ISOLATION AND CHARACTERIZATION OF HUMAN VASCULAR PLASMINOGEN ACTIVATOR

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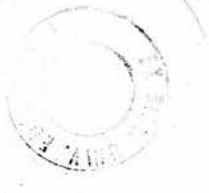
1979



ISOLATION AND CHARACTERIZATION OF HUMAN VASCULAR PLASMINOGEN ACTIVATOR

CONTENTS

DECLARATION

The work reported here was designed and carried out by myself with the following exceptions:

1 The production of the antisera and the radioactive labelling using the two lactoperoxidase methods, which was carried out by Dr J Dawes.

2 The SDS-polyacrylamide study of the soluble fibrin polymers which was carried out by Dr D Lane.

RODGER A ALLEN

ACKNOWLEDGEMENTS

I would like to acknowledge and sincerely thank Dr J D Cash and Dr D S Pepper for providing a stimulating environment in which to work and for their tireless enthusiasm and advice throughout the course of this research.

I would also like to thank Dr. H. Cameron, Professor A. Currie and Dr. A.J.M. Drennan for making the post mortem facilities available to me at the Royal Infirmary, Edinburgh, and Western General Hospital, Edinburgh, respectively.

Thanks are also due to Dr J Dawes for producing the antisera and also performing some radioactive labelling studies, and to Dr D Lane for performing the SDS-polyacrylamide electrophoretic studies on the soluble fibrin polymer preparations.

I would also like to thank Mrs A Malone for help in many areas including library and xerox work as well as for continually providing clean apparatus.

Finally I would like to record my great appreciation to Miss W Burns for her assistance in typing and checking the manuscript.

ABSTRACT

The methodology for the perfusion of the lower limbs of cadavers was improved to optimise yields. The most satisfactory perfusion fluid employed was 5% dextrose - 0.1% Triton X-100 - 10mM EDTA - 10u/ml Trasylol; this was used at 37°C. Originally the initial perfusate was rejected, but after protein and activity measurements it was found that although the specific activity was lower in these early perfusates, they contained the highest activity levels and thus all the perfusate was collected for purification. An average of 15,451 Ploug u/ml was extracted per cadaver. However, it was found impossible to predict which cadavers, in terms of age, cause of death and hours post mortem, would yield high levels of activator. Alternative sources of the activator were investigated but none proved as satisfactory as the cadaver perfusion.

The initial purification procedure involved affinity chromatography on lysine-Sepharose and protamine-Sepharose followed by gel filtration on Sephadex G-200 in a buffer containing 1.5M NaCl. Using this purification procedure a value of 54,545 was found for the molecular weight of the vascular plasminogen activator, 2.97nm for the Stokes radius, 7.22 Ficks for the diffusion coefficient, 4.40 Svedbergs for the sedimentation coefficient and a value of 1.18 for the frictional ratio.

PEG precipitation was investigated when it was realised that the activator was obtained from the cadaver in a high molecular weight complex that appeared to be more stable than the free enzyme. Purification by effecting a size change was investigated. The complexed activator eluted at V_o on Sepharose 6B but when re-run on Sephadex G-200 in a buffer containing 1M lysine the activator dissociated from the soluble fibrin carrier and eluted away from the high molecular weight contaminating protein which remained at V_o. This process often gave a very pure product but was not reproducible.

The final purification procedure developed involved PEG precipitation, followed by chromatography on hydroxyapatite, Sepharose 6B and finally on either lysine-Sepharose or fibrin-Sepharose.

Inhibition studies showed the vascular activator to be inhibited by the synthetic inhibitor Val-Pro-Arg-CH_2Cl, DFP, PMSF and DTT and not inhibited by Trasylol or iodoacetamide, whether tested on a fibrin plate or a synthetic chromogenic substrate. EACA gave some measure of inhibition on the fibrin plate but not with the synthetic substrate. The vascular activator is thus a serine protease that relies on disulphide bonds to maintain its tertiary structure for enzymic activity.

Urokinase differed in its inhibition profile from the vascular activator in that iodoacetamide, which had no

effect on the vascular activator, inhibited urokinase, and DTT which inhibited the vascular activator had no effect on urokinase. A human melanoma activator showed a similarity to the vascular activator in that it was not inhibited by iodoacetamide.

The best synthetic chromogenic substrates for the vascular activator were determined to be S-2322 and S-2444. However it was noted that for meaningful assays, samples must be free of all contaminating enzymic activity.

An apparent β electrophoretic mobility was determined for the activator, but this was later attributed to the soluble fibrin with which the activator was complexed. Immunological and radiolabelling studies were unsatisfactory mainly due to the insufficient amount of vascular activator present in purified and semi-purified preparations.

Soluble fibrin polymers were prepared in vitro and some of their properties investigated. A high affinity of the vascular activator for soluble fibrin polymers as well as insoluble fibrin was demonstrated; it was shown that urokinase and the melanoma activator did not possess this high affinity.

Finally poly-lysine was shown to accelerate the rate of activation of plasminogen by the vascular activator and human uterine tissue activator (both of which have high

affinity for fibrin) whereas it had no effect with urokinase and the melanoma activator (both of which have low affinity for fibrin). The possible explanations for this are discussed.

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1 THE DEVELOPMENT OF KNOWLEDGE OF FIBRINOLYSIS

The fact that blood is able to clot must have been one of the earliest physiological observations to be made: that it can reliquefy took much longer to discover. Hence the study of coagulation preceded the study of fibrinolysis, and consequently coagulation has been shown to be very complicated, but it would be wrong to assume that fibrinolysis is as simple as present concepts depict it to be. Indeed it is predictable that further research will reveal factors, as yet unknown, which take part in the fibrinolytic mechanism. Our present knowledge of fibrinolysis is on a par with that which pertained to coagulation at the beginning of this century. It is by no means improbable that by the end of the century the knowledge we now have of fibrinolysis will seem naïve, and that coagulation and fibrinolysis will be revealed as ranking in complexity.

There are remarkable similarities between the two mechanisms. Each involves an activating system which converts a precursor into an active proteolytic enzyme-thrombin in coagulation, plasmin in fibrinolysis - and each shares a common substrate, fibrinogen-fibrin. Fibrinolysis seems to be the physiological antithesis of coagulation; and perhaps both mechanisms should be regarded not as separate entities but as one whole coagulation-fibrinolysis system concerned with the

deposition and removal of fibrin.

The phenomenon of fibrinolysis was first observed over 200 years ago. Morgagni (1769) recorded an autopsy where the blood was entirely fluid. He described the case of a man he saw in 1725 who died shortly after being stabbed through the heart, and suggested that the fluidity of the blood was due to its dilution by the quantity of drink he had taken prior to being stabbed. Hunter (1794) records that in animals killed by lightning or electricity or in animals 'who are run very hard, and killed in such a state' the blood does not clot.

Fibrin stored in certain circumstances was noted to dissolve spontaneously (Denis, 1838; Plosz, 1873) and Green (1887) noted that when fibrin prepared from ox blood had dissolved when incubated in saline, it could not be clotted again by thrombin. Dastre (1893) observed that fibrin left in contact with the blood of dogs which had been subjected to the stress of repeated haemorrhage would lyse spontaneously. The destruction of fibrin was termed "fibrinolysis" by Dastre.

Gley and Le Bas (1897) published work showing that peptone injections produce incoagulability of the blood in hepatectomized dogs; Nolf (1904, 1905) reported that this phenomenon is associated with a marked increase in fibrinolytic activity that is due to irritation, by peptone, of the vascular wall and the consequent release

into the circulatory system of a substance that enhances normal fibrinolysis. He conjectured that the agent responsible for fibrinolysis is released from vessel walls, a suggestion fully confirmed by more recent studies. Whitehouse (1914), investigating the fluidity of menstrual blood, showed that it was actively fibrinolytic and considered that this activity was due to a tissue enzyme. Fleisher and Loeb (1915) investigated the fibrinolytic activity of a number of different tissues, and came to the conclusion that this depended on a proteolytic enzyme in the tissues concerned. However, Demuth and von Riesen (1928) thought that the reaction was more complicated, and suggested that the tissue enzyme activated a pro-enzyme present in the blood, a suggestion thatwas supported by the later observations of Fischer (1946) working with tissue culture.

In 1933 Tillett and Garner showed for the first time that broth cultures of beta haemolytic streptococci derived from patients, as well as filtrates from these cultures, are capable of rapidly liquefying normal human fibrin clots. In 1941 Milstone reported that fibrin prepared from highly purified fibrinogen was resistant to streptococcal fibrinolysis, but that lysis occurred when a small quantity of human globulin was incorporated in the test. A further factor, Milstone's "lytic factor", was evidently necessary for streptococcal fibrinolysis to take place. Christensen and MacLeod (1945) made the breakthrough which revealed the basis of the fibrinolytic mechanism and drew together

the hitherto unrelated observations on the behaviour of serum and of fibrin made during the preceding hundred years. These workers showed that plasma or serum contains an inactive enzyme precursor, plasminogen, which is converted to the active "proteolytic" enzyme plasmin, by an activator or kinase produced by the streptococcus, which they called streptokinase. Unless highly purified, fibrinogen preparations always contain plasminogen, and this explains why Milstone was unable to lyse purified fibrin unless the "lytic factor", ie plasminogen, was added to the test.

However, Geiger (1952) reported that an additional factor seemed to be required for the activation of certain animal plasminogens by streptokinase. These species differences were investigated further by Müllertz and Lassen (1953) who found that, although bovine plasminogen was not activated by streptokinase alone, it reacted if a small amount of normal human plasma globulin was added to the mixture. The plasmin then produced was derived from the bovine plasminogen, not the human globulin. It was therefore suggested by these workers that there is a "proactivator" in human plasma which is converted to "activator" by streptokinase and that plasminogen reacts with activator and not directly with streptokinase. Bovine plasma was considered to be deficient in proactivator, and is therefore not responsive to streptokinase.

Macfarlane (1937) conjectured that the stress of surgical operation causes fibrinolytic activity to appear in the blood.

Later with Biggs and Pilling (Biggs et al 1947) he showed that clots made from the diluted plasmas of healthy people who had undergone the stresses either of violent exercise or of the injection of adrenaline would lyse if incubated overnight; whereas clots made from the diluted plasmas of unstressed subjects remained intact. These workers concluded that stress causes fibrinolytic activity to appear in the blood, but that it is not normally present. However, Fearnley and Tweed (1953) using a rigid low-temperature technique between venepuncture and making dilute plasma clots, investigated 60 healthy people in the resting state and found fibrinolytic activity in nearly all of them. The nature of the factor responsible for endogenous fibrinolysis was unknown; it appeared to act as a plasminogen activator, and Fearnley and Lackner (1955) christened it "labile fibrinolytic component".

The fibrin plate method was originally described by Permin (1947) and later improved by Astrup and Müllertz (1952). Using this method, Albrechtsen (1957) demonstrated the presence of activators of plasminogen in most of the tissues of the body, the liver excepted. Fibrinolytic activity has also been demonstrated in a variety of body fluids, including milk, tears, saliva, various exudates, ascitic fluid, amniotic fluid and cerebrospinal fluid (Albrechtsen, Storm and Claassen, 1958).

From the fibrin plate method Todd (1959) developed the fibrinolytic autograph technique, with which he was able

to show that the endothelium and vasa vasorum of blood vessels are rich in plasminogen activator.

Throughout the course of the observations and investigations reviewed here, there have been many speculations, but few experiments, on the function of the fibrinolytic mechanism. It is postulated that the integrity of the endothelium depends on a fibrin layer on its outer or inner surfaces, thus excessive fibrinolysis causes haemorrhage, and deficient lytic activity will lead to fibrin accumulations and thus to thrombosis and atherosclerosis. During the past 20 years the fibrinolytic system has emerged as a mechanism active in its own right and the possible relationship of the fibrinolytic mechanism to other enzyme systems in the plasma is a fascinating field of inquiry which should be explored profitably now that many details of the different mechanisms have been clarified.

2 COMPONENTS OF THE FIBRINOLYTIC SYSTEM

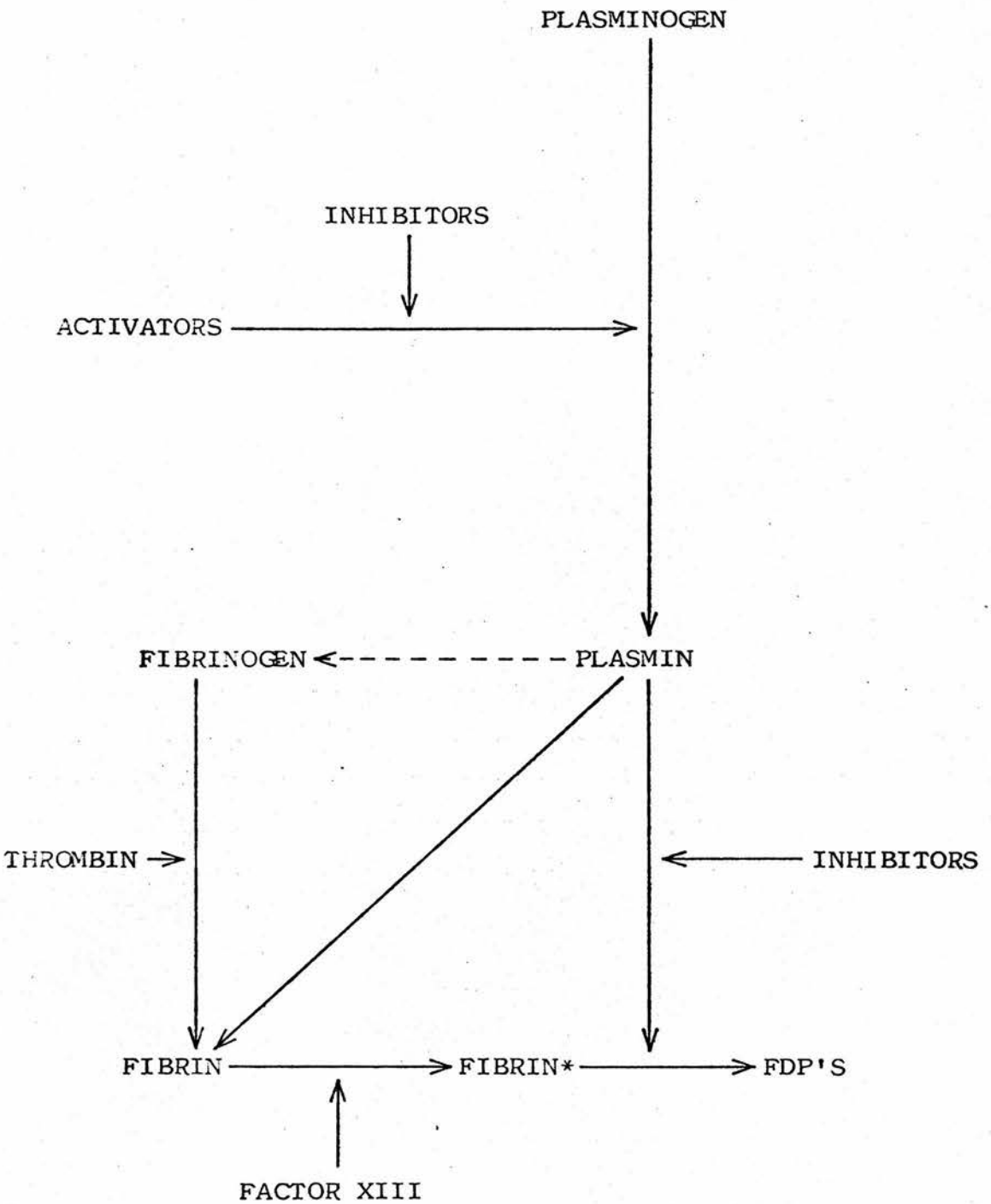
A PLASMINOGEN

a Structure and Properties

Plasminogen, a beta globulin (Robbins and Summaria, 1963) is the inactive precursor of the enzyme plasmin (see Fig 1). The presence of plasminogen in various human tissues was suggested by Kowalski et al (1958), and is present in the blood and most body fluids (Sherry, 1966).

The source of plasminogen has not yet been fully elucidated, although the liver and bone marrow eosinophils have been claimed to be important (Miller and Bale, 1954). Barnhart and Riddle (1963) suggested that plasminogen is transported from the bone marrow and released to the circulation and tissues when needed. More recent studies have suggested that the kidney could be a significant source (Highsmith and Kline, 1971). However, it is unlikely to be the only source in man as not all anephritic patients have low plasma plasminogen levels.

Plasminogen can be extracted from Cohn fraction III with strong mineral acid (Christensen and Smith, 1950; Kline, 1953) followed by a pH II denaturation of accompanying impurities. The purified proenzyme proved to be insoluble at neutral pH. The original technique was progressively improved first by abandoning the alkaline denaturation



* Cross-linked fibrin

FIG 1 SCHEME OF THE FIBRINOLYTIC ENZYME SYSTEM

step, but the most considerable improvement in purification came with the use of affinity chromatography, developed in 1970 by Deutsch and Mertz. They used lysine coupled to Sepharose, thus avioding the acidic extraction step, which probably produced several changes in the plasminogen molecule including changes in molecular weight and substrate specificity (Slotta et al, 1962). Plasminogen obtained by these more gentle purification techniques proved to be quite soluble at neutral pH, and considerable structural studies have been done with this material.

Plasminogen is a single chain molecule consisting of 840 amino acid residues (Wallén, 1978). If measures are taken to avoid proteolytic degradation during the preparation isolated plasminogen has glutamic acid in the N-terminal position (glu-plasminogen). If no such measures are taken, plasminogen with N-terminal lysine (lys-plasminogen) or valine (val-plasminogen) results. Incubation of glu-plasminogen with plasmin at 37°C for half an hour gives val- or lys-plasminogen, consisting of a single polypeptide chain with slower anodic mobility and lower molecular weight than glu-plasminogen (Claeys et al, 1973). In all the above cases asparagine is the C-terminal amino acid.

Physicochemical data indicate conformational differences between the two plasminogen forms. Glu-plasminogen appears to be larger in size than lys-plasminogen in SDS-acrylamide gel electrophoresis. The amino acid compositions of glu-

plasminogen and lys-plasminogen, and their major isolated isoelectric forms, are similar, but several amino acid residues (glutamic acid, alanine, isoleucine, phenylalanine, and lysine) have been found to be significantly higher in glu-plasminogen.

The molecular weights of glu- and lys-plasminogen have been variously quoted as:-

Glu-Plasminogen	Lys-Plasminogen	
93,000 ± 1,000	89,100 ± 1,400	(Claeys and Vermylen, 1974)
92,000	85,000	(Wiman and Wallén, 1975a)
83,800 ± 4,500	82,400 ± 3,300	(Robbins et al, 1975)

The work on the primary structure of human plasminogen is now practically finished, and it is therefore now possible to calculate a molecular weight from the amino acid composition. The molecular weights of glu-plasminogen and lys-plasminogen would be 88,365 and 80,100 respectively, if carbohydrates are not taken into account. Assuming a carbohydrate content of about 1.5% (Wiman and Wallén, 1975c) the values would be about 90,000 and 81,500 respectively (Wallén, 1978).

It has been shown that activation of lys-plasminogen occurs through the cleavage of a single arginine-valine bond, while activation of glu-plasminogen occurs in two steps, involving the removal of the N-terminal part of the molecule, as well as cleavage of the arginine-valine bond.

The release of peptide material from the N-terminal part of plasminogen is followed by significant changes in conformation that are reversible in the presence of an excess of the peptides (Sjöholm et al, 1973), indicating a noncovalent interaction with another site in plasminogen. The dissociation of this interaction, which occurs in the presence of certain omega amino acids is presumably responsible for the differences in properties between glu- and lys-plasminogen (Sjöholm et al, 1973; Wallén and Wiman, 1975b; Wiman and Wallén, 1975a). One site responsible for this intramolecular interaction has been localized to a heptapeptide sequence (-Ala^{45}-Phe^{46}-Gln^{47}-Tyr^{48}-His^{49}-Ser^{50}-Lys^{51}) situated in the N-terminal part of glu-plasminogen (Wiman and Wallén, 1975a). It is reasonable to assume that the other site that participates in the intramolecular interaction is the same as the site responsible for the interaction between plasminogen and lysine. This site, the lysine binding site (LBS), seems to form a stoichiometric 1:1 complex with omega amino acids resulting in marked conformational changes similar to those observed on the release of N-terminal peptides.

Dissociation of the internal noncovalent interaction in glu-plasminogen, either by proteolytic cleavage in the N-terminal part of the molecule, or by the addition of omega amino acids, causes a dramatic change in activation kinetics (Claeys and Vermylen, 1974). Thus, using urokinase as activator, lys-plasminogen is activated about 10 to 20 times faster than glu-plasminogen. While in vivo,

the half-life is 0.8 days for lys-plasminogen and 2-2.5 days for glu-plasminogen (Collen et al, 1975b).

Activation results in a two-chain monomer, in which the chains, heavy (A) and light (B), are connected by a single disulphide bond. It is from the N-terminal portion of plasminogen that the heavy (A) chain of plasmin derives and the light (B) chain originates from the C-terminal end of plasminogen.

Several groups have participated in work on the elucidation of the primary structure of human plasminogen (Claeys et al, 1976; Magnusson et al, 1976; Rickli et al, 1976; Robbins et al, 1975; Wiman and Wallén, 1975a, 1975b, 1975c; Wiman 1973, 1977), and a model, based on these studies, is shown in Fig 2.

Many different molecular forms of plasminogen exist as judged by electrophoretic criteria. Summaria et al (1972) found 8 bands when a preparation of human plasminogen was run in acrylamide gel electrophoresis at pH 8.4 in either 0.3M EACA or 8M urea. The S-carboxymethyl heavy (A) chain derivative of plasmin did not show multiple molecular forms whereas the derivatized light (B) chain did, but only when run in 8M urea.

Wallén and Wiman (1972) stated that pure preparations of plasminogen often contain up to 10 different forms, but they considered that only 6 of these forms had electro-

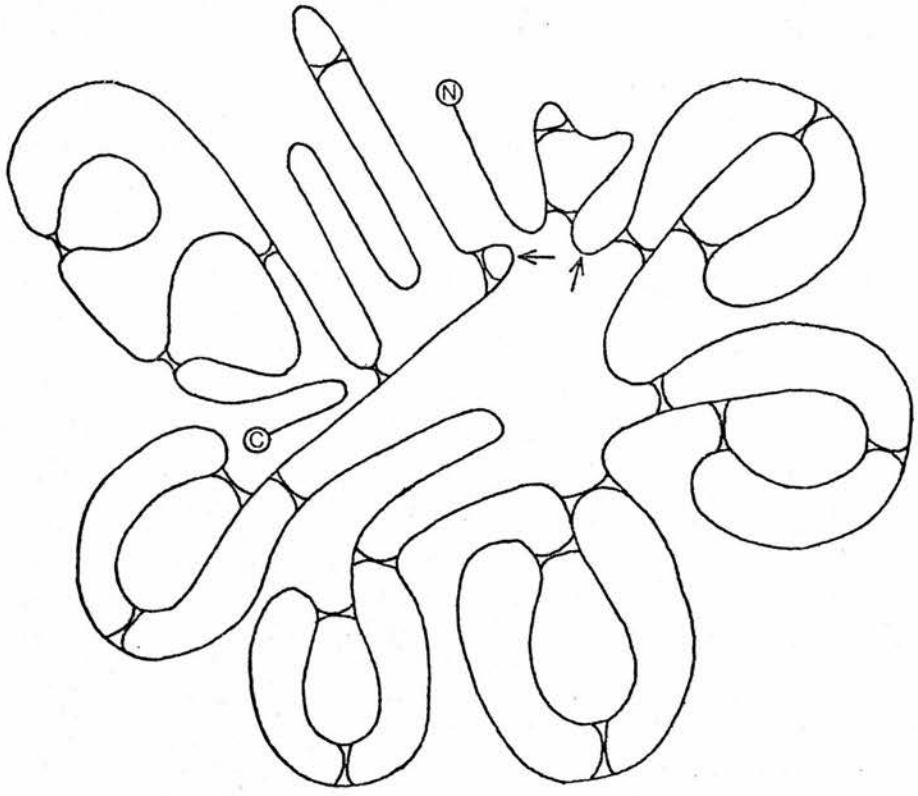


FIG 2 A MODEL OF PLASMINOGEN

The disulphide arrangement and the sites where proteolysis occurs on activation are shown by arrows (after Wallén, 1978).

phoretic properties identical to those in human plasma, and that the remainder were probably proteolytically degraded, while Collen (1973) considered that human plasminogen occurred in twelve main molecular forms in the circulation.

Furthermore, Summaria et al (1976) described two affinity forms of each zymogen when each were eluted from a L-lysine-Sepharose affinity chromatography column using a gradient of ε-aminocaproic acid (EACA). The four zymogen affinity chromatography forms glu-1-plasminogen; glu-2-plasminogen; lys-1-plasminogen and lys-2-plasminogen show distinct stepwise differences in their molecular size and charge. Glu-1-plasminogen is the largest and most acidic, while lys-2-plasminogen is the smallest and most basic. The proteolytically altered lys-1 and lys-2 forms appear to be specifically derived from the glu-1 and glu-2 forms respectively.

Although the sialic acid contents of the glu- and lys-forms appear to be similar, the isolated affinity chromatography forms show distinct differences. Sialic acid content of glu-1 and lys-1 are identical and substantially higher than glu-2 and lys-2 which are also identical. The charge differences of zymogen 1 and 2 forms may be related to sialic acid content.

The plasmin-derived carboxymethyl light (B) chains, from each of the four enzyme forms, appear to be identical,

whereas the carboxymethyl heavy (A) chains differ in molecular size, in the same sequence as their parent zymogens. It thus appears that the region of the molecule responsible for the differences in molecular size of the four zymogens is the N-terminal portion of the molecule.

Plasminogen can be completely desialylated without loss of proenzyme activity, suggesting that the sialic acid does not play an essential role in the function of plasminogen. However, Siefring and Castellino (1974) have found that desialylated rabbit plasminogen has a markedly shortened half-life.

Low molecular weight forms of plasminogen have been described. Paoni and Castellino (1975) have isolated a fully activatable plasminogen of molecular weight 50-55,000, by treating sheep plasminogen with sheep plasmin. Yecies and Kaplan (personal communication) have isolated a 42,000 molecular weight form of human plasminogen from fresh plasma. Formation of low-molecular weight plasminogen was diminished when DFP was incorporated into the purification procedure indicating that plasma proteolytic enzymes can degrade plasminogen without activating it.

As plasminogen is an inactive precursor, it has no active site. Thus binding to L-lysine Sepharose must be at a site or sites other than the active site; these are termed lysine binding sites. It would appear that one such site is found in the first triple disulphide loop (see Fig 2) in

the part of the molecule which corresponds to the heavy (A) chain of plasmin (Rickli and Otavsky, 1975). A fragment (6,000 daltons) containing the lysine binding site has been isolated and characterized (Wiman and Wallén, 1975c). The role of the lysine binding site in plasminogen is not certain but presumably it is involved in the interaction between plasminogen and fibrin. Holleman et al (1975) described a p-aminobenzamidine binding site distinct from the lysine binding site and on a different portion of the molecule. The p-aminobenzamidine site, presumably an arginine binding site in vivo, may also be involved in the plasminogen-fibrin interaction.

b Activation of Plasminogen

i Urokinase

Activation of human plasminogen has been considered a two-step process (Wiman and Wallén, 1973; Rickli and Otavsky, 1973; Walther et al, 1974) (see Fig 3). The first step of this process is cleavage of a peptide bond in the N-terminal region of the molecule resulting in the release of a peptide(s).

Wiman and Wallén (1973) activated glu-plasminogen (93,000) by passage through a column containing immobilized urokinase; they collected the effluent in an excess of the plasmin inhibitor PNPGB. They found that the released

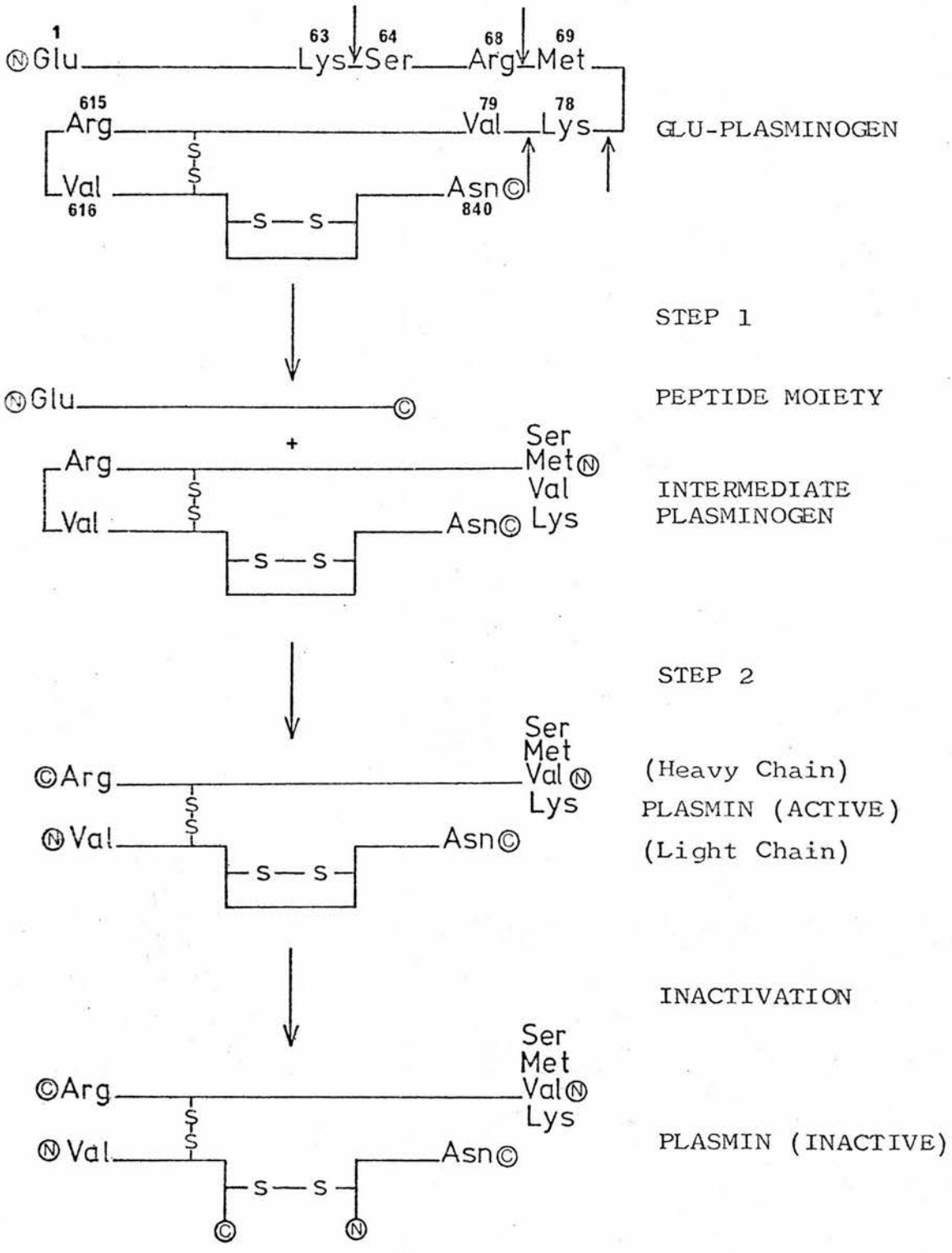


FIG 3 SCHEMATIC REPRESENTATION OF THE TWO-STEP ACTIVATION MECHANISM OF HUMAN PLASMINOGEN INDUCED BY UROKINASE

The arrows indicate the observed cleavage of peptide bonds (Rickli, 1975; Walther et al, 1974).

peptide material in the first step consisted of two activation peptides. Wiman (1973) called these API and APII, and showed that they occupied positions 1-63 and 64-68 respectively in intact glu-plasminogen. The two bonds cleaved to produce the peptides are lysine-serine (63-64) and arginine-methionine (68-69), and the result is an intermediate form of plasminogen of molecular weight 86,000 and N-terminal methionine (Wiman and Wallén, 1973).

The second step of activation to plasmin involves cleavage of an arginine-valine bond situated at about residue 600 (Robbins et al, 1967) resulting in a two-chain molecule connected by disulphide bridges. Wiman and Wallén (1973) found the intermediate plasminogen to yield plasmin with a light chain of molecular weight 25,000 and N-terminal valine, while the heavy chain was 63,000 and even though very sensitive to proteolytic degradation in its N-terminal region, evidence pointed to methionine as the true N-terminal amino acid residue, although serine was sometimes found in this position.

When activation of glu-plasminogen by urokinase is carried out in the presence of 0.01M TLCK, an 80,000 molecular weight lys-plasminogen intermediate is formed and an 8,200 molecular weight pre-activation peptide originating from the N-terminal of glu-plasminogen is released (Walther et al, 1974). According to Walther et al (1974), this intermediate then forms plasmin with a heavy chain of 55,000 and N-terminal of usually lysine, but sometimes valine,

and a light chain of 26,000. The heavy chain is fairly resistant to further proteolysis, but after prolonged periods may break down to give a molecular weight of 50,000; whereas the light chain can be further degraded to 18,000 and 6,500 moieties, with concomitant loss of plasmin activity.

A single-step activation mechanism has also been proposed. Violand and Castellino (1976) found that when human glu-plasminogen is activated by urokinase in the presence of pancreatic trypsin inhibitor, the plasmin formed exclusively contains a heavy chain derived intact from the original N-terminus of glu-plasminogen. Sodetz et al (1974) working with rabbit plasminogen found similar results. These studies strongly indicate that the N-terminal peptide(s) need not be released from glu-plasminogen prior to plasmin formation.

However, the findings of Thorsen (1975) that native glu-plasminogen is adsorbed to fibrin to a lesser degree than proteolytically degraded plasminogen, has attributed a physiological significance to the two-step activation mechanism. The formation of the intermediate activation product seems to be required to obtain plasminogen activation in situ on the fibrin clot in an environment, which is relatively free of plasmin inhibitors.

It can be questioned, however, whether the preactivation peptide bond and the activation peptide bond are cleaved in

vivo by the same enzyme. According to the hypothesis presented by Thorsen (1975), the preactivation bond should be cleaved freely in the circulation, while the activation peptide bond is essentially cleaved after adsorption of the intermediate to the fibrin matrix. There is some indirect evidence that the preactivation peptide bond is not primarily a urokinase-sensitive bond.

Sodetz et al (1974) questioned whether urokinase was capable of cleaving this N-terminal peptide, as a three-fold increase in the level of urokinase in the activation mixture in the presence of Trasylol, gave the same glutamic acid N-terminal heavy chain. Sodetz et al implicate the plasmin generated during the activation process as the species responsible for removal of the peptide; as in the absence of Trasylol the heavy chain of plasmin has an amino terminal sequence which differs from the original native plasminogen. Rickli (1975) also considers the variability of the N-terminal residue of both the intermediate plasminogen and the heavy chain of plasmin to be due to traces of plasmin causing proteolysis.

Summaria et al (1975) also used Trasylol in an attempt to distinguish between the action of urokinase and plasmin during the activation of plasminogen. They criticised the work of both Wiman and Wallén (1973) and Walther et al (1974) on the basis that the inhibition of plasmin by the methods employed was not instantaneous. Summaria et al used an excess of Trasylol in the activation mixture, as

plasmin is immediately inhibited upon formation. Using this system they found that urokinase does not cleave peptide bonds in the N-terminal portion of human plasminogen, and that the only bond that need be cleaved for the generation of plasmin is the arginyl-valine bond. These findings indicate that glu-plasmin does exist as an active enzyme.

Comparing the primary structure preceeding the preactivation bond (Arg-Lys-Ser-Ser-Ile-Arg.....Met) (Wiman, 1972) and the activation peptide bond (Lys-Lys-Cys-Pro-Gly-Arg..... Val) (Sottrup-Jensen et al, 1975) no homology could be found except for the arg residue in position 1, and the lys residue in position 5. If both peptide bonds were primarily cleaved by the same specific enzyme, a more pronounced homology would be expected.

Walther et al(1975) found that the activation peptide bond is cleaved 23 times faster by urokinase than the preactivation peptide bond, and Sodetz and Castellino (1975) observed that urokinase could not split the preactivation bond of rabbit plasminogen while it could hydrolyze the activation peptide bond. Studies by Castellino and Violand (1977) also indicate that urokinase is not capable of catalysis of the bond releasing peptide material from the N-terminus of glu-plasminogen, glu-plasmin or the native plasmin heavy chain (glu-H) isolated from glu-plasmin. On the other hand, plasmin readily accomplishes cleavage of this bond in all of the above molecules. The cleavage of

the arginine-valine bond, causing activation of plasminogen, is catalyzed only by urokinase in this system.

These data indicate that the preactivation peptide bond is not primarily a urokinase-sensitive bond, but that another enzyme may be involved in cleavage of this bond in vivo. The recent findings of Astrup and Fedderssen (1975) may be relevant. These authors found that proteolytic enzymes, probably belonging to the class of the cys-sensitive cathepsins, may also catalyse the cleavage of the preactivation bond.

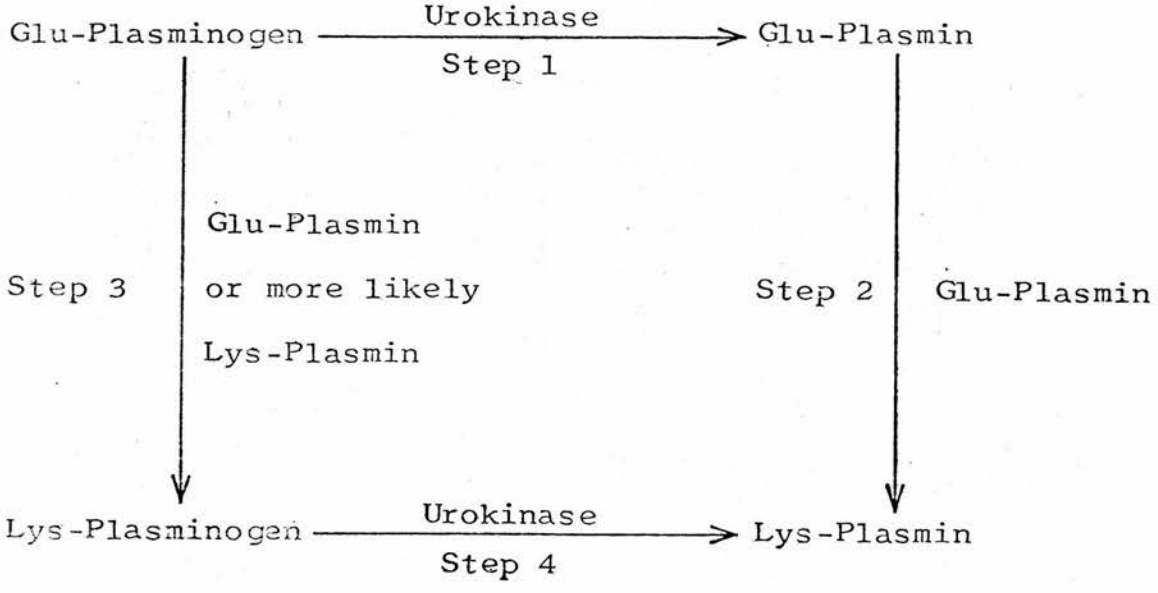
It has previously been accepted (Wiman and Wallén, 1973; Rickli and Otavsky, 1973; Walther et al, 1974) that cleavage of the preactivation peptide bond is the rate-limiting step during activation of plasminogen by urokinase.

However, many authors (Rickli and Otavsky, 1973; Walther et al, 1974) found that the intermediate accumulates in the first phase of activation. This discrepancy can be explained if it is accepted that cleavage of the preactivation peptide by itself is not the rate-limiting step but that the conformational change in the plasminogen molecule, induced by this cleavage, is rate-limiting.

Activation studies were performed with glu-plasminogen, lys-plasminogen and glu-plasminogen in the presence of EACA (causing the conformational change) (Claeys and Vermylen, 1974). Under the experimental conditions

selected, the intermediate proenzyme lys-plasminogen was fully activated whereas glu-plasminogen was only 50% activated. In the presence of EACA, glu-plasminogen behaved as the intermediate plasminogen, suggesting that the conformational change in the plasminogen molecule is rate-limiting.

The main pathway of formation of lys-plasmin is in dispute. Many authors favour lys-plasminogen as the intermediate, with the associated conformational change causing increased affinity for fibrin and the related enhancement of reaction rate. However, physical and kinetic studies led Castellino and Violand (1977) to propose a mechanism for the activation of glu-plasminogen by urokinase, see below:

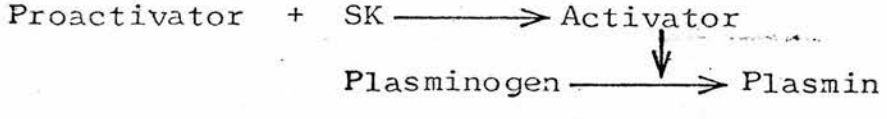


ii Streptokinase

There is no universally accepted theory on the activation of the human plasma fibrinolytic system by streptokinase (SK). Christensen (1945) demonstrated that the lysis of fibrin clots in the presence of streptokinase was attributable to an enzyme normally present in human serum or plasma in an inactive state, and not to streptokinase, which alone had no demonstrable proteolytic activity in this test system.

It was observed that streptokinase was able to activate the human euglobulin fraction, whereas it has no effect, or a very slight effect, on proenzyme in the euglobulin fraction of other species such as the cow and rabbit. Addition of traces of human euglobulins, containing human plasminogen, to bovine plasma made it susceptible to activation by streptokinase. These basic facts led to varying interpretations and the discussion of these different theories is still open.

Müllertz (1955) proposed the proactivator theory. According to this theory, bovine plasminogen preparations would contain large amounts of plasminogen and little or no proactivator



and thus bovine plasmin would not be formed upon addition

of streptokinase. Human plasminogen preparations would contain large amounts of proactivator and relatively small amounts of plasminogen, by addition of streptokinase, large amounts of activator and small amounts of plasmin would therefore be formed. Several attempts were made to isolate from human plasma both plasminogen and proactivator. Some authors postulated that human plasminogen was the proactivator itself (Ablondi and Hagan, 1957; Kline and Fishman, 1957). Supporting this latter view is the observation that in mixtures of streptokinase and human plasminogen, mainly plasmin was formed when plasminogen was in excess, and mainly activator when an excess of streptokinase was present. Zylber et al (1959) suggested that human plasmin and streptokinase combine to form a complex which is capable of converting bovine plasminogen to plasmin, thus excluding the existence of the proactivator, but rather considering it to be human plasminogen or plasmin.

Davies et al (1964) showed that an equimolar mixture of highly purified streptokinase and human plasminogen produced a complex of molecular weight 130,000, which was very close to the combined molecular weights found by the same authors for plasminogen (84,000) and streptokinase (47,000). They affirm that the observed complex could actually be a streptokinase-plasmin complex. Barg et al (1965) found that after an equimolar interaction of streptokinase and plasminogen a main product was identified, in electrophoresis, with a migration rate intermediate between those

of the two proteins. When either component was present in excess of a molar ratio 1:1, stainable protein corresponding to the excess was detected. The streptokinase-plasminogen complex undergoes a time and temperature dependant change generating an active site (McClintock and Bell, 1971).

Summaria and co-workers (1968) were able to distinguish between the human plasminogen-streptokinase complex and the human plasmin-streptokinase complex. It was possible to show, in the complex isolated at $0^{o}C$, that plasminogen was one of its components. After warming at $25^{o}C$, this plasminogen-streptokinase complex was quantitatively converted to a plasmin-streptokinase complex. The active site of the latter activator complex is single, and probably identical to the active site of plasmin. It was therefore suggested that during the complex formation a conformational modification of the plasmin molecule occurred, leading to a concomitant change in the enzymic specificity.

In 1969 Summaria et al showed that streptokinase could activate plasminogen directly, a previously controversial point, although probably the predominant mechanism of activation is via the plasmin(ogen)-streptokinase complex. Kline and Ts'ao (1971) also showed that the conversion of plasminogen to plasmin by catalytic amounts of streptokinase proceeded independantly of the availability of preformed plasmin. Spontaneous plasmin activity was removed

almost completely from human plasminogen by extraction with insolubilized soybean trypsin inhibitor (SBTI) and treatment with DFP.

Using the active site titrant, PNPGB, Reddy and Markus (1972) established that an active centre appears in the streptokinase-plasminogen complex even before proteolytic activity develops; that is before cleavage of the arginine-valine bond in plasminogen to produce the streptokinase-plasmin complex. The appearance of this active site was interpreted as being due to a streptokinase induced conformational rearrangement in the plasminogen moiety which makes available the proteolytic potential of the zymogen. It appears the transitory streptokinase-plasminogen complex plays a dominant role in the streptokinase activation of human plasminogen, as its formation does not depend on the presence of spontaneous plasmin.

Two distinct pathways of the streptokinase-mediated activation of human plasminogen were described by McClintock et al in 1974. Conversion to plasmin using catalytic amounts of streptokinase results in the cleavage of two characteristic peptide bonds yielding plasmin containing three peptide chains ($Plasmin_{60,\ 20,\ 5}$), the order of cleavage being exclusively $Plasminogen_{85}$ - $Plasminogen_{80,\ 5}$ - $Plasmin^{a}_{60,\ 20,\ 5}$ (where superscript "a" denotes a PNPGB-titratable active site and the subscripts indicate the approximate molecular weight, in thousands, of peptide chains involved.) Interaction of plasminogen with stoichiometric

amounts of streptokinase leads to the plasminogen activator complex streptokinase$_{37, 6, 4}$ - Plasmin$^{a}_{60, 20, 5}$. Bond cleavages of plasminogen within this complex occur entirely by the reactions plasminogen$^{a}_{85}$ - plasminogen$^{a}_{65, 20}$ - plasmin$^{a}_{60, 20, 5}$. This order is the reverse of that found in catalytic activations. The streptokinase peptide bond splits within the complex, occur in the order streptokinase$_{47}$ - streptokinase$_{43, 4}$ - streptokinase$_{37, 6, 4}$. End group studies of isolated peptides from the complex show the peptide orders to be N-SK$_6$-SK$_{37}$-SK$_4$-C for streptokinase$_{47}$ and N-Plg$_5$-Plg$_{60}$-Plg$_{20}$-C for plasminogen$_{85}$.

Native streptokinase does not activate bovine plasminogen. It has been well established, however, that human plasminogen or plasmin reacts with streptokinase to form an equimolar complex which is an activator of bovine plasminogen. Taylor and Beisswenger (1973) and Kline and Bowlds (1971) have reported that a streptokinase derivative virtually free of plasmin activity, can be isolated from the streptokinase-plasmin complex and that this modified streptokinase (SK^1) contains the active centre for the conversion of bovine plasminogen to plasmin. But Reddy and Kline (1975) used affinity chromatography to remove the last detectable trace of plasmin from the SK^1 preparation and found that bovine plasminogen activator activity disappeared. The addition of preformed plasmin restored bovine activator activity, supporting the theory of Reddy and Markus (1972) that streptokinase is not an enzyme but functions by inducing an active centre in human plasminogen

as a result of complex formation with it.

Robbins et al (1977) have isolated the human plasmin-derived light (B) chain and found it to be functionally active. It is both a proteolytic and esterolytic enzyme, but it has no plasminogen activator activity. It reacts with streptokinase to form an equimolar complex which has both human and bovine plasminogen activator activities. The binding site for streptokinase is located on the light (B) chain of plasmin. The light (B) chain-streptokinase complex is an effective activator in in-vitro clot lysis systems using crosslinked fibrin clots. The light (B) chain adsorbs to fibrin clots resulting in accelerated lysis of these clots by activators.

Reaction between streptokinase and plasminogen, when the former is in a molar excess, results in the extensive degradation of streptokinase (Brockway and Castellino, 1974; Summaria et al, 1974). The effect of this breakdown is to yield a modified plasmin molecule carrying none of the characteristics of streptokinase, yet retaining the capacity to activate both bovine and human plasminogen.

c The Effects of w-Aminocarboxylic Acids on the Activation and Conformation of Plasminogen, and Subsequent Effects on Fibrinolysis

The related w-aminocarboxylic acids, L-lysine, epsilon-

aminocaproic acid (EACA or 6-AHA), p-aminomethylbenzoic acid (PAMBA) and trans-4-aminomethylcyclohexanecarboxylic acid (trans-AMCHA) have similar effects on activation of plasminogen and on the catalytic effect of plasmin. Their relative potency increases in the order given and varies within a factor of 100, when determined on a molar basis (Baumgarten, 1972; Landmann, 1973; Lukasiewicz et al, 1968; Okamoto et al, 1968; Thorsen and Astrup, 1969; Thorsen and Müllertz, 1974).

Studies show that the native form of plasminogen, glu-plasminogen, undergoes a gross conformational change upon binding EACA and its analogues. Sedimentation velocity measurements show that lys-plasminogen, prepared by removal of a peptide of molecular weight 6-8,000, possesses a strikingly dissimilar gross conformation compared to glu-plasminogen (Violand et al, 1975). However, the overall conformation of lys-plasminogen is very similar to the glu-plasminogen-EACA complex, as shown by the circular dichroism spectra of the two species (Sjöholm et al, 1973). Although lys-plasminogen retains its full capacity for binding EACA, no additional gross conformational alteration results as a consequence of saturating the binding site of lys-plasminogen with this amino acid.

EACA and analogues, produce identical effects with glu- and lys-plasmin as with glu- and lys-plasminogen. These results illustrate the importance of the amino terminal region of glu-plasmin(ogen) in controlling the overall

conformation of the molecule as well as controlling the conformation achieved upon saturation by compounds such as EACA.

The effect of the amino acids on activator-induced fibrinolysis may greatly depend on the source of activator (Kok and Astrup, 1972; Thorsen and Astrup, 1969). The enhancement of fibrinolysis induced by the tissue activator over fibrinolysis induced by urokinase is related to the much higher affinity for fibrin of the tissue activator than of urokinase (Thorsen et al, 1972), associated with a marked accelerating effect of fibrin on the rate of plasminogen activation by the tissue activator (Camiolo et al, 1971; Wallén, 1978). The effect of EACA on urokinase-induced fibrinolysis in the presence of lys-plasminogen may mainly be due to an inhibition of the catalytic effect of plasmin. This is consistent with the fact that inhibition of fibrinolysis effected by urokinase, and inhibition of fibrinolysis effected by plasmin begin at the same concentrations of amino acid (between 10^{-5} and 10^{-4}M) (Thorsen et al, 1974). EACA mainly inhibits proteolysis of fibrin by competing with fibrin for sites on plasmin, which are different from the active centre (Iwamoto, 1975; Landmann, 1973) and most likely located on the heavy chain (Rickli and Otavsky, 1975). This prevents a functional interaction between the active centre on the plasmin light chain and fibrin. A much more pronounced inhibition by the w-aminocarboxylic acid of the activity of plasmin towards fibrin than towards fibrinogen, casein or synthetic esters (Ambrus et al, 1970;

Landmann, 1973; Lukasiewicz et al, 1968; Skoza et al, 1968; Thorsen et al, 1974) suggests that plasmin possesses specific substrate binding sites for fibrin. An effect of EACA on urokinase may only to a small extent contribute to the inhibition by the amino acid of urokinase-induced fibrinolysis because the dissociation constant for urokinase and amino acid (Geratz and Cheng, 1975; Landmann, 1973; Lorand and Condit, 1965; Walton, 1967) is 20-70 times the dissociation constant for plasmin and amino acid (Skoza et al, 1968).

The difference in effect of EACA on urokinase-induced lysis with native and proteolytically degraded plasminogen may mainly be explained by the difference in rate of activation of the two forms of plasminogen. EACA causes uniformly increasing inhibition of fibrinolysis (beginning between 10^{-5} and 10^{-4} EACA), whether the fibrinolysis is due to plasmin or urokinase in the presence of proteolytically degraded plasminogen. Thus it is evidence that the enhancement of urokinase-induced fibrinolysis (maximum of 10^{-3}M EACA) in the presence of native plasminogen is related to the increased rate of activation due to the complex formation between amino acid and plasminogen (Thorsen and Astrup 1974; Thorsen et al, 1974).

The effects of w-aminocarboxylic acids on fibrinolysis induced by activators may be summarized as follows:- (Thorsen, 1978)

1 They form reversible complexes with plasmin and plasminogen which leads to:
   a a dissociation of the complex between fibrin and the specific substrate binding sites on plasminogen;
   b a dissociation of the complex between fibrin and the specific substrate binding sites on plasmin;
   c conformational changes of the plasminogen molecule, leading to an increase in rate of activation.

2 They may react with activators and competitively inhibit their catalytic effect on plasminogen.

3 They may react with fibrin and change its structure.

The w-aminocarboxylic acids have a much higher affinity for plasmin(ogen) than for activators and fibrin. Consequently, the effect of the amino acids on fibrinolysis is mainly related to the effect on plasmin(ogen).

B PLASMIN

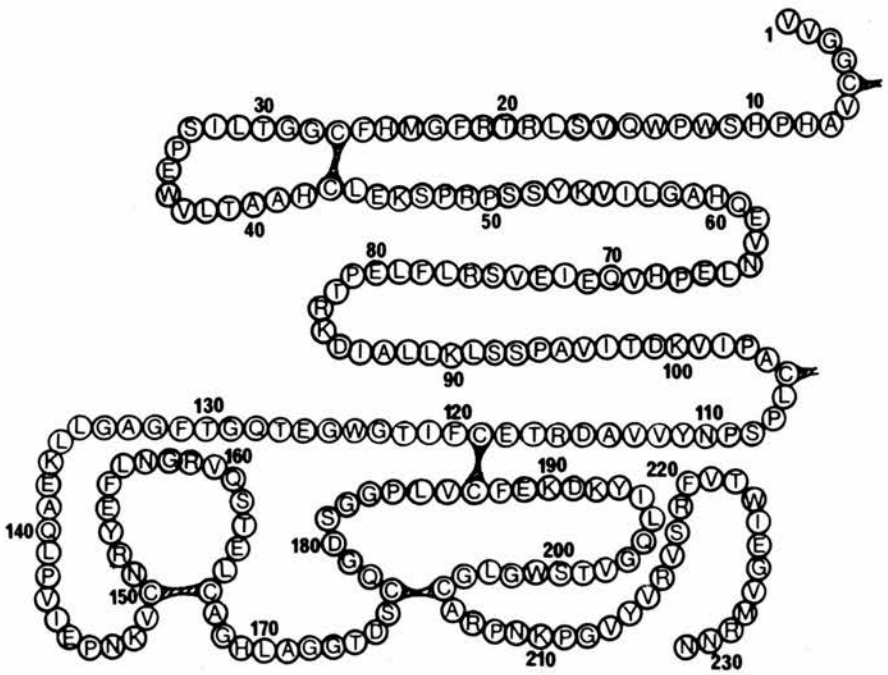
Plasmin is a proteolytic enzyme of the endopeptidase type with properties rather similar to trypsin. It cleaves, or hydrolyzes, proteins and peptides at arginyl and lysyl peptide bonds (Wallén and Iwanaga, 1968; Groskopf et al, 1968; Weinstein and Doolittle, 1972), basic amino esters (Weinstein and Doolittle, 1972; Sherry et al, 1966; Robbins et al, 1965) and amides (Christensen and Müllertz, 1974). Plasmin appears to have a preference for lysyl bonds in both protein and ester substrates (Weinstein and Doolittle, 1972).

Plasmin is a proteolytic enzyme of wide specificity, and can digest many proteins including fibrinogen and fibrin; in purified systems fibrinogen is digested as readily as fibrin, but in the presence of inhibitors the relative substrate specificity for fibrin is conferred on plasmin (Ratnoff, 1953). Other proteins digested by plasmin include prothrombin and Factor V (Alagille and Soulier, 1956), Factor VIII (Lewis et al, 1949), glucagon, ACTH and growth hormone (Mirskey et al, 1959) and certain components of complement (Pillemer et al, 1952). Plasmin when incubated with plasma in vitro can produce a bradykinin-like substance, and can also release a plasma kinin with vasodilator properties in vivo (Back et al, 1963). Why plasmin has such a wide specificity is not known but it may be of consequence for biological functions other than fibrinolysis.

Plasmin consists of two polypeptide chains of different size, a heavy and a light chain connected by at least one disulphide bond. A molecular weight of 48,800 is found for the heavy chain by Summaria et al (1971), 63,000 by Wiman and Wallén (1973), and 55,000 by Walther et al (1974); whereas evaluation of the molecular weight of the light chain differs only slightly from one laboratory to the other: 25,000 to 26,000. Like other proteases, plasmin undergoes autolytic degradation, which explains certain analytical difficulties.

The complete amino acid sequence of the heavy chain of human plasmin (residues 1-560) has now been established (Sottrup-Jensen et al, 1977). The complete sequence of the light chain is also accounted for, see Fig 4 (after Wallén, 1978).

A single DFP-sensitive serine residue (Summaria et al, 1967) and a single TLCK-sensitive histidine residue (Robbins et al, 1973) have been found in the active centre of human plasmin, and both residues are located on the light (B) chain of the enzyme (Summaria et al, 1967). The partial amino acid sequence of a 31-residue tryptic peptide containing the active centre serine residue was determined to be Val-Glx-(Ser-Thr, Glx)-Leu-(Gly, Ala)-His-Leu-Ala-Cys-Asn-(Thr, Gly, Gly)-Ser-Cys-Gln-Gly-Asp-Ser*-Gly-Gly-Pro-Leu-Val-Cys-Phe-Glu-Lys- (Groskopf et al, 1969). The N-terminal 21-residue sequence obtained from the light (B) chain and containing the active centre histidine residue



One Letter Amino Acid Abbreviations:

V = Val	P = Pro	R = Arg	K = Lys
G = Gly	S = Ser	T = Thr	Y = Tyr
C = Cys	W = Trp	F = Phe	N = Asn
A = Ala	Q = Gln	M = Met	D = Asp
H = His	L = Leu	E = Glu	I = Ile

FIG 4 THE AMINO ACID SEQUENCE OF THE LIGHT (B) CHAIN FROM HUMAN PLASMIN

The two incomplete disulphide bonds to the right (residues 5 and 105) represent the connections to the heavy (A) chain.

was determined to be His-Phe-Cys-Gly-Gly-Thr-Leu-Ile-Ser-Pro-Glu-Trp-Val-Leu-Ser-Ala-Ala-His*-Cys-Leu- (Robbins et al, 1973). This sequence contains the "histidine loop" of human plasmin, and it is homologous to the "histidine loop" sequences of other serine proteases.

C ACTIVATORS

a Vascular Activator

Vascular plasminogen activator, sometimes known as blood or plasma plasminogen activator, is the activator mainly responsible for physiological plasma fibrinolytic activity.

Blood fibrinolytic potential has a diurnal variation, with lower levels during the night (Fearnley et al, 1957; Rosing et al, 1970). This fluctuation appears to diminish with increasing age (Buckell and Elliott, 1959; Rosing et al, 1970). The plasminogen activator content of plasma falls precipitously during the latter part of the second trimester of pregnancy to return again to normal within minutes of placental separation (Bonnar et al, 1969; Åstedt, 1972). In the newborn the activator content is high but falls within hours of delivery (Ekelund et al, 1970).

i Localization

Certain organs may play significant roles in either the manufacture of circulating plasminogen activator or its removal. Thus the kidney (Januszko et al, 1966; Marsh, 1970), brain (Menon et al, 1970), stomach and intestine (Cox et al, 1967; Eras et al, 1970; Gans et al, 1971) have all been implicated as contributing to the activator input, whereas the liver and lung may play a role in its removal

(Januszko et al, 1966; Menon et al, 1968; Menon et al, 1969).

In 1959 Todd described his fibrinolytic autograph technique, which involved incubating a tissue section overlayed with a film of fibrin, thus enabling the lysis areas of the fibrin to be correlated with specific areas in the section. In most tissues, except the liver, zones of fibrinolysis appear on incubation. These zones are related almost exclusively to veins and venules. In the normal liver practically no lysis occurs, but in cirrhosis or carcinoma of the liver there is lysis around the newly-formed vessels in the scar tissue.

In 1967, Pandolfi et al showed that the fibrinolytic activity was higher in arm veins than in leg veins and that blood fibrinolysis stimulated by stasis is higher in the arms than the legs. Fibrinolytic activity in veins is localized mainly to the vasa vasorum of the adventitia (Pandolfi et al, 1967), the media is moderately active but the intimal cells are active only when detached (Pandolfi et al, 1968).

In 1969 Pandolfi et al studied the fibrinolytic activity in the vein walls of patients with thrombosis. The findings showed that the fibrinolytic activity of unaffected veins in the legs of patients with thrombosis is significantly lower than that of veins in normals. They concluded that low fibrinolytic activity of the vessel

walls predisposes to thrombosis.

There has been some dispute as to whether the vascular activator is generated by the endothelium or if the endothelial cells merely store activator that has been produced elsewhere. Lack and Ali (1964) raised the possibility that plasminogen activator was associated in some way with lysosomes. They fractionated rabbit and mouse kidney and liver and found plasminogen activator in the lysosomal fraction. Activity was enhanced by exposure to Triton X-100, saponin or endotoxin all of which are believed to release lysosomal enzymes.

Isacson and Nilsson (1969) studied fibrinolytic activity in biopsy specimens of superficial veins from patients who had been bilaterally nephrectomized and treated with haemodialysis for 3-15 months. They could not substantiate earlier reports of an essential role of the kidney in maintaining fibrinolysis of the blood and concluded that plasminogen activator forms in the vessel walls.

Further evidence that the endothelium can produce plasminogen activator was obtained by Pandolfi in 1970. Explants from human veins were cultured in a fibrinolytically inactive medium; after three weeks fibrinolytic activity was detected, by the fibrin overlay technique, confined to the vasa vasorum, suggesting that small blood vessels are able to synthesize plasminogen activator.

Generally more plasminogen activator is found in veins and venules than other vessels, but it is found in similar amounts in arteries and arterioles supplying very active tissues such as the retina, myocardium and kidney. In other systemic arteries endothelial activator seems less important and any activator seen is usually due to weaker activators released from leucocytes. Many kinds of cells may be made to release activator-like substances from lysosomes. The fibrinolytic activity of non-endothelial cells, however, tends to be weak and inconsistent, probably due to the release of non-specific proteolytic enzymes which, like trypsin, can activate plasminogen (Todd, 1972).

Noordhoek Hegt (1977) was able to correlate sites of low fibrinolytic activity with the presence of a fibrinolytic inhibitor, whereas inhibition was absent in areas showing strong fibrinolysis. Fibrinolysis is related to endothelial cells, while inhibition is related to smooth muscle cells. Thus differences in endothelial fibrinolysis may result from differences in inhibition caused by variations in the number of smooth muscle cells present locally.

ii Release

Systemic fibrinolysis and stress have been linked since the early observation of an association between violent

death and post mortem fluidity of blood. Subsequently exercise (Biggs et al, 1947), surgical operations (Macfarlane and Biggs, 1946), electroconvulsive therapy (Fantl and Simon, 1948), and even acute mental stress (Macfarlane and Biggs, 1946) were also shown to enhance the fibrinolytic potential in man. Systemic catecholamine release is a common feature of all these stimuli. Thus acute mental stress (Elmadjian et al, 1957), surgery (Franksson et al, 1954) and myocardial infarction (Wallace, 1968) have all been reported to be associated with a rise in circulating catecholamines.

Thus the study of local receptor systems began with catecholamine infusions; this was advanced by the introduction of a concept of specific catecholamine (adrenergic) receptor sites (Ahlquist, 1948), and the arrival of a group of compounds which selectively block these receptor sites (Fitzgerald, 1969). This approach has led to the definition of α- and β- adrenergic receptors in the body and to the concept that β-adrenergic receptors can be divided into β_1 and β_2 types, the former being associated with β-effects on the heart and intestinal muscle and the latter with muscle of the bronchi, uterus and vessel walls. Studies show that the plasminogen activator response to catecholamines appears to be a β-mediated phenomenon, and that the receptor involved is probably a β_2-type (Gader et al, 1973a). Evidence that the catacholamine-induced release of plasminogen activator is probably a direct effect on endothelial cell release has been obtained from

experiments in which intra-arterial epinephrine (adrenaline) was shown to stimulate a prompt increase in plasminogen activator content of the venous effluent of the infused limb (Cash, 1972).

The results of studies using β-adrenergic antagonists indicate that the catecholamine-mediated plasminogen activator response may be associated with three different receptor sites or three components of one site (Gader et al, 1974).

Studies during the early 1960's suggested that the hypothalamus and in particular the release of vasopressin might play a role in the control of fibrinolysis in man. Schneck and von Kaulla (1961) demonstrated that administration of intravenous pitressin, an animal posterior pituitary polypeptide extract which included vasopressin, was followed by a significant shortening of the euglobulin lysis time, suggesting a considerable release of plasminogen activator. Because pitressin contains several other polypeptides, it was not possible to conclude with certainty that the fibrinolytic response was related to vasopressin. However, this was recently verified using synthetic preparations of vasopressin (Gader et al, 1973b; Mannucci and Barbi, 1973). Unlike adrenaline, vasopressin does not appear to give rise to a local release of plasminogen activator when infused into a brachial artery (Cash, 1978). Therefore the conclusion is that the endothelial cells, at least in the forearms of humans, do not contain specific

vasopressin receptor sites. Moreover, the studies of Mannucci et al (1975) have clearly shown that the pathways by which adrenaline and vasopressin give rise to a release of plasminogen activator appear to be quite distinct, although Barker et al (1972) demonstrated a possible connection in that β-adrenergic stimulation may give rise to the release of vasopressin.

Benetato and Dumitrescu-Papahagi (1964) showed that following electrolytic lesions and hypothalamic ultrashort wave stimulation in man, there was a significant systemic plasminogen activator release. Apart from this there is no other clear evidence of central (CNS) control mechanisms associated with the peripheral release of plasminogen activator in man. Nevertheless, the way in which plasminogen activator is released in so many stressful events, including mental stress makes this seem highly probable. Moreover it has also been shown in man that prolonged mental stress can have a marked overriding influence on the subsequent fibrinolytic response to physical exercise.

Certainly adrenal medulla secretion of catecholamines is closely concerned with central mechanisms via neurogenic pathways and this provides a link with the brain. However, the magnitude of the role of catecholamines in plasminogen activator release, at least in the low stress situation, seems likely to be small.

It is unlikely that vasopressin is the natural specific neurohormone concerned with the physiological release of plasminogen activator. Firstly the doses of synthetic material required to produce a systemic plasminogen activator response appear to be too high to have physiological relevance, and secondly, there is no evidence of peripheral vasopressin plasminogen activator receptor sites, although it is conceivable that they might be present in endothelial cells other than those in the upper limb. However, a substance closely related structurally to vasopressin may well be important.

Cash (1978) hypothesized that the release of plasminogen activator from the endothelial cell with respect to normal daily activities may be explained on the basis of a localized (and therefore high concentration) release of a vasopressin-like substance in the hypothalamic area which triggers off the release of an as yet unknown neurohumoral substance that has a direct effect on the endothelial cell, and that the catecholamine pathway, for which there is good evidence for specific endothelial receptors, is only involved in particularly severe stressful situations.

iii Purification and Some Properties

Aoki and von Kaulla (1971a) were the first workers to describe the extraction of cadaveric vascular plasminogen

activator. They cannulated the femoral artery and vein, and perfused the vascular tree with warm buffered saline. They obtained an average of 9,621 CTA (Committee on Thrombolytic Agents) units per leg at an average of 14.5 units per ml. They observed no correlation of activator yield with age, cause of death, or time elapsed after death before extraction.

Aoki and von Kaulla (1971b) reported a molecular weight of 65,000 for the vascular activator, but Aoki (1974) later reported a value of 80,000; both values were determined by gel filtration. Aoki (1974) purified the crude vascular activator 200-fold by ammonium sulphate fractionation, zinc precipitation and ethanol fractionation followed by DEAE-cellulose chromatography and Sephadex G-200 gel filtration. Using this preparation he found that the activator was labile and rapidly lost its activity at pH values above 8 and below 4. The stability of the activator was dependant on the salt concentration of the medium in which the activator was dissolved; higher salt concentrations tended to stabilize the activator which was inactivated by dithiothreitol. The activator preparation did not possess any proteolytic activity when tested on fibrin, casein and denatured haemoglobin, but readily activated plasminogen to plasmin with zero-order kinetics.

Ogston et al (1976) partially purified a circulating plasminogen activator obtained after venous occlusion of the upper arm. No activator was recovered when plasma

obtained without venous occlusion was subjected to the same procedures. The activity of the activator preparations was markedly more stable at 37°C than that in the original plasma, and was inhibited by exposure to PMSF but not by exposure to TLCK, TPCK or iodoacetamide. Ogston et al also reported that the plasma activator was adsorbed, to a much greater extent than urokinase, to fibrin during its formation.

b Tissue Activator

Fibrinolytic activity of tissues was first reported by Fleisher and Loeb (1915). Astrup and Permin (1947) later demonstrated that the activity was due to the presence of plasminogen activators. Most tissues contain a certain amount of a plasminogen activator which is readily soluble in saline (Albrechtsen, 1957) and a certain amount of another activator which is less soluble and has to be extracted with potassium thiocyanate (Astrup and Stage, 1952). The former activator is a labile type, which might be derived from the tissues but could also be identical with the vascular plasminogen activator present in the blood. The latter activator, in contrast, is very stable and its content varies widely in different tissues.

The physiological role of these tissue activators is not clear, but presumably they play a role in the lysis of extravascular fibrin. The relationship of tissue activator

to vascular activator has been a subject of great interest, and it has been suggested that vascular activator is a product of the tissue activators. In view of the recent advances in knowledge concerning vascular activator this is unlikely and as proposed by Astrup (1975) it seems highly probable that there are separate humoral and cellular systems of plasminogen activation. The cellular system's role being the regulation of tissue repair and wound healing and the humoral system's role being the maintenance of vascular patency, with a contribution to wound healing.

Tissue activator is not extractable from tissues under physiological conditions and it is assumed that its insolubility in common aqueous media at neutral pH is due to its close association with certain intracellular elements, possibly the microsomes. Lack and Ali (1964) reported that the highest activity was in the lysosomal fraction.

A method has been developed for the assay of the tissue activator that is selective for the stable activator, since it makes use of an acid precipitation, which destroys the labile activator. In a survey of human organs Albrechtsen (1957) found that the organs could be grouped according to their average content. Some have high activity; uterus, adrenals, lymph nodes, prostate and thyroid are all above 300 u/g fresh tissue. Of medium activity are lung, ovary, pituitary, kidney, skeletal

muscle, all between 100 and 300 u/g. The heart and brain are between 30 and 100 u/g, and there is little or no activity in testes, spleen and liver. It is also seen that the activity of the individual specimens varies between extreme ranges. Albrechtsen's results on human material have been confirmed by Ende and Auditore (1961).

The greatest obstacle in the purification of tissue plasminogen activator has been the strong adherence of the activator to cellular components, making it insoluble under usual conditions. The inability to extract the activator from tissues with saline was observed by Fleisher and Loeb (1915) and has been confirmed by later investigations. Permin (1947) tried unsuccessfully to extract the activator with phosphate buffer ($^{N}/_{15}$, pH 5.6-8.3), acetic acid (1% and 5%), hydrochloric acid (0.01N), ammonium hydroxide (0.01N), sodium hydroxide (0.01N) and glycerol (87%) containing 0.5% acetic acid. The activator withstands drying with acetone and ether, and treatment with chloroform, indicating that it is free from lipids. The necessity of using concentrated solutions of potassium thiocyanate suggests that the activator is closely bound to other proteins by hydrogen bonding. One of the most important chemical properties of the tissue activator is its great stability at acid pH; at pH3 it is resistant to heating for 30 minutes at 70°C (Astrup and Sterndorff, 1956).

The major advances in purification of tissue activator

have been using the pig heart. Bachmann et al (1964) extracted the activator from acetone-dried pig heart powder with 0.3M potassium acetate pH4.2. Subsequent steps involved ammonium sulphate precipitations of pH4.2 and 50% saturation, and at pH8 and 35% saturation, followed by precipitation at low ionic strength in the presence of 0.3mM Zn^{2+}. Further substantial purification was obtained by gel filtration through Sephadex columns and precipitation with acetone. The method was reproducible and gave overall yields from 15-25%. The final preparations exhibited specific activities of 12,000-25,000 CTA u/mg protein, representing a 1,700-fold purification over the crude extract.

An improvement on the above procedures was reported by Wallén and Wiman in 1975a. Using the same initial steps, they took a fraction obtained between 14 and 66% saturation with ammonium sulphate dissolved in phosphate buffer at pH7 and adsorbed it with fibrin. The activity was eluted with 2M potassium thiocyanate, and then subjected to hydrophobic interaction chromatography on phenylethyl-Sepharose. The activity was eluted with a potassium thiocyanate gradient, yielding a preparation with a specific activity of 33,000 CTA u/mg.

A further improvement in the purification of pig heart tissue activator was obtained by two affinity adsorbtions involving two different sites in the activator molecule (Wallén and Rånby, 1977). Firstly the activator was

adsorbed to fibrin and eluted in two steps with potassium thiocyanate and phenylethylamine. This was followed by affinity chromatography on Sepharose-arginine. The final product gave a single band on SDS-polyacrylamide gel electrophoresis of molecular weight 63,000 dalton before reduction, and 31,000 dalton after reduction. The highly purified activator (specific activity ⩾ 150,000 CTA u/mg) was labile in neutral solutions of low ionic strength but was stabalized by increasing the ionic strength to 1. The firm and specific adsorbtion of tissue activator to fibrin is presumably of physiological importance, and attempts are being made to study sites in fibrin(ogen) with affinity for tissue activator. Preliminary experiments (Wallén and Rånby, 1977) indicate that one site is situated in the part of the Aα chain which is released at an early stage in the plasmin digestion of fibrin(ogen).

c Urinary Activator (Urokinase)

It has been known since 1885 that urine shows a proteolytic activity; an activity attributed by Sahli to formed proteolytic enzymes. Specific fibrinolytic activity was noted by Macfarlane and Pilling (1947), this effect being demonstrated by Williams (1951) to be due to the presence of a plasminogen activator in urine; the name urokinase was suggested by Sobel et al (1952).

Early purification of urokinase was by ammonium sulphate,

alcohol or acetone precipitation (Macfarlane and Pilling, 1947) or precipitation at pH4.5 in the cold (Celander et al, 1955). Celander et al (1955) also developed a foam technique in which urine was shaken by hand for two minutes to produce a foam in which the urokinase was concentrated between 10 and 40-fold.

Ploug and Kjeldgaard (1957) employed adsorption on silica gel, followed by chromatography on Amberlite IRC-50 to purify urokinase, although the preparations were still inhomogeneous by electrophoresis. Sgouris et al (1962) used the cation exchanger cellulose phosphate, an ethanol precipitation and the ion exchange resin XE-97; these procedures gave urokinase of specific activity 1,000 CTA u/mg, and electrophoresis on starch gel gave one major band associated with the activity, and four minor bands.

Lesuk et al (1965) prepared crystalline urokinase starting with aqueous solutions of highly purified material. Crystallization was induced by cautious adding of sodium chloride with gentle stirring. These procedures resulted in specific activities of 104,000 CTA u/mg protein and gave a molecular weight of 54,000 and an N-terminal amino acid residue of isoleucine (Lesuk et al, 1967).

Ogawa et al (1975) used a five-step purification procedure involving an initial adsorption on a synthetic acrylonitrile fibre followed by ammonium sulphate precipitation and three chromatography steps on Amberlite CG50, Sephadex G-100 and

CM Sephadex respectively, giving urokinase preparations with specific activities of over 215,000 CTA u/mg. Holmberg et al (1976) further purified commercial urokinase preparations by affinity chromatography on benzamidine-Sepharose.

According to Holmberg et al (1976) there are two molecular forms of urokinase; they have molecular weights of 54,000 and 31,000, whereas Ong et al (1976) claim molecular weights of 47,000 and 33,400. The high molecular weight form appears to be composed of two protein chains, one of which is the low molecular weight form, or light chain. When Holmberg et al (1976) ran their preparation in SDS gel electrophoresis after reduction with mercaptoethanol a 20,000 molecular weight band appeared. However, a monospecific antiserum was raised on immunization, thus it appears that the light chain of the 54,000 molecular weight urokinase contains only antigenic determinants that are present in the heavy chain. Holmberg et al also reported that electrophoretic mobility in agarose of both chains appeared to be similar. Ong et al (1976) determined the molecular weight of the light chain to be 18,600.

The high molecular weight form can be converted catalytically to the low molecular weight form (Ong et al, 1977); Lesuk et al (1967) also reported that urokinase could be cleaved by trypsin to give an active fragment of molecular weight 36,000. Some proteases, other than trypsin, were found to liberate still smaller active fragments which await further

characterization, while other proteases yield only inactive fragments.

It is likely that urokinase belongs in the class of enzymes utilizing the Asp His Ser triad for their catalytic action. The two active site residues so far identified, serine and histidine, are present in the low molecular weight form, and are located in the heavy chain of the high molecular forms (Ong et al, 1976).

Ong et al (1977) reported that at low plasminogen levels the high molecular weight form showed greater fibrinolytic activity than the low molecular weight form, suggesting that it may be more effective therapeutically. As a follow up to this Suyama et al (1977) showed that the concentration of high molecular weight urokinase necessary to induce 50% lysis of artificial thrombi was approximately half that of low molecular weight urokinase. Also Lormeau et al (1977) showed that high molecular weight urokinase is about 2.5-fold more active than low molecular weight urokinase towards glu-plasminogen.

Painter and Charles (1962) cultured monkey and dog kidney cells, and showed that they accumulated a fibrinolytic agent which was shown to be a soluble plasminogen activator with properties similar to urokinase. Bernik and Kwaan (1967) cultured explants from human kidneys and a plasminogen activator was produced and released by cells which emerged from small vessels. No significant fibrinolytic

activity was exhibited by tubular cells, fibroblasts, macrophages or lymphocytes in the outgrowths. The titre of the plasminogen activator in the culture media increased after repeated changes of fluid, indicating a continuous production and release of activator. This was found to be a function of living cells and was not observed in cells that died during culture. Preliminary immunological investigation of the activator showed it to be identical to urokinase. Barlow and Lazer (1972) using cultured human embryo kidney cells confirmed the identity of the activator with urokinase in all properties studied.

Primary cultures of kidney confirmed that this organ is a rich source of urokinase; it is produced in the early stages of gestation and in increasing amounts thereafter (Bernik and Kwaan, 1969). But the ability to produce urokinase is not limited to the kidney but is a function of cells distributed widely in body tissues. Activator antigenically identical to urokinase accumulated in culture supernates of faetal lung and ureter and in renal blood vessel cultures of adults.

Åstedt (1975) attempted to locate the actual site of production of urokinase in the kidney. Isolated glomeruli that were fibrinolytically inactive (by the fibrin slide technique) were obtained and cultured; during culture a marked release of fibrinolytic activators occurred. After culture histochemical examination showed the glomeruli to be fibrinolytically active. This means that the fibrinolytic

defence system of the draining pathways begins at least at the epithelial surface of the glomeruli.

Åstedt et al (1977) using organ and cell cultures of human faetal kidneys found that early cultures produced the high molecular weight urokinase, whereas late cultures produced low molecular weight urokinase. It is possible that the kidney cells lose their ability to link the two chains or, more probably, are not able to synthesize the light chain.

Bernik (1973) showed that deposition of fibrin on cells in culture was followed by a 2 to 6-fold increase in urokinase in the supernates and rapid disappearance of the fibrin. It appeared that the enhanced urokinase yields were mediated through plasmin and thrombin. Inactive material, which gave rise to urokinase upon exposure to proteolytic enzymes and which may represent a urokinase precursor, was found in cultures of kidney, lung, spleen and thyroid (Bernik, 1973). Nolan et al (1977) also found evidence for a trypsin-activatable proactivator in human embryonic kidney cell cultures.

Marder et al (1977) have shown clearly that the pharmacologic effect of tissue culture urokinase, from human kidney cells, is the same as that of urinary urokinase, and it is reasonable to expect that both materials will be equally effective therapeutically.

d Factor XII Mediated Fibrinolytic Activity

Iatridis and Ferguson (1961) and Niewiarowski and Prou-Wartelle (1959) showed that fibrinolytic activity could be generated in normal plasma (but only to a limited extent in factor XII deficient plasma) following incubation with kaolin after acidification and reduction in ionic strength. These and other observations have brought about considerable interest in the reaction sequence between the activation of factor XII and the activation of plasminogen, and in the interrelationships between this reaction and the activation of the coagulation, the kallikrein-kinin and the complement systems. It is now clear they are all interrelated and that factor XII occupies a key position in the reactions. The implications of this second humoral activator system on the overall fibrinolytic mechanism and the mechanism of thrombolysis in vivo, are still largely unknown.

Recently, Hageman factor (factor XII), in the presence of kaolin, has been ascribed a hitherto unsuspected function; that is, the direct activation of plasminogen (Goldsmith et al, 1978). Prior to this it was accepted that the interaction of a surface with at least three plasma proteins was a necessary requirement for factor XII dependant fibrinolysis. The three plasma proteins are factor XII, prekallikrein and high molecular weight (HMW) kininogen (see Fig 5). Plasmas that are deficient in any one of these components are defective in the conversion of plasminogen

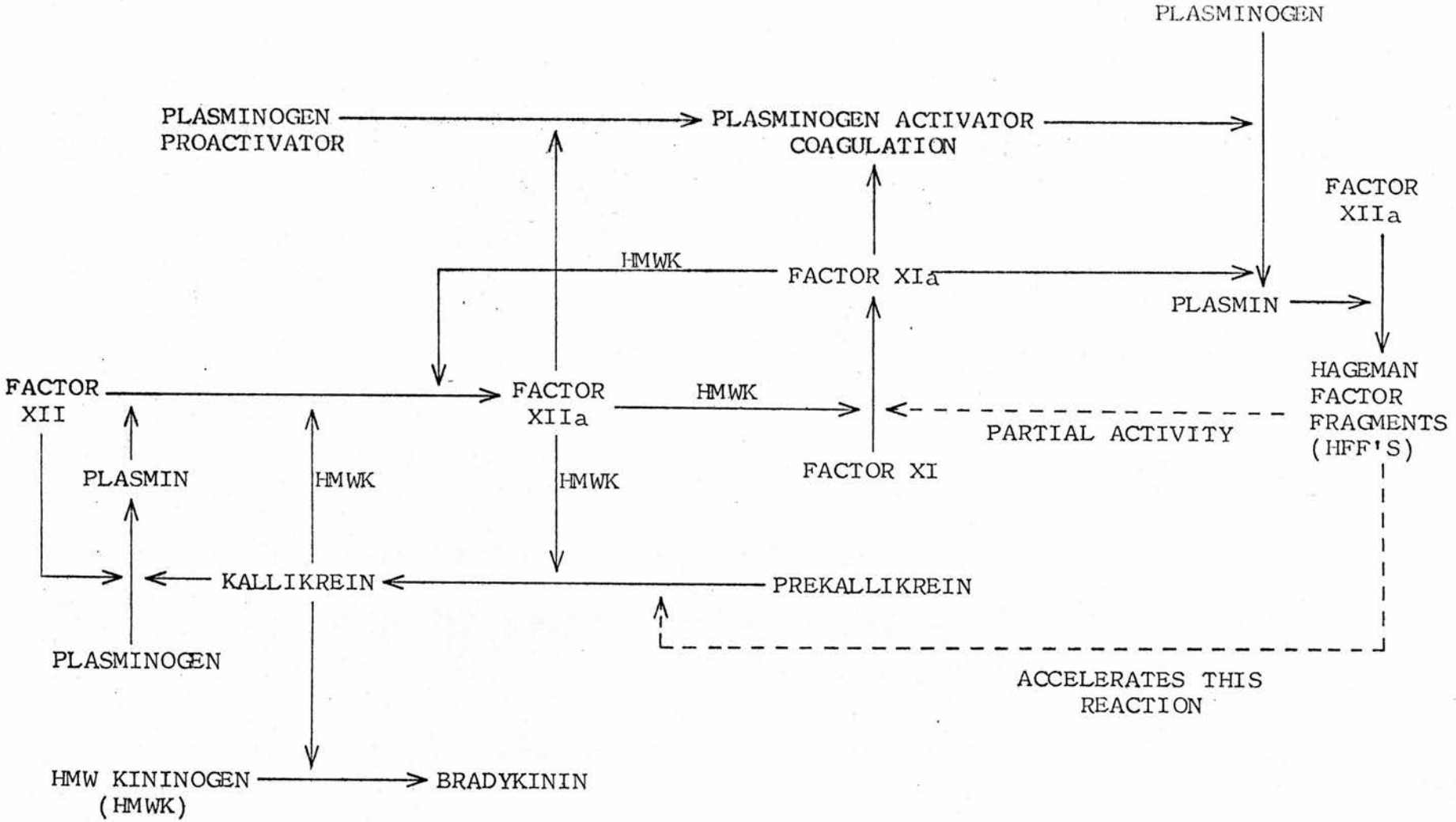


FIG 5 FACTOR XII MEDIATED PATHWAYS

to plasmin.

Prekallikrein and HMW kininogen circulate as a complex and molecular weight considerations suggest that the binding is 1mole/mole. This complex then interacts with factor XII when they are bound to a negatively charged surface. There is a reciprocal activation that occurs between factor XII and prekallikrein. Thus factor XIIa converts prekallikrein to kallikrein while kallikrein enzymatically activates factor XII. The reaction rate in both directions is augmented by HMW kininogen. The surface and HMW kininogen bring the two proenzymes together in an optimal fashion; however, it is not known which factor possesses the active site that initiates the reaction.

Kallikrein digests factor XII into two main fragments. The 28,000 molecular weight fragment contains the active site of factor XIIa, while the 52,000 molecular weight fragment is the portion that binds to surfaces. Initially cleavage occurs such that the two pieces are disulphide linked and bound to the surface and a secondary cleavage liberates the Hageman factor fragments. Kallikrein also digests the cofactor HMW kininogen to liberate bradykinin, and it also directly converts plasminogen to plasmin, with prekallikrein acting as a plasminogen proactivator.

In addition factor XI circulates bound to HMW kininogen and factor XI and Hageman factor also activate each other with HMW kininogen acting as a cofactor. However, the

activation of Hageman factor by factor XIa is only about 10% as effective as kallikrein.

Kaplan and Austen (1972) described a Hageman factor substrate that, upon activation, directly converted plasminogen to plasmin. This factor was clearly distinguished from factor XI, but was similar to prekallikrein in molecular weight and isoelectric point. Laake and Vennerod (1974) were unable to separate prekallikrein from plasminogen proactivator and concluded that these were one and the same molecule.

Kaplan et al (1976) when fractionating human plasma found two peaks of plasminogen proactivator activity, one fractionated along with prekallikrein, while the second was superimposable upon factor XI. This second activity may be factor XI or a different plasminogen activator that fractionates with factor XI.

A Hageman factor dependant factor that functions in fibrinolysis was reported by Ogston et al (1969) to be a γ-globulin of 160,000 molecular weight. Plasma adsorbed with crushed glass was found to possess a fibrinolytic defect that could be corrected by addition of the above plasma fraction. The properties of this factor most closely resemble the plasminogen activator associated with factor XI. However, glass-adsorbed plasma has been shown to be deficient in prekallikrein (Wuepper, 1973) and since the Hageman factor cofactor preparations utilized contain

prekallikrein (Ogston et al, 1969) it appears that the correction may merely represent the requirement for prekallikrein in the activation of Hageman factor.

The activation of plasminogen to plasmin by the Hageman factor dependant plasminogen activator appears to be similar to that seen with urokinase rather than streptokinase.

e Bacterial Activators

Plasminogen activator activity is produced by many microorganisms including streptococci, B coli, staphylococci, Claustridia and Pseudomonas pyocyanea. Of the bacterial activators, the most important is streptokinase produced by streptococci.

Streptokinase is an extracellular protein produced during the growth of several strains of beta-haemolytic streptococci, and it functions in the conversion of plasminogen to plasmin (see section 2Abii). Up to now the only substrates known for streptokinase are purified plasminogens and plasmins from human, monkey, baboon, chimpanzee, cat, dog and rabbit plasmas.

Streptokinase shares with other streptococcal proteins the ability to act as an antigen in man. Immune antibodies to streptokinase are present in all individuals, probably as

a result of previous streptococcal infection, but the amount of this inhibitor varies considerably throughout the population. Streptokinase reacts immediately with this antibody and is rendered biochemically inert; the complex formed is then cleared rapidly from the circulation.

Thus during the induction of a thrombolytic state with streptokinase, sufficient drug must be given to neutralise the circulating antibodies before its specific effect on the fibrinolytic enzyme system can take place (Johnson and McCarty, 1959). During treatment, the antistreptokinase titre remains essentially constant, but after 8-9 days it rises rapidly in most patients to 50-100 times the normal level, and returns to normal only after a period of 4-6 months or more.

The method of choice for purification of streptokinase depends upon the quality of the starting material. The most suitable starting material available commercially is Kabikinase. This material has plasma albumin as its major protein impurity, added as a stabilizer, this can be removed by blue-dextran-Sepharose chromatography (Castellino et al, 1976). Crude streptokinase can be purified by affinity chromatography on insolubilized DFP-plasmin (Comp, 1976). The bound streptokinase is eluted with citrate buffer at pH3. The streptokinase produced has a single N-terminal amino acid and runs as a single band on gel electrophoresis. Yields of over 95% have been obtained with no loss in streptokinase activity as

measured on fibrin agar plates.

Purified streptokinase is stable indefinitely in the lyophilized state or in aqueous solution in the frozen state. When thawed, the material is stable over several hours in an ice bath. The molecular weight of streptokinase has been determined by various groups, and values ranging from 45,000 (Brockway and Castellino, 1974) to 50,000 (Taylor and Botts, 1968) have been obtained.

Streptokinase is a protein consisting of a single subunit with very little carbohydrate and no lipid. The N-terminal amino acid sequence is NH_2-Ile-Ala-Gly-Pro-Glu-Trp-Leu-Leu-Asp-Arg-Pro-Ser and the C-terminal residue is lysine (Brockway and Castellino, 1974). The amino acid composition is consistant with the formula: Asp_{68}-Thr_{30}-Ser_{24}-Glu_{46}-Pro_{20}-Gly_{21}-Ala_{23}-Val_{23}-Met_{3}-Ile_{22}-Leu_{40}-Tyr_{20}-Phe_{15}-Lys_{33}-His_{9}-Arg_{21}-Trp_{1} for a molecular weight of 47,754 (De Renzo et al, 1967).

f Tumour-Associated Fibrinolysis

Since the early observation by Fischer (1925) that explants of chicken tumours caused lysis of plasma clots, many investigators have examined the association between raised levels of fibrinolysis and neoplastic transformation.

In 1973 Unkeless et al showed that chick embryo fibroblast

cultures develop fibrinolytic activity after transformation by Rous sarcoma virus (RSV); the same was found by Ossowski et al (1973) for chick, hamster, mouse and rat embryo fibroblast cultures transformed by either DNA or RNA viruses. This activity is not present in normal cultures of these cells and it does not appear after infection with either non-transforming strains of avian leukosis viruses or cytocidal viruses, and is therefore related to transformation rather than to virus infection or cell lysis.

Cells infected with a temperature-sensitive mutant of RSV and grown at 41°C are normal both by morphological and biochemical criteria, whereas these cells grown at 36°C are transformed by the same criteria (Kawai and Hanafusa, 1971). Changes occur very rapidly following temperature shifts, and the formation of plasminogen activator in such cultures is one of the earliest observable events associated with transformation (Christman et al, 1975b; Rifkin et al, 1975); indeed its appearance precedes morphological change (Ossowski et al, 1973).

Unkeless et al (1973) demonstrated the requirement for mRNA synthesis to precede plasminogen activator production following downward temperature shifts to conditions permissive for transformation, and Rifkin et al (1975) showed a comparable requirement for RNA synthesis to precede termination of activator formation in the converse situation, but the nature of the relevant RNA species is unknown.

Christman et al (1975a) demonstrated that plasminogen activator production is also co-ordinated with tumourogenicity. By growing a tumourogenic line of mouse melanoma cells in the presence of bromodeoxyuridine, it is possible to convert them to a non-tumourogenic state, and by removing the drug to allow reversal to the original state. A close link was observed between appearance of tumourogenicity and the production of plasminogen activator by these cells. Thus, circumstantial involvement of production of this enzyme with both tumourogenicity and transformation has been established.

Svanberg et al (1975) histochemically examined ovarian tumours obtained at laparotomy for local fibrinolytic activity and also determined the fibrin(ogen) degradation products (FDP's) in the serum. The fibrinolytic activity was confined mainly to vessels of both malignant and benign tumours; however FDP's were found in the serum of 13 out of 14 patients with malignant tumours, but in none with benign tumours. The difference in occurrence of FDP's in patients with malignant and benign tumours might be due to the invasive growth of the malignant tumours with the entrance of thromboplastic substances, fibrinolytic activators or locally formed FDP's into the bloodstream. Determination of FDP's may therefore make it possible to differentiate between malignant and non-malignant tumours. FDP's are found invariably and in high concentration in malignant ascitic fluid (Åstedt et al, 1971; Svanberg and Åstedt, 1975), and the fibrinolytic activity in malignant

ascitic fluid is due to an activator rather than plasmin (Svanberg and Åstedt, 1975).

Several plasminogen activators have been partially or completely purified. They are all serine proteases. Christman and Acs (1974) have purified an activator from transformed hamster cells to apparent homogeneity. It has a molecular weight of 50,000 and it consists of subunits linked by disulphide bridges. The subunit containing the active site has a molecular weight of 25,000. Evidence suggests that the enzyme is probably firmly associated with the plasma membrane of transformed cells as well as being released into the medium.

Unkeless et al (1974a) have partially purified an activator from RSV transformed chicken fibroblasts. It is an arginine-specific protease of 39,000 molecular weight. The cell factor that is detected in the supernatent culture fluid also occurs in a cell-associated form in transformed but not in normal cells. This form is tightly bound in a postnuclear cellular particulate fraction and can be solubilized by SDS or Triton X-100.

Rifkin et al (1974) isolated two activators from human melanoma cells; the major component has a molecular weight of 48-50,000 and the minor species 60,000.

Wu and Yunis (1976) using a human pancreatic carcinoma cell line have purified an activator 2,000-fold. The purified

enzyme shares many properties with urokinase including a molecular weight of 54,000, electrophoretic mobility, heat stability, inhibition by DFP, stability over a wide pH range and its mode of activation of plasminogen. The intracellular enzyme is membrane bound and can be solubilized by SDS or Triton X-100. Solubilized activator has a similar molecular weight to that of the secreted enzyme as determined by SDS gel electrophoresis.

Thus the presence of plasminogen activator within transformed cells could serve a regulatory function, while the presence of this enzyme in the surrounding medium affords the transformed cell a mechanism for changing its chemical milieu, an action by which it might exert a profound effect either on its own fate or that of its normal neighbours.

g Miscellaneous Plasminogen Activators

i Polysaccharide Sulphate- and Flufenamate-Activated Fibrinolysis

The first observations about polysaccharide sulphates and activation of fibrinolysis are those of Ungar and Mist (1949). Some acid mucopolysaccharides like hyaluronic acid, chondroitin sulphuric acid and heparin produced fibrinolytic activity in the euglobulin fraction of guinea pig serum, when added at low ionic strength before euglobulin precipitation at acid pH. This activity is

similar to that produced by peptone, cellulose sulphate, dextran sulphate and a number of synthetic or semi-synthetic polysaccharides. A number of these substances are also active in human plasma.

Martin (1975) attributed the activity elicited in human euglobulins, in vitro, by a pentosan sulphate to the increased amount of precipitable material and to the higher plasminogen content of the euglobulin fraction. However, on top of this, pentosan sulphate can elicit fibrinolytic activity after in vivo administration, both in the euglobulin fraction and in whole plasma (Coccheri et al, 1963). Moreover, antiplasmin activity of plasma is decreased by these substances (Coccheri et al, 1963).

It is possible that these negatively charged substances induce a physical separation between plasminogen activators and their inhibitors, allowing availability of free fibrinolytic activity, thus changes in the precipitation pattern of proteins and in the amount of plasminogen and activators recovered in the euglobulin precipitate may be a consequence of the alterations induced by the polysaccharides rather than being the mechanism primarily responsible for the production of fibrinolytic activity.

Astrup and Rosa (1974) described a potent fibrinolytic system, activated by polysaccharide sulphates, occurring in human blood. This system was found to generate considerably more activity than produced by glass activation

of factor XII, and in fact Astrup and Rosa showed the system to be activatable in the absence of Hageman factor.

The same system or mechanisms may be operating in the flufenamate induction of fibrinolysis. Tomikawa and Abiko (1973) described the isolation of a latent plasminogen activator activity from human plasma by gel filtering the supernatent of a euglobulin precipitate on Sephadex G-200 followed by isoelectric focusing. The component was immunologically different from B_1C-globulin, plasminogen and plasmin (thus excluding the possibility of an antiplasmin-plasmin complex), and was present in the ratio 3:7 in the euglobin: euglobulin supernatent, although it could only be detected in the supernatent after removal of antiplasmins. Plasminogen activator activity was liberated from the component by treatment with flufenamate.

There have been few reports on the mechanism of this chemical induction of fibrinolysis. It has been postulated that inactivation of antiplasmin (von Kaulla, 1963) or antiactivators (Aoki and von Kaulla, 1969a) or a possible modification of B_1C-globulin (Aoki and von Kaulla, 1969b) may be responsible. Baumgarten et al (1970) also showed that serum proteins were required for chemically induced fibrinolysis. Kluft (1978) has shown that the extrinsic activator (vascular activator) is resistant to C1-inactivator and that no extra resistant activity is generated in vitro with dextran sulphate. However, there is also present an activity that is susceptible to C1-inactivator. This

activity appears to originate from intrinsic proactivator systems, and it is this susceptible intrinsic activity that can be fully revealed by the combined effects of dextran sulphate and flufenamate. Kluft also points out that the extrinsic activity has the capacity, at least in vitro, to initiate activation of the intrinsic system, although it is not clear whether the active agent is plasmin or plasminogen activator. A mechanism such as this in vivo would have great implications for current concepts of thrombolysis.

ii Blood Cells

Unkeless et al (1974b) reported that cultured thioglycollate-stimulated peritoneal macrophages synthesize, accumulate, and continually release high levels of plasminogen activators, whereas cultures of unstimulated macrophages do not. The higher specific activity of released as opposed to cell-associated enzyme suggests that the activators are actively secreted. The major macrophage plasminogen activator is a serine protease of molecular weight 48,000, and thus resembles the comparable enzyme released by virally transformed fibroblasts. Macrophages release a second plasminogen activator, of molecular weight 28,000, that is also a serine protease.

Kopitar et al (1974) isolated a plasminogen activator from disrupted pig leukocytes with the aid of DEAE-chromatography,

gel filtration and CM-cellulose chromatography. The molecular weight of the activator was determined by gel filtration to be 28,000-30,500, and the activity was inhibited 100% when tested on a fibrin plate with 10^{-2}M EACA and 20 KIE/ml Trasylol.

Granelli-Piperno et al (1977) reported that purified human polymorphonuclear leukocytes (PMN's) secrete a plasminogen activator. Results show that the activator production by PMN's is controlled by agents that affect inflammation, and that this control is not shared by other lytic enzymes known to be associated with these cells. This suggests a particular role for plasminogen activator in the response pattern of PMN's and also supports the concept, previously developed for macrophages, that the secretion of activator is correlated with cell migration in vivo.

iii Body Fluids

As well as the well documented plasminogen activators present in blood, urine and many tissues in the body Albrechtsen et al (1958) found activity in cerebrospinal fluid, ascitic fluid, synovial fluid, hydrocele fluid, lymph and fluid from blisters. Sweat appeared inactive. Storm (1955) found lacrymal fluid to contain small amounts of a plasminogen activator and large amounts of a pro-activator, which is converted into a plasminogen activator

by streptokinase. A similar system was described in human milk by Astrup and Sterndorff (1953). According to Okamoto et al (1974) plasminogen activator activity was higher in colostrum than in mature milk, was inhibited by AMCHA and had a molecular weight of 80,000.

There is a proactivator present in human saliva, but there has been some dispute as to whether or not a plasminogen activator is present. Albrechtsen and Thaysen (1955) found an activator, whereas Nitta et al (1967) did not. Moody (1976) consistently found plasminogen activator activity in mixed native saliva, but eliminated a soluble activator, showing the activity to reside in the epithelial cells of the salivary pellet.

Oshiba et al (1977) showed human bile to contain a plasminogen activator, termed bilokinase, as well as a proactivator. The bilokinase activity of human bile was weak in comparison with that of certain other species and no significant relationship between biliary and plasma fibrinolysis was observed.

h Comparison of Activators

As the first pure preparations of a plasminogen activator were of urokinase, most of the comparitive work has been against this enzyme. Ali and Evans (1968) showed rabbit kidney cytokinase to be very similar, if not the same, as

human urokinase. The molecular weight is similar, the inhibition profile the same and starch gel electrophoretic behaviour identical. Barlow and Lazer (1972) compared the activator isolated from cultured human embryo kidney cells with urokinase. They found that physical, chemical and immunological studies showed all properties of the two activators as being identical. The comparison between urokinase and the cultured kidney cell activator was extended to include the activator associated with ovarian tumour cultures by Åstedt and Holmberg (1976, 1978). All three activators gave two active peaks on Sephadex G-100 gel filtration at molecular weights 35,000 and 52,000. The activators from the kidney and ovarian carcinoma cultures were both neutralized by anti-urokinase IgG fractions, and all three gave lines of identity between each other in double diffusion experiments.

Kok and Astrup (1969) described a difference in molecular weight between urokinase (32,000 and 54,000) and tissue activator from pregnant hog ovaries (60,000). Thorsen and Astrup (1969) compared the activators using the inhibitors EACA, AMCHA and PAMBA. Inhibition of porcine tissue activator increased uniformly with increasing concentrations of inhibitor, whereas with urokinase, an early phase of inhibition at low inhibitor concentrations was followed by a phase of enhancement of fibrinolysis, turning into a second phase of inhibition. This biphasic inhibition of urokinase distinguishes it from porcine tissue activator. However, EACA produces a slightly biphasic pattern of

inhibition with plasminogen activator from human uterine tissue (Kok and Astrup, 1972).

Nieuwenhuizen et al (1977) used synthetic substrates to demonstrate a difference between tissue activator and urokinase with respect to their substrate requirements. Tissue activator has a pronounced preference for a substrate with a C-terminal arginyl residue, whereas urokinase has a less pronounced preference for this, and a blocked N-terminal amino group is a minimal substrate requirement for the tissue activator, whereas it is not essential for urokinase.

Camiolo et al (1971), Thorsen et al (1972) and Thorsen and Astrup (1976) noted differences in the fibrin affinity of the activators. Whereas urokinase was reported to be slightly, or not at all, adsorbed on fibrin (Back et al, 1961; Blix, 1962; Brakman and Astrup, 1963) other activators were found to be strongly adsorbed. This applies to tissue activator from the pig heart (Brakman and Astrup, 1963), the activator in human blood produced by exercise (Blix, 1962), the activator produced in human blood by streptokinase (Blix, 1962; Brakman and Astrup, 1963; Lassen, 1958), and the activator activity present in human cadaver blood (Müllertz, 1953b).

A thorough investigation of various activators was carried out using antiserum against purified human urokinase by Kucinski et al in 1968. The anti-urokinase neutralized

purified human urokinase, native urokinase in urine and the activator (urokinase) from human kidney cultures. They found partial cross-reaction against urokinase from primates such as the rhesus monkey and baboon; and also against rhesus monkey kidney culture supernatents. However, results established the lack of immunological identity of human urokinase with streptokinase, staphylokinase, urokinase from non-primate mammals (eg dog, pig, rabbit and guinea pig), tissue activator and tissue culture supernatents of porcine origin, human milk activator, human adrenal tissue activator and the human vascular activator.

A further comparison between the human vascular activator and urokinase was made by Aoki and von Kaulla (1971b). They found the molecular weights to be different, determining urokinase to be 55,000 and the vascular activator to be 65,000. The Km values for both activators were the same for the activation of plasminogen, but different for the hydrolysis of AGLMe. Both enzymes are inhibited, to different degrees, by the placental urokinase inhibitor. Furthermore, an immunological distinction between urokinase and the vascular activator was revealed using the IgG fraction of a urokinase antiserum. Whereas this fraction inhibited urokinase up to a dilution of 1:2,560, it had no effect on the vascular activator.

D PROACTIVATOR

Streptokinase is a good activator of plasminogen in the human fibrinolysin system (Tillet and Garner, 1933), but not in other species (Norman, 1957; Meyers and Burdon, 1957; Sherry, 1954; Chattopadhyay and Cliffton, 1965). The species difference was further investigated by Müllertz and Lassen (1953), who found that bovine plasminogen was not activated by streptokinase alone, but reacted if a small amount of human plasma globulin was added. Accordingly, it was suggested that human plasma contains a proactivator, which is converted to an activator by streptokinase. Bovine plasma, and, to some extent, that of other animal species, was thought to be deficient in proactivator, and hence not responsive to streptokinase. However, the controversy as to whether human proactivator has a separate identity to human plasminogen is still open to debate.

Kline (1966) reported that when the streptokinase concentration was carefully controlled a strong covarience relationship was found between the plasminogen and proactivator concentration in 12 normal human plasma samples. Kline suggested that the lack of such control had led earlier investigators to the erroneous conclusion that they had demonstrated the presence of a proactivator other than plasminogen or plasmin in human plasma.

Markus and Werkheiser (1964) and Werkheiser and Markus (1964)

suggested that two types of plasminogen-SK complexes can be formed, one of which acts primarily as an activator of bovine plasminogen, whereas the other can activate both human and bovine plasminogen. Ling et al (1965) have also suggested that an SK-plasmin complex may be responsible for activation of bovine plasminogen.

However, a great deal of work has been done by Takada and Coworkers which supports the view that there is at least one proactivator distinct from plasminogen. Takada Y et al (1969) found two peaks of proactivator activity when they fractionated human plasma or its euglobulin on Sephadex G-200. They termed these proactivators A and B. Proactivator A was of large molecular weight and could be isolated free of plasminogen, whereas proactivator B was smaller and contaminated with plasminogen.

Takada Y and Ambrus (1969) reported that proactivator A, in the presence of streptokinase, lyses standard bovine fibrin plates, but not heated bovine plates. Whereas without streptokinase, there is no lysis of either type of bovine plate. Proactivator A plus streptokinase does not induce caseinolysis, but does activate human, bovine and guinea pig plasminogen in caseinolysis tests. They also demonstrated that fibrinolytic activity of proactivator A-streptokinase shows no covarience with plasminogen or plasmin, and immunodiffusion showed no cross-reactivity between proactivator A and various human plasminogen preparations.

Takada A et al (1970) showed that small amounts of streptokinase converted the proactivator to activator, which in turn converted human plasminogen to plasmin, but the same amounts of streptokinase could scarcely activate plasminogen in the absence of added proactivator. Very large amounts of streptokinase, however, activated plasminogen equally well in the presence or absence of proactivator.

Takada A et al (1972) determined a molecular weight of 300,000, by gel filtration, for the proactivator. It migrates in the α_2 region in electrophoresis, but there is no cross-reaction with either anti-α_2 Macroglobulin or anti-β-lipoprotein.

Thus it appears that experimental evidence is accumulating to support the concept that there is a functional proactivator molecule in plasma distinct from plasminogen.

E PHYSIOLOGICAL INHIBITORS

Proteolytic activity is generated following activation of the fibrinolytic system and to ensure that this activity is not generated inappropriately, the organism has a system of antiproteinases. Inhibitors of the fibrinolytic system may act at two levels, either by inhibiting formed plasmin or by inhibiting plasminogen activation.

a Inhibitors of Plasmin

i α_2-Antiplasmin

Prior to the discovery of α_2-antiplasmin, it was generally believed that α_2-macroglobulin and α_1-antitrypsin were the main plasmin inhibitors in human plasma. However, Collen et al (1975a) showed that gel filtration of urokinase or streptokinase activated fresh human plasma, containing a trace of radiolabelled plasminogen, gives two radioactive peaks, one eluted at the void volume, and one just before the globulin peak. The first peak was identified as a plasmin-α_2-macroglobulin complex. The second peak had a molecular weight of 120-140,000 and did not cross-react with antisera against any of the main protease inhibitors in plasma.

In 1976 Collen, Müllertz and Clemmensen, and Moroi and Aoki all described the new inhibitor, α_2-antiplasmin.

Collen (1976) showed that the complex in the second peak, referred to above, reacted with antisera to plasminogen and with antisera to the complex, adsorbed with purified plasminogen. The latter antisera also reacted against a single component in plasma, and specific removal of this component from plasma by immunoadsorption resulted in the disappearance of the fast-acting antiplasmin activity, whereas α_2-macroglobulin was found to represent the slow-reacting plasmin-neutralizing activity.

Gallimore (1975) partially purified a fibrinolytic inhibitor from human serum that he called inter-α-antiplasmin. Further work (Hedner and Gallimore, 1977; Gallimore and Hedner, 1977) indicates that this preparation contains a mixture of α_2-antiplasmin and the inhibitor of plasminogen activation described by Hedner (1973), which in turn is different from α_2-antiplasmin (Hedner and Collen, 1976).

Upon activation in plasma, the formed plasmin is first preferentially bound to α_2-antiplasmin. Only upon complete activation of plasminogen (concentration in plasma 1.5μM), resulting in saturation of the α_2-antiplasmin (concentration in plasma 1μM), is the excess plasmin neutralized by α_2-macroglobulin (Collen, 1976). In the presence of normal concentrations of these two inhibitors the other plasma protease inhibitors do not play a role in the inactivation of plasmin (Collen, 1976; Müllertz, 1974).

To date four methods have been described for the purification of α_2-antiplasmin from plasma. Moroi and Aoki (1976) used a combination of ammonium sulphate precipitation, DEAE-Sephadex chromatography, affinity chromatography on insolubilized plasminogen and hydroxyapatite chromatography of plasminogen-depleted plasma, to purify the inhibitor to homogeneity. Wiman and Collen (1977) used chromatography on insolubilized plasminogen, DEAE-Sephadex and insolubilized concanavalin A. This procedure resulted in a higher yield and a more stable inhibitor preparation. Müllertz and Clemmensen (1976) partially purified the inhibitor by ammonium sulphate precipitation, then sequential chromatography on DEAE-Sephadex, insolubilized concanavalin A, gel filtration and a further DEAE-Sephadex. Collen et al (1978) isolated the inhibitor by immunoadsorption chromatography and gel filtration; however, the product was mainly in an inactive form.

α_2-antiplasmin is a single chain glycoprotein of 65-70,000 molecular weight estimated by SDS-gel electrophoresis and ultracentrifugation (Moroi and Aoki, 1976; Wiman and Collen, 1977). On electrophoresis it migrates as an α_2-globulin. The amino acid composition reported by Moroi and Aoki (1976) and that reported by Wiman and Collen (1977) correspond well for most amino acids, and the estimated carbohydrate content of the molecule was determined as 11.7% and 13.7% respectively. The inhibitor is immunochemically different from α_1-antitrypsin, α_2-macroglobulin C_1-esterase inhibitor, antithrombin III, α_1-antichymotrypsin,

and inter-α-trypsin inhibitor (Moroi and Aoki, 1976; Wiman and Collen, 1977) and from the inhibitor of plasminogen activation described by Hedner (Wiman and Collen, 1977; Hedner and Collen, 1976). The inhibitor is stable in solution above pH 6.3 but is rapidly inactivated below pH 6.0, and the N-terminal amino acid sequence has been determined as Asn-Gln-Glu-Gly (Wiman and Collen, 1977).

In purified systems (Moroi and Aoki, 1976; Wiman and Collen, 1977) and in plasma (Collen, 1976; Müllertz, 1974) antiplasmin forms a 1:1 stoichiometric complex with plasmin that is devoid of protease or esterase activity and that cannot be dissociated by denaturing agents such as urea, guanidine HCl, or dodecyl sulphate. SDS-gel patterns indicate that complex formation occurs by strong interaction between the light (B) chain of plasmin and the inhibitor. Moroi and Aoki (1977b) reported that the complex is dissociated by hydrazine, indicating an ester bond between the active-site serine residue of plasmin and antiplasmin. A peptide of molecular weight 11-14,000 may be released after formation of the complex. This finding has led to the assumption that inhibition occurs by hydrolysis of a specific peptide bond in the inhibitor followed by esterification between the newly formed C-terminal residue of the inhibitor and the active-site serine residue of plasmin (Collen, 1976; Moroi and Aoki, 1976; Müllertz and Clemmensen, 1976). It has been shown that the release of this polypeptide is not an essential step in complex formation but the result of hydrolysis of the complex by

free plasmin (Wiman and Collen, 1977).

Antiplasmin was also shown to react with trypsin and slowly with urokinase in purified systems (Moroi and Aoki, 1976). When trypsin, chymotrypsin, thrombin and plasmin were added to normal plasma, antiplasmin played a role in the binding of plasmin and, to a lesser extent, trypsin, but not of thrombin or chymotrypsin (Edy and Collen, 1977).

The principal role of antiplasmin seems to be the very fast and irreversible neutralization of plasmin in the circulation. However, antiplasmin also interferes with the binding of plasminogen to fibrin and thus may have an additional antifibrinolytic effect (Moroi and Aoki, 1977a).

ii α_2-Macroglobulin

The molecular weight of α_2-macroglobulin is variously quoted in the range 725,000-845,000 and occurs at a concentration of 2-5.8μM in human plasma. It can bind trypsin, thrombin, kallikrein, chymotrypsin and elastase, as well as plasmin and many other enzymes. None of the other inhibitors of proteinases from plasma, or other sources, share the apparent capacity of α_2-macroglobulin to react with this whole functional group of enzymes. Inactivated forms of the proteinases and zymogens are not bound by α_2-macroglobulin (Barrett and Starkey, 1973), thus plasminogen and DFP-plasmin are not bound (Steinbuch, 1975).

The trapped enzyme is irreversibly bound to the α_2-macroglobulin molecule, and experiments to show any reversibility of the binding, with reappearance of free enzyme and native α_2-macroglobulin have failed (Barrett et al, 1974).

Barrett and Starkey (1973) concluded that the available evidence pointed to α_2-macroglobulin binding proteinases in general, in equimolar ratios. The idea that all proteinases are bound in the same ratio is supported by the evidence that α_2-macroglobulin can be saturated with one enzyme, so that it is unable to bind another. This is demonstrated when α_2-macroglobulin is presaturated with plasmin preventing inhibition of thrombin (Ganrot and Nilehn, 1967); and pretreatment of α_2-macroglobulin with trypsin prevented the binding of chymotrypsin and vice versa (Berthillier et al, 1968).

α_2-macroglobulin forms proteolytically inactive, but esterolytically active complexes with plasmin, trypsin and thrombin (Ganrot, 1967). Typically the 'bound' enzyme may show 80-100% of normal activity with low molecular substrates, but little or none against large proteins. Furthermore, the bound enzyme is protected from other high molecular weight inhibitors, thus protecting its remaining activity against low molecular weight substrates. Soybean trypsin inhibitor (SBTI) in molar excess almost completely inhibits the proteolytic and esterolytic activity of plasmin, but it does not inhibit

the esterolytic activity of the α_2-macroglobulin-plasmin complex (Ganrot, 1967).

Harpel and Mosesson (1973) showed that after complex formation with α_2-macroglobulin in plasma, plasmin retained less than 0.1% of its fibrinogenolytic activity. However they considered α_2-macroglobulin may modulate enzyme-substrate interactions, such as these, by providing a mechanism for the preservation and protection of a portion, albeit small, of the enzymic activity in the presence of other circulating inhibitors.

Barrett and Starkey (1973) put forward the 'trap hypothesis' concerning the molecular mechanism of interaction of α_2-macroglobulin with proteinases. They suggest that binding is initiated by a proteolytic attack on the α_2-macroglobulin molecule, and that this results in a conformational change such that the enzyme molecule is irreversibly trapped within the α_2-macroglobulin molecule. The inhibition of enzymic activity results from the steric hindrance of access of substrates to the enzyme in its enclosed environment.

Physiologically the most important function of α_2-macroglobulin appears to be this trapping mechanism of proteinases, rather than their inhibition. The importance lies in the extreme rapidity with which α_2-macroglobulin complexes are cleared from the circulation, perhaps as a result of the recognition of the altered α_2-macroglobulin

conformation, by mononuclear phagocytes. Complexes of α_2-macroglobulin with plasmin or trypsin are cleared from the circulation in minutes, as compared with many days for native α_2-macroglobulin (Nilehn and Ganrot, 1967). In this way the protein plays an important role in the regulation of extracellular proteolytic activity resulting from coagulation and fibrinolysis, as well as the release of proteinases from granulocytes and other cells in inflammation (Barrett and Starkey, 1973). α_2-macroglobulin has been identified on the luminal surface of endothelial cells in sections of normal human arteries, veins and lymphatics by an indirect immunofluorescent technique (Becker and Harpel, 1976). The location at the surface of the vessel wall suggests a protective role against potentially injurous intravascular proteases.

iii α_1-Antitrypsin

α_1-antitrypsin has a molecular weight of approximately 55,000 (Gallimore, 1975) and occurs at a concentration of 35-71μM in human plasma (Müllertz, 1978). It inhibits trypsin, chymotrypsin, elastase, plasmin (Schwick et al, 1967), thrombin (Rimon et al, 1966) and kallikrein (Clemmensen et al, 1976). It was claimed by Schwick et al (1967) that α_1-antitrypsin represents 90% of the anti-proteolytic activity of plasma, and it was regarded as the slow reacting antiplasmin.

The inhibition of trypsin and chymotrypsin by α_1-antitrypsin was instantaneous and a stoichiometric reaction, whereas the inhibition of plasmin and thrombin was time-dependant and of a non-stoichiometric nature (Rimon et al, 1966). It was suggested by Rimon et al (1966) that the latter inhibition mechanism may be an enzymatic inactivation. This inactivation was irreversible in the sense that no enzymatic activity could be recovered after acid dissociation of the enzyme-inhibitor complex.

Crawford and Ogston (1974) showed α_1-antitrypsin to inhibit the caseinolytic and fibrinolytic activity of plasmin and, in contrast to α_2-macroglobulin, it also inhibits esterase activity. They also reported that the fibrinolytic and esterase activities of urokinase were not affected by concentrations of α_1-antitrypsin that caused marked inhibition of plasmin. However, Clemmensen and Christensen (1976), using purified reagents, showed that the esterolytic activity of urokinase was inhibited in a strong time-dependant manner by α_1-antitrypsin. Complex formation was demonstrated by crossed immunoelectrophoresis and by SDS-polyacrylamide electrophoresis. The latter method revealed the formation of 1:1 and 2:1 ratios of enzyme-inhibitor complexes. Clemmensen et al (1976) went on to show that α_1-antitrypsin appears to be identical to the inhibitor of urokinase-induced fibrinolysis that appears during late pregnancy, and that although it inhibits urokinase, it does not inhibit porcine tissue activator.

Raised levels of α_1-antitrypsin are found in plasma in conditions with tissue damage. High levels are found in patients with infection, with malignant disease and in renal disease, in acute pancreatitis, in pregnancy and in diabetes (Hedner, 1974).

iv C_1-Inactivator

C_1-inactivator has a molecular weight of 105,000 and is found at a concentration of 1.4-3.3μM in human plasma (Müllertz, 1978). C_1-inactivator is capable of inhibiting plasmin, factor XIIa (and its active fragments) and kallikrein (Trumpi-Kalshoven, 1978). It is present in the euglobulin precipitate and may be the cause of variability (Kluft, 1976).

Working with purified reagents in vitro, Trumpi-Kalshoven (1978) reported that once the plasmin-C_1 inactivator complex is formed, it does not dissociate in the presence of fibrin; this results in inhibition. However, if plasmin is generated in the presence of fibrin and C_1-inactivator, it has a higher affinity for fibrin and thus little or no inhibition occurs.

Several investigators have found a decreased potency of inhibitors when fibrinogen was converted into fibrin, which resulted in the observation that fibrinolysis can occur under conditions where fibrinogen is not degraded. The

inhibition of plasmin by C_1-inactivator correlates with this observation. However, there is complete inhibition of plasmin after the visible lysis of fibrin (Trumpi-Kalshoven, 1978).

v Antithrombin III

Antithrombin III has a molecular weight of 62,000 and occurs at a concentration of 3.5-6.3μM in human plasma (Müllertz, 1978). Antithrombin III is the principal inhibitor of thrombin in human blood and recently its ability to inhibit factor Xa and other coagulation factors has been demonstrated (Crawford et al, 1975).

Highsmith and Rosenberg (1974) studied the interaction of purified human antithrombin III and plasmin in the presence and absence of heparin. Antithrombin III is a progressive, time-dependant inhibitor of the proteolytic and esterolytic activities of plasmin. Incubation of plasmin with antithrombin III for 15-30 minutes resulted in 90-100% inhibition of both activities of the enzyme. The presence of heparin dramatically accelerated the rate of interaction, with nearly complete inhibition within 30 seconds. SDS-gel electrophoresis of reduced and non-reduced proteins indicated that antithrombin functions as a potent antiplasmin by forming an undissociable complex which is stable in the presence of denaturing or reducing agents or both. This complex represents a 1:1

stoichiometric combination of enzyme and inhibitor. Heparin increases the rate of formation of this complex without affecting its dissociability or stoichiometry.

In the absence of heparin, less than 1% of plasma antithrombin III participates in the binding of plasmin, but as the heparin concentration is increased, more antithrombin III binds to plasmin (Telesforo et al, 1975). In the range of therapeutic anticoagulation (1-2u heparin/ml plasma), 2.5-3.8% plasma antithrombin III is used in the binding of plasmin, which corresponds to the binding of 6-9% of generated plasmin (Telesforo et al, 1975). Crawford and Ogston (1975) reported that antithrombin III does not influence the activity of plasminogen activators from tissue, blood or urine.

vi Inter-α-Trypsin Inhibitor

Inter-α-trypsin inhibitor has a molecular weight of 180,000 (Gallimore, 1975) and occurs at a concentration of 1.2-4.4μM in human plasma (Müllertz, 1978). It inhibits plasmin, but provides only a minor contribution to the total antiplasmin activity of blood.

b Inhibitors of Plasminogen Activation

Much evidence has been put forward for the existence of

inhibitors acting both as antiplasmins and as inhibitors of plasminogen activation, although assay methods, generally relying on the action of formed plasmin, make it difficult to distinguish between the two.

However, it is reported that a selective increase in the inhibitors acting on plasminogen activation has been demonstrated in patients with severe thrombotic disease (Brakman et al, 1966; Nilsson et al, 1961; Pandolfi et al, 1970). In patients this inhibition does not correlate with α_2-macroglobulin, α_1-antitrypsin (Hedner and Nilsson, 1971) or with α_2-antiplasmin (Hedner et al, 1978).

The activation inhibitor was shown to be an α_2-globulin, which was acid- and heat-labile (Hedner, 1973). After gel filtration on Sephadex G-200 inhibitory activity appeared in two peaks, one in the high molecular weight region and one just ahead of the albumin peak (Hedner et al, 1970). It was found that the second, low molecular weight, peak varied with the serum activity of the inhibitor.

This low molecular weight inhibitor has been purified by passage through haemoglobin-Sepharose (to remove haptoglobin), DEAE-Sephadex chromatography, gel filtration on Sephadex G-200 and preparative gel electrophoresis (Hedner, 1973). The molecular weight of the α_2-globulin was 75,000 and it did not react with antisera against α_2-macroglobulin, α_1-antitrypsin, antithrombin III, C_1-inactivator, inter-α-trypsin inhibitor or antichymotrypsin.

The inhibitor mainly inhibited plasminogen activation and an antiserum was readily raised that was capable of neutralising the inhibitor in serum (Hedner, 1973).

Hedner and Collen (1976) provided immunological evidence for the non-identity of the antiactivator described above and the α_2-antiplasmin described by Collen et al (1975a); the antisera against the antiactivator and that against α_2-antiplasmin reacting with two different components in human plasma. A cautionary note was added by Hedner et al (1978) pointing out that the antiserum against Collen's antiplasmin was raised against a complex and not against the purified protein itself. A structural change of the protein may, therefore, be responsible for a changed immunochemical behaviour; this seems unlikely, however, as Collen (1976) was able to remove the fast-reacting antiplasmin activity from plasma by immuno-adsorption and this would not be the case if his preparation of antiserum only recognized the complexed form of α_2-antiplasmin.

An additional inhibitor with a molecular weight of 80,000 has recently been described by Gallimore (1975). This inhibitor inhibits both plasmin- and urokinase-induced clot lysis and has been called inter-α-antiplasmin. However, it has been shown by Gallimore and Hedner (1977) that partially purified inter-α-antiplasmin contains proteins cross-reacting with both antiactivator and α_2-antiplasmin antisera.

A further question mark was raised against Hedner's antiactivator by Collen et al (1977), who reported that normal plasma showed inhibition in a clot lysis assay with urokinase, but surprisingly this activity was virtually absent in antiplasmin-depleted plasma containing a normal amount of the inhibitor of plasminogen activation described by Hedner (1973). However this anomoly was resolved when it was later reported that Hedner's inhibitor was a fast reacting inhibitor of factor XIIa (Hedner, 1978).

Aoki and von Kaulla (1971c) described the isolation of an 80,000 molecular weight moiety with antiactivator activity. However, this preparation also exerted some antiplasmin activity and with further work it appeared more likely that it was α_2-antiplasmin (Moroi and Aoki, 1976). Beattie et al (1976) described two inhibitors of plasminogen activation by urokinase when they fractionated plasma by gel filtration on Sephadex G-200. The low molecular weight inhibitor was separate from the principal protease inhibitors of plasma, and although active in a clot lysis system, failed to inhibit the esterase activity of urokinase. Inhibitors of plasminogen activation have also been partially purified from human placenta (Uszynski and Abildgaard, 1971; Kawano and Uemura, 1971), and platelets (Ogston et al, 1973; Moore et al, 1975; Joist, 1977).

There still remains some confusion concerning the number and type of inhibitors of fibrinolysis present in the α_2-globulin fraction of human plasma. However, it appears

that there are at least two inhibitors of fibrinolysis migrating in the α_2-globulin region (Hedner and Collen, 1976; Gallimore and Hedner, 1977; Gallimore, 1978).

3 FIBRIN : FORMATION AND LYSIS

A FIBRINOGEN

Fibrinogen is one of the largest proteins found in normal plasma. Estimates of the molecular weight of circulating fibrinogen (bovine or human) range from 245,000 to 700,000. However, values below 300,000 probably indicate determinations made on partially catabolized forms of the molecule. There is now general agreement that more "native" forms of the molecule have molecular weights in the range 325,000-345,000 (Caspary and Kekwick, 1957; Endres and Scheraga, 1971), and are found in plasma in concentrations ranging from 200-500mg per 100ml plasma.

N-terminal analyses of fibrinogen indicate three types of polypeptide chains (Blombäck et al, 1966), and when fibrinogen is reduced and run in SDS-polyacrylamide electrophoresis, it separates into three chains with molecular weights 63,000, 56,000 and 47,000 (McKee et al, 1970). Comparison of the molecular weight and the results of quantitative N-terminal amino acid determinations has revealed that the molecule consists of six chains, two of each type, and thus the native molecule exists as a dimer. The three chains are termed $A\alpha$, $B\beta$ and γ; A and B refer to the respective N-terminal peptides, removed by thrombin during fibrin formation.

Although it was known that native fibrinogen was a dimer,

little was known about how the two halves were linked together. The observation that fibrinogen's molecular weight did not change in denaturing solvents suggested that the chains were held together by covalent linkages. Since reductive cleavage of disulphide bridges severed all covalent connections between chains (McKee et al, 1966), the covalent bridging must be accomplished solely by interchain disulphide bonds.

Blombäck et al (1968) used cyanogen bromide cleavage to prepare a fragment that contained the N-terminal regions of all three types of fibrinogen subunit chains, joined by interchain disulphide bonds. This structure was termed the N-terminal disulphide knot (N-DSK), and Blombäck proposed that this was the site at which both halves of the fibrinogen molecule were joined together. This idea was confirmed by Marder et al (1972) from studies on the degradation products of fibrinogen produced by plasmin (see Fig 6). They found that fragment E, the plasmin resistant degradation product, was almost identical with the disulphide knot region and similarly contained the N-terminal portions of all six polypeptide chains. The amino acid sequence of each of the constituent chains of the N-terminal disulphide knot is now known, as are the probable locations of the inter- and intrachain disulphide bridges (Wallén, 1971; Blombäck and Blombäck, 1972; Blombäck, 1969). Doolittle et al (1977) reported that human fibrinogen contains 29 disulphide bonds, only three of which are involved in holding the two dimeric

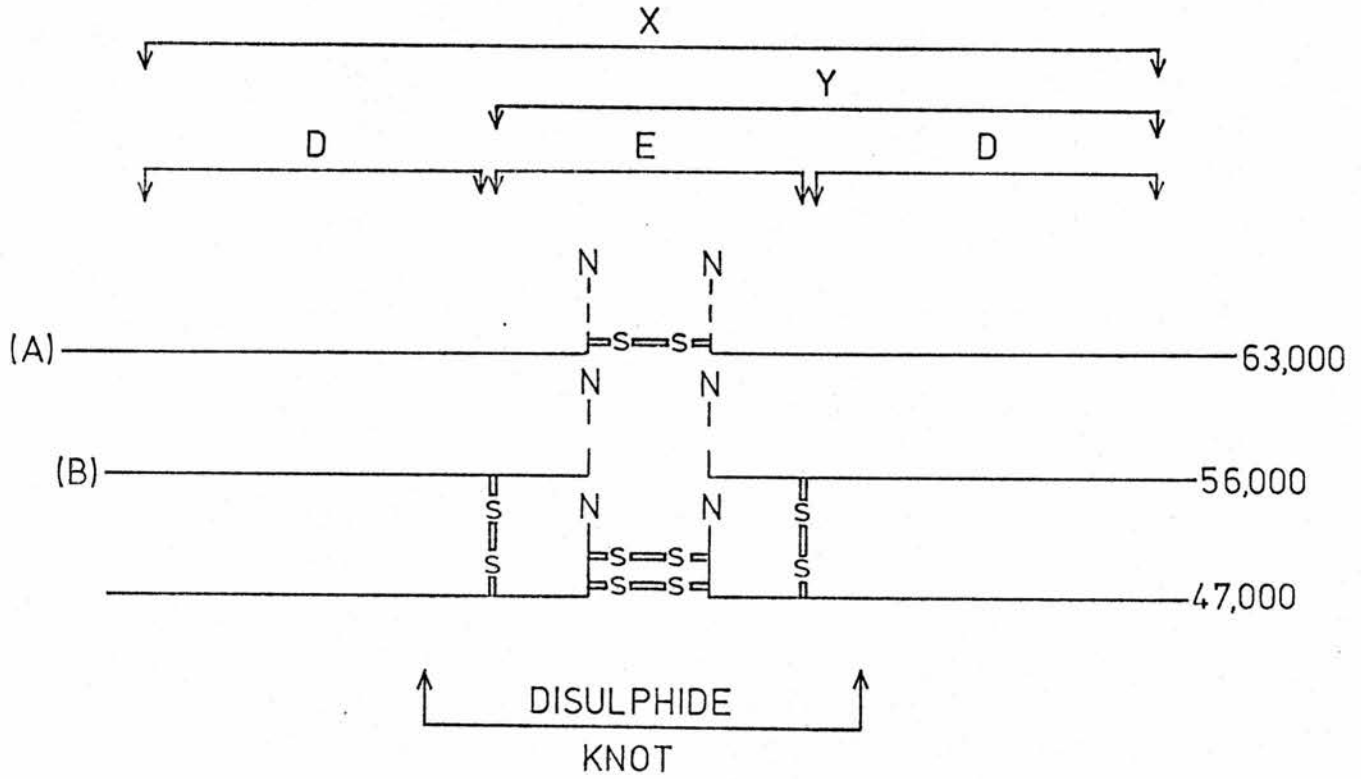


FIG 6 DIAGRAMMATIC REPRESENTATION OF THE FIBRINOGEN MOLECULE

The dotted N-terminal regions represent fibrinopeptides A and B. X, Y, D and E are the plasmic degradation products.

halves of the molecule together.

Henschen et al (1977) have reported the complete amino acid sequence of the γ-chain consisting of 409 residues. Bouma et al (1977) also working on the γ-chain noticed significant homologies with the α- and β-chains, especially with regard to the arrangement of certain key cysteine residues. Thus, the sequence at residues γ 135-139 is Cys-Gln-Glu-Pro-Cys and γ 19-23 is Cys-Pro-Thr-Thr-Cys; the occurrence of similar pentapeptide skeins in the α- and β-chains, in each case separated by about the same number of residues, has both structural and evolutionary connotations.

B THROMBIN

Thrombin, the natural coagulant of fibrinogen, appears in blood following activation of the clotting system. Mann et al (1973) have shown that there are at least three forms of thrombin, α, β and γ. The β and γ forms are degradation products of the α form. The molecular weight of these different species are 39,000 (α) and 28,000 (β and γ). The specific esterase activity of these three forms of thrombin are the same, while only the α form has proteolytic activity on fibrinogen (Mann et al, 1971).

The amino acid composition and primary sequence of α-thrombin have been published (Magnusson, 1971). α-thrombin is a two-chain protein with a light chain of 49 residues connected by a disulphide bridge to the heavy chain which is made up of 260 amino acid residues. The heavy chain also contains the serine active centre and a carbohydrate residue.

C ACTION OF THROMBIN ON FIBRINOGEN - FORMATION OF FIBRIN

Once thrombin is generated, it specifically cleaves two pairs of unique arginine-glycine peptide bonds in fibrinogen and initiates development of a fibrin clot (see Fig 7). Cleavage by thrombin releases the N-terminal portions of four of the chains as the A and B peptides, which have molecular weights of 1,536 and 1,552 respectively (Blombäck et al, 1966).

When thrombin catalyzes the release of fibrinopeptides A and B from fibrinogen, fibrinopeptide A (FPA) must appear, followed, after a lag phase, by fibrinopeptide B (FPB), for polymerization to occur. Preferential release of FPB by an enzyme in copperhead snake venom (Agkistrodon contortrix) does not result in normal polymer formation unless FPA is also removed (Shainoff and Dardik, 1977). Thus, it has been proposed that two sets of polymerization sites are involved in fibrin formation (Blombäck and Blombäck, 1972); one set, A:a, is activated on release of FPA and results in end-to-end polymerization; the other, B:b, is activated on release of FPB and results in lateral aggregation.

The removal of FPB acts as a switch; after it is removed fibrin monomers assemble rapidly to form thick fibres and gelation is fast, but when this peptide is still attached, there is a slow assembly of thin fibres and

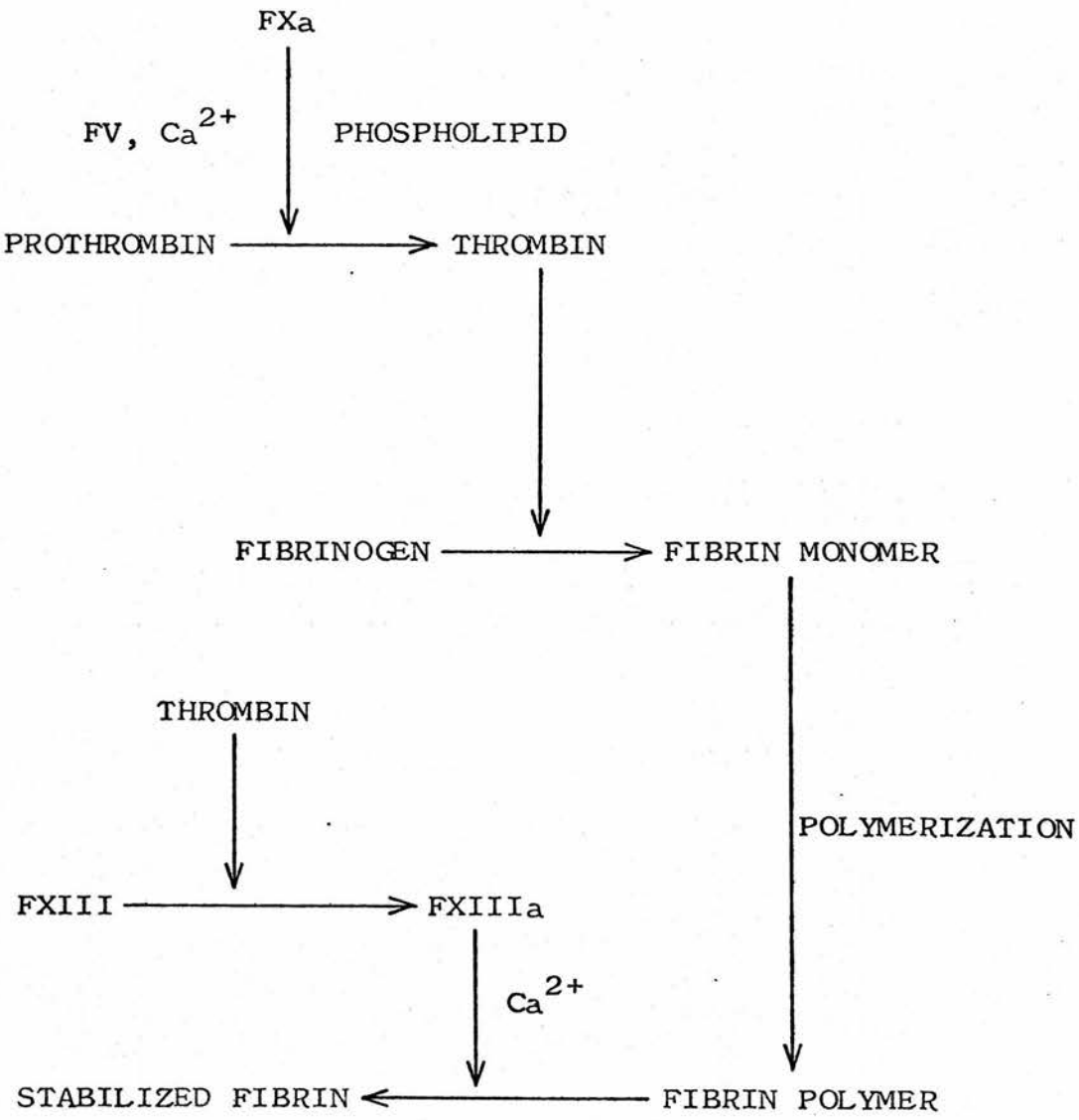


FIG 7 PATHWAY OF FIBRIN FORMATION

(Coagulation abbreviated to show the common pathway only.)

gelation is delayed (Shen et al, 1977). Shen et al (1977) speculate that this delay may well be critical for the complete digestion, by plasmin, of fibrin formed during in vivo defibrination with Ancrod (removes FPA), and of fibrin produced by very small amounts of thrombin (which would still contain FPB), and that slow release of FPB is part of a control mechanism for the regulation of fibrin formation and prevention of intravascular coagulation.

D FACTOR XIII - CROSSLINKING OF FIBRIN

Although purified fibrin is soluble in urea, fibrin formed in the physiological situation is not soluble in urea (Robbins, 1944). The difference in behaviour is due to the presence of factor XIII (Lorand and Jacobsen, 1958) in the physiological situation. Human plasma factor XIII has a molecular weight of 320,000 and is composed of two types of subunit, an a chain of 75,000 daltons and a b chain of 85,000 daltons. The native factor XIII has an a_2b_2 subunit structure (Schwartz et al, 1973). The activation of factor XIII by thrombin and other proteolytic enzymes is accompanied by a decrease in the molecular weight of the a chain of 4,000 daltons, which becomes enzymatically active, while the b chain remains unchanged. Activated factor XIII (factor XIIIa) stabilizes fibrin by enzymatically forming e-(γ-glutamyl) lysine cross-links between γ-chains and, secondarily, α-chains of fibrin, by transamidation. Under suitable conditions, approximately six cross-links per mole fibrin are formed, provided the fibrin substrate under study contains a high proportion of intact α chains (Finlayson et al, 1972). Of those six cross-links, two occur rapidly between γ chains of fibrin, which on reduction of the fibrin yield γ-γ dimers, and four occur more slowly between the α-chains of fibrin, reduction yielding α-polymers. The β-chain of fibrin is not involved in the cross linking reaction.

Doolittle et al (1972) have identified the residues involved

in the formation of the γ-γ dimer and have found that γ-chains lie in an antiparallel arrangement. While Fretto et al (1977) reported that crosslink acceptor sites of the α-chain reside in the middle third of the chain.

E LYSIS OF FIBRIN(OGEN)

Müllertz (1953a) reported that plasmin from post mortem blood split pure preparations of fibrinogen and fibrin at nearly the same rates. Subsequently, Marder et al (1969) looked more closely at the plasmin digestion of fibrinogen (see Fig 8) and found that two high molecular weight derivatives were produced on digestion. The first to appear is fragment X, molecular weight in the region of 240,000. This fragment is slowly, but almost completely (85%) clottable by thrombin. Fragment X then breaks down to give fragments Y and D. Fragment Y, molecular weight in the region of 155,000, is non-clottable by thrombin. This fragment breaks down further to give another fragment D, and fragment E, the plasmin-resistant core, molecular weight approximately 50,000.

The N-terminal amino acid residues of plasmin-digested human fibrinogen were determined by Marder et al (1972) and compared with undigested fibrinogen. Fibrinogen and fragments X, Y and E had primarily alanine and tyrosine N-terminal residues, whereas fragment D clearly had a different complement of N-terminal residues and was thought to derive from the C-terminal part of the parent molecule. Marder et al (1972) also compared fragment E with the disulphide knot, a chemical derivative of fibrinogen, containing the N-terminal region of the parent molecule. Fragment E showed striking similarity of N-terminal amino acid residues and antigenic determinants with the N-terminal

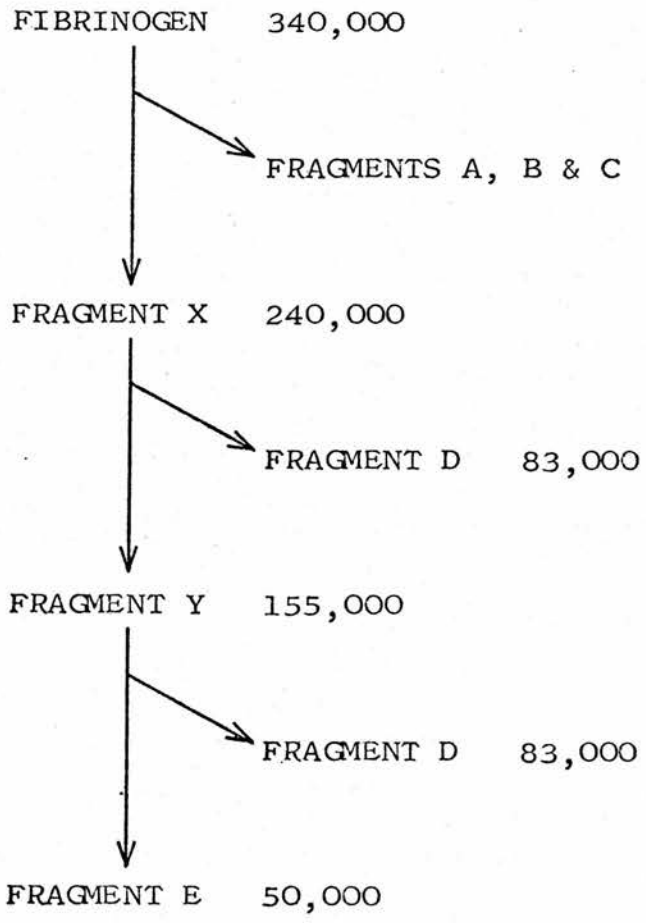


FIG 8 SCHEMATIC REPRESENTATION OF THE PLASMIC DIGESTION OF FIBRINOGEN

disulphide knot.

Furlan and Coworkers (Furlan and Beck, 1972; Furlan et al, 1975a; Furlan et al, 1975b) further elucidated the events in the degradation of fibrinogen by plasmin (see Figs 9A and 9B). They found that in the early phase of plasmic hydrolysis large polypeptides are progressively being split from the C-terminal part of the α-chain. These can be seen by PAGE of reduced fibrinogen chains, following plasmin digestion, as polypeptide remnants of various size (14-58,000). Thus fragment X was found to be a heterogenous population of molecules differing in the length of their α-chains. Early X fragments seem to have intact β-chains as opposed to late X fragments, in which fibrinopeptide B is missing. The β and γ chains are generally more resistant to attack than the α-chains.

Fragment X splits into one molecule of fragment Y and one molecule of fragment D. Fragment D is split assymetrically from fragment X, as previously suggested by Marder et al (1969), one half of fragment X remaining unchanged. According to the degradation scheme of Marder et al (1969) at least three peptide bonds must be broken in order to produce fragments Y and D. It is conceivable that these bonds are not split simultaneously. The same argument is also valid for conversion of Y into D and E. A sequential splitting of the individual polypeptide chains, while the split remnants are still held together by disulphide bridges, will result in a mixture of products, having the

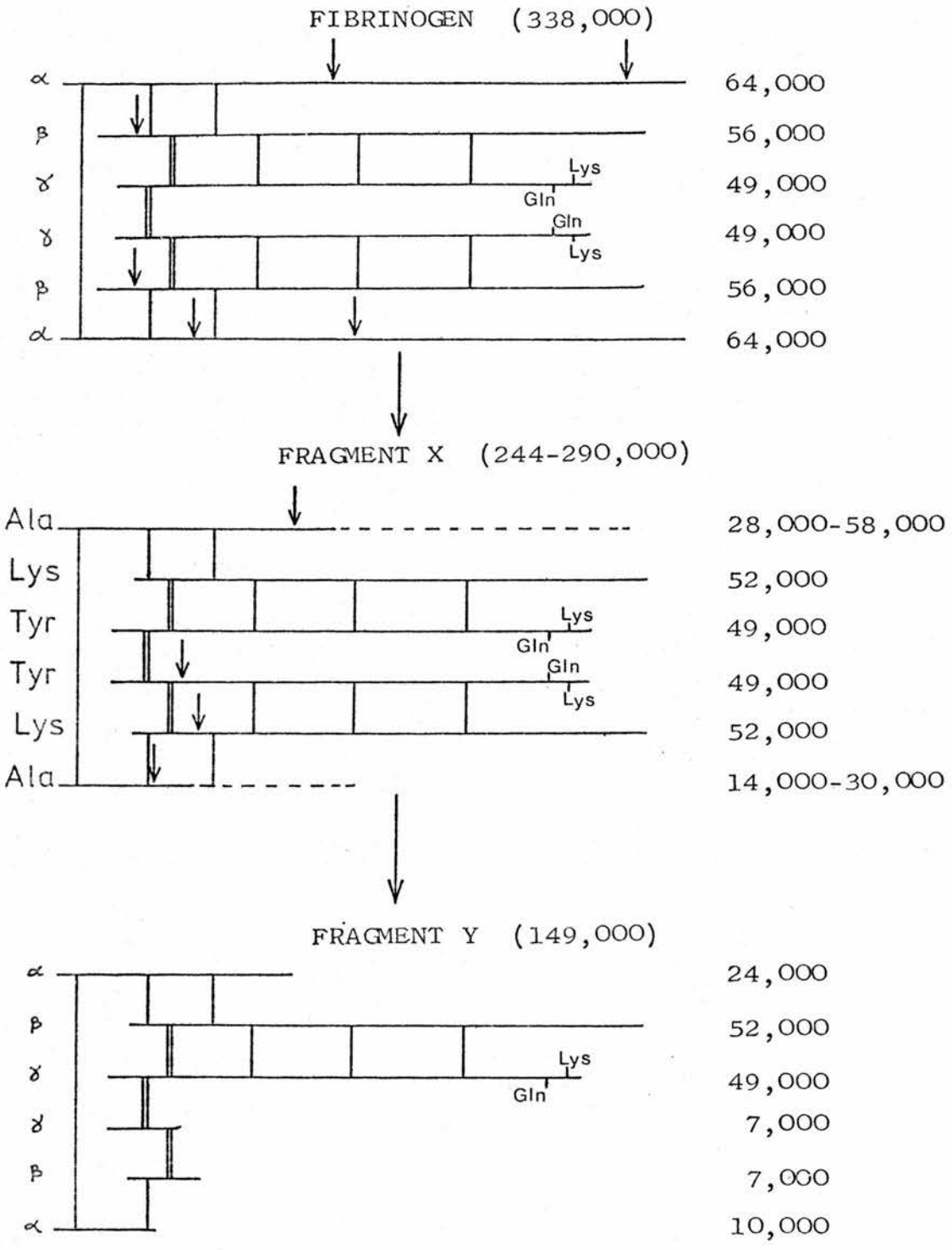


FIG 9A PROPOSED SCHEME OF PLASMIC DEGRADATION OF FIBRINOGEN TO FRAGMENT Y

Vertical lines represent disulphide bonds.
Small arrows show sites of proteolytic cleavage.
Potential F XIII cross-linking sites on the γ-chains (-GLN-LYS) are indicated.

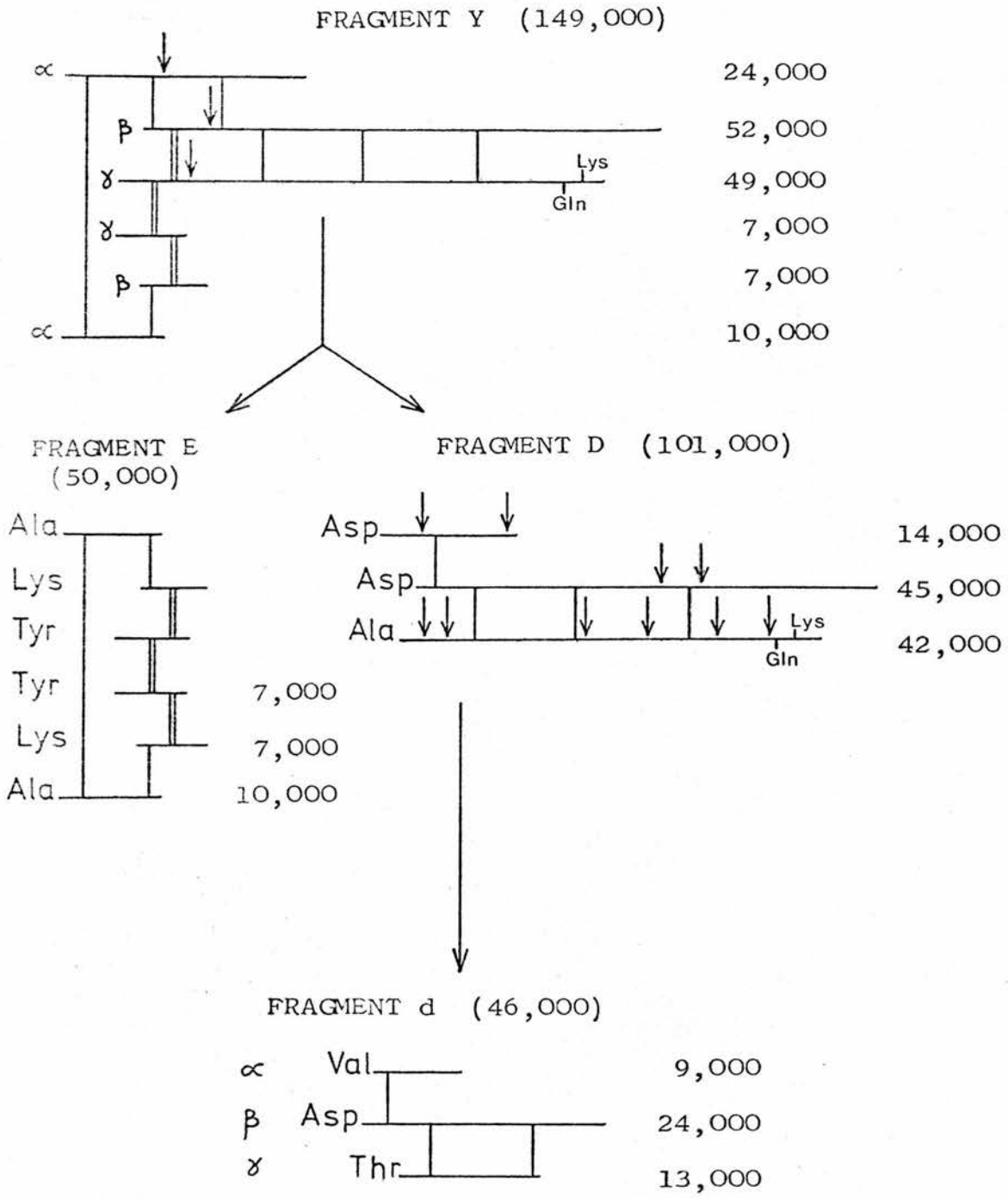


FIG 9B PROPOSED SCHEME OF PLASMIC DEGRADATION OF FRAGMENT Y

Vertical lines represent disulphide bonds.
Small arrows show sites of proteolytic cleavage.
Potential F XIII cross-linking sites on the γ-Chains (-GLN-LYS) are indicated.
(After Furlan et al, 1975b.)

same molecular weights but different patterns of disulphide-reduced chains.

Y fragments are composed of one molecule of fragment E and one molecule of monomeric fragment D, linked together by either one, two or three peptide bonds. It appears that the splitting of fragment Y into fragments D and E starts at the α-polypeptide chain; from then on both β and γ-chains appear to be equally susceptible to plasmin attack.

Fragment D, previously thought to be plasmin-resistant, breaks down further to a 46,000 molecular weight species, termed fragment d, which like fragment E is plasmin-resistant (Furlan et al, 1975a).

Fragments released by plasmin from non-crosslinked fibrin were electrophoretically similar to those found during plasmin-fibrinogen interaction, namely X, Y, D and E (Gaffney, 1973; Pizzo et al, 1973a). The only difference being that fragments X, Y and E from the fibrin digest will not contain fibrinopeptide A, whereas it may still be present in the fragments from fibrinogen, depending on the extent of digestion with plasmin.

The digestion of crosslinked fibrin yields core fragments called D dimer (Gaffney, 1973) and E. Another component has been identified as a complex of D dimer and fragment E, which is dissociable on SDS gel-electrophoresis. The D dimer has been shown to contain the crosslinked γ chain

remnants of the originating fibrin molecules. These crosslinked peptides have been variously reported to have molecular weights from 63,000 (Gaffney and Brasher, 1973) to 81,000 (Pizzo et al, 1973b).

On digestion of fibrin containing covalent inter-molecular γ chain crosslinks, one would expect to see crosslinked, high-molecular-weight fragments (eg X, Y). However, although possibly formed at some stage during the lytic process, they are probably not soluble and thus are not observed during SDS-gel electrophoresis of fibrin lysates. These fragments should indeed be held together in a polymer-like form by the combination of two plasmin-resistant covalent types of bonding, the E-located (N-terminal) disulphide bonds joining the two halves of the fibrinogen dimer together, and the D-located, factor XIII-mediated amide bonds binding the γ chains of adjacent fibrin molecules together. The complete estrangement of these two forms of mutually supportive covalent bonds in the crosslinked fibrin is necessary for the lysis of fibrin, and thus only D dimer and E are observed as major products during the digestion of crosslinked fibrin.

The α chain crosslinks render fibrin resistant to lysis by plasmin (Gaffney and Brasher, 1973). The preferential attack by plasmin on the polypeptide chains of fibrinogen, non-crosslinked fibrin and γ-γ crosslinked fibrin is in the order α, β and γ and the chain preference of plasmin for totally-crosslinked fibrin (containing γ and α crosslinks)

is in the order β or γ and then crosslinked α chains (Gaffney, 1973; Pizzo et al, 1973a; Pizzo et al, 1973b). Indeed, the lysis of the crosslinked α chains of fibrin seem to represent a rate-limiting step during the digestion of fibrin clots.

4 THE NORMAL FIBRINOLYTIC MECHANISMS IN VIVO

There are two principal theories of the mechanism of thrombus dissolution by the fibrinolytic system. Alkjaersig et al (1959) suggested that as fibrin polymerizes, plasminogen is adsorbed preferentially to the fibrin and is available in large quantities within a thrombus which is comparitively free of inhibitors. When an activator enters the circulation it diffuses into the clot converting the plasminogen to plasmin in situ and so promotes lysis. They reported that thrombolysis by exogenous plasmin action appeared to be relatively unimportant. Ambrus and Markus (1960), however, proposed that when plasmin forms in the circulation naturally or during infusion of an activator it is normally bound to the excess inhibitor present in blood. They suggested that this plasmin-inhibitor complex is reversible and dissociates in the presence of fibrin, its preferred substrate, so allowing the plasmin to bring about fibrin dissolution by "external lysis".

A third possible suggestion (Gottlob and Blumel, 1968; Sharp, 1975) is that induced activator in the circulation binds or adsorbs to the fibrin in a thrombus and that circulating plasminogen is converted to plasmin within the thrombus, thus initiating fibrinolysis.

Part of the controversy is due to the conflicting reports on the binding of plasminogen by fibrin. Some groups have

found little or no affinity between the two proteins; Fantl (1962) demonstrated that plasminogen levels are essentially the same in plasma and serum, and that the plasminogen content of thrombi formed in vitro is no greater than the amount contained in entrapped serum. Others have found high affinity between plasminogen and fibrin (Ball et al, 1971; Strachen et al, 1974; Cederholm-Williams, 1977a). Warren et al (1973) reported that the naturally occurring fibrin clot has been shown to contain sufficient plasminogen laid down during the clotting process to ensure its own dissolution on conversion to plasmin. Strachen et al (1974) showed that although substantial amounts of plasminogen are bound in the forming thrombus, this uptake is not total and that additional circulating plasminogen can be taken up without a proportional loss of intrinsic plasminogen.

The concept of activator binding as a mechanism of clot dissolution is not new; Müllertz (1953b) showed the post mortem activator to be strongly bound to fibrin and Dalal et al (1969) were able to elute plasminogen activator from a fresh unorganized native human thrombus. Other activators also reported as having fibrin affinities include the activator produced on exercise (Blix, 1962), porcine tissue activator (Thorsen et al, 1972) and the streptokinase-plasmin(ogen) activator (Lassen, 1958), although Cederholm-Williams (1977b) reported that fibrin binding is interfered with by the continued association of streptokinase with plasmin(ogen). In contrast, however,

urokinase is generally considered not to possess this high fibrin affinity (Blix, 1962; Ball et al, 1971; Thorsen et al, 1972).

As well as the fibrinolytic enzyme system's role in the removal of pathological intravascular and extravascular fibrin, it has been suggested that even under physiological conditions there is a dynamic equilibrium between coagulation, constantly laying down fibrin to seal defects in the endothelium, and fibrinolysis, eliminating such deposits after they have served their haemostatic function (Astrup, 1956). The finding of activator activity in normal blood (Flute, 1960), the presence of small amounts of measurable fibrinogen-fibrin related antigen in normal serum (Merskey et al, 1969), more specific fibrin/fibrinogen degradation products in plasma (Gordon et al, 1973), and the presence of fibrinopeptide A in normal plasma (Nossel et al, 1973) are all in favour of this hypothesis.

Alternatively, it has been suggested that fibrinogen degradation by low grade plasmin activity in vivo is a major pathway for fibrinogen catabolism. Mosesson (1973) claimed conclusive evidence that fibrinogenolysis in rabbits accounts for 25% of the fibrinogen turnover, and suggested no less a role for this pathway in man.

The basis of this suggestion has been attacked by others. Collen et al (1974) pointed out that artefactual fibrinogen

degradation during preparation may have accounted for some of the breakdown ascribed to fibrinogenolysis. Collen et al (1974) found less than 5% of the Aα chains to be degraded in a sample of fibrinogen prepared by affinity chromatography, with no special protective procedures, from fresh frozen plasma. Collen et al also point out that Mosesson has assumed that the coagulation-fibrinolysis balance in humans and in rabbits operates at similar rates; this may not be valid.

Müllertz (1974) employed urokinase-activated human plasma to study the relationship of the activation process to the lysis of fibrin and fibrinogen. At low doses of urokinase degradation of fibrinogen was low or absent, but fibrinolysis occurred within 30 minutes. At higher doses of urokinase fibrinolysis occurred in 10 minutes, whereas fibrinogenolysis progressed between 3 and 10 hours.

Gurewich et al (1974a, 1974b, 1975) working with exercise-induced activator, venous occlusion activator and the activator obtained from cadaver limbs reported that fibrinogen was not degraded in plasma by these activators whereas fibrin was rapidly lysed, they deduced that significant fibrinogenolysis does not occur. They also found that these activators did not induce lysis of soluble fibrin monomer complexes, whereas lysis did occur of protamine sulphate-precipitated fibrinogen and protamine sulphate or ethanol-induced gels of fibrin monomer. They

explained these results by saying a solid phase was necessary to dissociate a hypothesized activator-inhibitor complex.

However, in contrast to these last conclusions McKillop et al (1975) studied in vivo production of soluble fibrin complexes during defibrination by Ancrod infusion. SDS-polyacrylamide gel electrophoresis showed that the component units of the soluble complexes were markedly deficient in intact Aα chain when compared with the uncomplexed material. They suggested that preferential digestion of fibrin may occur while it is still in the soluble form. Konttinen et al (1973) studied streptokinase-activated plasma and found that degradation of soluble fibrin monomer complexes proceeded at a much higher rate than with fibrinogen. They concluded that the fibrinolytic system has an essentially higher affinity to monomers than to fibrinogen, but that the specificity of the monomers as substrate may be limited to the early steps of degradation. Further evidence of the susceptibility of soluble fibrin to degradation was provided by Gaffney (1978) who studied prolonged Ancrod defibrination in man. The monomeric form of fragment D, rather than D dimer, was found in plasma and, since γ-γ crosslinking of fibrin occurs in conjunction with fibrin insolubilization under physiological conditions, this allows the conclusion that plasmin-mediated digestion of soluble fibrin polymers is the likely plasma defibrinating mechanism induced by Ancrod

infusion (Gaffney and Brasher, 1974).

The proteolytic enzyme plasmin has a broad specificity, although in vivo its main target is fibrin. Various hypotheses have been put forward to explain this specificity, but results of subsequent research have often cast doubts on the value of these theories. However, during the past few years specific interactions at the molecular level have been demonstrated between the different components of the fibrinolytic system. These findings enabled Wiman and Collen (1978b) to formulate a molecular model for the regulation of fibrinolysis in vivo. In purified systems it was found that glu-plasminogen, the native form has a weak affinity for fibrin, whereas the partially degraded, lys-plasminogen has a stronger affinity (Thorsen, 1975). It was later demonstrated that this interaction is mediated by way of structures, in the plasminogen molecule, known as lysine-binding sites (Wiman and Wallén, 1977). Rákóczi et al (1978) recently determined that approximately 4% of native and 8% of partially degraded plasminogen was specifically adsorbed to the fibrin, when a whole plasma system was clotted.

Kinetic and structural work both indicate that the plasminogen molecule contains at least two lysine-binding sites (Wiman and Wallén, 1977; Iwamoto, 1975; Sottrup-Jensen et al, 1977). The higher affinity of the partially degraded form of plasminogen for fibrin might be explained by the fact that both its lysine-binding sites are available,

whereas in native plasminogen one of them is occupied by an interaction with a specific site in the N-terminal part of the molecule (Wiman and Wallén, 1975a).

Fibrin markedly enhances the activation of plasminogen by tissue activator (Wallén, 1977). This seems to be due to the adsorption of both plasminogen and activator to the fibrin network. Plasmin added to, or formed in, plasma is very rapidly neutralised by antiplasmin, which is responsible for all of the fast-acting plasmin-neutralising activity of plasma. Antiplasmin and plasmin form a very stable stoichiometric 1:1 complex which is devoid of enzymatic activity (Moroi and Aoki, 1976; Wiman and Collen, 1977). Antiplasmin can also bind to insolubilized plasminogen and can be specifically displaced by EACA, suggesting that this interaction is also mediated by way of the lysine-binding sites in the plasminogen molecule. Accordingly, it has been found in purified systems that fibrin and antiplasmin compete for the binding to plasminogen (Moroi and Aoki, 1977a). In plasma systems, however, the binding of plasminogen to fibrin is not significantly influenced by antiplasmin (Rákóczi et al, 1978).

Plasmin which is bound to fibrin and involved in its degradation, in contrast to free plasmin, is only slowly inactivated by antiplasmin (Wiman and Collen, 1978a). However, once formed, the plasmin-antiplasmin complex does not seem to dissociate in the presence of fibrin (Wiman and

Collen, 1978a).

Thus, a molecular model for physiological fibrinolysis can be deduced, which explains the restricted action of plasmin in vivo. The system seems to be regulated at two levels: localised plasminogen activation at the fibrin surface; and sequestration of the formed plasmin from circulating antiplasmin. The lysine-binding sites in the plasminogen molecule play a key role in these regulations. When fibrin is formed, a small amount of plasminogen is specifically bound to it by way of its lysine-binding site(s). Plasminogen activator present in the blood or released from the vascular endothelium is adsorbed on the fibrin surface and efficiently activates the adsorbed plasminogen. The formed plasmin has its lysine-binding site(s) occupied in complex formation with fibrin and is also involved in fibrin degradation by way of its active site, and would therefore only react very slowly with antiplasmin. In contrast, plasmin which is released from digested fibrin is rapidly and irreversibly neutralised by antiplasmin in the circulation.

It can be seen from this model of physiological fibrinolysis that the potential causes of a defective fibrinolytic system may prove to be multitudinous, and thus therapeutic approaches could be equally wide ranging. Present knowledge of the biochemistry and physiology of the fibrinolytic system does suggest that we are beginning to unveil a system of immense importance, the functions of which may

ultimately prove to be more far-reaching than could possibly have been envisaged.

Plasminogen, being a precursor of humoral origin is ubiquitously available for activation in the organism, and thus fibrinolysis becomes involved in many physiological and pathological processes. The fibrinolytic system is of fundamental importance in maintaining the patency of the vascular system. Furthermore it appears to play a role in neoplasia, tissue repair and certain types of inflammation. If this is so, then the effects of defective fibrinolysis may also prove to be multitudinous. Thus the importance and the potential reward for advances in this area become self-evident.

Page

CHAPTER 2

4/

1 PERFUSION OF CADAVERS

A METHODOLOGY

The method of perfusion developed by Aoki and von Kaulla (1971a) was, with minor modifications, used initially. The leg is incised, parallel to, and about 1 centimetre above, the inguinal ligament, from the pubis to the iliac crest of the ilium. A further incision is then made from the midpoint of the first, to a point about 15 centimetres down the leg, over Hunters canal and taking care to avoid the saphenous vein. The minor venous branches are tied off, and the femoral vein and artery dissected out. The femoral artery is then cannulated with the tip of the cannula above the profunda artery and a ligature proximal to the cannula. The femoral vein is also cannulated and ligatured in a similar position.

The perfusion fluid is run into the femoral artery from a height of four feet and the outflow is collected from the femoral vein. In both cases a modified administration set is used, with a bore of approximately 5mm. The leg is massaged as the vascular system is perfused.

In the original method the perfusate was not collected until the fluid was nearly colourless. However, when sequential aliquots of perfusion fluid were tested for

activity and protein content (see Fig 10) it was found that the initial vascular perfusates, although of low specific activity, contained the greatest amount of plasminogen activator activity and thus it was decided to collect the total perfusate.

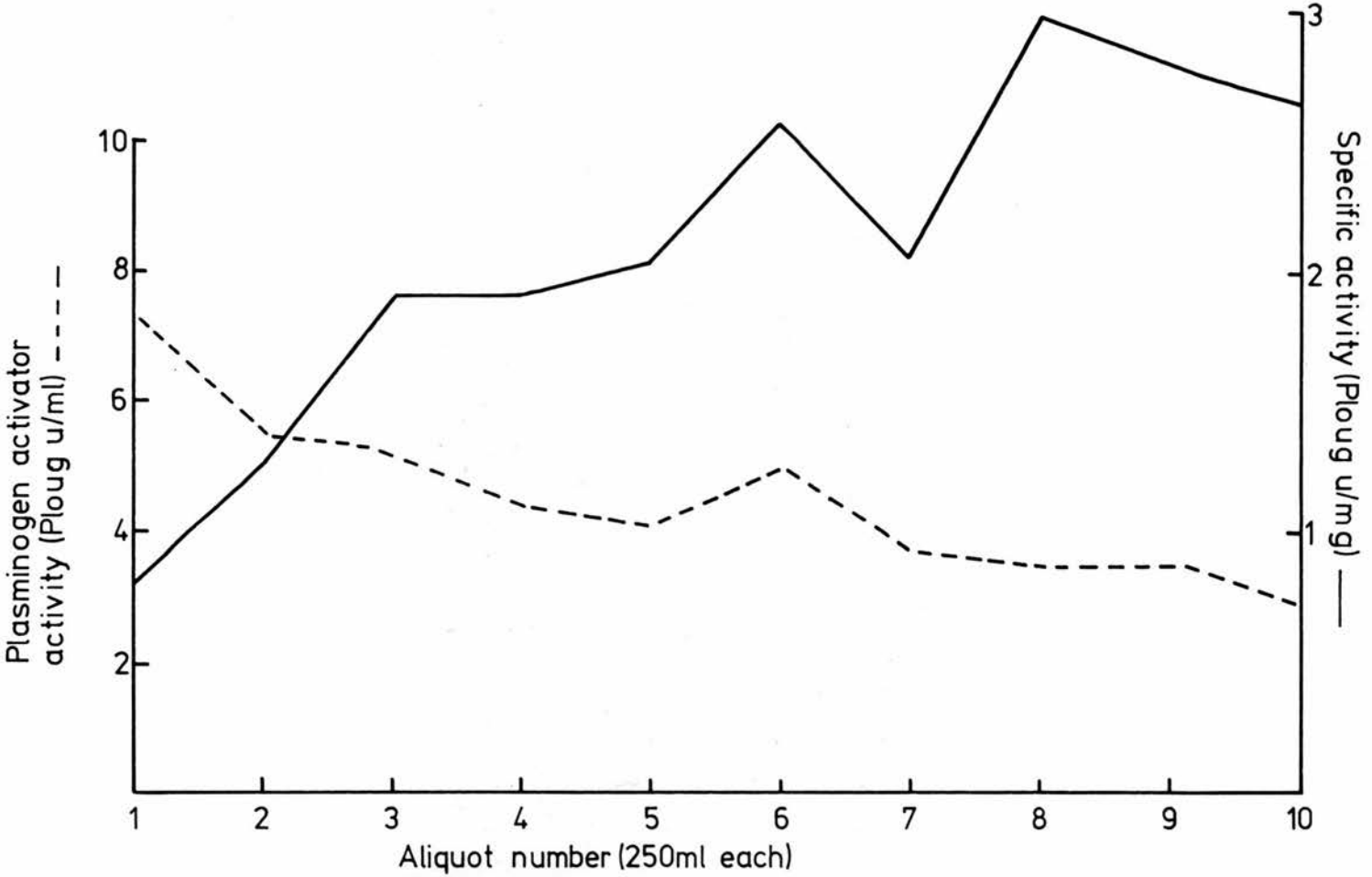


FIG 10 GRAPH SHOWING THE ACTIVATOR ACTIVITY AND SPECIFIC ACTIVITY EXTRACTED IN SEQUENTIAL ALIQUOTS OF A CADAVER PERFUSION

B DETAILS OF CADAVERS AND RESPECTIVE PERFUSATES

The lower limbs of nearly 80 cadavers have been perfused during the course of this work. The protein and activator content of the perfusates, as well as age, sex and cause of death of the first 60 cadavers are given in Table 1. Trasylol, a plasmin inhibitor, was incorporated into the perfusion fluid used with the remaining cadavers, thus excluding them from activator activity, and thus specific activity, determinations.

CADAVER NO	SEX	AGE	HOURS PM	PRIMARY CAUSE OF DEATH	PROTEIN CONC mg/ml	TOTAL PROTEIN g	ACTIVATOR ACTIVITY u/ml	TOTAL ACTIVATOR ACTIVITY u	SPECIFIC ACTIVITY u/mg
B1	M	50	62	Subarachnoid Haemorrhage	0.4	1.4	10	40,500	28.9
B2	M	59	35	Myocardial Infarction	0.6	1.3	2.1	5,000	3.9
B3	F	73	43	Sub-Dural Haemorrhage	0.3	1.6	1.0	5,000	3.1
B4	F	49	30	Chronic Renal Failure	0.3	1.1	1.8	7,830	7.1
B5	M	65	28	Chronic Renal Failure	1.6	2.9	7.1	12,972	4.5
*B6	F	71	34	Pulmonary Embolism (+DVT)	0.2	0.2	2.8	2,610	13.1
B7	F	65	44	Breast Carcinoma	0.3	0.7	12.4	32,100	45.9
B8	M	46	44	Cardiac Arrest	0.6	3.0	2.0	10,835	3.6
B9	F	53	30	Breast Carcinoma	0.3	0.5	2.1	3,100	6.2
B10	M	57	30	Myocardial Infarction	0.9	2.2	14.4	36,000	16.4
B11	F	51	30	Cardiac Failure; Chronic Renal Congestion	1.1	1.4	4.4	5,460	3.9

TABLE 1 DETAILS OF CADAVERS AND PERFUSATES

CADAVER NO	SEX	AGE	HOURS PM	PRIMARY CAUSE OF DEATH	PROTEIN CONC mg/ml	TOTAL PROTEIN g	ACTIVATOR ACTIVITY u/ml	TOTAL ACTIVATOR ACTIVITY u	SPECIFIC ACTIVITY u/mg
B12	M	60	45	L Ventricular Hypertrophy; Intestinal Obstruction	1.7	3.2	6.5	12,350	3.9
B13	F	55	46	Metastatic Carcinoma of the Lung	1.6	1.6	9.0	8,820	5.5
B14	F	75	56	Pulmonary Embolism	1.8	4.9	11.2	31,360	6.4
B15	M	82	37	Respiratory and Cardiac Failure	2.4	17.4	4.1	30,258	1.7
B16	M	78	30	Myocardial Infarction	ND	ND	ND	ND	ND
B17	M	63	34	Carcinoma of Bladder	3.1	4.9	6.4	10,240	2.1
B18	M	76	35	Myocardial Infarction; Lung Carcinoma	2.2	8.6	4.3	16,856	2.0
B19	M	67	39	Lung Carcinoma; Gastric Ulcer; Peritonitis	ND	ND	ND	ND	ND
B20	M	62	48	Cardiac Arrest Whilst Undergoing Coronary Bypass Surgery	23.7	19.2	12.0	10,560	0.6

TABLE 1 (Cont) DETAILS OF CADAVERS AND PERFUSATES

CADAVER NO	SEX	AGE	HOURS PM	PRIMARY CAUSE OF DEATH	PROTEIN CONC mg/ml	TOTAL PROTEIN g	ACTIVATOR ACTIVITY u/ml	TOTAL ACTIVATOR ACTIVITY u	SPECIFIC ACTIVITY u/mg
B21	F	67	48	Cardiac Valve Disease	12.4	3.7	4.8	1,440	0.4
B22	F	85	48	Bronchopneumonia; Renal Carcinoma	7.6	5.3	7.7	5,390	1.0
B23	M	72	50	Lung Carcinoma	9.2	9.7	15.0	15,750	1.6
B24	F	57	49	Lung Carcinoma	12.7	20.1	15.9	25,154	1.3
B25	M	73	37	Septicaemia	18.4	4.4	0	0	0
B26	M	66	30	Bronchogenic Carcinoma	25.0	6.0	57.0	13,680	2.3
B27	F	81	30	Myocardial Infarction; Pulmonary Embolism	1.8	5.4	13.5	40,635	7.5
B28	F	74	57	Rheumatoid Arthritis; Perforated Bowel	1.5	2.0	28.0	36,960	18.5
B29	F	72	30	Cerebral Haemorrhage	1.9	6.7	4.4	15,400	2.3
B30	M	73	28	Carcinoma of Bladder	2.8	6.8	5.4	13,000	1.9
B31	F	53	16	Cerebral Haemorrhage	7.4	8.8	5.1	6,069	0.7
B32	F	70	60	Renal Failure	9.8	26.5	4.1	11,070	0.4

TABLE 1 (Cont) DETAILS OF CADAVERS AND PERFUSATES

CADAVER NO	SEX	AGE	HOURS PM	PRIMARY CAUSE OF DEATH	PROTEIN CONC mg/ml	TOTAL PROTEIN g	ACTIVATOR ACTIVITY u/ml	TOTAL ACTIVATOR ACTIVITY u	SPECIFIC ACTIVITY u/mg
B33	M	66	42	Myocardial Infarction	12.8	10.9	17.5	14,875	1.4
B34	M	70	36	Metastatic Carcinoma of the Lung	10.2	14.7	1.6	2,304	0.2
B35	M	72	48	Myocardial Infarction; Heart Failure	9.3	8.7	7.6	7,068	0.8
B36	M	74	48	Bronchopneumonia	7.0	12.5	2.5	4,450	0.4
B37	F	32	24	Chronic Myeloid; Leukaemia	14.4	9.2	6.6	4,191	0.5
B38	M	79	30	Pulmonary Embolism	2.5	7.1	8.2	23,329	3.3
B39	F	78	48	Obstructive Jaundice due to Carcinoma of Colon	5.6	14.9	2.4	6,372	0.4
B40	M	59	26	Pulmonary Embolism and Heart Failure	3.8	12.5	1.1	3,606	0.3
B41	F	72	31	Myocardial Infarction	9.3	11.5	8.1	10,040	0.9
B42	M	49	48	Heart Failure	8.4	25.2	4.8	14,400	0.6
B43	M	81	21	Pneumothorax; Carcinoma Bronchus	5.4	21.1	6.6	25,740	1.2

TABLE 1 (Cont) DETAILS OF CADAVERS AND PERFUSATES

CADAVER NO	SEX	AGE	HOURS PM	PRIMARY CAUSE OF DEATH	PROTEIN CONC mg/ml	TOTAL PROTEIN g	ACTIVATOR ACTIVITY u/ml	TOTAL ACTIVATOR ACTIVITY u	SPECIFIC ACTIVITY u/mg
B44	M	22	4	Astrocytoma	13.9	18.0	7.1	9,230	0.5
+B45	M	71	63	Bronchopneumonia	24.7	29.8	41.0	49,405	1.7
B46	F	53	31	Astrocytoma; Bronchopneumonia	6.0	6.6	11.5	12,650	1.9
B47	F	76	43	Myocardial Infarction	4.9	8.8	13.0	23,400	2.7
B48	M	57	10	Carcinoma of Bronchus	7.4	27.8	6.8	25,500	0.9
B49	F	51	41	Carcinoma of Bronchus	11.4	12.5	7.9	8,690	0.7
B50	F	70	20.5	Carcinoma of Colon; Cirrhosis of Liver	3.6	9.9	3.9	10,725	1.1
B51	M	52	27	Myocardial Infarction	15.0	8.9	9.2	5,428	0.6
B52	M	45	2	Lung Carcinoma; Pleurisy	15.6	15.6	7.5	7,500	0.5
B53	F	62	24	Carcinoma of Oesophagus	17.2	21.0	9.2	11,224	0.5
B54	M	34	25.5	Aortic Stenosis	11.4	8.6	25.0	18,750	2.2
B55	F	71	24	Bronchopneumonia; Carcinoma of Thyroid Gland	12.8	18.6	38.5	55,825	3.0

TABLE 1 (Cont) DETAILS OF CADAVERS AND PERFUSATES

CADAVER NO	SEX	AGE	HOURS PM	PRIMARY CAUSE OF DEATH	PROTEIN CONC mg/ml	TOTAL PROTEIN g	ACTIVATOR ACTIVITY u/ml	TOTAL ACTIVATOR ACTIVITY u	SPECIFIC ACTIVITY u/mg
B56	F	62	48	Bronchopneumonia; Carcinoma of Gastric-Oesoph-ageal Junction	9.3	7.8	19.0	15,960	2.1
B57	M	65	20	Carcinoma of Rectum; Acute Myocardial Infarction; Bronchopneumonia	17.2	10.5	23.0	14,030	1.3
B58	F	60	24	Carcinoma of Bronchus	11.0	11.0	15.5	15,500	1.4
B59	M	75	24	Myocardial Infarction	12.1	24.2	12.5	25,000	1.0
B60	M	56	7	Carcinoma of Bronchus	14.0	49.0	11.0	38,500	0.8

* Upper Limb Perfusion

\+ Contaminated With Plasmin (Not Included In Averages)

ND Not Determined

TABLE 1 (Cont) DETAILS OF CADAVERS AND PERFUSATES

C PERFUSION FLUIDS TESTED

Various perfusion fluids have been tested during the course of this work. They are described below, along with any disadvantages encountered in their use.

a Saline

0.9%($^{w}/_{v}$) saline was used from viaflex containers, which was very convenient. The saline, as with all the perfusion fluids, was used at 37°C. This was to provide a thermal "shock" in an attempt to release plasminogen activator, from the endothelial cells coating the inside of the vasculature. The saline worked well; it is of physiological strength and thus osmotically compatible, so no flow problems were encountered, except those caused by thrombus blocking the vessels, which was minimised by rejecting cadavers with a previous history of thrombotic problems.

However, a serious disadvantage in using saline was that in order to apply the perfusate to some ion-exchange or affinity chromatography columns, dialysis was first required to lower the sodium chloride concentration or an ammonium sulphate precipitation followed by resuspension in a buffer of lower ionic strength. These processes are time consuming, which is not acceptable when dealing with a labile molecule, and losses are unavoidable when

using dialysis membranes.

b Distilled Water

Use of distilled water overcame the ionic strength problem, so that perfusates could be applied, after centrifugation, directly to ion exchange or affinity chromatography columns. However, flow problems were encountered with distilled water that had not been present with saline. These problems were probably caused, at least in part, by the endothelial cells swelling up, due to water entering them, causing blockage of the smaller vessels. Also, the distilled water caused lysis of the red blood cells, effectively giving a perfusate of lower specific activity.

c 5% Dextrose

This was the best fluid employed. It can be applied directly to chromatographic columns, with no prior treatment, and also no flow problems were encountered during its use.

Although initially the 5% dextrose was used alone, subsequently Triton X-100, a mild detergent, was added in an attempt to disrupt the endothelial cells. Various concentrations of Triton X-100 (0.1%, 0.2% and 0.4%) were investigated and 0.1% gave the best activator yields and

flow properties.

Various other additives were tested during the course of the work. Fibrin formation after perfusion occasionally caused problems, especially when very "fresh" cadavers (<12 hours post mortem) were perfused; so heparin (1u/ml and 10u/ml) and EDTA (10mM) were added to the perfusion fluid in an attempt to prevent fibrin formation, and to abolish crosslinking of any fibrin formed, respectively. The heparin did not prove very successful; it was found more effective to avoid cadavers less than about 12 hours post mortem. However, the EDTA was retained and it has recently been reported as a stabilizing agent for activator (Radcliffe and Heinze, 1978).

Trasylol, a plasmin inhibitor, was latterly added to the perfusion fluid for two reasons. Firstly, to avoid the degradation of the soluble fibrin polymers, believed to be the species that the vascular activator is bound to when obtained from the cadaver. The complex with soluble fibrin polymers is desirable in that it confers a measure of stability on the activator. Secondly, the Trasylol also inhibits plasmin from degrading the activator itself.

Thus the final perfusion fluid that was employed was 5% dextrose-0.1% Triton X-100-10mM EDTA-10u/ml Trasylol; this was used at 37^{o}C.

D DISCUSSION

Table 2 shows the wide range of protein and activator activity extracted from the cadavers and the resultant range of specific activities obtained as a crude starting material for purification. The plasminogen activator extracted varied from nothing, in one cadaver, up to 55,825 Ploug units, the average extracted being 15,451 Ploug units per cadaver. With such a wide range of values it was hoped that it would have been possible to predict "good" cadavers in terms of yield and then select only the ones that were likely to give high activator levels. However, by referring to Tables 3, 4 and 5 it can be seen that this was not possible.

Table 3 shows the average activator levels extracted from cadavers of varying lengths of time after death. There seems to be a general trend that the longer the time elapsed after death, the less the activator obtained. However, the highest average activator extracted was obtained from the group of over 49 hours post mortem.

Table 4 shows the average activator levels extracted from cadavers of various age groups. It would appear from the data that the age of the cadaver makes no difference to the activator yield, except that again the oldest group, 81-90 years, yielded the highest amount of activator. However, statistics on only 4 cadavers in this age group may well have distorted the figures.

	Protein Concentration (mg/ml)	Total Protein (g)	Activator Activity (u/ml)	Total Activator (u)	Specific Activity (u/mg)
Range	0.2 - 25	0.2 - 49	0 - 57	0 - 55,825	0 - 45.9
Average	7.5	10.4	9.9	15,451	4.0

TABLE 2 RANGE AND AVERAGE VALUES OF PROTEIN AND PLASMINOGEN ACTIVATOR EXTRACTED FROM CADAVERS AND THEIR SPECIFIC ACTIVITES

Hours Post Mortem	Average Activity Extracted	No of Cadavers
0 - 12	20,183	4
13 - 24	18,700	9
25 - 36	13,489	19
37 - 48	11,776	18
49	32,409	6

TABLE 3 AVERAGE PLASMINOGEN ACTIVATOR ACTIVITY EXTRACTED FROM CADAVERS OF VARIOUS HOURS POST MORTEM

Age Group	Average Activity Extracted	No of Cadavers
40	10,724	3
41 - 50	16,213	5
51 - 60	14,122	15
61 - 70	12,399	13
71 - 80	18,113	16
81 - 90	25,506	4

TABLE 4 AVERAGE PLASMINOGEN ACTIVATOR ACTIVITIES EXTRACTED FROM CADAVERS OF VARIOUS AGE GROUPS

Cause of Death	Average Activity Extracted	No of Cadavers
Brain Haemorrhage	16,742	4
Cancer	14,766	15
Renal Failure	10,624	3
Myocardial Infarction Pulmonary Embolism	16,828	11
Septicaemia	0	1

TABLE 5 AVERAGE PLASMINOGEN ACTIVATOR ACTIVITIES EXTRACTED FROM CADAVERS WITH DIFFERENT CAUSES OF DEATH

Table 5 shows the average activator levels obtained from cadavers with different causes of death. Again as can be seen no distinct pattern emerges from the cadavers studied. Only one cadaver failed to yield any activator at all; the cause of death in this case was septicaemia. It is unknown whether any real significance can be attached to this result as only one cadaver with this cause of death was studied.

Somewhat surprisingly, the highest yield of activator was obtained from cadavers with myocardial infarction and pulmonary embolism as their cause of death, both of which are generally associated with a tendancy to thrombosis. It has been demonstrated that this is related to low levels of the vascular activator, thus it was a little surprising to find high yields from these cadavers. This could be explained by assuming that a faulty release mechanism, rather than a low level of production, causes the low levels of activator, such that perfusion of the vascular system would still be able to harvest the physiologically unavailable activator; alternatively it could be that these patients had, before death, been on fibrinolytic therapy.

Other factors that could explain the wide variation in yields and the absence of any predictable pattern could include the type of death endured by the patient. It is well known that in cases of sudden or violent death high fibrinolytic activities are found in the blood, thus if the activator were largely released into the blood on

death, the hours elapsing between death and perfusion of the vascular system would provide ample time for the degradation of the activator in an environment laden with proteases. Whereas in cases of slow or quiet deaths, the activator may remain in the relative safety of the endothelial cell until extracted, although in some cases a lingering death appears to result in the exhaustion of the endothelial cell.

Another uncontrollable variable between cadavers is the volume of the perfusate obtained. Thus it could be that not all the activator is extracted in some situations in which the vascular system is blocked with thrombus, prematurely terminating the perfusion.

But probably the biggest factor in affecting the yield obtained is insoluble fibrin, either preformed or formed during or after perfusion. Apart from causing flow problems during the perfusion, fibrin avidly adsorbs vascular activator and reduces the yield.

2 INITIAL PURIFICATION PROCEDURE

A TREATMENT OF CRUDE PERFUSATE

The crude perfusate is centrifuged as a first step, to remove red blood cells and other debris, including any thrombus present. The perfusate is centrifuged at 10,000g and 4°C for 30 minutes, although neither the speed or time are critical.

Initially, the ammonium sulphate precipitation step of Aoki and von Kaulla (1971a) was used, as the saline perfusate could not be applied direct to certain ion-exchange or affinity chromatography columns. Solid ammonium sulphate was slowly added, with mechanical stirring, to a saturation of 45% (277g/litre). This was then left stirring overnight at 4°C to precipitate.

The precipitate, after being removed by centrifugation, was resuspended in a minimum amount of buffer, initially containing sodium chloride, which was subsequently reduced in concentration, in two stages, by dialysis. The gradual reduction in sodium chloride concentration was designed to overcome solubility problems; however, this was very time consuming and was associated with large losses of activity.

The ammonium sulphate step gave variable results, probably again due to solubility problems. The activator activity retained in the resuspended precipitate generally varied

from 60-80%, although sometimes less than this was recovered, and approximately 30% of the protein reappeared in the resuspended precipitate, representing a two-fold purification. With 25-30% of the total protein reproducibly appearing in the resuspended precipitate, even if 100% of the activity was retained in this portion, this would still only represent a 3 to 4-fold purification.

In an attempt to overcome solubility problems and activator losses, the ammonium sulphate precipitation step was pH controlled. The pH after adding the ammonium sulphate to the perfusate, in the region of pH5.0-5.5, was adjusted to pH7.5. However, this did not have the desired effect and the ammonium sulphate step was discontinued when the perfusion fluid was changed to distilled water and then 5% dextrose. With both of these fluids it is possible to apply the perfusates, after centrifugation, directly to ion-exchange or affinity chromatography columns.

B PURIFICATION PROCEDURE (AND COMPARATIVE BEHAVIOUR OF OTHER FIBRINOLYTIC COMPONENTS)

Various chromatography media were tested before a satisfactory combination was evolved for the purification of the activator. DEAE-cellulose, later available as DEAE-agarose, was investigated initially. The activator had a low affinity for this gel, eluting at approximately 0.1M sodium chloride. This resulted in very little purification as all the bound protein eluted early in the sodium chloride gradient. In one run the pH of the buffers was increased from pH7.4 to 8.5 in an attempt to improve binding, but this was unsuccessful.

CM-cellulose and heparin-Sepharose were tried, although neither gave good results. The activator did not separate from bound protein using heparin-Sepharose, while no activity was recovered from the CM-cellulose column, possibly due to the pH (6) of the acetate buffers that the column was eluted with. The behaviour of the activator was also investigated on various gel filtration media including Ultrogel AcA 22, Ultrogel AcA 34, Biogel A5M and Sepharose 4B.

The initial purification procedure developed is shown in Figures 11, 12 and 13. The crude perfusate after centrifugation was adsorbed directly with lysine-Sepharose gel while stirring for 1 hour at $4^{o}C$. The gel was then removed and washed on a Buchner funnel with 0.025M tris buffer

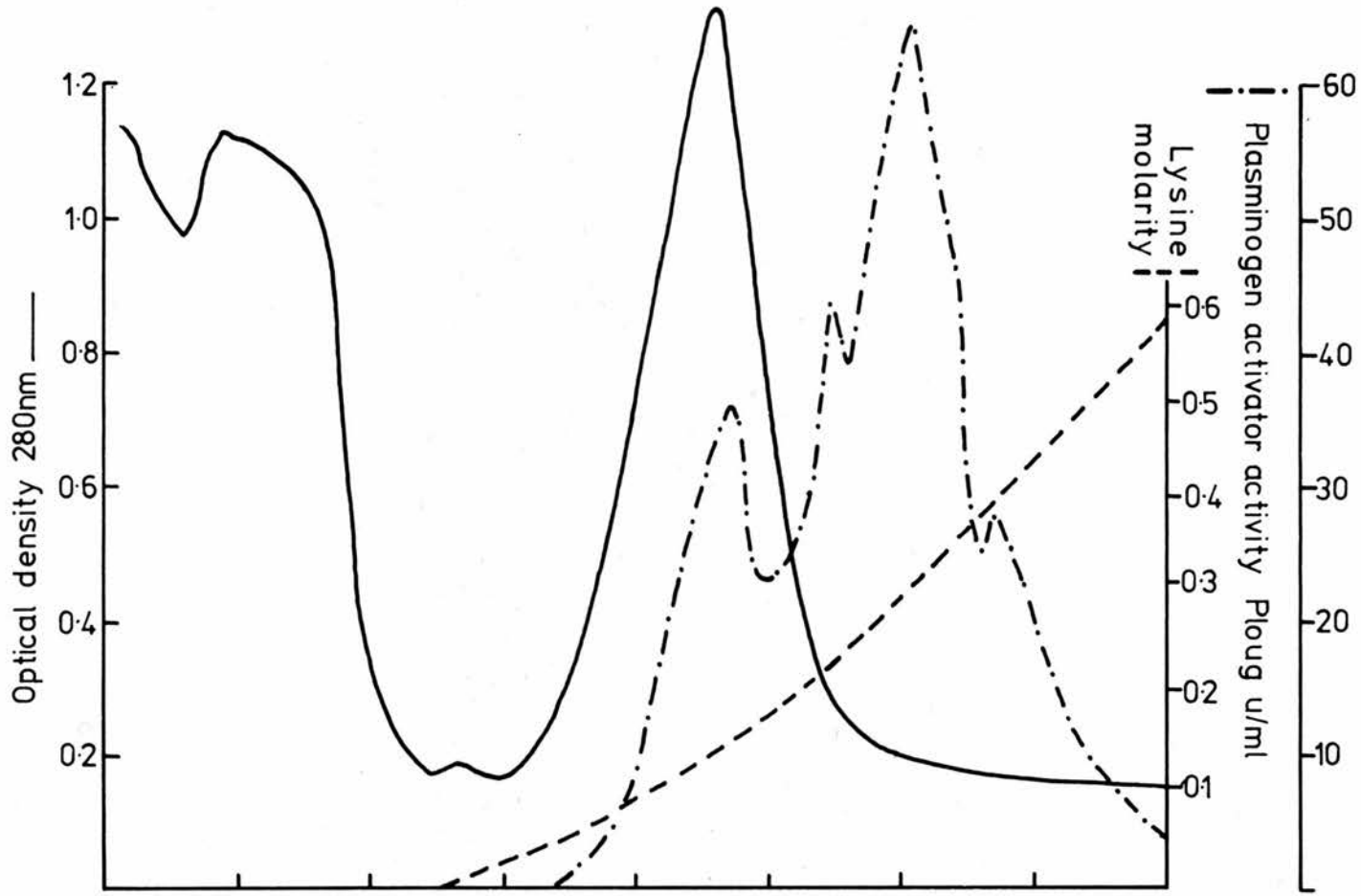


FIG 11 LYSINE-SEPHAROSE

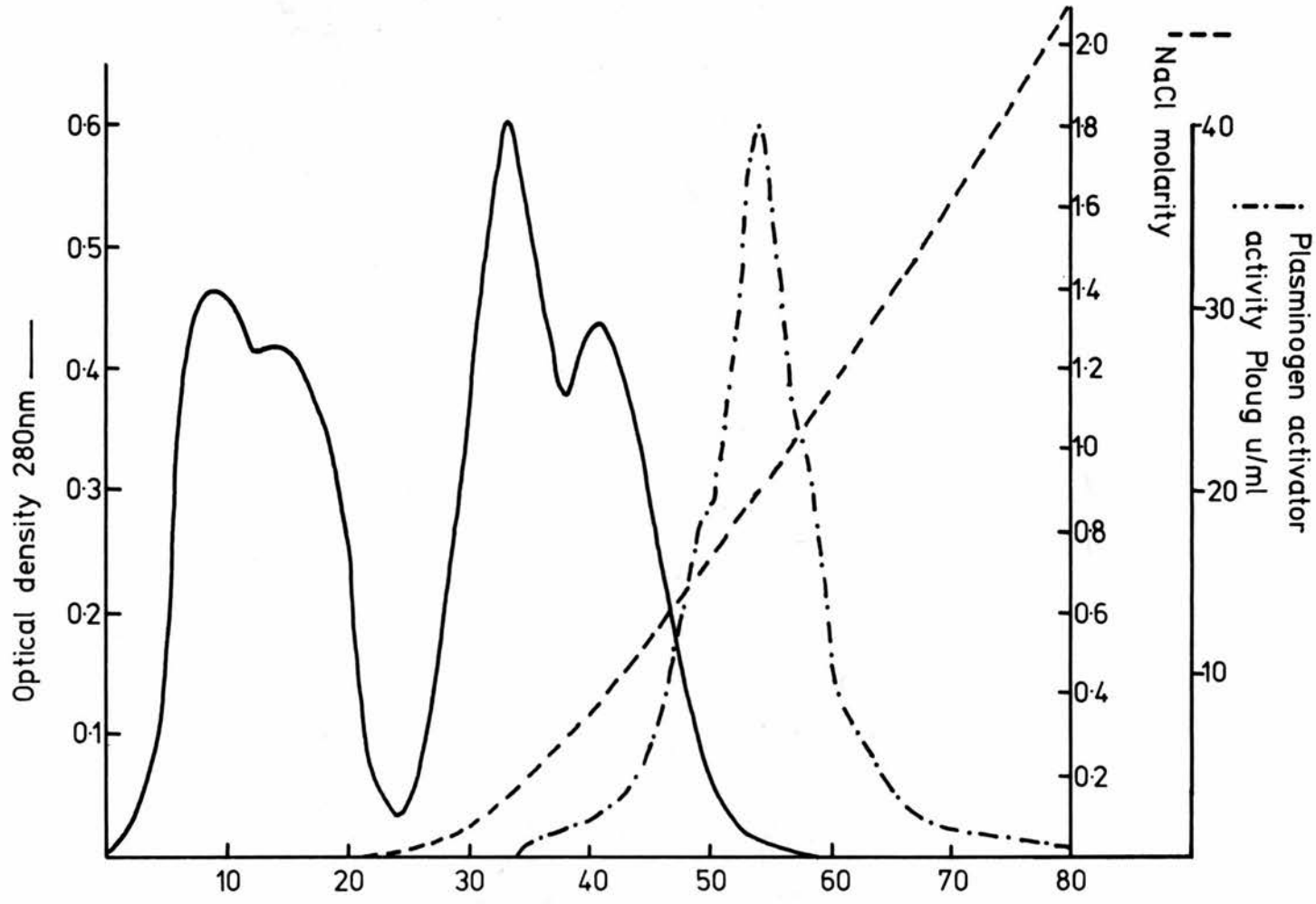


FIG 12 PROTAMINE-SEPHAROSE

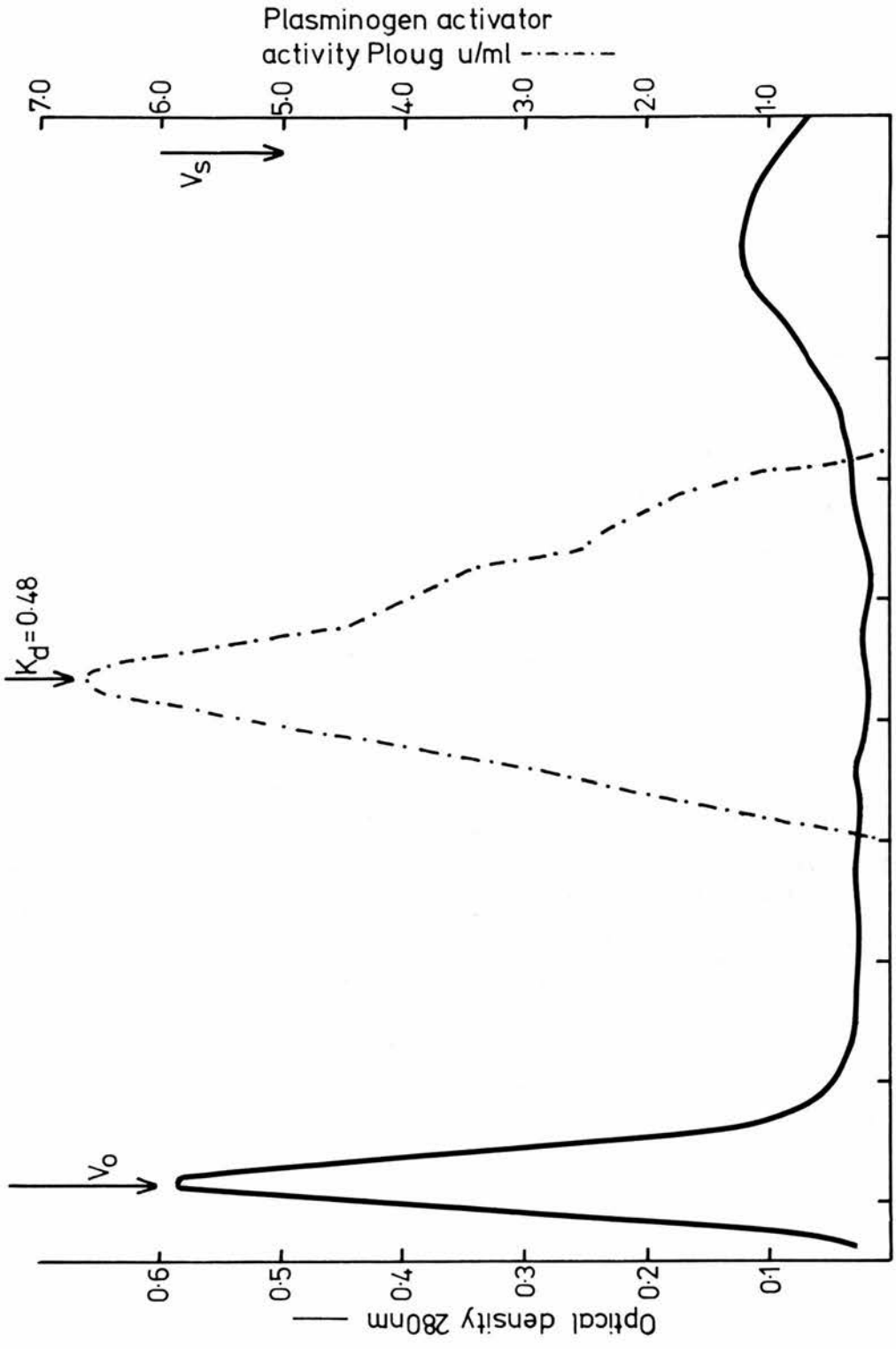


FIG 13 SEPHADEX G-200

(pH7.5). The wet gel was repacked into a column (2.5 x 15cm) and eluted at 4°C with an exponential gradient of 0-0.5M lysine in 0.025M tris (pH7.5) at 30ml/hour. 7.5ml fractions were collected and were assayed for protein in a spectrophotometer at 280nm, the lysine concentration was determined in every fifth fraction by measuring the refractive index in an Abbé refractometer, and the fractions were also screened on normal (containing plasminogen) fibrin plates to assay for plasminogen activator activity and on plasminogen-free fibrin plates to screen for non-specific or direct (due to plasmin) proteolysis. The activator activity was expressed in Ploug units after calibrating the plates with Leo urokinase. The activator reproducibly eluted in two peaks at 0.15M lysine (range 0.11-0.17M) and 0.32M lysine (range 0.28-0.34M).

The active fractions were pooled and concentrated to 5ml using an Amicon ultrafiltration system; this was diluted with 0.025M tris buffer (pH7.5) and again concentrated to 5ml to reduce the lysine concentration of the sample. The concentrated fraction was applied to a protamine-Sepharose column (2.5 x 25cm) and eluted at 4°C with an exponential salt gradient from 0-2.0M NaCl (see Fig 12) in 0.025M tris (pH7.5) at 30ml/hour. The fractions were again screened at 280nm and on normal and plasminogen-free fibrin plates; the NaCl concentration was determined by conductivity measurements.

The activator eluted from protamine-Sepharose at 0.9M NaCl

(range 0.75-1.04M). The active peak was pooled, concentrated to 5ml and applied to a Sephadex G-200 column (2.5 x 100cm) and eluted at 4°C with 0.025M tris pH7.5 containing 1.5M NaCl (see Fig 13). Void volume and solvent volume (V_o and V_s respectively) were determined by adding blue dextran and glucose to the sample before chromatography. The activator activity eluted at a K_d of 0.48, and was not associated with any detectable peak of absorbance when measured at 280 or 220nm.

For comparative purposes the plasminogen activator appearing in the blood after venous stasis was purified according to the same scheme outlined above. 130mls of blood were collected from two arms, of the same subject, after 15 minutes of venous occlusion. The blood was collected into citrate and after centrifugation the plasma was purified on lysine-Sepharose, protamine-Sepharose and Sephadex G-200.

Only one peak appeared on lysine-Sepharose, eluting at 0.38M lysine with a peak activator activity of 6 Ploug units/ml. The activator eluted from protamine-Sepharose at 1.1M NaCl with a peak activity of 25 units/ml, and eluted from Sephadex G-200 with a K_d of 0.32 and a peak activity of 3 units/ml.

The binding of plasminogen activator, plasminogen and plasmin to the same batch of lysine-Sepharose was compared. The plasminogen activator eluted at 0.30M lysine, plasmin

(Kabi) eluted at 0.24M lysine and plasminogen (Kabi) eluted at 0.22M lysine.

C DISCUSSION

The vascular perfusate is most easily applied to lysine-Sepharose by bulk adsorption, as very often large volumes are involved. Both 1 hour and overnight adsorptions were investigated and the shorter adsorption time proved adequate.

As already stated the activator eluted reproducibly in two peaks from lysine-Sepharose; one peak associated with the bound protein and one, with a higher affinity, associated with very little protein. Various theories have been considered to explain the elution of the activator at two distinct lysine molarities. Firstly, as lysine has two amino groups it is conceivable that when it is linked to the Sepharose, two sites of different affinity for the activator, may be produced. However, this is unlikely as the pK values are different for the two amino groups (pK for α-amino group = 9.2, and for ϵ-amino group = 10.7) and thus, at the pH (9) used for the reaction, lysine should be coupled mainly through the α-amino group. Another possibility considered was that the lower affinity binding was due to the cyanogen bromide linkage acting as an ion exchanger. However, this was disproved by preparing a lysine-Sepharose column using an adipic acid dihydrazide (ADH) linkage, which still gave two peaks of activator. A more feasible explanation could be that the lower affinity activator represents a partially degraded molecule.

However, the most probable explanation is that the two peaks represent complexed and free vascular activator. The lower affinity material would represent activator in a complex with some sort of carrier, later believed to be soluble fibrin polymers (see Section 3 of this Chapter and Chapter 4), and the higher affinity material would represent free activator. Both of these peaks when run separately on Sephadex G-200 in 0.025M tris buffer (pH7.5) elute in the V_o position, indicating a larger molecular weight than the native molecule. This elution position was expected for the low affinity peak, hypothesised to be an activator complex, but was initially surprising for the higher affinity material. However, it is quite likely that sufficient carrier molecules from the first peak tail into the second peak, to allow recombination of the activator and carrier on removal of lysine (the binding of the activator to fibrin is believed to be mediated through lysine binding sites). In fact a preparation of soluble fibrin polymers (SFP's) was later found to bind to lysine-Sepharose (see Chapter 4) and elute at a lysine molarity similar to the low-affinity peak of activator, and sufficient tailing of the SFP's was observed on elution to account for the above explanation.

More supportive evidence for this theory was obtained when the low-affinity peak alone was pooled, concentrated and the lysine concentration lowered sufficiently to allow the sample to be re-applied to the lysine-Sepharose column. On elution, two peaks were again observed. This would

suggest that on applying a sample of activator containing soluble fibrin to a lysine-Sepharose column, there is competition between the soluble fibrin and the insolubilised lysine for the lysine binding site of the activator. A state of equilibrium is reached whereby some of the activator is bound via the SFP's and some is bound directly to the lysine-Sepharose.

However, whether or not this latter explanation is substantiated, the two peaks, when tested separately in the remainder of the purification procedure, behaved identically. Thus all the active fractions eluting from lysine-Sepharose are pooled and treated as one.

Various concentrations of NaCl (0.025M-2.0M) were incorporated into the tris buffer of the Sephadex G-200 step, before 1.5M NaCl proved to be the most satisfactory. The problem to be overcome was the apparent heterogeneity of the activator in the last stage of purification, when the G-200 column was operated in a buffer with physiological salt concentration. The V_o or high molecular weight elution positions of the activator were thought to be due to aggregation. Also the activator eluted after V_s on several occasions due to an interaction with the gel matrix. However, a concentration of 1.5M NaCl in the buffer effected the elution of activator as a homogenous peak. It was later realised that the soluble fibrin polymers, of various molecular weights, were causing some of the variation in elution positions. 1.5M NaCl obviously broke this

association down, but later 1M lysine was found to be more effective, as the binding would be mediated through the lysine binding sites.

The activator appearing in the blood after venous stasis was purified in the same scheme as the cadaveric activator and generally behaved in an identical manner. However, there were some slight, but possibly significant, differences. The venous occlusion activator eluted in only one peak from lysine-Sepharose, which corresponded to the high affinity material from the cadaver. The absence of the low-affinity peak can be explained in two ways. If it is accepted that a degraded molecule is the cause of the low-affinity of cadaveric activator then it is not surprising that this was absent when the source of the activator was as "fresh" as venous occlusion plasma. Alternatively, if the "complex-theory" is the correct one then again it is quite likely that there would be very little or no SFP's present in normal plasma, and thus the activator would bind to lysine predominantly in the free form.

The venous occlusion activator eluted from lysine and protamine columns at slightly higher lysine and NaCl molarities respectively, than the reported ranges for the cadaveric activator. Also it gave a lower K_d value on Sephadex G-200 indicating a larger molecular weight. Thus it could be that the venous occlusion activator represents a more native molecule than the cadaveric activator. The venous occlusion activator also appeared to give a better

percentage recovery as the peak activity value of the lysine-Sepharose step was only 6 Ploug u/ml and yet a peak value of 3u/ml was obtained from the Sephadex G-200 run. Experience in the purification of the cadaveric activator shows that the activity of the peak fractions of the G-200 run is approximately one-tenth that of the lysine-Sepharose run.

Finally the binding of the activator, plasminogen and plasmin to lysine-Sepharose, was compared. All three molecules are believed to possess lysine binding sites, and differences in their binding to lysine may reflect their relative affinities for fibrin. The activator had the highest affinity, eluting at 0.30M lysine, while plasmin and plasminogen eluted at 0.24M and 0.22M respectively. The higher affinity of the activator over plasminogen, for fibrin, was confirmed by later studies.

In conclusion, the purification scheme works very well, giving a product with biological activity but no detectable protein either by absorption at 280nm or 220nm or when run on polyacrylamide gel electrophoresis and stained. Many experiments, which will be described in later sections, were carried out on this material.

3 IMPROVED PURIFICATION PROCEDURE

A INTRODUCTION

Although purification of the vascular plasminogen activator on lysine-Sepharose, protamine-Sepharose and Sephadex G-200 gives a pure product, the final yields obtained are very small indeed.

Thus after the observation that a sample of concentrated (20-fold) crude perfusate when run on Sephadex G-200 eluted at V_o, it was realised that the vascular activator was being obtained from the cadaver in the form of a complex. As it appeared that this complex was more stable than the free enzyme, studies were initiated to purify the activator in its complexed form, using dissociation of the complex only as a last step. To these ends polyethylene glycol precipitation was investigated since it is known to preferentially precipitate larger molecules.

B POLYETHYLENE GLYCOL PRECIPITATION

Addition of polyethylene glycol (PEG) to a mixture of proteins will cause the high molecular weight species to come out of solution first, and on increasing concentrations of PEG, more protein, of progressively lower molecular weight, will precipitate out. This is because, with increasing concentrations of PEG, the effective amount of solvent available to maintain the proteins in solution is progressively reduced. Thus as the activator is obtained as a high molecular weight complex from the cadaver, PEG precipitation was investigated.

Initially PEG 6000 was employed. 8% PEG 6000 was sufficient to bring 90-100% of the activator down in the precipitate. However, this was soon replaced by PEG 400 which gave a slightly better purification; the results were more reproducible and PEG 400, as a liquid, is easier to use than the solid PEG 6000.

PEG 400 was investigated at various concentrations (5-35%) in 5ml aliquots of a crude perfusate. Folin protein assays and fibrin plate assays for activator activity were carried out on the supernatents (after precipitation) and resuspended precipitates. As can be seen by reference to Table 6, 15% PEG 400 causes precipitation of all of the activator activity associated with the least amount of protein.

Final Conc PEG 400	% Activator in ppt	% Protein in ppt
5%	45.5	2.3
10%	54.6	1.9
15%	100	2.7
20%	100	4.9
25%	100	7.7
30%	100	10.8
35%	100	13.3

TABLE 6 EFFECT OF VARIOUS CONCENTRATIONS OF PEG 400 ON THE PERCENTAGE OF ACTIVATOR AND PROTEIN PRECIPITATED

Table 7 gives the results obtained using 15% PEG 400 with the crude perfusate of one cadaver, given as an example. Under 4% of the total protein is precipitated, whereas nearly 80% of the activator occurs in the resuspended precipitate. Actually 100% of the activator activity is precipitated, as there is no detectable activity in the supernatent after precipitation, but only 80% of this is resuspended. The remaining activity can probably be accounted for by the losses associated with binding to insoluble fibrin, sometimes seen to form on precipitation. However this still represents a 20-fold purification with an increase of the specific activity from 0.43 to 8.8 Ploug units per mg.

Thus PEG 400 was introduced as the first step in the purification procedure. To the centrifuged crude perfusate is added PEG 400 to a final concentration of 15%. This is stirred overnight at 4°C to facilitate precipitation. The precipitate formed is removed by centrifugation and is generally resuspended in 100-250ml (depending on amount of precipitate) of 25mM tris, 1mM EDTA 3.8mM borate 0.15M NaCl at pH8.8. However if the sample is to be applied to a particular column, the precipitate may be resuspended in the relevant starting buffer.

	Protein Concentration mg/ml	Total Protein g	Activator Activity Ploug u/ml	Total Activator Activity Ploug Units	Specific Activity u/mg
Crude Perfusate Vol = 2,655ml	5.6	14.9	2.4	6,372	0.43
Post PEG Precipitation Supernate Vol = 3,122ml	4.5	14.1	0	0	0
Resuspended PEG Precipitate Vol = 120ml	4.8	0.58	42.0	5,040	8.8

TABLE 7 FIGURES RELATING TO 15% PEG 400 PRECIPITATION (FIGURES FOR ONE CADAVER GIVEN AS AN EXAMPLE)

C PURIFICATION PROCEDURES (AND COMPARITIVE BEHAVIOUR OF OTHER FIBRINOLYTIC COMPONENTS)

In the initial procedure developed the PEG precipitate was resuspended in as small a volume of 25mM tris, 1mM EDTA 3.8mM borate, 0.15 NaCl (pH8.8), as possible. The sample, with glucose added as a V_s marker, was then applied to a Sepharose CL-6B column (5 x 80cm). This was eluted at 4°C with 25mM tris, 1mM EDTA, 3.8mM borate, 0.15M NaCl, pH8.8; 30ml fractions were collected at 125ml/hour (see Fig 14). The fractions were screened for protein at 280nm and for activator activity on normal and plasminogen-free fibrin plates. The active fractions, eluting at V_o, were pooled and concentrated to 5ml by ultrafiltration; to this was added lysine, to a final concentration of 1 molar, and glucose as a V_s marker. This sample was then applied to a Sephadex G-200 column and eluted at 4°C with 0.025M tris - 1M lysine (pH7.5) buffer; 7.5ml fractions were collected at 15ml/hour (see Fig 15). The activator eluted with a K_d of 0.42 and was not associated with any detectable peak of absorbance when measured at 280 or 220nm.

This purification, by effecting a size change of the activator activity using lysine as the dissociating agent, gives an excellent purification in just two chromatographic steps. However, the purification obtained is very much dependant on the V_o elution position in the Sepharose 6B step. Unfortunately the activator activity, although

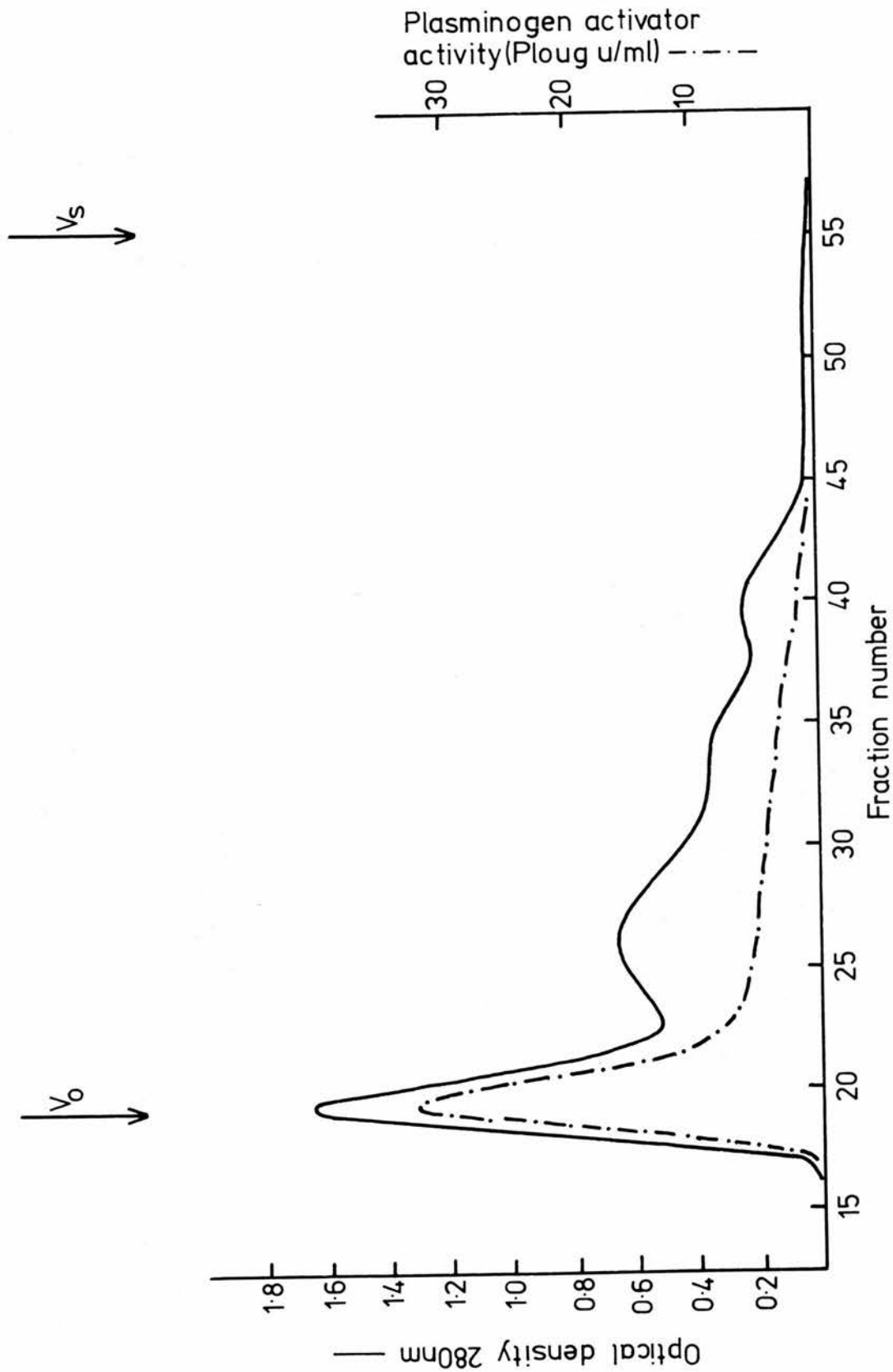


FIG 14 SEPHAROSE 6B

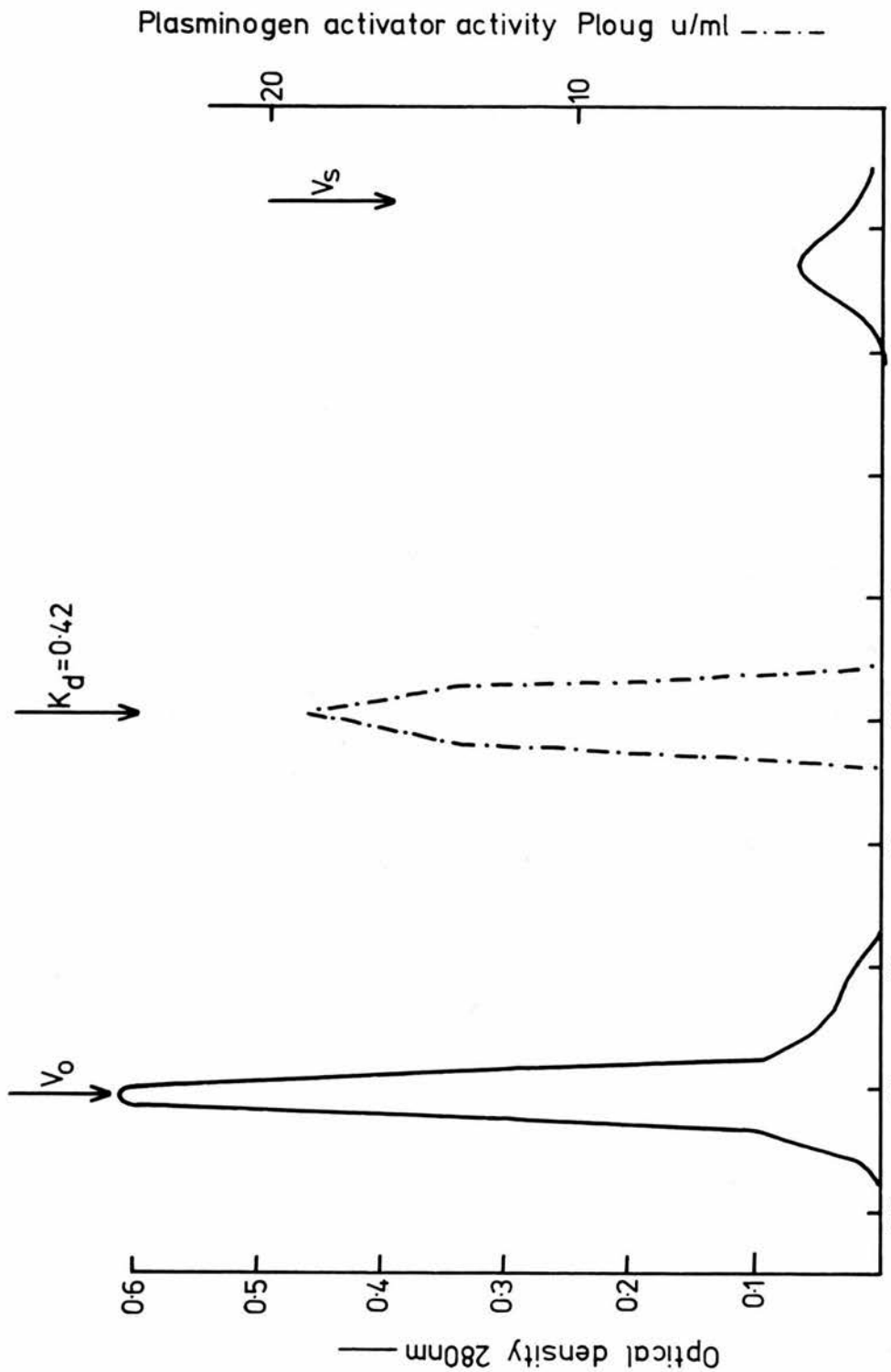


FIG 15 SEPHADEX G-200

always of higher molecular weight than free activator, is often heterogeneous and elutes in a broad peak, or numerous peaks. Thus alternative methods were investigated.

The purification of a resuspended PEG precipitate was investigated on octyl-Sepharose, Lens culinaris-Sepharose and hydroxyapatite chromatography. The activator complex did bind tightly to the first two columns, but was difficult to elute, and on elution the activator activity was not associated with fibrinogen related protein. It appeared that the Lens culinaris column was binding the carrier protein so strongly that, for elution, the activator had to be dissociated from its carrier (1M NaCl or lysine was able to do this), leaving the fibrin bound to the column. Hydroxyapatite, however, gave the most satisfactory results, being easy to handle and giving a good purification, and was thus used as the first step after PEG precipitation.

The resuspended PEG precipitate was applied to the hydroxyapatite column (7 x 15cm). The adsorbed protein was eluted with an exponential gradient of 5-500mM Na_2HPO_4 at pH7 (see Fig 16). 30ml fractions were collected at 150ml/hour and screened for protein (at 280nm) activator activity (normal and plasminogen-free plates) and the phosphate gradient was measured by conductivity. The activator activity eluted as a single peak at 230mM phosphate (range 200-270mM). The active fractions were pooled, concentrated by ultrafiltration to approximately 10ml, glucose added and the sample applied to a Sepharose CL-6B

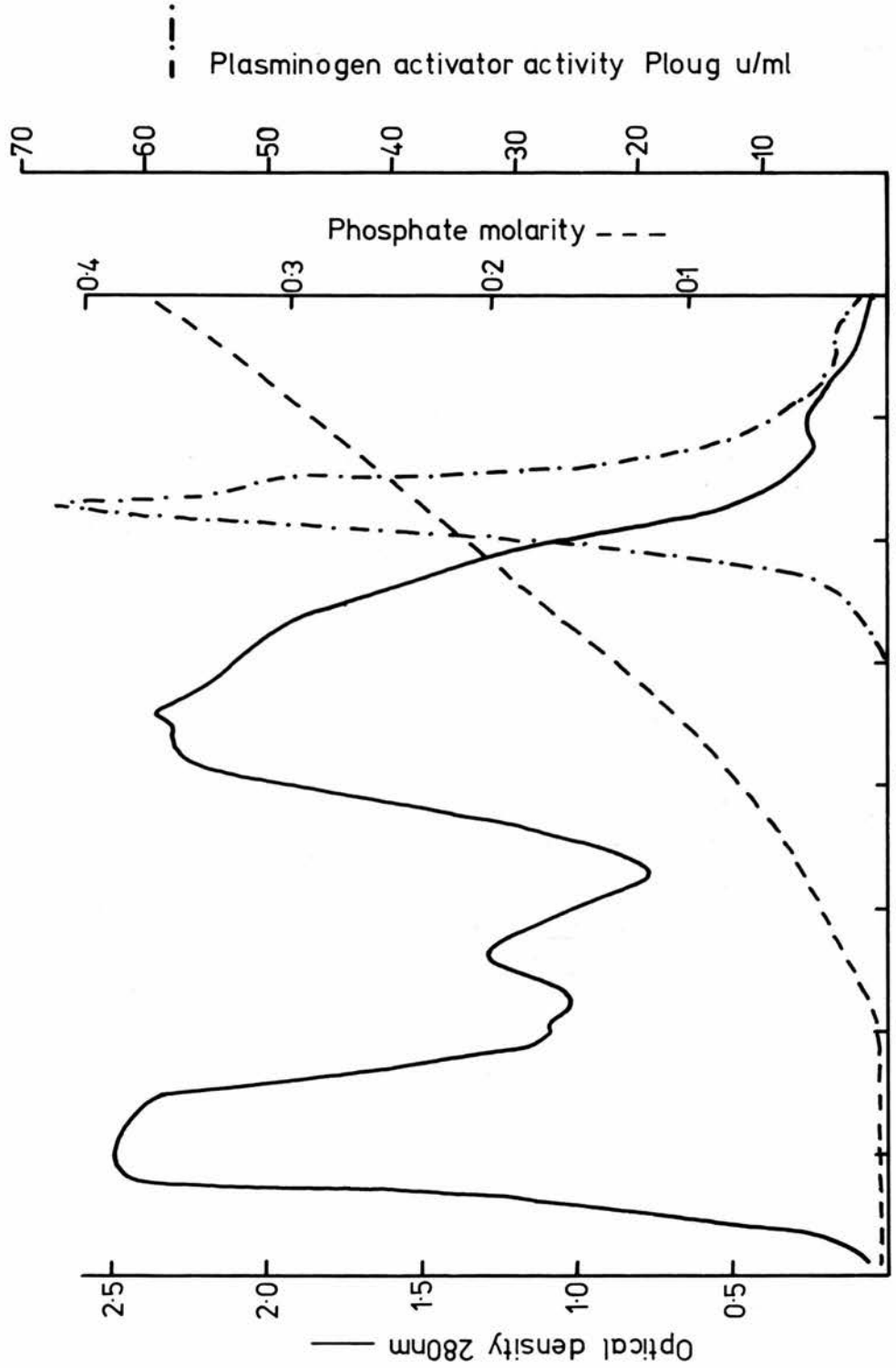


FIG 16 HYDROXYAPATITE

column (5 x 80cm). The sample was eluted at 4°C with 25mM tris, 1mM EDTA, 3.8mM borate, 0.15M NaCl pH8.8 (see Fig 17). The fractions were assayed for protein and activator activity. The elution position of the activator activity at this stage was variable, but always showed that the activator was still in the complexed form.

The active fractions were pooled, concentrated by ultrafiltration, then diluted two-fold with 0.025M tris (pH7.5) to reduce the effect of saline, before being applied to lysine-Sepharose (2.5 x 15cm) at 4°C. The adsorbed protein was eluted with an exponential gradient of 0-1M lysine in 0.025M tris, pH7.5 (see Fig 18). 7.5ml fractions were collected at 30ml/hour and screened for protein, activator activity and the lysine gradient was determined by refractometry. The activity elutes from lysine-Sepharose at approximately 0.32M lysine, as previously determined. The activity at this stage can sometimes be seen to be associated with a very small peak of protein.

An alternative to this last step in the purification is provided by fibrin-Sepharose. Fig 19 shows the excellent purification obtained when a resuspended PEG precipitate is applied direct to fibrin-Sepharose. The majority of the protein does not bind to the column, and the small amount that does bind has a lower affinity for fibrin than the activator, which elutes at 0.44M lysine. A column prepared by coupling soluble fibrin polymers (see Chapter 4,

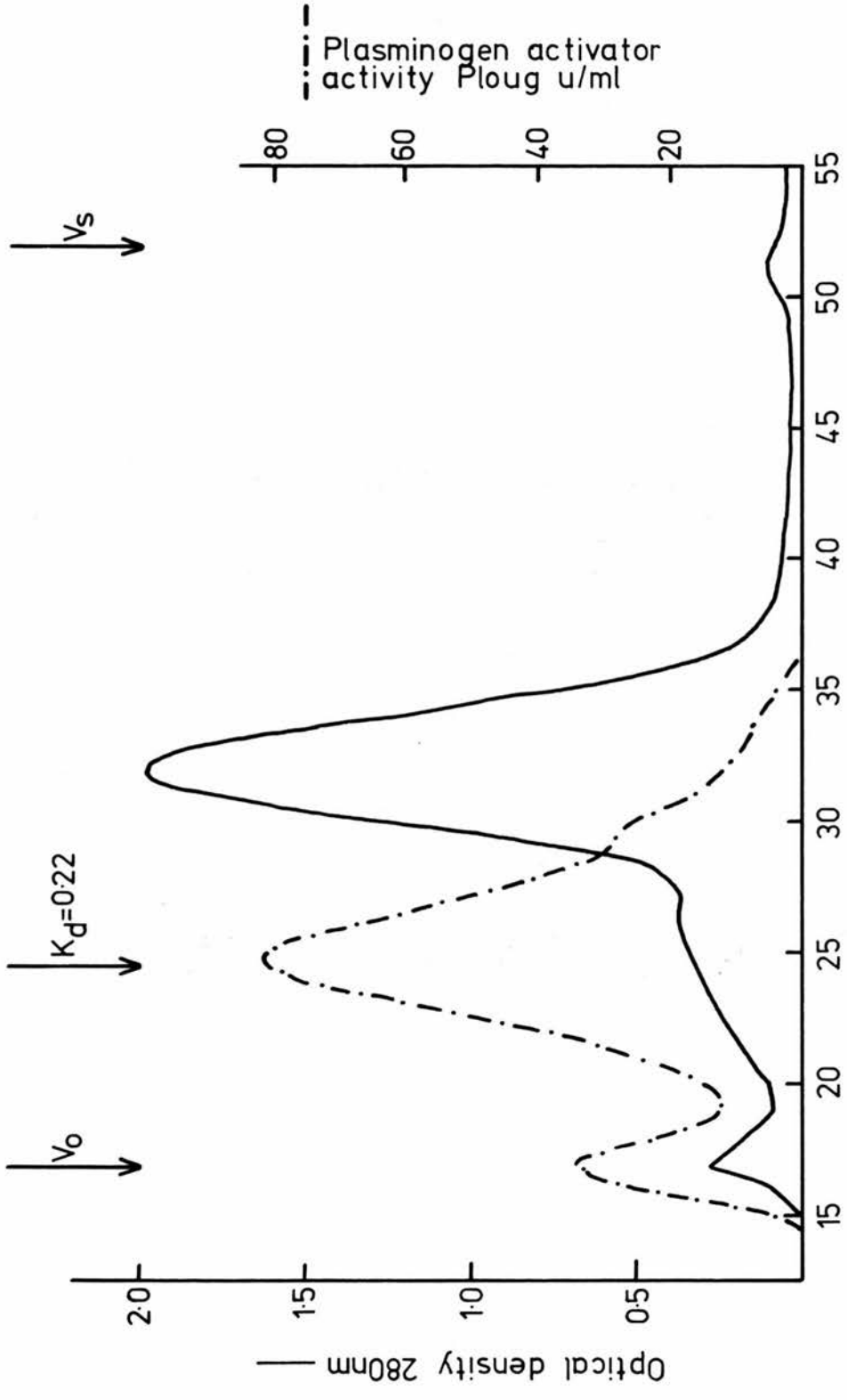


FIG 17 SEPHAROSE 6B

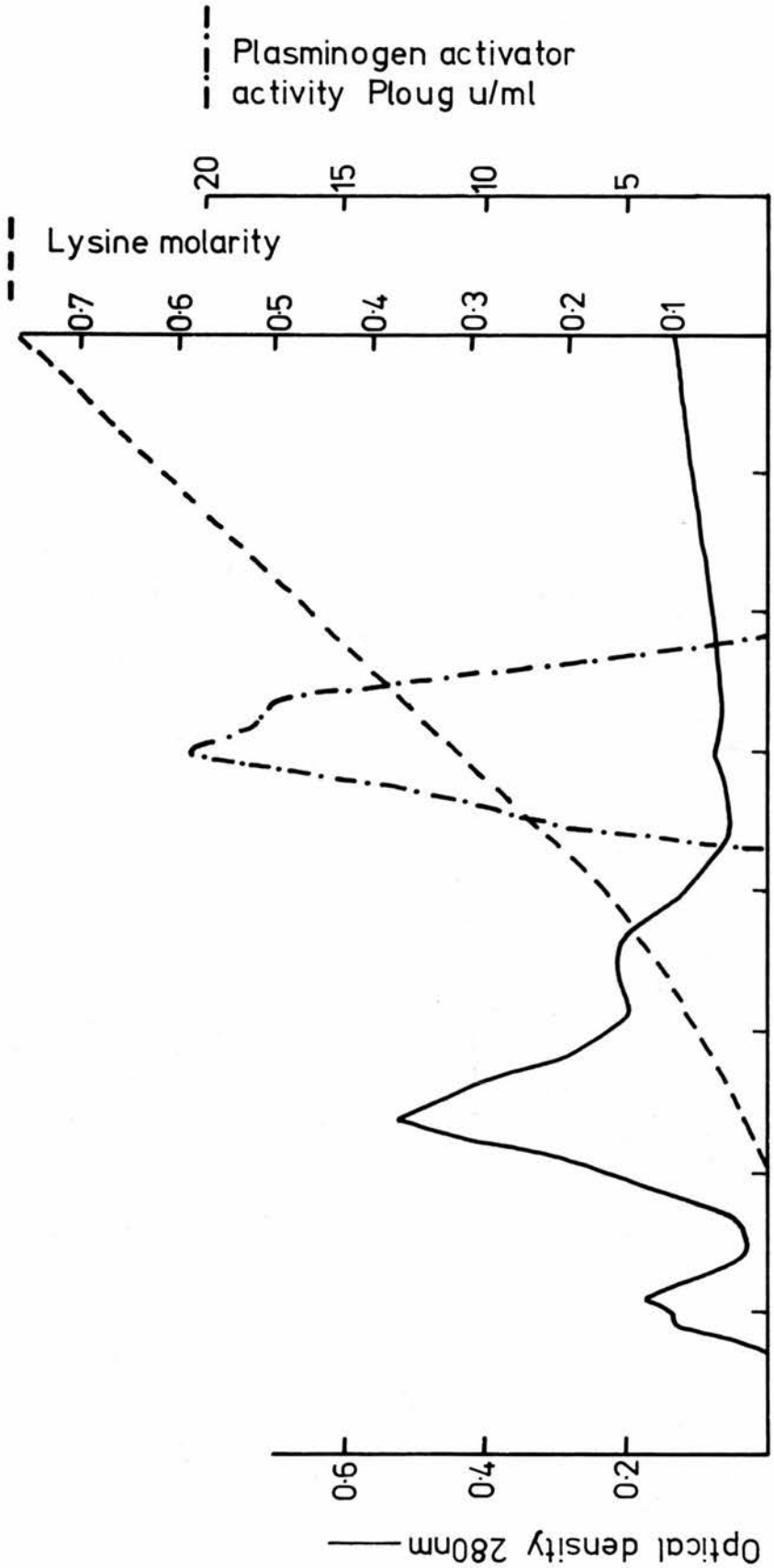


FIG 18 LYSINE-SEPHAROSE

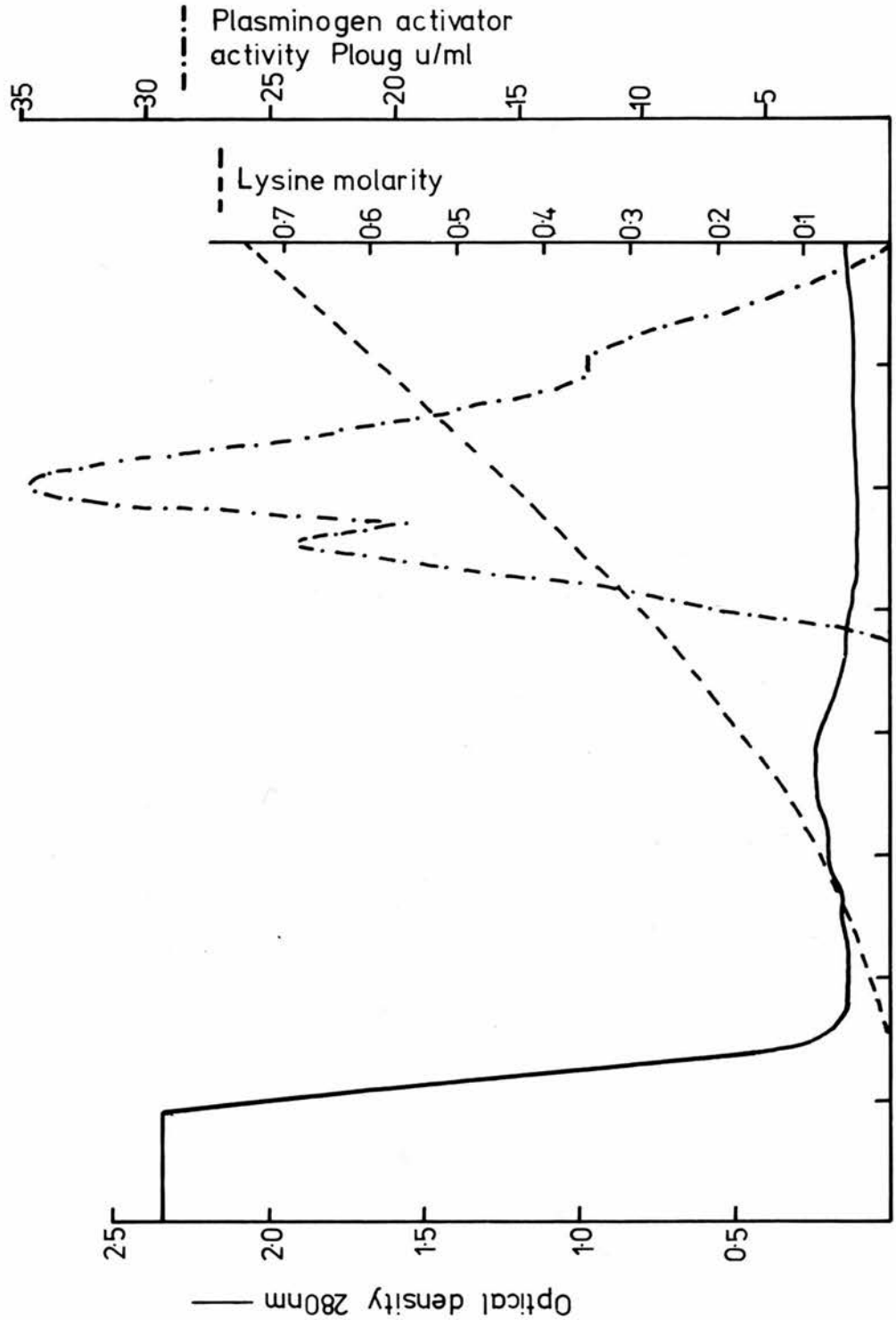


FIG 19 FIBRIN-SEPHAROSE

Section 1) to Sepharose, gave a very similar profile to fibrin-Sepharose, the activator activity eluting at 0.41M lysine.

One further ligand that is worthy of mention is benzamidine. A resuspended PEG precipitate was purified on lysine-Sepharose, and it is the high affinity peak that is further purified on benzamidine-Sepharose, shown in Fig 20. The adsorbed protein is eluted by an exponential lysine gradient from 0-1M in 0.025M tris (pH7.5). The activator elutes at 0.6M lysine (range 0.57-0.62M lysine), and some interesting comparisons have been made on various fibrinolytic components using this column (see later).

Some interesting comparisons were made between the vascular activator and other fibrinolytic components, with regard to their behaviour in the purification procedures developed. A sample of cadaveric vascular activator (crude eluate, a gift from Dr A Todd) was purified in the scheme developed for our own activator. It behaved in an identical manner, being wholly precipitated by 15% PEG 400, binding to hydroxyapatite and eluting at 255mM phosphate, within the range obtained for our activator. It also gave two peaks of activator activity on Sepharose CL-6B, one at V_o and one with a K_d of 0.22, which shows it was in a complexed form and is consistent with the behaviour observed with our own activator. Unfortunately insufficient activity was present to carry out the final step on lysine or fibrin-Sepharose, but its behaviour in the first three steps was

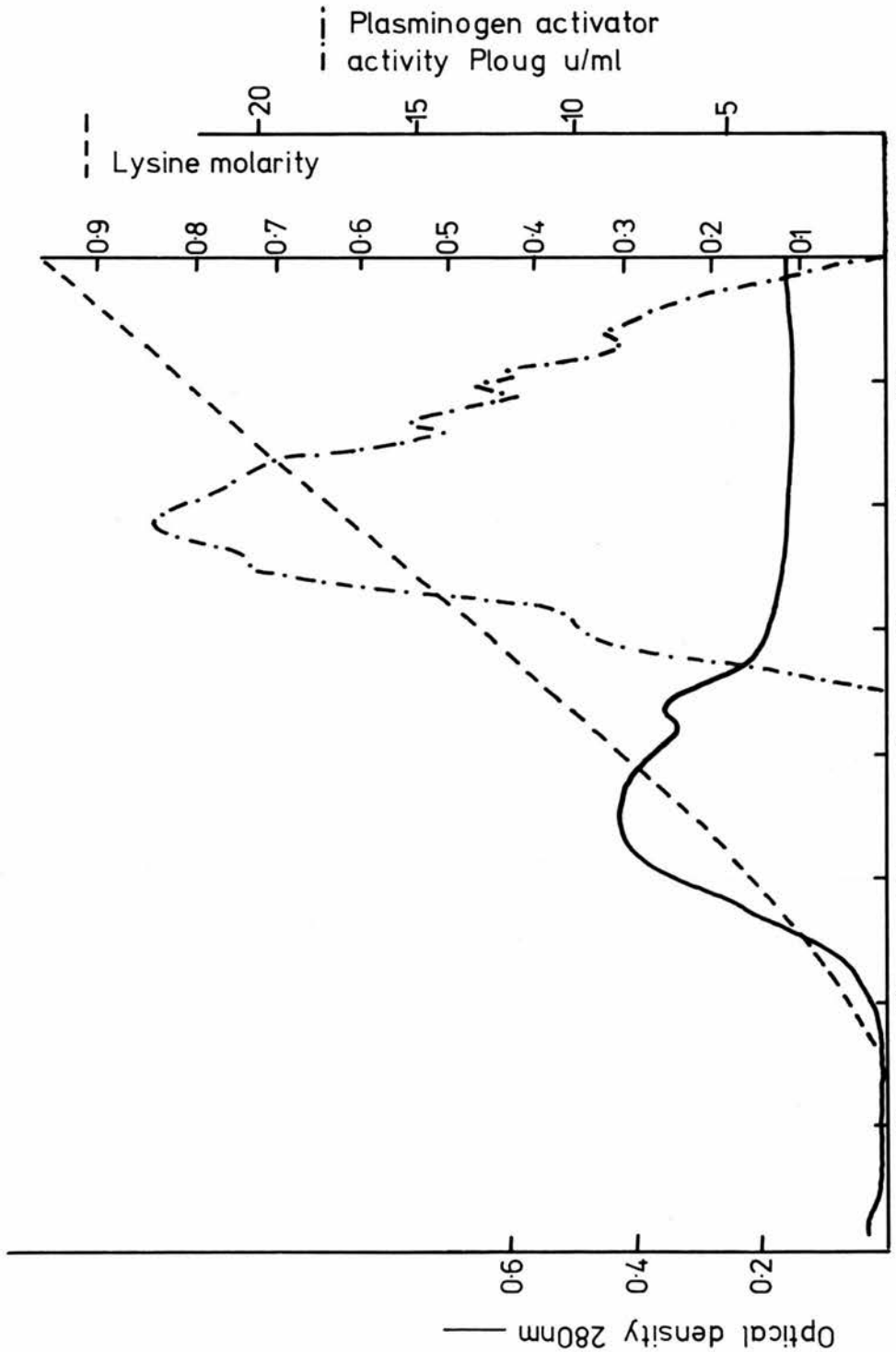


FIG 20 BENZAMIDINE-SEPHAROSE

identical to our own activator.

The precipitation of plasminogen by 15% PEG 400 was studied in two ways. Firstly, a sample of radioactive plasminogen was added to a crude perfusate and samples were taken before and after precipitation and of the resuspended PEG precipitate for counting. Secondly, plasminogen was determined in the crude perfusate and resuspended PEG precipitate by Laurell rocket electrophoresis, using dilutions of normal plasma as standards. The results were contradictory; 74.7% of the added ^{125}I-plasminogen was precipitated and appeared in the resuspended PEG precipitate, whereas only 37.3% of the intrinsic plasminogen was in the resuspended PEG precipitate.

When the resuspended PEG precipitate containing ^{125}I-plasminogen was applied to a hydroxyapatite column, the plasminogen eluted slightly before the activator at 210mM phosphate (the activator activity eluted at 235mM phosphate). When ^{125}I-plasminogen was incorporated in a sample of crude activator and applied to Sepharose CL-6B the plasminogen eluted with a K_d of 0.43, after the activator (K_d = 0.37), indicating a slightly lower molecular weight; however the elution position indicates a molecular weight higher than native plasminogen, thus it must be complexed. The comparison between plasminogen and activator on lysine-Sepharose has already been discussed in the previous section.

^{125}I-plasminogen has also been compared with the vascular

activator in its binding to fibrin- and SFP-Sepharose columns. In both cases plasminogen has a lower affinity than the activator. Plasminogen elutes at 0.29M lysine from fibrin-Sepharose, and 0.36M lysine from SFP-Sepharose, whereas the activator elutes at 0.44M and 0.41M lysine respectively.

An interesting comparison emerges when binding to benzamidine-Sepharose is compared. As already stated the activator elutes from benzamidine-Sepharose at 0.6M lysine (see Fig 20). In contrast plasminogen (Kabi) elutes at 0.14M lysine, plasmin (Kabi) elutes at 0.82M lysine and an activator present in melanoma culture supernatent (gift from Dr G Barlow) eluted in two peaks at 0.30M and 0.62M lysine. The melanoma activator did not show any binding to lysine-, fibrin- or SFP-Sepharose.

D DISCUSSION

PEG precipitation has proved to be a very satisfactory purification step. Apart from reducing the volume of sample from 3 litres or more to 100mls, it also appears to stabilize the activity. Also it gives a very useful 20-fold purification; this compares favourably with the 2 to 4-fold purification obtained with ammonium sulphate precipitation.

The studies on the precipitation of plasminogen by 15% PEG 400, as stated, were contradictory. However, the more accurate value is provided by the radioactivity precipitated, rather than by the Laurell rocket technique. The plasminogen, iodinated by the chloramine T method, showed that 74.7% of the counts were precipitated, whereas only 37.3% of the intrinsic plasminogen antigen in the perfusate appeared in the resuspended PEG precipitate. The actual value of plasminogen in the crude perfusate (3500mls) was 49% of that in normal plasma and a value of 320% that of normal plasma was obtained in the resuspended PEG precipitate (200mls). One explanation for the higher value of the radiolabelled plasminogen is that the chloramine T iodination may have damaged the molecule, or induced a conformational change (possibly by damage) that gave the molecule a higher affinity for the precipitated fibrin. The figure obtained for the precipitation of the iodinated plasminogen is almost exactly twice that obtained for the intrinsic plasminogen. By coincidence Rákóczi et al (1978) found

the same ratio for the relative affinity of lys- and glu-plasminogen for fibrin. However, the actual figures were 8% and 4% respectively.

Another possibility is that some of the plasminogen was converted to plasmin during iodination, or activated to plasmin after addition to the perfusate. This would tend to increase the radiolabel precipitated (due to the higher affinity of plasmin for fibrin) and also decrease the immunoreactivity towards the anti-plasminogen used in the Laurell rocket electrophoresis technique. It is worth noting that while only a fraction of the plasminogen is precipitated by 15% PEG 400, 100% of the activator is precipitated. This provides another indication of the relative affinities of the two for fibrin.

The purification of the activator by PEG precipitation and then chromatography on Sepharose 6B and Sephadex G-200 was the shortest purification scheme developed and the most satisfactory from the point of view of achieving maximum purification. However, it was less satisfactory from the point of view of reproducibility. It made use of a molecular weight change for purification. The activator, while complexed with high molecular weight soluble fibrin polymers, eluted at V_o on Sepharose 6B. When re-run on Sephadex G-200 in a buffer containing 1M lysine, the contaminating protein still elutes at V_o, while the activator, dissociated from the SFP's, elutes in its normal position (K_d 0.4-0.5), separated from the

protein. For the purification to work, the activator complex must elute at V_o on Sepharose 6B (to enable separation from the protein on dissociation); unfortunately this is not always so.

The activator is obtained from the cadaver in a complex with SFP's, and these are subject to degradation. Thus generally what is seen is a heterogeneous collection of carrier molecules exhibiting a range of molecular weights. Trasylol was incorporated into the perfusion fluid, and later in the column buffers, in an attempt to avoid proteolytic breakdown of the SFP's by plasmin. This was partially successful, but produced assay problems. One further problem encountered was that the maximum sample size that could be applied to the Sepharose 6B column used (5 x 80cm) was 50ml. The PEG precipitate could not always be resuspended in 50ml and sometimes had to be run as two samples. However, if the method was being used routinely the column dimensions could be scaled up.

The K_d of the activator purified by this latter method is slightly lower than the K_d of the activator produced by the initial purification procedure. This represents a slightly larger molecular size and could be a result of the greater stability of the complexed, as opposed to the free enzyme.

The final purification scheme developed involved a PEG precipitation and then chromatography on hydroxyapatite,

Sepharose 6B and lysine-Sepharose or fibrin-Sepharose. One advantage of this scheme was that the samples could be applied to the subsequent column without prior treatment; this was not the case in the initial purification scheme (lysine-Sepharose, protamine-Sepharose, Sephadex G-200). The yields with both methods using dissociation of the complex as a last step, were superior to the yields of the initial purification scheme. A value of 20 Ploug u/ml was generally obtained at the peak of the last step of the improved procedures, and although this was measured in lysine buffers (an inhibitor - this work) it is still 2-3 times the values obtained by the initial procedure.

The activator showed a higher affinity than plasminogen to fibrin- and SFP-Sepharose. The activator eluted at 0.44M and 0.41M lysine respectively, whereas plasminogen eluted at 0.29M and 0.36M lysine respectively. This, as expected, mirrors the relative affinity of the two for lysine-Sepharose (activator 0.3M lysine, plasminogen 0.22M lysine); also as expected the affinity for the lysine binding site of fibrin is higher than the lysine ligand which attempts to mimic it.

The results of the binding to 4-amino-benzamidine-Sepharose are interesting. Holleman et al (1975) reported a p-aminobenzamidine binding site on plasminogen that was separate from the lysine binding site. This was considered not to be an active site, or potential active site interaction, as PNPGB inhibited plasmin behaved identically to

plasminogen when adsorbed to lysine- or p-aminobenzamidine-Sepharose. The p-aminobenzamidine site, presumed to be an arginine site in vivo, was implicated in the interaction with fibrin.

The results obtained with the binding studies of plasminogen, plasmin, vascular activator and melanoma activator to 4-aminobenzamidine-Sepharose indicate that the arginine binding site does not play a dominant role in the interaction with fibrin. Of the proteins tested, plasminogen, plasmin and the vascular activator are known to have a high affinity for fibrin and all bind to lysine-Sepharose. However, although plasmin (elutes at 0.82M lysine) and vascular activator (elutes at 0.6M lysine) bind strongly to 4-aminobenzamidine-Sepharose, plasminogen (0.14M lysine) is only weakly bound. Conversely, the melanoma activator, which does not bind to lysine-, fibrin-, or SFP-Sepharose, binds quite strongly to 4-aminobenzamidine-Sepharose, eluting in two peaks at 0.3M and 0.6M lysine. Urokinase is another example that has a low affinity for fibrin, but binds quite strongly to 4-aminobenzamidine-Sepharose. Thus it would appear that the lysine binding site is the controlling influence in the binding to fibrin. A more likely interpretation of benzamidine binding therefore is that it does reflect binding at the active site of the serine proteases (plasmin, vascular activator and melanoma activator) since plasminogen does not show strong binding.

4 ALTERNATIVE SOURCES OF HUMAN PLASMINOGEN ACTIVATOR INVESTIGATED

A UMBILICAL CORD

An umbilical cord was obtained fresh from a caesarian delivery. The cord was cannulated and one litre of 0.15M NaCl was passed through its length and the effluent collected. The first 50-100ml was run through, and then thrombin was added to the remaining saline to a final concentration of 5u/ml, which was then also run through the cord.

The 1 litre of effluent was collected and spun, before ammonium sulphate was added to 45% saturation; this was stirred overnight at 4^{o}C to precipitate. The precipitate was removed by centrifugation and resuspended in 20ml 0.15M NaCl. Samples were taken for fibrin plate and folin (protein) assays. The resuspended precipitate contained 1 Ploug u/ml of activator activity with a specific activity of 0.5u/mg.

B MILK

10ml of unheated human milk was de-fatted by centrifugation, before samples were applied to a normal and plasminogen-free plate. The milk was active giving a value of 47 Ploug u/ml on the normal fibrin plate, of which 16u/ml was contributed by a direct proteolytic enzyme rather than an activator, as evidenced by the plasminogen-free plate.

C SALIVA

Samples of pooled saliva were centrifuged and separated into a pellet and supernatent. The pellet was frozen (-40°C) and thawed twice, suspended in distilled water and sonicated for 1 minute. Samples of the resuspended sonicated pellet and the supernatent were applied to a normal and plasminogen-free fibrin plate.

The supernatents tested gave no lysis on either type of plate; the treated salivary pellets gave values of 1-5 Ploug u/ml on normal plates and no lysis on plasminogen-free plates.

D ASCITIC FLUID

Samples of ascitic fluid were tested from 12 patients with ovarian carcinoma or alcoholic cirrhosis of the liver. The ascitic fluid and its euglobulin were tested on normal and plasminogen-free fibrin plates. Very little (<1 Ploug u/ml) or no activity was found in any of the samples.

E LUNG

One lobe of human lung was washed free of blood with saline and then chopped up and homogenized in 250ml of saline. This was magnetically stirred for 1 hour and then ammonium thiocyanate was added to a final concentration of 2M. This was stirred for a further 1 hour before being centrifuged and dialysed against two changes of saline. The final product contained a heavy precipitate, which was removed by centrifugation. The supernatent when tested gave an activity of less than 1 Ploug u/ml on the normal fibrin plate and no lysis on the plasminogen-free plate.

F CULTURED MELANOMA SUPERNATENT

The supernatent of cultured human melanoma cells was the gift of Dr G Barlow. This was tested on normal and plasminogen-free fibrin plates. The melanoma activator was very active giving a value of 150 Ploug u/ml, of which only 3 Ploug u/ml was due to a direct proteolysis. A number of experiments were carried out on this activator which will be described later.

G CRYOPRECIPITATE

22 units of cryoprecipitate were taken, the supernatent removed and the precipitates added to 100ml 25mM tris, 1mM EDTA, 3.8mM borate, 0.15M NaCl pH8.8 containing 10u/ml heparin. This was kept at 37°C for half an hour to solubilize the precipitate. To this was added 15% PEG 400 and the mixture stirred overnight at 4°C. The precipitate was removed by centrifugation and redissolved in 100ml 25mM tris, 1mM EDTA, 3.8mM borate, 0.15M NaCl pH8.8. All the activity was precipitated, as the supernatent was inactive, while the resuspended PEG precipitate gave an activity of 6 Ploug u/ml, giving a total of 600 Ploug units from 22 units of cryoprecipitate.

H INDUCED ACTIVITY IN BLOOD

Three procedures that induce raised levels of the vascular activator in the blood were investigated. They were venous occlusion (15 minutes), exercise (15 minutes) and drug infusion (adrenalin 300µg over 30 minutes or DDAVP 20µg over 30 minutes). In each case the plasma and euglobulin precipitate were tested on normal and plasminogen-free fibrin plates and in each case an increase of activity over a control plasma sample was noted. However, the increases were not of sufficient magnitude, bearing in mind the limitations on sample size, to be of any use for a source of activator activity for purification.

I DISCUSSION

The majority of the alternative sources of plasminogen activator investigated gave very little activator activity. However, two sources did give high yields - human milk (47 Ploug u/ml) and the melanoma supernatent (150 Ploug u/ml). Unfortunately the amount of human milk that could be obtained would be very much less than required to attempt a purification of activator from this source.

The cultured melanoma supernatent was a valuable source of activator activity and many experiments have been carried out with this activator (which will be reported elsewhere). However, fundamental differences from the cadaveric vascular activator render it only useful for comparitive purposes. Thus the cadaver remained the best source of plasminogen activator for the purpose of purification.

Page

CHAPTER 3

5/

1 PHYSICOCHEMICAL STUDIES

A CALIBRATION OF SEPHADEX G-200 AND DETERMINATION OF STOKES RADIUS (r_s) AND DIFFUSION COEFFICIENT (D)

The Sephadex G-200 column was calibrated with standards of known Stokes radius (r_s) under identical elution conditions that were employed for the last step of the initial purification procedure. The column (2.5 x 100cm) was run at 15ml/hour at 4°C in 25mM tris, 1mM EDTA, 3.8mM borate, 1.5M NaCl pH7.5. Three standards were used, ^{125}I-labelled albumin, ovalbumin and cytochrome c. The distribution coefficients (K_d) determined for the standards were 0.387, 0.517 and 0.715 respectively. These values of the K_d were converted to the inverse error function ($erfc^{-1}(1-K_d)$) by reference to the "Tables of the Error Function and Its Derivatives" (1954). The calibration curve shown in Fig 21 was then constructed by plotting the known r_s values of the standards against the value obtained for $erfc^{-1}(1-K_d)$.

The activator purified by the initial purification procedure (Fig 13) had a K_d of 0.48 (inverse error function = 0.500); this gives a value of 2.97nm for the Stokes radius of the activator by interpolation on the calibration curve (Fig 21). The diffusion coefficient (D) for the activator was determined by substitution of the r_s value into the Stokes-Einstein equation and a value for D of 7.22 Ficks was obtained.

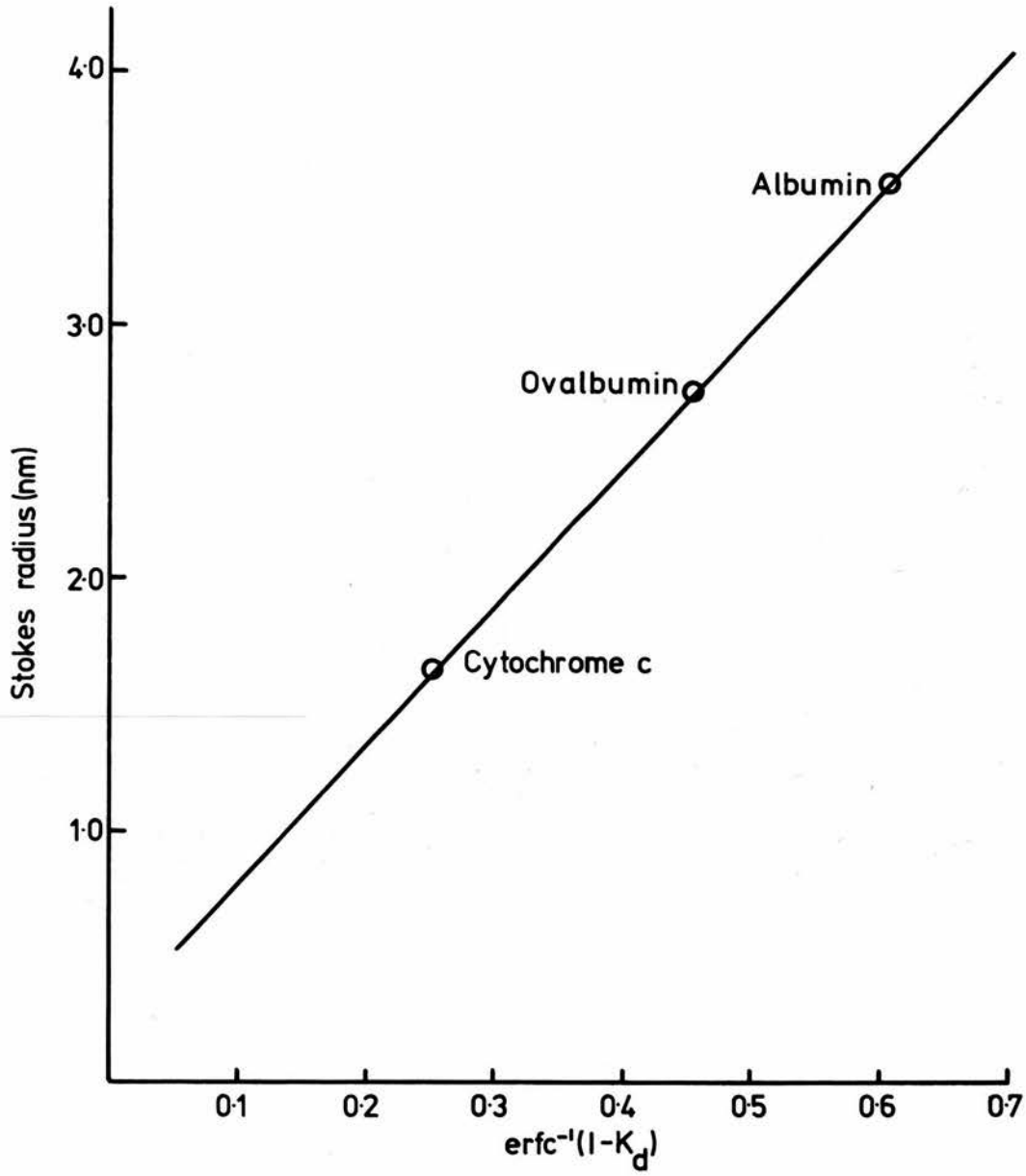


FIG 21 CALIBRATION OF SEPHADEX G-200

The Stokes radius of the activator purified from venous occlusion plasma, which had a K_d of 0.32 ($erfc^{-1}(1-K_d$ = 0.703), can be read from the calibration curve (Fig 21) and has a value of 4.08nm. This corresponds to a value of 5.26 Ficks for D.

The same calibration curve can also be used to determine r_s for the activator purified by PEG precipitation, Sepharose 6B and Sephadex G-200 (Fig 15), although the last step of this was carried out in 0.025M tris-1M lysine pH7.5. V_o and V_s are unaffected by changes in ionic strength and pH over a wide range, and the properties of the gel are unaffected by the change in solvent. This activator had a K_d of 0.42 ($erfc^{-1}(1-K_d)$ = 0.570), which corresponded to values of 3.35nm and 6.4 Ficks for r_s and D respectively.

B DETERMINATION OF SEDIMENTATION COEFFICIENT

Linear sucrose density gradients, over the range 6% - 30% $^{w}/_{v}$, were made up in 25mM tris, 1mM EDTA, 3.8mM borate, 1.5M NaCl pH7.5. Three 6ml gradients were set up in 12 x 70mm polycarbonate tubes, and allowed to diffuse at room temperature for approximately 4 hours.

The samples were layered on the surface of the gradient. Gradients 1 and 2 (Figs 22 and 23 respectively) contained 20µl ^{125}I-albumin (10mg/ml), 100µl cytochrome c (10mg/ml) and 100µl of an activator preparation. Gradient 1 contained an activator sample from the high-affinity peak shown in Fig 11, while Gradient 2 contained an activator sample from Fig 13. Gradient 3 (Fig 24) contained 20µl ^{125}I-albumin (10mg/ml) and 200µl ovalbumin (10mg/ml). The gradients were centrifuged at 4°C and 200,000g for 20 hours, then fractionated and assayed for radioactivity protein (folin assay) and activator activity.

The distances sedimented from the origin were measured from the graphs (Figs 22-24) and plotted against the known $S^{o}_{20,w}$ values of the standards, to construct a standard curve (Fig 25). From this curve an $S^{o}_{20,w}$ value of 4.40 Svedberg's was obtained for the activator.

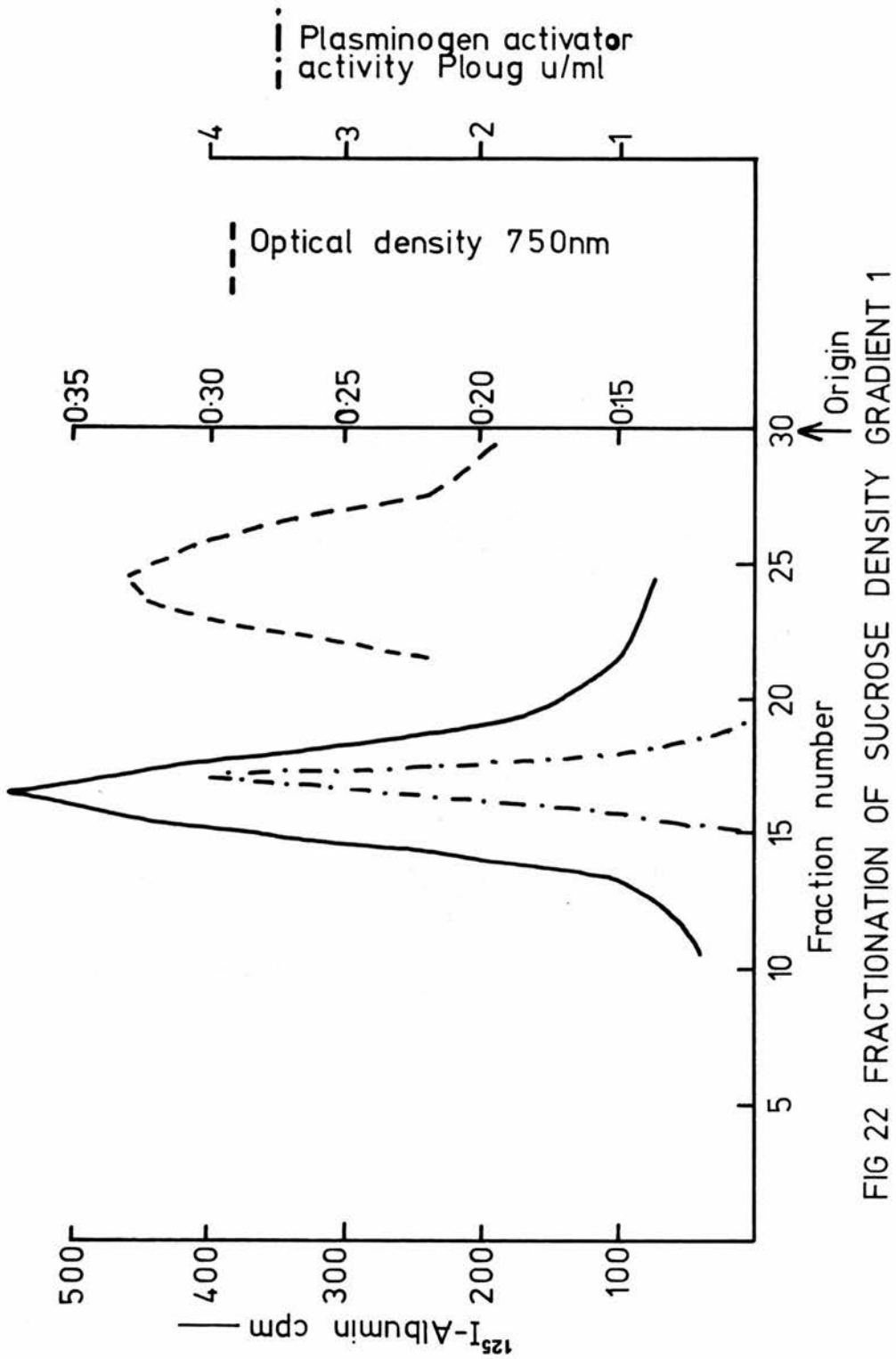


FIG 22 FRACTIONATION OF SUCROSE DENSITY GRADIENT 1

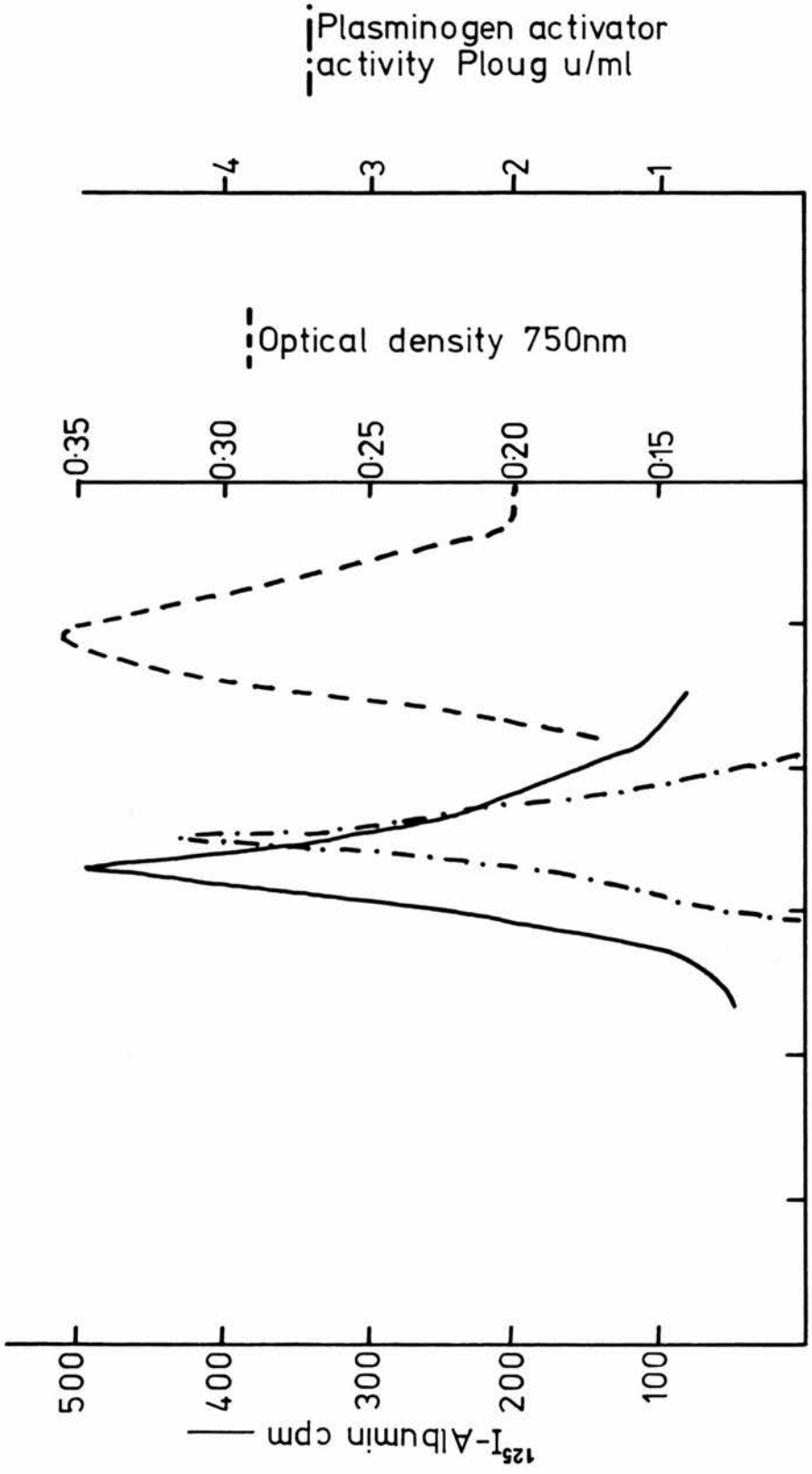


FIG 23 FRACTIONATION OF SUCROSE DENSITY GRADIENT 2

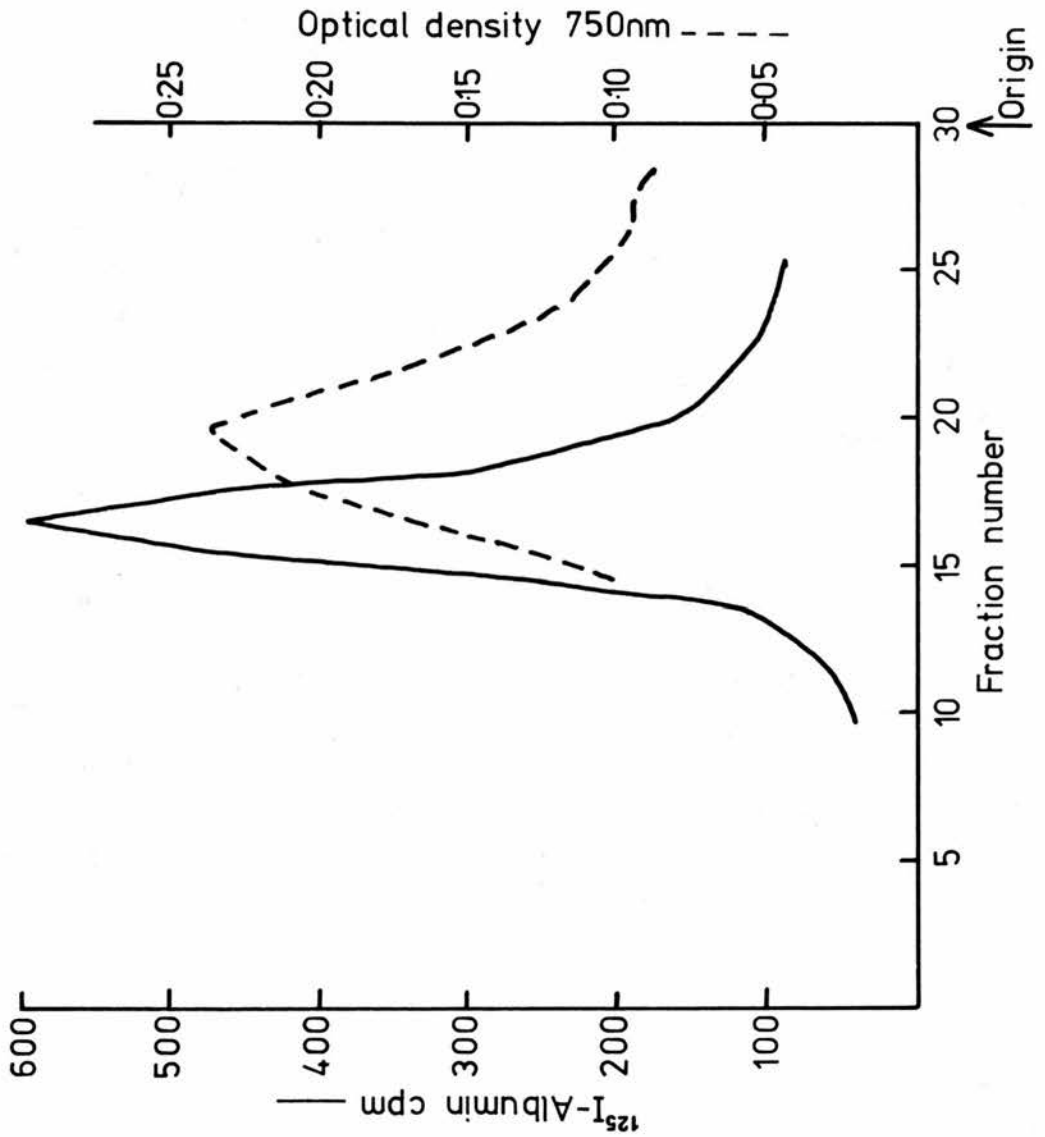


FIG 24 FRACTIONATION OF SUCROSE DENSITY GRADIENT 3

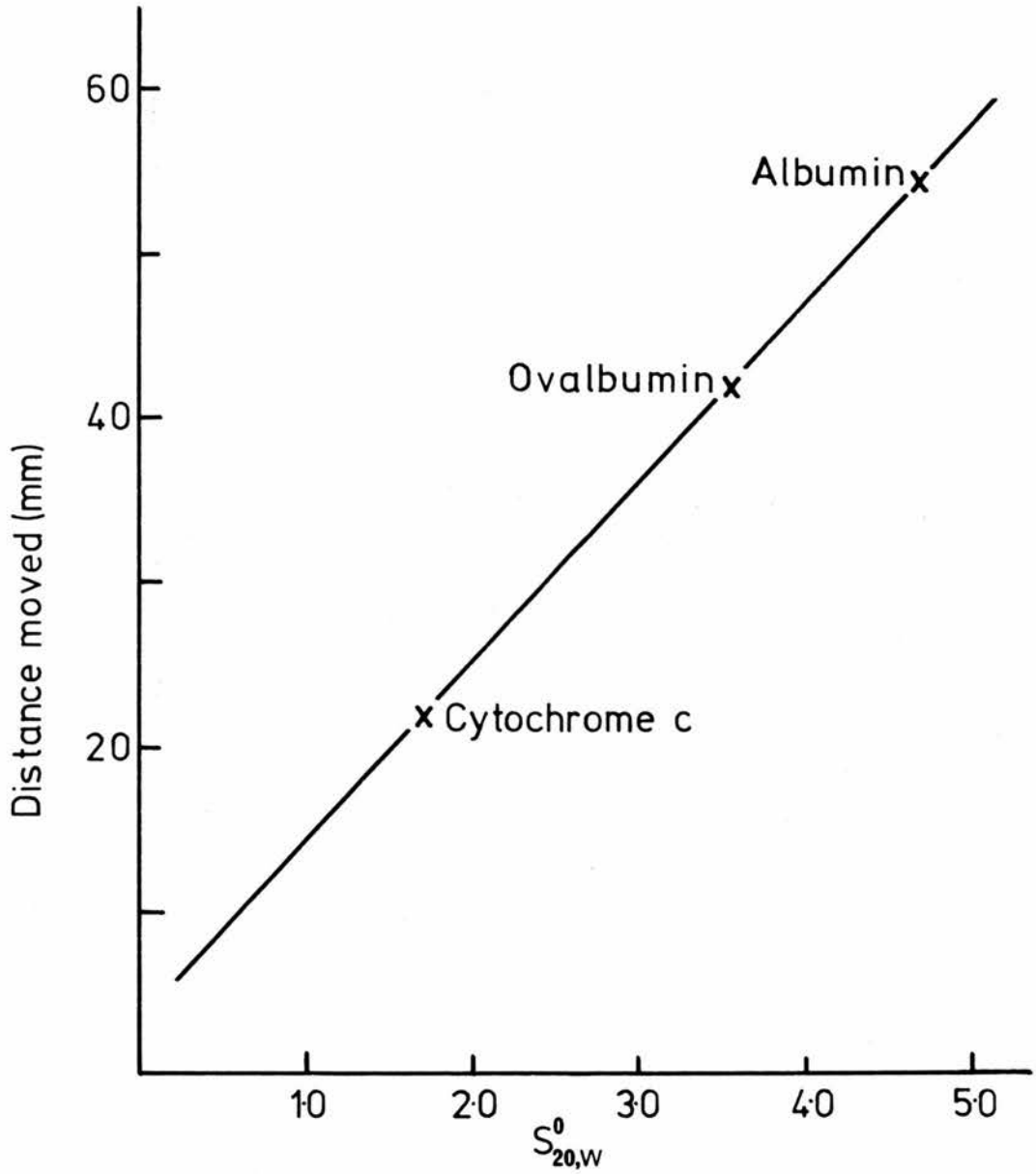


FIG 25 CALIBRATION OF SUCROSE DENSITY GRADIENTS WITH STANDARDS OF KNOWN $S^0_{20,w}$

C CALCULATION OF MOLECULAR WEIGHT AND FRICTIONAL RATIO

The molecular weight was calculated from the sedimentation and diffusion coefficients by the Svedberg equation. A value of 54,545 was determined for the molecular weight of the plasminogen activator shown in Fig 13, and a value of 1.18 was calculated for the frictional ratio ($^{f}/_{fo}$).

D DISCUSSION

The physical data determined for the vascular plasminogen activator purified by the initial purification method (Figs 11-13) is shown in Table 8. The accuracy of the determined r_s value is dependant on the elution position of the molecule in question and the accuracy of the r_s values of the standards. Ideally the molecule whose r_s value is to be determined should elute with a Kd as near to 0.5 as possible. Various gels of different pore size can be used to achieve this. The Sephadex G-200 column was ideal for the activator which eluted with a Kd of 0.48.

The molecular weight of the activator purified by the initial purification scheme (Figs 11-13) was determined to be 54,545. An accurate estimate of the molecular weights of the activator in the improved purification scheme (Figs 14 and 15) and the venous occlusion activator could not be made as the sedimentation coefficients of these activators were not determined. However, as accurate diffusion coefficients were calculated for these activators, a rough estimate of molecular weight can be made using the $S^{o}_{20,w}$ value determined for the activator in Fig 13. This gives molecular weights of 61,500 for the activator produced by the improved purification scheme (Figs 14 and 15) and approximately 75,000 for the activator produced by venous occlusion.

Stokes Radius (r_s) nm	2.97
Diffusion Coefficient ($D^{o}_{20,w}$) Fick's	7.22
Sedimentation Coefficient ($S^{o}_{20,w}$) Svedberg's	4.40
Molecular Weight (Mr)	54,545
Frictional Ratio ($^{f}/_{fo}$)	1.18

TABLE 8 PHYSICAL PROPERTIES OF THE VASCULAR PLASMINOGEN ACTIVATOR

The frictional ratio of 1.18 calculated for the activator shows that the molecule is of compact shape. Globular proteins generally have values up to about 1.3; values greater than 1.3 cannot be accounted for by hydration and indicate asymmetry of the molecule.

In the range of sucrose concentrations used to determine the sedimentation coefficient, the viscosity increases in an almost linear manner; only at higher concentrations does the viscosity increase become significantly non-linear. $S^{o}_{20,w}$ refers to the sedimentation coefficient corrected for density and viscosity to water at 20°C. The method used is self-correcting for temperature deviations from 20°C. This means that the determination of the sedimentation coefficient at 4°C by comparison with standards of known $S^{o}_{20,w}$, also run at 4°C, gives $S^{o}_{20,w}$ directly. Also temperature differences within the gradient have no effect on the results obtained; the accuracy of the results are dependant mainly on the accuracy of the $S^{o}_{20,w}$ values of the standards.

2 ELECTROPHORETIC STUDIES

A POLYACRYLAMIDE ELECTROPHORESIS

Polyacrylamide gradient gels, of 4-30% acrylamide, were used to investigate many activator preparations, both pure and impure.

The samples to be run were concentrated (if necessary) firstly by ultrafiltration under pressure and then by using Minicon A25 concentrators (Amicon). Using these the samples could be reduced in volume to 25µl or less. To this was added 10µl of a bromophenol blue/sucrose mixture, before the sample was applied to the gel. The bromophenol blue acts as a marker enabling visualisation of the movement of the leading edge during electrophoresis, and the sucrose is to increase the density of the sample to avoid mixing with the tank buffer.

The gels were normally run at 100 volts (although 50 volts and 75 volts were also used). The buffer was circulated through a cooling tank at $4^{o}C$.

After electrophoresis the gels were placed in distilled water for half an hour to remove the buffer salts. They were then sliced into two equal halves (front - back); one half could then be stained for protein while the other half was assayed for activator activity. The stain used was 0.04% Coomassie blue G-250 in 3% $HClO_4$. The activator

activity was assayed in a number of ways. Firstly the gel was simply overlayed with fibrin (the same reagents were used as for the fibrin plate), incubated at 37°C, and lysis visualised with bromothymol blue dye in 50% ($^{v}/_{v}$) methanol. The activity was also localized by slicing the gel into 30 equal slices (approximately 2.5mm wide) and extracting the activity by macerating each slice into 0.5ml 0.025M tris-1M NaCl pH7.5 and freezing and thawing. The extracts were then screened on fibrin plates (see Fig 26a). Finally a radiolabelled sample of activator was run, the gel was cut into 15 equal slices (approximately 5mm wide) and each slice counted for iodine-125 (see Fig 26b).

The purest samples always showed no detectable protein bands on staining, but still had biological activity. The activator activity was not sharply localized, but was usually smeared from the point of application down through the gel. Fig 26a shows the result of a fibrin plate screen of activator extracted from 30 slices of a polyacrylamide gel; as can be seen all the activity is in the first 5 slices from the point of application.

Fig 26b shows the γ-count of 15 slices of a polyacrylamide gel after an iodinated activator sample had been run. Again most of the counts appeared in the first two slices from the point of application, but also a small peak appears at slice number 11. This could represent labelled activator but also could be a labelled impurity, such as albumin.

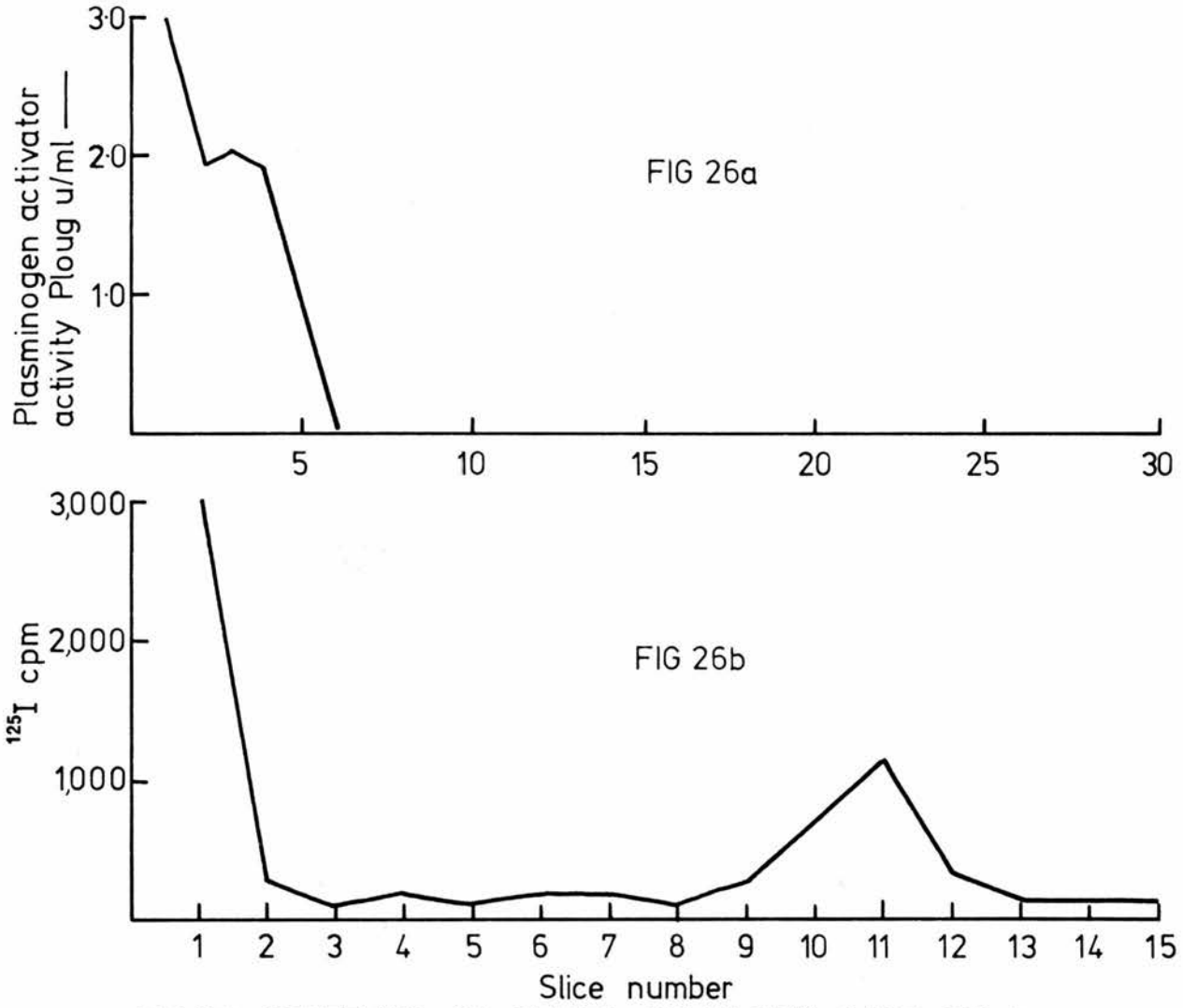


FIG 26 SCREENING OF SLICED POLYACRYLAMIDE GELS

At first the reason for the majority of the activity remaining at or near the point of application was thought to be aggregation. It was only later when it was realised that the activator was obtained complexed with soluble fibrin polymers that it was clear that this could also explain the activator's behaviour on polyacrylamide gel electrophoresis. It would only require a trace of soluble fibrin to recombine with the activator when lysine was removed to reform an activator complex of high molecular weight and account for its slow mobility in the gel. Furthermore, if the activator was combined with soluble fibrin polymers (SFP), the heterogeneity of the activator, due to the breakdown of the carrier fibrin polymers, would account for the smearing of activity observed when the gel was overlayed with fibrin.

B CELLULOSE ACETATE ELECTROPHORESIS

Cellulose acetate electrophoresis was used to investigate the mobility of the activator.

Concentrated crude perfusates and an iodinated activator sample with plasma added were run, and in every case the fibrinolytic activity and γ-counts were located at the origin. Originally this was thought to indicate γ-mobility of the activator; however this could also be explained by the activator existing in a complex with soluble fibrin. This would certainly be the case with the crude perfusate samples, thus the limited porosity of cellulose acetate and the slow electrophoretic migration of fibrin(ogen) would combine to give γ-mobility.

C AGAROSE GEL ELECTROPHORESIS

Electrophoresis of the activator was carried out in 1% agarose gels.

Duplicate samples were run to allow, after division of the gel, staining for protein as well as fibrin overlay for location of activator activity. Fibrin overlay revealed the activator activity was again at the origin.

D DISCUSSION

Originally it was thought that the γ-mobility determined for the activator was a true observation as mobility was the same whether the substrate was cellulose or agarose, and in 0.5M tris as well as 0.025M tris. However when it was appreciated that the activator was being obtained as a complex with soluble fibrin polymers, the electrophoretic behaviour of the activator became explicable.

The heterogeneity and inability to sharply localize the activator activity was directly attributable to the breakdown profile of the carrier fibrin polymers, giving the activator activity a wide range of molecular weights on polyacrylamide electrophoresis. The high molecular weight complexes could also be responsible for the apparent γ-mobility on cellulose acetate and agarose gel electrophoresis.

3 BEHAVIOUR WITH VARIOUS CHROMOGENIC SUBSTRATES

Synthetic chromogenic substrates are a series of tri- and tetra-peptide structures terminated in a p-nitroanilide (pNA) group (see Table 9). The synthetic substrates, to some extent, mimic the susceptible bond(s) in the natural substrates of a range of enzymes. The enzyme cleaves the synthetic substrate releasing the pNA group giving rise to a change in optical density at 405nm.

Various synthetic chromogenic substrates have been investigated for use as an assay system for the vascular plasminogen activator. The method for the determination of activity is based on the difference in optical density between the pNA formed and the original substrate. The rate of pNA formation, measured by the increase in optical density per second at 405nm, is proportional to the enzymatic activity.

Fig 27 compares the activity of a sample of activator on various chromogenic substrates. The activator sample used in this comparison was a resuspended PEG precipitate purified on lysine-Sepharose; it had an activity of 50 Ploug u/ml of which approximately 5 Ploug u/ml were later found to be due to plasmin contamination. This sample is also contaminated by a non-specific enzyme activity which cleaves most of the synthetic substrates tested, even in the presence of 10mM DFP. However this activity is not active fibrinolytically.

S-2227	H-D-glutamyl-L-glycyl-L-arginyl-p-nitroanilide HCl
S-2238	H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilide 2HCl
S-2251	H-D-valyl-L-leucyl-L-lysine-p-nitroanilide HCl
S-2302	H-D-prolyl-L-phenylalanyl-L-arginyl-p-nitroanilide HCl
S-2322	H-D-L-valyl-L-glycyl-L-arginyl-p-nitroanilide HCl
S-2444	L-pyroglutamyl-L-glycyl-L-arginyl-p-nitroanilide HCl

TABLE 9 THE COMPOSITION OF SOME SYNTHETIC CHROMOGENIC SUBSTRATES

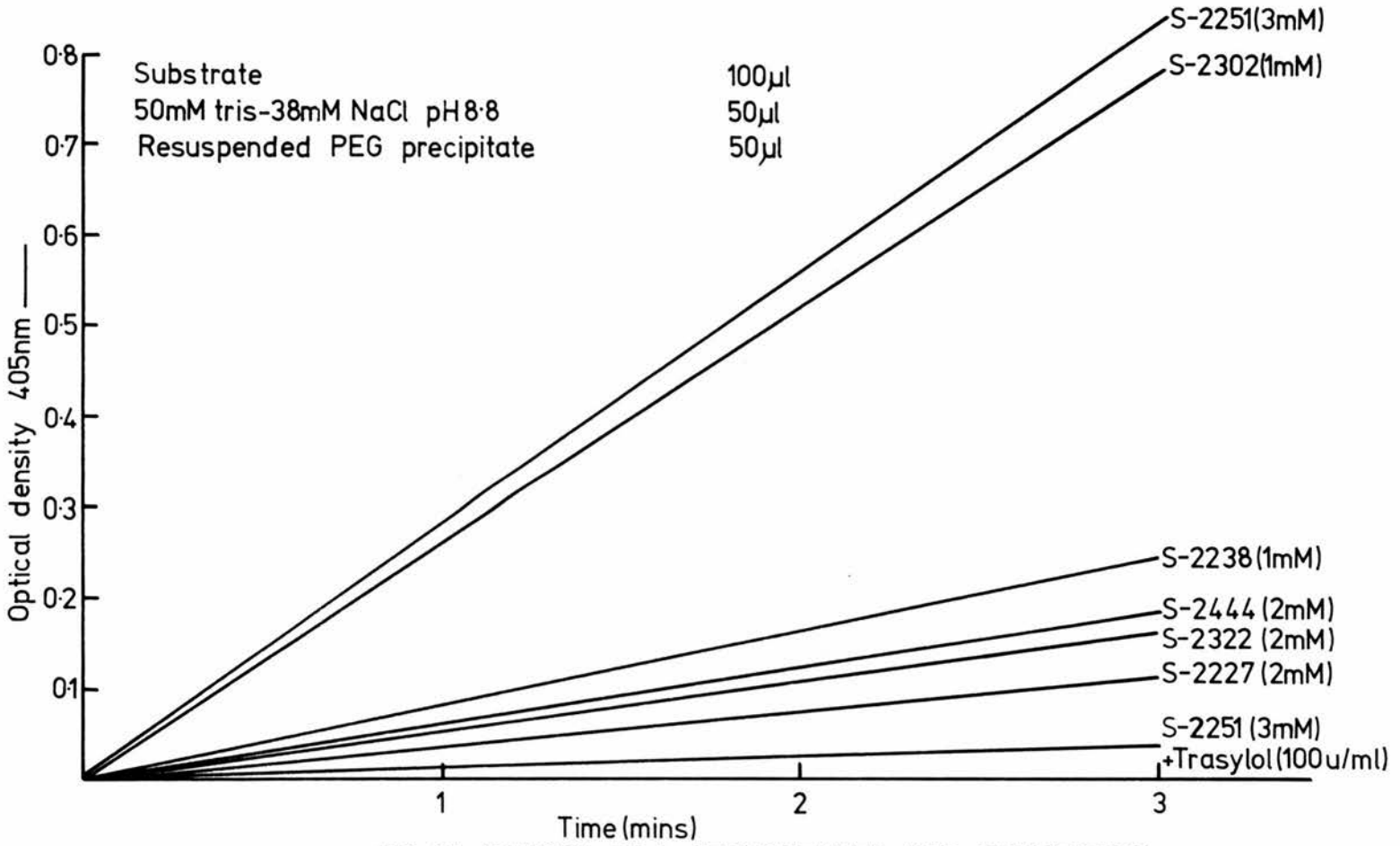


FIG 27 ACTIVITY OF A RESUSPENDED PEG PRECIPITATE ON VARIOUS CHROMOGENIC SUBSTRATES

Thus at first glance at Fig 27 it would appear that S-2251 (plasmin substrate) and S-2302 (kallikrein substrate) were the best substrates for the vascular activator. However S-2251 was used at 3mM, whereas the other substrates were used at 1 or 2mM and also when the activator sample was tested on normal and plasminogen-free fibrin plates it was found to be contaminated with plasmin. When the reaction was set up again containing the plasmin inhibitor Trasylol at a final concentration of 100 kiu/ml the change in optical density in 3 minutes with S-2251 was the lowest obtained with any of the substrates tested. (Trasylol does not inhibit the activity of the vascular activator on synthetic substrates - see next Section.) So the large change in optical density was due to the trace plasmin contamination which has a very high activity towards S-2251.

The high change in optical density obtained with S-2302, although only used at 1mM, can largely be attributed to the uncharacterized non-specific activity present in many of the activator samples tested. Reference to Table 10 shows that S-2302 is particularly susceptible to this activity. The same observation can be made for S-2238 (thrombin substrate), although the change in optical density is much smaller. This leaves S-2444, S-2322 and S-2227, all urokinase substrates, as the best for the vascular activator.

Table 10 compares the activity of a resuspended PEG precipitate (giving no lysis on a plasminogen-free fibrin

SUBSTRATE	CONC	Δ O.D. AT 405nm IN 3 MINS		% ACTIVITY THAT IS DFP SENSITIVE
		- DFP	+ 10mM DFP	
S-2322	2mM	1.06	0.12	88.7%
S-2444	2mM	0.75	0.19	74.7%
S-2227	2mM	0.26	0.08	69.2%
S-2238	1mM	0.87	0.44	49.4%
S-2251	3mM	0.86	0.52	39.5%
S-2302	1mM	0.78	0.54	30.7%

TABLE 10 ACTIVITY OF A RESUSPENDED PEG PRECIPITATE WITH VARIOUS CHROMOGENIC SUBSTRATES (WITH AND WITHOUT 10mM DFP)

plate) on various synthetic chromogenic substrates. The non-specific activity appears to be resistant to DFP at 10mM (1 hour at RT). Thus by comparing the change in optical density, with and without 10mM DFP, the relative activity of the vascular activator (sensitive to 10mM DFP) for the various substrates can be ascertained. As already stated S-2302, S-2251 and S-2238 have low specificity for the activator as shown by only 30.7%, 39.5% and 49.4% respectively of the activity being inhibited by 10mM DFP. Whereas over 88% and nearly 75% of the cleavage obtained with S-2322 and S-2444 respectively, is due to a DFP inhibitable activity, that is probably largely, if not entirely, represented by the vascular activator.

This non-specific activity was first encountered when attempting to quench the activity of various plasminogen activators with a range of antisera, and measuring the residual activity on chromogenic substrates. Rather than quenching the activity, the antisera-treated activators had far greater optical density changes than controls that had not been treated with antisera. This activity was still present when IgG fractions were prepared by protein A affinity chromatography. The activity was also present in normal human serum and rabbit serum and was only partially inhibited by 10mM or 20mM DFP either after 1 hour or overnight incubations. However, 1-1$\frac{1}{2}$ hour incubation at 56^{o}C removed nearly all the activity from both normal human serum and IgG fractions of antisera. By contrast very little of this uncharacterized activity was present

in normal human plasma.

An attempt was made to screen the fractions of column eluates using chromogenic substrates. However this was largely unsuccessful due to the presence of non-specific activities. The fractions obtained on elution of a resuspended PEG precipitate from hydroxyapatite were screened with S-2444 and two peaks of activity were obtained. One broad peak that did not bind and one smaller peak that overlapped the vascular activator as judged by a fibrin plate screen. A similar result was obtained when S-2444 was used to screen a lysine-Sepharose elution; in this case the sample was activator remaining in the supernatent after fibrin formation (from added fibrinogen and thrombin) in a resuspended PEG precipitate. The peak that did not bind could be due to the added thrombin. When the fractions from the hydroxyapatite run (above) were further purified on lysine-Sepharose, the fractions were screened with S-2322. Only one peak of activity was obtained and this did not bind to lysine-Sepharose. However the activity of the vascular activator was low (as judged by a fibrin plate screen) and was possibly too low to be detected by S-2322 with the incubation time used.

Thus it would appear that for the synthetic chromogenic substances to be of use in screening fractions of column eluates the non-specific enzymatic activities must firstly be identified and secondly specific inhibitors be incorporated into the assays. So far none have been found to be entirely satisfactory.

4 INHIBITION STUDIES

A USING THE FIBRIN PLATE

The vascular activator has been further characterized by treatment with a range of inhibitors. Both purified and crude activator samples (after excluding plasmin contamination on a plasminogen-free fibrin plate) have been investigated, and the results obtained are the same.

When purified samples of activator were used, 2µl of inhibitor were added to 0.2ml of sample. Whereas when crude samples of activator were used, generally resuspended PEG precipitates, the volumes used were scaled up, making dialysis easier. In these cases 100µl of the inhibitor was added to 0.9ml sample and this was incubated for 18 hours at 4°C. Then Tween 20 was added to a final concentration of 0.1% and the mixture dialysed at RT for 6 hours, against 2 (x 5 litre) changes of 25mM tris, 1mM EDTA, 3.8mM borate, 0.15M NaCl pH8.8. In some cases a longer period of dialysis was required and this was against further changes of the same buffer.

Precipitation problems were encountered with some of the inhibitors using dimethylsulphoxide (DMSO) as solvent. These were set up again using 0.5ml sample and 5µl of a more concentrated inhibitor solution. The same incubation and dialysis conditions were used.

After dialysis, the mixtures and urokinase standards were screened on fibrin plates, incubated for 20 hours at 37°C. Solvent controls of distilled water and DMSO were set up and treated in exactly the same way as the tests, to correct for any non-specific effects.

Table 11 gives the percentage inhibition obtained.

INHIBITOR	FINAL CONCENTRATION	DURATION OF DIALYSIS (HRS)	% INHIBITION
Val-Pro-Arg CH_2Cl	0.1mM	6	98
Trasylol	100kiu/ml	6	92
		30	53
		54	4
*SBTI	10ug/ml	6	32
		30	29
		54	25
EACA	10mM	6	11
		30	16
L-Lysine	10mM	6	16
		30	0
L-Arginine	10mM	6	0
DFP	10mM	6	98
PMSF	10mM	6	87
	1mM	6	22
DTT	10mM	6	80
TPCK	10mM	6	34
	1mM	6	0
TLCK	10mM	6	3
	1mM	6	0
Iodoacetamide	10mM	6	0
*PNPGB	10mM	6	30-100
	1mM	6	70-100

* See text for explanation of values obtained.

TABLE 11 PERCENTAGE INHIBITION OF THE VASCULAR ACTIVATOR ASSAYED ON THE FIBRIN PLATE

B USING CHROMOGENIC SUBSTRATES

To obtain a meaningful inhibition profile of vascular activator with synthetic chromogenic substrates the sample of activator used must be free of any non-specific activities which are likely to cleave the substrate and also be affected by the inhibitors. For most of the studies S-2322 was used; however S-2444 was used with the melanoma activator.

The inhibitor was added to the sample and incubated at 37°C. At 30 minute intervals 50µl of this reaction mixture was removed and added to 50µl buffer and 100µl chromogenic substrate (also at 37°C). Approximately 150µl of this was transferred to a micro cuvette in a thermostatted cell (37°C) and the change in optical density at 405nm recorded at regular time intervals. The inhibition obtained after half an hour, one hour, one and a half hours and two hours incubation altered very little.

Inhibition of the vascular activator is shown in Figs 28 and 29. Inhibition of urokinase (Figs 30 and 31) and the melanoma activator (Fig 32) was also investigated for comparitive purposes.

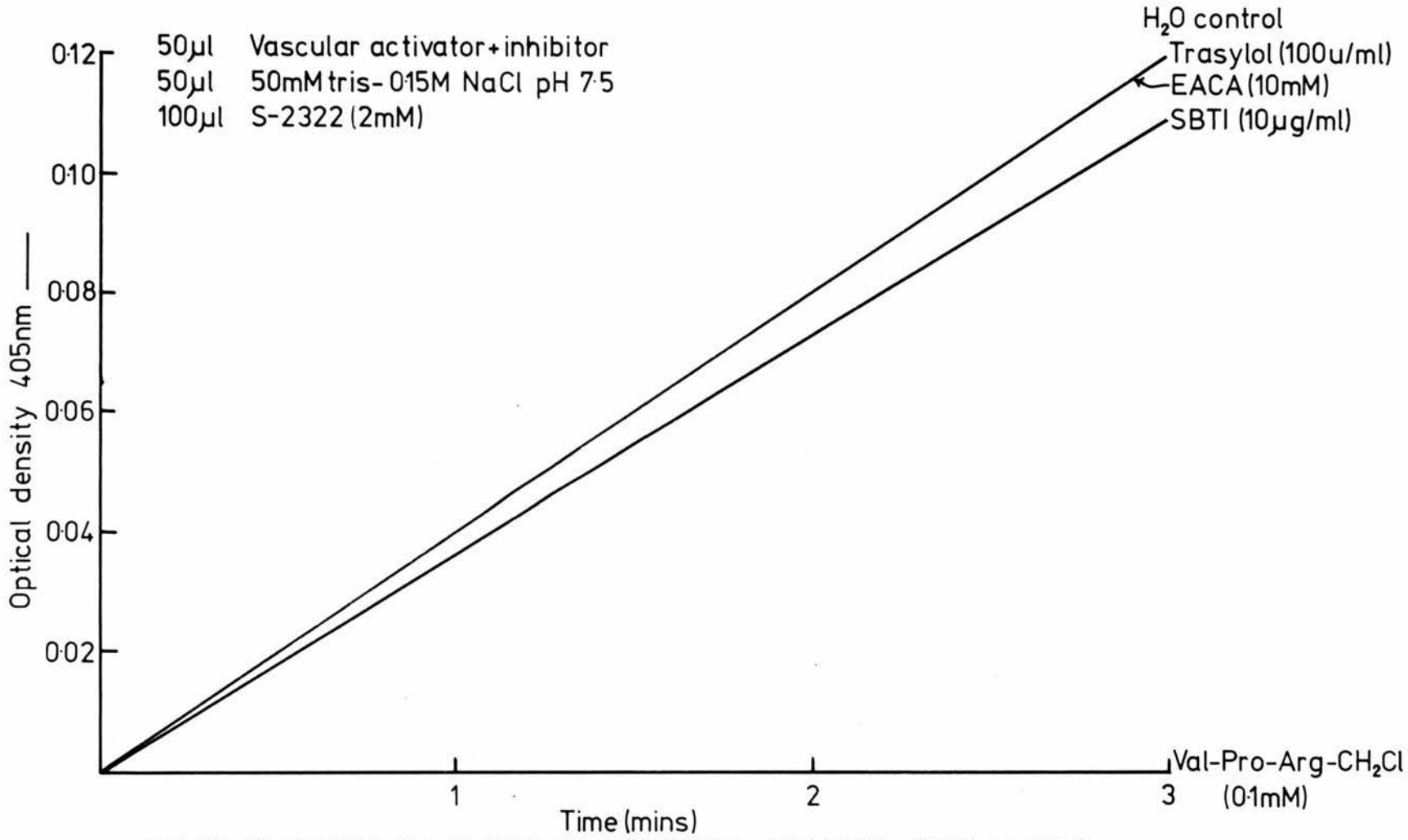


FIG 28 INHIBITION OF VASCULAR ACTIVATOR ASSAYED WITH S-2322

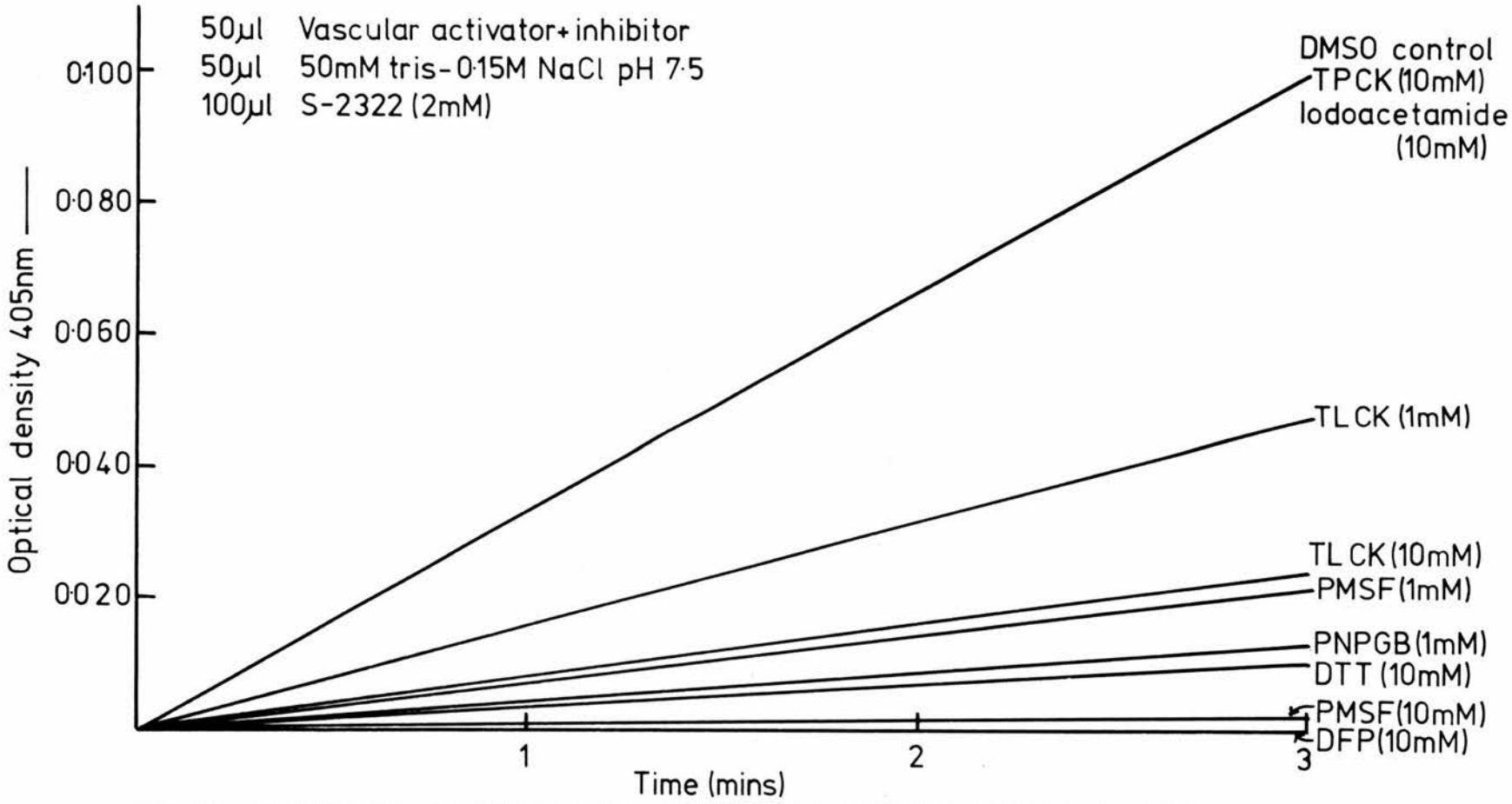


FIG 29 INHIBITION OF VASCULAR ACTIVATOR ASSAYED WITH S-2322

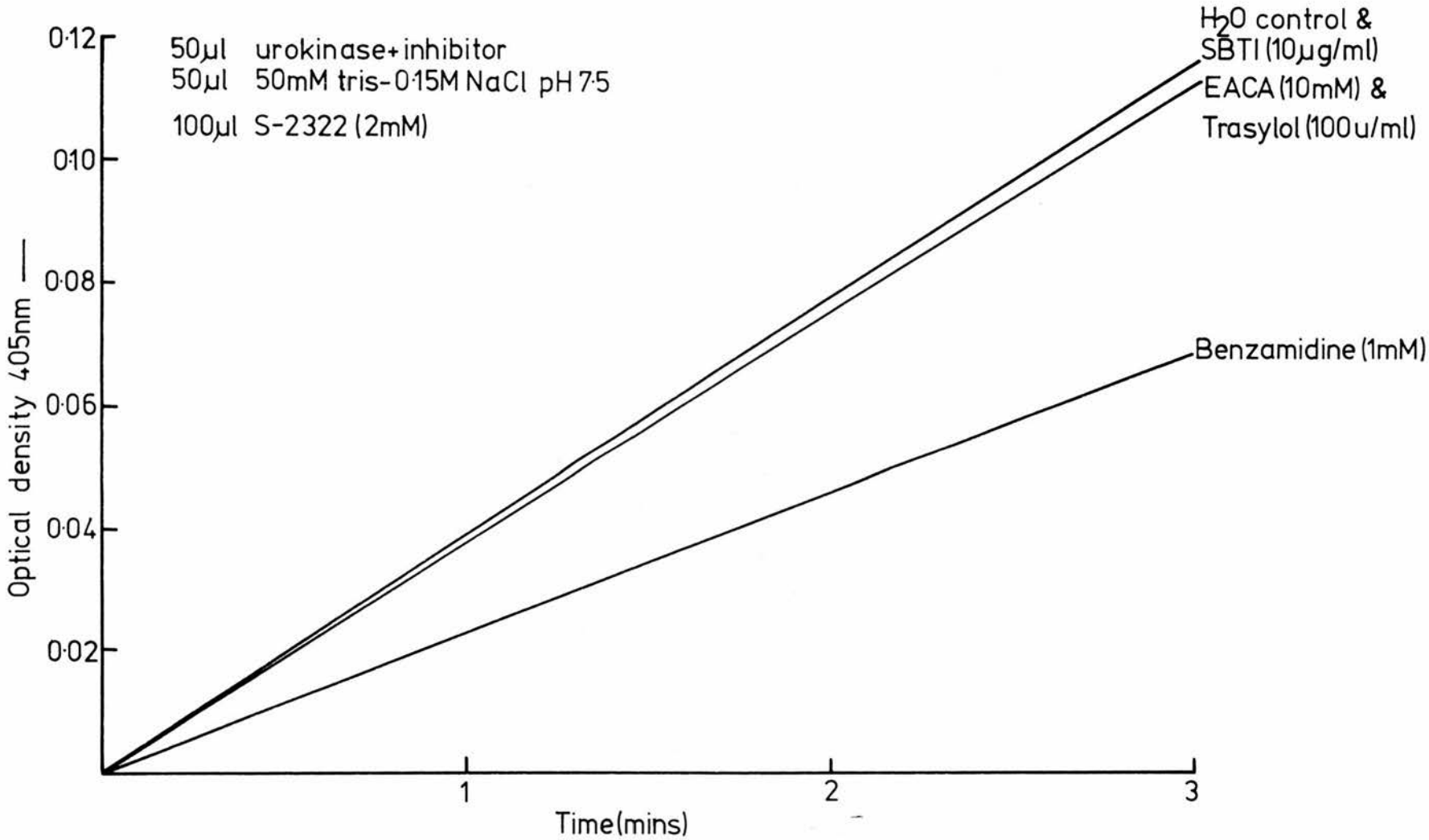


FIG 30 INHIBITION OF UROKINASE ACTIVITY ASSAYED WITH S-2322

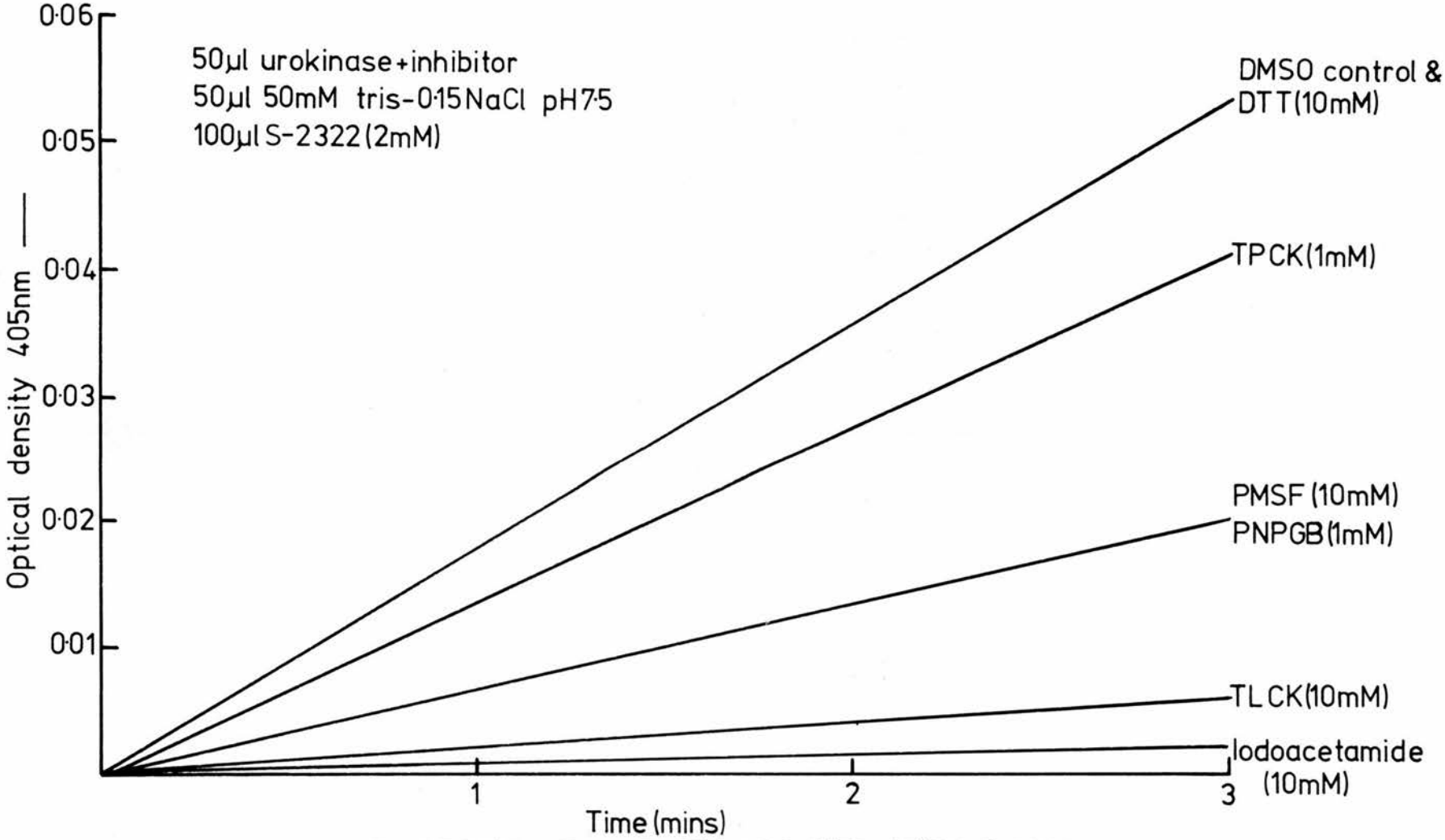


FIG 31 INHIBITION OF UROKINASE ACTIVITY ASSAYED WITH S-2322

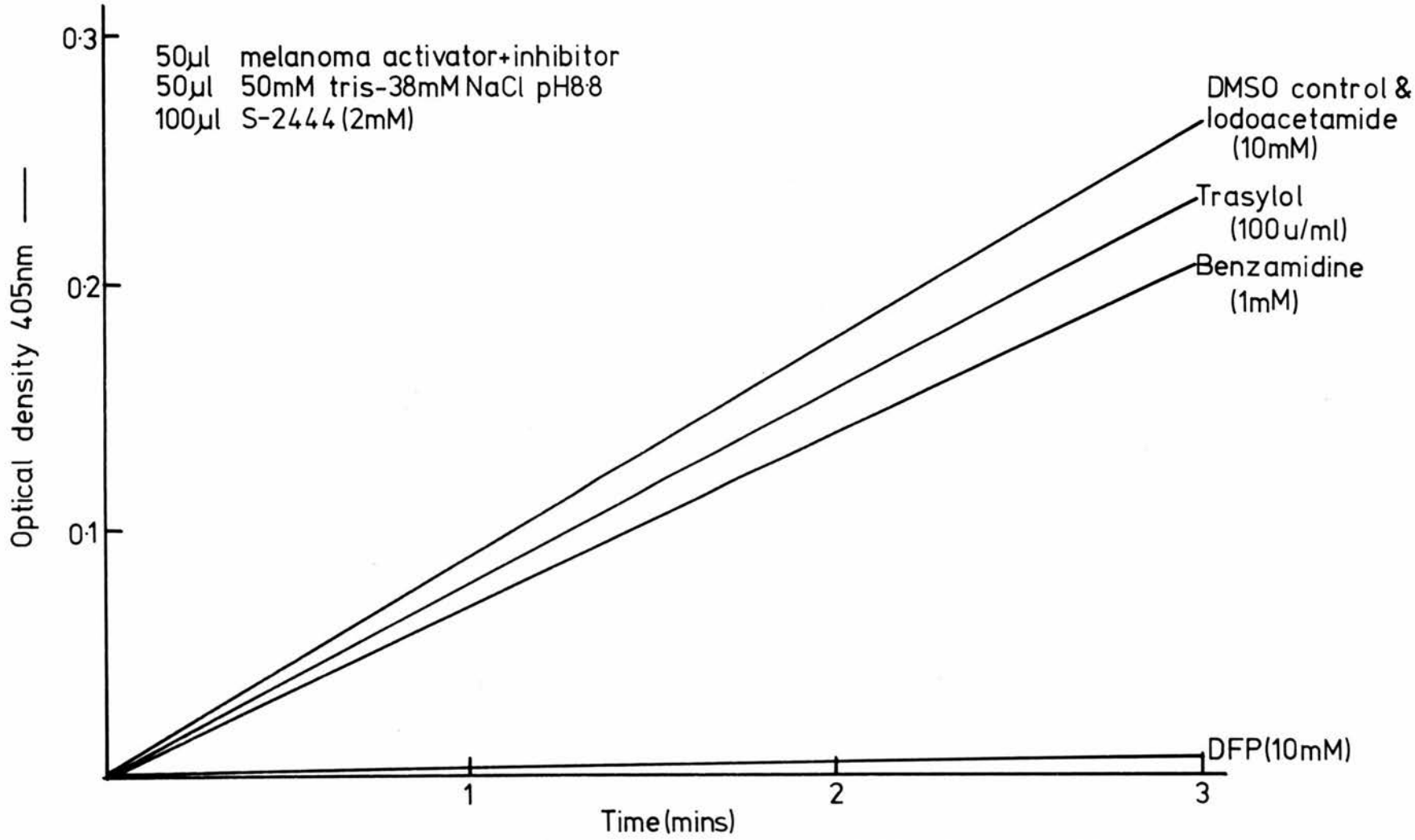


FIG 32 INHIBITION OF THE MELANOMA ACTIVATOR ASSAYED WITH S-2444

C DISCUSSION

Strong inhibition of the vascular activator was obtained on the fibrin plate with the synthetic inhibitor Val-Pro-Arg-CH_2Cl (gift from Dr Shaw), with the serine protease inhibitors DFP and PMSF and also with the disulphide bond reducing agent DTT.

Strong inhibition (92%) was found with Trasylol after 6 hours dialysis; however this was reduced to 53% after 30 hours and 4% after 54 hours dialysis. Trasylol is very slow to dialyse due to its molecular weight (6,500); the molecular weight cut off point of the dialysis tubing is in the region of 14,000. The apparent inhibition seen initially is mediated through Trasylol's inhibition of the plasmin formed in the fibrin plate. The same situation occurs with SBTI, which is also a plasmin inhibitor. The molecular weight of SBTI is 24,000 and thus is non-dialysable.

A modest inhibition is seen with EACA which appears to increase slightly on further dialysis. If this sample was applied to a fibrin plate, prior to dialysis, then considerable inhibition would result. Some activity may still be measurable as the EACA (molecular weight 131.18) diffuses through the fibrin plate more quickly than the vascular activator (molecular weight 54,500) and this tends to lower its local concentration. However if EACA is incorporated into the fibrin plate at 10mM, then total

inhibition is seen, of all but the most active samples. Trans-AMCHA gives the same inhibition at a 10-fold lower concentration. EACA affects the components of the fibrinolytic system in a number of ways. It can induce a conformational change in the native plasminogen molecule that causes it to be more easily activated to plasmin. It is also involved in the interactions of plasminogen and the vascular activator with fibrin, which are mediated through the lysine binding sites. L-lysine exerts a similar effect, although EACA is effective at a lower concentration. The slight inhibition seen with L-lysine after 6 hours dialysis, is abolished after further dialysis. However this does have implications for the screening of column fractions, when the elution gradient is of L-lysine and the fractions are screened on fibrin plates without dialysis. No inhibition was seen with L-arginine.

TLCK, a trypsin inhibitor that also inhibits plasmin, gave no inhibition at 1mM and only 3% at 10mM. However, TPCK, a chymotrypsin inhibitor, although giving no inhibition at 1mM gave 34% inhibition at 10mM; the significance of this remains unclear. Precipitation problems were encountered with some of the DMSO-soluble inhibitors, TPCK was one of these; another was PNPGB (plasmin active site titrant). The results with PNPGB were very variable. 10mM PNPGB gave 30-100% inhibition and 1mM gave 70-100% inhibition, but an immediate yellow colour and a heavy precipitate were always obtained, and the inhibition

observed could be due to precipitation of activator activity rather than inhibition.

Most importantly, no inhibition was seen with 10mM iodoacetamide which clearly distinguishes the vascular activator from urokinase, which is totally susceptible to iodoacetamide.

The inhibition profile of the vascular activator determined with S-2322 (Figs 28 and 29) was generally consistent with that obtained using the fibrin plate, although there were a few discrepancies. Strong inhibition was again obtained with the synthetic inhibitor (Val-Pro-Arg-CH_2Cl), DFP, PMSF and DTT. No inhibition was seen with Trasylol and only very slight inhibition with SBTI, in both cases their plasmin-inhibition could not be exercised in this system. There was no inhibition seen with EACA using chromogenic substrates, as the lysine binding sites play no part in the interaction between the vascular activator and S-2322. As with the fibrin plate screen, the vascular activator was unaffected by 10mM iodoacetamide and 1mM PNPGB caused considerable inhibition. The discrepancies involved TLCK and TPCK. TLCK which had shown 0% and 3% inhibition (at 1mM and 10mM respectively) on the fibrin plate, gave 52% and 76% inhibition respectively, using chromogenic substrates; and TPCK which at 10mM gave 34% inhibition using fibrin plates, but gave no inhibition when used at the same concentration on S-2322. The significance of these results, if any, is not known, but solvent artefacts

are a possibility.

The inhibition profile of urokinase (Leo) determined with S-2322 (Figs 30 and 31) produced some interesting comparisons with the vascular activator. As with the vascular activator, Trasylol, EACA and SBTI showed little or no inhibitory activity. 10mM PMSF and 10mM TLCK gave considerable inhibition of urokinase, as with the vascular activator. However, the most interesting differences involved 10mM iodoacetamide and 10mM DTT. Iodoacetamide which gave no inhibition of the vascular activator, either on fibrin plates or with S-2322, gave 96% inhibition of urokinase; and conversely DTT which gave 80-90% inhibition of the vascular activator gave no inhibition of urokinase.

The inhibition profile of the melanoma activator determined with S-2444 (see Fig 32) was similar to the vascular activator and urokinase in that it showed no inhibition with Trasylol and strong inhibition with DFP. However, the melanoma activator was <u>not</u> inhibited by 10mM iodoacetamide which implies a closer relationship with the vascular activator than with urokinase.

Thus in conclusion the vascular activator is a serine protease that relies on disulphide bonds to maintain its tertiary structure for enzymic activity. EACA and lysine show slight inhibition of the vascular activator on the fibrin plate due to an interaction with the lysine binding sites, and no inhibition is seen with iodoacetamide.

Urokinase does not rely on disulphide bonds for its activity, and in contrast to the vascular activator and melanoma activator is inhibited by iodoacetamide.

5 IMMUNOLOGICAL STUDIES

A INHIBITION OF ACTIVATORS BY VARIOUS ANTISERA

The quenching of a range of activator activities by various antisera was investigated. Two substrates were used to measure residual activity; one was the synthetic chromogenic substrate S-2444, the other was the fibrin plate.

The activator and antiserum to be tested were mixed and incubated for 1 hour at 37°C in a 1:1 ratio. A control of activator and saline was treated in the same way. After incubation 50µl of each of the incubation mixtures was taken and added to 50µl 50mM tris, 38mM NaCl pH8.8 and 100µl 2mM S-2444 (also at 37°C). After mixing, 150µl of this was transferred to a thermostatted (37°C) micro-cuvette and the change in optical density at 405nm recorded at regular time intervals. A further 30µl of each of the incubation mixtures was taken and activity measured on normal fibrin plates calibrated with Leo urokinase.

a Using Chromogenic Substrates

In every case where measurement of residual activity was made on S-2444, the activator-antiserum mixture gave an <u>increased</u> rate of cleavage over the activator-saline control.

This was due to a non-specific activity present in the antisera, which was very active on S-2444 and was not inhibited by 10mM DFP. In an attempt to remove the non-specific activity, IgG fractions of the antisera were prepared by affinity chromatography on protein A-Sepharose, but this was unsuccessful. The non-specific activity was however susceptible to incubation at 56°C for 1 hour (85% inhibited). The non-specific activity was also present in normal human serum and was 75%, 92% and 96% inhibited after half an hour, one hour and one and a half hours respectively, at 56°C. However, even when the heat-treated IgG fractions of the antisera were used to quench the various activator activities, the residual activity, when measured on S-2444, was still greater than the controls. Thus the results are not presented.

b Using the Fibrin Plate

The results obtained using the fibrin plate are summarised in Table 12. Two antisera were raised against partially purified vascular activator preparations. The first antiserum (anti-VPA 1) was raised against a perfusate purified on lysine-Sepharose and Sephadex G-200, and the second antiserum (anti-VPA 2) was raised against a re-suspended PEG precipitate purified on lysine-Sepharose. The anti-human urokinase and anti-pig heart tissue activator antisera were gifts from Dr B Åstedt and Dr P Wallén

FIBRINOLYTIC ACTIVITY / ANTISERUM FRACTION	ANTI-VPA 1 IgG	ANTI-VPA 1 HEAT TREATED IgG (56°C,1 HR)	ANTI-VPA 2 IgG	ANTI-UROKINASE IgG	ANTI-PIG HEART TISSUE ACTIVATOR
Melanoma Activator	0	14	44	27	100
Urokinase	S T I M U L A T I O N (2x)	(2x)	(5x)	38	100
Purified Vascular Activator	NT	0	21	NT	NT
Resuspended PEG Precipitate	47	8	37	6	100
Pig Heart Tissue Activator	NT	30	46	NT	NT
Plasmin	NT	49	41	NT	100

TABLE 12 INHIBITION (%) OF VARIOUS ACTIVITIES WITH A RANGE OF ANTISERA, ASSAYED BY FIBRIN PLATE

respectively. The pig heart tissue activator (PHTA) was a gift from Dr E Cole.

The IgG fraction of anti-VPA 1 appears to inhibit a resuspended PEG precipitate (47%), has no effect on the melanoma activator and stimulates urokinase activity by a factor of 2. However, when the IgG fraction of this antiserum had been heated at 56°C for 1 hour (to remove the non-specific activity) its inhibitory activity against a resuspended PEG precipitate (a different sample) was reduced to 8% and it gave no inhibition of a purified sample of vascular activator. This fraction gave 14% inhibition of the melanoma activator and 30% inhibition of PHTA. Furthermore, it gave 49% inhibition of plasmin.

The IgG fraction of anti-VPA 2 gave some inhibition of all the activities, except with urokinase when a 5-fold stimulation was observed. The inhibition of the purified vascular activator and the resuspended PEG precipitate (21% and 37% respectively) were lower than the inhibition of plasmin (41%), the melanoma activator (44%) and PHTA (46%).

The IgG fraction of anti-urokinase gave 38% inhibition of urokinase, 27% inhibition of the melanoma activator and 6% inhibition of a resuspended PEG precipitate. Thus the inhibition did appear to be directed mainly against urokinase. The anti-PHTA gave complete inhibition of the melanoma activator, urokinase, a resuspended PEG precipitate and plasmin.

B GEL DIFFUSION STUDIES

Four antisera were set up against a range of antigens. In each case the IgG fraction of the antiserum was used. The IgG fraction of anti-VPA 1 gave strong reactions with normal human serum, normal human plasma, a resuspended PEG precipitate, ascitic fluid, plasminogen and plasmin, but gave no cross-reaction with the melanoma activator, urokinase, PHTA, rabbit serum or the buffer control. The IgG fraction of anti-VPA 2 gave similar results.

The IgG fraction of anti-PHTA only reacted with PHTA and gave no cross-reaction with human serum, a resuspended PEG precipitate, the melanoma activator, urokinase or plasmin. The IgG fraction of anti-urokinase did not give a precipitation reaction with any of the antigens tested, including urokinase.

C DISCUSSION

Partially purified preparations, rather than the most pure samples, were used to raise antisera against the vascular activator. This was because the amounts of activator present in the purest preparations were thought to be insufficient for the raising of antisera. Thus it is not surprising that the antisera do not appear to be very specific.

From the gel diffusion results it can be seen that the anti-VPA antisera raised give precipitation reactions with a number of components in normal human plasma, normal human serum and ascitic fluid as well as with resuspended PEG precipitates. Anti-VPA 2 clearly reacts with two antigens in a partially purified sample of vascular activator. Both antisera cross-react with plasmin(ogen) and are quite likely to recognise fibrin(ogen), as this certainly would have been a contaminant in the samples used to raise the second batch of antiserum, and possibly also the first.

The most likely antigens that are being recognised in the ascitic fluid are fibrin(ogen), the degradation products (FDP's) and plasmin(ogen). No plasminogen activator activity was found in any of the ascitic fluids tested on fibrin plates. It is presumably these same antigens, among others, that are recognised in normal human serum, normal human plasma and the resuspended PEG precipitates.

However, no cross-reaction was noted with the melanoma activator, urokinase or PHTA.

The IgG fraction of anti-VPA 1 gives 47% inhibition of the activity of a resuspended PEG precipitate, while giving no inhibition of the melanoma activator. The reaction between the antiserum fraction and the resuspended PEG precipitate was accompanied by an immediate precipitation. Thus, the inhibition seen could at least partly be explained by precipitation of fibrin, either immunologically or otherwise, and the associated precipitation of activator activity due to its affinity for fibrin.

When the IgG fraction of anti-VPA 1 was incubated at 56°C for 1 hour (to inactivate the non-specific S-2444 activity) and then re-tested on fibrin plates with the activators, some changes occurred. 14% inhibition of the melanoma activator appeared (Table 12), and the inhibition of a resuspended PEG precipitate dropped from 47% to 8%. However this was a different resuspended PEG precipitate, and if the inhibition was due to precipitation of fibrin, it could be that less was present in this particular sample. Alternatively, an unknown thermolabile protease inhibitor could have been responsible for the effect. However, this fraction still gave inhibition of PHTA (30%) and plasmin (49%).

The IgG fraction of anti-VPA 2 gave inhibition of the melanoma activator (44%) and PHTA (46%). This was unexpected

as no cross-reaction with these antigens was noted when run in gel diffusion. However, the concentrations of the antigens may have been too low to detect a reaction. This fraction gave 37% and 21% inhibition of a resuspended PEG precipitate and a partially purified sample of vascular activator respectively. However this antiserum, as did the first raised, quenched the activity of plasmin, and this raises the possibility that any quenching of the activator activities, as judged by the fibrin plate, may be due to inhibition of formed plasmin.

The IgG fractions of both anti-VPA antisera produced a stimulation of the activity associated with urokinase. There was a two-fold increase with anti-VPA 1 and a five-fold increase with anti-VPA 2. This is difficult to explain but could be due to the conversion of urokinase to a more active form by a factor present in the IgG fraction. However if this was the case, the agent is most likely to be an enzyme, and the enhancing effect is still present after incubation at 56^{o}C for one hour. Other possibilities include the presence of a cofactor in the IgG fraction or that urokinase may activate a proactivator. As can be seen from the foregoing discussion the vascular activator antiserum inhibition studies are presently unsatisfactory.

The anti-PHTA gave 100% inhibition of the melanoma activator, urokinase, a resuspended PEG precipitate and plasmin when assayed on a fibrin plate. However the whole antiserum was used and not an IgG fraction, thus the inhibition

seen is most probably due to a plasmin inhibitor in the serum acting on the plasmin formed in the fibrin plate. The IgG fraction gave a precipitation reaction with PHTA in gel diffusion and no reaction with human serum, a resuspended PEG precipitate, the melanoma activator, urokinase or plasmin. This substantiates the idea that the inhibition seen with the whole antiserum was not entirely immunological.

The IgG fraction prepared from anti-urokinase inhibits urokinase 38% (although no precipitation reaction was seen in gel diffusion), the melanoma activator 27% and the activity of a resuspended PEG precipitate 6%. These values suggest that the melanoma is immunologically more like urokinase than the vascular activator, which is in contrast to inhibition with iodoacetamide, which indicated a closer relationship between melanoma activator and the vascular activator than melanoma activator and urokinase.

6 RADIOACTIVE LABELLING OF THE ACTIVATOR

A IODINATION

Three different methods were used in an attempt to iodinate (^{125}I) the vascular activator. They were the chloramine T method, succinimide ester iodination, and the double enzyme method using lactoperoxidase and glucose oxidase. The activator preparations that were initially used for iodination were purified on lysine-Sepharose, protamine-Sepharose and Sephadex G-200. However later resuspended PEG precipitates, purified on lysine-Sepharose were used as a source of partially purified vascular activator. The preparations after labelling were run on Sephadex G-25 to separate labelled protein, which elutes at V_o, from uncoupled radioactivity.

The labelled preparations were run in the chromatographic systems used for purification of the activator. When the purified activator labelled by the chloramine T method was added to a crude perfusate and adsorbed to lysine-Sepharose approximately 30% of the counts were found to bind and on elution with an exponential lysine gradient the radioactivity was associated with the low affinity peak of vascular activator. However, when this was further purified on protamine-Sepharose, although 75% of the radioactivity did bind, it eluted at a lower NaCl concentration than the vascular activator activity, as judged by fibrin plates. When the partially purified

vascular activator sample was labelled by the chloramine T method no binding to lysine- or benzamidine-Sepharose was observed.

When a purified activator sample was labelled by the succinimide ester iodination, applied to Sephadex G-200 and eluted in a buffer containing 1.5M NaCl, a large peak (75,000cpm) appeared at V_o, a smaller peak (9,000cpm) at V_s and two very small peaks (2,000 and 1,500cpm) with K_d values of 0.42 and 0.58 respectively. A partially purified sample of activator was labelled by the succinimide ester iodination and then applied to protamine-Sepharose but no binding was observed. A further partially purified sample of the activator was labelled by the double enzyme method (lactoperoxidase/glucose oxidase), but showed no binding to lysine- or benzamidine-Sepharose.

Kabi plasminogen was iodinated by the three methods mentioned above as well as by the lactoperoxidase/H_2O_2 method. The best results, in terms of the binding of radioactivity to lysine-Sepharose, were obtained with the chloramine T and double enzyme methods, where $\frac{1}{3}$-$\frac{1}{2}$ of the radioactivity generally was seen to bind. Kabi plasmin was also labelled by the double enzyme method. When this was run on lysine-Sepharose only $\frac{1}{3}$ of the radioactivity was adsorbed and eluted with a lower affinity than unlabelled plasmin.

B DFP LABELLING

Partially purified samples of vascular activator were reacted with both ^{3}H- and ^{32}P-DFP to label the active site. The conditions of the reaction were varied to optimise the reaction. Labelled DFP was used alone and also used in conjunction with cold DFP to increase the concentration to 10mM. The reaction time was varied from one hour to overnight and carried out at 4°C, RT as well as 37°C and at pH7.5. The "labelled" activator was evaluated by chromatography on lysine-, protamine-, fibrin-Sepharose, Sepharose 6B and Sephadex G-200.

Generally the "labelled" activator showed no binding to lysine-, fibrin- or protamine-Sepharose. However on one occasion, when radioactive DFP alone had been reacted with the vascular activator at RT for 22 hours, a peak of radioactivity did bind to lysine-Sepharose which was associated with the low affinity peak of vascular activator as determined on fibrin plates. However when this peak was run on protamine-Sepharose no binding of radioactivity was observed. When the labelled activator was run on Sephadex G-200 in a tris buffer containing 1.5M NaCl, the radioactivity eluted at V_o. However when run on Sepharose 6B in a tris buffer containing 1M lysine, the radioactivity eluted at V_s.

Kabi plasmin was reacted with ^{3}H-DFP with and without cold DFP (to a final concentration of 10mM) for either one

hour at 37°C or overnight at RT and then the binding of radioactivity to lysine-Sepharose was tested. In each case approximately 99.9% of the radioactivity did not bind, and a very small peak of about 200cpm was eluted from lysine-Sepharose with an exponential lysine gradient, generally in the position occupied by unlabelled plasmin.

C DISCUSSION

The attempts to radiolabel the activator were not very successful. The most successful method of iodination was chloramine T; with this method 30% of the radioactivity did bind to lysine-Sepharose, when applied in a crude perfusate, and eluted in the same position as the low affinity peak of activator activity. This low percentage binding to lysine-Sepharose could be due to damage of the lysine binding sites on iodination. A similar proportion of labelled plasminogen was also seen to bind to lysine-Sepharose, although again the chloramine T method gave the best results. When the lactoperoxidase/ H_2O_2 method was used to label Kabi plasminogen no radioactivity was adsorbed when applied to lysine-Sepharose, and when the double enzyme method was used to label Kabi plasmin $\frac{1}{3}$ of the radioactivity bound, but demonstrated a lower affinity than unlabelled Kabi plasmin for lysine-Sepharose. Damaged or modified lysine binding sites could also explain the lowered affinity, or lack of affinity of labelled Kabi plasminogen and plasmin for lysine-Sepharose.

When the radioactivity that eluted from lysine-Sepharose (with the low affinity activator peak) was further purified on protamine-Sepharose, 75% of the radioactivity did bind but eluted earlier in the NaCl gradient than the vascular activator activity. An arginine binding site has been implicated in the in vivo interaction of plasminogen with fibrin (Holleman et al, 1975), and may be involved in the

binding of the activator to fibrin. Alternatively protamine-binding may correlate with benzamidine-binding which was thought possibly to bind via the active site (this work). Whichever is the case, damage of these sites could be the cause of the lower affinity of the activator after labelling. Alternatively the labelled material may have been an impurity in the original sample used for iodination.

When activator, labelled by the succinimide ester method, was run on Sephadex G-200 in a buffer containing 1.5M NaCl, four peaks of radioactivity were obtained at V_o, V_s and two with K_d values of 0.42 and 0.58. The V_o peak was the largest comprising 86% of the total radioactivity. This could be due to aggregation or possibly an interaction of the labelled activator with soluble fibrin polymers, although the 1.5M NaCl in the buffer should have broken this association, unless the labelled activator has a higher affinity for fibrin than the native enzyme. The V_s peak (10% of the total radioactivity) may be due to 'free' activator interacting with the gel matrix. One of the two peaks with K_d values of 0.42 and 0.58 could represent labelled activator with the other being a labelled impurity or both could be activator, with one species being degraded. However each peak only represents approximately 2% of the total radioactivity and would not be very useful for further experiments. Another activator sample labelled by the succinimide ester method showed no binding to protamine-Sepharose. The final method of labelling employed, the

double enzyme method, abolished binding to lysine-Sepharose and benzamidine-Sepharose.

Other reasons for the lack of success in iodinating the activator include the low amounts of the activator present in the samples used and possibly also the absence of aromatic residues in the activator.

Only one sample of activator labelled with ^{3}H-DFP showed any binding to lysine-Sepharose, when applied in a crude perfusate, and again as with the iodinated material, it was associated with the low affinity peak of vascular activator activity. However, when this radioactivity was run on protamine-Sepharose no binding was observed. But, if protamine-binding is via the active site it would be expected that DFP would abolish this binding.

When a DFP-labelled sample of activator was run on Sephadex G-200 in a buffer containing 1.5M NaCl, the radioactivity eluted at V_o. Again any interaction between soluble fibrin polymers and the activator should be abolished by 1.5M NaCl, unless the DFP-labelled activator has an increased affinity for soluble fibrin. In contrast, when samples were run on Sepharose 6B in a buffer containing 1M lysine, the radioactivity eluted at V_s. The 1M lysine may overcome any association of the labelled activator with soluble fibrin polymers, but not with the gel matrix.

If it is assumed that interaction with DFP does not interfere

with the lysine binding sites then the poor binding of radioactivity to lysine-Sepharose of DFP-labelled plasmin, when 0.5mg of pure Kabi plasmin is used, makes the chances of success with the vascular activator very small indeed. However, the activity of the activator sample can be seen to be inhibited by the DFP and thus some radioactivity must be incorporated into the active site. It is, of course, quite possible that binding of DFP to the active site does in some way interfere with binding to lysine-Sepharose.

Fluorescent dansyl fluoride was also used in an attempt to label the active site of the vascular activator, but no binding of fluorescent activator to lysine- or fibrin-Sepharose was observed. The reasons for lack of success with dansyl fluoride would be the same as with the iodinations and DFP labelling.

1 PRODUCTION AND PROPERTIES OF SOLUBLE FIBRIN POLYMERS

A THEORY

The action of thrombin on fibrinogen produces fibrin monomer by removal of fibrinopeptides A and B (see Fig 33). Fibrin monomer has two polymerization sites, and under the action of factor XIII (gift from Dr J Holbrook) in the presence of calcium ions, will form insoluble fibrin. However, when fibrinogen is digested by plasmin it produces the characteristic fragments X, Y, D and E. Fragments Y and D are both polymer chain terminators as they only possess one polymerization site.

By varying the digestion time of fibrinogen by plasmin, it is possible to vary the ratio of Y and D fragments to unchanged fibrinogen and X fragment. If the ratio of fibrinogen and fragment X to fragments Y and D (F:Y) is 1:1 then on average a polymer consisting of 4 fragments (Y-F-F-Y) will be produced on addition of thrombin. If the ratio is 2:1 then Y-F-F-F-F-Y will be produced, and so on. Thus the length of polymer produced is related to the digestion time employed.

The desired digestion time can be determined either by a clottability trial or by monitoring the change in optical density at 320nm of the digestion mixture. The clottability trial was carried out by setting up the digestion mixture of fibrinogen and urokinase (to produce plasmin from

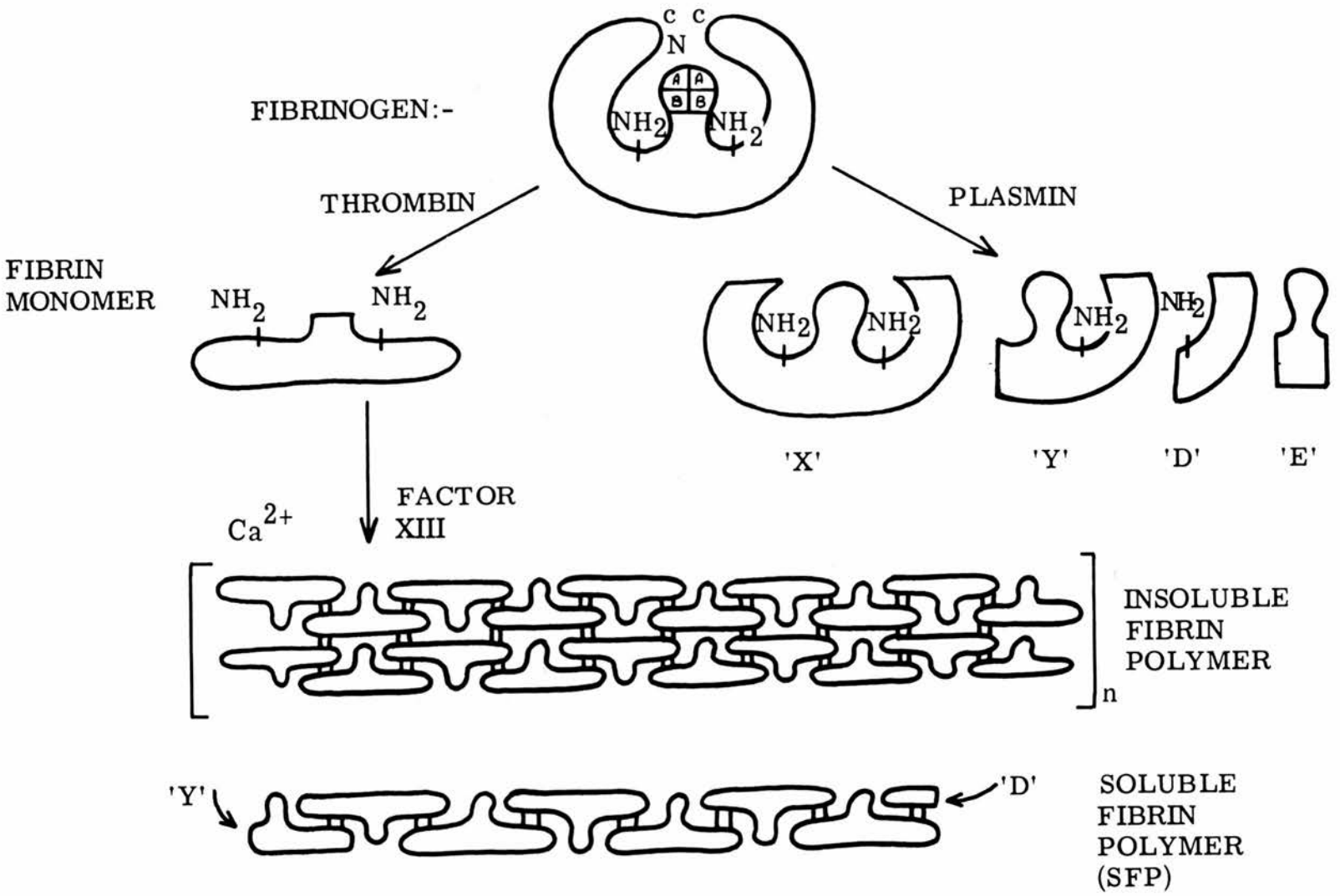


FIG 33 THEORY OF PRODUCTION OF SOLUBLE FIBRIN POLYMERS

plasminogen associated with the fibrinogen). 0.1ml samples of this were removed at half minute intervals, which were added to a mixture of thrombin, EACA and Trasylol at final concentrations of 5u/ml, 10mM and 1000 kiu/ml respectively. The desired digestion time was the one that rendered the fibrinogen just non-clottable. Alternatively, a sample of the digestion mixture can be transferred to a cuvette, in a thermostatted cell ($37^{o}C$), and the optical density change at 320nm recorded at half minute intervals. As the fibrinogen is degraded, the light scattering effect of the high molecular weight molecule diminishes, which is accompanied by a decrease in optical density at 320nm. This decrease flattens off to a plateau when the fibrinogen molecule is completely broken down, thus the desired digestion time can be taken from a point just prior to the plateau portion of the graph.

Radioactive fibrinogen labelled with ^{125}iodine may be included in the digestion mixture, or alternatively may be added at the end of the digestion, prior to addition of thrombin and cross-linking.

B METHODOLOGY

The soluble fibrin polymers (SFP's) are produced according to the scheme below:

Digestion	45ml 1% ($^{w}/_{v}$) Fibrinogen 5ml Urokinase (100 Ploug u/ml)		Incubate at 37°C for 16 minutes

Stop		Final Concentrations
Add:	Trasylol	1,000kiu/ml
	EACA	10mM

Polymerization		Final Concentrations	
Add:	Thrombin	5u/ml	Incubate at 37°C for 30 minutes
	F XIII	0.5u/ml	
	$CaCl_2$	10mM	

The mixture, after 30 minutes at 37°C, is then centrifuged to remove any insoluble fibrin that may have formed, and can then be applied to a Sepharose 6B or 4B column to separate the high molecular weight polymers from degradation products.

C CHROMATOGRAPHIC BEHAVIOUR

The mixture obtained at the end of the polymerization incubation was centrifuged, glucose added as a V_s marker, and the sample applied to Sepharose 6B (5 x 80cm). This was eluted at 4°C with 25mM tris, 1mM EDTA, 3.8mM borate, 0.15M NaCl, pH8.8 containing Trasylol at 10kiu/ml (see Fig 34). 30ml fractions were collected at 125ml/hour.

The molecular weight of the SFP's can also be determined on Sepharose 4B. Fig 35 shows the results when a sample of the V_o peak, obtained on Sepharose 6B, is applied to Sepharose 4B (2.5 x 100cm) and eluted at 4°C with 25mM tris, 1mM EDTA, 3.8mM borate, 0.15M NaCl pH8.8 containing Trasylol at 10kiu/ml. 8ml fractions were collected at 35ml/hour.

Samples of SFP's obtained as V_o peaks from Sepharose 6B were applied to hydroxyapatite and lysine-Sepharose chromatography columns, to determine their behaviour on chromatographic systems used in the purification of the vascular activator. The elution of an SFP sample from a hydroxyapatite column (2.5 x 10cm) is shown in Fig 36. The SFP's were eluted with an exponential phosphate (Na_2HPO_4) gradient from 5-500mM at pH7. 8ml fractions were collected at 80ml/hour. The SFP's eluted in one peak at 200mM phosphate.

Fig 37 shows the elution of a sample of SFP's from lysine-

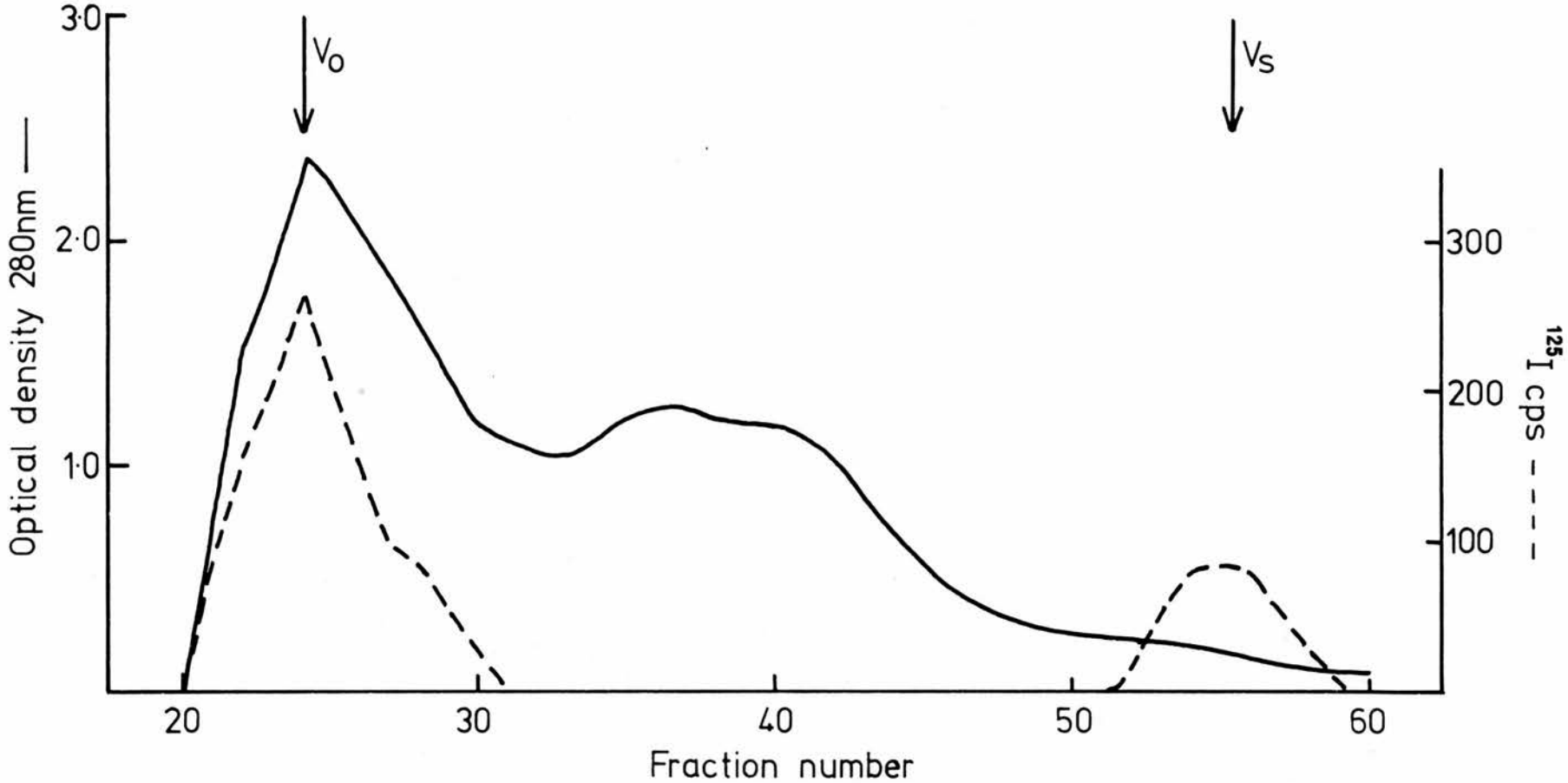


FIG 34 CHROMATOGRAPHIC BEHAVIOUR OF SFP'S ON SEPHAROSE 6B

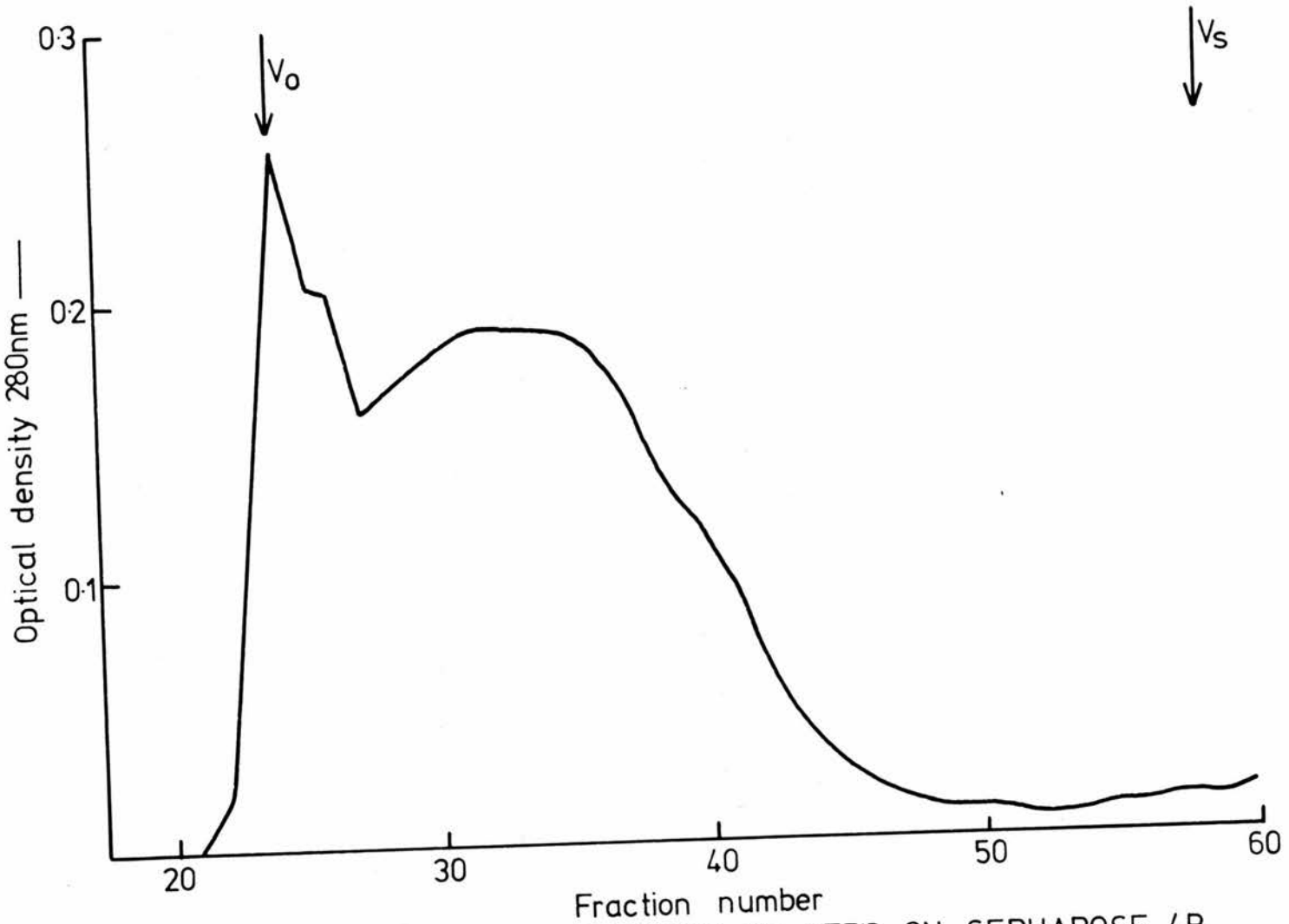


FIG 35 CHROMATOGRAPHIC BEHAVIOUR OF SFP'S ON SEPHAROSE 4B

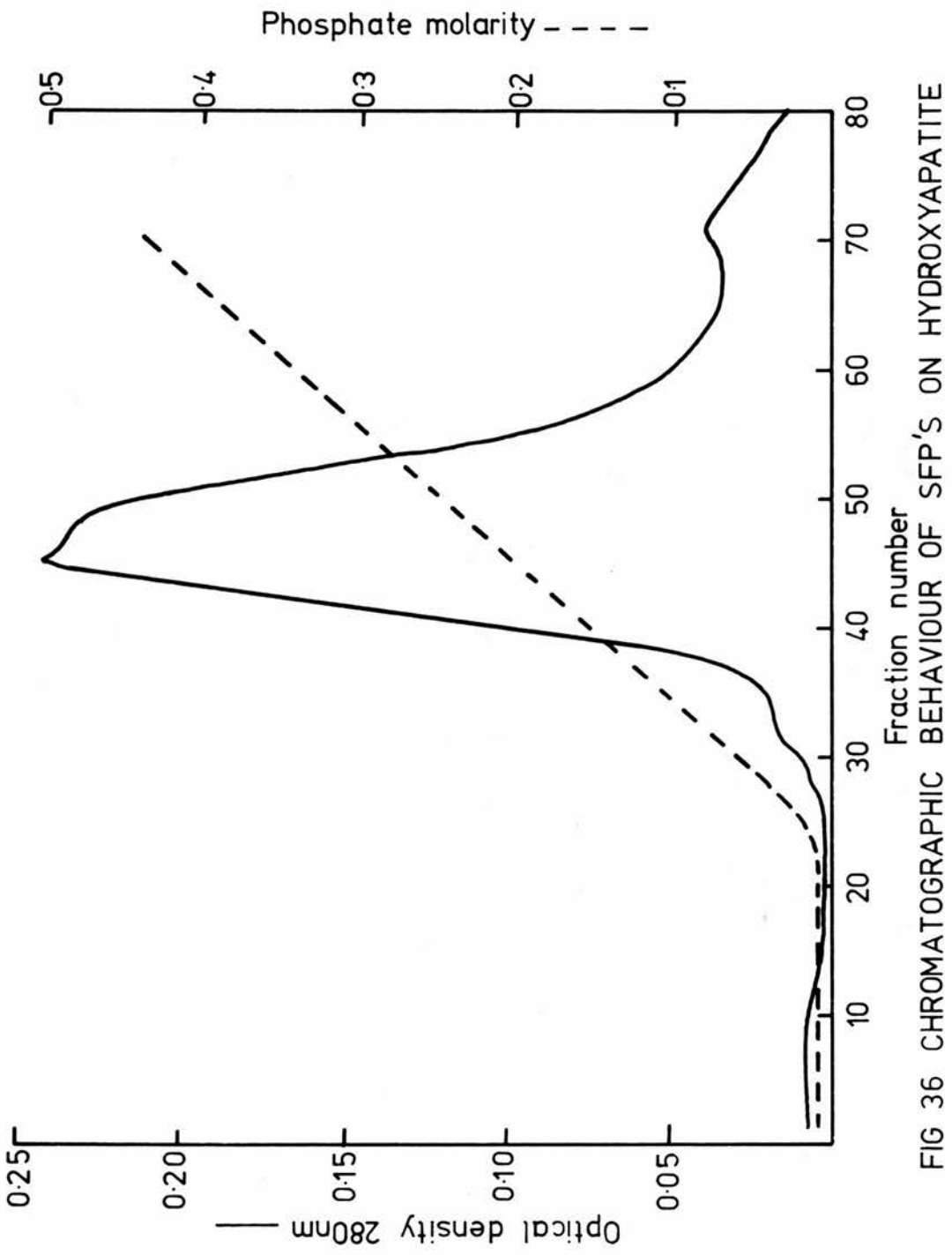


FIG 36 CHROMATOGRAPHIC BEHAVIOUR OF SFP'S ON HYDROXYAPATITE

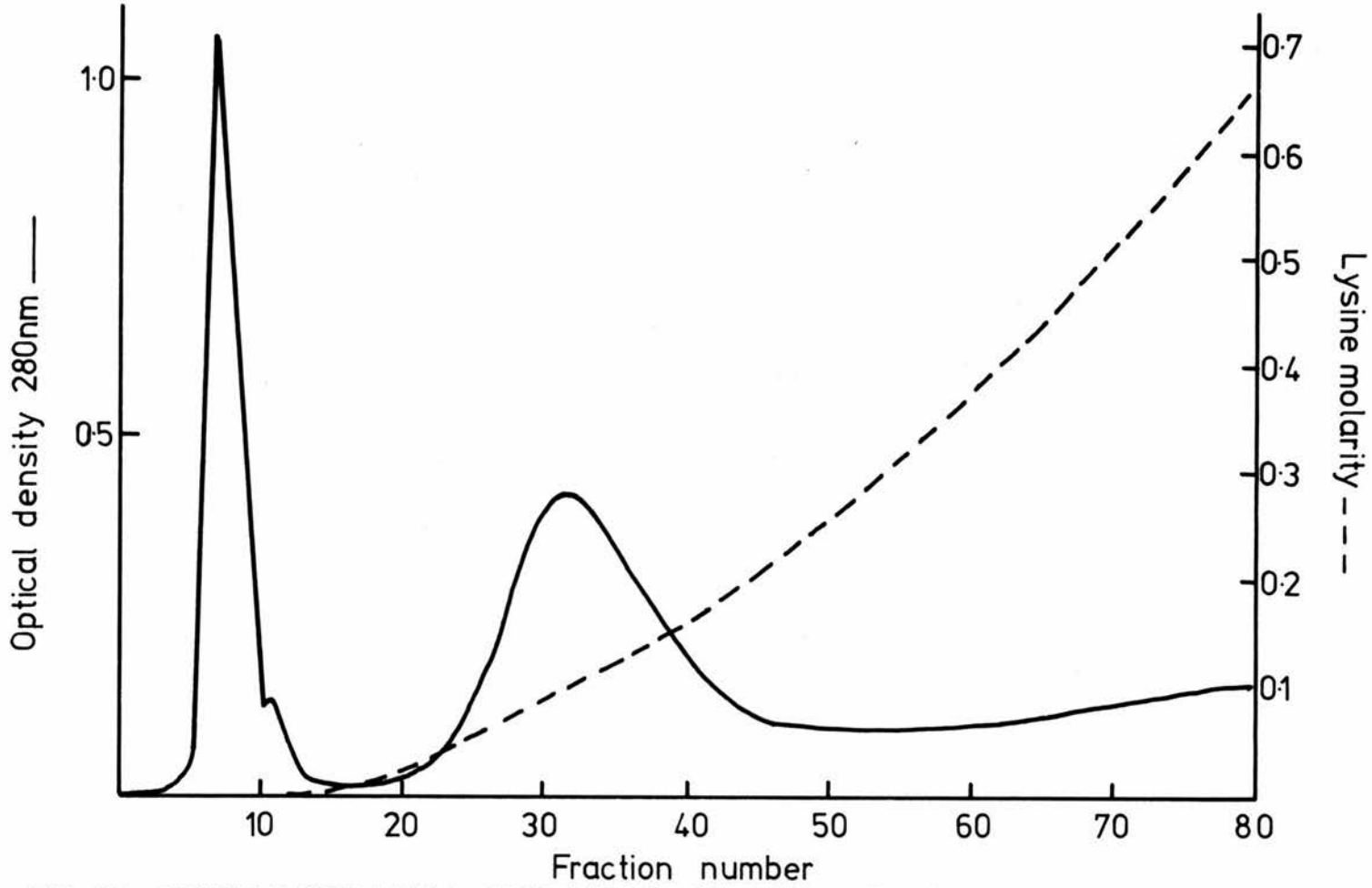


FIG 37 CHROMATOGRAPHIC BEHAVIOUR OF SFP'S ON LYSINE-SEPHAROSE

Sepharose (2.5 x 15cm) with an exponential gradient of 0-1M lysine in 0.025M tris (pH7.5) at 4°C. 7ml fractions were collected at 30ml/hour. One peak of SFP's did not bind while the other eluted at 0.1M lysine.

A Staphylococcus aureus clumping test (see Methods Section) for fibrin(ogen) related molecules was carried out on the peak obtained on hydroxyapatite chromatography and the two peaks obtained on lysine-Sepharose chromatography, and all gave positive reactions.

D ELECTROPHORETIC BEHAVIOUR

Unreduced and reduced (with β-mercaptoethanol) samples of SFP's from the V_o peaks of Sepharose 6B and 4B were run on SDS-polyacrylamide gel electrophoresis. The unreduced samples were run on 5% polyacrylamide gels along with a sample of X, Y, D and E fragments, produced by plasmin digestion of fibrinogen. The reduced samples were run on 7% polyacrylamide gels along with a reduced sample of Kabi fibrinogen.

The high molecular weight material was mostly of molecular weight greater than fragment X and fibrinogen, and there was a trace of fragments Y and D present. The chain data from the reduced samples suggested X and Y like material cross-linked mainly by γ chains to form γ dimers, as there was barely any α-polymers in the high molecular weight region of the gel. Thus it appears that the polymers were largely devoid of intact α chains and were γ chain cross-linked X fragments, terminated by incorporation of Y and D fragments into the polymer.

E DISCUSSION

The production of SFP's was quite a short and simple process and reproducible if all the conditions were kept constant. One problem encountered was the lability of stock solutions of urokinase, even when stored at -40°C; this was overcome by storing the urokinase in liquid nitrogen at -196°C.

As mentioned earlier, ^{125}I-fibrinogen can be incorporated into the SFP's. If the radiolabelled fibrinogen is added at the start of the digestion with plasmin, the radioactivity follows the protein profile when run on Sepharose 6B. However if the radiolabelled fibrinogen is added at, or near, the end of the digestion, the majority of the radioactivity is incorporated into the V_o peak on Sepharose 6B (see Fig 34).

A good yield of high molecular weight SFP's is generally obtained from a digest, but all subsequent operations after polymerization and cross-linking must be carried out in Trasylol at 10kiu/ml. If this is not done the polymers are prone to degradation.

All the SFP's bound to the hydroxyapatite column, and eluted in one peak at 200mM phosphate. This is within the range reported for the elution of the vascular activator from hydroxyapatite, and suggests that the binding of the activator to hydroxyapatite is through

soluble fibrin.

The SFP's were divided into a non-binding and a bound peak on lysine-Sepharose, although this could merely be due to overloading of sample on the column. Again the bound peak eluted at a value (0.1M lysine) close to the value reported for the elution of the low affinity peak of the activator from lysine-Sepharose, and supports the hypothesis that the low affinity peak of vascular activator on lysine-Sepharose is indeed a soluble fibrin-activator complex.

As already stated both peaks obtained on lysine-Sepharose gave a positive Staphylococcus aureus clumping test, and both peaks when re-run on Sepharose 6B eluted at V_o, and as will be seen in the next Section, the vascular activator has an equally high affinity for both of them. The identical behaviour of the two peaks in all but lysine binding suggests that overloading did produce the non-binding peak.

2 INTERACTION OF VARIOUS ACTIVATORS WITH FIBRIN

A AFFINITY OF VARIOUS ACTIVATORS FOR SOLUBLE FIBRIN POLYMERS

Binding studies were carried out to judge the relative affinities of the vascular activator, urokinase and the melanoma activator, for SFP's.

The top half of Fig 38 shows a fairly typical separation of a resuspended PEG precipitate on Sepharose 6B in 25mM tris, 1mM EDTA, 3.8mM borate, 0.15M NaCl pH8.8. However, if the activator eluting between fractions 20 and 30 is pooled, and a sample of this mixed with a sample of SFP's taken from a V_o peak on Sepharose 6B, when this mixture is re-run on the same column under identical conditions, the vascular activator activity now elutes at V_o as shown in the bottom half of Fig 38. This same high affinity of vascular activator for SFP's has been demonstrated for both peaks obtained when SFP's are applied to lysine-Sepharose (see last Section).

The same procedure was adopted for the melanoma activator. When run alone on Sepharose 6B the melanoma activator eluted with a K_d of 0.76. When re-run with SFP's approximately 8% of the total activity eluted at V_o, while the remainder eluted with an unaltered K_d. Urokinase was similarly tested; when run alone the activity eluted with a K_d of 0.77. When re-run with SFP's <u>all</u> the activity

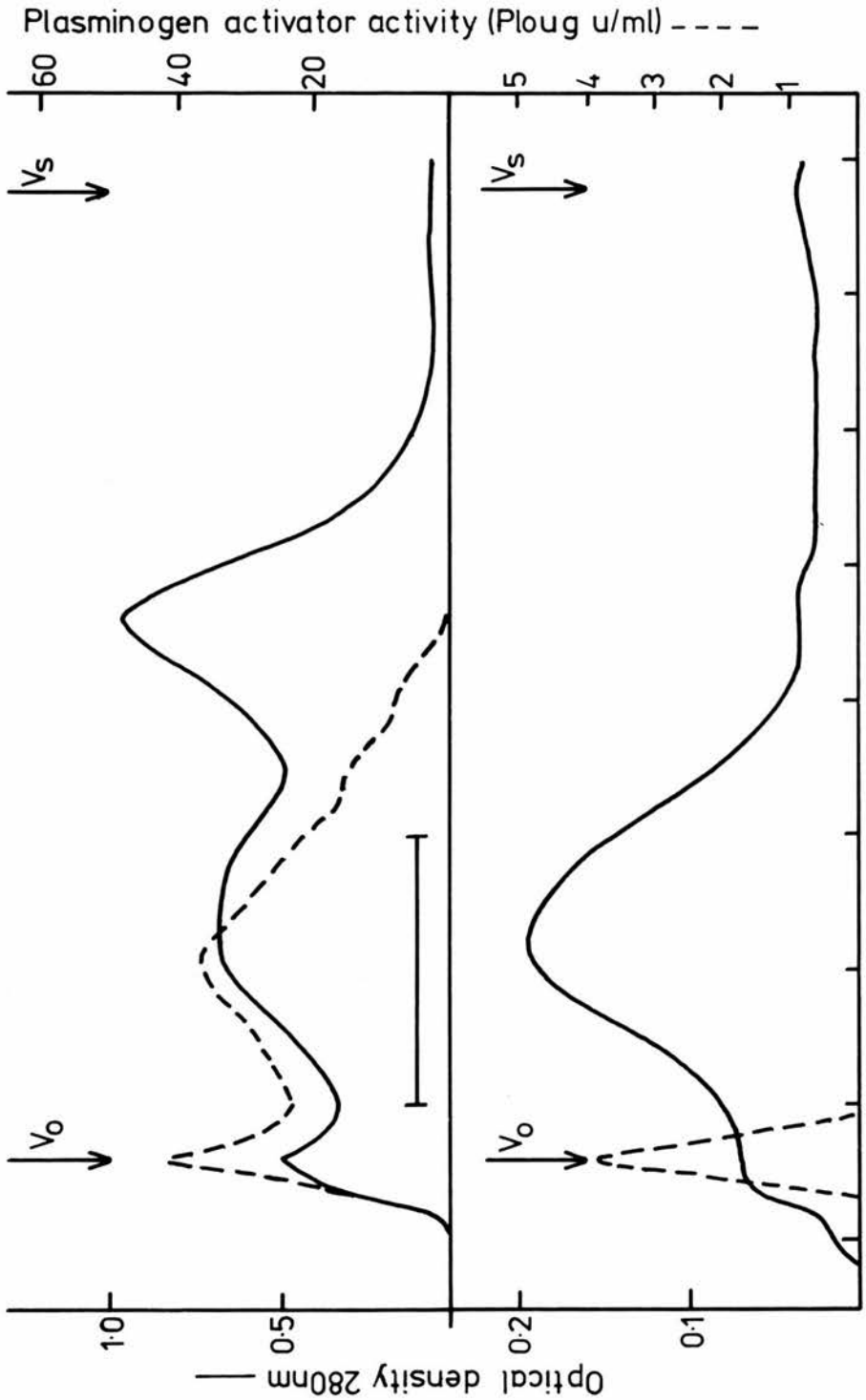


FIG 38 THE AFFINITY OF THE VASCULAR ACTIVATOR FOR SFP'S

eluted with an unaltered K_d, with no activity at V_o. The column, buffer and conditions used for both the melanoma and urokinase affinity studies were identical to those used for the vascular activator studies above.

B AFFINITY OF VARIOUS ACTIVATORS FOR FIBRIN CLOTS

The affinity of the vascular activator, urokinase and the melanoma activator for fibrin formed in situ was tested. The vascular activator sample used was a resuspended PEG precipitate (160 Ploug u/ml), urokinase (Leo) was used at 250 Ploug u/ml and the melanoma activator had a value of 150 Ploug u/ml.

To 50ml samples of each activator was added 10mls of 1% fibrinogen and 0.2ml thrombin (50u/ml); this was allowed to clot for half an hour at RT before being centrifuged. The activity of the supernatent (1) was determined on a fibrin plate. To the clot was added 50ml of a solution of 1M NaBr and 1M lysine; this was magnetically stirred for half an hour at RT. The solution was then centrifuged and again the activity present in the supernatent (2) determined on a fibrin plate.

The activity in supernatent 1 represents the unbound activity, while the activity in supernatent 2 represents the activity that has bound to the fibrin, and been eluted from the clot by the 1M lysine. The results of the binding studies are given in Table 13. As can be seen the vascular activator has a very high affinity for fibrin giving 75% binding. Urokinase only gave 17% binding, while the melanoma gave 5% binding.

ACTIVATOR	% BOUND TO FIBRIN CLOT
Vascular Activator	75
Urokinase	17
Melanoma Activator	5

TABLE 13 PERCENTAGE OF VARIOUS ACTIVATORS BINDING TO FIBRIN FORMED IN SITU

C DISCUSSION

The results clearly show that the vascular activator has a far greater affinity for both SFP's and insoluble fibrin than urokinase or the melanoma activator.

The vascular activator sample used in both cases was a resuspended PEG precipitate, thus the activator would already have been in a complex with soluble fibrin. The sample used for the SFP-binding experiments on Sepharose 6B eluted after V_o (fractions 20-30), but was obviously of higher molecular weight than uncomplexed vascular activator. Thus the vascular activator shows a preference for the higher range molecular weight SFP's over the lower molecular weight soluble fibrin it was already bound to. Similarly the activator shows a higher affinity for insoluble fibrin (or perhaps the affinity is for the soluble phase present during the formation of insoluble fibrin), than the soluble fibrin with which it was already complexed. The higher affinity could be due to the presence of a more complete binding site in the larger polymers or insoluble fibrin, or that the binding site only attains the desired configuration when certain bonds are present and intact. Alternatively, the polymer (whether soluble or insoluble) may provide a series of binding sites in the correct orientation, which become less attractive to the activator as the polymer is broken down.

The elution of the vascular activator from the formed

fibrin clot in the affinity experiments, was put to further practical use. It was possible to retrieve activator, that would have otherwise been lost, from fibrin clots forming spontaneously during purification procedures, usually at the concentration steps. A useful purification can also be effected by forming a fibrin clot in a crude perfusate or resuspended PEG precipitate, removing it by centrifugation, and then breaking up the clot and stirring it in a buffer containing 1M NaBr (to help break up the fibrin clot) and 1M lysine (to elute the activator from the fibrin).

3 A SYNTHETIC CARRIER?

A EFFECT OF POLY-LYSINE ON THE RATE OF ACTIVATION OF PLASMINOGEN

The effect of poly-D-lysine on the rate of production of plasmin from plasminogen, using various activators, was investigated.

Fig 39 shows the effect of poly-D-lysine (M Wt 144,000) used at 0.1mg/ml (final concentration = 0.046μM) on a mixture of purified vascular activator, plasminogen and S-2251. The S-2251 measures the plasmin formed. As can be seen from Fig 39, in the presence of 0.1mg/ml poly-D-lysine the amount of plasmin produced is increased approximately 4-fold. However this increase is seen to be more when the effect of poly-D-lysine on plasmin is studied. The cleavage of S-2251 by plasmin (1cu/ml) is inhibited 50% by poly-D-lysine at a final concentration of 0.046μM. Thus the true increase in the amount of plasmin produced, by activation of plasminogen by the vascular activator, in the presence of 0.046μM poly-D-lysine (M Wt 144,000) is in the region of an 8-fold increase.

Thus a range of concentrations of poly-D-lysine was used with the vascular activator and plasminogen to determine the optimum range of concentrations. Fig 40 shows the effect of the range from 0.02mg/ml to 0.1mg/ml (0.0092-0.046μM) and Fig 41 shows the effect of the range 0.2mg/ml

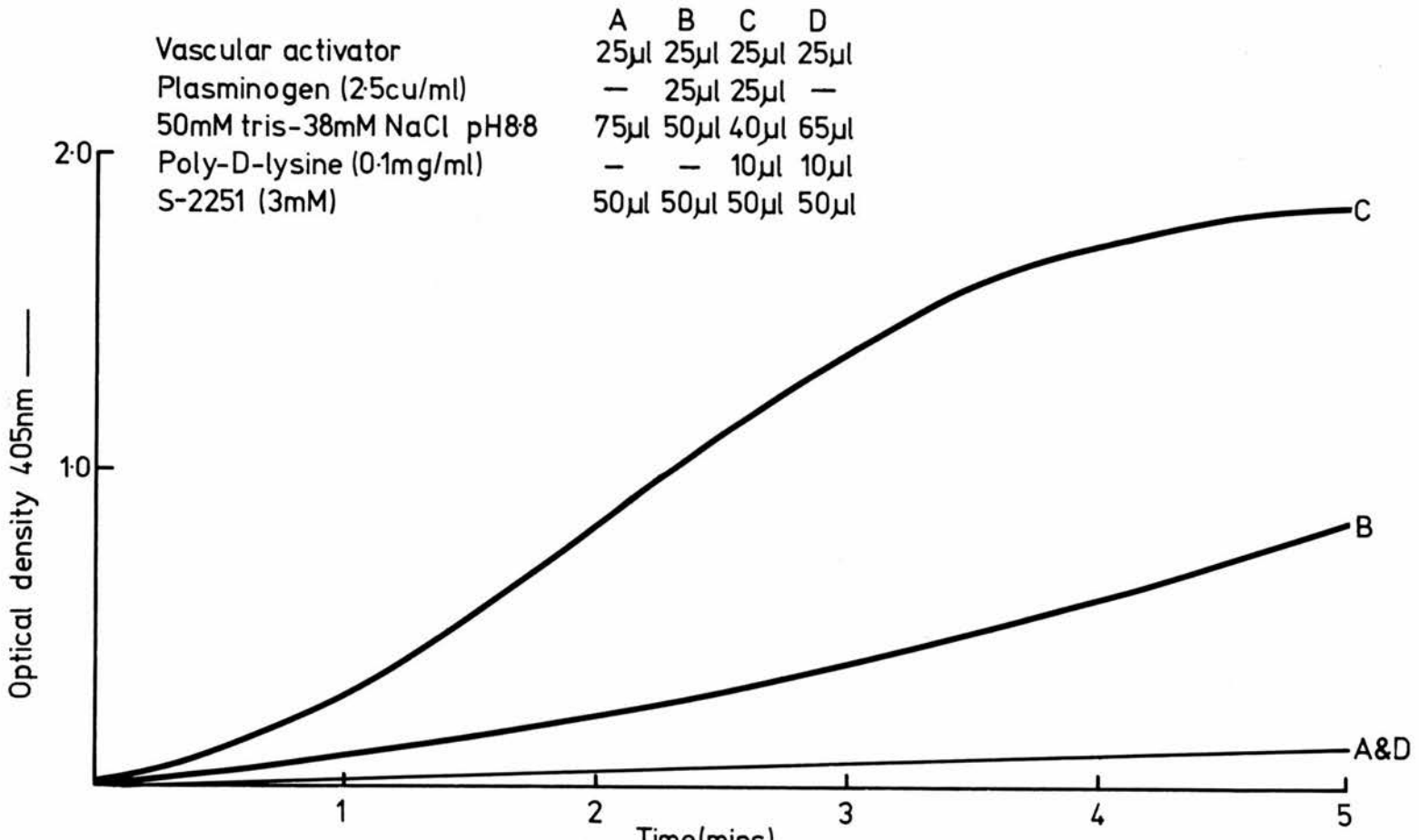


FIG 39 EFFECT OF POLY-D-LYSINE ON RATE OF ACTIVATION OF PLASMINOGEN BY THE VASCULAR ACTIVATOR

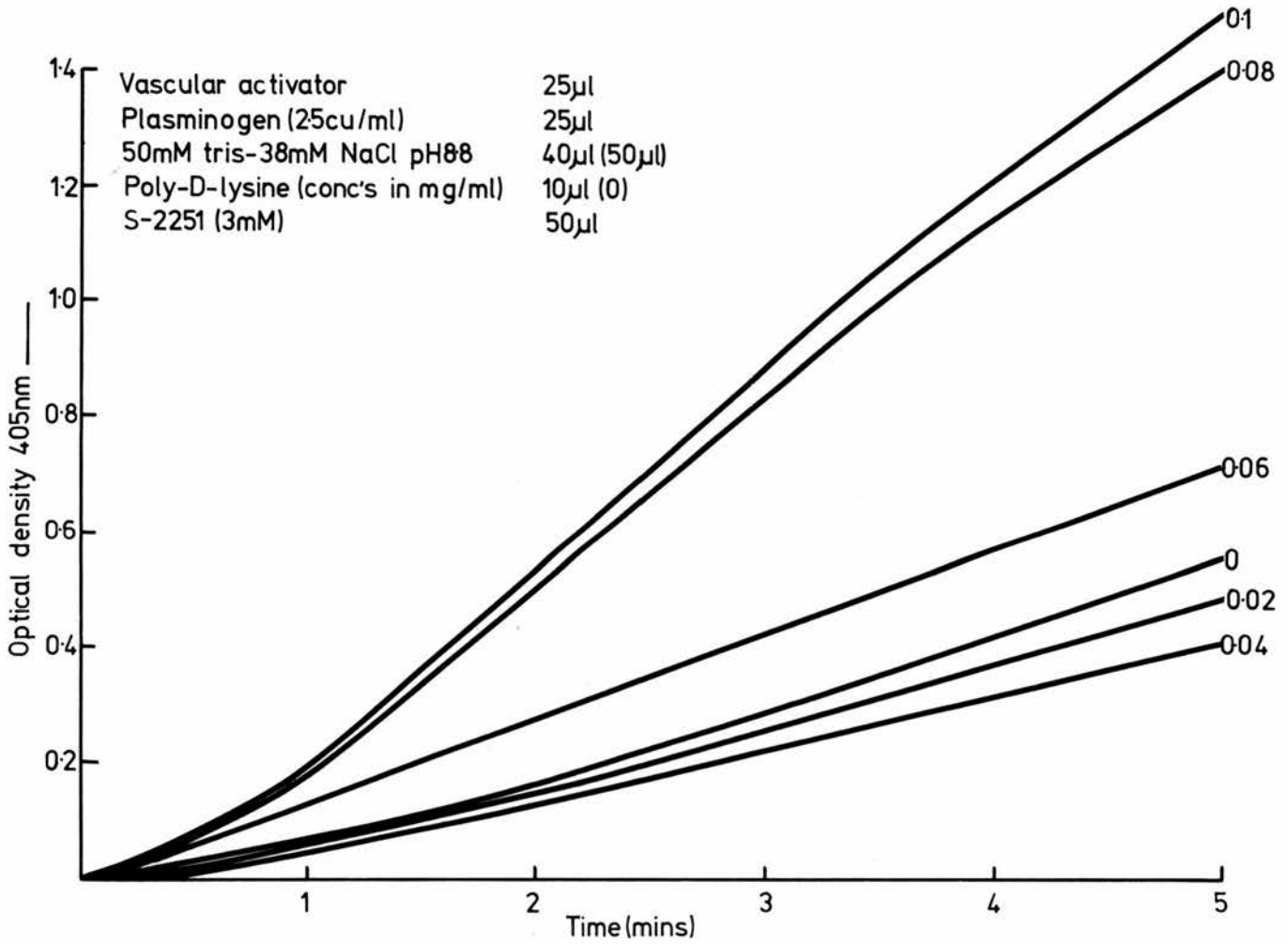


FIG 40 EFFECT OF VARIOUS CONCENTRATIONS OF POLY-D-LYSINE ON RATE OF ACTIVATION OF PLASMINOGEN BY THE VASCULAR ACTIVATOR

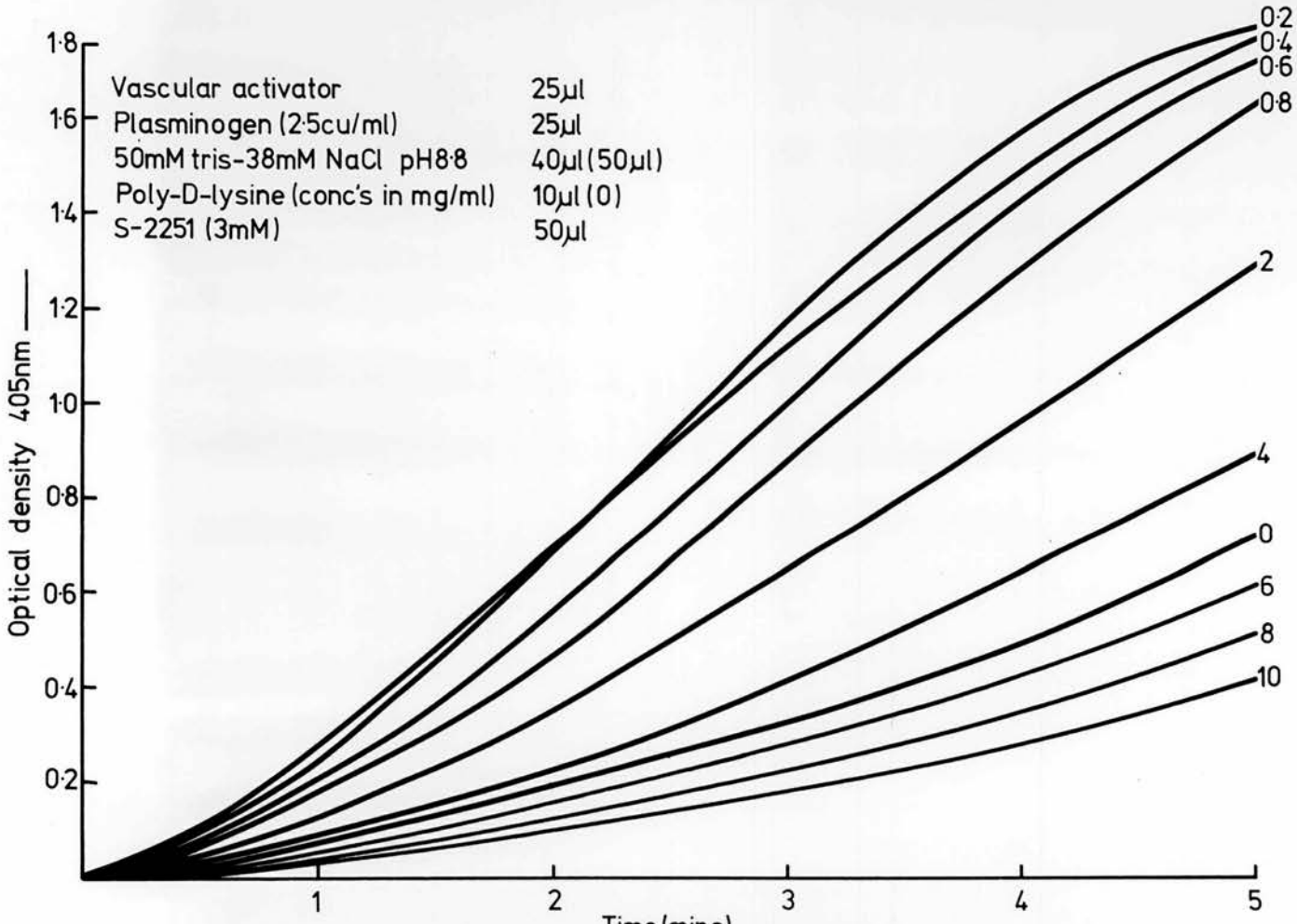


FIG 41 EFFECT OF VARIOUS CONCENTRATIONS OF POLY-D-LYSINE ON RATE OF ACTIVATION OF PLASMINOGEN BY THE VASCULAR ACTIVATOR

to 10mg/ml (0.092-4.6μM). The range 0.01μg/ml to 0.01mg/ml was also tested (not shown), but the effects of these concentrations fell between 0.02mg/ml and no poly-D-lysine (Fig 40), as would be expected.

The range of concentrations that give an overall increase is 0.06-4mg/ml (0.028-1.84μM). However the range would be wider if an allowance was made for the plasmin inhibition by poly-D-lysine. The biggest increases occur in the range 0.08-0.8mg/ml (0.037-0.37μM), and 0.1mg/ml (0.046μM) is the most effective. Overall inhibition is seen with concentrations up to 0.04mg/ml (0.018μM) and over 6mg/ml (2.76μM).

The effect of poly-D-lysine at a final concentration of 0.046μM was then investigated on a range of activators. The melanoma activator showed a 13% increase in plasmin production after 5 minutes in the presence of poly-D-lysine. This effect would be masked somewhat by the inhibitory effect of poly-D-lysine on plasmin. When streptokinase was tested, an initial small increase in the rate of production of plasmin, was overtaken by the plasmin inhibitory effect later, and after 5 minutes the amount of plasmin produced with or without poly-D-lysine was approximately the same. When urokinase was tested an overall inhibition was seen that could be accounted for by plasmin inhibition, and thus no enhancing effect was present.

An 11 to 12-fold increase in the amount of plasmin produced was obtained with human uterine tissue activator (gift from Dr D Rijken) in the presence of poly-D-lysine (see Fig 42) used at 0.1mg/ml. The effects of poly-L-lysines (used at 0.1mg/ml) of various molecular weights, were also investigated, on human uterine tissue activator. As can be seen by reference to Fig 42, the response to poly-L-lysine appeared to be related to its molecular weight. The higher the molecular weight, the greater the amount of plasmin produced.

Finally a resuspended PEG precipitate was tested with and without poly-D-lysine (0.1mg/ml). The poly-D-lysine showed approximately 33% inhibition. This means that there was a slight enhancement, but that this was swamped by the inhibitory effect on plasmin.

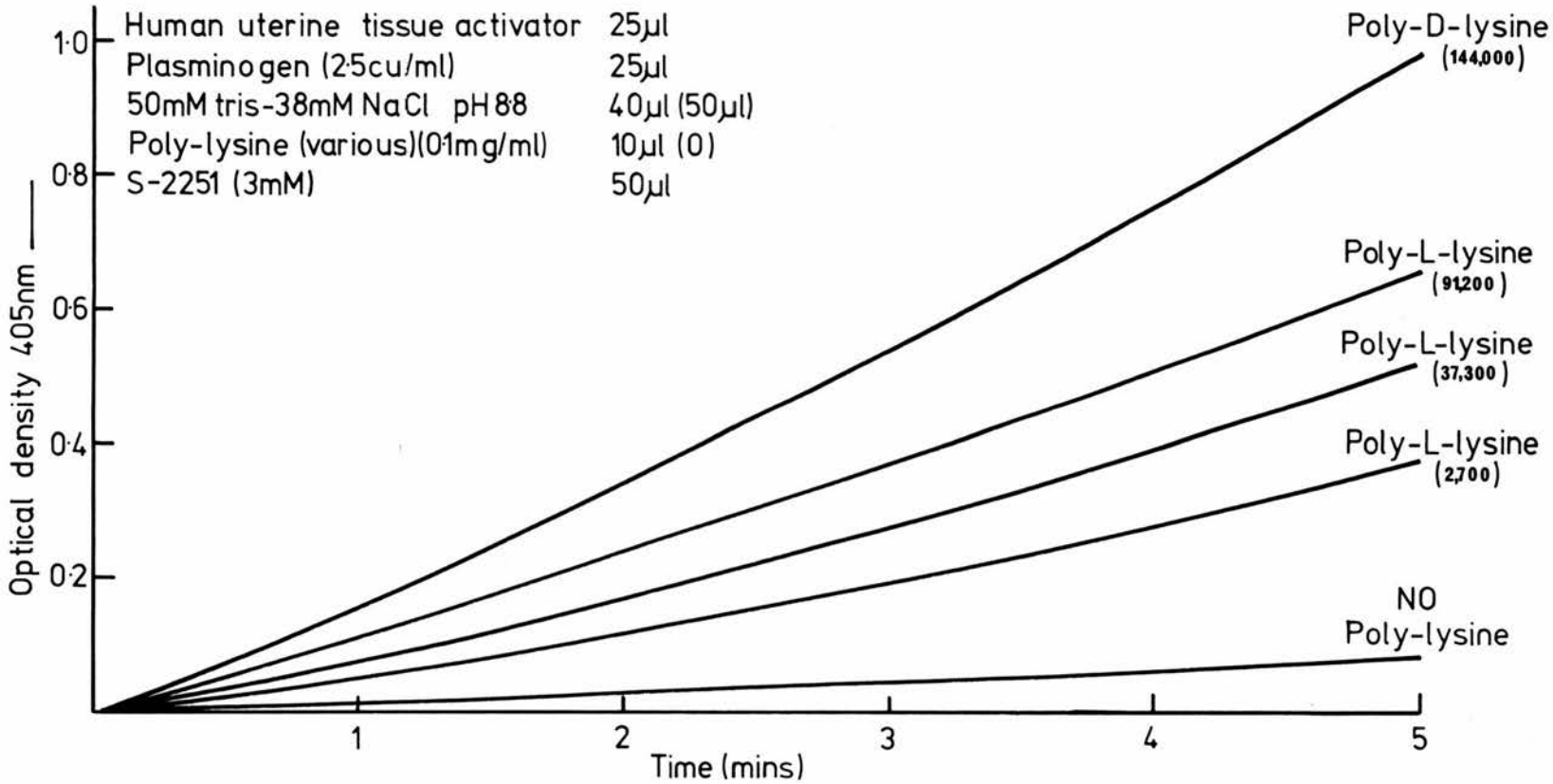


FIG 42 EFFECT OF VARIOUS POLY-LYSINE'S ON THE RATE OF ACTIVATION OF PLASMINOGEN BY HUMAN UTERINE TISSUE ACTIVATOR

B DISCUSSION

Poly-D-lysine appears to have a far greater effect on the purified vascular activator and human uterine tissue activator than the other activators tested. Bearing in mind that poly-D-lysine inhibits plasmin (Kabi, 1cu/ml) approximately 50%, the enhancement with the vascular activator is in the region of 8-fold, and with the uterine tissue activator 22 to 24-fold. The melanoma activator shows a slight overall enhancement, streptokinase no overall effect and urokinase is inhibited by an amount that can be accounted for by plasmin inhibition.

The greater enhancement associated with the vascular activator over the other activators tested reflects the affinities found of these activators for SFP's and fibrin. The human uterine tissue activator also has a high affinity for fibrin (Dr D Rijken - personal communication). It appears that the poly-lysine may be acting as a synthetic carrier, partly mimicing the binding sites present on SFP's and fibrin. Thus if the vascular activator and uterine tissue activator, with their high affinity for fibrin also bind to poly-lysine they may undergo a conformational transition giving a more active enzyme. The other activators not having this high affinity for fibrin, would not benefit from the presence of poly-lysine. If this is the case then it may go some way to explaining why an overall inhibition of 33% was seen when the activator sample was a resuspended PEG precipitate. SFP's

would be present in this sample and presumably the vascular activator would show a preference for SFP's over poly-D-lysine, and thus very little effect would be seen, except for the inhibitory effect of poly-D-lysine on plasmin. It is unlikely that plasminogen possesses the affinity for poly-lysine that is seen for fibrin. Plasminogen appears to require a 6-carbon chain terminated in a COOH group for binding. These requirements are fulfilled by EACA and lysine but not by poly-lysine. In poly-lysine the COOH group is lost during polymerization and thus the ability to bind plasminogen would not be expected.

We have, on several occasions, attempted to demonstrate an enhancing effect with SFP's. In some cases an increase in the rate of production of plasmin does occur in the presence of SFP's, using the same system as with poly-lysine. However, often they show an inhibitory effect. One explanation could be that the natural substrate of the formed plasmin is fibrin, and thus with SFP's present a substrate is introduced to compete with S-2251; whereas when poly-D-lysine is used, the enhancement remains the same but there is no competition with S-2251. Bovine plasmin is known to degrade poly-L-lysine (Rigbi, 1957) but not poly-D-lysine.

A range of poly-L-lysines of molecular weights 2,700 to 91,200 were investigated, with a different sample of vascular activator, and no enhancement was found. However, with this particular sample no effect was found with

poly-D-lysine either, possibly due to contamination with SFP's. More work is required here.

However, enhancement was demonstrated with poly-L-lysines (used at 0.1mg/ml) of different molecular weights when used with human uterine tissue activator. The enhancement of plasmin produced appeared to be related to the molecular weight of the poly-L-lysine, the poly-L-lysine of highest molecular weight (91,200) having the greatest effect.

It is difficult to compare the effects of poly-L and poly-D-lysines as preparations of the same molecular weight were not available. Poly-D-lysine induced a greater response, but this may have been due to the molecular weight of 144,000 as opposed to the 91,200 of the poly-L-lysine; alternatively it could be due to the fact that the plasmin formed may degrade the poly-L-lysine but not affect the poly-D-lysine. Bovine plasmin is known to degrade poly-L-lysine (Rigbi, 1957) but not poly-D-lysine. The same may be true for human plasmin.

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CHAPTER 5

5/

1 ASSAY METHODS

A PLASMINOGEN ACTIVATOR

This is assayed on a fibrin plate prepared from the following reagents:

1 6ml 1% ($^{w}/_{v}$) human fibrinogen (Kabi Pharmaceuticals, Grade L)

2 24ml 0.05M tris, 0.15M NaCl pH7.5

3 0.6ml Thrombin (Parke Davis) 10 Iu/ml

The fibrinogen is mixed with the buffer, then the thrombin is added, the mixture thoroughly mixed and immediately 25ml transferred into a 10 x 10cm square plastic petri dish on a levelled table. This is left at room temperature for 30 minutes. The petri dish can be marked off into 16 (or 25, depending on the level of activity expected) equal sections, and 30µl of each sample applied to the centre of each section using a micro-syringe. The plates are calibrated with urokinase (Leo Pharmaceuticals) from 1-100 Ploug u/ml. The plates are then incubated on a levelled table at 37°C overnight.

The lysis areas are visualised by adding a few drops of 0.1% bromothymol blue in 50% ethanol, 0.25M NaOH. Two perpendicular diameters of the lysis areas are measured and the average of these for the urokinase standards are plotted against the log of the urokinase concentrations (in Ploug u/ml). The unknown plasminogen activator activities

can then be read from the graph using the average diameter of the lysis areas of the test samples.

The activator converts the plasminogen (associated with the fibrinogen) to plasmin, and the subsequent lysis is measured. Thus it must be noted that the assay is not specific for activator; plasmin applied to the plate or contaminating the activator sample will also cause lysis. However if samples suspected of being contaminated with plasmin are screened in parallel on normal fibrin plates and plasminogen-free fibrin plates, prepared using plasminogen-free fibrinogen (see Section 3D), then the latter will detect lysis due to non-specific or direct proteolysis (eg plasmin) and deduction of this value from the value obtained on normal fibrin plates will give a value for the plasminogen activator activity.

B PLASMIN

Plasmin was detected in two ways. Firstly on plasminogen-free fibrin plates (30µl samples), prepared as normal fibrin plates, except that the fibrinogen used had been passed through a lysine-Sepharose column (to remove plasminogen).

Alternatively the synthetic substrate S-2251 was used to screen column eluate fractions containing only plasmin. 0.1ml of the sample was added to a tube containing 50µl S-2251 (3mM). This was incubated at 37°C until a yellow colour became visible; this could be measured at 405nm.

C PLASMINOGEN

Two assays were used in this work for the detection of plasminogen in column eluate fractions. In both assays the samples (0.1ml) were added to 0.1ml streptokinase (Lederle) and incubated for half an hour at 37°C. Then either 30µl of each was screened on plasminogen-free fibrin plates, or 0.1ml of each was added to 50µl S-2251 (3mM) and incubated at 37°C until a yellow colour developed, which was measured at 405nm.

The Laurell electroimmunoassay was used for the determination of plasminogen antigen. 1% agarose was prepared in 25mM tris, 1mM EDTA, 3.8mM borate pH8.8. 0.1ml Behringwerke anti-plasminogen was added to 12.5ml of 1% agarose, which was poured onto an 8 x 8cm glass plate on a level surface and allowed to set. 5µl samples were applied to wells cut along one edge of the gel and the plate was run overnight at 120 volts. The tank buffer was the same as the gel buffer. Serial dilutions of normal plasma were run as standards.

After electrophoresis the gel was dried under weighted filter paper and stained in 0.1% Coomassie brilliant blue R250 (Sigma) in 40% methanol, 10% acetic acid, 50% H_2O, destained in the solvent and the 'rocket' heights measured.

D PROTEIN

The folin assay was used for protein estimations. The reagents used were as follows:

Reagent A (Stable for 24 hrs)	Copper Citrate	1ml
	2% Na_2CO_3 (anhyd) in 0.1M NaOH	49ml
Reagent B	Folin reagent (diluted 1:1 with distilled H_2O)	

To 0.5ml sample or standard was added 2.5ml reagent A. This was mixed and left 10 minutes at room temperature then 0.25ml reagent B was added. This was mixed and left 30 minutes at room temperature before being read at 750nm. The range of the assay is 5-200µg, and human serum albumin was the standard used.

E SOLUBLE FIBRIN POLYMERS

(STAPHYLOCOCCUS AUREUS CLUMPING TEST)

Staphylococcus aureus (Strain Newman D_2C) was made up at 10mg/ml. It was mixed vigorously (Vortex mixer) for at least one minute, before being allowed to stand for 5 minutes. The suspension was freshly prepared for each assay and must be used within 30 minutes of being made.

50µl of test sample and 50µl cell suspension were spread on a glass plate and then rotated for two minutes before being viewed over a black background. Clumping was scored from - (no visible clumping) to ++++ (maximum clumping). Controls of the cell suspension against distilled water and against 1% fibrinogen were set up. All the reagents must be at room temperature and have no visible signs of bacterial growth to be sure that no false positives occur.

The Staphylococcus aureus clumping test was used to assay column eluates when investigating the chromatographic behaviour of soluble fibrin polymers. The higher molecular weight polymers tend to give a stronger clumping reaction.

2 GEL FILTRATION CHROMATOGRAPHY

A PREPARATION OF THE GEL FILTRATION COLUMN

Sepharose 6B and 4B were obtained as preswollen aqueous suspensions. Sephadex G-25 and G-200 were obtained as dry powders and swollen in approximately five times their water-regain weight of appropriate buffer at room temperature for three days. Fines consisting of fractured and undersized gel beads that tend to reduce the flow rate and affect separation, were removed by allowing the gel beads to sediment freely in a two litre measuring cylinder. As the beads settle, the supernatent containing the fines was pumped off. This process was repeated until the majority of fines were removed (usually ten times).

A glass column open at one end and with a Teflon stopcock at the other was filled with appropriate buffer and clamped in a vertical position. A spherical glass bead was dropped to the bottom of the column to prevent sand entering the stopcock and outlet tubing. Sufficient acid washed sand to cover the rounded portion of the column was added, such that after sedimentation the sand level would just reach the parallel portion of the column.

A two litre Imhoff sedimentation cone was connected to the top of the column through a rubber bung and firmly clamped. The gel slurry was poured into the cone and a variable speed peristaltic pump (LKB 12000 Varioperpex) was connected

to the bottom of the column, the stopcock opened and pumping started. The flow rate was immediately measured and adjusted to the correct value for the gel type in use. Pumping was continued for 12 hours which was sufficient for the gel to sediment and form a packed gel bed. Excess solution remaining in the cone was removed and the top 2cm of the gel bed stirred and allowed to sediment, thus forming a perfectly flat bed surface. A reservoir containing the desired eluant was connected to the top of the column and pumping continued until at least one column volume of buffer had been eluted, at which point the apparatus was ready for use. Two types of fraction collectors were used depending on the size of the column employed. The Gilson microcol TDC 80 was able to collect 80 fractions of 10ml, whereas the LKB 7000 Ultrorac fraction collector was used with the larger columns; this was capable of collecting 200 fractions of 30ml.

B CALIBRATION OF SEPHADEX G-200 IN TERMS OF STOKES RADIUS AND THE INVERSE ERROR FUNCTION OF THE DISTRIBUTION COEFFICIENT

The Stokes radius (r_s) is defined in the Stokes-Einstein equation as follows:

$$r_s = \frac{kT}{6\pi\eta D}$$

where k = Boltzmann's constant

T = Absolute temperature

η = Solvent viscosity

D = Diffusion coefficient

and is a measure of the hydrodynamic radius of the molecule in solution. It has been shown that the Stokes radius of a molecule in solution can be related to its behaviour in gel filtration through the inverse error function of the distribution coefficient (K_d) by the relationship:

$$r_s = a_o + b_o \, \text{erfc}^{-1} (1-K_d) \text{ (Ackers, 1967)}$$

where a_o and b_o are constants relating to the particular column in use. erfc^{-1} is the inverse error function from "Tables of the Error Function and its Derivatives" (1954).

A plot of r_s against $\text{erfc}^{-1} (1-K_d)$ for molecules of unknown Stokes radius will allow the latter to be determined if the column has been previously calibrated with compounds of known r_s.

Since r_s and D are readily interconverted through the Stokes-Einstein equation, D values can be calculated from measured r_s values and used in the Svedberg equation for the calibration of molecular weights.

K_d is calculated from the equation:

$$K_d = \frac{(V_e - V_o)}{(V_s - V_o)}$$

where V_e = Elution volume of the compound.

V_o = Void volume, which is the elution volume of a compound sufficiently large to be completely excluded from the gel matrix. This is equal to the volume of liquid exterior to the gel beads.

V_s = Solvent volume; this is the total volume of liquid both outside and inside the gel beads and is the elution volume of a compound sufficiently small to be totally free to enter the gel matrix.

Compounds of intermediate size between those eluting at V_o and V_s are partially excluded from the gel matrix and consequently elute at intermediate volumes (V_e) depending on their size. K_d values range from 0 to 1; values greater than 1 are not possible for simple gel filtration but do occur when adsorption of the solute onto the gel matrix occurs. V_o is generally measured by determining the elution volume of Dextran Blue 2000, a dyed macromolecular dextran of molecular weight 2×10^6. V_s is measured by

determining the elution volume of glucose, detected with Clinistix (Ames).

3 AFFINITY CHROMATOGRAPHY

A COUPLING OF LIGANDS VIA THE CNBr LINKAGE

The general method for coupling a ligand to CNBr-activated Sepharose is given below. The quantities given are for coupling 100g ligand to 1 litre of gel.

200g cyanogen bromide (Koch-Light) were dissolved in approximately 120ml N-methylpyrrolidone and added slowly, with stirring, to 1 litre of gel in a large beaker surrounded by an ice-water-salt mixture. The 1 litre of gel was suspended in 1 litre of 5M K_2HPO_4 pH12. When all the CNBr was added the reaction mixture was retained at 4°C with stirring for 45 minutes.

The gel was then washed with 5 litres cold distilled water, slurried in 1 litre 0.1M $NaHCO_3$-0.5M NaCl pH9 and 100g of solid ligand added. The pH was re-adjusted to 9 (if necessary) and this was left at room temperature overnight with gentle stirring.

Then ethanolamine was added to a final concentration of 1 molar and left for four hours at 20°C. The gel was then filtered off on a Buchner funnel, washed with distilled water and put through the washing cycle three times, using:

0.1M sodium acetate - 1M NaCl pH4

distilled water

0.1M boric acid - 1M NaCl pH8

distilled water.

B COUPLING LIGANDS VIA THE ADIPIC ACID DIHYDRAZIDE (ADH) LINKAGE

Sepharose activated with CNBr (as above) was treated with an equal volume of 0.5M ADH in 0.2M $NaHCO_3$ at pH9. The suspension was gently shaken for 15 hours at 4°C and an additional 4-6 hours at room temperature. The hydrazido-Sepharose was washed with 10 volumes of 1M NaCl over a coarse sintered glass funnel and incubated with 1M glycine at pH9 for 4 hours at room temperature. The substituted Sepharose was extensively washed with 1M NaCl until ADH was no longer detected in the washings. The hydrazido-Sepharose derivative is stable for months if stored at 4°C in the presence of 0.02% NaN_3.

To couple ligands to hydrazido-Sepharose, 10mls (for example) was taken and suspended in 8ml distilled water and 2ml 1M HCl. The suspension was cooled in an ice-bath for 30 minutes and while stirring, 2ml of an ice-cold solution of 1M $NaNO_2$ was added dropwise over a 1 minute period. The suspension was stirred for an additional 2-3 minutes, then rapidly (1-2 minutes) filtered with suction on a previously cooled (4°C) coarse sintered glass funnel and washed with 20-30ml 5mM HCl at 4°C. The ligand to be coupled (1g), in 0.2M $NaHCO_3$ pH8, was added while being stirred with a glass rod. The suspension was transferred to a polyethylene vial and shaken gently for 15 hours at 4°C. The substituted Sepharose was washed extensively with 1M NaCl, and finally transferred to the appropriate buffer before use.

C PREPARATION OF THE IgG FRACTION OF ANTISERA

5ml of antiserum was applied to a protein A-Sepharose column (1 x 20cm) in 0.025M tris pH7.5 and the column was then well washed in the same buffer. Then 2ml 0.15M glycine HCl pH2.1 in neutral red indicator dye was applied to the column and washed through with 0.025M tris pH7.5. 1ml fractions were collected. As the fractions containing the red dye (also contains IgG) were collected enough 1M tris pH9.5 was added to turn the indicator dye to brown-orange (pH7). Collection of 1ml fractions was continued until all the dye had been washed through the column. The coloured fractions were pooled and concentrated (dilution and re-concentration removed the dye) in an Amicon ultrafiltration system to give the IgG fraction.

D PREPARATION OF PLASMINOGEN-FREE FIBRINOGEN

1g of human fibrinogen (Kabi Pharmaceuticals) was made up in 100ml of a buffer of final concentration 0.3M NaCl - 0.4% sodium citrate pH7.5 (the freeze-dried Kabi material already contained 1g NaCl and 0.1g sodium citrate). This was applied to a lysine-Sepharose column (2.5 x 20cm) in 0.3M NaCl - 0.4% sodium citrate pH7.5 and 8ml fractions collected at 35ml/hour. The fractions were screened for protein at 280nm, and the fractions containing plasminogen-free fibrinogen were pooled and could be concentrated (if necessary) to 100ml to give the same concentration of fibrinogen (1%) as is used to prepare a fibrin plate. The lysine-Sepharose column has to be regenerated after use with a wash in a buffer containing 1M lysine to remove the bound plasminogen.

The plasminogen-free plate is made in the same way as the normal fibrin plate except that plasminogen-free fibrinogen is used. The plate was always tested for absence of plasminogen when used by applying 30µl urokinase (100 Ploug u/ml). This gave no lysis. To check that the sensitivity in normal and plasminogen-free fibrin plates was the same when set up in parallel a 30µl sample of plasmin (5cu/ml) was applied to both types of plate and the lysis areas compared.

4 DETERMINATION OF SEDIMENTATION COEFFICIENTS BY SUCROSE DENSITY GRADIENT ULTRACENTRIFUGATION

The behaviour of a solute in a gravitational field can be described in the following way. In the condition where a density difference between the solute and solvent occurs the effective mass (m_{eff}) can be expressed as:

$$\begin{aligned} m_{eff} &= m - m_{\text{solvent displaced}} \\ &= m - m/dp \\ &= m - m\bar{v}p \\ &= m\,(1-\bar{v}p) \end{aligned}$$

where m = mass of the protein molecule

d = density of the protein molecule

p = solvent density

$\bar{v}$ = partial specific volume of the protein (ie the reciprocal of its density).

Since $f = ma$

where f = force

m = mass

a = acceleration

then in a centrifuge rotor rotating with an angular velocity of w radians/second a solute molecule will experience a centrifugal force Ø per molecule such that at distance x a point is reached when:

$$\text{Ø} = mw^2x(1-\bar{v}p).$$

The centrifugal force exerted on the protein molecule in

the gravitational field is counterbalanced by the resisting force of the solvent due to its viscous resistance which is given by:

$$\text{resisting force} = f\,dx/dt$$

where f = frictional coefficient.

Thus the sedimenting molecule reaches constant velocity.

Since $f = kT/D$

where k = Boltzmann's constant

T = Absolute temperature

D = Translational diffusion coefficient

then when resisting and gravitatonal forces are equal

$$kT/D \cdot dx/dt = m(1-\bar{v}\rho)$$

Multiplying through by Avogadros number (N) and rearranging gives the equation originally derived by Svedberg:

$$M = RT/D(1-\bar{v}) \cdot \frac{dx/dt}{w^2 x}$$

where M = molecular weight

R = Gas constant.

The quantity $\frac{dx/dt}{w^2 x}$ is determined in the ultracentrifuge and is termed the sedimentation coefficient s. The unit of s is usually expressed as one Svedberg (S) thus

$$s = 1 \times 10^{-13}\ \text{Sec}^{-1} = 1S.$$

The Svedberg equation is usually written in the form

$$M = RTs/D(1-\bar{v}\rho).$$

The centrifuge used was an MSE superspeed 65 preparative ultracentrifuge with a titanium 3 x 6.5ml swing-out rotor (MSE type 59587) capable of producing 420,000g at its maximum radius at a speed of 60,000rpm. 6ml thin walled polycarbonate centrifuge tubes (MSE type 59203) were used to contain the gradients which were produced by over-layering 0.4ml of 30% $^{w}/_{v}$ sucrose with 1.4ml each of 24%, 18%, 12% and 6% sucrose solutions in 25mM tris, 1mM EDTA, 3.8mM borate, 1.5M NaCl pH8.8. The gradients were left for four hours at room temperature to diffuse. The protein to be investigated as well as standards were over-layered on the gradient and centrifuged for 20 hours.

The gradients were fractionated with a peristaltic pump connected at one end to a pasteur pipette which was carefully lowered to the bottom of the gradient. Pumping was then begun and 30 equal fractions collected for each gradient. The fractions were analysed for protein, radioactivity and/or biological activity and graphs constructed of the latter versus the number of fractions moved (sedimentation distance) from the meniscus (Figs 22-24). A standard curve was then constructed (Fig 25) of the number of fractions moved versus $S^{o}_{20,w}$ (sedimentation coefficient corrected for density and viscosity to water at 20°C), from which the unknown $S^{o}_{20,w}$ could be read using the determined sedimentation distance. The standard curve was linear which was slightly surprising due to the non-linear viscosity characteristics of the sucrose gradient. This was explained by inspection of a graph of viscosity

versus concentration for sucrose solutions. In the range of concentrations used the viscosity increases in an almost linear manner and this is counterbalanced by the increase in 'g' with radius. Only at higher concentrations does the viscosity increase become significantly non-linear.

As already stated the determination of the sedimentation coefficient at 4°C by comparison with standards of known $S^{o}_{20,w}$ also run at 4°C gives $S^{o}_{20,w}$ directly. A further advantage is that temperature variation with time during the run and also temperature differences within the gradient have no effect on the results obtained since internal standards were used.

5 CALCULATION OF THE FRICTIONAL RATIO

f/f_o is defined as the ratio of the frictional coefficient of the hydrated solute, f, to that of the anhydrous spherical molecule, fo.

Using the Stokes equation

$$f = 6\pi\eta r$$

where η = coefficient of viscosity

r = radius of the solute

then $$f/f_o = 6\pi\eta r / 6\pi\eta r_o$$

The molecular volume V_m of an anhydrous spherical solute can be expressed as

$$V_m = \frac{4}{3}\pi r_o^3$$

or since the partial specific volume $\bar{v}$ refers to the anhydrous sphere, then

$$V_m = \bar{v}M/N$$

$$4\pi r_o^3/3 = \bar{v}M/N \qquad \text{and} \qquad r_o = \frac{3\bar{v}M^{\frac{1}{3}}}{4\pi N}$$

$$f/f_o = \frac{r}{\frac{3\bar{v}M^{\frac{1}{3}}}{4\pi N}}$$

The quantity r is the Stokes radius of the hydrated molecule and can be found from the diffusion coefficient using the Stokes-Einstein equation or can be measured by gel filtration.

$f/_{fo}$ is always greater than unity and is a measure of hydration and asymmetry. $f/_{fo}$ values range from 1.1 to over 2. Globular proteins have values ranging up to about 1.3. Ratios in excess of 1.3 cannot be accounted for solely by hydration and indicate asymmetry of the molecule and/or an expanded non-compact structure.

6 PREPARATION OF ANTISERA

The antisera were raised in 3-5kg New Zealand white rabbits. 0.5ml of the vascular plasminogen activator and 0.5ml Freunds complete adjuvant was injected sub cutaneously at four sites (two above the scapula and two on the inside of the thigh). Six weeks later a boost injection of 0.5ml activator and 0.5ml Freunds incomplete adjuvant was administered at the same sites. Ten days later the rabbit was bled from the lateral ear vein. 50mls of blood was collected. This was left at $4^{o}C$ (to retract the clot) for 24 hours before the serum (20mls) was decanted.

7 OUCHTERLONY IMMUNODIFFUSION

A molten solution of 1.5% agarose in 25mM tris, 1mM EDTA, 3.8mM borate, 0.15M NaCl pH8.8 was prepared by heating in a boiling water bath for 20 minutes. 12.5ml of this solution was poured onto an 8 x 8cm glass plate placed on a level surface and left to set for one hour.

A central well was cut and six peripheral wells at a distance of 5mm. The antiserum (20µl) was placed in the central well and a range of antigens (20µl) in the surrounding wells. The plate was left to diffuse at room temperature for 48 hours.

The gel was then dried under weighted filter paper, washed overnight in a large volume of saline, dried as before and then washed in distilled water. The gel was then dried, under a piece of filter paper, at approximately 50°C for 1-2 hours before being stained in 0.5% amido black 10B in 45% ethanol, 10% acetic acid (15 minutes) and destained in the solvent.

8 CONCENTRATION OF PROTEIN SOLUTIONS

Concentration of protein solutions was carried out by gas pressure ultrafiltration. The apparatus was manufactured by the Amicon Corporation. Various membrane porosities are available. In this work PM10, PM30 and XM50 were generally used; they retain molecules of molecular weight greater than 10,000, 30,000 and 50,000 respectively. The PM10 and PM30 membranes were operated at 50psi, while the XM50 was used at 25psi. The XM50 membrane was used for solutions of the activator known to be in a high molecular weight complex with soluble fibrin.

The solution could be stirred magnetically while the system was pressurised thus eliminating the possibility of the formation of a thin layer of highly concentrated protein above the membrane. The apparatus is available in various capacities of which 5ml, 50ml, 400ml and 2 litre versions were used.

The system was used at $4^{o}C$ as well as room temperature, although the flow rate was reduced at the lower temperature. Occasionally fibrin precipitation was a problem; this slows the flow rate down, but more importantly binds the vascular activator.

P ELECTROPHORESIS

A POLYACRYLAMIDE GEL ELECTROPHORESIS

Polyacrylamide gel gradient slabs of 4-30% acrylamide were equilibrated before use in the electrophoresis tank at 400V/50mA. This was to replace the gel buffer with tank buffer. The tank buffer used was generally 0.5M tris, 0.02M EDTA, 0.075M borate pH8.8. However a $^{1}/_{5}$th dilution of this was also employed.

To 25µl of the concentrated sample was added 10µl of a bromophenol blue/sucrose mixture before being applied to the gel. The gels were normally run at 100 volts (although 50 volts and 75 volts were also used). The buffer was circulated through a cooling tank at 4°C.

After electrophoresis the gels were placed in distilled water for half an hour to remove the buffer salts before either being stained in 0.04% Coomassie blue G-250 in 3% $HClO_4$, overlayered with fibrin or screened for radioactivity (see Text).

B SODIUM DODECYL SULPHATE - POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

The method used was that described by Weber and Osborn (1969). The samples to be run in the SDS-PAGE system (see Text) were incubated at 37°C for two hours in 0.01M sodium phosphate buffer pH7 containing 1% SDS and 1% β-mercaptoethanol.

After incubation, the samples with added bromophenol blue and a drop of glycerol were applied to 7% polyacrylamide gels in 10cm glass tubes of 6mm internal diameter. The buffer employed was 0.05M NaH_2PO_4-0.14M Na_2HPO_4-0.2% SDS. The gels were run at a constant current of 8mA/tube before being stained in Coomassie brilliant blue R250 in 40% $^{v}/_{v}$ methanol, 10% $^{v}/_{v}$ acetic acid, 50% $^{v}/_{v}$ distilled water and destained in the same solvent.

C CELLULOSE ACETATE ELECTROPHORESIS

Cellulose acetate strips (Cellogel, Reeve Angel) were immersed in the electrophoresis buffer for ten minutes, then carefully blotted to remove excess liquid before the samples were applied with a Pasteur pipette drawn out to a fine diameter. The strips were 2.5 x 17cm and the samples (5µl) with added bromophenol blue were applied across the strip about 6cm from the cathodic end.

The tank buffer used was 0.5M tris, 0.02M EDTA, 0.075M borate pH8.8. This was also used at a $^{1}/_{5}$th dilution. The samples were run at 50-100 volts and 1mA/strip for 2-3 hours. A plasma standard was generally run as a reference of the mobility of the various classes of proteins. Once run the strip was either stained for protein with 1% napthalene black, 50% methanol, 5% acetic acid (and destained in 50% methanol, 5% acetic acid), assayed for activator activity by fibrin overlay or cut into 1mm slices and counted for ^{125}iodine.

D AGAROSE GEL ELECTROPHORESIS

Electrophoresis of the activator was carried out in 1% agarose gels. Samples (15µl) and standards were applied, with bromophenol blue, onto pieces of filter paper which were placed into slit wells 1cm long in the agarose. These slits were situated nearer the cathodic end of the plate.

The gel was run in 0.5M tris, 0.02M EDTA, 0.075M borate pH8.8 at 200V and 25mA for four hours. Staining was accomplished with 1% napthalene black in 50% methanol, 5% acetic acid and was destained in the same solvent.

10 IODINATION METHODS

A CHLORAMINE T

The vascular activator solution to be labelled was concentrated in a Minicon A25 concentrator (Amicon) to 10µl (ideally this should contain about 5µg of protein). To this was added 5µl carrier-free Na ^{125}I (100mCi/ml, Amersham) and 10µl chloramine T (5mg/ml). This was shaken and after 1 minute 200µl $Na_2S_2O_5$ (0.6mg/ml) and 0.1ml KI (10mg/ml) was added and mixed. The mixture was chromatographed on Sephadex G-25 to separate the labelled protein from other components of the mixture.

The column was eluted in 0.05M Na_2HPO_4 pH7.5 containing 2% horse serum. This is to overcome any interactions between the labelled protein and the gel matrix.

B SUCCINIMIDE ESTER (N-SUCCINIMIDYL 3-(4-HYDROXYPHENYL) PROPIONATE) - SHPP

Iodinated succinimide ester was obtained in benzene from Amersham. The benzene was evaporated in a stream of nitrogen gas. To the dried ester was added 10µl of the activator sample in 0.1M borate buffer pH8.5 and this was placed in an ice-bath for 30 minutes. To obviate subsequent conjugation to carrier proteins, unchanged ester was reacted with 0.5ml of 0.2M glycine-0.1M borate buffer pH8.5 for 5 minutes at room temperature.

The mixture was then run on Sephadex G-25 eluted with 25mM tris, 1mM EDTA, 3.8mM borate, 0.15M NaCl, 0.3% $^{w}/_{v}$ gelatin pH8.8. The gelatin was to avoid adsorption to the gel and co-elution of adsorbed hydroxyphenyl propionic acid.

C LACTOPEROXIDASE/H_2O_2

To 10µl of the vascular activator solution (120u/ml) was added 30µl 0.5M Na_2HPO_4 pH7.5, 30µl solid phase lactoperoxidase (Worthington) and 10µl $Na^{125}I$ (100mCi/ml, Amersham). To this was added 5µl H_2O_2 (made by diluting 10µl of "100 volumes" H_2O_2 to 100ml with distilled water) and the mixture kept at room temperature for 30 minutes with occasional shaking. The reaction was stopped by dilution, by addition of 200µl 0.1M Na_2HPO_4 pH7.5.

The mixture was then run on Sephadex G-25 in 0.05M Na_2HPO_4 pH7.5.

D LACTOPEROXIDASE/GLUCOSE OXIDASE

The procedure for this method is identical to the latter except that the H_2O_2 is replaced by 30µl solid phase glucose oxidase (Worthington) and 5µl 0.5% D-glucose.

ABBREVIATIONS

ADH	Adipic Acid Dihydrazide
trans-AMCHA	trans-4-Aminomethylcyclohexanecarboxylic Acid
DFP	Diisopropylfluorophosphate
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
HMWK	High Molecular Weight Kininogen
PAMBA	p-Aminomethylbenzoic Acid
PEG	Polyethylene Glycol
PHTA	Pig Heart Tissue Activator
PMSF	Phenylmethylsulphonylflouride
PNPGB	p-Nitrophenylguanidinobenzoate
RSV	Rous Sarcoma Virus
SBTI	Soybean Trypsin Inhibitor
SDS	Sodium Dodecyl Sulphate
SFP	Soluble Fibrin Polymer
TLCK	N-α-p-Tosyl-L-Lysine Chloromethyl Ketone
TPCK	L-1-Tosylamide-2-Phenylethylchloromethyl Ketone
VPA	Vascular Plasminogen Activator

PUBLISHED WORK

PEPPER D S, ALLEN R A In Progress in Chemical Fibrinolysis and Thrombolysis, Vol 3; Editors Davidson J F, Rowan R M, Samama M M, Desnoyers P C; Raven Press, New York, 1978, 91-98. Isolation and Characterization of Human Cadaver Vascular Endothelial Activator.

A reprint of the article is appended at the end of this thesis.

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