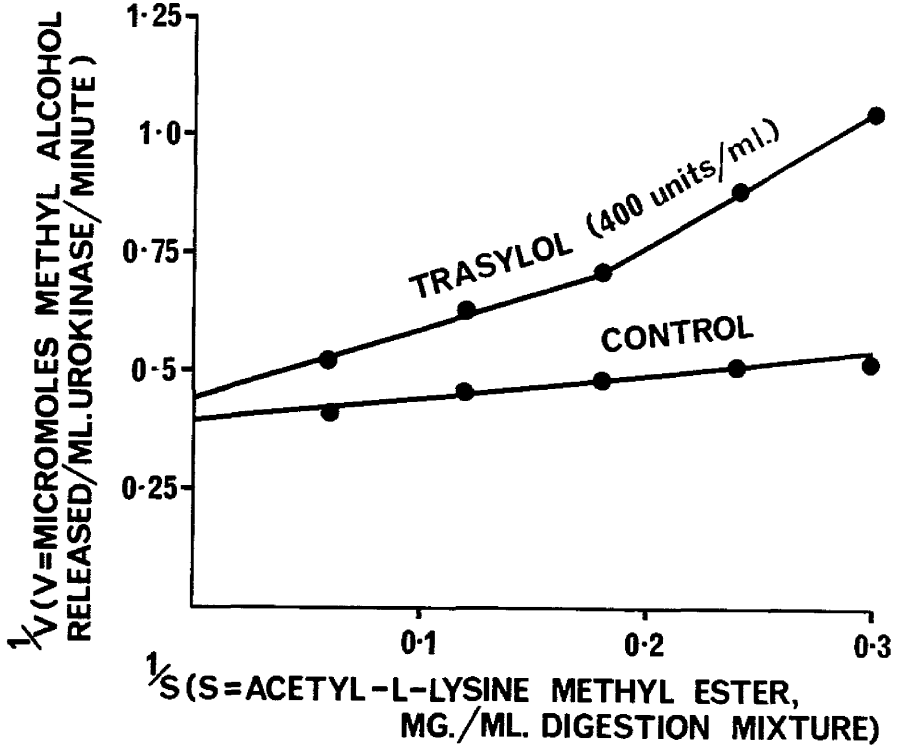


Figure 34 shows Lineweaver-Burk plots demonstrating the inhibitory effect of Trasylol on the esterolytic activity of urokinase. A fivefold variation in substrate concentration was used. The figure is constructed from the data of table 31.

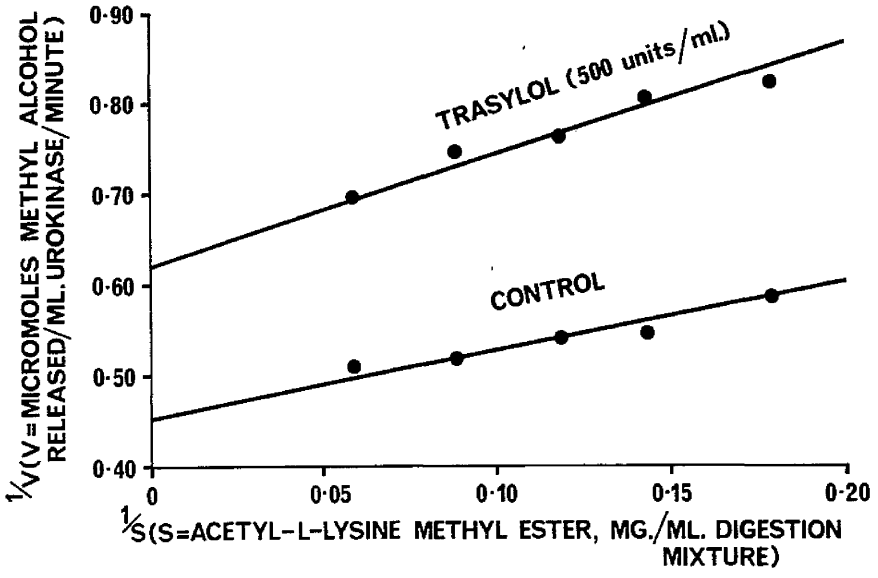


Figure 35 shows Lineweaver-Burk plots demonstrating that Trasylol is a 'mixed' competitive and non-competitive inhibitor of the esterolytic activity of urokinase. A threefold variation in substrate concentration was used. The figure is compiled from the data of table 32.

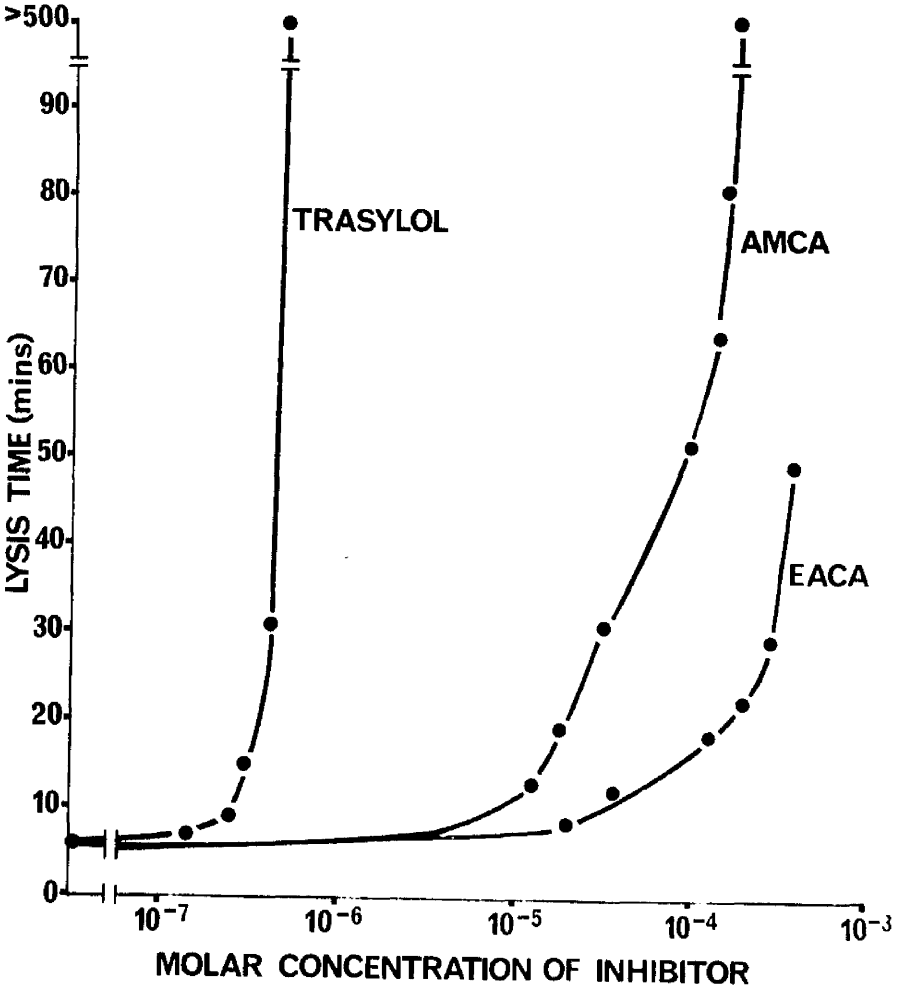


Figure 36 shows a comparison of the antifibrinolytic effect of Trasylol, AMCA and EACA in an <u>in vitro</u> system - the urokinase sensitivity test with bank plasma as substrate. The horizontal scale is in four logarithmic cycles. The figure is prepared from data of table 33.

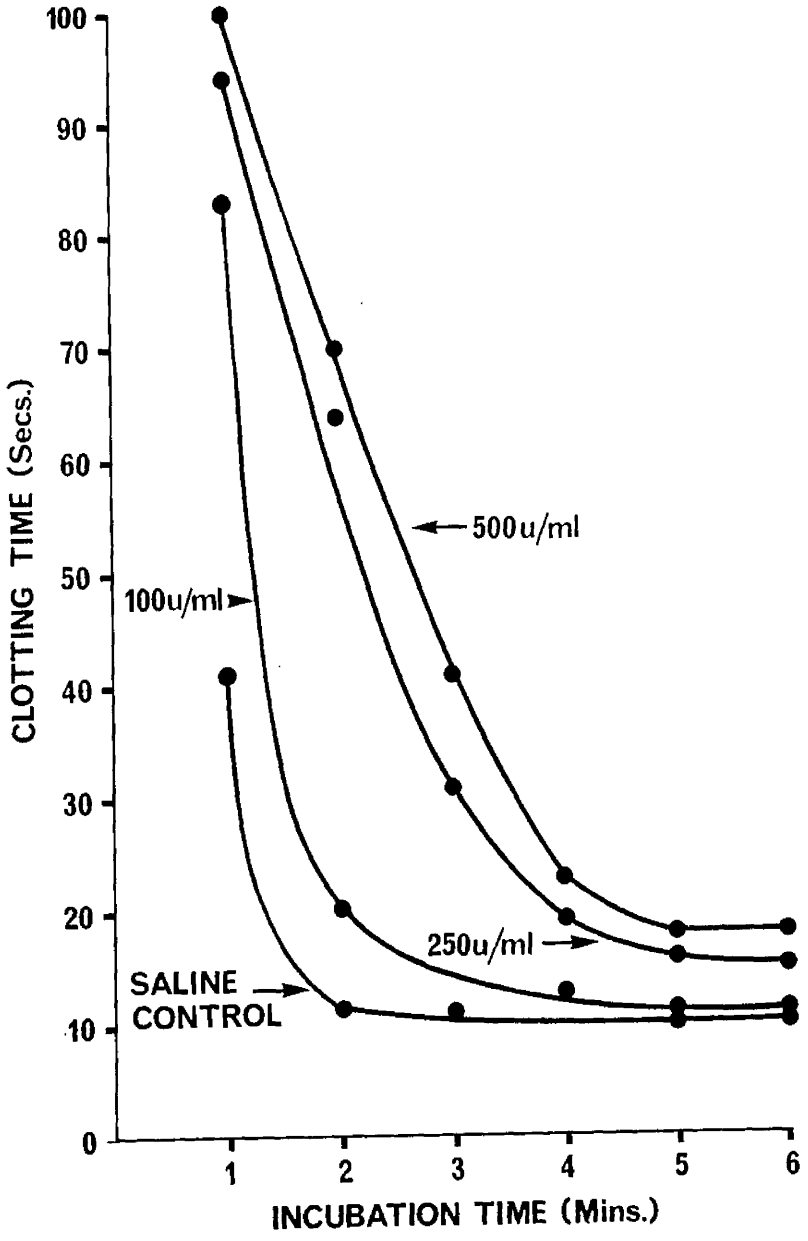


Figure 37 shows the effect of Trasylol <u>in vitro</u> in the incubation mixture of the thromboplastin generation test. The figure is prepared from the data of table 35.

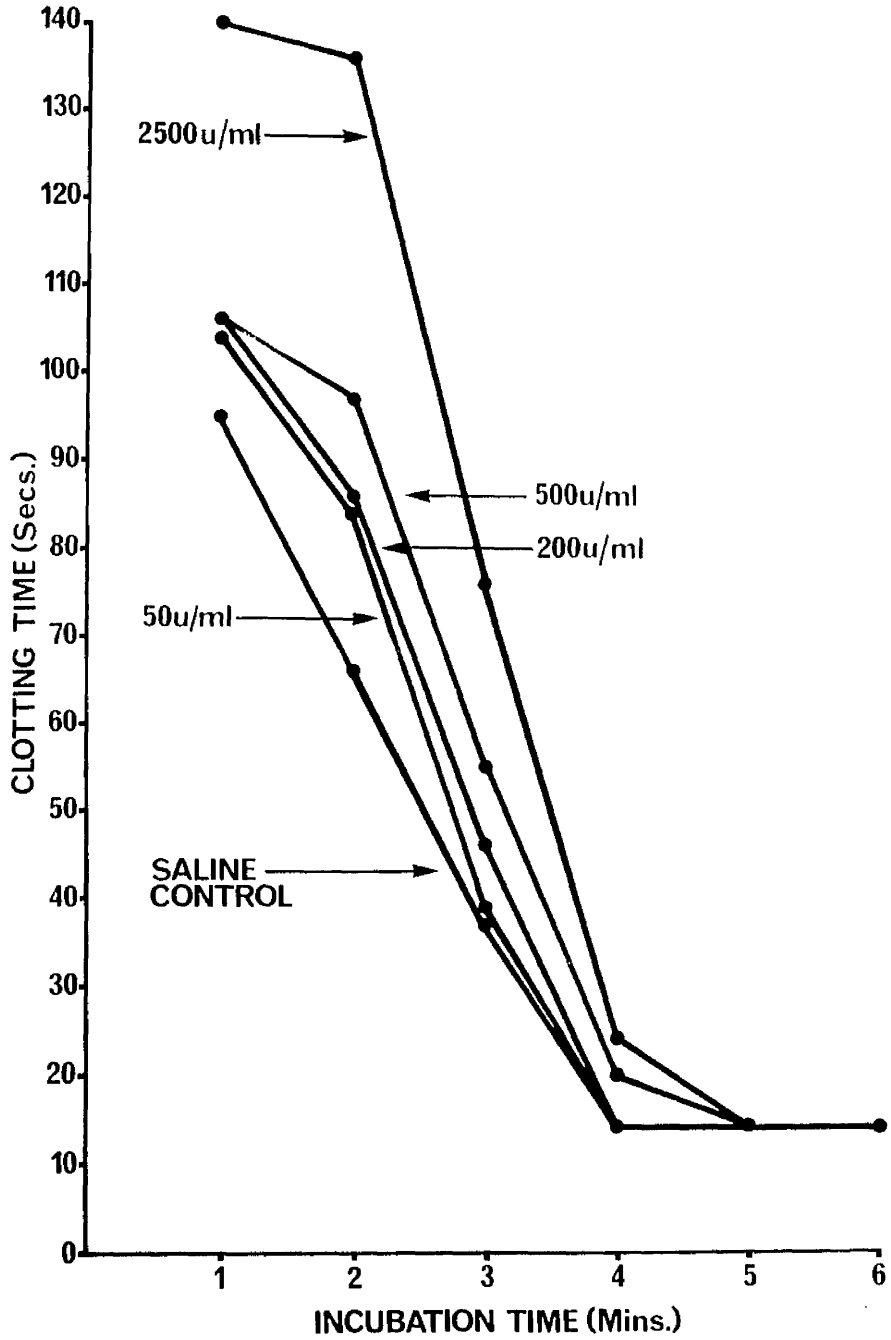


Figure 38 shows the effect of Trasylol in vitro in the substrate system of the thromboplastin generation test. The figure is prepared from the data of table 37.

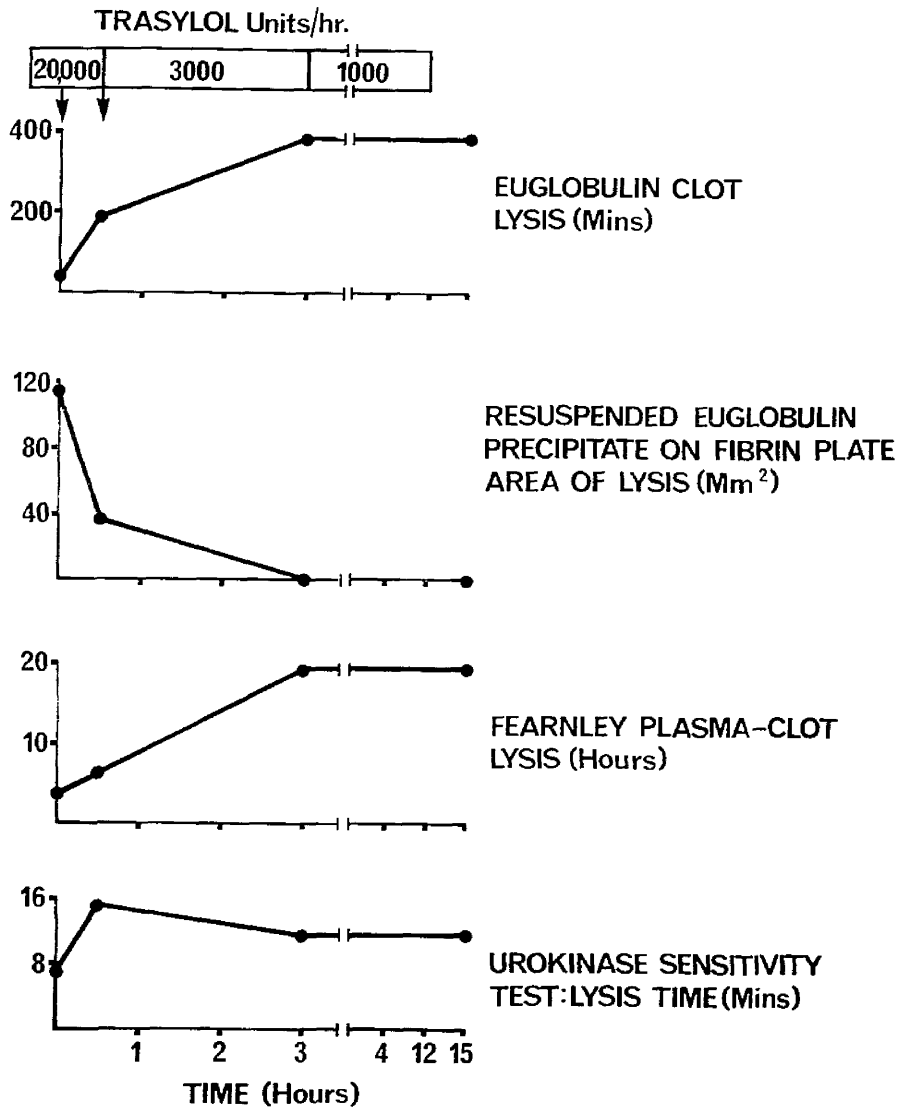


Figure 39 shows the effects of a Trasylol infusion in a normal subject. This figure is prepared from part of the data of table 41.

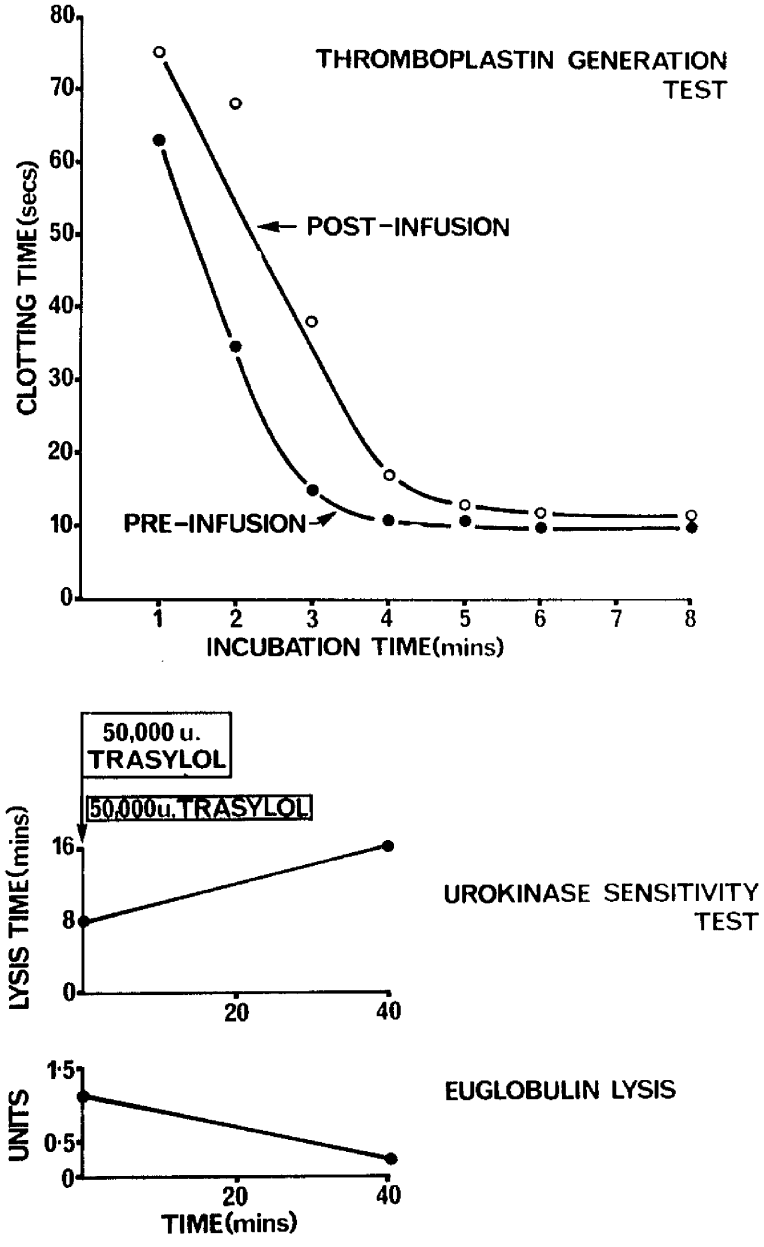


Figure 40 shows the effects in a normal subject of a single injection of Trasylol followed by an infusion of Trasylol given over 30 minutes. This figure displays in part the data of table 42.

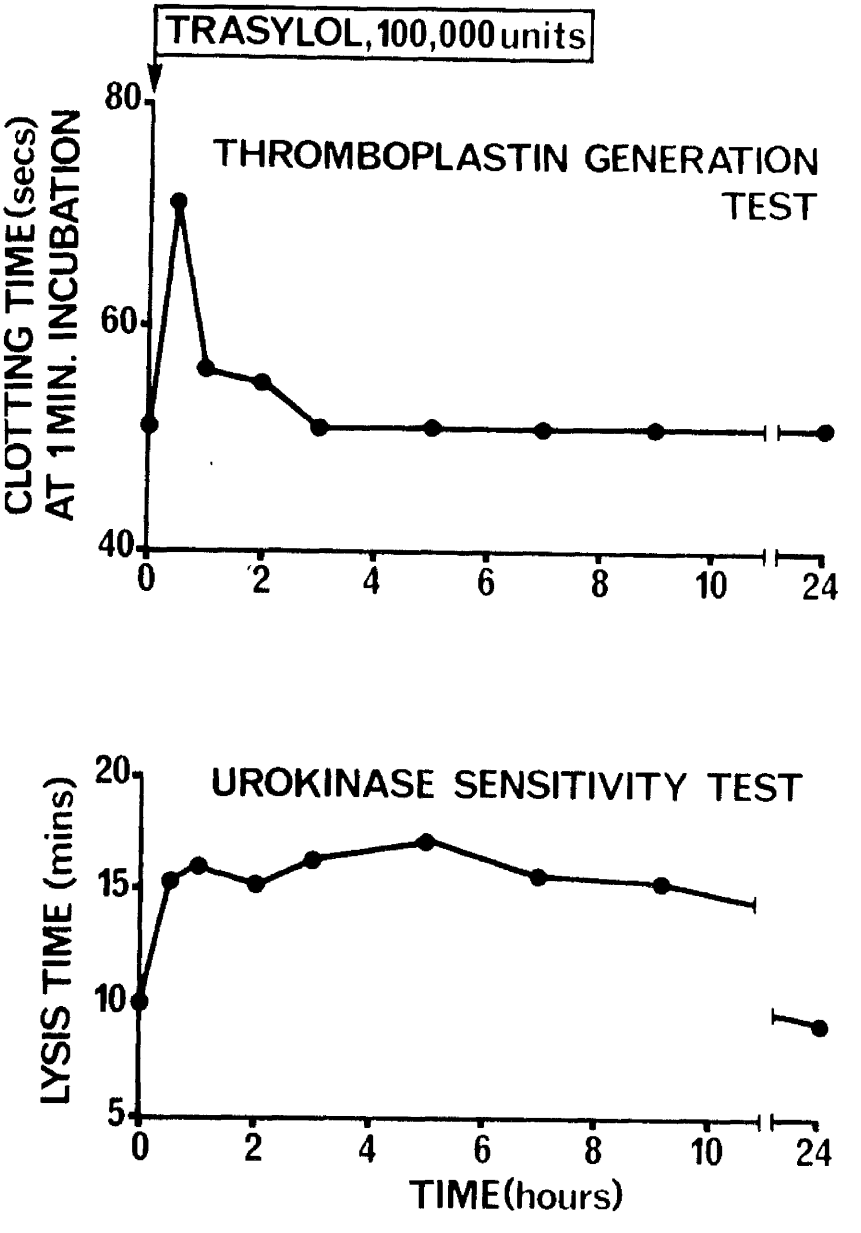


Figure 41 shows the effect of a single intravenous injection of Trasylol on the thromboplastin generation test and the urokinase sensitivity test of a normal subject. The figure graphically displays in part the data of table 43.

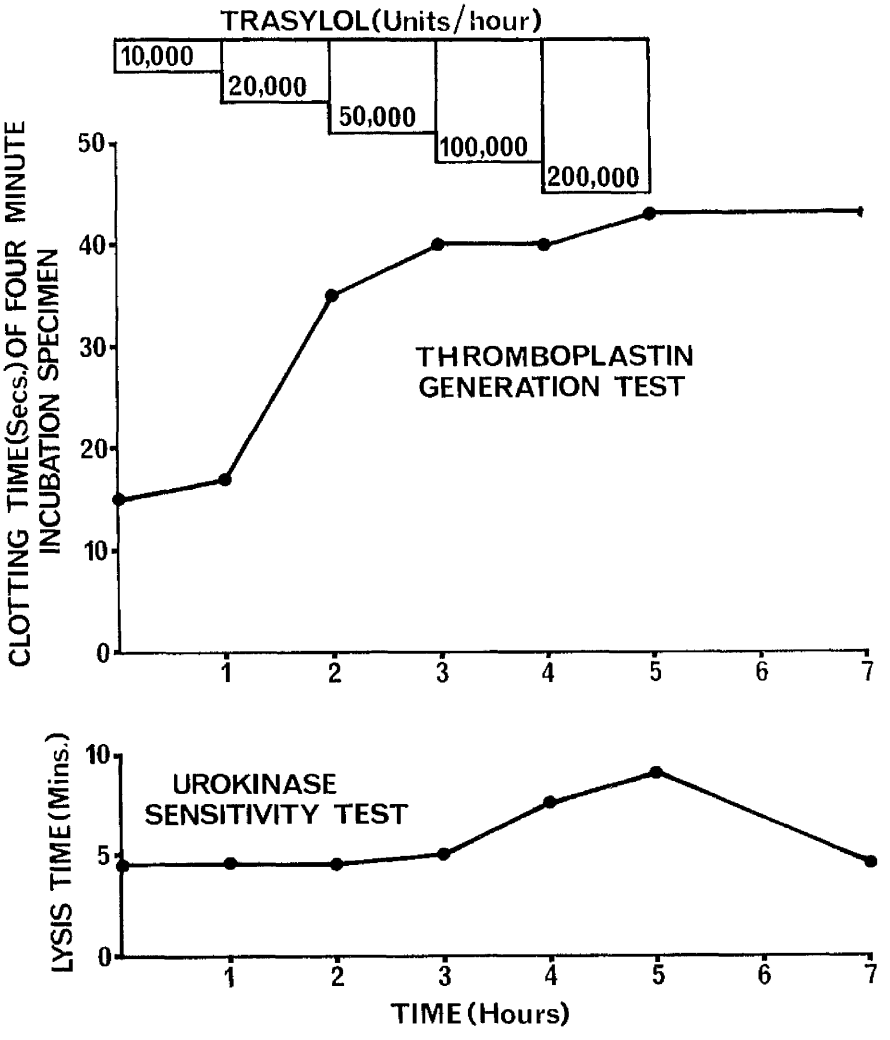


Figure 42 shows the effect of an infusion of Trasylol on the thromboplastin generation test and the urokinase sensitivity test of a normal subject. The figure graphically displays in part the data of table 44.

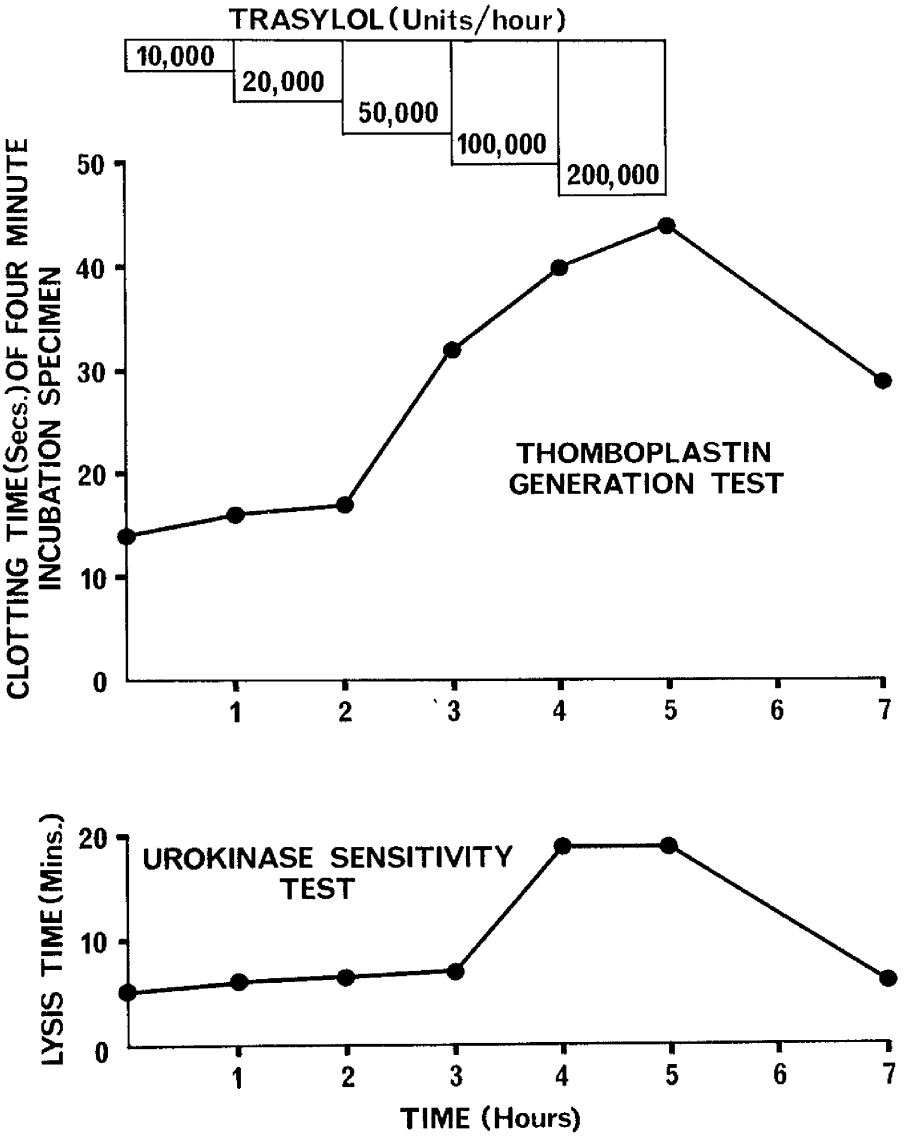


Figure 43 shows the effect of an infusion of Trasylol on the thromboplastin generation test and the urokinase sensitivity test of a normal subject. The figure graphically displays in part the data of table 45.

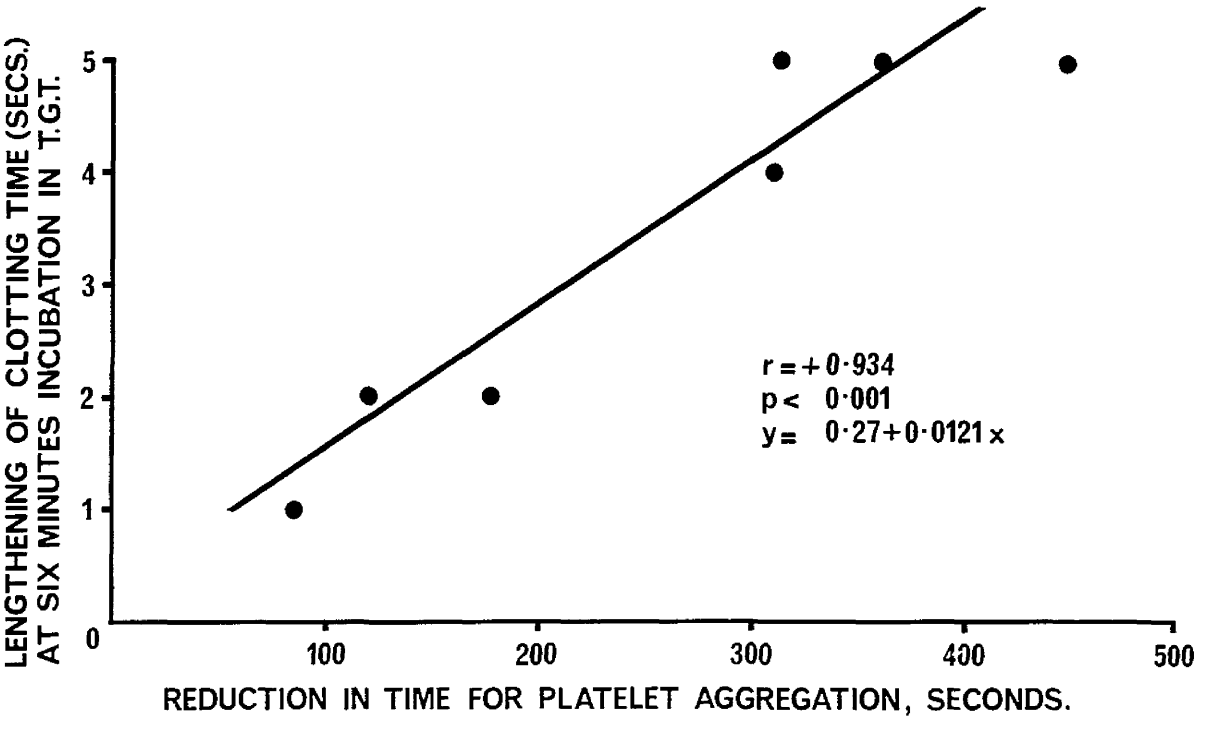


Figure 44. Platelet aggregation was enhanced and thromboplastin generation impaired after Trasylol injection in each of 7 subjects (tables 46 and 47). Shown here is the correlation between the reductions in times for platelet aggregation and the prolongations of substrate clotting times at 6 minutes incubation in the thromboplastin generation test.

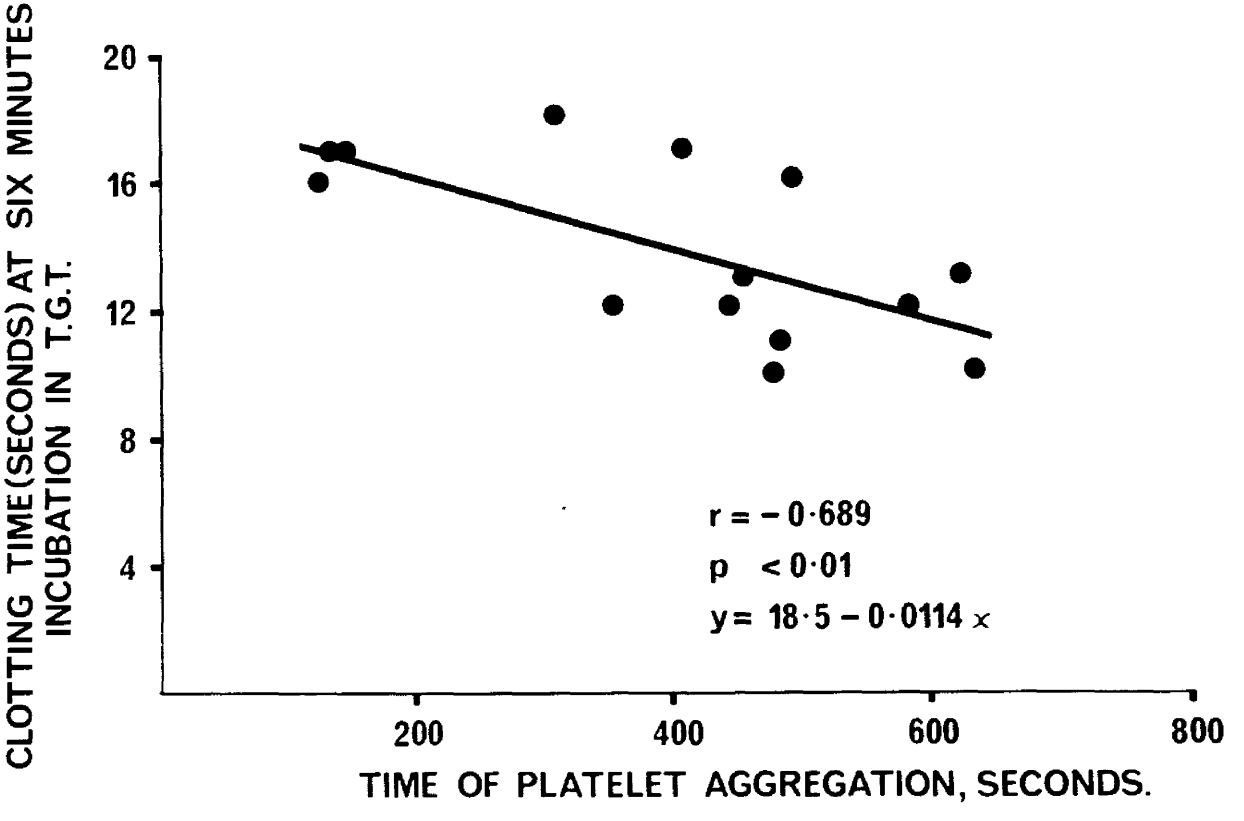


Figure 45 shows the correlation between times for platelet aggregation in each of 7 subjects before and after Trasylol injection and clotting times of substrate plasmas at 6 minutes incubation in the corresponding thromboplastin generation tests. The correlation is calculated and the figure constructed from the data of tables 46 and 47.

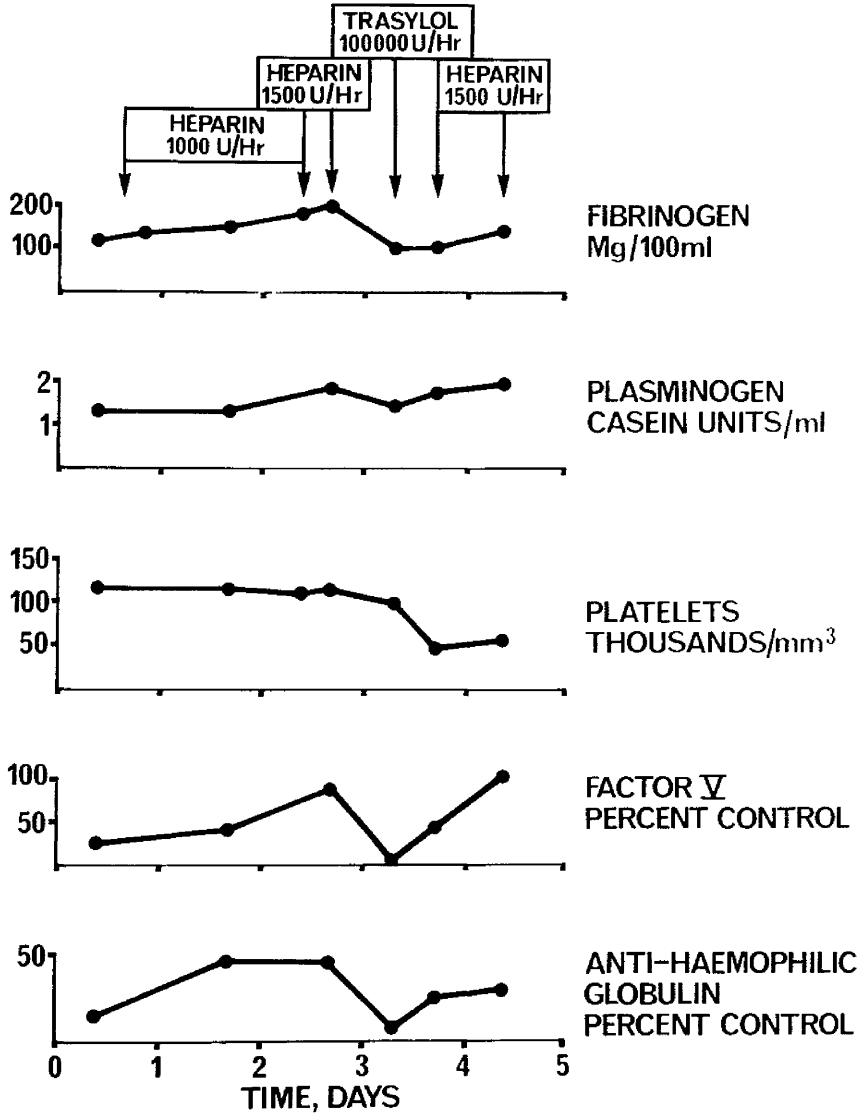


Figure 46 shows the effect in a patient with a defibrination syndrome of a heparin infusion compared with a Trasylol infusion. The figure is prepared from the data of table 49.

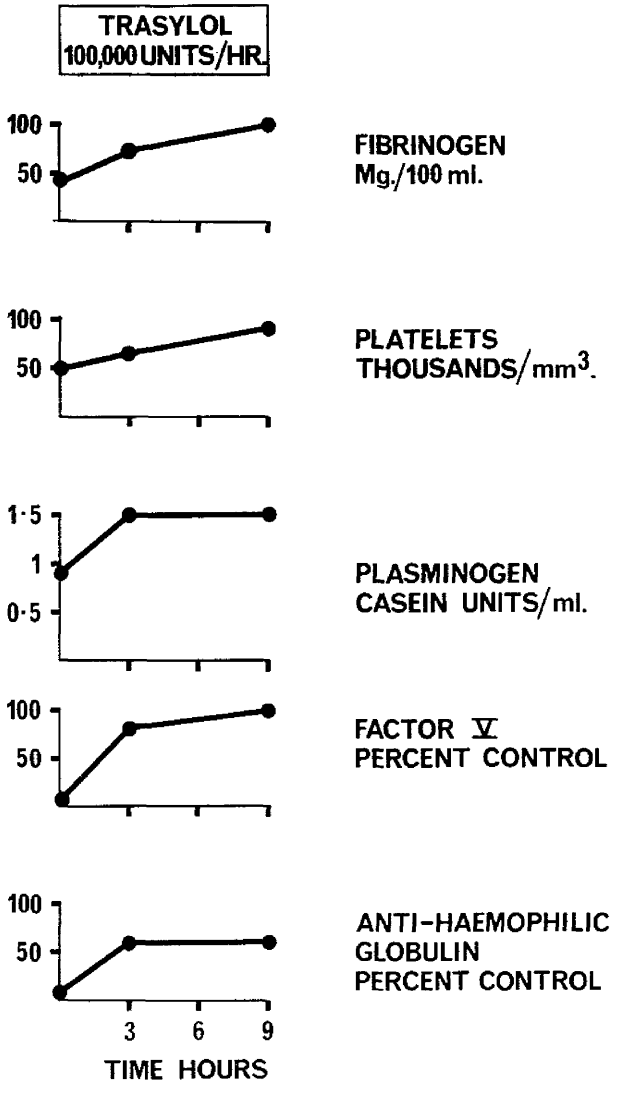


Figure 47 shows the effect in a patient with a defibrination syndrome of a Trasylol infusion. The figure is prepared from the data of table 50.

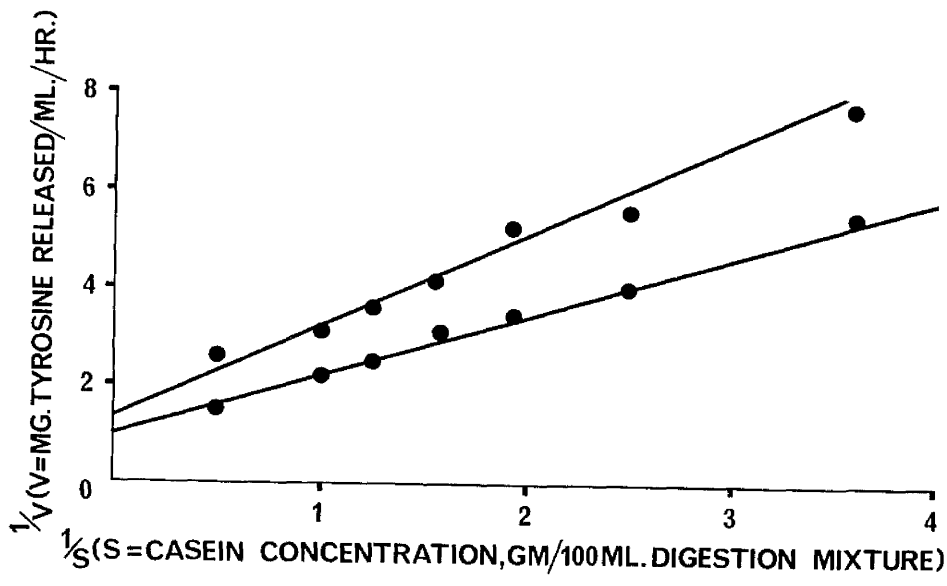


Figure 48 shows Lineweaver-Burk plots demonstrating that Trasylol is a non-competitive inhibitor of the proteolytic activity of trypsin. The upper plot is the experiment in which Trasylol was incorporated and the lower plot is the control experiment. The plots are constructed from the data of table 51.

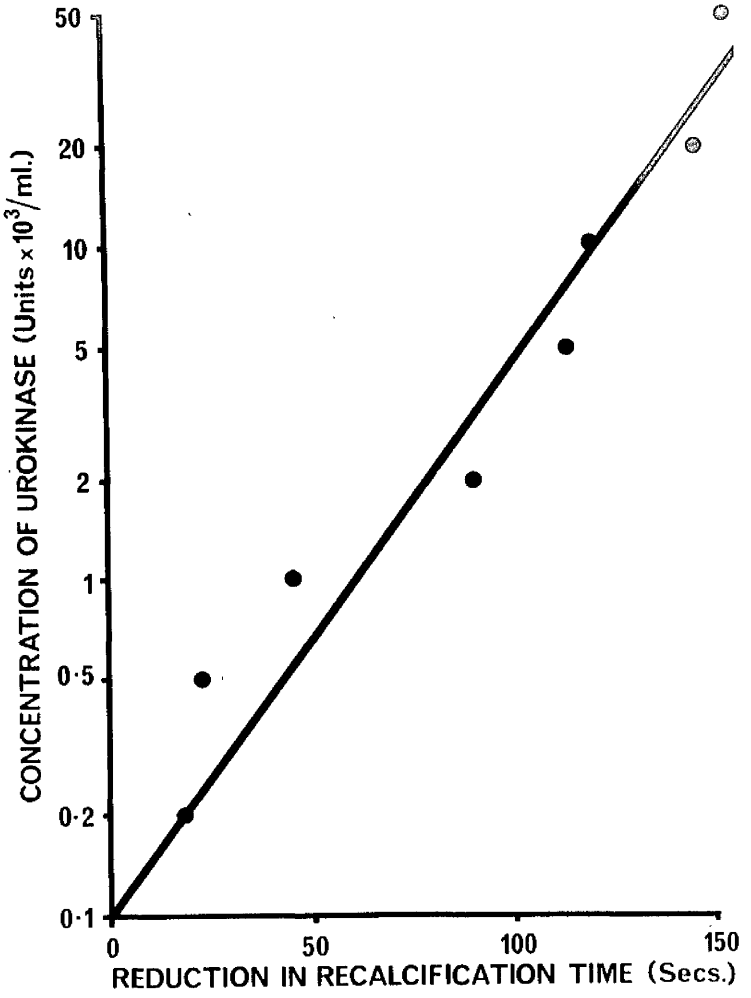


Figure 49 shows reduction in clotting time with increasing amounts of dialysed urokinase added to silicone normal plasma before recalcification. The figure is prepared from table 55.

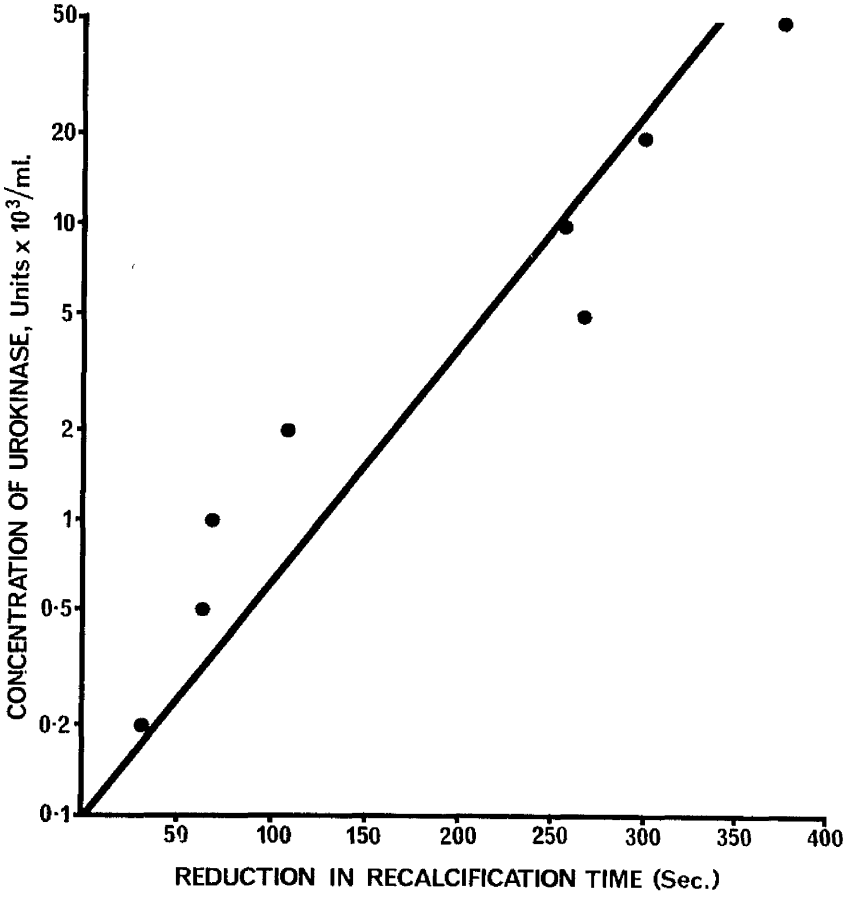


Figure 50 shows reduction in clotting time with increasing amounts of pure urokinase added to silicone normal plasma before recalcification. The figure is prepared from table 55.

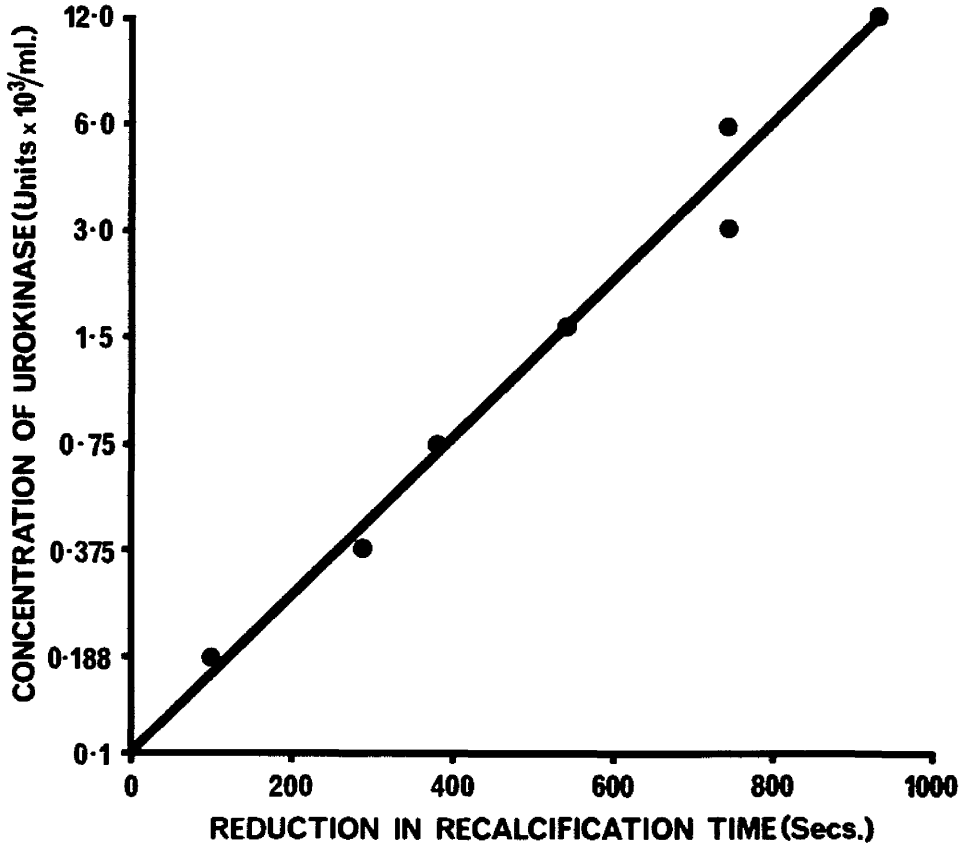


Figure 51 shows reduction in clotting time with increasing amounts of dialysed urokinase added to Hageman factor deficient plasma before recalcification. The figure is prepared from table 56.

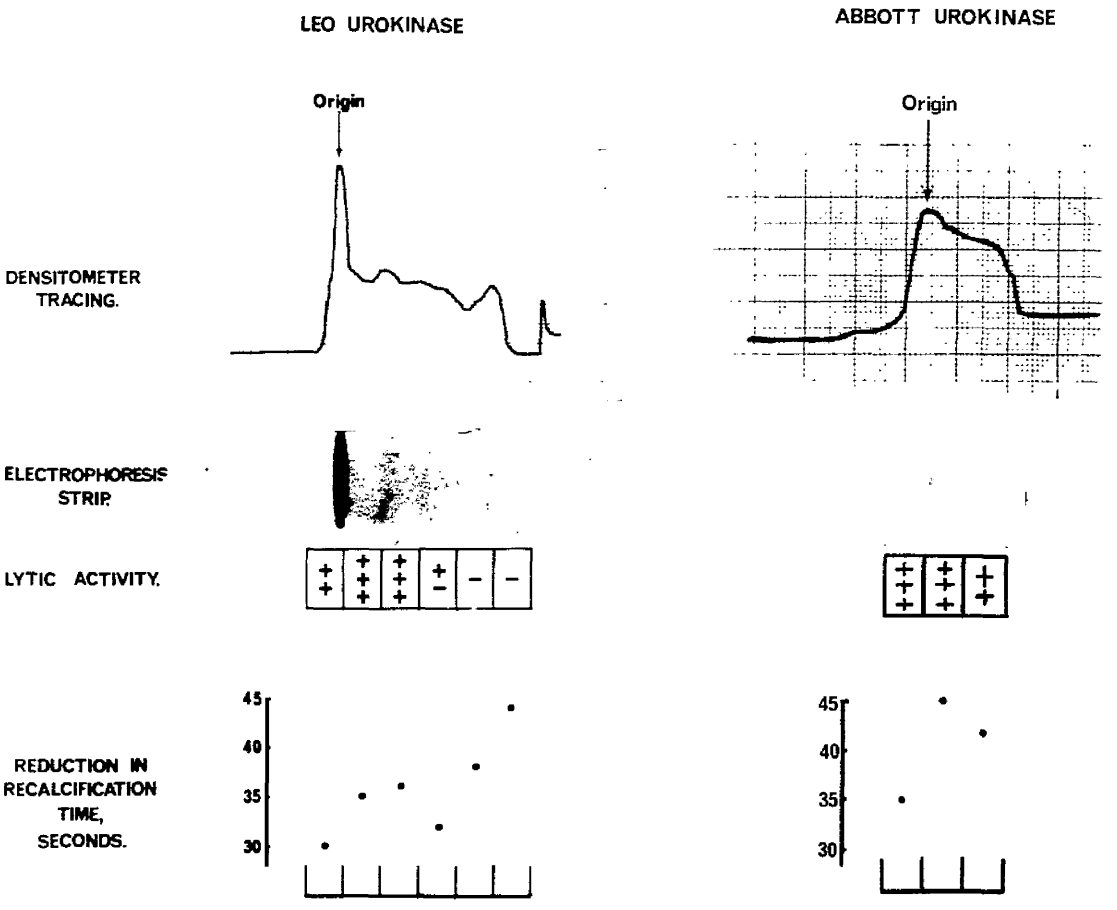


Figure 52 shows electrophoresis of two preparations of urokinase, Leo urokinase and Abbott urokinase, on cellulose acetate paper in an acetate buffer system (0.1 Molar, pH 4.5). Leo urokinase separated into at least 4 components with differing levels of fibrinolytic activity and differing coagulative properties. Abbott urokinase was a homogeneous preparation and it was not possible to separate fibrinolytic and coagulative properties.

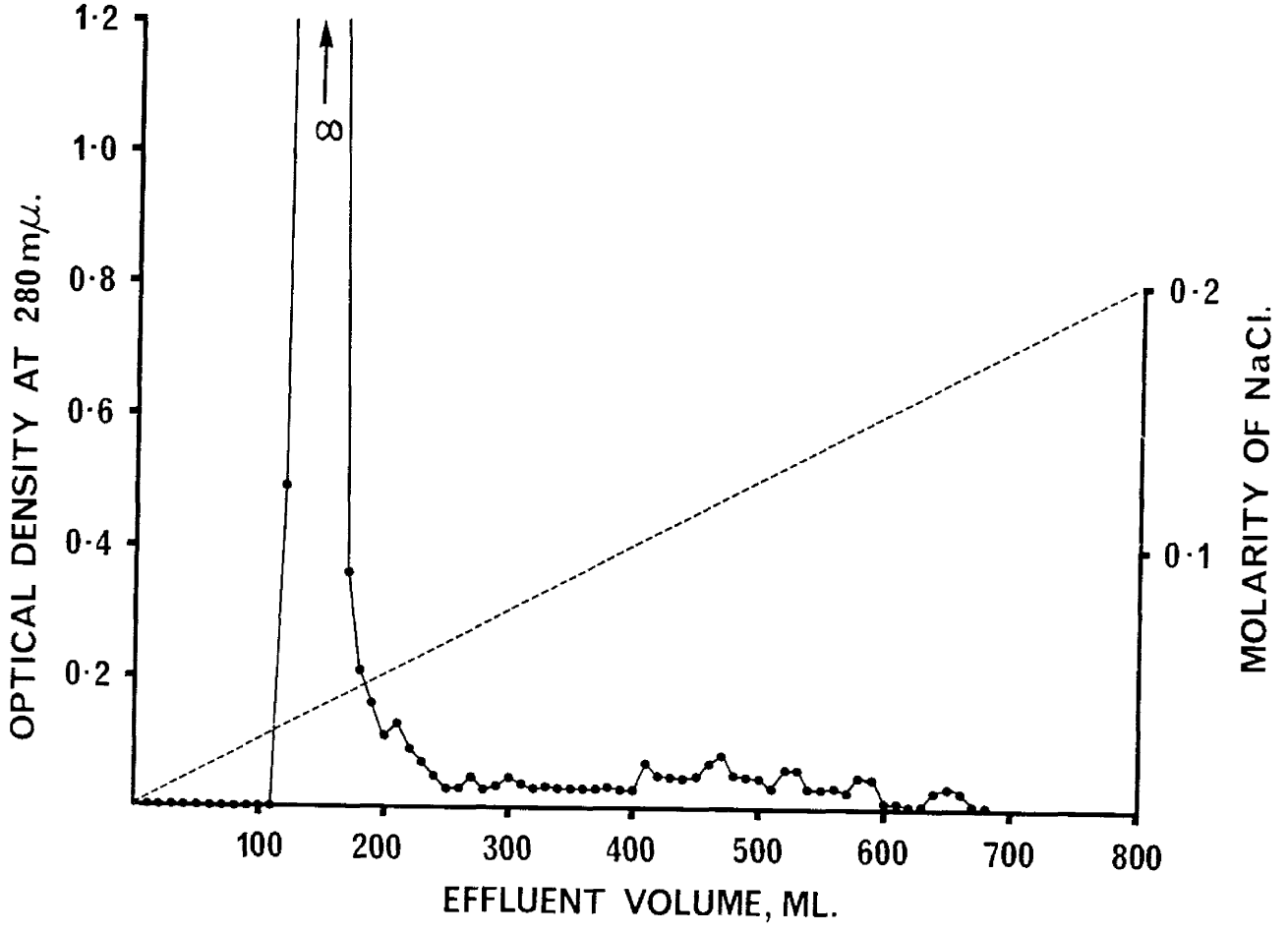


Figure 53 illustrates graphically, fractionation, on a DEAE-cellulose column, of a fibrinogen degradation product prepared by incubating at 37°C for 10 minutes a mixture of fibrinogen, plasminogen and urokinase. Fractionation was achieved using a linear concentration gradient of sodium carbonate, 0.1 Molar to sodium carbonate, 0.1 Molar and sodium chlorate, 0.2 Molar.

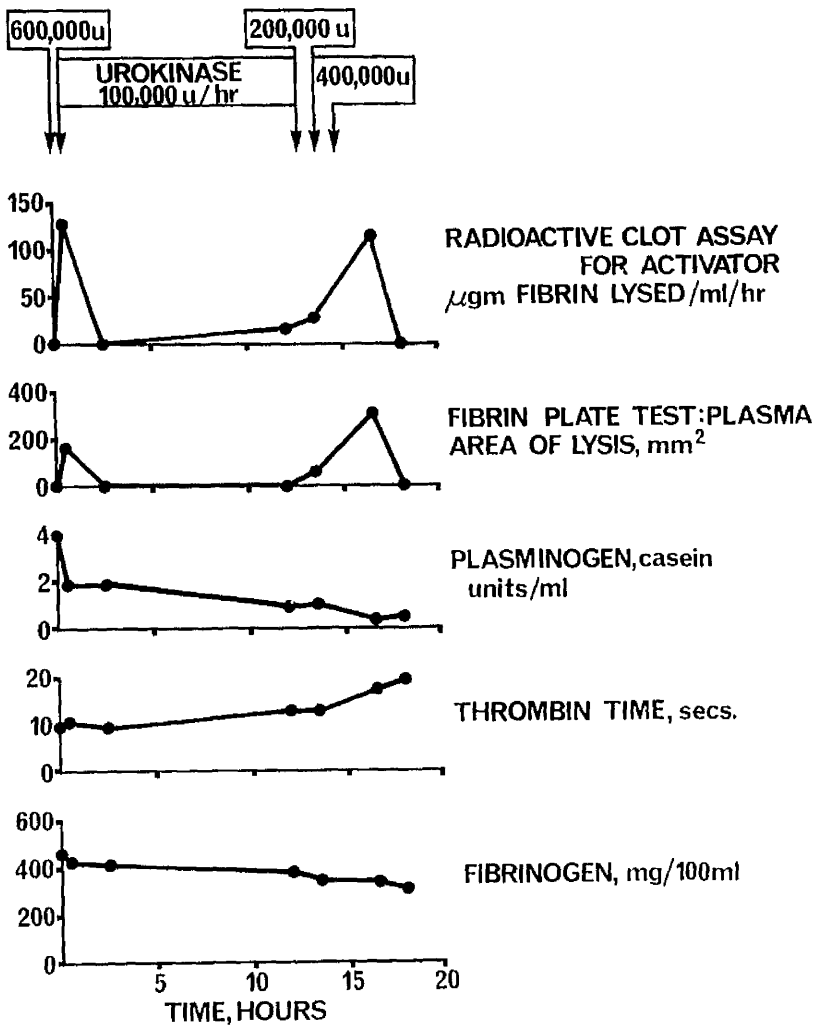


Figure 54 shows the effect on the fibrinolytic enzyme system of an infusion of urokinase. The figure is prepared from table 63.

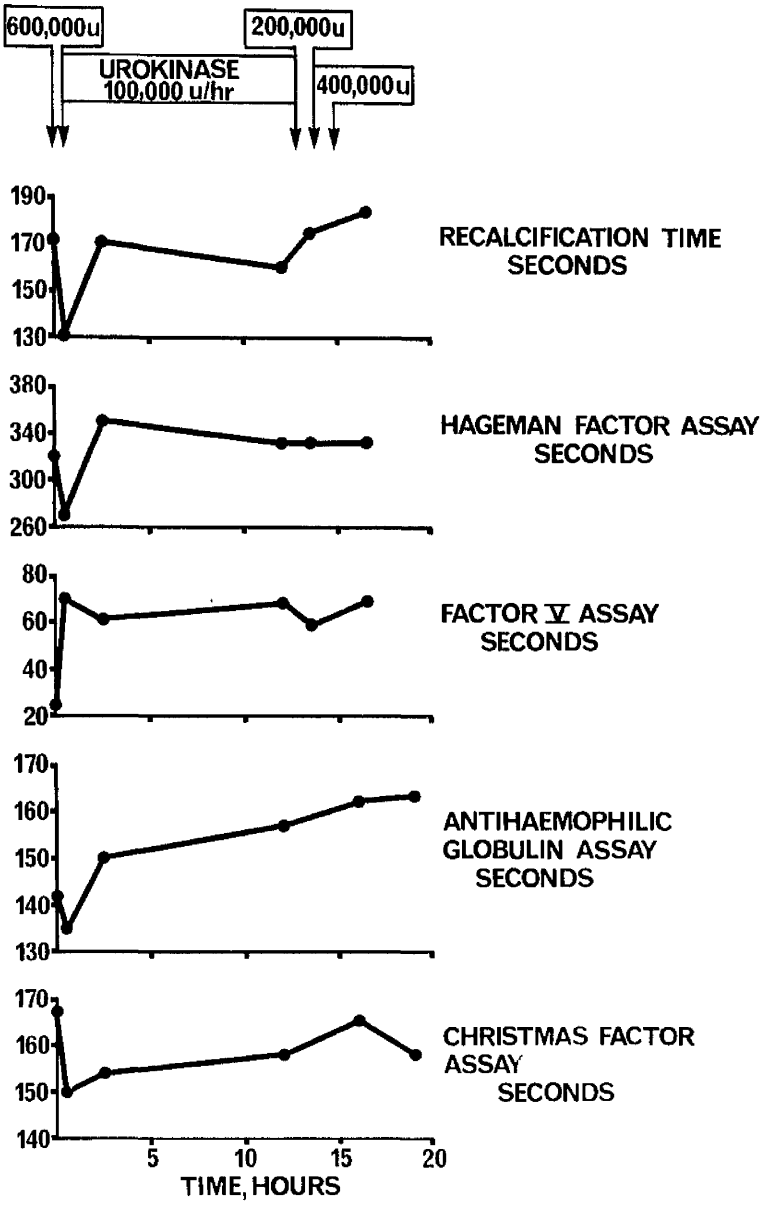


Figure 55 shows the effect on the coagulation system of an infusion of urokinase. The figure is prepared from table 64.

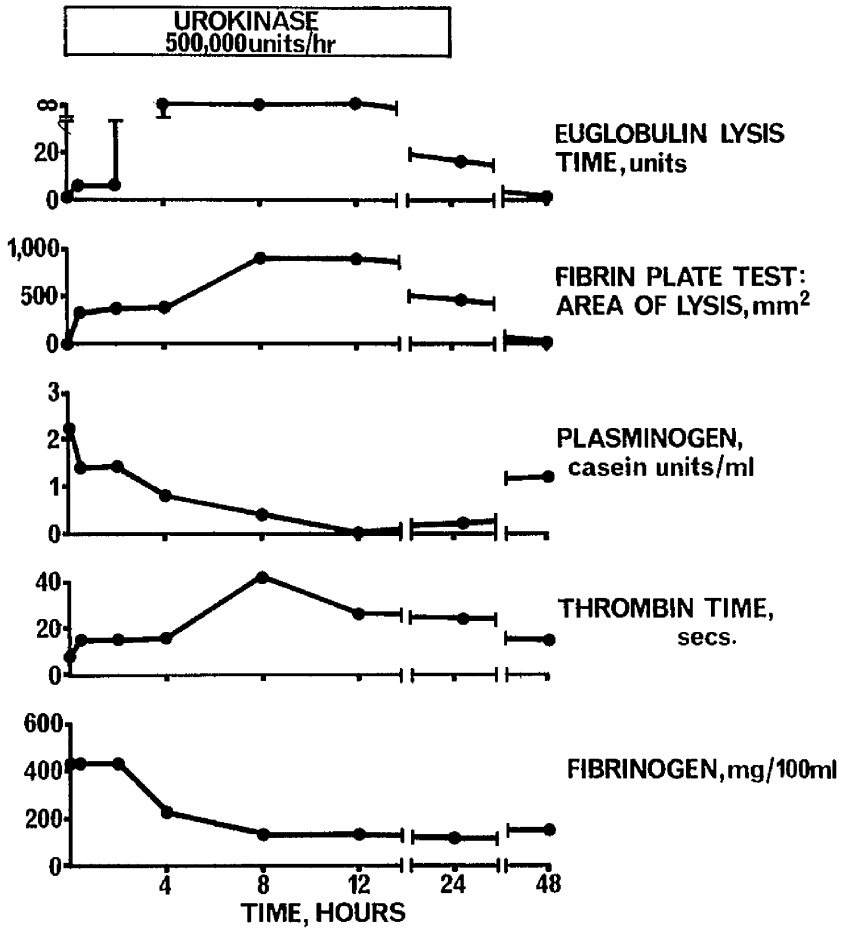


Figure 56 shows the effect on the fibrinolytic enzyme system of an infusion of urokinase. The figure is prepared from table 65.

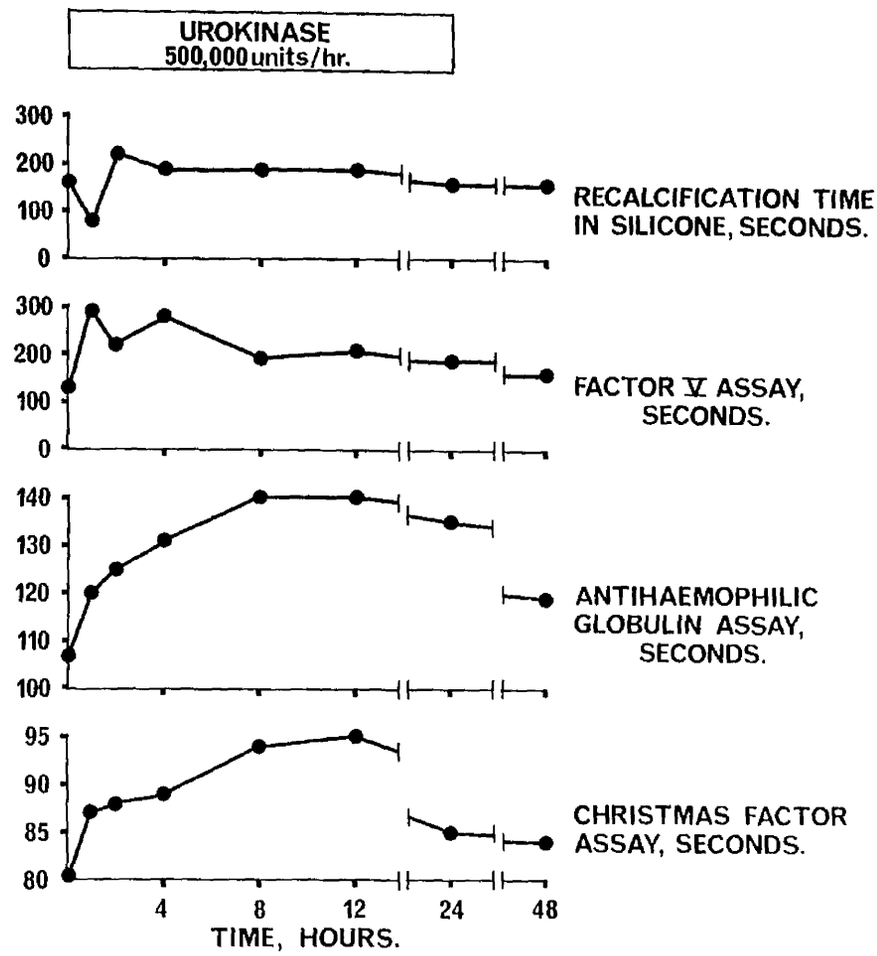


Figure 57 shows the effect on the coagulation system of an infusion of urokinase. The figure is prepared from table 66.

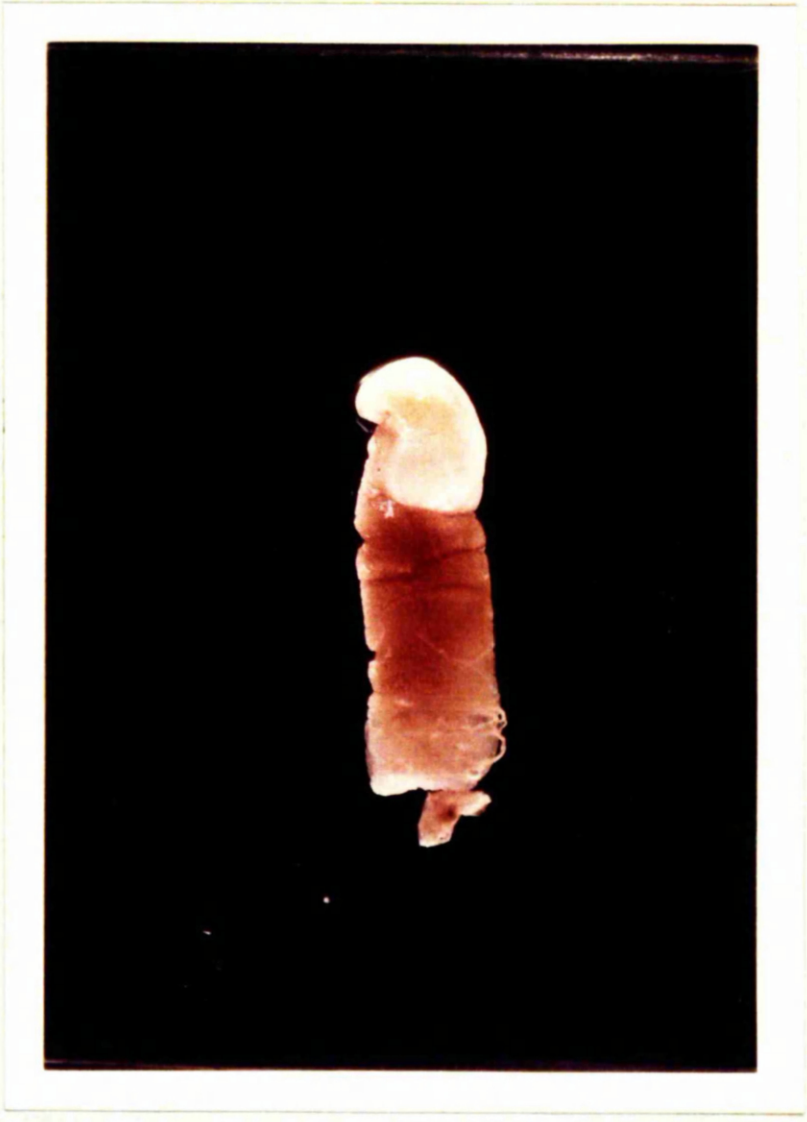


Figure 58 shows an artificial thrombus made in a Chandler's tube by rotation of 15 ml whole blood for 1 hour. The white head and the fibrin tail can be seen.

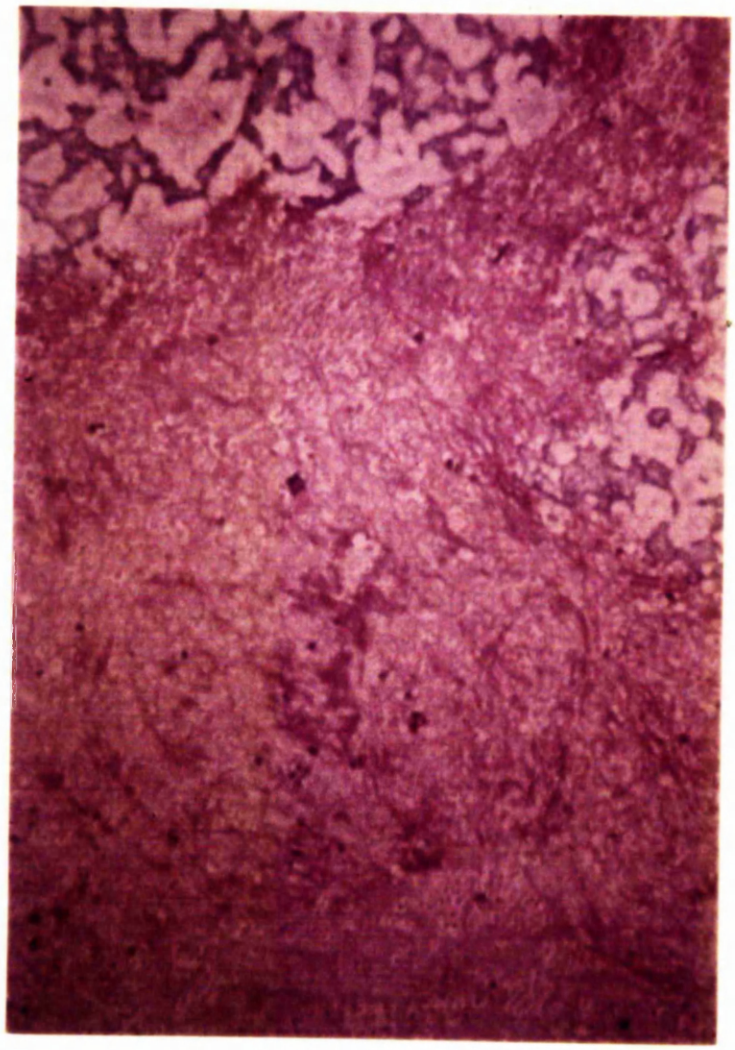


Figure 59 shows a longitudinal section through a Chandler thrombus. The contrast between the platelet head and the fibrin tail can be seen. Original magnification x 500. Stain - Picro Mallory. (Photomicrograph prepared by Dr. F. Walker).

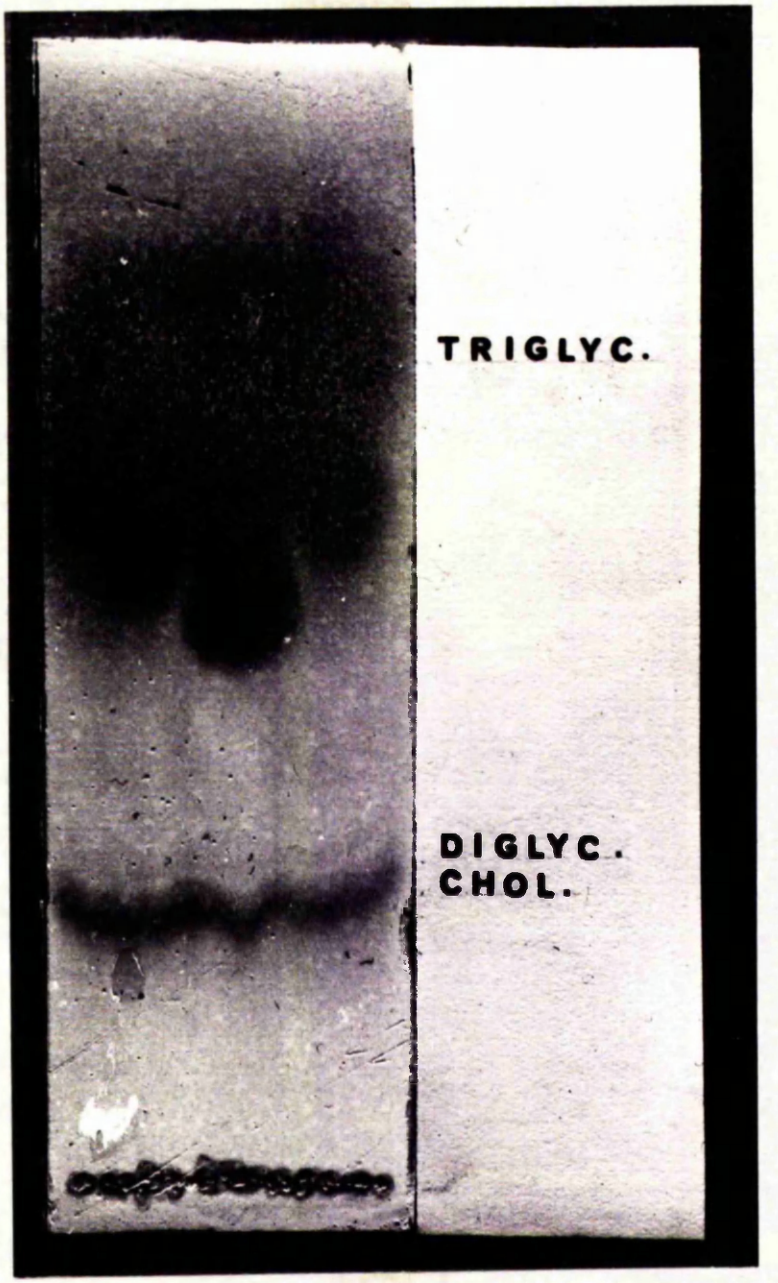


Figure 60 shows a thin layer chromatogram of double cream. The solvent system was petroleum ether-diethyl ether - acetic acid 60:40:1. The chromatogram shows that most of the lipid in the double cream was triglyceride with traces of diglyceride and cholesterol.

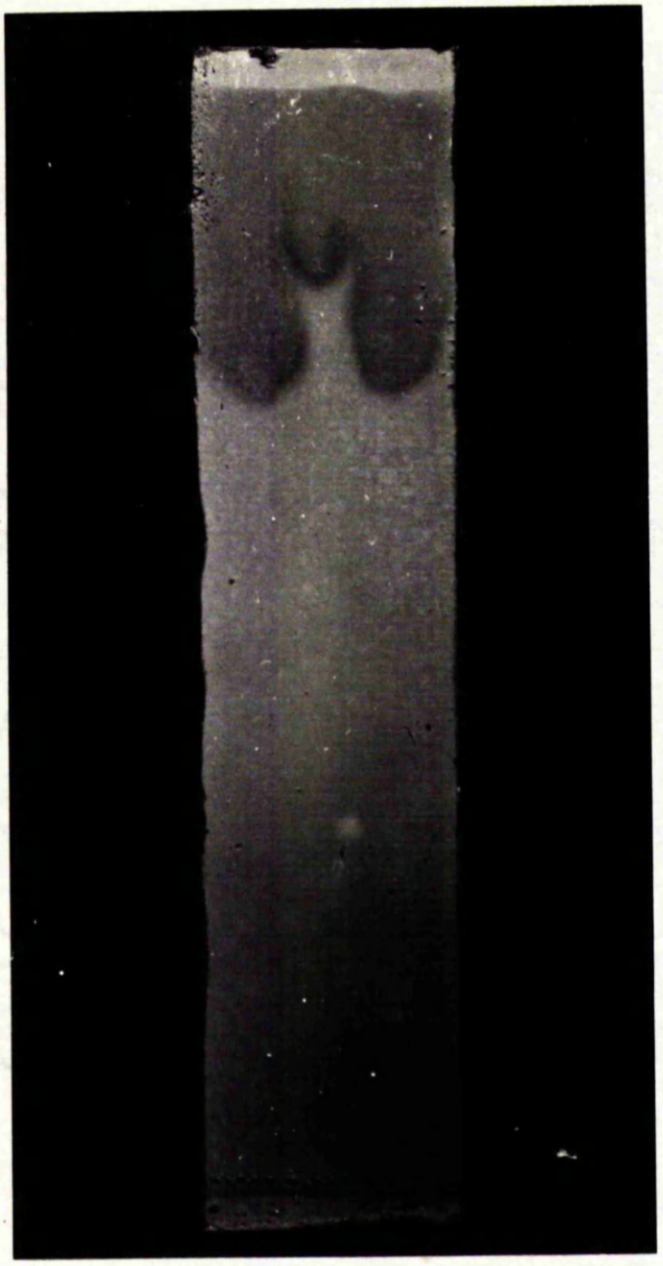


Figure 61 shows a thin layer chromatogram of double cream. The solvent system was chloroform-methanol-water 65:25:4. In this system, neutral lipids, cholesterol and any free fatty acids appear accumulated at the top of the lane as shown here. Phospholipids in this system should appear in the ascending order, lysolecithin, sphingomyelin, lecithin and phosphatidylethanolamine, this last being well behind the other lipids at the top of the lane. As shown, no phospholipid was detected in double cream.

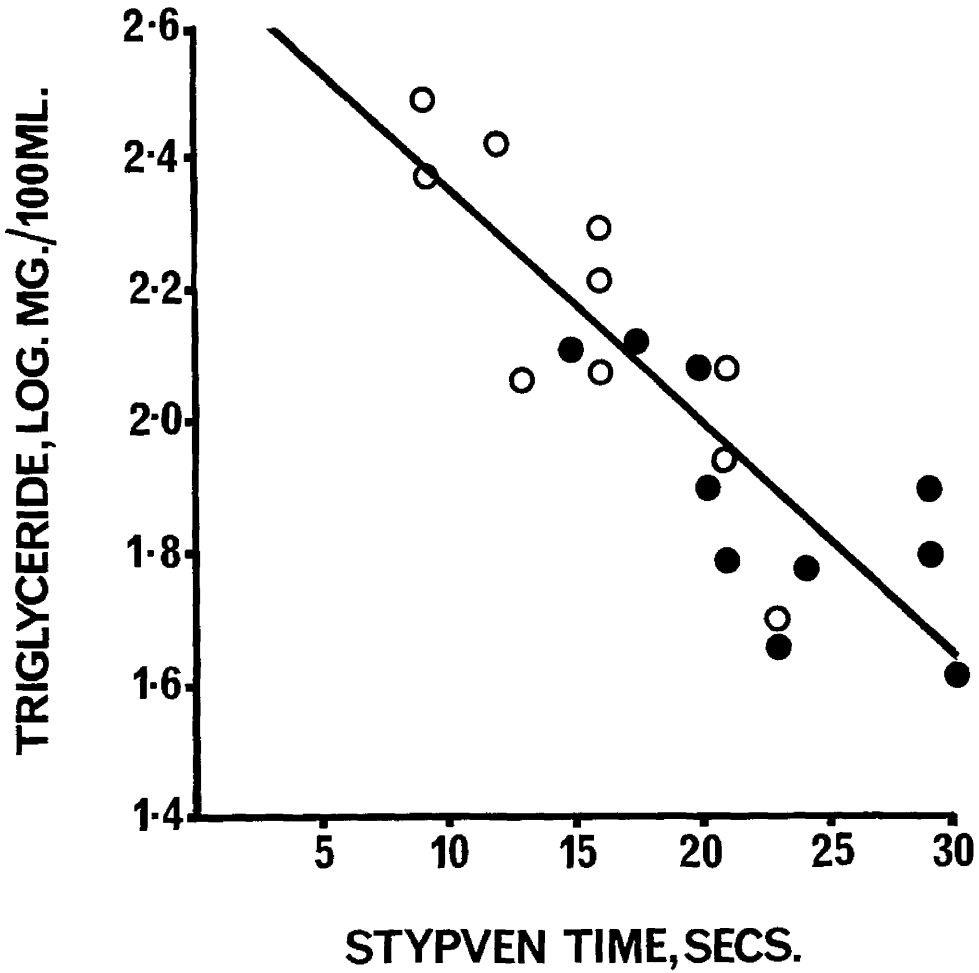


Figure 62 shows the correlation between 'Stypven' times and triglyceride levels expressed as logarithms. The solid circles represent the results observed after the low fat breakfast and the open circles the results after the high fat breakfast. The figure was prepared from the data of tables 78 and 81.

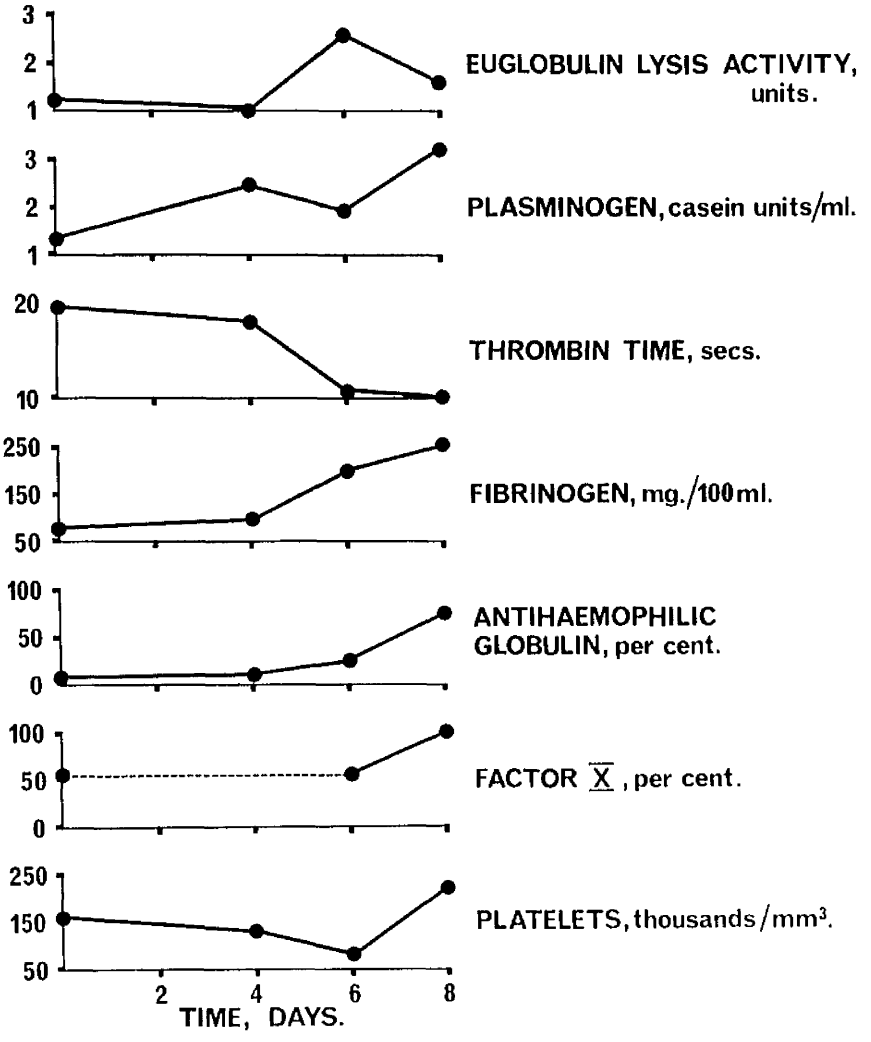


Figure 63 shows the results of fibrinolytic and coagulation assays in patient 1, chapter 8. The patient, in whom the defibrination syndrome was diagnosed, had a retained dead foetus. Delivery of the foetus occurred on day 5. Therapy with EACA was administered from day 1 until 12 hours after the delivery. The figure is prepared from the data of table 86.

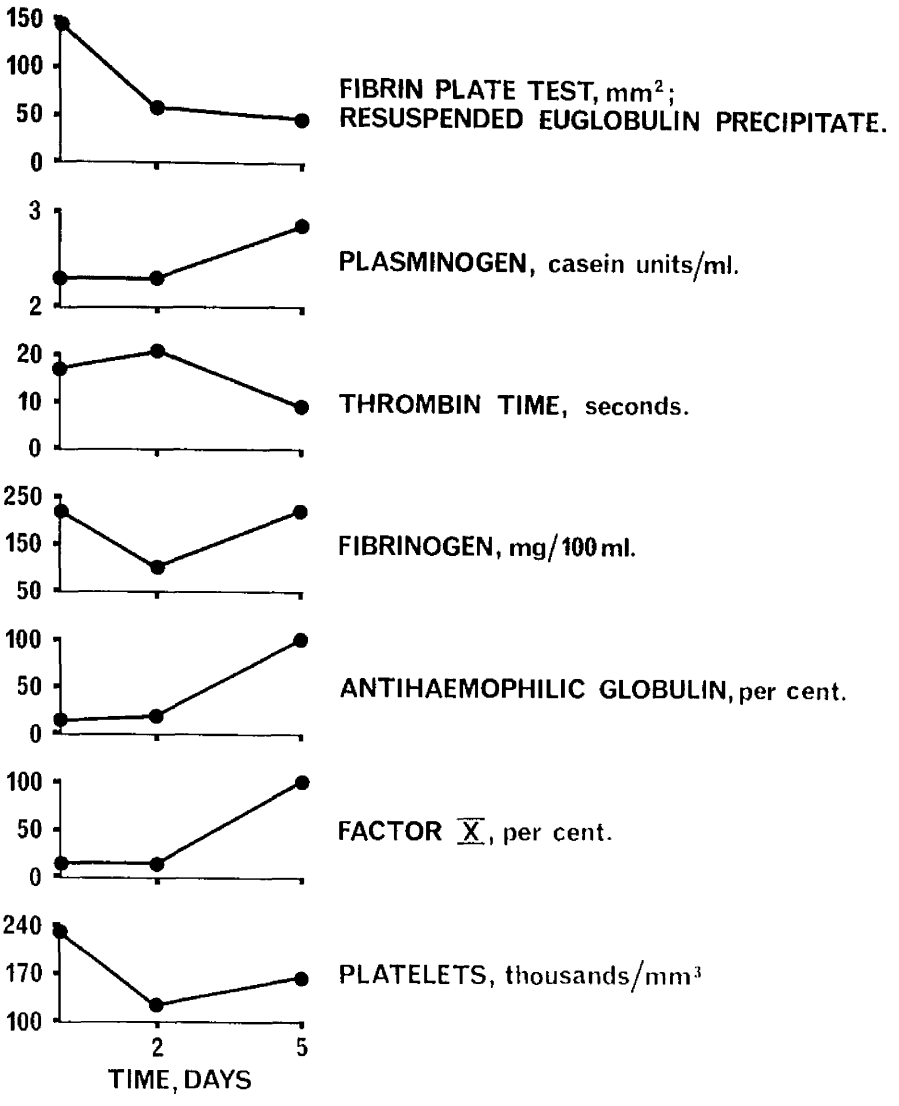


Figure 64 shows the results of fibrinolytic and coagulation assays in patient 2, chapter 8. The patient, in whom the defibrination syndrome was diagnosed, had a retained dead foetus. Delivery of the foetus occurred on day 4. Therapy with EACA was administered from day 1 until 12 hours after the delivery. The figure is prepared from the data of table 87.

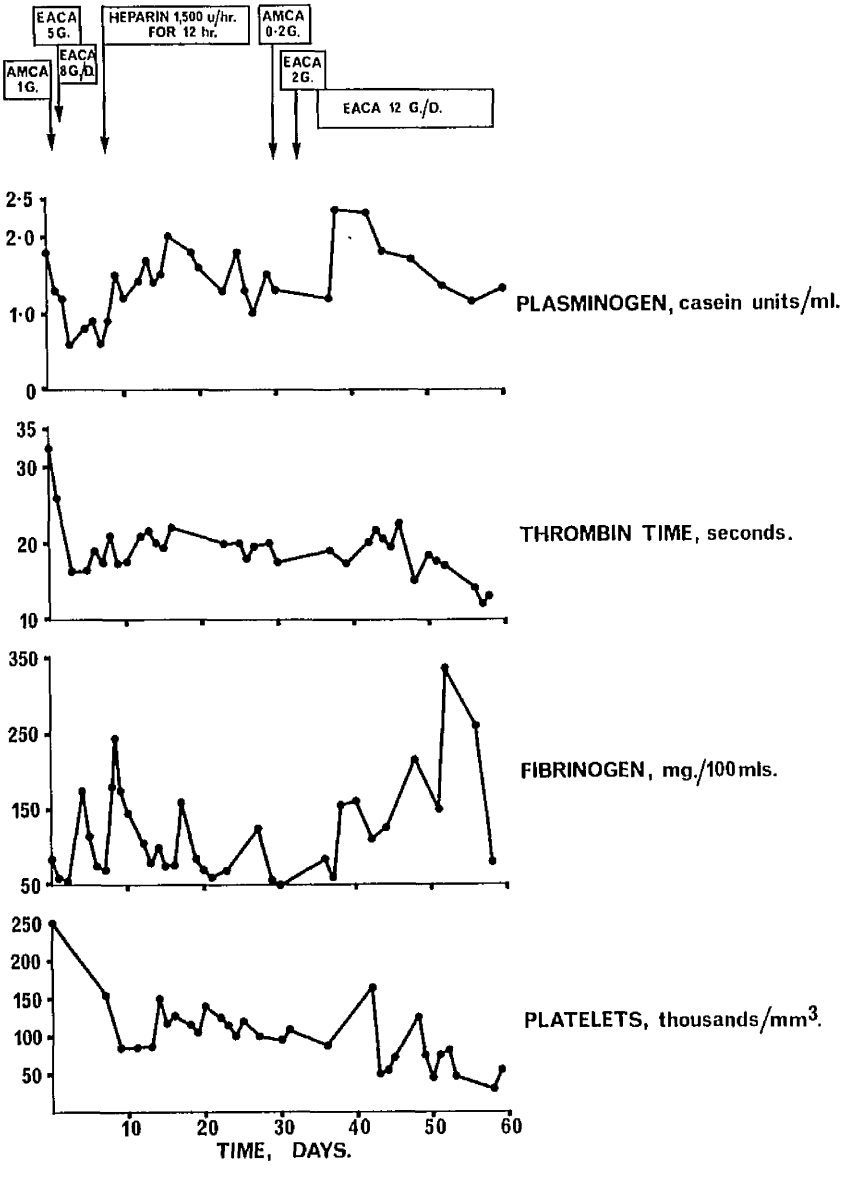


Figure 65 shows the results of some fibrinolytic and coagulation assays and the therapy administered in patient 3, chapter 8. This patient, in whom the defibrination syndrome was diagnosed, had a carcinoma of breast with metastases.

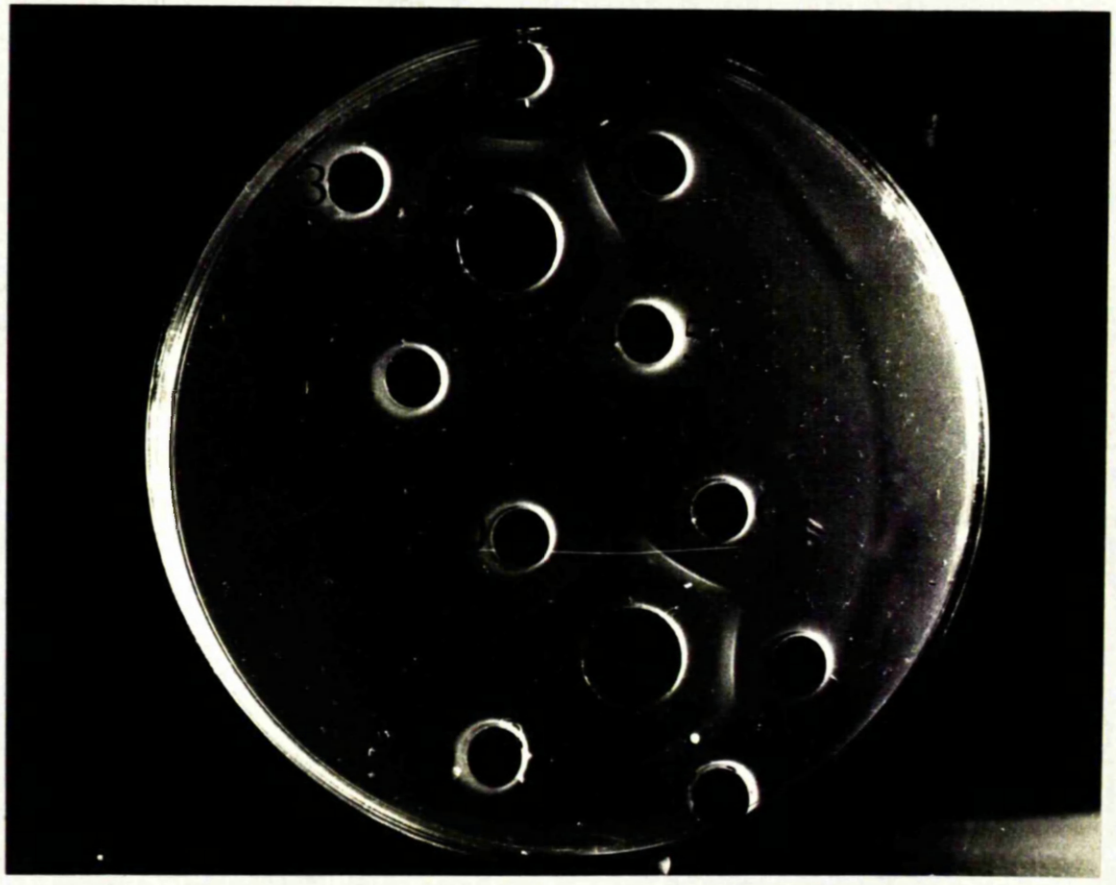


Figure 66 shows immunodiffusion of serum of patient 3, chapter 8 with anti-human fibrin serum in the centre wells. Wells 1 and 6 contained plasma; wells 2 and 7 contained human fibrinogen (4 mg/100 ml); wells 3, 4, 5, 8, 9 and 10 contained the patient's serum collected on days 3, 9, 12, 14, 15 and 17 (figure 65).

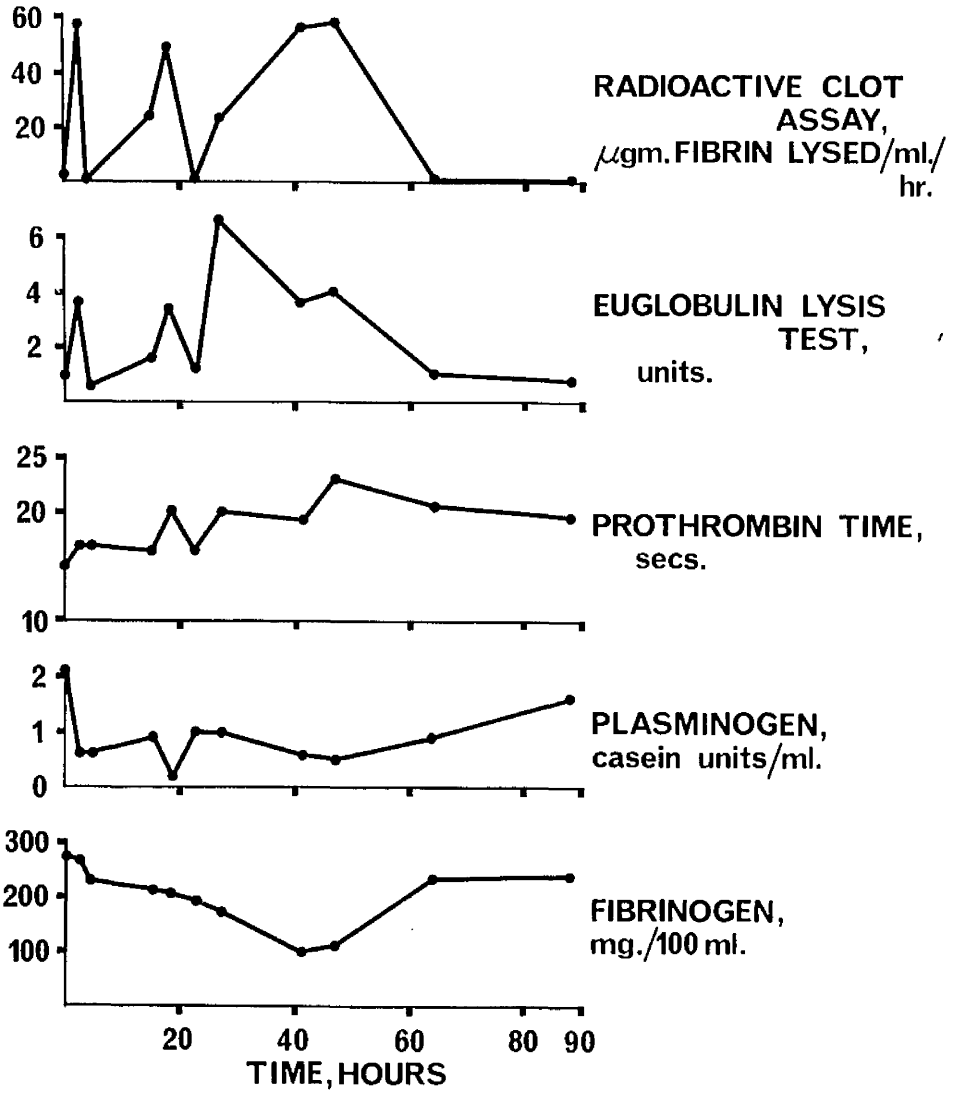


Figure 67 shows the results of fibrinolytic assays in patient 6, chapter 8. This patient had an infusion of urokinase. The dosage rate was 850,000 Ploug units over 2½ hours; 20,000 Ploug units/hour for 2 hours; 40,000 Ploug units/hour for 17½ hours. At 22 hours, there was a break in the infusion for 2½ hours. The infusion was then resumed at a rate of 80,000 Ploug units/hour for a further 23½ hours. The figure is prepared from the data of table 89.

TABLES

Table 1. Use of glycine buffer with acid-haemoglobin substrate

Molar concentration of inhibitor in acid-haemoglobin substrate	pH of acid-haemoglobin substrate	
	With glycine	Without glycine
AMCHA		
8×10^{-5}	2.0	2.5
8×10^{-4}	2.0	2.6
2×10^{-3}	2.0	2.8
4×10^{-3}	2.0	3.4
7×10^{-3}	2.2	3.8
7×10^{-2}	3.4	4.5
EACA		
2×10^{-4}	2.0	2.2
2×10^{-3}	2.0	2.4
5×10^{-3}	2.0	3.4
1×10^{-2}	2.1	3.7
2×10^{-2}	2.4	4.7
2×10^{-1}	2.9	5.9

Shown is the effect of glycine as a buffer for the acid-haemoglobin substrate when increasing concentrations of AMCHA or EACA were added to it.

Table 2. Inhibition by AMCHA of caseinolytic activity of bank plasma

Molar concentration of AMCHA in digestion mixture	Optical density in caseinolytic assay	Per cent inhibition of caseinolytic activity
AMCHA added before activation		
0	0.165, 0.166, 0.167	-
3.2×10^{-5}	0.165, 0.165, 0.169	0
6.4×10^{-5}	0.160, 0.165, 0.170	0.6
9.6×10^{-5}	0.166, 0.166, 0.166	0
1.6×10^{-4}	0.130, 0.140, 0.150	16
3.2×10^{-4}	0.094, 0.101, 0.105	40
6.4×10^{-4}	0.075, 0.075, 0.085	53
9.6×10^{-4}	0.049, 0.050, 0.060	68
1.3×10^{-3}	0.043, 0.045, 0.050	72
2.1×10^{-3}	0.025, 0.025, 0.030	84
AMCHA added after activation		
0	0.155, 0.165	-
4.0×10^{-5}	0.135, 0.155	9
8.0×10^{-5}	0.145, 0.150	7.5
1.6×10^{-4}	0.150, 0.150	5
3.2×10^{-4}	0.145, 0.155	5
6.4×10^{-4}	0.148, 0.152	7
9.6×10^{-4}	0.150, 0.150	5
1.3×10^{-3}	0.148, 0.150	7
2.1×10^{-3}	0.156, 0.164	2.5
3.2×10^{-3}	0.138, 0.142	12.5
6.4×10^{-3}	0.128, 0.130	19

Shown is the effect in a caseinolytic assay system of increasing concentrations of AMCHA added to bank plasma before activation with streptokinase and after activation with streptokinase but prior to plasmin assay. These results are illustrated in figure 4.

Table 3 Inhibition by EACA of caseinolytic activity of bank plasma

Molar concentration of EACA in digestion mixture	Optical density in caseinolytic assay	Per cent inhibition of caseinolytic activity
EACA added before activation		
0	0.150, 0.150	-
3.2×10^{-5}	0.150, 0.151	0
1.6×10^{-4}	0.140, 0.142	6
3.2×10^{-4}	0.123, 0.123	18
6.4×10^{-4}	0.111, 0.112	25
3.2×10^{-3}	0.091, 0.094	38
6.4×10^{-3}	0.075, 0.077	49
1.3×10^{-2}	0.057, 0.059	61
1.9×10^{-2}	0.035, 0.037	63
1.0×10^{-1}	0.025, 0.026	83
EACA added after activation		
0	0.140, 0.140	-
3.2×10^{-5}	0.133, 0.135	4
1.6×10^{-4}	0.126, 0.128	9
3.2×10^{-4}	0.140, 0.140	0
6.4×10^{-4}	0.136, 0.136	3
3.2×10^{-3}	0.138, 0.142	0
6.4×10^{-3}	0.121, 0.123	13
1.3×10^{-2}	0.120, 0.121	14
1.9×10^{-2}	0.112, 0.114	19

Shown is the effect in a caseinolytic assay system of increasing concentrations of EACA added to bank plasma before activation with streptokinase and after activation with streptokinase but prior to plasmin assay. These results are illustrated in figure 5.

Table 4. Inhibition by AMCHA and by EACA of proteolytic activity of plasmin: kinetics of these reactions

Concentration of substrate, casein: gm./100 ml. digestion mixture. (S).	Reaction velocity in caseinolytic assay: mg. tyrosine released/ml./hour. (V)	$1/S$	$1/V$
Control experiment: no inhibitor			
2.00	1.30	0.50	0.8
1.60	1.10	0.63	0.9
1.40	0.90	0.72	1.1
0.80	0.80	1.25	1.3
0.40	0.40	2.50	2.5
0.28	0.31	3.57	3.2
Experiment with 0.2 Molar AMCHA incorporated			
2.00	0.45	0.50	2.2
1.60	0.41	0.63	2.4
1.40	0.40	0.72	2.5
0.80	0.38	1.25	2.6
0.40	0.29	2.50	3.4
0.28	0.23	3.57	4.3
Experiment with 0.4 Molar EACA incorporated			
2.00	0.37	0.50	2.7
1.60	0.34	0.63	2.9
1.40	0.32	0.72	3.1
0.80	0.25	1.25	4.0
0.40	0.19	2.50	5.3
0.28	0.16	3.57	6.3

The double reciprocal plots shown in figure 6 for AMCHA and in figure 7 for EACA are compiled from this table.

Table 5. Inhibition by AMCHA and by EACA of plasminogen activation by streptokinase: kinetics of these reactions

Concentration of substrate plasminogen: casein units/ml added to digestion mixture. (S)	Reaction velocity in caseinolytic assay: casein units plasminogen activated/ml./minute. (V)	1/S	1/V
Control experiment: no inhibitor			
23.3	0.132, 0.134	0.04	7.5
18.7	0.131, 0.133	0.05	7.6
14.0	0.117, 0.117	0.07	8.5
9.3	0.115, 0.115	0.11	8.7
4.7	0.110, 0.114	0.21	8.9
2.3	0.070, 0.074	0.43	13.9
Experiment with 0.05 Molar AMCHA incorporated			
23.3	0.084, 0.085	0.04	11.8
18.7	0.076, 0.077	0.05	13.1
14.0	0.062, 0.062	0.07	16.1
9.3	0.041, 0.042	0.11	24.1
4.7	0.032, 0.034	0.21	30.3
3.5	0.025, 0.025	0.29	40.0
2.3	0.019, 0.020	0.43	51.3
Experiment with 0.10 Molar EACA incorporated			
23.3	0.104, 0.104	0.04	9.6
18.7	0.091, 0.093	0.05	10.9
14.0	0.083, 0.087	0.07	11.8
9.3	0.062, 0.064	0.11	15.9
4.7	0.035, 0.035	0.21	28.6
3.5	0.026, 0.028	0.29	37.0
2.3	0.020, 0.021	0.43	48.8

The double reciprocal plots shown in figure 8 for AMCHA and in figure 9 for EACA are compiled from this table.

Table 6. Assay of plasmin: effect of 0.05 Molar AMCHA and of 0.10 Molar EACA

Concentration of plasmin: casein units/ml. added to digestion mixture	Optical density in caseinolytic assay.	Optical density in caseinolytic assay with 0.05 molar AMCHA incorporated.	Optical density in caseinolytic assay with 0.10 Molar EACA incorporated
6.0	0.454, 0.462	0.455, 0.460	0.457, 0.459
4.0	0.303, 0.305	0.304, 0.306	0.304, 0.304
2.0	0.151, 0.152	0.152, 0.152	0.150, 0.152
1.0	0.071, 0.074	0.072, 0.073	0.073, 0.075
0.5	0.037, 0.038	0.038, 0.038	0.037, 0.038

Shown are the results of caseinolytic assays of varying concentrations of plasmin alone and of the same concentrations assayed in the presence of 0.05 Molar AMCHA and 0.10 Molar EACA.

Table 7. Inhibition by AMCA of plasminogen activation by streptokinase: kinetics of the reaction

Concentration of substrate, plasminogen: casein units/ml. added to digestion mixture. (S)	Reaction velocity in caseinolytic assay: casein units plasminogen activated/ml/minute. (V)	1/S	1/V
	Control experiment; no inhibitor		
23.3	0.138	0.04	7.2
9.3	0.118	0.11	8.5
6.7	0.112	0.15	8.9
4.7	0.105	0.21	9.5
3.6	0.093	0.28	10.8
	Experiment with 0.02 Molar AMCA incorporated		
23.3	0.062	0.04	16.1
9.3	0.033	0.11	30.3
6.7	0.025	0.15	40.0
4.7	0.019	0.21	52.6
3.6	0.014	0.28	71.4

The double reciprocal plots shown in figure 10 are compiled from this table.

Table 8. Inhibition by AMCHA and by EACA of proteolytic activity of trypsin: kinetics of these reactions

Concentration of substrate casein: gm./100 ml. digestion mixture. (S)	Reaction velocity in caseinolytic assay: mg. tyrosine released/ml./hour. (V).	1/S	1/V
	Control experiment: no inhibitor		
2.00	1.04	0.50	0.96
1.40	0.99	0.72	1.01
0.80	0.95	1.25	1.05
0.40	0.90	2.50	1.11
0.28	0.88	3.57	1.14
	Experiment with 0.2 Molar AMCHA incorporated		
2.00	0.65	0.50	1.54
1.40	0.63	0.72	1.59
0.80	0.56	1.25	1.79
0.40	0.54	2.50	1.85
0.28	0.52	3.57	1.92
	Experiment with 0.4 Molar EACA incorporated		
2.00	0.68	0.50	1.47
1.40	0.63	0.72	1.59
0.80	0.59	1.25	1.69
0.40	0.56	2.50	1.79
0.28	0.52	3.57	1.92

The double reciprocal plots shown in figure 11 for AMCHA and in figure 12 for EACA are compiled from this table.

Table 9. Inhibition by AMCHA and by EACA of trypsinogen activation by enterokinase: kinetics of these reactions

Concentration of substrate, trypsinogen mg/100 ml digestion mixture. (S)	Reaction velocity in caseinolytic assay, optical density readings. (V)	$\frac{1}{S}$	$\frac{1}{V}$
Control experiment; no inhibitor			
50	0.680	0.020	1.5
30	0.530	0.033	1.9
20	0.420	0.050	2.4
10	0.286	0.100	3.5
8	0.210	0.125	4.8
5	0.167	0.200	6.0
Experiment with 0.05 Molar AMCHA incorporated			
50	0.405	0.020	2.5
30	0.320	0.033	3.1
20	0.204	0.050	4.9
10	0.180	0.100	5.6
8	0.125	0.125	8.0
5	0.070	0.200	14.3
Experiment with 0.10 Molar EACA incorporated			
50	0.400	0.020	2.5
30	0.275	0.033	3.6
20	0.170	0.050	5.9
10	0.125	0.100	8.0
8	0.083	0.125	12.0
5	0.060	0.200	16.7

The double reciprocal plots shown in figure 13 for AMCHA and in figure 14 for EACA are compiled from this table.

Table 10. Assay of trypsin: effect of 0.05 Molar AMCHA and of 0.10 Molar EACA

Concentration of trypsin: mg/100 ml of digestion mixture.	Optical density in caseinolytic assay.	Optical density in caseinolytic assay with 0.05 Molar AMCHA incorporated	Optical density in caseinolytic assay with 0.10 Molar EACA incorporated
50	0.620	0.630	0.622
30	0.270	0.264	0.268
20	0.190	0.198	0.190
10	0.112	0.110	0.116
5	0.070	0.074	0.070

Shown are the results of caseinolytic assays of varying concentrations of trypsin alone and of the same concentrations assayed in the presence of 0.05 Molar AMCHA and of 0.10 Molar EACA.

Table 11. Inhibition of pepsin by AMCHA

compared with inhibition by EACA

Molar concentration of AMCHA in digestion mixture	Optical density in pepsin assay	Per cent inhibition of pepsin
0	0.350, 0.350	-
1×10^{-3}	0.350	0
5×10^{-3}	0.320	9
1×10^{-2}	0.300	14
2×10^{-2}	0.265	24
3×10^{-2}	0.160	54
4×10^{-2}	0.105	70
5×10^{-2}	0.060	83
6×10^{-2}	0.050	86

Molar concentration of EACA in digestion mixture	Optical density in pepsin assay	Per cent inhibition of pepsin
2×10^{-2}	0.350	0
3×10^{-2}	0.185	47
4×10^{-2}	0.150	57
5×10^{-2}	0.080	77
6×10^{-2}	0.070	80
7.5×10^{-2}	0.060	83
1×10^{-1}	0.045	87

Shown is the effect of increasing concentrations of AMCHA and of EACA on the action of pepsin on an acid-haemoglobin substrate. The data are also shown in figures 15 and 16.

Table 12. Effect of AMCA, compared with that of AMCHA and of EACA, in the urokinase sensitivity test.

Molar concentration of inhibitor in digestion mixture	Lysis time in urokinase sensitivity test
AMCA	
0	4 mins 30 secs
4.7×10^{-7}	15 mins 45 secs
9.4×10^{-7}	22 mins 30 secs
2.0×10^{-6}	31 mins 18 secs
4.7×10^{-6}	66 mins
7.5×10^{-6}	>500 mins
AMCHA	
0	6 mins
3.5×10^{-6}	6 mins 40 secs
7.1×10^{-6}	7 mins. 30 secs
1.7×10^{-5}	14 mins 40 secs
3.4×10^{-5}	21 mins 10 secs
6.8×10^{-5}	28 mins 10 secs
1.7×10^{-4}	48 mins 10 secs
EACA	
0	5 mins 45 secs
2.8×10^{-5}	8 mins
5.6×10^{-5}	11 mins 10 secs
1.1×10^{-4}	17 mins 35 secs
2.8×10^{-4}	21 mins
4.4×10^{-4}	27 mins 35 secs
5.6×10^{-4}	46 mins 15 secs

Shown is the antifibrinolytic effect of increasing concentrations of AMCA, AMCHA and EACA in the urokinase sensitivity test. These results are displayed graphically in figure 17.

Table 13. Effect of AMCA, compared with that of PAMBA, in the urokinase sensitivity test.

Molar concentration of inhibitor in digestion mixture	Lysis time in urokinase sensitivity test
AMCA	
0	4 mins
4.7×10^{-7}	15 mins
9.4×10^{-7}	22 mins 30 secs
2.0×10^{-6}	30 mins
4.7×10^{-6}	64 mins
PAMBA	
0	6 mins
1.75×10^{-6}	7 mins 30 secs
3.5×10^{-6}	9 mins 15 secs
4.25×10^{-6}	10 mins 30 secs
8.5×10^{-6}	16 mins
1.75×10^{-5}	23 mins
3.5×10^{-5}	28 mins 30 secs
1.75×10^{-4}	70 mins

Shown is the antifibrinolytic effect of increasing concentrations of AMCA and PAMBA in the urokinase sensitivity test. The data are shown graphically in figure 18.

Table 14. Thrombin clotting time and one-stage prothrombin time: effect of AMCHA.

Molar concentration AMCHA/ml plasma	0	1.0×10^{-2}	2.5×10^{-2}	3.0×10^{-2}
Clotting time, seconds, in thrombin clotting time	11.5, 12.0	11.6, 11.8	11.8, 11.8	12.5, 13.0
Clotting time, seconds, in one-stage prothrombin time	17.0, 17.0	17.0, 17.0	17.0, 18.0	20.0, 20.0

Shown is the effect of increasing concentrations of AMCHA in the thrombin clotting time and the one-stage prothrombin time. The first column shows the saline controls.

Table 15. Thromboplastin generation test: effect of AMCHA.

Incubation time, minutes	Clotting times, seconds, at varying AMCHA concentrations				
	Molar concentration AMCHA/ml incubation mixture				
	0	1.0×10^{-3}	1.0×10^{-2}	2.5×10^{-2}	3.0×10^{-2}
1	58	58	58	59	61
2	37	36	37	40	42
3	21	22	23	25	26
4	15	15	16	17	19
5	12	11	12	15	16
6	11	11	11	14	15

Shown is the effect of increasing concentrations of AMCHA added to the incubation mixture in the thromboplastin generation test. The first column shows the saline control.

Table 16. Effect of 2 gm AMCHA on the urokinase sensitivity test of a normal subject

Time of withdraval of plasma sample, hours after ingestion	Lysis time in urokinase sensitivity test		
	after 2 gm AMCHA	after dialysis	after dilution
0	8 mins	18 mins	18 mins
¼	12 mins 20 secs	17 mins 50 secs	22 mins 10 secs
½	20 mins 30 secs	18 mins 30 secs	28 mins 20 secs
1	31 mins	18 mins 20 secs	36 mins 10 secs
2	39 mins 30 secs	18 mins 30 secs	42 mins
3	28 mins 50 secs	18 mins 10 secs	34 mins 30 secs
5	26 mins 30 secs	18 mins	32 mins 40 secs
7	12 mins 50 secs	18 mins 25 secs	22 mins 35 secs
12	8 mins	18 mins 25 secs	18 mins 20 secs
24	8 mins 30 secs	18 mins 20 secs	18 mins 20 secs

Shown in the first column is the effect, on the urokinase sensitivity test of a normal subject, of ingestion of 2 gm AMCHA. These results, along with those of urokinase sensitivity tests on a control day, are shown in figure 19. The plasma samples, withdrawn after ingestion of AMCHA, were dialysed and the second column gives the results of the effect of dialysis on the urokinase sensitivity tests. A volume increase of the samples occurred during dialysis. The third column shows the results of testing undialysed samples, which had been diluted by the amount of this volume increase, in the urokinase sensitivity test.

Table 17. Effect of 1 gm AMCHA, compared with that of 2 gm EACA on the urokinase sensitivity test of a normal subject

Time of withdrawal of plasma sample, hours after ingestion	Lysis time in urokinase sensitivity test	
	after 1 gm AMCHA	after 2 gm EACA
0	6 mins. 30 secs.	8 mins.
$\frac{1}{6}$	7 mins. 30 secs.	9 mins. 20 secs.
$\frac{1}{2}$	11 mins.	17 mins. 30 secs.
1	19 mins. 50 secs.	20 mins. 20 secs.
2	22 mins.	22 mins. 30 secs.
3	17 mins. 25 secs.	17 mins. 20 secs.
5	13 mins. 30 secs.	14 mins. 30 secs.
7	11 mins. 30 secs.	7 mins.
12	6 mins. 30 secs.	7 mins.
24	6 mins. 30 secs.	7 mins.

Shown is the effect, on the urokinase sensitivity test of a normal subject, of ingestion of 1 gm AMCHA, compared with the effect of 2 gm EACA. Each compound was ingested on different days by the same subject. The results are displayed graphically in figure 20.

Table 18. Ingestion of 1 gm AMCHA compared with ingestion of 2 gm EACA; effect of dialysis

Time of withdrawal of plasma sample, hours after ingestion	Lysis time in urokinase sensitivity test			
	AMCHA samples		EACA samples	
	after dialysis	after dilution	after dialysis	after dilution
0	15 mins.20 secs.	15 mins.30 secs.	16 mins.	16 mins.10 secs.
¼	15 mins.30 secs.	17 mins.	15 mins.50 secs.	18 mins.30 secs.
½	15 mins.	20 mins.20 secs.	16 mins.50 secs.	21 mins.
1	15 mins.55 secs.	31 mins.50 secs.	16 mins.20 secs.	33 mins.30 secs.
2	15 mins.	35 mins.25 secs.	16 mins.30 secs.	36 mins.
3	15 mins.50 secs.	30 mins.10 secs.	16 mins.48 secs.	32 mins. 5 secs.
5	15 mins.20 secs.	24 mins.	16 mins.30 secs.	24 mins.30 secs.
7	15 mins.	19 mins.50 secs.	16 mins.50 secs.	15 mins.50 secs.
12	15 mins.30 secs.	15 mins.10 secs.	16 mins.	16 mins.
24	15 mins.20 secs.	15 mins.	16 mins.10 secs.	15 mins.40 secs.

Aliquots of the samples for which lysis times are shown in Table 17, were dialysed. Aliquots of the samples were diluted by the volume increase due to dialysis. Shown are the results of testing the samples thus manipulated in the urokinase sensitivity test.

Table 19. Effect of 200 mg AMCA, compared with that of 2 gm EACA, on the urokinase sensitivity test of a normal subject

Time of withdrawal of plasma sample, hours after ingestion	Lysis time in urokinase sensitivity test,	
	after 200 mg AMCA	after 2 gm EACA
0	5 mins. 30 secs.	8 mins.
¼	7 mins.	9 mins. 30 secs.
½	17 mins. 30 secs.	17 mins. 35 secs.
1	19 mins. 30 secs.	20 mins. 50 secs.
2	20 mins.	23 mins. 50 secs.
3	-	16 mins. 20 secs.
5	19 mins. 40 secs.	9 mins. 40 secs.
7	14 mins. 5 secs.	7 mins. 20 secs.
12	12 mins. 5 secs.	-
24	5 mins. 30 secs.	8 mins.

Shown is the effect, on the urokinase sensitivity test of a normal subject, of ingestion of 200 mg. AMCA, compared with the effect of 2 gm EACA. Each compound was ingested on different days by the same subject. The results are illustrated graphically in figure 21.

Table 20. Effect of 200 mg AMCA, compared with that of 1 gm AMCHA, on the urokinase sensitivity test of a normal subject.

Time of withdrawal of plasma sample, hours after ingestion	Lysis time in urokinase sensitivity test, after 200 mg AMCA	after 1 gm AMCHA
0	5 mins	6 mins 30 secs
¼	7 mins	7 mins 30 secs
½	17 mins 30 secs	11 mins
1	18 mins 50 secs	20 mins
2	19 mins 20 secs	22 mins 30 secs
3	-	19 mins 50 secs
5	18 mins 50 secs	13 mins 30 secs
7	14 mins	11 mins 30 secs
12	12 mins	6 mins 30 secs

Shown is the effect, on the urokinase sensitivity test of a normal subject, of ingestion of 200 mg AMCA, compared with the effect of 1 gm AMCHA. Each preparation was ingested by the same subject on different days. The data are shown graphically in figure 22.

Table 21. Effect of 200 mg AMCA on the urokinase sensitivity tests of five normal subjects

Time of withdrawal of plasma sample, hours after ingestion	Lysis time in urokinase sensitivity test				
	Subject number				
	1	2	3	4	5
0	5 mins	10 mins 20 secs	10 mins	11 mins 30 secs	10 mins 20 secs
¼	7 mins	18 mins 25 secs	12 mins 50 secs	11 mins 50 secs	16 mins 40 secs
½	17 mins 30 secs	24 mins 18 secs	26 mins	12 mins	24 mins 50 secs
1	18 mins 30 secs	35 mins 5 secs	32 mins	16 mins 50 secs	27 mins 50 secs
2	18 mins 50 secs	34 mins 10 secs	26 mins	22 mins	30 mins
3	18 mins 30 secs	34 mins 20 secs	26 mins	26 mins 50 secs	27 mins 20 secs
5	18 mins 30 secs	35 mins 20 secs	26 mins	26 mins 30 secs	27 mins 15 secs
7	14 mins	35 mins	22 mins 25 secs	16 mins 10 secs	24 mins
12	12 mins	23 mins 20 secs	19 mins	14 mins 20 secs	21 mins
24	5 mins	10 mins	10 mins	11 mins	10 mins

Shown is the effect, on the urokinase sensitivity tests of five normal subjects, of ingestion of 200 mg AMCA.

Table 22. Effect of 200 mg AMCA, compared with that of 2 gm EACA, on the Fearnley dilute plasma clot lysis time of a patient with hypofibrinogenaemia

Time of withdrawal of plasma sample, hours after ingestion	Lysis time, minutes, of Fearnley dilute plasma clot		
	after 200 mg AMCA	on control day	after 2 gm EACA
0	220	110	225
1½	320	125	-
3¼	450	104	285
4	-	-	298
5¼	340	145	515
7	240	-	345
12	195	-	200
24	200	170	180

Shown, in a patient who had hypofibrinogenaemia associated with accelerated lysis of Fearnley dilute plasma clots, is the effect of ingestion of 200 mg AMCA, compared with that of 2 gm EACA. The findings on a control intervening day, when no fibrinolytic inhibitor was administered, are also given. These results are displayed graphically in figure 23.

Table 23. Estimation of plasma levels of AMCA

Control curve		Test samples		
Molar concentration of AMCA/ml plasma	Lysis time, seconds, in urokinase sensitivity test	Time of withdrawal of plasma sample, hours after ingestion	Lysis time, seconds, in urokinase sensitivity test	Derived Molar concentration of AMCA/ml plasma
0	360	0	360	0
2.0×10^{-6}	422	$\frac{1}{4}$	504	4.4×10^{-6}
4.0×10^{-6}	490	$\frac{1}{2}$	1266	1.65×10^{-5}
8.0×10^{-6}	668	1	1332	1.7×10^{-5}
1.2×10^{-5}	890	2	1585	2.0×10^{-5}
1.6×10^{-5}	1202	5	1332	1.7×10^{-5}
2.0×10^{-5}	1585	8	1020	1.4×10^{-5}
		12	840	1.2×10^{-5}
		24	360	0

Shown, on the left, are the data for the control curve for estimation of plasma levels of AMCA. Shown, on the right, are the estimated plasma levels of AMCA, following the ingestion of 200 mg AMCA by the subject whose pre-ingestion plasma was used to prepare the control cure. The control curve is illustrated graphically in figure 24.

Table 24. Inhibition by Trasylol of caseinolytic activity of bank plasma

Concentration of Trasylol, units/ml. of incubation mixture	Optical density in caseinolytic assay	Per cent inhibition of caseinolytic activity
Trasylol added before activation		
0	0.250, 0.250	-
0.2	0.239, 0.241	4
0.4	0.216, 0.224	12
1.0	0.180, 0.180	28
2.0	0.140, 0.140	44
4.0	0.100, 0.110	58
8.0	0.032, 0.034	87
10.0	0.028, 0.028	99
Trasylol added after activation		
0	0.163, 0.163	-
0.2	0.100, 0.110	36
0.4	0.083, 0.083	49
1.0	0.078, 0.080	52
2.0	0.075, 0.075	54
4.0	0.038, 0.042	75
8.0	0.008, 0.008	95
10.0	0.005, 0.005	99

Shown is the effect in a caseinolytic assay system of increasing concentrations of Trasylol added to bank plasma before activation with streptokinase and after activation with streptokinase but prior to plasmin assay. These results are illustrated in figures 25 and 26.

Table 25. Inhibition by Trasylol of proteolytic activity of plasmin with sevenfold variation in substrate concentration: kinetics of the reaction

Concentration of substrate, casein: gm/100 ml digestion mixture. (S)	Reaction velocity in caseinolytic assay: mg tyrosine released/ml/hour. (V)	1/S	1/V
Control experiment without Trasylol			
2.00	0.97	0.50	1.0
0.80	0.61	1.25	1.6
0.50	0.41	2.00	2.4
0.40	0.32	2.50	3.1
0.33	0.29	3.03	3.5
0.285	0.25	3.50	4.0
Experiment with Trasylol, 5 units/ml digestion mixture, incorporated			
2.00	0.65	0.50	1.5
0.80	0.43	1.25	2.3
0.50	0.32	2.00	3.1
0.40	0.29	2.50	3.5
0.33	0.23	3.03	4.4
0.285	0.16	3.50	6.3
Experiment with Trasylol, 8 units/ml digestion mixture, incorporated			
2.00	0.22	0.50	4.6
0.80	0.20	1.25	5.0
0.50	0.18	2.00	5.6
0.40	0.145	2.50	6.9
0.33	0.11	3.03	9.1
0.285	0.09	3.50	11.1
Experiment with Trasylol, 10 units/ml digestion mixture, incorporated			
2.00	0.072	0.50	13.9
0.80	0.054	1.25	18.5
0.40	0.045	2.50	22.2
0.33	0.036	3.03	27.8
0.285	0.018	3.50	55.6

The double reciprocal plots shown in figures 27 and 28 are compiled from this table.

ble 26. Inhibition by Trasylol of proteolytic activity of plasmin with ourfold variation in substrate concentration: kinetics of the reaction

ntration of substrate, asein: gm/100 ml stion mixture. (S)	Reaction velocity in caseinolytic assay: mg tyrosine released/ml/hour. (V)	1/S	1/V
Control experiment without Trasylol			
2.00	0.83	0.50	1.2
1.00	0.69	1.00	1.5
0.80	0.54	1.25	1.9
0.65	0.43	1.54	2.3
0.53	0.35	1.89	2.9
Experiment with Trasylol, 5 units/ml digestion mixture, incorporated			
2.00	0.41	0.50	2.4
1.00	0.36	1.00	2.8
0.80	0.32	1.25	3.1
0.65	0.26	1.54	3.9
0.53	0.23	1.89	4.4

The double reciprocal plots shown in figure 29 are compiled from this table.

Table 27 Inhibition by Trasylol of [illegible]

with sevenfold variation in substrate concentration: kinetics of the reaction

Concentration of substrate plasminogen: casein units/ml added to digestion mixture. (S)	Reaction velocity in caseinolytic assay: casein units plasminogen activated/ml/minute. (V)	1/S	1/V
Control experiment without Trasylol			
23.3	0.135, 0.137	0.04	7.4
11.7	0.133, 0.135	0.09	7.5
7.9	0.127, 0.128	0.13	7.8
5.8	0.117, 0.117	0.17	8.5
4.7	0.100, 0.102	0.21	9.9
3.7	0.093, 0.100	0.27	10.4
2.8	0.080, 0.083	0.36	12.3
Experiment with Trasylol, 2 units/ml digestion mixture, incorporated			
23.3	0.123, 0.123	0.04	8.1
11.7	0.110, 0.112	0.09	9.0
7.9	0.100, 0.103	0.13	9.9
5.8	0.070, 0.072	0.17	14.1
4.7	0.047, 0.047	0.21	21.3
3.7	0.042, 0.042	0.27	23.8
2.8	0.030, 0.030	0.36	33.3
Experiment with Trasylol, 5 units/ml digestion mixture, incorporated			
23.3	0.108, 0.113	0.04	9.1
11.7	0.087, 0.087	0.09	11.5
7.9	0.073, 0.078	0.13	13.4
5.8	0.052, 0.053	0.17	19.0
4.7	0.023, 0.025	0.21	41.7
2.8	0.018, 0.020	0.36	52.6
Experiment with Trasylol, 10 units/ml digestion mixture, incorporated			
23.3	0.080, 0.085	0.04	12.1
11.7	0.060, 0.067	0.09	15.7
7.9	0.047, 0.050	0.13	20.6
5.8	0.022, 0.022	0.17	45.5
4.7	0.018, 0.018	0.21	55.6
3.7	0.012, 0.012	0.27	83.3
2.8	0.003, 0.003	0.36	333.3

The double reciprocal plots shown in figure 30 are compiled from this table.

Table 28. Inhibition by Trasylol of plasminogen activation by streptokinase with threefold variation in substrate concentration: kinetics of the reaction

Concentration of substrate, plasminogen: casein units/ml added to digestion mixture. (S)	Reaction velocity in caseinolytic assay: casein units plasminogen activated/ml/minute. (V)	$\frac{1}{S}$	$\frac{1}{V}$
	Control experiment without Trasylol		
23.3	0.123, 0.127	0.04	8.0
18.7	0.122, 0.123	0.05	8.2
16.3	0.117, 0.117	0.06	8.5
14.0	0.115, 0.117	0.07	8.6
11.7	0.113, 0.113	0.09	8.8
9.3	0.108, 0.108	0.11	9.3
7.9	0.107, 0.107	0.13	9.4
	Experiment with Trasylol, 5 units/ml digestion mixture, incorporated		
23.3	0.105, 0.105	0.04	9.5
18.7	0.097, 0.100	0.05	10.2
16.3	0.092, 0.092	0.06	10.9
14.0	0.083, 0.087	0.07	11.8
11.7	0.077, 0.080	0.09	12.7
9.3	0.070, 0.073	0.11	14.0
7.9	0.063, 0.067	0.13	15.4

The double reciprocal plots shown in figure 31 are compiled from this table.

Table 29. Inhibition by Trasylol of plasminogen activation by streptokinase with threefold variation in substrate concentration: kinetics of the reaction

Concentration of substrate, plasminogen: casein units/ml added to digestion mixture. (S)	Reaction velocity in caseinolytic assay: casein units plasminogen activated/ml/minute. (V)	1/S	1/V
Control experiment without Trasylol			
18.0	0.036	0.06	27.8
12.6	0.028	0.08	35.7
9.0	0.022	0.11	45.5
7.2	0.020	0.14	50.0
6.1	0.015	0.16	66.7
Experiment with Trasylol, 17 units/ml digestion mixture, incorporated			
18.0	0.027	0.06	37.0
12.6	0.022	0.08	45.5
9.0	0.015	0.11	66.7
7.2	0.013	0.14	76.9
6.1	0.010	0.16	100.0

The double reciprocal plots shown in figure 32 are compiled from this table.

Table 30. Inhibition by Trasylol of plasminogen activation by urokinase: kinetics of the reaction

Concentration of substrate, plasminogen: casein units/ml added to digestion mixture. (S)	Reaction velocity in caseinolytic assay: casein units plasminogen activated/ml/minute. (V)	1/S	1/V
Control experiment without Trasylol			
17.50	1.37	0.06	0.73
12.25	1.35	0.08	0.74
8.75	1.30	0.11	0.77
7.00	1.28	0.14	0.78
5.95	1.23	0.17	0.81
Experiment with Trasylol, 5 units/ml digestion mixture, incorporated			
17.50	1.27	0.06	0.79
12.25	1.18	0.08	0.85
8.75	1.12	0.11	0.89
7.00	1.03	0.14	0.97
5.95	0.98	0.17	1.02

The double reciprocal plots shown in figure 33 are compiled from this table.

Table 31. Inhibition by Trasylol of the esterolytic activity of urokinase on AcLMe with fivefold variation in substrate concentration: kinetics of the reaction.

Concentration of substrate, AcLMe; mg/100 ml digestion mixture. (S)	Reaction velocity in esterolytic assay; µ moles methyl alcohol released/ml urokinase/minute. (V).	1/S	1/V
Control experiment without Trasylol			
16.7	2.42	0.06	0.41
8.3	2.13	0.12	0.47
5.6	2.11	0.18	0.47
4.2	1.92	0.24	0.52
3.3	1.91	0.30	0.52
Experiment with Trasylol, 400 units/ml digestion mixture, incorporated			
16.7	1.91	0.06	0.52
8.3	1.60	0.12	0.63
5.6	1.39	0.18	0.72
4.2	1.12	0.24	0.89
3.3	0.94	0.30	1.06

The double reciprocal plots shown in figure 34 are compiled from this table

Table 32. Inhibition by Trasylol of the esterolytic activity of urokinase on AcLMe with threefold variation in substrate concentration: kinetics of the reaction

Concentration of substrate, AcLMe: mg/ml digestion mixture. (S)	Reaction velocity in esterolytic assay: μ moles methyl alcohol released/ml urokinase/minute	1/S	1/V
Control experiment without Trasylol			
16.9	1.95	0.06	0.51
11.3	1.93	0.09	0.52
8.5	1.85	0.12	0.54
7.0	1.82	0.14	0.55
5.6	1.70	0.18	0.59
Experiment with Trasylol, 500 units/ml digestion mixture, incorporated			
16.9	1.44	0.06	0.69
11.3	1.34	0.09	0.75
8.5	1.32	0.12	0.76
7.0	1.24	0.14	0.81
5.6	1.22	0.18	0.82

The double reciprocal plots shown in figure 35 are compiled from this table.

Table 33. Effect of Trasylol, compared with that of AMCA and of EACA, in the urokinase sensitivity test.

Molar concentration of inhibitor in digestion mixture	Lysis time in urokinase sensitivity test
Trasylol	
0	6 mins
1.5×10^{-7}	6 mins 40 secs
3.8×10^{-7}	9 mins 10 secs
4.5×10^{-7}	15 mins
6.0×10^{-7}	31 mins
6.8×10^{-7}	>500 mins
AMCA	
0	6 mins
1.0×10^{-5}	13 mins
2.5×10^{-5}	19 mins 20 secs
5.0×10^{-5}	31 mins
1.0×10^{-4}	51 mins 30 secs
1.5×10^{-4}	64 mins
2.0×10^{-4}	81 mins
2.5×10^{-4}	>500 mins
EACA	
0	6 mins
2.8×10^{-5}	8 mins
5.6×10^{-5}	11 mins 15 secs
1.1×10^{-4}	17 mins 35 secs
2.8×10^{-4}	21 mins
4.4×10^{-4}	27 mins 35 secs
5.6×10^{-4}	46 mins 20 secs

Shown is the antifibrinolytic effect of increasing concentrations of Trasylol, AMCA and EACA in the urokinase sensitivity test. These results are plotted in figure 36.

Table 34. Thrombin clotting time and one-stage prothrombin time: effect of Trasylol.

Trasylol concentration, units/ml plasma	0	50	100	250	500	2,500	5,000
Clotting time, seconds	13.2	11.8	12.8	12.0	11.8	11.8	11.2
in thrombin clotting time	13.4	12.6	13.0	12.8	12.6	12.4	11.4
Clotting time, seconds,	23.6	23.4	23.2	24.0	23.8	23.4	24.0
in one-stage prothrombin time	24.2	24.0	23.4	24.0	24.0	24.0	24.2

Shown is the effect of increasing concentrations of Trasylol in the thrombin clotting time and the one-stage prothrombin time. The first column shows the saline controls.

Table 35. Thromboplastin generation test: effect of Trasylol in incubation mixture.

Incubation time, minutes	Clotting times, seconds, at varying Trasylol concentrations			
	Trasylol concentration, units/ml incubation mixture			
	0	100	250	500
1	41	83	94	100
2	11	20	64	70
3	11	11	31	41
4	12	12	19	23
5	10	11	16	18
6	10	11	15	18

Shown is the effect of increasing concentrations of Trasylol added to the incubation mixture in the thromboplastin generation test. The first column shows the saline control. The results are graphically displayed in figure 37.

Table 36. Thromboplastin generation test: effect of Trasylol in incubation mixture.

Incubation time, minutes	Clotting times, seconds, at varying Trasylol concentrations					
	Trasylol concentration, units/ml incubation mixture					
	0	50	80	100	200	500
1	47	46	47	50	100	106
2	45	45	45	46	75	84
3	39	38	38	41	57	62
4	22	22	22	27	35	44
5	12	12	12	16	22	33
6	12	12	12	15	16	27
8	12	12	12	15	16	22
10	12	12	12	14	16	22
12	12	12	12	14	15	22
14	12	12	12	14	15	22
16	12	12	12	14	15	22

Shown is the effect of increasing concentrations of Trasylol added to the incubation mixture in the thromboplastin generation test. The first column shows the saline control.

Table 37. Thromboplastin generation test:

effect of Trasylol added to substrate plasma.

Incubation time, minutes	Clotting times, seconds, at varying Trasylol concentrations							
	Trasylol concentrations, units/ml substrate plasma							
	0	40	50	100	200	500	1,000	2,500
1	95	100	104	104	106	106	106	140
2	66	75	84	86	86	97	97	136
3	37	37	39	45	46	55	55	76
4	14	14	14	14	14	20	24	24
5	14	14	14	14	14	14	14	14
6	14	14	14	14	14	14	14	14

Shown is the effect of Trasylol added to the substrate plasma in the thromboplastin generation test. The first column shows the saline control. The data are displayed in part in figure 38.

Table 38. Platelet aggregation in Chandler's tube: effect of Trasylol added in vitro to plasma

Subject number	Time for platelet aggregation, seconds	
	Control	With Trasylol 300 units/ml plasma
1	630	805
2	552	928
3	572	797
4	588	977
5	568	1273
6	582	735
7	544	1058
Mean	577	939
S.D.	± 28	± 186

Shown is the effect on platelet aggregation of Trasylol added to plasma from each of seven subjects. Mean values and standard deviations are also shown. The mean time for platelet aggregation, when Trasylol was added to each plasma in a concentration of 300 units/ml, is prolonged significantly compared with the mean time for platelet aggregation in the absence of Trasylol ($t = 4.797$, $0.001 < p < 0.005$).

Table 39. Platelet aggregation in Chandler's tube: effect of Trasylol added *in vitro* to plasma

Subject number	Time for platelet aggregation, seconds	
	Control	With Trasylol 40 units/ml plasma
1	545	605
2	501	578
3	260	290
4	410	284
5	141	381
6	653	810
7	473	621
8	430	921
9	390	420
10	590	805
Mean	439	572
S.D.	± 152	± 227

Shown is the effect on platelet aggregation of Trasylol added to plasma from each of ten subjects. Mean values and standard deviations are also shown. The mean time for platelet aggregation, when Trasylol was added to each plasma in a concentration of 40 units/ml, is prolonged significantly compared with the mean time for platelet aggregation in the absence of Trasylol ($t = 2.543$, $0.02 < p < 0.05$)

Table 40 Platelet aggregation in Chandler's tube: effect of Trasylol incubated in vitro with plasma

Subject number	Time for platelet aggregation, seconds	
	Control	With Trasylol 40 units/ml plasma
1	275	255
2	292	286
3	260	255
4	188	358
5	247	335
6	340	480
7	240	171
8	330	390
9	320	267
10	440	320
Mean	293	312
S.D.	± 69	± 86

Shown is the effect on platelet aggregation of Trasylol incubated for 10 minutes with plasma from each of ten subjects. Mean values and standard deviations are also shown. The mean time for platelet aggregation, when Trasylol was incubated with plasma in a concentration of 40 units/ml, does not differ significantly from the mean time for platelet aggregation in the absence of Trasylol ($t = 0.625$, $0.5 < p < 0.6$).

Table 41 Biochemical effects of Trasylol infusion

	Time of sampling, hours			
	0	½	3	15
One-stage prothrombin time; seconds	15.8	15.4	15.0	14.0
Thrombin clotting time; seconds	10.5	9.6	10.4	9.6
Euglobulin clot lysis time; minutes	41	190	380	382
Fearnley plasma clot lysis time: minutes	230	382	1,200	1,200
Urokinase sensitivity test, lysis time; minutes, seconds	7,9	15,4	10,35	10,27
Fibrin plate test; mm^2; plasma.	49	0	0	0
resuspended euglobulin precipitate	110	36	0	0
Platelet count thousands/mm^3	170	157	207	212
Thromboplastin generation test Incubation time, minutes	Clotting times, seconds			
1	8.8	8.8	8.8	8.4
2	8.8	8.6	8.8	8.4
3	8.4	8.6	8.2	8.4
4	8.4	8.0	8.2	8.4
5	8.2	8.0	8.2	8.4
6	8.2	8.0	8.2	8.4

Shown is the effect in a normal subject of an intravenous infusion of Trasylol on various fibrinolytic and coagulation tests. The dosage schedule of Trasylol was 10,000 units in 30 minutes; 3,000 units/hour for two and one-half hours and 1,000 units/hour for 9 hours. The data are displayed in part in figure 39.

Table 42. Biochemical effects of Trasylol infusion

	Time of sampling, minutes	
	0	40
Euglobulin lysis; units	1.1	0.2
Urokinase sensitivity test, lysis time; minutes, seconds	8,39	16,39
Platelet count; thousands/mm^3	200	186
Thromboplastin generation test. Incubation time, seconds	Clotting times, seconds	
1	63	76
2	35	68
3	15	38
4	11	17
5	11	13
6	10	12
8	10	12

Shown is the effect in a normal subject of an intravenous infusion of Trasylol. The dosage schedule of Trasylol was 50,000 units as a single injection followed by 50,000 units over 30 minutes. The data are displayed in part in figure 40.

Table 43. Effect of Trasylol injection on the thromboplastin generation test, urokinase sensitivity test and platelet count

Incubation time, minutes	Time of sampling, hours: 0	½	1	2	3	5	7	9	24
	Thromboplastin generation test — Clotting times, seconds								
1	51	71	56	55	51	51	51	51	51
2	36	62	41	38	36	35	36	35	36
3	17	39	26	20	17	17	18	17	17
4	11	21	11	11	11	11	11	11	11
5	11	13	11	11	11	11	11	11	11
6	11	11	11	11	11	11	11	11	11
	Urokinase sensitivity test — Lysis time; minutes, seconds								
	9,40	1,25	16,0	15,20	16,20	17,15	15,35	15,25	9,20
	Platelet count — Thousands/mm^3								
	268	262	258	273	265	275	260	261	260

Shown is the effect of a single intravenous injection of Trasylol, containing 100,000 units, on the thromboplastin generation test, urokinase sensitivity test and platelet count of a normal subject. The dataare displayed in part in figure 41.

Table 44. Effect of Trasylol infusion on the thromboplastin generation test, urokinase sensitivity test and platelet count

Incubation time, seconds	Time of sampling, hours: 0	1	2	3	4	5	7
	Thromboplastin generation test: Clotting time, seconds						
1	71	77	80	80	80	80	81
2	55	62	64	68	68	75	77
3	32	39	58	60	60	62	62
4	15	17	35	40	40	43	43
5	15	16	16	20	20	22	22
6	15	15	15	15	15	15	15
	Urokinase sensitivity test: Lysis time; minutes, seconds						
	4,30	4,30	4,30	5,0	7,30	9,0	4,30
	Platelet count: Thousands/mm^3						
	308	318	310	320	302	312	316

Shown is the effect in a normal subject of a five hour intravenous infusion of Trasylol on the thromboplastin generation test, urokinase sensitivity test and platelet count. The Trasylol infusion was administered at a progressively increasing dosage; 10,000 units in the first hour; 20,000 units in the second hour; 50,000 units in the third hour; 100,000 units in the fourth hour and 200,000 units in the fifth hour. The data are displayed in part in figure 42.

Table 45. Effect of Trasylol infusion on the thromboplastin generation test, urokinase sensitivity test and platelet count

Incubation time, minutes	Time of sampling, hours: 0	1	2	3	4	5	7
	Thromboplastin generation test						
	Clotting time, seconds						
1	50	52	54	56	56	58	51
2	29	44	44	53	56	56	50
3	14	30	34	47	50	52	47
4	14	16	17	32	40	44	29
5	12	14	17	23	23	23	20
6	12	14	14	18	18	18	17
	Urokinase sensitivity test						
	Lysis time; minutes, seconds						
	5,0	6,5	6,35	6,40	19,0	19,0	6,10
	Platelet count						
	Thousands/mm^3						
	210	200	218	205	210	195	204

Shown is the effect in a normal subject of a five hour intravenous infusion of Trasylol on the thromboplastin generation test, urokinase sensitivity test and platelet count. The Trasylol infusion was administered at a progressively increasing dosage; 10,000 units in the first hour; 20,000 units in the second hour; 50,000 units in the third hour; 100,000 units in the fourth hour and 200,000 units in the fifth hour. The data are displayed in part in figure 43.

Table 46. Effect of Trasylol injection on platelet aggregation measured in the Chandler's tube

Subject number	Time for platelet aggregation, seconds	
	Before Trasylol	After Trasylol
1	480	360
2	636	448
3	495	410
4	455	145
5	486	125
6	586	137
7	623	311
Mean	537	277
S.D.	± 75	± 139

Shown for each of seven subjects is the effect of a single intravenous injection of Trasylol, containing 100,000 units, on platelet aggregation measure in the Chandler's tube. Mean values and standard deviations are also shown. The mean time for platelet aggregation 30 minutes after the Trasylol injection was significantly less than the mean time for platelet aggregation immediately pre-injection ($t = 4.976$, $0.001 < p < 0.005$)

Table 47. Effect of Trasylol injection on the platelet count

Subject number	Platelet count, thousands/mm^3	
	Before Trasylol	After Trasylol
1	300	301
2	355	350
3	398	402
4	243	238
5	208	211
6	383	380
7	278	275
Mean	309	308
S.D.	± 72	± 72

Shown for each of seven subjects is the effect of a single intravenous injection of Trasylol, containing 100,000 units, on the platelet count. Mean values and standard deviations are also shown. The mean platelet count immediately before the Trasylol injection is almost identical with the mean platelet count 30 minutes after the Trasylol injection.

Table 48. Thromboplastin generation test: effect of Trasylol injection.

	Thromboplastin generation test Clotting times, seconds					
Incubation time, minutes	1	2	3	4	5	6
Subject number						
1	43	42	21	10	10	10
	52	45	24	13	12	12
2	45	36	21	10	10	10
	49	44	34	16	13	12
3	75	68	59	41	17	16
	75	69	63	41	21	17
4	61	63	43	34	19	13
	61	61	61	46	26	17
5	46	44	26	17	11	11
	62	62	46	26	18	16
6	53	41	24	15	12	12
	60	50	45	35	23	17
7	63	59	33	16	13	13
	69	61	52	31	22	18

Shown for each of 7 subjects are the results of thromboplastin generation tests carried out before a single intravenous injection of Trasylol containing 100,000 units and 30 minutes after the injection. The results of the test before Trasylol injection for each subject are recorded above the results of the test after Trasylol injection. At 6 minutes incubation, the mean clotting time before Trasylol injection was 12.1 ± 2.1 seconds and after Trasylol injection was 15.6 ± 2.5 seconds. The prolongation of clotting times after Trasylol injection is significant ($t = 5.277$, $0.001 < p < 0.005$).

Table 49. Effect of heparin infusion compared with Trasylol infusion in a patient with the defibrination syndrome.

	Time of sampling, hours							
	0	11	31½	48	55	72½	79½	96
Fibrinogen, mg/100 ml	115	137	158	175	195	100	100	137
Plasminogen, casein units/ml	1.3	-	1.3	-	1.8	1.4	1.7	1.9
Thrombin clotting time, seconds	15	15	13	7	7	15	13	13
Anti-haemophilic globulin, per cent	15	-	45	-	45	7½	25	32
Factor V, per cent	25	-	40	-	85	6	42	100
Platelet count, thousands/mm^3	116	115	110	112	-	98	45	54
Christmas factor, per cent	100	-	100	-	100	100	100	100

Shown is the effect, in a patient with hypofibrinogenaemia associated with metastatic carcinoma, of a heparin infusion compared with a Trasylol infusion. Heparin infusion was administered for 55 hours; 1,000 units/hour for 48 hours then 1,500 units/hour for 7 hours. No therapy was given thereafter for 17½ hours. Tradylol infusion was then administered; 100,000 units/hour for 7 hours. A further heparin infusion, 1,500 units/hour for 16½ hours, followed the Trasylol infusion. These results are displayed in part in figure 46.

Table 50. Effect of Trasylol infusion in a patient with the defibrination syndrome

	Time of sampling, hours		
	0	3	9
Fibrinogen mg/100 ml	45	75	100
Plasminogen casein units/ml	0.9	1.5	1.5
Thrombin clotting time, seconds	20	18	15
One-stage prothrombin time, seconds	21	17	16
Anti-haemophilic globulin, per cent	5	60	60
Factor V, per cent	8	80	100
Platelet count thousands/mm^3	50	63	90

Shown is the effect, in a patient with hypofibrinogenaemia associated with metatastic carcinoma, of Trasylol infusion. The infusion rate was 100,000 units/ hour for 9 hours. These results are displayed in part in figure 47.

Table 51. Inhibition by Trasylol of proteolytic activity of trypsin: kinetics of the reaction

Concentration of substrate, casein: gm/100 ml digestion mixture. (S)	Reaction velocity in caseinolytic assay: mg tyrosine released/ml/hour. (V)	1/S	1/V
Control experiment without Trasylol			
2.00	0.67	0.50	1.5
1.00	0.46	1.00	2.2
0.80	0.40	1.25	2.5
0.64	0.32	1.56	3.1
0.52	0.29	1.92	3.4
0.40	0.25	2.50	4.0
0.28	0.18	3.57	5.6
Experiment with Trasylol, 100 units/ml. digestion mixture, incorporated			
2.00	0.38	0.50	2.6
1.00	0.32	1.00	3.1
0.80	0.28	1.25	3.6
0.64	0.24	1.56	4.2
0.52	0.19	1.92	5.3
0.40	0.18	2.50	5.6
0.28	0.13	3.57	7.7

The double reciprocal plots shown in figure 48 are compiled from this table.

Table 52. Inhibition by Trasylol of trypsin added to bank plasma

Concentration of trypsin, μg/ml plasma	Optical density in trypsin assay		Per cent inhibition of trypsin activity
	Without Trasylol	With Trasylol	
0	0	0	-
10	0	0	-
20	0	0	-
50	0.020, 0.020	0	100
100	0.020, 0.020	0	100
120	0.020, 0.020	0	100
160	0.020, 0.020	0	100
200	0.036, 0.038	0.020, 0.022	43
400	0.064, 0.066	0.035, 0.045	38
500	0.090, 0.090	0.045, 0.055	44
600	0.110, 0.110	0.080, 0.090	23
700	0.150, 0.155	0.104, 0.105	31
800	0.175, 0.185	0.105, 0.115	39
900	0.275, 0.275	0.195, 0.205	27
1,000	0.360, 0.370	0.245, 0.255	32

Shown is the activity in a caseinolytic assay system of increasing concentrations of trypsin, assayed in the presence of bank plasma. Shown also is the effect of Trasylol, 40 units/ml of plasma, on the activity of the same concentrations of trypsin assayed in the presence of bank plasma.

Table 53. Anti-tryptic activity in plasma: effect of Trasylol injection

Subject number	Optical density in trypsin assay, before Trasylol	after Trasylol
1	0.105, 0.115	0.030, 0.030
2	0.170, 0.180	0.140, 0.150
3	0.175, 0.185	0.150, 0.150
4	0.160, 0.166	0.154, 0.156
5	0.150, 0.150	0.140, 0.044
6	0.300, 0.300	0.300, 0.300
7	0.140, 0.142	0.112, 0.118
Mean	0.174	0.134
S.D.	± 0.060	± 0.089

Shown are the results of assay of trypsin, 1,000 µgm/ml of plasma, added to plasmas obtained from each of 7 normal subjects immediately before the intravenous injection of Trasylol, 100,000 units, and 30 minutes after the injection. Mean values and standard deviations are also shown. Mean activity of trypsin in plasma after Trasylol injection is significantly less than mean trypsin activity in plasma before Trasylol injection ($t = 3.451$, $0.01 < p < 0.02$)

Table 54. Effect of dialysed urokinase and pure urokinase substituted for human brain in the one-stage prothrombin time

Plasma number	Recalcification time, seconds			
	Human brain	Dialysed urokinase	Pure urokinase	Saline
1	77	75	76	107
2	79	77	75	116
3	84	84	86	101
4	78	72	77	95
5	92	86	88	110
6	86	69	74	96

Shown are the results of substituting 10,000 C.T.A. units/ml solutions of dialysed and pure urokinase for a 1:500 dilution of human brain extract in the one-stage prothrombin time. The last column gives the saline controls. EACA was present in the system in a concentration of 10^{-2} Molar to inhibit lysis.

Table 55. Coagulative activity of dialysed urokinase and pure urokinase added to silicone plasma.

Concentration of urokinase in test system, units/ml plasma	Recalcification time, seconds	
	with dialysed urokinase	with pure urokinase
0	446	621
50	445	618
200	428	589
500	413	557
1,000	401	552
2,000	354	512
5,000	332	353
10,000	326	366
20,000	299	320
50,000	294	245

Shown is the effect of increasing concentrations of dialysed urokinase and pure urokinase on the recalcification time of silicone plasma. In each case a saline control is shown. The test system contained EACA at a concentration of 10^{-2} Molar to inhibit lysis. The data for the dialysed urokinase are graphically displayed in figure 49 and the data for the pure urokinase in figure 50.

Table 56. Coagulative activity of dialysed urokinase added to Hageman-factor deficient plasma.

Concentration of urokinase in test system, units/ml plasma.	Recalcification time of Hageman-factor deficient plasma, seconds
0	1,316
94	1,324
188	1,225
375	1,026
750	935
1,500	778
3,000	571
6,000	574
12,000	395

Shown is the effect of increasing concentrations of dialysed urokinase on the recalcification time of Hageman-factor deficient plasma. A saline control is also shown. The test system contained EACA in a concentration of 10^{-2} Molar to inhibit lysis. The data are graphically displayed in figure 51.

Table 57. Coagulative activity of two separate batches of dialysed urokinase added to fresh normal plasma.

Concentration of urokinase in test system, units/ml plasma	Recalcification time of fresh normal plasma, seconds.	
	urokinase	Further purified urokinase
0	416	385
50	414	377
100	412	375
200	406	372
500	404	351
1,000	379	349
2,000	368	340
5,000	368	330
10,000	353	310

Shown is the effect of increasing concentrations of two separate batches of dialysed urokinase on the recalcification time of fresh normal plasma. A saline control is also shown. The test system contained EACA in a concentration of 10^{-2} Molar to inhibit lysis.

Table 58. The effect of dialysed and pure urokinase in the thromboplastin generation test with deficiency of antihaemophilic globulin and Christmas factor.

Incubation time, minutes		Clotting times, seconds					
		1	2	3	4	5	6
Normal plasma Normal serum Platelet substitute	No urokinase	17	17	16	16	15	13
Haemophilic plasma	No urokinase	64	55	43	40	40	40
Normal serum	Dialysed urokinase	52	50	37	35	33	33
Platelet substitute	Pure urokinase	52	36	35	35	34	33
Normal plasma	No urokinase	91	89	82	82	79	75
Christmas serum	Dialysed urokinase	85	85	75	60	55	45
Platelet substitute	Pure urokinase	85	81	73	60	55	51

Shown is the effect of dialysed urokinase and pure urokinase in partially correcting the defect due to deficiency of antihaemophilic globulin and Christmas factor in the thromboplastin generation test. Where urokinase was added, its concentration was 4,000 C.T.A. units in the incubation mixture. EACA was present in the test system at a concentration of 10^{-2} Molar to inhibit lysis.

Table 59. The effect of dialysed and pure urokinase in the thromboplastin generation test with deficiency of platelets.

Incubation time, minutes	Clotting times, seconds 1	2	3	4	5	6
Normal plasma) Normal serum) No urokinase Normal platelets)	15	15	13	13	13	13
Normal plasma) No urokinase	98	98	98	97	96	96
Normal serum) Dialysed urokinase	74	73	72	66	54	48
No platelets) Pure urokinase	81	73	67	65	63	60
Normal plasma 1 in 4) Serum diluted 1 in 4) No urokinase Normal platelets)	19	19	19	19	17	17
Normal plasma 1 in 4) No urokinase	94	66	43	33	33	33
Serum diluted 1 in 4)Dialysed urokinase	79	54	30	20	20	20
Platelets diluted 1 in 3) Pure urokinase	63	56	37	26	25	24

Shown is the effect of dialysed urokinase and pure urokinase in partially correcting the defect due to deficiency of platelets in the thromboplastin generation test. Where urokinase was added, its concentration was 6,000 C.T.A. units in the incubation mixture without platelets and 4,000 C.T.A. units with platelets diluted one in three. EACA was present in the test system at a concentration of 10^{-2} Molar to inhibit lysis.

Table 60. The effect of dialysed urokinase in the thromboplastin generation test, substituted for plasma and serum.

Incubation time, minutes	Clotting time, seconds 1	2	3	4	5	6
Normal plasma Normal serum Normal platelets	63	35	13	11	11	11
Saline Normal serum Normal platelets	88	87	87	86	84	80
Urokinase Normal serum Normal platelets	63	56	53	50	49	47
Normal plasma Saline Normal platelets	93	92	91	90	90	90
Normal plasma Urokinase Normal platelets	76	75	71	58	55	34

Shown is the effect of dialysed urokinase in partially correcting the defect due to absence of plasma and serum in the thromboplastin generation test. Where urokinase was added, its concentration was 6,000 C.T.A. units in the incubation mixture. EACA was present in the test system at a concentration of 10^{-2} Molar to inhibit lysis.

Table 61. Effect of urokinase in antihaemophilic globulin and Christmas factor assays.

ANTIHAEMOPHILIC GLOBULIN ASSAY

	Control dilution curve, per cent dilution of control plasma						
	100	50	20	10	5	2	0
Clotting time, seconds of substrate plasma	161	178	200	215	225	243	285

	Substitutions for 100 per cent control plasma		
	Haemophilic plasma	Pure urokinase	Dialysed urokinase
Clotting time, seconds, of substrate plasma	271	243	244

CHRISTMAS FACTOR ASSAY

	Control dilution curve, per cent dilution of control plasma						
	100	50	20	10	5	2	0
Clotting time, seconds, of substrate plasma	147	155	179	184	192	203	268

	Substitutions for 100 per cent control plasma		
	Christmas plasma	Pure urokinase	Dialysed urokinase
Clotting time, seconds, of substrate plasma	264	205	204

Shown are the effects of pure and dialysed urokinase substituted for 100 per cent of the control plasma in antihaemophilic globulin and Christmas factor assays. In each assay, control dilutions were made from a pool of 10 normal plasmas. In each assay, the controls and substitutions were prepared and recalcified simultaneously. Urokinase was present in each system in a concentration of 2,000 C.T.A. units. Both forms of urokinase had closely similar effects. Each preparation had the same effect in both assays as the 2 per cent dilutions of the control plasma. EACA, 10^{-2} Molar, was present in the systems to inhibit lysis.

Table 62. Effect on platelet aggregation of fibrinogen degradation products added in vitro to plasma.

Subject number	Time for platelet aggregation, seconds	
	control	with fibrinogen degradation products
1	533	303
2	418	286
3	270	242
4	556	289
5	255	237
6	678	356
7	629	342
Mean	477	294
S.D.	± 168	± 45

Shown are the times for platelet aggregation in the Chandler's tube of plasma from each of 7 normal subjects and the times for platelet aggregation of the same plasmas to which had been added fibrinogen degradation products in a concentration of 180 μg/ml plasma. Mean values and standard deviations are also shown. In the presence of fibrinogen degradation products, platelet aggregation was significantly enhanced compared with the control ($t = 3.874$, $0.005 < p < 0.01$).

Table 63. Effects of urokinase infusion on the fibrinolytic enzyme system.

	Time of sampling, hours						
	0	½	2½	12	13½	16½	18
Radioactive clot assay, μgm fibrin lysed/ml plasma/hour	0	126.7	0	15.5	29.4	113.9	0
Fibrin plate test, mm^2;							
plasma	0	163	0	0	53	313	-
resuspended euglobulin precipitate	75	145	0	0	33	76	185
Euglobulin lysis activity, units	1.61	1.44	1.62	1.20	2.14	4.61	9.68
Plasminogen, casein units/ml	3.9	1.8	1.8	0.9	1.0	0.4	0.5
Thrombin time, seconds	9.8	10.1	9.2	12.3	12.3	17.8	18.8
Fibrinogen, mg/100 ml	465	430	423	380	350	333	315

Shown in one subject is the effect on the fibrinolytic enzyme system of an intravenous infusion of urokinase. The infusion rate was 600,000 C.T.A. units in 30 minutes; 100,000 C.T.A. units/hour for 12½ hours; 200,000 C.T.A. units for 1 hour and 400,000 C.T.A. units for 1 hour. The data are displayed in part in figure 54.

Table 64. Effects of urokinase infusion on the coagulation system.

	time of sampling, hours					
	0	½	2½	12	13½	16½
Recalcification time, seconds	171	130	170	160	175	185
Hageman factor assay, seconds	320	270	350	333	333	333
Factor V assay, seconds	25	71	61	68	59	69
Antihaemophilic globulin assay, seconds	143	135	150	157	162	163
Christmas factor assay, seconds	167	150	154	158	165	158
One-stage prothrombin time, seconds	22.3	20.0	19.8	22.2	24.8	25.6
Platelet count, thousands/mm^3	495	399	660	662	562	552

Shown in one subject is the effect of an intravenous infusion of urokinase. The infusion rate was 600,000 C.T.A. units in 30 minutes; 100,000 C.T.A. units/hour for twelve hours; 200,000 C.T.A. units for the next hour and 400,000 C.T.A. units for the final hour. EACA was present in all clotting systems in a concentration of 10^{-2} Molar to inhibit lysis. The data are displayed in part in figure 55. The dilution curves for the various assays are shown in table 67.

Table 65. Effects of urokinase infusion on the fibrinolytic enzyme system

	Time of sampling, hours							
	0	1	2	4	8	12	24	48
Fibrin plate test, mm^2; plasma	0	325	361	391	900	900	442	-
Euglobulin lysis activity, units	0.97	6.0	6.0	∞	∞	∞	15.8	2.6
Plasminogen, casein units/ml	2.2	1.4	1.4	0.8	0.4	0	0.2	1.2
Thrombin time, seconds	8.5	15.0	16.0	16.0	43.0	26.5	23.0	15.0
Fibrinogen, mg/100 ml	434	432	424	225	130	145	115	140

Shown in one subject is the effect on the fibrinolytic enzyme system of an intravenous infusion of urokinase administered at a rate of 500,000 C.T.A. units/hour for 24 hours. The data are displayed in part in figure 56.

Table 66. Effects of urokinase infusion on the coagulation system

	Time of sampling, hours							
	0	1	2	4	8	12	24	48
Silicone recalcification time, seconds	160	83	226	187	189	185	165	160
Factor V assay, seconds	136	295	224	286	194	207	197	160
Antihaemophilic globulin assay, seconds	107	120	125	131	140	140	135	119
Christmas factor assay, seconds	80	87	88	89	94	95	85	84
One-stage prothrombin time, seconds	22.5	25.0	27.5	30.0	43.0	26.5	23	15
Platelets, thousands/mm^3	275	175	240	290	262	290	295	375

Shown in one subject is the effect on the coagulation system of an intravenous infusion of urokinase. The infusion rate was 500,000 C.T.A. units/hour for 24 hours. EACA was present in all clotting systems in a concentration of 10^{-2} Molar to inhibit lysis. The data are displayed in part in figure 57. The dilution curves for the various assays are shown in table 67.

Table 67. Dilution curves for coagulation factor assays performed on plasma samples obtained during two urokinase infusions.

Per cent dilution	100	50	25	12	6
Hageman factor dilution curve, secs	320	362	380		
Factor V dilution curve, secs	25 136	31 158	40 178	46 188	50 203
Antihaemophilic globulin dilution curve, secs	143 107	162 120	179 129	198 137	215 143
Christmas factor dilution curve, secs	167 80	178 88	190 102	107	116

Shown are dilution curves for the coagulation factor assays performed for two subjects who were given urokinase infusions. Each dilution curve was made with the pre-infusion plasma of each subject. The Hageman factor assay was carried out for the subject whose data are shown in table 64. The other assays were carried out for both subjects. Those dilution curves recorded on the upper lines pertain to the subject whose data are shown in table 64 and those on the lower lines for the subject whose data are shown in table 66. EACA was present in all clotting systems at a concentration of 10^{-2} Molar.

Table 68. Effect of intravenous injection of urokinase on platelet aggregation measured in Chandler's tube.

Subject number	Time for platelet aggregation, seconds,	
	Before urokinase	After urokinase
1	570	465
2	460	260
3	630	582
4	530	306
5	430	350
6	435	450
7	490	470
Mean	506	412
S.D.	± 74	± 112

Shown for each of seven normal subjects are the results of measuring platelet aggregation in the Chandler's tube immediately before the intravenous injection of urokinase, 80,000 C.T.A. units, and 10 minutes after the injection. Mean values and standard deviations are also shown. The mean time for platelet aggregation after the urokinase injection is significantly less than the mean time before the injection ($t = 2.800$, $0.02 < p < 0.05$)

Table 69. Effect of intravenous injection of urokinase on euglobulin lysis activity

Subject number	Euglobulin lysis activity, units,	
	Before urokinase	After urokinase
1.	0.97	1.92
2	0.81	1.61
3	0.83	0.88
4	1.46	2.61
5	0.64	1.50
6	0.60	1.20
7	1.11	1.58
Mean	0.92	1.61
S.D.	± 0.30	± 0.55

Shown for each of seven normal subjects are the results of euglobulin lysis tests, immediately before the intravenous injection of urokinase, 80,000 C.T.A. units, and 10 minutes after the injection. Mean values and standard deviations are also shown. Mean euglobulin lysis activity after the urokinase injection is significantly greater than the mean activity pre-injection ($t = 5.145$, $0.001 < p < 0.005$).

Table 70 Effect of intravenous injection of urokinase on platelet count

Subject number	Platelet count, thousands/mm^3	
	Before urokinase	After urokinase
1	250	245
2	240	210
3	210	210
4	220	200
5	410	400
6	229	231
7	398	383
Mean	279.6	268.4
S.D.	± 86.0	± 85.5

Shown for each of seven normal subjects are the results of platelet counts immediately before the intravenous injection of urokinase, 80,000 C.T.A. units, and 10 minutes after the injection. Mean values and standard deviations are also shown. The mean platelet count after the urokinase injection is significantly less than the mean platelet count immediately pre-injection ($t = 2.575$, $0.02 < p < 0.05$).

Table 71. Content of the low fat breakfast and of the high fat breakfast

	Carbohydrate, gm	Protein, gm	Fat, gm
Low fat breakfast			
Cornflakes, 1 cup	22.1	1.7	0.2
Stewed fruit, ½ cup	10.0	-	-
White bread, 1 slice	15.8	2.3	0.4
Marmalade, 1 teaspoon	10.0	-	-
Black coffee + sugar, 1 teaspoon	5.0	-	-
High fat breakfast			
Low fat breakfast	62.9	4.0	0.6
Double cream, 270 ml.	8.1	6.2	94.5

Table 72. Plasminogen levels after the low fat breakfast and after the high fat breakfast

Subject number	Plasminogen, casein units/ml	
	Control	After fat
1	2.24, 2.26	2.31, 2.39
2	2.89, 2.91	2.60, 2.60
3	2.59, 2.61	2.57, 2.63
4	2.09, 2.11	2.19, 2.21
5	2.25, 2.35	2.59, 2.61
6	2.07, 2.13	2.30, 2.30
7	2.20, 2.20	2.28, 2.32
8	2.15, 2.35	1.90, 1.90
9	2.18, 2.22	2.23, 2.25
10	2.85, 2.85	2.30, 2.30
Mean	2.375	2.344
S.D.	± 0.299	± 0.218

Shown for each subject are the results of duplicate plasminogen assays after each type of breakfast. Mean values and standard deviations are also shown. Plasminogen levels after each type of breakfast do not differ significantly ($t = 0.375$, $0.7 < p < 0.8$).

Table 73 Fibrinogen levels after the low fat breakfast and after the high fat breakfast.

Subject Number	Fibrinogen, mg/100 ml	
	Control	After fat
1	272, 278	235, 245
2	394, 408	373, 387
3	380, 380	396, 404
4	363, 367	360, 360
5	236, 244	248, 252
6	160, 160	166, 174
7	158, 162	168, 172
8	147, 153	150, 150
9	200, 210	202, 208
10	190, 200	200, 200
Mean	253.0	252.5
S.D.	±97.0	±94.0

Shown for each subject are the results of duplicate fibrinogen assays for each type of breakfast. Mean values and standard deviations are also shown. Fibrinogen levels after each type of breakfast are almost identical ($t = 0.098$, $0.90 < p < 0.95$).

Table 74. Euglobulin lysis tests after the low fat breakfast and after the high fat breakfast

Subject number	Euglobulin lysis activity, units	
	Control	After fat
1	1.33	0.94
2	1.25	0.91
3	2.70	1.15
4	2.50	1.22
5	1.88	1.18
6	0.92	0.77
7	1.67	1.65
8	3.16	1.90
9	1.63	1.29
10	0.20	0.03
Mean	1.72	1.10
S.D.	± 0.88	± 0.51

Shown for each subject are the results of euglobulin lysis tests after each type of breakfast. Shown also are mean values and standard deviations. Mean euglobulin lysis activity after the high fat breakfast is significantly less than mean euglobulin lysis activity after the low fat breakfast ($t = 3.573$, $0.005 < p < 0.01$).

Table 75. Urokinase sensitivity tests after the low fat breakfast and after the high fat breakfast

Subject number	Urokinase sensitivity test, units	
	Control	After fat
1	0.97	0.76
2	1.77	1.38
3	1.12	0.74
4	1.25	0.97
5	2.31	1.71
6	0.68	0.68
7	0.65	0.48
8	0.67	0.60
9	0.48	0.54
10	0.60	0.59
Mean	1.05	0.85
S.D.	± 0.59	± 0.38

Shown for each subject are the results of urokinase sensitivity tests after each type of breakfast. Mean values and standard deviations are also shown. The mean increased resistance to lysis of the standard plasma clots formed in this test after the high fat breakfast is significant compared with the finding after the low fat breakfast ($t = 3.087$, $0.01 < p < 0.02$).

Table 76. Lysis, measured as reduction in radioactivity, of Chandler thrombi formed after the low fat breakfast and after the high fat breakfast

Subject number	Lysis of Chandler thrombi, per cent reduction in radioactivity	
	Control	After fat
1	67.8	29.3
2	66.0	64.6
3	67.1	35.2
4	35.8	31.0
5	62.0	35.3
6	28.3	25.4
7	38.4	30.7
8	41.1	22.7
9	48.1	46.4
10	31.6	30.0
Mean	48.6	35.1
S.D.	± 15.7	± 12.2

Shown for each patient is the per cent reduction in radioactivity of ^{131}I-tagged Chandler thrombi formed after each type of breakfast and subjected to lysis by streptokinase for 24 hours in an artificial circulation. Mean values and standard deviations are also shown. The mean reduction in radioactivity of thrombi formed after the high fat breakfast is significantly less than the mean reduction in radioactivity of thrombi formed after the low fat breakfast ($t = 3.024$, $0.01 < p < 0.02$)

Table 77. Lysis, measured as a reduction in weight, of Chandler thrombi formed after the low fat breakfast and after the high fat breakfast.

Subject number	Lysis of Chandler thrombi, per cent reduction in weight	
	Control	After fat
3	90.3	73.7
4	71.0	61.9
5	70.2	64.8
6	72.6	66.5
7	54.6	28.0
8	74.9	66.7
9	78.8	57.0
10	52.8	49.3
Mean	56.5	46.8
S.D.	± 12.3	± 14.3

Shown for subjects number 3-10 is the per cent weight loss of Chandler thrombi formed after each type of breakfast and subjected to lysis by streptokinase for 24 hours in an artificial circulation. Mean values and standard deviations are also shown. Mean weight loss of thrombi formed after the high fat breakfast is significantly less than mean weight loss of thrombi formed after the low fat breakfast ($t = 4.054$, $0.001 < p < 0.005$).

Table 78. Russell viper venom (Stypven) clotting times after the low fat breakfast and after the high fat breakfast

Subject number	Clotting time, seconds	
	Control	After fat
1	14.7, 14.9	9.0, 9.0
2	17.2, 17.6	9.0, 9.4
3	29.0, 29.0	21.0, 21.0
4	20.2, 20.4	11.9, 12.1
5	24.0, 24.0	15.5, 16.5
6	19.8, 20.2	16.0, 16.0
7	30.0, 30.0	22.8, 23.2
8	28.4, 29.6	21.0, 21.0
9	21.0, 21.0	16.0, 16.0
10	23.0, 23.0	12.6, 13.4
Mean	22.9	15.6
S.D.	± 5.2	± 4.9

Shown for each subject are Russell viper venom (Stypven) clotting times after each type of breakfast. Mean values and standard deviations are also shown. Each clotting time was measured in duplicate. The mean clotting time after the high fat breakfast is significantly shorter than the mean clotting time after the low fat breakfast ($t = 12.726$, $p < 0.001$).

Table 79. Platelet aggregation in the Chandler's tube after the low fat breakfast and after the high fat breakfast

Subject number	Time for platelet aggregation, seconds	
	Control	After fat
1	521	588
2	512	603
3	589	665
4	330	514
5	624	699
6	519	683
7	550	605
8	530	614
9	608	570
10	430	490
Mean	521	603
S.D.	± 87	± 68

Shown for each subject are the times for platelet aggregation in the Chandler's tube after each type of breakfast. Mean values and standard deviations are also shown. The mean time for platelet aggregation after the high fat breakfast is significantly prolonged compared with the finding after the low fat breakfast ($t = 4.263$, $0.005 < p < 0.01$)

Table 80. Platelet counts after the low fat breakfast and after the high fat breakfast

Subject number	Platelet count, thousands/mm^3	
	Control	After fat
1	200	272
2	171.5	198
3	230	228
4	196	258
5	250	228
6	200	201
7	183	150
8	250	256
9	174	201
10	342	332
Mean	219.7	232.4
S.D.	± 51.6	± 50.2

Shown for each subject are platelet counts after each type of breakfast. Mean values and standard deviations are also shown. Platelet counts after each type of breakfast do not differ significantly ($t = 1.178$, $0.2 < p < 0.3$).

Table 81. Serum triglyceride levels after the low fat breakfast and after the high fat breakfast

Serum number	Serum triglycerides, mg/100 ml	
	Control	After fat
1	128	304
2	130	239
3	62	87
4	83	256
5	59	193
6	118	162
7	41	50
8	78	120
9	61	117
10	45	114
Mean	81	164
S.D.	± 34	± 82

Shown for each subject are serum triglyceride levels after each type of breakfast. Mean values and standard deviations are also shown. The mean serum triglyceride level after the high fat breakfast is significantly higher than the mean level after the low fat breakfast ($t = 4.427$, $0.001 < p < 0.005$).

Table 82. Serum phospholipid levels after the low fat breakfast and after the high fat breakfast

Subject Number	Serum phospholipids, mg/100 ml	
	Control	After fat
1	209	211
2	186	225
3	204	263
4	210	188
5	165	218
6	233	306
7	241	293
8	225	239
9	239	274
10	203	226
Mean	212	244
S.D.	± 24	± 38

Shown for each subject are serum phospholipid levels after each type of breakfast. Mean values and standard deviations are also shown. The mean serum phospholipid level after the high fat breakfast is significantly higher than the mean level after the low fat breakfast ($t = 3.596$, $0.005 < p < 0.01$)

Table83. Plasma free fatty acid levels after the low fat breakfast and after the high fat breakfast

Subject number	Plasma free fatty acids, μe/litre	
	Control	After fat
1	389	703
2	962	1018
3	620	944
4	518	972
5	500	925
6	546	833
7	481	722
8	296	1258
9	666	1182
10	629	963
Mean	561	952
S.D.	± 180	± 177

Shown for each subject are plasma free fatty acid levels after each type of breakfast. Also shown are mean values and standard deviations. The mean plasma free fatty acid level after the high fat breakfast is significantly higher than the mean level after the low fat breakfast ($t = 5.213$, $p < 0.001$).

Table 84. Serum cholesterol levels after the low fat breakfast and after the high fat breakfast

Subject number	Serum cholesterol, mg/100 ml	
	Control	After fat
1	227	247
2	201	195
3	174	152
4	179	209
5	201	165
6	296	296
7	242	210
8	215	214
9	292	220
10	199	188
Mean	223	210
S.D.	± 43	± 41

Shown for each subject are serum cholesterol levels after each type of breakfast. Mean values and standard deviations are also shown. Cholesterol levels after each type of breakfast do not differ significantly ($t = 1.417$, $0.10 < p < 0.20$).

Table 85. Plasma thrombi triglyceride content after the low fat breakfast and after the high fat breakfast.

Subject number	Plasma thrombus triglyceride content, mg/100 ml	
	Control	After fat
1	0.31	0.36
2	0.27	0.80
3	0.10	0.27
4	0.14	0.26
5	0.00	0.24
6	0.07	0.44
7	0.06	0.00
8	0.23	0.17
9	0.34	0.24
10	0.01	0.09
Mean	0.15	0.29
S.D.	± 0.13	±0.22

Shown for each subject is the triglyceride content of the plasma thrombi formed after each type of breakfast in the experiment to measure platelet aggregation. Mean values and standard deviations are also shown. The mean triglyceride content of the plasma thrombi formed after the high fat breakfast is higher than the mean value after the low fat breakfast but the difference is not significant ($t = 2.109$, $0.05 < p < 0.1$).

Table 86. Fibrinolytic and coagulation assays in patient 1, chapter 8.

	Time of sampling, days			
	0	4	6	8
Fibrin plate test mm^2: plasma	0	0	0	9
resuspended euglobulin precipitate	0	4	100	30
Radioactive clot assay	0	0	0	0
Euglobulin lysis activity, units	1.2	1.0	2.6	1.6
Plasminogen, casein units/ml	1.35	2.45	1.90	3.20
Thrombin time, seconds	19.5	18.0	10.6	10.0
Fibrinogen, mg/100 ml	76	91	195	257
One-stage prothrombin time, seconds	19.5	19.0	15.0	12.0
Tame assay, per cent	25	33	50	-
Factor V assay,	10	10	-	30
Factor VII assay, per cent	100	100	100	100
Antihaemophilic globulin assay, per cent	7	10	25	75
Christmas factor assay, per cent	75	75	75	75
Factor X assay, per cent	55	-	55	100
Platelets, thousands/mm^3	160	130	85	220

Shown are the results of fibrinolytic and coagulation assays in patient 1, chapter 8. This patient had had an intrauterine death at least 7 weeks preceding investigation. Therapy with EACA was commenced immediately after investigation on day 0. Delivery of a stillborn infant occurred on day 5 and therapy with EACA was discontinued 12 hours post-delivery, on the evening of day 5. The data are displayed in part in figure 63.

Table 87. Fibrinolytic and coagulation assays in patient 2, chapter 8.

	Time of sampling, days 0	2	5
Fibrin plate test, mm^2; plasma	0	0	0
resuspended euglobulin precipitate	146	56	44
Radioactive clot assay	0	0	0
Euglobulin lysis activity, units	0.78	0.78	0.50
Plasminogen, casein units/ml	2.3	2.3	2.85
Thrombin time, seconds	17.0	21.0	9.7
Fibrinogen, mg/100 ml	218	98	221
One-stage prothrombin time, seconds	14.7	13.4	14.5
Factor V assay, per cent	25	-	25
Factor VII assay, per cent	100	100	100
Antihaemophilic assay, per cent	15	20	100
Christmas factor assay, per cent	100	100	100
Factor X assay, per cent	15	12	100
Platelets, thousands/mm^3	237	127	160

Shown are the results of fibrinolytic and coagulation assays in patient 2, chapter 8. This patient had had an intrauterine death at least 6 weeks preceding investigation. Therapy with EACA was commenced immediately after investigation on day 0. Delivery of a stillborn infant occurred on day 4 and therapy with EACA was discontinued 12 hours post-delivery, on the evening of day 4. The data are displayed in part in figure 64.

Table 88. Fibrinolytic and coagulation assays in patient 3, chapter 8.

	Time of sampling, days	
	0	58
Fibrin plate test, mm^2; plasma	0	0
resuspended euglobulin precipitate	49	66
Euglobulin lysis activity, units	1.85	2.0
Plasminogen, casein units/ml	1.8	1.3
Thrombin time, seconds	33	16
Fibrinogen mg/100 ml	85	80
One-stage prothrombin time, seconds	22	20
Factor V assay, per cent	100	12
Antihaemophilic globulin assay, per cent	100	
Christmas factor assay, per cent	100	
Platelets, thousands/mm^3	260	53

Shown are the results of fibrinolytic assays in patient 3, chapter 8. This patient had a carcinoma of breast with metastases. Other data for this patient are shown in figures 23 and 65.

Table 89. Biochemical effects of urokinase infusion

	Time of sampling, hours										
	0	2½	4½	15½	18½	22½	27½	41½	47	64	88
adioactive clot assay, gm fibrin lysed/ml lasma/hour	0	58	0	24	50	0	24	56	58	0	0
uglobulin lysis activity, nits	0.91	3.60	0.63	1.60	3.40	1.20	3.50	6.70	4.00	0.97	0.83
lasminogen, casein units/ml	2.1	0.6	0.6	0.9	0.2	1.0	1.0	0.6	0.5	0.9	1.6
hrombin time, seconds	8	11	12	10	10	12	12	12	12	8	8
ibrinogen, mg/100 ml	275	263	230	210	205	198	174	100	112	235	239
latelets, thousands/mm^3	195	217	196	155	207	197	210	140	218	165	290
ntihaemophilic globulin assay, per cent control	100	80	50	60	50	10	3	3	-	-	-

Shown in case 6, chapter 8 are the biochemical effects of an intravenous infusion of urokinase. The infusion rate was 850,000 Ploug units in 2½ hours; 20,000 Ploug units/hour for 2 hours; 40,000 Ploug units/hour for 17½ hours. There was a break in the infusion at 22 hours for 2½ hours. At 24½ hours the infusion was restarted at the rate of 80,000 Ploug units/hour for a further 23½ hours. The data are displayed in part in figure 67.

Table 90. Relationship between fibrinogen concentration and thrombin clotting time.

Concentration of fibrinogen, mg/100 ml	Thrombin clotting time, seconds
400	11
350	12
300	13
250	15
200	17
150	25
100	30
50	60
25	100

Shown is the relationship, in a purified system, between fibrinogen levels and thrombin clotting time.

Table 91. Relationship between fibrinogen concentration and lysis time.

Concentration of fibrinogen, mg/100 ml	Plasma clot lysis time
400	15 mins
350	14 mins
300	13 mins 30 secs
250	11 mins 20 secs
200	9 mins
150	6 mins 10 secs
100	3 mins 45 secs
50	2 mins 30 secs
25	1 min 50 secs

Shown is the relationship, in a purified system containing a standard amount of urokinase and plasminogen, between fibrinogen levels and clot lysis times.

Table 92. Plasminogen levels in plasma and serum

Subject Number	Plasminogen, casein units/ml	
	Plasma	Serum
1	3.6, 3.8	3.55, 3.6
2	4.7, 4.7	4.7 , 4.7
3	3.35, 3.45	3.35, 3.45
4	4.2, 4.6	4.4, 4.4
5	5.0, 5.0	5.0, 5.0
6	3.05, 3.15	3.25, 3.3
7	3.9, 3.9	3.8, 3.8
8	2.7, 3.1	3.0, 3.0
9	3.05, 3.15	3.4, 3.45
10	2.8, 2.8	3.0, 3.0
11	3.7, 3.7	3.9, 3.9
Mean	3.7	3.8
S.D.	$\pm$ 0.7	$\pm$ 0.7

Shown are the results of duplicate assays of plasminogen in plasmas and sera from 11 normal subjects. Mean values and standard deviations are also shown. The mean plasminogen level in plasma does not differ significantly from the mean level in serum ($t = 1.862$, $0.05 < p < 0.10$).