CHARACTERIZATION OF A PLASMINOGEN ACTIVATOR FROM HUMAN MELANOMA CELLS CULTURED IN VITRO

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ABSTRACT

In this thesis I describe the work that I have done on the isolation and characterization of a plasminogen activator, Mel-PA, that is released by human melanoma cells cultured *in vitro*. This enzyme was compared to the urinary plasminogen activator, urokinase.

The human melanoma cell line, RPMI-7272, (also referred to as the "Bowes" melanoma cell line) released large amounts of Mel-PA into the surrounding medium when cultured under serum-free conditions. A subline of these cells (Bowes II) was developed that was capable of continuous growth in the absence of serum. These cells released only one type of plasminogen activator with a molecular weight of approximately 70 000 daltons.

A technique was developed in which plasminogen activators were separated electrophoretically and detected in polyacrylamide gel slabs containing the co-polymerized substrates, plasminogen and gelatin. The technique was compared with the zymographic procedure developed by Granelli-Piperno and Reich (62) using fibrin-plasminogen-agarose underlays.

Mel-PA was concentrated and partially purified by affinity chromatography on benzamidine-sepharose. This preparation was used to prepare rabbit antisera to the enzyme. These antibodies inhibited the activity of plasminogen activators released by all melanoma cells but had no effect on urokinase. Antibodies to urokinase had no effect on Mel-PA. A survey of human plasminogen activators and their distribution by immunochemical and electrophoretic techniques showed that tissue extracts and body fluids, with the exception of normal urine, contained mixtures of Mel-PA- and urokinase-type enzymes. Urine of patients with some types of renal disease also contained a Mel-PA type enzyme.

A study of the distribution of plasminogen activators in tissues and body fluids obtained from a number of animals showed that all mammals examined had two immunochemically distinct plasminogen activators that corresponded, in their distribution, to the urokinase-like and Mel-PA-like enzymes of man. Antibodies to human Mel-PA cross-reacted with the corresponding enzyme in all mammals tested, whereas antibodies to human urokinase were species specific.

The seeds of the South African legume, Erythrina latissima, contain a 20 000 dalton protein that functioned as an inhibitor of Mel-PA, plasmin, and trypsin, but had no effect on urokinase. During its reaction with the enzymes the inhibitor was cleaved by Mel-PA and trypsin but not by urokinase. The susceptible bond was straddled by an intrachain disulphide bridge. The inhibitor bound reversibly to Mel-PA and could therefore be used to develop an affinity reagent for a one-step purification procedure for Mel-PA in melanoma cell harvest fluids.

Purified preparations of Mel-PA could be shown to comprise both active enzyme (two chain form) and pro-enzyme (one chain form). The one chain form could be converted to the two chain form by treatment with plasmin. It could also be shown that fibrinogen and fibrin contained a contaminating protease that was capable of converting pro-Mel-PA to Mel-PA.

A comparative study of the kinetic behaviour of Mel-PA and urokinase showed numerous differences between the catalytic activities of these two enzymes. Mel-PA was capable of binding to fibrinogen insolublilized on a plastic surface whereas urokinase did not. The presence of fibrinogen enhanced the plasminogen activating activity of Mel-PA but had no effect on urokinase activity.

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ABBREVIATIONS

Ab	-	antibody
AMC	-	amino methyl coumarin
BPTI	-	basic pancreatic trypsin inhibitor (Kunitz)
BSA	-	bovine serum albumin
DFP	-	diisopropylfluorophosphate
DME	-	Dulbecco's modified Eagle medium
DMSO	-	dimethylsulphoxide
EDTA	-	ethylene diamine tetra-acetate
FCS	-	foetal bovine serum
FU	-	fluorometric unit
IgG	-	immunoglobulin G
Kdalton	-	kilodalton
Mel-PA	-	melanoma plasminogen activator
MW	-	molecular weight
NPGB	-	nitrophenyl guanidinobenzoate
PA	-	plasminogen activator
PBS	-	phosphate buffered saline (8mM Na_2HPO_4; 1.5 mM KH_2PO_4; 0.14 M NaCl; 2.7 mM KCl)
RPMI	-	Roswell Park Memorial Institute culture medium 1640
SDS	-	sodium dodecyl sulphate
TCA	-	trichloracetic acid
TD	-	Tris Dulbecco's saline (24.8 mM Tris HCl pH 7.4 containing 0.1mM Na_2HPO_4, 5 mM KCl, 0.14M NaCl)
TPA(PMA)	-	12-O-tetradecanoylphorbol-13-acetate
Tris	-	tris(hydroxymethyl)-aminomethane
T-T(0.1)	-	0.1M Tris HCl pH 8.1 containing 0.1% Triton X-100
UK	-	urokinase

INTRODUCTION

Biological mechanisms for the liquefaction of blood clots have been known and studied for at least a century. Possibly the earliest systematic studies and definitive reports were those of Sahli in 1885 (1) who found that urine dissolved blood clots and Loeb in 1898 (2) who showed that regenerating epithelial cells *in vivo* caused dissolution of clotted plasma. Dastre in 1893 (3) was the first to use the term "fibrinolysis" for this phenomenon. Barker in 1908 (4) identified a "proteolytic factor" associated with fibrinolysis and Demuth and von Riesen (1928)(5), while studying cells cultured *in vitro*, suggested that the lysis of the fibrin clot substrate by these cells involved the activation of a pro-enzyme present in the surrounding plasma-containing medium. Similar observations were made by Fischer in 1946 (6) who also worked with cells cultured *in vitro*. At approximately the same time, Tillet and Garner (7) discovered a fibrinolytic agent in culture filtrates of beta haemolytic streptococci. In 1941 Milstone (8) showed that lysis of fibrin by this agent depended on the presence of a "lytic factor" in human plasma. Although these early observations generated a great deal of research interest for their possible relevance to the control and management of thrombosis, work on fibrinolysis was constrained by a lack of expertise in protein chemistry, a limited knowledge of enzyme action and relatively crude techniques.

It was not until the mid 1940's that Kaplan (9) and Christensen (10) demonstrated that the "lytic factor" of Milstone was a powerful proteolytic enzyme capable of digesting fibrin, fibrinogen, casein and gelatin. This "lytic factor" was named "plasmin", and its inactive precursor "plasminogen" by Christensen and MacLeod in 1945 (11).

In 1915, Fleisher and Loeb (12) demonstrated that a wide variety of tissues could induce fibrinolysis, but it was only in 1947 that Astrup

and Permin (13) showed that this fibrolytic activity of tissues was due to the activation of plasminogen by a "tissue factor" which was later named "tissue activator of plasminogen" (14,15) or "plasminogen activator". At the same time Goldhaber et al (16) made a similar observation with tumour cells cultured in vitro.

In recent years, advances in technical and conceptual knowledge have brought us to a stage at which a number of facts regarding fibrinolytic systems may be regarded as definitely established. These relate to plasminogen, its activation to plasmin, and inhibitors of the enzymes involved in fibrinolysis. These I propose to discuss briefly as introductory material to the substance of this thesis, identifying those respects in which I believe my results have contributed to knowledge of the subject.

Native human plasminogen is a 92 000 dalton glycoprotein containing 2% carbohydrate and a single polypeptide chain with glutamic acid as its NH_2-terminal residue (17,18). During the activation of plasminogen by plasminogen activators a single Arg-Val bond is cleaved to form plasmin with a heavy chain (67 000 daltons) and a light chain (25 000 daltons) connected by disulphide bridges (17,19). Plasmin is a serine-histidine protease with its active site located on the light chain (20,17).

The primary structures of plasminogen and plasmin have been elucidated by the studies of several groups (21, 22, 23, 24, 25, 26, 17). Plasminogen is composed of three distinct domains, namely a "heavy chain", a "light chain" (these two giving rise to the heavy and light chains respectively of plasmin) and "preactivation peptides". The heavy chain has an interesting structure that results from the presence of a series of triple, intrachain disulphide bonds (27). These give rise to a series of loops in the chain referred to as "kringles" from their similarity to the Scandinavian pastry by that name. The "kringle" region shows extensive homology

with prothrombin (25,27). The light chain of plasminogen shows extensive homology with chymotrypsin (23) and contains the active serine site (20,23).

During the activation of Glu-plasminogen to plasmin, the plasminogen molecule is also cleaved at two sites in the amino terminal part of the molecule. Cleavage at these points (Arg 67-Met 68; Lys 76-Lys 77; or Lys 77-Val 78) results in the release of preactivation peptides with a combined molecular weight of 8 000 daltons (21,28,29,30) and in formation of an alternative form, Lys-plasminogen, with a molecular weight of 85 000 daltons and with an amino terminal of lysine. Since the release of the amino terminal peptides from Glu-plasminogen causes a conformational change in the molecule that facilitates the activating cleavage at a susceptible Arg-Val (560-561) bond, into the two chain plasmin molecule, the term "preactivation peptide" or "PAP" was given (31,32,33,34,29). It has been established, however, that the release of PAP from Glu-plasminogen is not necessary for the generation of plasmin by the action of urokinase (35,36) although it has been shown that activation occurs at a much faster rate with Lys-plasminogen (29,32,33,34). The cleavage of the bonds necessary for the release of PAP has been ascribed mainly to the action of plasmin (37,36). A diagrammatic presentation of the sequence of events is shown in Fig. 1.

Purification of plasminogen from plasma usually yields both Glu- and Lys-plasminogen (18,19,38). Since the addition of protease inhibitors during purification gives a better yield of Glu-plasminogen (47), it is now felt that Lys-plasminogen originates from partial proteolytic degradation of Glu-plasminogen during the isolation procedures (18,38).

The biochemistry of plasminogen is noteworthy in several other functional aspects. Firstly the kringle region of the heavy chain contains 5 "lysine binding sites" through which plasminogen binds strongly to fibrin, to lysine analogues such as EACA and to the inhibitor α_2 antiplasmin (24,39, 40,41,42,43,44). This capacity for reversible binding to lysine is

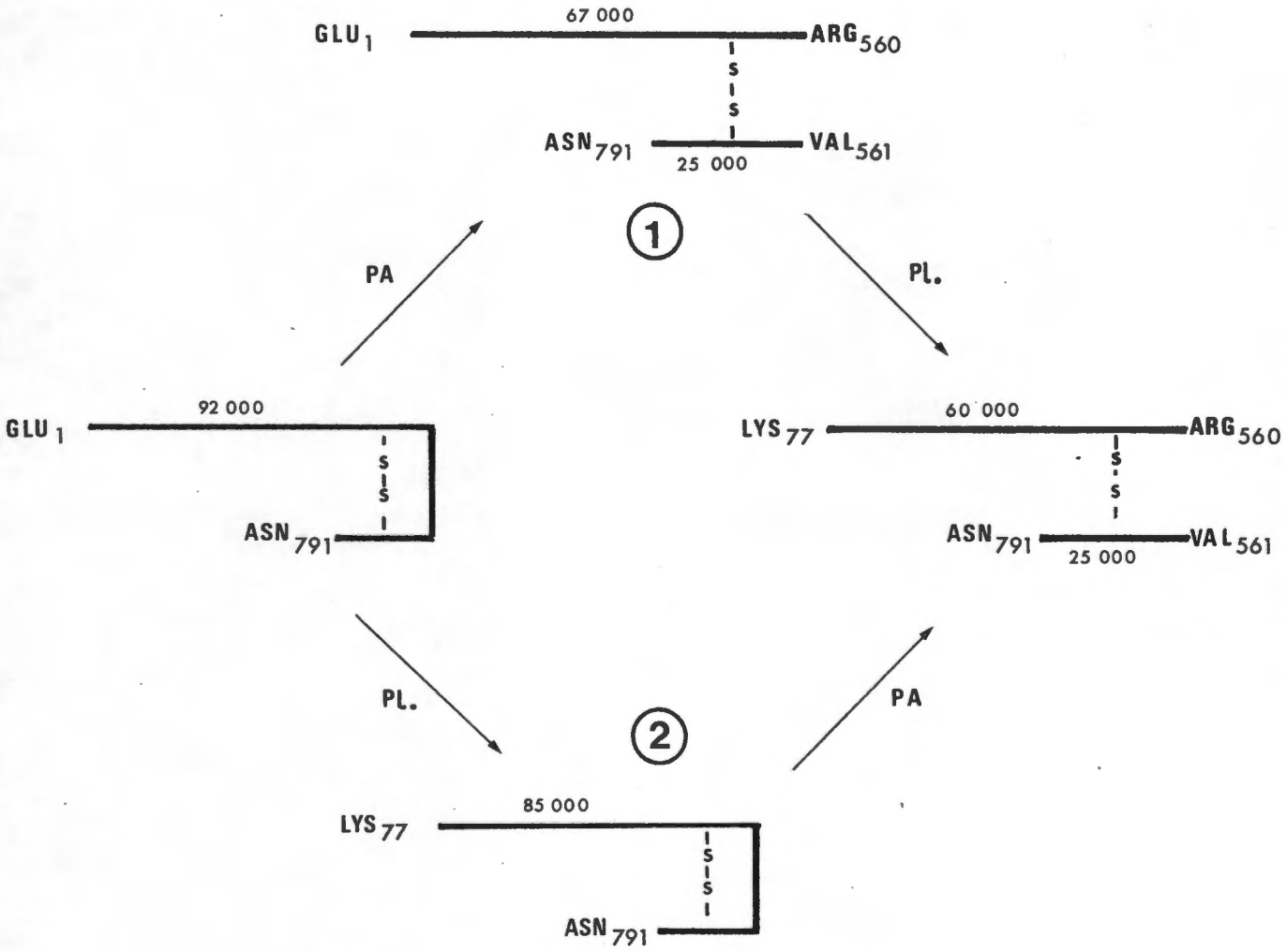


Figure 1 Sequence of reactions occurring during the conversion of plasminogen to plasmin.

Two pathways of plasminogen activation ((1) and (2)) have been proposed, with pathway (2) being favoured *in vivo* (60).

exploited in the use of affinity chromatography with sepharose-lysine for the purification of plasminogen (45). The binding of EACA at low concentration is known to facilitate activation of Glu-plasminogen to plasmin (31,46). and binding to fibrin or α_2 antiplasmin may have important implications for fibrinolytic regulation in vivo that I discuss in a later chapter.

Secondly, plasminogen represents approximately .25% of the total protein present in human plasma (48) and thus constitutes a very considerable pool of potential proteolytic activity. Considered in conjunction with the inducible nature of plasminogen activator release, the implications of this fact for biological mechanisms that involve regulated local proteolysis are considerable.

Thirdly, the metabolic turnover of plasminogen in man is relatively rapid. A daily fractional turnover rate of 55% (48) suggests that the constant levels of the zymogen found in plasma represents an equilibrium between synthesis and loss through continuous activation and degradation. This in turn may be taken to imply a need for such a mechanism to handle continuous thrombus deposition in the human vascular tree.

Plasminogen activators of mammalian origin comprise a group of divers serine proteases that have been demonstrated in many tissues, body fluids and secretions. They are also released by a wide variety of cells cultured in vitro (Table 1).

The different human plasminogen activators first came into comparison when they were detected separately in urine (49,50). and in tissues (13). Since, at the time, techniques were not available for the definitive molecular identification of the enzymes from these two sources, they were referred to as "urokinase" (49) and "tissue plasminogen activator" (14,15) and it was tacitly agreed that they should be regarded as different until proved otherwise (14). The discovery of plasminogen activators in

TABLE 1

OCCURRENCE OF PLASMINOGEN ACTIVATORS IN HUMANS AND ANIMALS

Source	Reference
Tissues	
Uterus	68,55
Lung	52,69,70
Prostate	52,70,71
Colon	52,72
Kidney	198,73,74
Heart	198,75,76,77
Intestine	198
Blood vessels	198,79,80
Leucocytes	81,82,83,84
Ovary	85
Erythrocytes	86
Chondrocytes	87
Mammary glands	88
Body fluids and secretions	
Serum and plasma	89,90,91,66,92,93
Bile	94
Seminal plasma	95,96
Saliva	97
Tears	198
Milk	98
Urine	99-102
Cells cultured in vitro	
Normal cells	103-106,51
Transformed or neoplastic cells	107-118,61,106

blood vessels and in plasma added "vascular plasminogen activator" and "blood plasminogen activator" to the list.

Thus, a classification of plasminogen activators developed that was based upon the origins of the enzyme rather than upon more satisfactory biochemical criteria. This classification was to some extent justified by the finding that urokinase and tissue plasminogen activator were, in all probability, not the same since they were antigenically dissimilar (51,52, 53,54,55,56) and had different binding affinities for fibrin (46,57,58,59). Vascular plasminogen activators and blood plasminogen activators occupied an uncertain position. In a comprehensive review of the subject presented as recently as 1979, Collen stated that "the plasminogen activator found in blood probably represents released vascular plasminogen activator and that these activators are similar or identical to the tissue activator, but different from urokinase" (60).

The successful application of more advanced analytical and preparative techniques to the study of human plasminogen activators has brought us to the stage where it is now possible to say with certainty that normal urine contains plasminogen activator activity, more than 95% of which is attributable to a number of enzymes that are exclusive in the sense that they all belong to the same immunochemically-defined family (198). There is thus reasonable justification for retaining the term "urokinase" to describe the major fraction of these urinary plasminogen activators.

Melanoma cells cultured <u>in vitro</u> release plasminogen activators that are similarly exclusive in that they all show immunochemical cross-reactivity with each other but not with urokinase (61). Since urokinase enzymes and the enzymes released by melanoma cells are antigenically unrelated there is justification for referring to the latter group as melanoma-plasminogen activators or Mel-PA.

This thesis is concerned for the most part, with the study of

Mel-PA's and in particular plasminogen activators released by a cultured melanoma cell line, RPMI-7272.

These cells proved to be a convenient source of enzyme for the fact that they released no plasminogen activators of the urokinase type. In Chapter I of this thesis I describe the procedures used to cultivate these melanoma cells and to isolate a partially purified plasminogen activator from the conditioned medium in which these cells had been grown.

In Chapter II I describe a technique that I developed for the analysis and characterisation of plasminogen activators separated by electrophoresis in polyacrylamide gel slabs containing co-polymerized substrates. This technique was compared with the zymographic procedures developed by Granelli-Piperno and Reich (62) using plasminogen-fibrin-agarose underlays.

Partially purified enzyme isolated from melanoma cell cultures was used to prepare rabbit antisera. These antibodies inhibited all Mel-PA's but did not inhibit urokinase. Conversely, antibodies prepared to urokinase inhibited the urinary enzyme but had no effect upon Mel-PA.

Having serological reagents that distinguished clearly between Mel-PA and urokinase and electrophoretic techniques for the characterisation of these enzymes according to their molecular weights, I was able to undertake a comprehensive survey of human plasminogen activators and their distribution. These studies showed that plasminogen activators present in tissue extracts were invariably mixtures of the urokinase and Mel-PA type enzymes. The same was true for plasminogen activators that could be identified in a variety of body fluids and secretions. Normal urine was found to contain virtually only urokinase with very little of the Mel-PA type enzyme. Urine obtained from patients with renal disease, however, contained considerably more of the Mel-PA. A study of plasminogen activators present in tissues and body fluids obtained from a variety of animal species showed that, with the

exception of the pig, all mammals examined contained plasminogen activators of the two immunochemically distinct types that corresponded in their distribution to the Mel-PA and urokinase found in the human situation.

The interesting fact also emerged that antibodies to human Mel-PA cross-reacted with the corresponding enzyme in all mammals studied. Antibodies to human urokinase, however, were species specific. The pig proved to be an interesting exception to the general rule in that porcine urinary plasminogen activator was consistently inhibited by antibodies to human Mel-PA. These combined electrophoretic and immunochemical studies are described in Chapter III.

In Chapter IV I present the results of work that I did to identify a 20 000 dalton protein present in the seeds of the South African Erythrina species. This compound functioned as an inhibitor of Mel-PA and plasmin, but had no effect on urokinase. It bound reversibly to Mel-PA and could thus be used to develop an affinity reagent for the one step purification of Mel-PA from melanoma cell harvest fluids. Studies with radioactive inhibitor showed that the compound was cleaved by Mel-PA at a susceptible bond straddled by an intrachain disulphide bridge. Trypsin cleaved at the same bond, but urokinase had no effect.

Mel-PA purified by benzamidine- or Erythrina-inhibitor-affinity chromatography could be shown to comprise both active enzyme and pro-enzyme. The latter could be converted to active Mel-PA by treatment with plasmin or by incubation with fibrinogen and fibrin. These studies, together with the more extensive investigations that I did to define kinetic parameters of Mel-PA and the way these differed from those of urokinase, are presented in Chapter V. In this chapter and in Chapter VI I also produce evidence to show that the well known effect of fibrin as a "catalyst" of plasminogen activation is due, on the one hand, to the tendency of fibrin to bind to Mel-PA and, on the other hand, to the contaminating presence in most preparations of fibrinogen and fibrin

of a serine protease that converts pro-Mel-PA to Mel-PA.

In Chapter VI I discuss methods for the measurement of plasminogen activators and the reasons for the different results that are obtained when different methods are used. I conclude that Mel-PA is best standardized using a direct assay in which enzyme activity is linearly related to the rate of hydrolysis of fluorogenic or chromogenic synthetic substrates. Under standard conditions 1 pmol of active sites of Mel-PA as measured by labelling with radioactive DFP, would hydrolyse 9.8 pmol of substrate/min. Other assay procedures are related to this standard method.

Although urokinase and Mel-PA type enzymes could be identified as the major if not the only plasminogen activators in tissues and most body fluids, blood plasma could be shown by electrophoretic and immunochemical techniques to contain plasminogen dependent fibrinolytic and caseinolytic enzymes that are different from either of these two groups. The situation in blood is made more complex by the observation that normal plasma contains urokinase in a pro-active form (63); it contains Mel-PA after exercise and venous occlusions (64,65,66); and it contains a number of potent inhibitors of plasmin and possibly plasminogen activators (67).

The fact that blood, urine and tissues are so easily shown to contain varying mixtures of plasminogen activators makes it inappropriate to retain such terms as "tissue plasminogen activator", "urokinase" or "blood plasminogen activator". Since techniques are now available for the definitive identification of each of the components of these mixtures, it is therefore high time that a standard and internationally accepted nomenclature was adopted.

The need to bring order to the subject of plasminogen activation is particularly pressing in view of their potential usefulness as thrombolytic agents and the increasing extent to which they are being seen as agents for achieving regulated, local and limited proteolysis in a wide range of physiologically important situations that have no obvious

relationship to fibrinolysis. If, for example, we see reason to agree that plasminogen activators are involved in such diverse processes as ovulation (119-122), mammary involution (88) trophoblast implantation (123) and embryogenesis (124), as well as inflammatory processes (81,84,125) we should also be able to agree on which particular enzyme it is that is responsible for each of these functions.

CHAPTER I

ISOLATION AND CONCENTRATION OF THE PLASMINOGEN ACTIVATOR SYNTHESIZED BY MELANOMA CELLS CULTURED IN VITRO.

It is now well established (51,126,61,62,117,127,128,129,115) that human cells cultured *in vitro* release plasminogen activators that may be divided into two distinct types on the basis of their immunochemical, physical and biochemical characteristics.

The first type is in all respects similar to urokinase in that it comprises a mixture of plasminogen dependent proteases with major proteases having molecular weights of 60 000 and 32 000 daltons. These are all inhibited by antibody to urokinase.

The second type is made up of plasminogen activators with a predominant molecular weight species of 70 000 daltons. These are not inhibited by antibody to urokinase. Most cell lines derived from human malignant melanomas release exclusively the activator of this non-urokinase type (61,117,115).

I therefore felt that a melanoma cell culture would provide a useful source of material for the purification of the non-urokinase activator since it would avoid the problems attendant upon separation of two closely related and similar enzymatic functions.

Of the several melanoma cell lines available to me, the RPMI-7272 line was selected for this purpose since these cells produce relatively large quantities of the enzyme and are easily maintained in culture.

For the sake of convenience I refer to the non-urokinase type of plasminogen activator released by melanoma cells as "melanoma plasminogen activator" or "Mel-PA".

In Chapter III I present the work that I did to characterize

Mel-PA and to demonstrate its close similarity to the "tissue activator" first described by Astrup and Permin in 1947 (13). My results are essentially in accord with those presented by Rijken and Collen (130) in a paper published while my studies were in progress. These workers used enzyme derived from the same cell line as did I. In view of the close agreement between our two laboratories on the nature of Mel-PA and for reasons which I discuss in further detail in Chapter III, I shall continue to refer to this enzyme as "Mel-PA" and not as "tissue activator".

In the present chapter I report the results of experiments that were performed to define conditions for cell culture and isolation that would provide optimal yields of enzyme under the most convenient circumstances. While this work was in progress, I was at the same time involved in studies to examine assay techniques, to characterize the activator and to purify the enzyme. For the sake of orderly presentation I have relegated these results to other chapters. Since, however, they had an important bearing on my experimental approaches to defining optimal conditions, it is appropriate for me to make brief mention at this stage of two observations that are more completely documented in other chapters.

In the first instance, I found that melanoma cells released plasminogen activator both as the active enzyme and as a pro-activator which could be converted, by limited proteolysis, to the active form of the enzyme. Evidence for this fact comes from the data presented in Chapter V, in which I showed that plasminogen activator content of harvest fluids could be increased by treatment with plasmin. In the present chapter, therefore, when I describe the effect of culture conditions on enzyme release, these results take into account the fact that both enzyme and pro-enzyme were involved.

Secondly, I found it possible to prepare an affinity reagent for the one-step purification of Mel-PA by coupling an inhibitor from the seed

of the South African tree, Erythrina latissima to sepharose-CL. This work is described in detail in Chapter IV.

MATERIALS AND METHODS

Cultivation of melanoma cells

The cell line (RPMI-7272) used for these studies was isolated in 1974 by Dr. G. Moore, Denver Hospital, Denver, Colorado from a primary culture of a malignant melanoma removed from a female patient. It is also known as "Bowes melanoma" and has been referred to as such in the literature (130). The subculture that I obtained was generously provided by Dr. E. Reich of the Rockefeller University, New York.

Stock cultures of these cells were maintained in disposable plastic petri dishes (100 mm or 60 mm, Falcon No. 3003 and 3002) at 37°C in a humid atmosphere of 95% air/5% CO_2. The cells grew as adherent monolayers in RPMI 1640 tissue culture medium supplemented with 10% heat-inactivated (56°C; 30 min) foetal bovine serum (FCS), and antibiotics (300 µg/ml penicillin; 200 µg/ml streptomycin; and 10 µg/ml of tylocine).

The cells were passaged at confluence (approximately 4 x 10^5 cells/cm^2) by trypsinisation and reseeding at 5 x 10^5 cells/100 mm dish. After aspirating the medium the cells were incubated in 0.25% Trypsin (Difco, 1:250) in TD at 37°C for 5 min. Detached cells were dispersed by gentle pipetting and the suspension was added to an equal volume of medium containing FCS to neutralize the protease. The cells were then washed by centrifugation at 350g for 5 min, resuspended and reseeded into fresh dishes.

Dormant stocks were maintained in liquid nitrogen according to the following procedure: Healthy cultures were trypsinized and suspended at 2 x 10^6 cells/ml in RPMI 1640 medium containing 10% FCS and 10% DMSO at 0°C. The suspension was distributed as 1 ml aliquots into 3 x 12,5 mm

screw cap nylon storage vials (Nunc, Cat. No. 1078). These were then frozen in an automatic liquid nitrogen cooled freezing apparatus of our own manufacture. This was programmed to freeze the cell suspension according to the protocol of Farrant et al (131), in which suspensions were cooled as rapidly as possible ($-4^{o}C$/min) to a temperature of $-26^{o}C$; they were then maintained at this temperature for a period of 20 minutes after which they were frozen rapidly to a temperature of $-60^{o}C$. At this point they were immersed in liquid nitrogen in a storage container.

To reestablish cultures from frozen cells a vial was removed from liquid nitrogen and thawed rapidly by immersion, with gentle manual agitation, in a $37^{o}C$ waterbath. As soon as the last visible ice crystals had melted, the contents of the vial were added to 10 ml complete tissue culture medium in a 100 mm plastic dish. This was then placed in a CO_2 incubator. After allowing 6 hours for the cells to adhere, the DMSO was removed by washing and the cells were fed with fresh medium.

While this work was in progress, a subline of the Bowes melanoma cells was developed, which would adhere, proliferate and synthesize plasminogen activator in the complete absence of serum. To distinguish these cells, I refer to them as "Bowes II" cells. The serum-dependent cells from which they were derived, I refer to as "Bowes I" cells.

Bowes II were maintained as adherent monolayer cultures in tissue culture flasks (Costar Cat. No. 3150). Monolayers were covered with 50 ml of RPMI 1640 medium supplemented with tylocine and antibiotics but without serum. These cells were passaged at confluence by vigorous tapping. Cells dislodged in this mechanical fashion were suspended in serum-free tissue culture medium at a concentration of approximately 10^6 cells/ml and used to reseed fresh flasks at approximately 5×10^7 cells/flask.

A detailed description of this unique serum independent Bowes II line is shortly to be published in association with Dr. E.L. Wilson with

whom credit for development of the line rests. For the purpose of this thesis it is appropriate to record only the essential and relevant attributes summarized in the Results section.

Collection of conditioned medium.

Observations made by ourselves (106) have shown that it is preferable to collect harvest fluids in the absence of serum since in this way the effect of serum inhibitors on the enzyme are avoided. It is also known that plasminogen activators in dilute serum-free solution are notoriously liable to loss by adsorption to plastic surfaces, by denaturation, or by proteolysis. Albumin (106), gelatin (108), salt (132) detergents (108,130,68) or protease inhibitors or arginine (90, 108,134) have been variously used to counteract these losses. For my particular purposes it was necessary to study the effects of conditions of storage on the enzyme.

Protocols for the collection and storage of harvest fluids are therefore presented and considered in the Results section.

Preparation of aminobenzamidine-sepharose.

Meta-aminobenzamidine was prepared from meta-nitro benzamidine by catalytic hydrogenation according to the method of Hixson and Nishikawa (135) as described in detail in the Appendix (A1.7).

Sepharose CL-4b was activated with CNBr according to the method of Nishikawa et al (136) as detailed in the Appendix (A.1.7).

Aminobenzamidine was linked to the CH-sepharose by carbodiimide coupling according to the method of Hixson and Nishikawa (135) in which meta-aminobenzamidine (8g) in 40 ml 0.2M NaCl, pH 4.75 and 1-ethyl-3-(3'-dimethyl amino-propyl) carbodiimide HCl (EDC; 5g) in H_2O were added

to 100 ml of settled CH-sepharose, and the pH was maintained at 4.75 with 2M HCl. The reaction was continued at 4°C for 24 hr, after which the matrix was washed sequentially with 0.01M HCl - 0.5M NaCl; 0.01M NaOH - 0.5M NaCl; and 0.2M NaCl.

Preparation of zinc chelate-sepharose.

Zinc-chelate-sepharose was prepared according to Rijken et al (130) as follows:

Sepharose CL-4b (300 ml) was washed with distilled water on a sintered glass filter and mixed with 300 ml of 1,4-butanediol diglycidyl ether and 300 ml 0.6M NaOH containing 2 mg/ml sodium borohydride. The suspension was incubated at room temperature for 16 hr with stirring. The activated matrix was then collected under suction on a sintered glass funnel and thoroughly washed by the passage of 50 litres of distilled water. It was then mixed with 200 ml of 2M sodium carbonate, pH 11.0 containing 40g iminodiacetic acid disodium salt. The suspension was rotated for 3 days at room temperature, harvested under suction and then washed with 30 litres of water.

The matrix was resuspended in 2.5 litres of 7.3 mM $ZnCl_2$ and incubated for 1 hr at room temperature. After washing with 5 litres of distilled water and equilibration in 0.02M Tris HCl, pH 7.5 containing 1M NaCl and 0.01% Triton, the matrix was ready to use.

The matrix could be regenerated by washing consecutively with 0.05M EDTA pH 8.0; 0.05M ammonium hydrogen carbonate, pH 10.5; distilled water; 7.3 mM $ZnCl_2$; and 0.02M Tris-HCl pH 7.5 in 0.01% Triton, 1M NaCl.

Assay for plasminogen activator.

Plasminogen activator activity was quantitated by one of two techniques. In the first I measured the time course of plasminogen-dependent release of radioactive fibrin-degradation peptides from ^{125}I-labelled fibrin deposited as an insoluble coating on to plastic surfaces (106).

In the second, I measured the increase in fluorescence that resulted from the direct hydrolysis of the synthetic substrate Cbz-Gly-Gly-Arg-AMC (133). This method, although less sensitive had the advantage of plasminogen independence. It could also be used in the presence of plasmin inhibitors, such as Trasylol, that did not inhibit Mel-PA.

Both of these techniques are described in detail in the Appendix (A3) and are considered fully in Chapter V and VI..

SDS-polyacrylamide gel electrophoresis.

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis in gel slabs was carried out according to the method of Maizel (137). Plasminogen activator activity in gels was detected by the zymographic technique of Granelli-Piperno and Reich (62) or by the method of Heussen and Dowdle (138)(see Appendix A2).

Autoradiography of gels containing ^{3}H-labelled proteins was performed according to the method of Bonner and Laskey (139)(Appendix A.2.5).

RESULTS

Cultivation of RPMI 7272 melanoma cells.

The results of preliminary experiments and considerations of convenience, economy and available facilities, led to the adoption of a standard tissue culture system in which cells were grown as adherent monolayers in 75 cm^2 or 150 cm^2 tissue culture flasks. These containers had the advantage over others, such as petri dishes, that harvest fluids could readily be collected by decantation and it was thus easier to maintain flasks free of bacterial and fungal contamination. Under usual circumstances 30 such flasks yielding approximately 1.5 litres of conditioned medium per day, constituted a manageable and convenient operation.

The appearance of the cell monolayers is shown in the photographs presented in Figure 1.1. Bowes I cells in presence of serum had a polygonal appearance (Fig. 1.1.a). At confluence they tended to pile up in local clusters which detached from the monolayers in viable floating groups. After 4 days of serum deprivation, Bowes I cells appeared less well spread (Fig. 1.1b). The cytoplasm became vacuolated and the cells detached readily as confluent sheets. These changes were reversible within 24 hr by serum replacement. Bowes II cells that had been passaged 13 times in absence of serum over a period of 5 months grew as adherent monolayers (Fig. 1.1c). The cells were less well spread than Bowes I cells. At confluence they too tended to detach from the dish as viable clusters which could easily be dispersed as suspensions. The addition of serum to these cells resulted in a pronounced morphological change (Fig. 1.1d) that came about in a matter of hours. Individual cells spread more extensively; abundant mitotic figures became apparent and the cultures came once more to resemble Bowes I cells from which they were originally derived but had a slightly flatter appearance.

Figure 1.1 Morphological appearance of Bowes I and Bowes II melanoma cells cultured in vitro.

The photographs show the appearance, under phase contrast microscopy, of adherent monolayers of

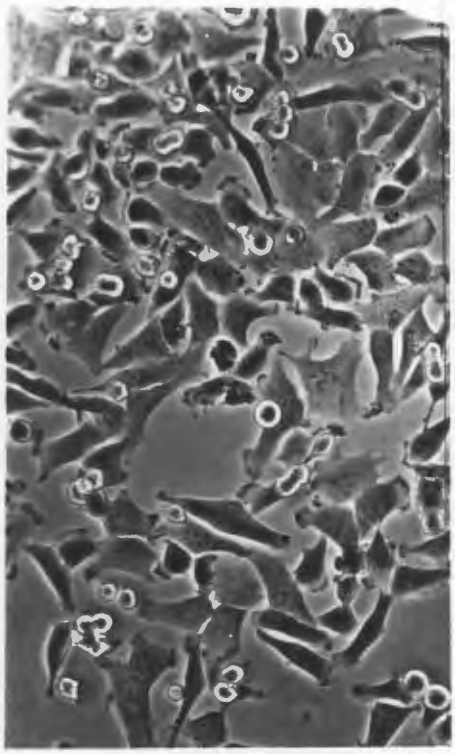
(a) Bowes I cells in RPMI/10% FCS;
(b) Bowes I cells in RPMI after 5 days of serum deprivation
(c) Bowes II cells cultured in RPMI without serum and
(d) Bowes II cells to which medium containing 10% FCS had been added 3 days previously.

Bowes I cells in serum had a polygonal morphology with dendritic processes typical of many melanoma cell lines. Mitotic cells were frequent. When confluent, cells tended to aggregate and to separate from the adherent monolayer (Figure 1.1a). Serum deprivation for 5 days caused the cells to round up and to retract their processes (Figure 1.1b).

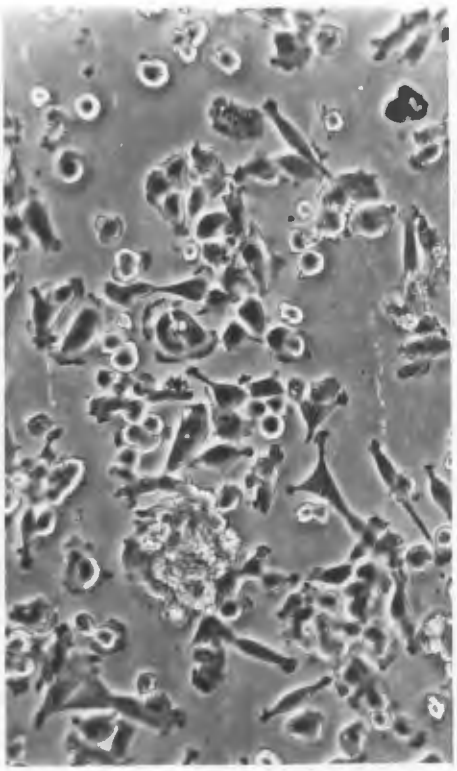
Bowes II cells showed an appearance similar to Bowes I after serum deprivation, although mitoses were noticeably more frequent. Addition of serum to Bowes II cells produced a marked morphological change. The cells became large and flat and were quite distinct from the original Bowes I cells grown in serum.

Figure 1.1

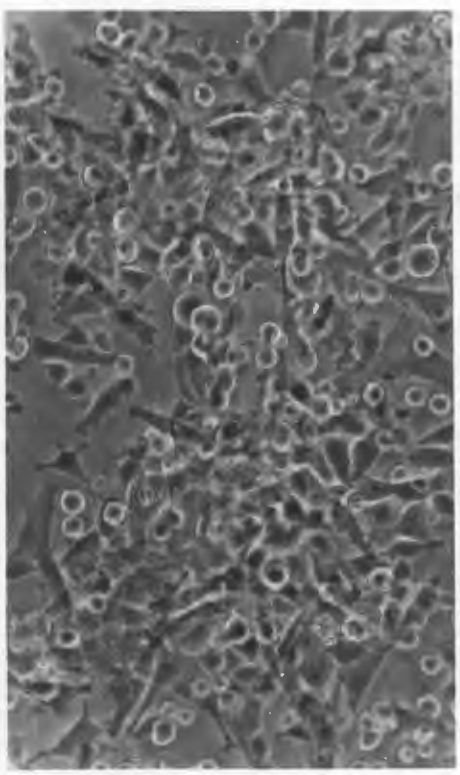
a



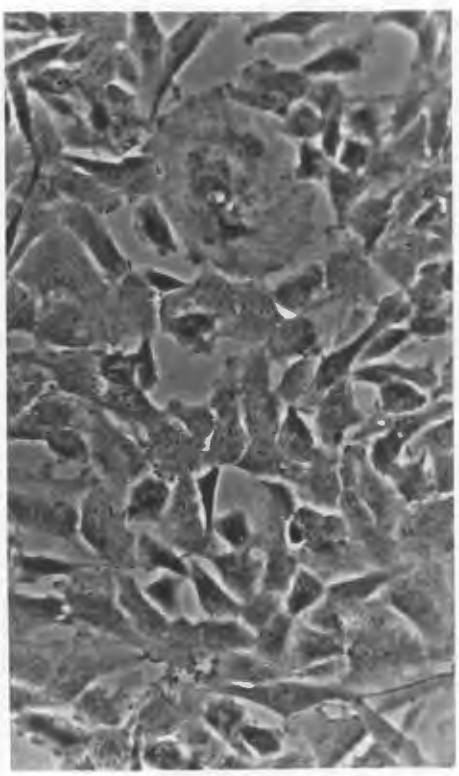
b



c



d



As is evident from the graphs presented in Fig. 1.2, Bowes I cells under standard culture conditions grew with a doubling time of 27.3 hours to reach maximum adherent cell density of 5×10^5 cells/cm^2. If medium was not replenished frequently after confluence had been reached or if the cells were not passaged, rapid deterioration of the cultures resulted. Removal of serum from Bowes I cultures resulted in a rapid and complete cessation of growth.

Bowes II cells in the absence of serum maintained a healthy appearance and grew with a doubling time of 5.7 days. Addition of serum to Bowes II cells resulted in a rapid increase in proliferative rate with a doubling time of 2.1 days.

Collection and storage of conditioned medium.

Conditioned medium containing plasminogen activator was collected in one of two ways, depending upon whether the enzymes were derived from Bowes I or Bowes II cells.

In the case of Bowes I, cells were seeded into 75 cm^2 tissue culture flasks (Falcon Cat. No. 3024) at a density of $2\text{-}5 \times 10^5$ cells, and allowed to grow to near confluency in medium supplemented with 10% FCS. The cultures were then washed once and covered with 20 ml of serum free medium. After 24 hr the medium was collected and fresh medium was added. The process of taking sequential 24 hr, serum free harvest fluids could be repeated 3-4 times before the cells degenerated and tended to detach from the plastic substrate. Vitality and adherence was readily restored by treatment for 24 hours with medium containing 10% serum.

When Bowes II cells were used as the source of enzyme, they were seeded at 5×10^7 cells per 150cm^2 flask (Costar Cat. No. 3150) and covered with 50 ml of medium. The cells proliferated slowly in serum free medium and were hence plated at near confluence. The taking of 24-hour

Figure 1.2 Growth curves of Bowes I and Bowes II melanoma cells in vitro.

A. Bowes I melanoma cells were seeded in RPMI supplemented with 10% FCS on 35 mm dishes at 5×10^4 cells/dish. Medium was changed and cells in replicate cultures were counted at 24 hr intervals for the indicated period of time (•——————•). After 5 days the medium in some cultures (- - - - - -) was replaced with serum-free RPMI to observe the effects of serum deprivation on cell number.

B. Bowes II melanoma cells were seeded either in RPMI at 2.5×10^6 cells/35 mm dish (- - - - -) or in RPMI supplemented with 10% FCS at 1.9×10^5 cells/35 mm dish (•———•). Medium was changed every 48 hr and cells in replicate cultures were counted at the indicated times.

The doubling times, calculated from the exponential part of the growth curves, were as follows:

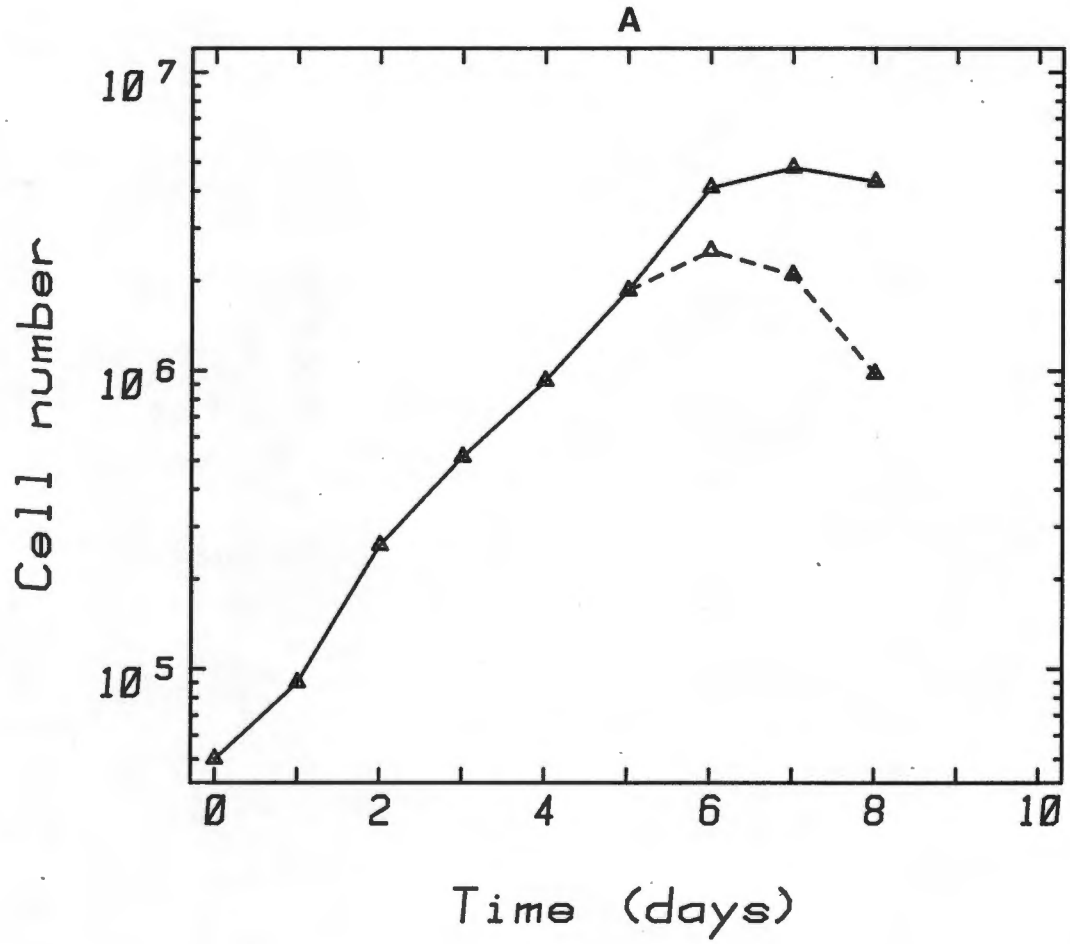
Bowes I cells in RPMI/10% FCS - 1.1 days

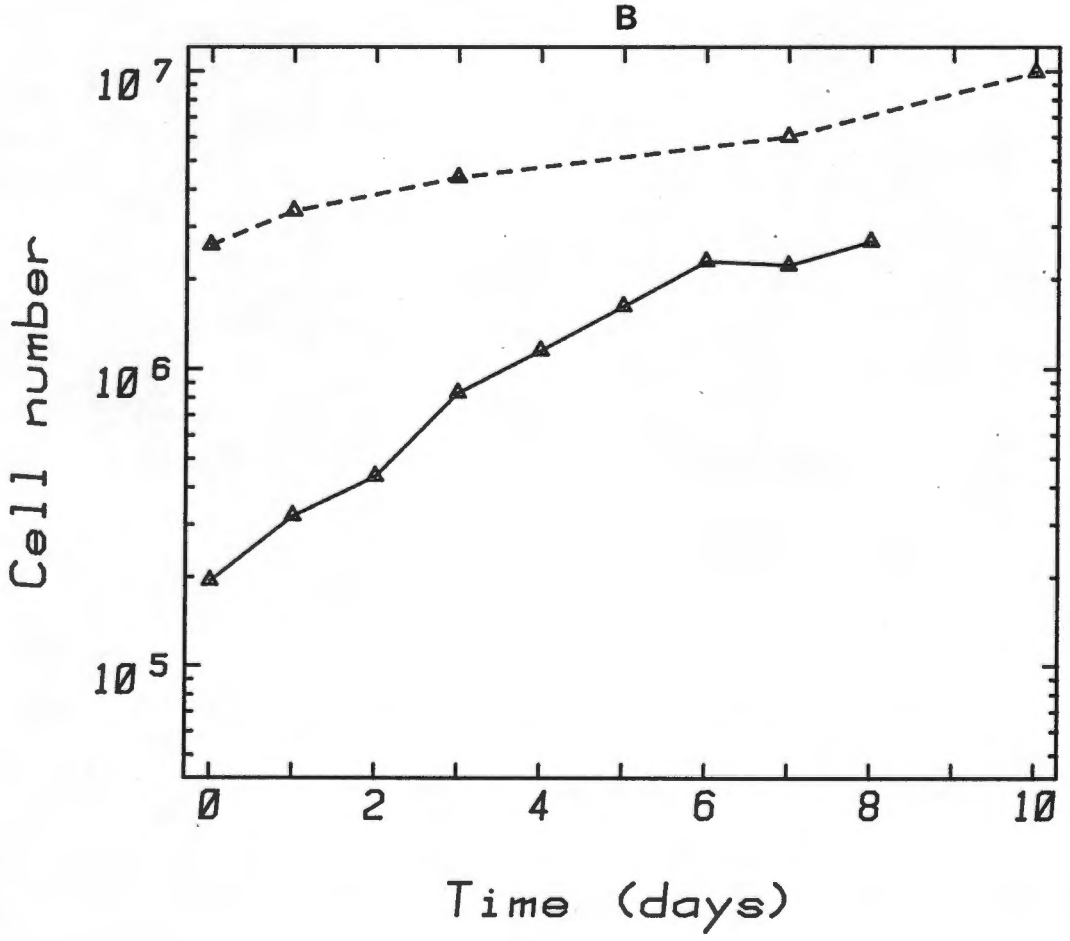
Bowes II cells in RPMI - 5.7 days

Bowes II cells in RPMI/10% FCS - 2.1 days

Although Bowes II cells in the absence of serum, did not attain saturation density, it can be seen that they would have achieved a higher density at confluence than did Bowes I cells in the presence of serum.

Figure 1.2


A
Cell number
10 7
10 6
10 5
0
2
4
6
8
10
Time (days)


B
Cell number
10 7
10 6
10 5
0
2
4
6
8
10
Time (days)

serum free harvest fluids started immediately when cells had become adherent. Daily collection of 50 ml from each flask were taken until confluence of the monolayers necessitated passaging. This usually amounted to approximately 2 weeks.

Mel-PA containing harvest fluids were centrifuged at 2000 rpm for 5 min to remove whole cells and cellular debris. To define optimal conditions for the storage of harvest fluid prepared in this way, the activity of Mel-PA was determined after treatment at different temperatures for various times. The activities were compared with those of harvest fluids kept at -20°C and thawed just before assay. Fig. 1.3 shows that all Mel-PA activity was lost after incubation for 20 hr at 4°C or for 4 hr at 37°C. Methods to prevent this loss of activity were therefore explored.

As is shown in Fig. 1.4 the presence of 0.4 mg/ml of protease- and inhibitor-free bovine serum albumin (PIF-BSA) in harvest fluids greatly preserved enzyme activity.

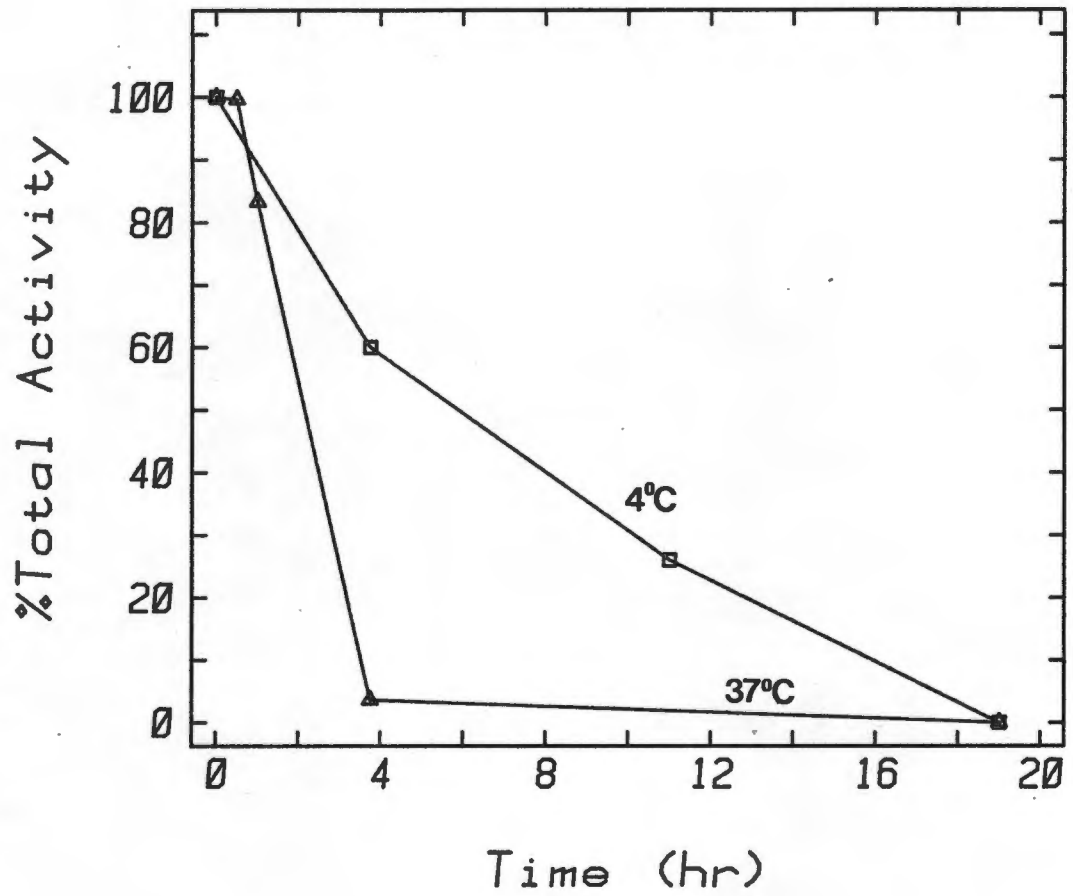
Nonionic detergents such as Tween 80 and Triton X-100 have been reported by others to stabilize plasminogen activator activity (108,130,68). The effect of Triton X-100 on Mel-PA activity was therefore tested. Results given in Table 1.1 show that Mel-PA activity can also be preserved in all cases by the addition of the nonionic detergent, Triton X-100, provided it was added to the container either with the enzyme or before it. Addition after the enzyme had been added and shortly before assay abrogated the protective effect of the detergent. These time relationships led me to conclude that the detergent was acting by inhibiting losses of enzyme by absorption to the wall of the tube.

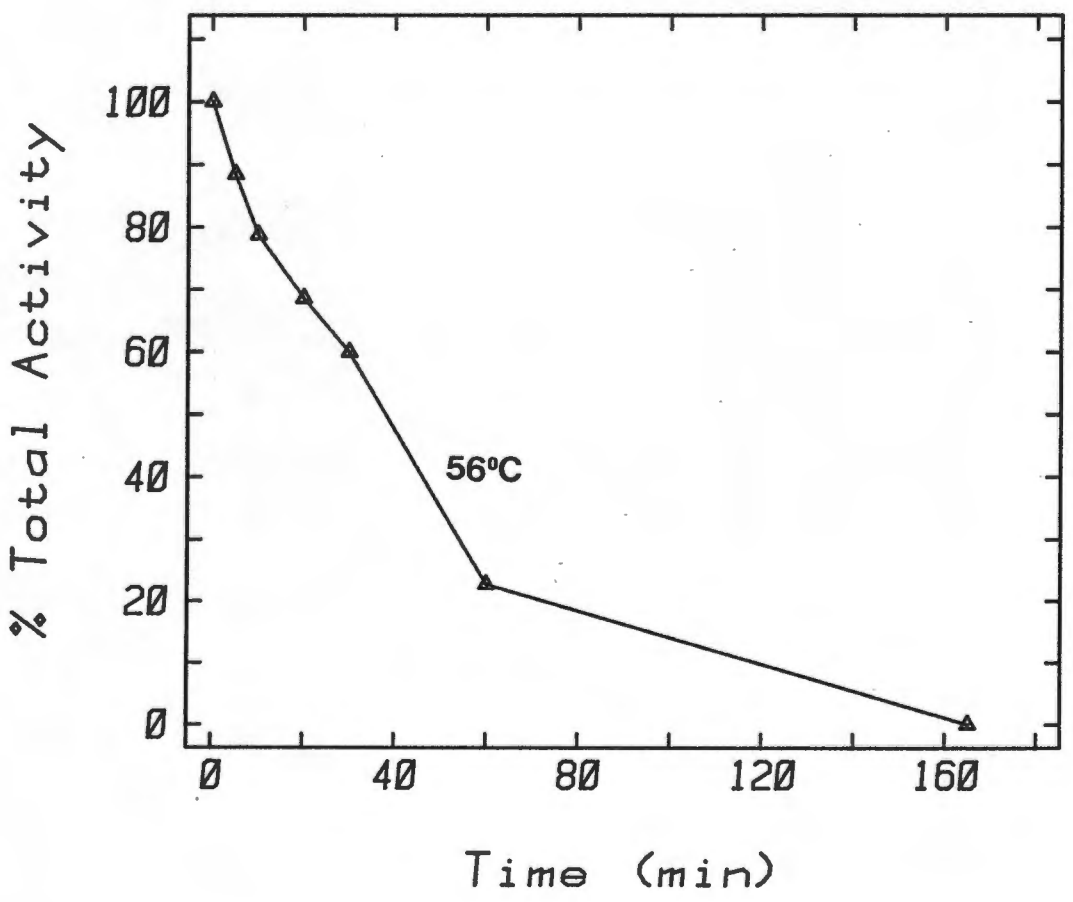
Since I was loath to add a foreign protein, and in particular, one such as albumin with a molecular weight so close to that of Mel-PA, I elected to stabilise the enzyme with nonionic detergents i.e. either 0.1%

Figure 1.3 Thermostability of plasminogen activator in harvest fluids.

Harvest fluid from melanoma cells was stored at -20°C in 100 µl volumes. Samples were thawed and incubated in duplicate for the times and at the temperatures shown. After incubation residual enzyme activity was measured in the ^{125}I-fibrin assay and expressed as a percentage of that present in solutions maintained at -20°C.

Figure 1.3


%Total Activity
100
80
60
40
20
0
0
4
8
12
16
20
4°C
37°C
Time (hr)


% Total Activity
100
80
60
40
20
0
0
40
80
120
160
56°C
Time (min)

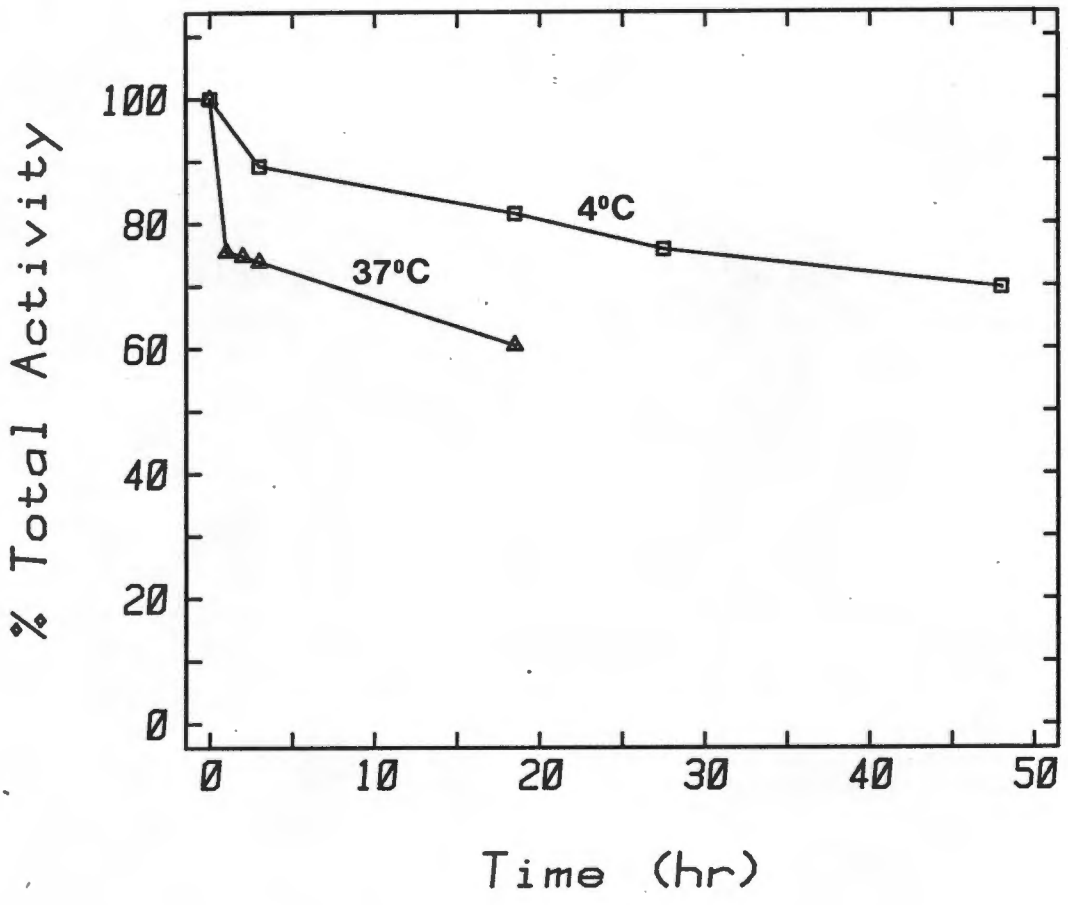


Figure 1.4 Thermostability of plasminogen activator in the presence of bovine serum albumin.

The effect of temperature on Mel-PA was determined using the same protocol as that described in the legend to Fig. 1.3. In this case, however, the samples were adjusted to contain 0.4 mg/ml of BSA before incubation at 4°C or 37°C for the times shown.

TABLE 1.1 Effect of Triton X-100 on stability of plasminogen activator.

Triton conc.	Condition of incubation or storage				
%(v/v)	20°C, 4hr	20°C, 20hr	4°C,20hr	-20°C(stored) (a)	-20°C(stored) (b)
0	80(c)	160	175	185	210
.0001	255	215	335	220	150
.001	555	365	425	440	315
.01	610	390	480	440	200

(c) Figures in the body of the table refer to plasminogen activator activity (% T/30 min/ml) in solutions incubated or stored as indicated.

To 450 µl of conditioned medium from melanoma cells was added 50 µl of 0.1%, 0.01% or 0.001% of Triton X-100 in H_2O or 50 µl of H_2O only. The mixtures were frozen and stored at -20°C.

For the experiment, samples were thawed and incubated at 20°C or 4°C for the times shown.

Residual plasminogen activator activity was then measured in the ^{125}I-fibrin assay.

Comparison tubes (columns a and b) were kept at -20°C and thawed immediately before assay without incubation. In samples under column (a) the Triton was added before freezing and storage; in (b) the Triton was added after thawing and before assay.

Triton X-100 or 0.1% Tween 80.

The stability of Mel-PA in harvest fluids in the presence of 0.1% Triton X-100 at various pH's is shown in Fig. 1.5. As can be seen, the enzyme was stable at neutral pH at 4°C for 48 hr. The highest activity was maintained at pH 5.5 - 6.0. Harvest fluids were therefore acidified to this pH before storage at -20°C. A decline in activity was noticed below pH 4.5 and above pH 8.0.

Using the standard protocol for the collection and storage of harvest fluids, the results summarized in Tables 1.2 and 1.3 were obtained.

Bowes I cells at a density of 1.3×10^5 cells/cm^2 could be relied upon to release approximately 1200 UK units of plasminogen activator in 24 hr when cultivated under 2 ml of medium/10^6 cells. Of this enzyme 90% was active. The remainder was in the form of proactivator. Bowes I cells in the presence of 10% serum grew with a doubling time of 27.3 hr. One 75 cm^2 flask of adherent Bowes I cells at confluence could be sustained with cyclical serum deprivation for harvest fluid collection for an indefinite period provided infection of the cultures could be avoided or cell detachment and death due to serum deprivation or superconfluence was guarded against. Generally speaking one flask of Bowes I cells could be expected to provide harvest fluids for approximately 1 month.

Bowes II cells proliferated more slowly, with a doubling time of approximately 136.8 hr in the absence of serum. One 150 cm^2 bottle carrying a confluent monolayer of 4×10^5 cells/cm^2 and covered with 0.8 ml medium/10^6 cells could be relied upon to produce approximately 3700 UK units of plasminogen activator in 24 hr, of which 10% was in the form of active plasminogen activator and 90% in the form of pro-plasminogen activator.

The slow growth rate in the face of active protein synthesis made these cells very much easier to work with. They showed no tendency to detach from the plastic. On average each 150 cm^2 bottle continued to

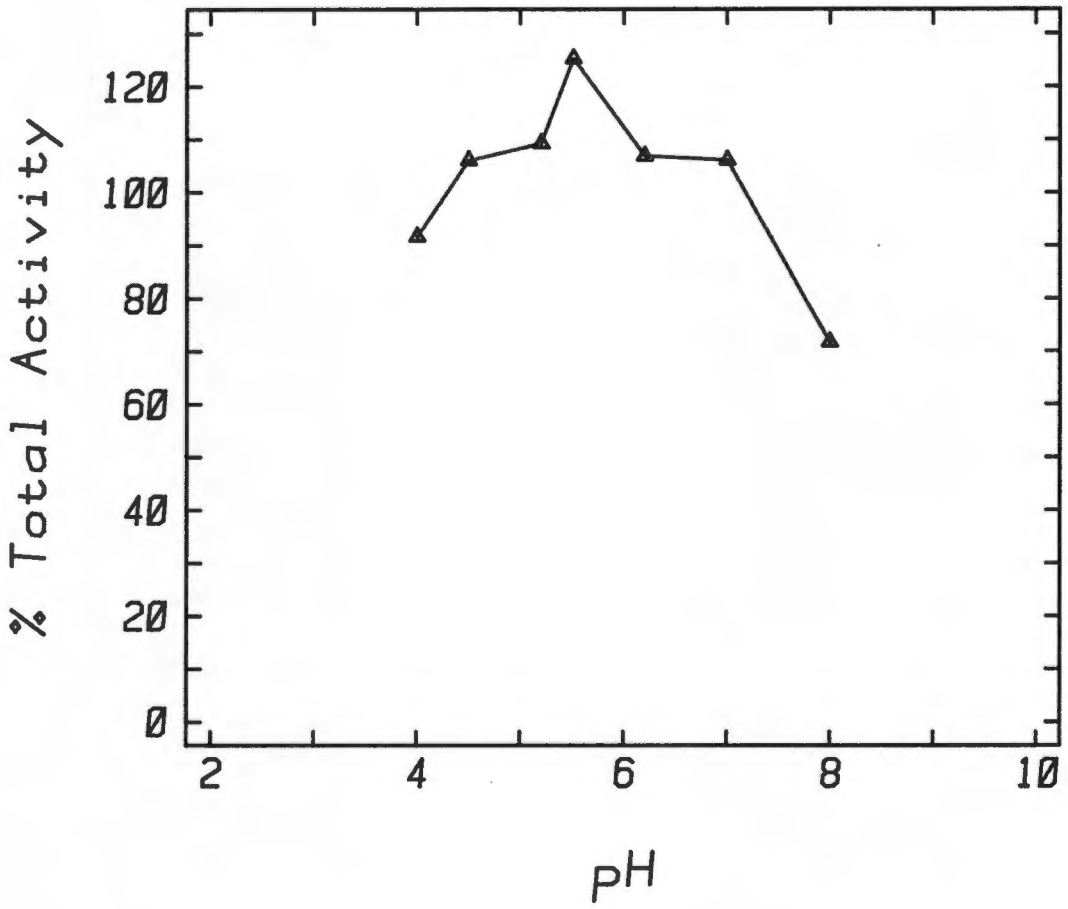


Figure 1.5 Stability of plasminogen activator at different pH's in the presence of 0.1% Triton X-100.

Harvest fluid samples containing 0.1% Triton X-100 were adjusted to different pH values by the careful addition of 0.1M HCl or 0.1M NaOH. They were incubated at 4°C for 48 hr after which the pH values were readjusted to pH 7.2. Plasminogen activator activity was assayed in the ^{125}I-fibrin assay. The results are expressed as the percentage activity in the samples relative to that in harvest fluid containing Triton X-100 and incubated for the same length of time but without adjustment of the pH. Note that optimal stability was observed at a pH of approximately 5.5.

TABLE 1.2 Plasminogen activator release by Bowes I and Bowes II melanoma cells grown in tissue culture flasks.

Cell type	Time (days)	PA activity (UK u/ml)	Total PA (UK u)
Bowes I	1	57.7	1154
	2	62.7	1254
	3	69.0	1380
	4	48.8	976
Bowes II	1	71.2	3560
	2	73.2	3660
	3	79.0	3950
	4	77.0	3850

Harvest fluids from Bowes I cells grown in 75 cm^2 flasks (1.3×10^5 cells/cm^2; 20 ml/flask) or Bowes II cells grown in 150 cm^2 flasks (4×10^5 cells/cm^2; 50 ml/flask) were taken every 24 hr over 4 days. Plasminogen activator activity was measured in the ^{125}I-fibrin assay. Note that PA release stayed constant over the 4 day period in the case of Bowes II cells.

In the case of Bowes I cells, PA activity increased slightly over the first 3 days, but then decreased on the 4th day. By this time most of the cells had detached from the surface, and RPMI supplemented with 10% FCS had to be added to restore adherence.

Note: Since the ^{125}I-fibrin assay measures both Pro PA and PA activity (cf Chapter 6) the results given for PA activity refer to total enzyme activity.

Table 1.3 Plasminogen activator release by Bowes I and Bowes II cells

Cell type	Days in culture	Cell density (cells$\times 10^5 \times cm^{-2}$)	Plasminogen activator release ($FU/10^6$ cells/24 hr) Total activity[(a)]	PA(%)	Pro PA(%)[(b)]
Bowes I	1	1.04	46.62	87.5	12.4
	2	1.27	51.96	92.1	7.9
	3	1.43	64.86	83.9	16.1
Bowes II	1	3.88	26.06	12.7	87.3
	2	3.88	25.10	12.4	87.5
	3	4.01	24.40	10.9	88.9

Bowes I cells were plated at 5×10^5 cells per replicate 35 mm dish in 2 ml RPMI containing 10% FCS. After 24 hr the medium was replaced with serum free RPMI and 24 hr harvest fluids were collected for 3 consecutive days. At the end of each 24 hr period the cell number per plate was counted.

Bowes II cells were plated at 3×10^6 cells per 35 mm dish in 2 ml RPMI.

Plasminogen activator activity was determined using the fluorometric assay with Cbz-Gly-Gly-Arg-AMC as substrate.

(a) Total PA activity was measured after incubation of 295 µl harvest fluid with 5 µl plasmin (0.1 mg/ml) for 60 min at room temperature. Plasmin was inhibited by Trasylol for the assay.

(b) The amount of proactivator (Pro PA) was estimated by subtracting the activity measured without plasmin activation from the total activity.

produce enzyme in useful daily quantities for a period of 3-6 months.

Attempts were made to increase the yield of plasminogen activator from melanoma cells by the addition of inducing agents such as TPA, dexamethasone or retinoic acid. None of these were successful on any of the melanoma cells tested (Table 1.4).

Effects of volume of serum-free medium on Mel-PA secretion by melanoma cells.

Considerations of economy, convenience and enzyme stability made it desirable to keep harvest fluid volumes as small as possible without compromising cellular viability or the rate of enzyme synthesis by the cultured cells.

Several experiments were therefore performed in which harvest fluids were prepared by covering replicate monolayer cultures with different volumes of serum free medium. In a typical experiment Bowes I cells were seeded at 5×10^5 cells/100 mm dish in 10 ml of DME containing 10% FCS. When near confluence, the cells were washed twice with serum-free medium. After 24 hr the conditioned medium was harvested for assay of plasminogen activator activity and the number of cells per dish were counted.

The results summarized in Table 1.5 showed that the rate of enzyme release per cell was relatively independent of medium volume:cell number ratio. Thus 5.7×10^6 cells covered by 5 ml of medium released an amount of 34 UK u/10^6 cells/24 hr, whereas 5.6×10^6 cells covered with 20 ml of medium released 40 UK u/10^6 cells/24 hr. It was felt that 10 ml of medium per 5×10^6 cells provided a satisfactory compromise between the conflicting interests of cell viability and enzyme concentration. All subsequent experiments, therefore, used this ratio for medium volume:cell number.

Although similar formal experiments were not performed on Bowes II cells, my impression was that a lower optimal ratio for medium volume:cell number could be used.

TABLE 1.4 Effect of tetradecanoyl phorbol acetate, retinoic acid and dexamethasone on plasminogen activator release by melanoma cells.

Cell line	Plasminogen activator release (% of control)		
	TPA (10 ng/ml)	RA (10^{-6}M)	Dex (10^{-6}M)
UCT-Mel 1	27	164	143
UCT-Mel 2	100	29	143
UCT-Mel 3	100	100	100
UCT-Mel 5	0	290	100
UCT-Mel 6	100	208	34
UCT-Mel 7	100	143	78

Melanoma cells were seeded at semiconfluence in medium supplemented with FCS. The following day, the test compounds were added at the concentration indicated in the table. The cultures were maintained in the presence of these compounds for 48 hr with one medium change after 24 hr. They were then washed and 24 hr harvest fluids were taken for measurement of plasminogen activator in the ^{125}I-fibrin assay. In the table plasminogen activator release is expressed as the percentage of control values obtained with cells that were not exposed to the compound.

(I am grateful to Miss E. Hoal for permission to use these results. They came from experiments she performed during the course of her work for her Ph.D thesis).

TABLE 1.5 The effect of volume of serum-free medium on plasminogen activator secretion by melanoma cells.

Volume (ml)	UK u/ml	Cells/dish	UK u/10^6 cells
5	39.3±2.5	5.70×10^6	34.5
10	23.7±3.1	5.60×10^6	42.3
15	15.6±1.1	5.10×10^6	46.1
20	10.4±0.8	5.20×10^6	40.0

Melanoma cells were grown to near confluency, washed twice in serum-free DME and incubated in different volumes of serum-free DME. After 24 hr conditioned medium was harvested for assay of plasminogen activator and the number of cells per dish was counted.

The effects of different media and substrata on the growth of melanoma cells and plasminogen activator release.

To optimise medium and culture substratum, Bowes I melanoma cells were seeded into tissue culture (Falcon Cat. No. 3003) or standard plastic bacteriology petri dishes at a density of 5×10^5 cells/100 mm dish. In all cases the cells were suspended in 10 ml medium supplemented with 10% FCS. Different media were used, including minimal Eagle's medium (MEM), MEM-Spinners medium (MEM-S), Dulbecco's modified Eagles medium (DME) or RPMI-1640 medium. These media were buffered with bicarbonate or Hepes buffer as indicated in Table 1.6.

The cells were incubated in the presence of 10% foetal calf serum for a total of 72 hr, with a medium change after 24 hr. After 72 hr in the presence of serum, the cells were washed and covered with 10 ml of the corresponding serum-free medium and incubated for a further 48 hr period after which time the harvest fluid was taken for plasminogen activator assay. The results of these experiments summarised in Table 1.6 show that optimal cell growth was observed in bicarbonate-buffered RPMI or DME in both tissue culture and bacteriology dishes.

Optimal activator production (in terms of enzyme/ml of harvest fluid) was similarly observed in bicarbonate-buffered RPMI or DME.

The detrimental effects of Hepes buffer are evident from the data, and particularly so when Hepes-RPMI was used in combination. In bacteriology dishes cell growth was arrested; in tissue culture dishes the cells detached and died.

Spinner medium, which might have been used to cultivate cells in suspension, failed to support growth as well as did RPMI or DME. For subsequent work RPMI was used.

It is of interest to note that cells from which serum was with-

TABLE 1.6 The effect of different media on the growth of melanoma cells and plasminogen activator release.

	Medium	No. of cells/ml x 10^5 24 hr+FCS	72hr+FCS	48hr-FCS	PA Activity (c) (%T/hr/ml)
a)	MEM	0.42	2.40	7.8	9550
	MEM-H	0.92	2.6	3.7	8350
	MEM-S	0.72	2.8	3.8	9050
	MEM-S-H	0.80	2.0	1.2	3600
	RPMI	0.98	3.1	8.6	13950
	RPMI-H	0.93	1.7	0	1500
	DME	1.20	2.9	8.5	13400
	DME-H	1.10	1.8	3.8	9100
b)	MEM	0.17	2.6	9.9	11600
	MEM-H	0.53	1.2	2.2	
	MEM-S	0.66	3.0	4.2	11800
	MEM-S-H	0.43	1.7	1.0	
	RPMI	0.78	3.0	14.0	15100
	RPMI-H	0.50	1.2	0.9	
	DME	0.84	2.8	6.9	13350
	DME-H	0.13	1.6	2.7	

a) - Cells were plated in Falcon tissue culture dishes (cat. No. 3003)

b) - Cells were plated in bacteriology petri dishes

c) - Enzyme activity is expressed as the percentage total cpm released from ^{125}I-fibrin coated wells in 1 hr.

Abbreviations: MEM - minimum essential medium (Eagle)

MEM-S - minimum essential medium for suspension culture

DME - Dulbecco's modified Eagle medium

H - HEPES (N-2-hydroxyethylpiperazine - N'-2 ethanesulfonic acid)

drawn continued to proliferate at approximately the same rate as they had done in the presence of serum. This "serum-independent" proliferation lasted for approximately 48 hr and required that the medium be bicarbonate-buffered RPMI 1640, MEM or DME.

Since the doubling time of Bowes II cells in serum-free medium was considerably longer than 48 hr (Figure 1.2), I presume that either growth factors present in serum continue to exact their effects on the Bowes I cells for some time after their withdrawal or, alternatively, serum depletion was not complete. It is, in fact, not difficult to show that despite extensive washing with medium, it is very difficult to remove all traces of residual serum.

Large volumes of harvest fluid were required as starting material for the purification of Mel-PA and attempts were therefore made to cultivate melanoma cells on a large scale. Since the melanoma cells seemed capable of growth and Mel-PA synthesis when maintained in bacteriological petri dishes that impaired attachment, cultivation of these cells in suspension in spinner flasks was explored.

A 250 ml spinner flask (Bellco, Cat. No. 1960-00250) was inoculated with melanoma cells at a concentration of 7.0×10^4 cells/ml in a total volume of 250 ml of RPMI/10% FCS equilibrated with 5% CO_2 in air. The culture was kept at 37°C and stirred magnetically at 30 rpm.

At 24 hr intervals 100 ml of the suspension was removed. After taking a 100 µl sample for a cell count, the cells were collected from each 100 ml aliquot by centrifugation at 350g for 5 min, resuspended in 100 ml of fresh medium, and returned to the flask.

After 6 days of cultivation in medium supplemented with serum, all cells were taken out of the flask, washed in serum-free medium and added back in 250 ml RPMI. Medium was again changed every 24 hr when the cell

number was determined and plasminogen activator activity was measured.

There was a slow, steady increase in cell number over the 6-day period of growth in medium supplemented with 10% FCS (Fig. 1.6). After change to serum-free medium, there was a slight decline in cell number which then stabilized at 4.3×10^5 cells/ml. Plasminogen activator secretion per cell remained constant during the 4 day period in serum-free meidum. On the basis of these results it was decided to attempt the mass cultivation of melanoma cells in 1 litre spinner flasks in RPMI medium. For this purpose a system was devised for changing the medium without having to remove the cells from the flask. This is depicted diagrammatically in Fig. 1.7. Medium was pumped out of the flask at a rate of 5-10 ml/hr and replaced at the same rate with fresh medium which had been equilibrated with 5% CO_2/air. The cells were retained by a glass tube filled with glass wool at the outlet. This type of filter proved superior to the various types of Millipore filters in that position, since the latter became clogged with cells very rapidly. Despite the improvised glass wool filter, however, cells escaped through the outlet and accumulated in the tubing thus necessitating frequent changes. Contamination of the effluent with microorganisms consequently became a major problem and led to my abandoning this method of collecting harvest fluid. Furthermore, the Mel-PA concentration in these harvest fluids was very low, varying from 4 UK u/ml to 6 UK u/ml.

A second method of mass cultivation of melanoma cells on Melanex sheets in Sterilin bulk cell culture vessels (ICRF spiral system)(140) was unsuccessful. The melanoma cells did not seem to multiply on the Melanex substrate, nor was I successful in inducing the cells to grow on "Cytodex" beads (Pharmacia)(141).

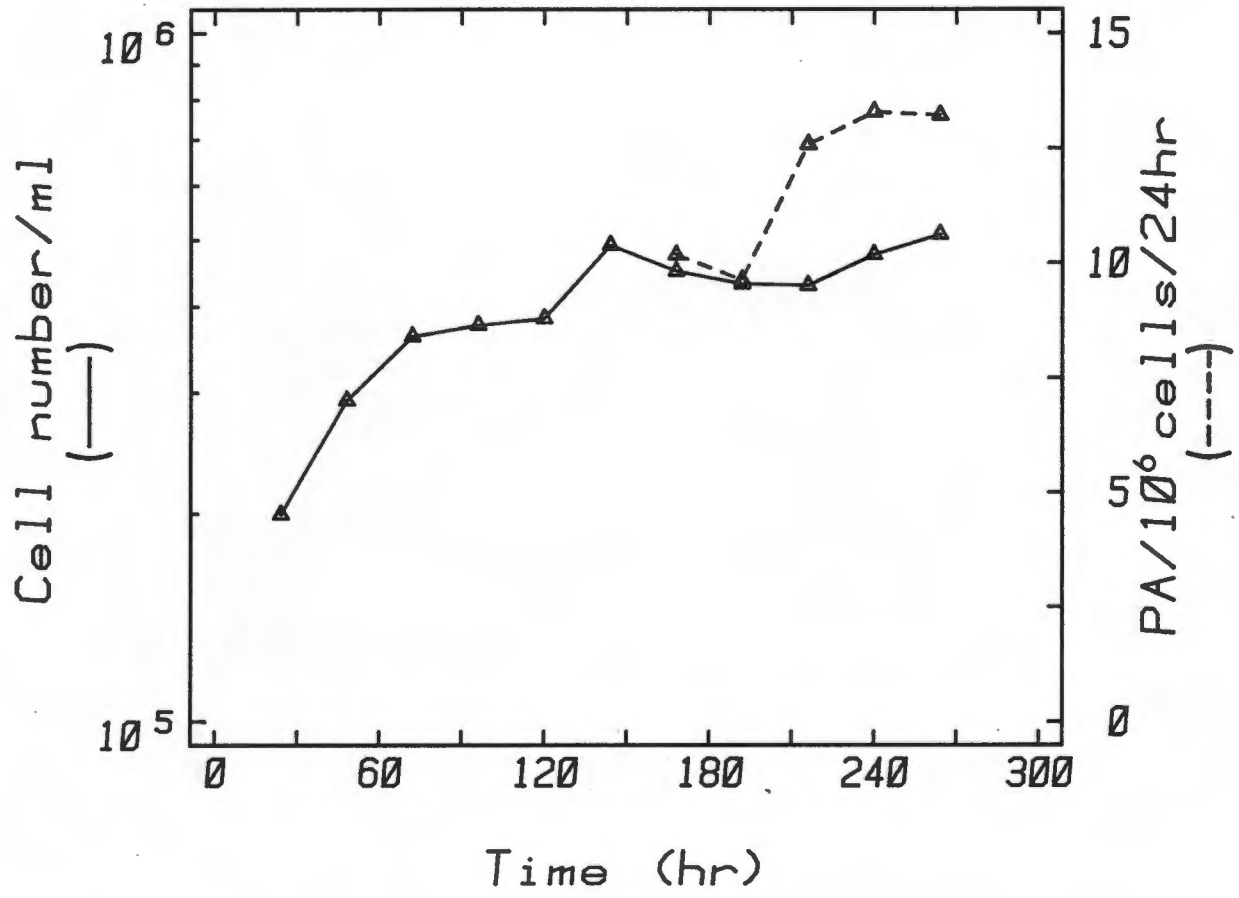


Figure 1.6 Plasminogen activator release and proliferation exhibited by Bowes I cells grown in a spinner flask.

A 250 ml spinner flask was inoculated with melanoma cells at a concentration of 2 x 10^5 cells/ml in a total volume of 250 ml of RPMI/10% FCS. At 24 hr intervals 100 ml of the suspension was removed, the cells were collected by centrifugation and returned to the flask after resuspension in 100 ml of fresh medium. Cells were counted in 100 µl aliquots. After 6 days of cultivation the medium was replaced by 250 ml RPMI without serum. Medium was again changed every 24 hr at which time the cell number was determined and plasminogen activator activity was measured. PA activity is expressed as %T/hr x 10^3 (see Appendix A.3).

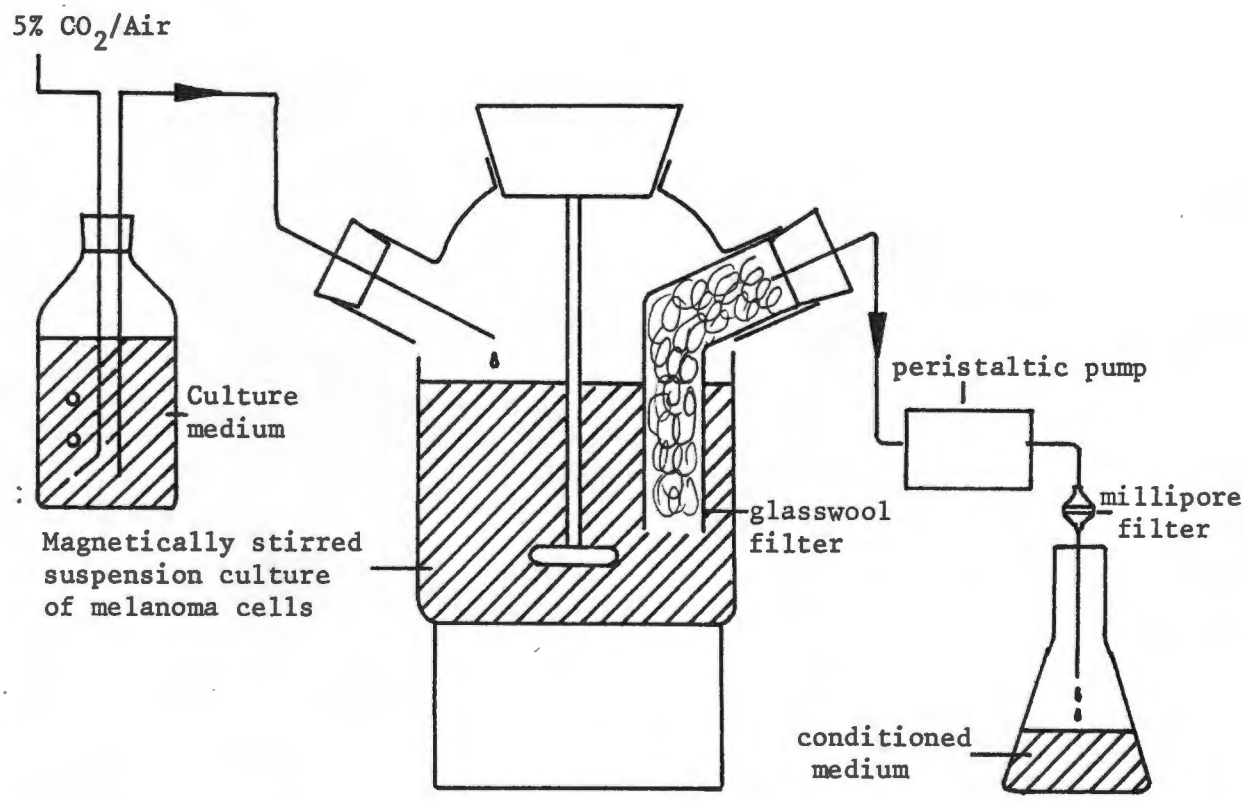


Figure 1.7 Mass cultivation of melanoma cells in a spinner flask

A 1 litre spinner flask was inoculated with melanoma cells at a concentration of 5×10^5 cells/ml in a total volume of 1 litre of RPMI/10% FCS. The flask was kept at 37°C and stirred magnetically at 30 rpm. Medium was pumped out of the flask at a rate of 5-10 ml/hr and replaced at the same rate with fresh medium that had been equilibrated with 5% CO_2 in air. The cells were retained in the culture vessel by an outlet glass tube plugged with glass wool.

Electrophoretic analysis of proteins released by RPMI-7272 cells.

To obtain some idea of the extent to which plasminogen activator synthesis occupied the protein synthetic machinery of RPMI-7272 melanoma cells and, in an attempt to determine whether or not these cells released more than one species of serine-protease, protein synthesis was measured by incorporation of radioactive leucine and serine proteases released by labelling with ^{3}H-labelled DFP.

Harvest fluids containing biosynthetically labelled proteins and ^{3}H-DFP labelled proteases were precipitated with 10% TCA and the precipitated proteins were redissolved in SDS. These solutions were analysed by electrophoresis followed by autoradiography or counting of the sliced gel. The results of these experiments are presented in Fig. 1.8 and Fig. 1.9. Detailed technical aspects are described in the legends to the figures.

As can be seen from the results, only one 70 000 dalton DFP-labelled protease could be detected. Many species of labelled protein however were discernible, varying in molecular weight from > 100 000 to 10 000. Of these the 70 000 molecular weight component corresponding to the DFP-labelled enzyme constituted approximately 3%.

Plasminogen-dependent fibrinolytic activity migrated electrophoretically with the same mobility as the DFP-labelled protein.

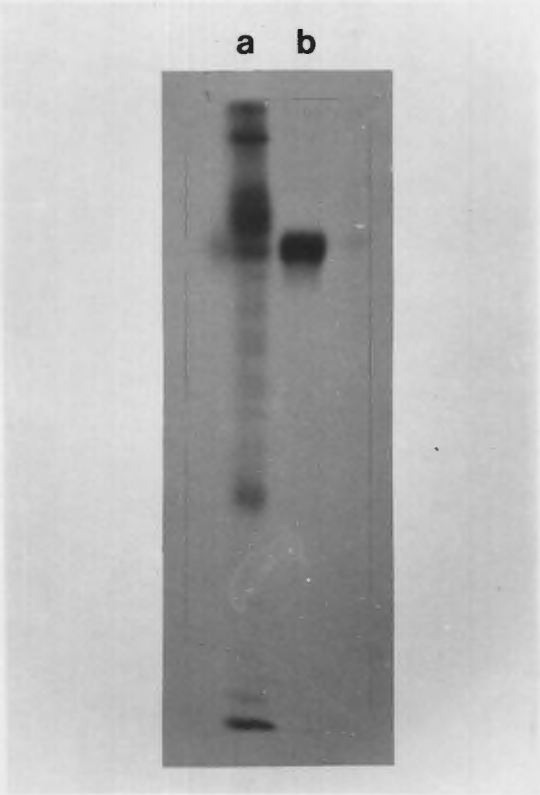


Figure 1.8 Autoradiographs of ^{3}H-leucine- and ^{3}H-DFP-labelled proteins in harvest fluid.

Proteins released by melanoma cells were labelled biosynthetically by cultivation in the presence of ^{3}H-leucine. Serine proteases released by these cells were labelled by incubation with ^{3}H-DFP. Details of the methods used are given in the legend to Fig. 1.9.

Radioactive protein samples were analysed by electrophoresis (see legend to Fig. 1.9) and auto-fluoro-radiography. The photograph above depicts the results. Track (a): Total protein released (^{3}H-leucine labelled). Track (b): Serine protease labelled with ^{3}H-DFP.

Figure 1.9

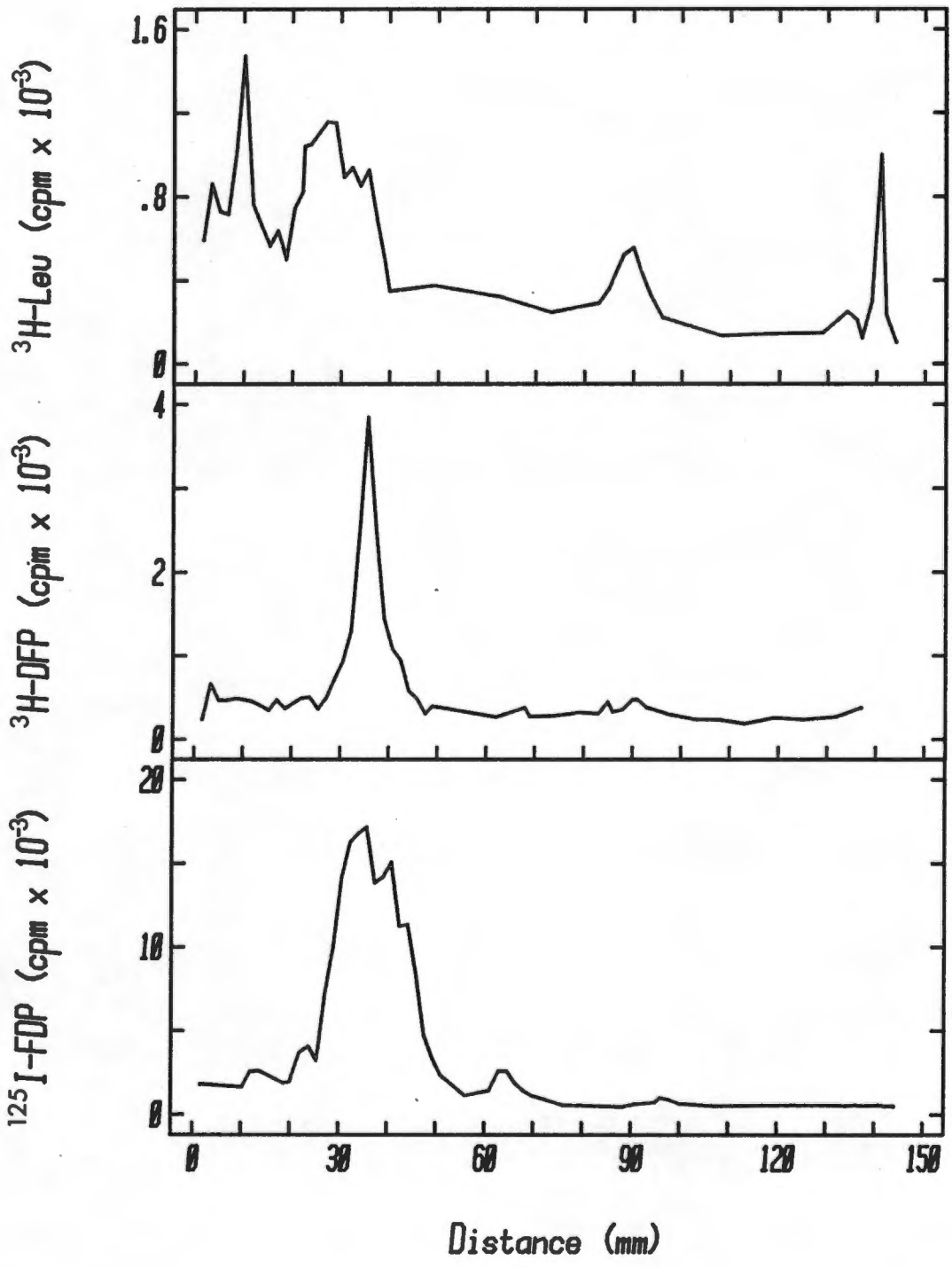
Figure 1.9 Electrophoretograms of ^{3}H-leucine labelled proteins, ^{3}H-DFP labelled proteins and plasminogen activator activity in harvest fluid.

Proteins in 5 ml of harvest fluid from melanoma cells that had been cultured in the presence of 10 µCi ^{3}H-leucine (48 Ci/mmol, Amersham TRK 170) for 24 hr were precipitated with 10% TCA and redissolved in 100 µl of SDS-sample buffer (0.0625M Tris HCl pH 6.8 containing 2.5% SDS and 10% glycerol). Two samples of 50 µl each were electrophoresed in a 6-15% linear gradient of SDS polyacrylamide (120 x 170 x 1 mm). After electrophoresis, the gel was sliced into 1.7 mm slices, and each slice was incubated in 1 ml Soluene 350 (Packard Inst. Corp., 6003038) at 50°C for 3 hr. Four ml of Dimilume (Packard 6003037) was then added and each slice was counted after 24 hr at 4°C, in a β scintillation counter.

^{3}H-DFP (100 µl; 3.9 Ci/mmol, 5 mCi/ml) was added to 1 ml of a non-radioactive harvest fluid sample that had been concentrated 160x to contain 3200 UK u/ml of Mel-PA. The mixture was incubated at room temperature for 22 hr and then precipitated with 10% TCA. The precipitate was redissolved in 100 µl sample buffer and two samples of 50 µl each were electrophoresed. Radioactivity measurement was done in the same way as described for ^{3}H-leucine-labelled proteins.

Plasminogen activator activity was determined in slices of a gel track that had been loaded with 100 µl of the identical harvest fluid that had been used for ^{3}H-DFP-labelling. After electrophoresis the gel was incubated in 2.5% Triton X-100 for 30 min and then sliced. Each slice was added to a ^{125}I-fibrin coated Linbro plate well together with 200 µl 0.1M Tris HCl containing 10 µg/ml human plasminogen. Radioactivity release was determined after 1 hr incubation at 37°C.

Autoradiographs of the ^{3}H-leucine and ^{3}H-DFP labelled proteins are presented in Fig. 1.8.


1.6
.8
0
^{3}H-Leu (cpm x 10^{-3})
4
2
0
^{3}H-DFP (cpm x 10^{-3})
20
10
0
^{125}I-FDP (cpm x 10^{-3})
0
30
60
90
120
150
Distance (mm)

Concentration of Mel-PA by chromatography on aminobenzamidine-sepharose.

The finding that benzamidine is a reversible inhibitor of plasminogen activators (142) led to the development of benzamidine-agarose as an affinity reagent for the purification of these enzymes from human (99,74,126), avian (109) or murine (108) sources. I therefore investigated the usefulness of this procedure for the purification of Mel-PA from melanoma cell harvest fluids.

In an early experiment, 100 ml of harvest fluid was adjusted to pH 7.0 and passed through a 5 ml column of aminobenzamidine-sepharose that had been equilibrated at 4°C with 0.01M potassium phosphate buffer pH 7.4 containing 0.1% Triton X-100. All Mel-PA in the harvest fluid was adsorbed but little activity could be recovered from the column by elution either with 0.1M benzamidine, pH 7.4 or with a solution of 1 mM HCl (pH 2.8) containing 0.2M NaCl and 0.1% Triton. Since binding of macromolecules to their affinity matrices is known to increase with time and to diminish with increasing temperature, subsequent experiments were carried out at room temperature and batchwise adsorption of the activator was achieved by tumbling the harvest fluid with a small volume of the benzamidine-sepharose. The matrix was then recovered by centrifugation and packed into a suitable column for elution.

The capacity of the benzamidine sepharose for adsorption of Mel-PA was tested by adding 50 ml of harvest fluids to 0.1, 1.0, 1.5 and 2.0 ml of matrix in plastic centrifuge tubes and tumbling these for 30 min at room temperature. Elution was carried out using 0.1M sodium acetate pH 3.5, containing 0.01% Triton. Although approximately 90% of the Mel-PA in 50 ml harvest fluid could be adsorbed by 1.5 and 2.0 ml of matrix, the recovery of activity was low (13% and 11.8% respectively). Addition of 10% ethylene glycol to the acetate eluting buffer increased the recovery of Mel-PA activity to 26.1%. Electrophoretic analysis of the enzyme

containing fractions showed that affinity chromatography had failed to achieve any substantial measure of purification since most proteins present in the harvest fluids were adsorbed and eluted together with Mel-PA.

In the hope that I would achieve purification of the activator, I added 0.5M arginine, a benzamidine analogue, to an eluting buffer containing 0.1M sodium acetate pH 4.5; 0.1% Triton X-100; and 10% ethylene glycol. Before elution the column was washed with 0.01M potassium phosphate pH 7.6 containing 0.01% Triton X-100 and 10% ethylene glycol. Although recovery of the activator activity from the column was increased to 74.1% no further purification was achieved as judged by SDS-polyacrylamide electrophoresis (Fig. 1.10). The minimum concentration of arginine found to be required for elution of Mel-PA in this system was 0.5M.

The method published by Danø et al (108) was used, with slight modifications, in a further attempt to reduce losses and improve purity. Amino-benzamidine sepharose that had been equilibrated with 0.01M sodium acetate, pH 5.5, containing 0.1% Triton and 1 mM $ZnCl_2$ was added in 1.5 ml volumes to 50 ml centrifuge tubes. The tubes were then filled with harvest fluid which had been adjusted to pH 5.5 and to contain 0.1% Triton X-100 and 1 mM $ZnCl_2$. After incubation for 60 min at room temperature with occasional inversion, the tubes were centrifuged at 250xg for 5 min and the supernatant was removed. Equilibrating buffer (50 ml) was added and the procedure repeated. The matrix was then resuspended in equilibrating buffer containing 0.2M NaCl and 10% ethylene glycol and packed into a 2 x 3 cm column at a flow rate of 240 ml/hr. The matrix was washed with buffer until all visible traces of the phenol red present in the tissue culture medium had been washed from the gel. Bound protein was then eluted with 0.5M arginine in 0.1M sodium acetate at pH 5.5 containing 10% ethylene glycol, 0.1% Triton and 1 mM $ZnCl_2$. The column was eluted at a flow rate of 50 ml/hr and fractions of 5 ml were collected. Those with the highest

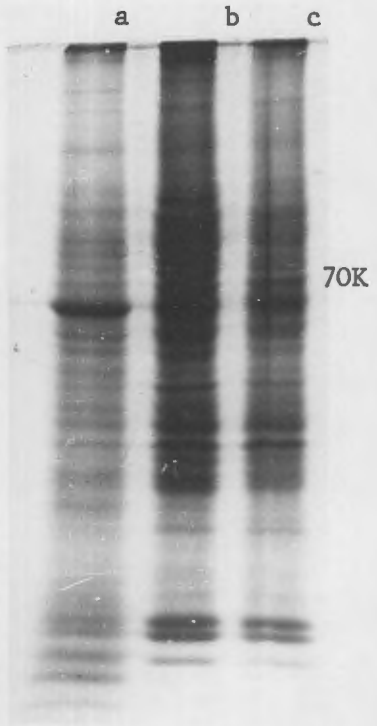


Figure 1.10 SDS polyacrylamide gel electrophoresis of proteins before and after benzamidine-sepharose chromatography.

The photograph shows the fixed and stained polyacrylamide slab gel tracks containing electrophoresed proteins from:-

(a) 2 ml of harvest fluid from Bowes I cells
(b) and (c) 1 ml of pooled enzyme-containing fractions eluted from benzamidine sepharose

In (b) the harvest fluid was added to the affinity-matrix equilibrated with 0.05M sodium phosphate buffer, pH 7.6 containing 0.1% Triton X-100. The enzyme was eluted with a solution of the following compositions:
Triton X-100:- 0.1%; ethylene glycol:- 10%;
and arginine:- 0.5M.

In (c) the harvest fluid was added to a benzamidine agarose affinity gel that had been equilibrated with 0.1M sodium acetate pH 5.5 containing 0.1% Triton X100 and 1 mM $ZnCl_2$. Enzyme fractions were eluted with the same buffer to which 0.5M arginine and 10% ethylene glycol had been added.

Samples were prepared for electrophoresis by precipitating with 10% TCA. The precipitates were dissolved and boiled in 25 µl SDS sample buffer; 10 µl aliquots were then electrophoresed in 6-15%-linear gradient-polyacrylamide gels containing 0.1% SDS (Appendix A.2). Note that neither method of benzamidine-agarose chromatography produced any useful degree of purification.

plasminogen activator activity were pooled. Usually 30 ml of eluate was obtained from 500 ml of harvest fluid.

The recovery of activity from such columns ranged from 60% to 70% without substantially better purification (Fig. 1.10). Since the recovery was lower than that obtained with the phosphate system used previously, this method was abandoned.

Although none of the above methods using amino-benzamidine sepharose resulted in any purification of Mel-PA, the following procedure was finally adopted as a useful means of concentrating the proteins present in the large volumes of harvest fluid.

Aminobenzamidine sepharose (5 ml; pre-equilibrated with 0.05M potassium phosphate buffer pH 7.0 containing 0.1% Triton X-100) was added to 45 ml of harvest fluid adjusted to contain 0.1% Triton X-100 to a pH of 7.0. Adsorption was carried out for 30 min at $4^{o}C$ with tumbling of the tubes, after which they were centrifuged at 360xg for 5 min and the supernatant discarded. A fresh batch of harvest fluid was added and the procedure repeated five times. In this way, Mel-PA from 225 ml of harvest fluid could be quantitatively adsorbed on to 5 ml of affinity gel. After the last adsorption, the matrix from 8 tubes was packed into 2.5 x 12 cm perspex column and eluted at a flow rate of 75 ml/hr.

Adsorbed protein was eluted with 0.5M arginine in 0.01M sodium acetate pH 4.5 containing 0.1% Triton X-100 and 10% ethylene glycol. Five ml fractions were collected. Plasminogen activator activity in each fraction was measured with the ^{125}I-fibrin assay. Active fractions were pooled and stored in the presence of 0.5M arginine, 0.01M Na acetate at $-20^{o}C$.

Other methods of concentrating harvest fluids were tried including dialysis against Sephadex G200, dialysis against ammonium acetate and lyophilization, and concentration in an Amicon concentrator using various

membranes. The results are summarised in Table 1.7 and show that none of these procedures yielded as much active enzyme as did chromatography on benzamidine sepharose.

Concentration and partial purification of Mel-PA using zinc-chelate sepharose and Concanavalin A sepharose.

These very useful procedures were taken, with minor modifications from those described by Rijken and Collen (130). They exploit the tendency of Mel-PA to bind to its reversible inhibitor, Zn^{++}, and the fact that Mel-PA is a glycoprotein which binds to Concanavalin A.

Zinc-chelate-sepharose was prepared as described in detail in the Methods section and used to pack a column measuring 5 x 15 cm. The adsorbent was equilibrated with 0.02M Tris HCl pH 7.5 containing 1M NaCl and 0.01% Triton X-100.

Serum-free conditioned medium was made 1M with respect to NaCl, 0.1% with respect to Triton X-100, and the pH was adjsuted to 7.5. A volume of 10 litres of serum-free harvest fluid was applied to the zinc-chelate-sepharose at a constant rate of 200 ml/hr.

The column was then washed with equilibration buffer (1.5 litre) and the adsorbed protein was eluted with 900 ml of a linear gradient of 0 to 0.06M imidazole in equilibration buffer at a rate of 100 ml/hr. Ten ml fractions were collected. Enzyme containing fractions were pooled as indicated in Fig. 1.11 to give 400 to 450 ml of solution containing 950-1200 UK u/ml of Mel-PA activity. This represented a recovery of approximately 80-90% of enzyme activity. SDS-polyacrylamide gel electrophoresis at this stage showed the presence of numerous contaminating proteins (Fig. 1.11).

Commercial Con A-sepharose (Pharmacia) was packed into a 1.2 x 10cm column and equilibrated with 0.01M sodium phosphate buffer pH 7.5 containing

Table 1.7 Methods of concentrating proteins present in harvest fluids.

Method	PA activity recovered (% T/60)	Volume after concentration (ml)	% recovery
Dialysis against 0.1M ammonium acetate pH 6.5 and lyophilization	53 250	1.0	16.0
Dialysis against dry Sephadex G200	122 500	4.5	36.80
Ultrafiltration on Amicon XM 50 membrane	88 875	5.1	26.70[a)]
Amicon YM 10	162 509	7.8	48.82
Amicon YM 30	49 798	3.4	14.96[a)]

Harvest fluid (50 ml) containing 0.1% Triton was concentrated by the methods listed in the table.

Plasminogen activator activity was measured before and after concentration. The total activity contained in the harvest fluid was 332 875% T/60 min as measured in the ^{125}I-fibrin assay.

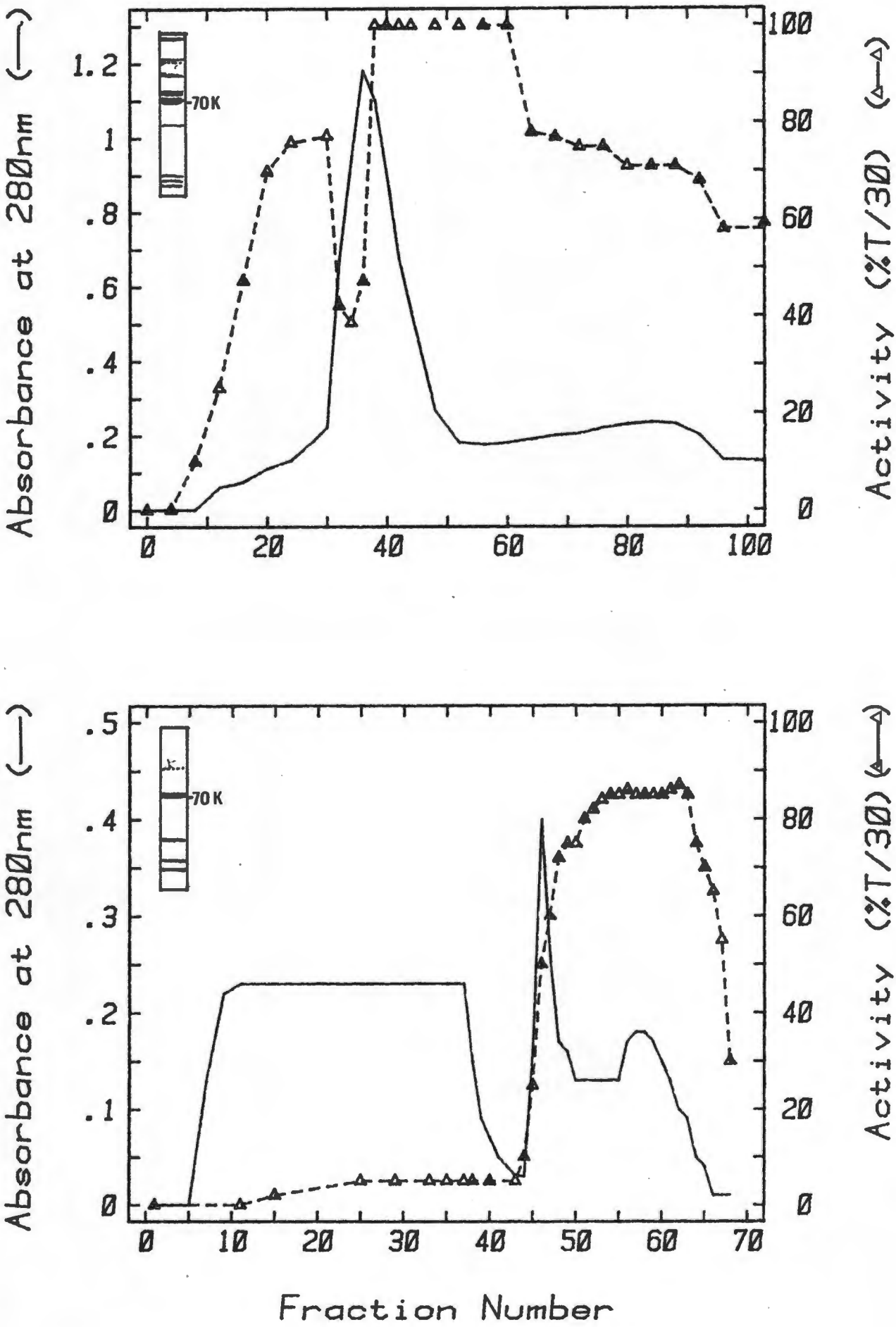
a) Plasminogen activator activity was detected in the ultrafiltrates.

Figure 1.11

Figure 1.11 Chromatography of Mel-PA on zinc-chelate sepharose and concanavalin A sepharose.

a) Serum free harvest fluid was applied to the zinc-chelate sepharose and bound Mel-PA was eluted as described in the text. Fraction 36 to 76 were pooled to give 425 ml of solution containing 1050 UK u/ml of Mel-PA activity. The insert diagram shows the protein pattern of the pool after SDS-polyacrylamide gel electrophoresis.

b) Pooled enzyme containing fractions from the zinc-chelate chromatography step (400 ml) were applied to a Con A-sepharose column and bound material was eluted as described in the text. Fraction 47 to 65 were pooled to give 120 ml of solution containing 1550 UK u/ml of Mel-PA activity. The preparation was contaminated with Con A as shown by SDS-polyacrylamide gel electrophoresis (diagram).


Absorbance at 280nm (—)
Activity (%T/30) (△—△)
70K
1.2
1
.8
.6
.4
.2
0
100
80
60
40
20
0
0
20
40
60
80
100
Absorbance at 280nm (—)
Activity (%T/30) (△—△)
70K
.5
.4
.3
.2
.1
0
100
80
60
40
20
0
0
10
20
30
40
50
60
70
Fraction Number

1M NaCl and 0.1% Triton. Pooled enzyme containing fractions from the zinc-chelate chromatography step (total volume approximately 400 ml) were then applied at a flow rate of 10 ml/hr. The column was washed with equilibrating buffer and the adsorbed protein was eluted using 200 ml of a linear gradient of 0 to 0.4M α methyl-D-mannoside and 0 to 2M KSCN in 0.01M phosphate buffer pH 7.5 containing 1M NaCl and 0.1% Triton. Fractions of 5 ml were collected and the active fractions were pooled to give 120 - 150 ml of solution containing approximately 1500 - 1600 UK u/ml of Mel-PA. This represented an overall recovery of 40% and a recovery of 50 - 60% for the Con A sepharose step.

SDS-polyacrylamide gel electrophoresis at this stage gave results shown in Fig. 1.11. After Con A sepharose chromatography the plasminogen activator preparation appeared to be relatively pure save for the presence of contaminating Con A that had leached from the column.

DISCUSSION

The work described in this chapter was undertaken to define optimal conditions for obtaining starting material for the purification of Mel-PA. Although other cell types available to me could be shown to release a similar plasminogen activator (Table 1.8), the consistency with which melanoma cell lines behaved in this regard made these most attractive as a source of enzyme. Furthermore, melanoma cells are well known for the ease with which they can be cultivated in vitro.

As is evident from the data in Table 1.8, the melanoma cell line RPMI-7272 (Bowes) produced more enzyme activity, in terms of units/10^6 cells/24 hr, than did any of the other melanomas. I therefore concentrated my efforts on these cells.

Very fortunately for me a unique subline of RPMI-7272 cells was established in the laboratory while this work was in progress. These cells

Table 1.8 Plasminogen activators secreted by cultured human cells

Cell line	Plasminogen activator species[a]				Plasminogen activator ($UK\ u/10^6$ cells/24 hr)
		Mel-PA		UK	
	100 00	70 000	60 000	60 000	
UCT-Mel 1	+	+++[b]	++		19
UCT-Mel 2	+	+++	+		4
UCT-Mel 3	+	+++	++		9
UCT-Mel 4a	+	++	+		31
UCT-Mel 4b	+	+++	++		50
UCT-Mel 5	++	+++	++		7
Melanoma (M-127)	+++	++			ND
Melanoma (M-170)	+	+++			ND
Bowes	+	+++	+		70
Ca.breast		+++	+		19
Malignant teratoma		+		+++	30
Sarcoma of uterus		+		+++	26
Glioblastoma		+		+++	8.9

The data were obtained from Wilson et al (61), Wilson and Dowdle (106), and Hoal (151).

a) The immunochemical type of PA is expressed as UK-type (UK) or Mel-PA-type (Mel-PA). The molecular weights given are approximate.

b) The relative abundance of the various molecular weight species is based upon the area of fibrinolytic zones and the time taken for development of lysis in the zymographic method of Granelli-Piperno and Reich (62).

adhere and remain healthy indefinitely in the absence of serum while continuing to synthesize plasminogen activator at a steady and useful rate. Apart from obvious considerations of economy in foetal calf serum and absence of contaminating serum proteins or inhibitors, use of these cells had an additional advantage in their low proliferative rate in the absence of serum. This meant that cultures require passaging only infrequently and one flask could be "milked" for several months.

Unfortunately neither TPA nor any of the other compounds known to enhance PA release by cells in vitro (143-150) had any useful effect on plasminogen activator released by melanoma cells. Christman et al (147) made a similar observation and noted that synthesis of melanoma type activator was not as readily modulatable as was that of urokinase.

In devising a strategy for obtaining adequate amounts of Mel-PA for isolation and purification, it was necessary to take into account the very small amounts of enzyme released into the medium and the very low concentrations of active protein I could expect. It has been estimated, for example, that serum-free culture fluids from MSV-transformed 3T3 cells contain approximately 15 nmol/l of plasminogen activator (108). By labelling harvest fluid with ^{3}H-DFP of known specific activity (Chapter V) I was able to estimate, as a very rough approximation, that conditioned serum-fre medium collected routinely in large volumes from Bowes melanoma cells contained approximately 10-20 nmol/l. This meant that it would be necessary to collect and concentrate large volumes of harvest fluid in order to obtain sufficient pure protein for adequate biochemical characterization.

In attempting to develop the culture of Bowes melanoma cells on the production scale necessary to meet this requirement, I hoped that "Melanex" spirals or "Cytodex" beads would offer the convenience of a large surface area for cell adherence without the inconvenience of a

proportionate increase in volume of the culture vessel. Unfortunately, however, neither of these substrata proved propitious for satisfactory cell viability or function under the conditions of serum deprivation and medium composition that I wished to use.

A serious problem that confronted me at an early stage in the work was the apparent instability of Mel-PA in harvest fluids. Others have encountered similar difficulties and have found that the addition of nonionic detergents (108,130,68), high salt concentrations (132) or reversible protease inhibitors such as $ZnCl_2$, benzamidine, arginine and trasylol (93,108,134,90) have proved useful. I was able to reduce losses very effectively by the addition of Triton X-100 and by acidifying the sample.

Chromatography on benzamidine-sepharose provided an efficient and rapid means of concentrating the proteins present in the harvest fluids and it proved useful as a preliminary means of preparing the activator for storage. The slightly acid conditions and the high concentration of arginine (0.5M) may have contributed an additional stabilizing influence.

The improvement in recovery of the enzyme that resulted from the addition of ethylene glycol to the eluting buffer could probably be attributed to non specific hydrophobic interactions between the enzyme and the affinity matrix.

The tenacity with which the activator bound to the benzamidine-sepharose column militated against effective purification with this reagent since the drastic measures (low pH, ethylene glycol, arginine and high salt concentration) that were needed to release the enzyme also released contaminating proteins. Goldfarb et al (109) working with chicken plasminogen activator and Danø et al (108) who purified the murine enzyme, achieved very satisfactory purifications with benzamidine-sepharose and others have found that urokinase-type activators could be eluted from

benzamidine columns with low pH buffers without the need for arginine or benzamidine in the eluant (99,74). Although I have not studied the matter systematically or thoroughly, my own experience would confirm these observations since I have been able to elute 81% of urokinase added to an affinity column with 0.1M acetate buffer pH 3.5.

Despite the fact that affinity chromatography may be more effective for urokinase type activator purification, Holmberg et al (99) have pointed out that benzamidine-sepharose chromatography does not provide the "one-step" purification of urokinase from tissue culture medium or urine that one would hope for. This was certainly true for Mel-PA, and Zn-chelate affinity chromatography was not much better in this regard.

The recovery of activity from the Zn-chelate column ranged from 80-90% (i.e. only slightly better than the recoveries of 75-85% obtained after affinity chromatography on benzamidine sepharose using the phosphate system) and the concentration of 950-1200 UK u/ml after the Zn chelate column was similar to that (850-1600 UK u/ml) achieved by chromatography on benzamidine sepharose. SDS-PAGE showed that both preparations were still heavily contaminated with other proteins (Fig. 1.10 and Fig. 1.11).

The advantage of the Zn-chelate chromatography step for the concentration of proteins in harvest fluids was that a large volume (10 litres) of harvest fluid could be processed at one time with very little attention and in less time (3½ days) than was required for the benzamidine sepharose chromatography step (5 days). Eluted pooled proteins from both columns had to be dialysed or processed further before they could be used in assays for the characterization of Mel-PA. Enzyme containing fractions from the benzamidine sepharose contained high concentrations of arginine, and those from the Zn chelate column contained imidazole. Both of these compounds interfere in protein assays and in enzyme assays.

Chromatography of proteins obtained from the Zn-chelate column on Con A sepharose yielded 120-150 ml of a 1600 UK u/ml solution of Mel-PA. The recoveries were 50-60%. Very good purification was achieved as shown by the protein pattern on SDS-PAGE. Unfortunately, however, the activator preparation was heavily contaminated with Con A and therefore could not be used unless it was further processed either by SDS-PAGE (Chapter III) or by gel filtration chromatography.

CHAPTER II

ELECTROPHORETIC ANALYSIS OF PLASMINOGEN ACTIVATORS IN SDS-POLYACRYLAMIDE GELS CONTAINING COPOLYMERIZED SUBSTRATES.

The study of the various forms of plasminogen activators occurring in tissue, in body fluids, and those released by cells cultured *in vitro*, has been greatly aided by electrophoresis in polyacrylamide gels containing SDS. Using this technique, the number as well as an approximate molecular size of plasminogen activators in minute amounts of sample could be determined. Two general techniques have been employed for the detection of enzyme bands in such gels after electrophoresis.

In the first, exemplified by the approach of Danø, and Reich (152) polyacrylamide slab gel tracks containing electrophoresed enzymes were frozen and cut into 1.1 mm slices. Each ordered slice was then assayed for plasminogen-dependent lysis of ^{125}I-labelled fibrin and the results were used to construct a profile of enzymatic activity in the track. The small amount of SDS in each slice was diluted in the reaction mixture to concentrations that did not interfere significantly with the assay.

The second procedure by Granelli-Piperno and Reich (62), exploited the fact that the catalytic activity of plasminogen activators was reversibly inhibited by SDS in the electrophoresis gel and could be restored by removal of the SDS from the polyacrylamide slab by incubating in aqueous Triton X-100. Bands of plasminogen activator activity could then be localized by a zymographic procedure in which the polyacrylamide slab was overlaid on an agar gel layer containing the two sequential substrates, plasminogen and fibrin. Plasminogen activator bands were visible under dark background illumination, as localized dark zones of fibrinolysis against an opaque white background of undegraded fibrin.

I have developed a modification of the Granelli-Piperno and Reich

technique that offers certain advantages for the electrophoretic analysis of plasminogen activators in SDS-polyacrylamide gels. It is based upon the observation (a) that gelatin is a sensitive and satisfactory substrate for plasmin and (b) that if gelatin and plasminogen are copolymerized into the matrix of the SDS-polyacrylamide gel at the time of casting they are retained during subsequent electrophoresis of enzyme samples so providing *in situ* substrates for separated bands of concentrated plasminogen activator activity. The necessity for separate fibrin-plasminogen-agar indicator gels is dispensed with. This approach was suggested by the paper of Hochstrasser and Schorn (153) who used azocasein-polyacrylamide gels for the electrophoretic analysis of plasminogen-independent proteases.

METHODS

Source of enzymes.

To illustrate the usefulness of this method I have analysed urokinase and plasminogen activators released into serum free tissue culture medium by a variety of normal and neoplastic human cells cultured *in vitro*. Cultures were established and maintained as described in Chapter I for the melanoma cells and as described by Wilson and Dowdle (106).

Human urokinase was obtained commercially from Leo Laboratories Limited, Hayes, Middlesex.

Human plasminogen was prepared from human plasma by lysine-sepharose affinity chromatography (45) and stored as a 1.2 mg/ml solution in PBS at -20°C, as described in the Appendix (A1.3).

Electrophoretic procedure.

Preliminary experiments led to the adoption of the following standard procedure for the electrophoretic analysis of most mammalian plasminogen activators.

Polyacrylamide resolving gel slabs measuring 60 x 70 x 1 mm were cast from a mixture prepared from stock solutions as follows:

(i)	Acrylamide (30g%) and bisacrylamide (1g%) in distilled water	3.30 ml
(ii)	1.5M Tris-HCl, pH 8.8, containing 0.4 g% SDS	2.25 ml
(iii)	Purified human plasminogen: 1.2 mg/ml in phosphate-buffered saline (PBS)	0.1 ml
(iv)	Gelatin: 1 g% distilled water	0.9 ml
(v)	Ammonium persulfate (100 mg/ml in water)	0.02 ml
(vi)	TEMED (as supplied)	0.01 ml
(vii)	Water	2.45 ml
	Total volume	9.03 ml

The resolving gels were carefully overlaid with 0.05% gelatin in 0.3M Tris-HCl, pH 8.8 and allowed to polymerize at room temperature. When polymerization was complete, a stacking gel with castellated sample wells was cast on top of the resolving gel from the following mixture of stock solutions:

(i)	Acrylamide (30 g%) and bisacrylamide (1g%) in distilled water	0.2 ml
(ii)	0.5M Tris-HCl, pH 6.8 containing 0.4% SDS	0.25 ml
(iii)	Ammonium persulfate (100 mg/ml in water)	0.02 ml
(iv)	TEMED (as supplied)	0.01 ml
(v)	Water	1.55 ml
	Total volume	2.03 ml

Reservoir buffer (0.025M Tris, 0.192M glycine-NaOH, pH 8.5, 0.1% SDS) was added to the upper and lower reservoirs and samples were added to the sample wells in a final volume of 5 - 50 µl of solution containing 2.5 g% SDS, 10 g% sucrose, and 4 µg/ml phenol red to serve as a tracking dye.

Electrophoresis was performed at 4°C at a constant current of 8 mA. When the tracking dye front had reached the bottom of the resolving gel (after approximately 3.5 hr) the gel was removed and shaken gently at room temperature for 1 hr in 2.5% Triton X100 in water to remove SDS. The gel slabs were then transferred to a bath containing 0.1M glycine-NaOH, pH 8.3, and incubated at 37°C for 3-5 hr.

The gels were then fixed and stained by immersion for 1 hr in a 0.1% solution of amido black in methanol:acetic acid:water (30:10:60). The gels were destained in methanol:acetic acid:water (30:10:60).

Note that the resolving gel routinely contained a final total concentration of acrylamide (%T) of 11% and that the stacking gel did not contain gelatin or plasminogen. Plasminogen was omitted from the resolving gel to detect plasminogen-independent proteases.

Since microbial contamination of electrophoretic buffers and other solutions occasionally contributed bacterial proteases, all solutions were autoclaved or sterilized by filtration and stored under conditions that minimized contamination.

RESULTS

When the standard procedure described under Methods was used for the electrophoretic analysis of solutions containing plasminogen activators, well-resolved bands of enzyme activity were seen as clear zones of proteolysis against a blue background of undegraded stained gelatin. When plasminogen was omitted from the resolving gel mixture no proteolytic bands were observed in electrophoretic tracks containing urokinase or cell harvest fluid samples whereas trypsin and elastase showed clearly defined bands of plasminogen-independent proteolysis (Fig. 2.1).

Results of preliminary experiments that were performed for the development of the method can be summarized as follows:

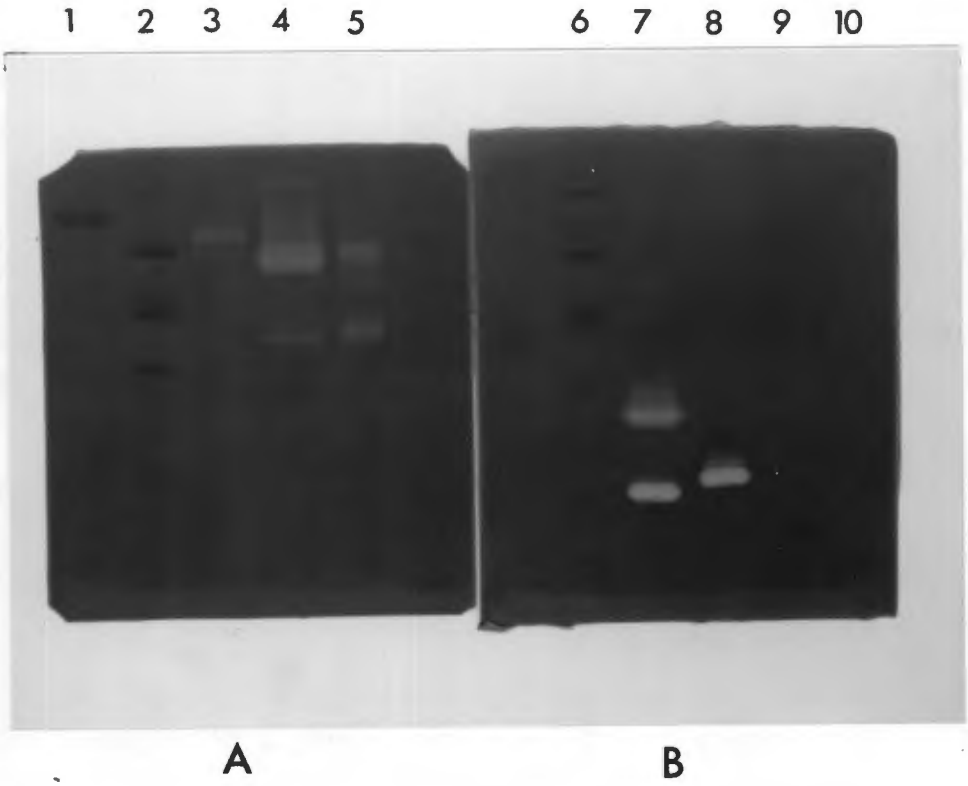


Figure 2.1 Identification of plasminogen activators and proteases in 11% polyacrylamide gel slabs containing gelatin.

Gel slabs containing 11% polyacrylamide, 0.1% SDS and 0.1% co-polymerized gelatin were cast with (gel A) and without (gel B) 13 µg/ml co-polymerized plasminogen. Samples were electrophoresed as follows: track 1, lactoperoxidase (78 000); tracks 2 and 6, reference protein mixture comprising phosphorylase B (94 000), bovine serum albumin (67 000), ovalbumin (43 000) and carbonic anhydrase (30 000); track 3, 5µl of serum-free melanoma cell harvest fluid; track 4 and 9, 5 µl of serum-free foreskin epithelium harvest fluid; track 5 and 10, 1 x 10^{-3} Ploug units of commercial urokinase; track 7, 6.2 x 10^{-3} µ commercial elastase; track 8, 2.5 x 10^{-3} µ trypsin.

After electrophoresis the gels were washed in Triton X100, incubated in glycine-NaOH buffer at 37°C for 3 hr and fixed, stained and destained as described under Methods. Note that gelatinolysis by urokinase and epithelial cell proteases were plasminogen dependent. (cf. tracks 4 and 5 and 9 and 10).

The gelatin substrate tended to migrate out of the resolving gel if the total polyacrylamide concentration was less than 5% or if the ratio of bisacrylamide to acrylamide was less than 1:30.

Electrophoresis at room temperature instead of at 4°C frequently resulted in "trails" of gelatinolysis in electrophoretic tracks containing plasminogen activators. This phenomenon may have been due to incomplete inhibition of the enzymes by the SDS in the gel with the result that proteolysis proceeded during electrophoresis at higher temperatures. Alternatively, ionic discontinuities due to salts in the samples may have resulted in local heating during electrophoresis with "melting" of the gelatin in sample tracks. Whatever the cause of this phenomenon, it could be obviated by electrophoresis in the cold.

The removal of SDS by Triton X-100 before incubation in buffer was an absolute necessity - no bands of activity could be detected without this step. Incubation of gels in different buffers after the Triton wash showed that 0.1M glycine HCl pH 8.3 was the most suitable as shown in Fig. 2.2a.

Incubation of gel strips containing identical samples in buffers ranging from pH 2.5 - 10.4 showed that activity was completely inhibited below pH 7.5 and above pH 8.9 (Fig. 2.2b). Using a glycine buffer ranging in pH from pH 8.2 to pH 9.6, optimum gelatinolysis was observed between pH 8.2 to 8.4.

Incorporation of 0.25% powdered skim milk as a source of casein instead of gelatin yielded the same pattern of proteolytic bands as seen in gelatin gels.

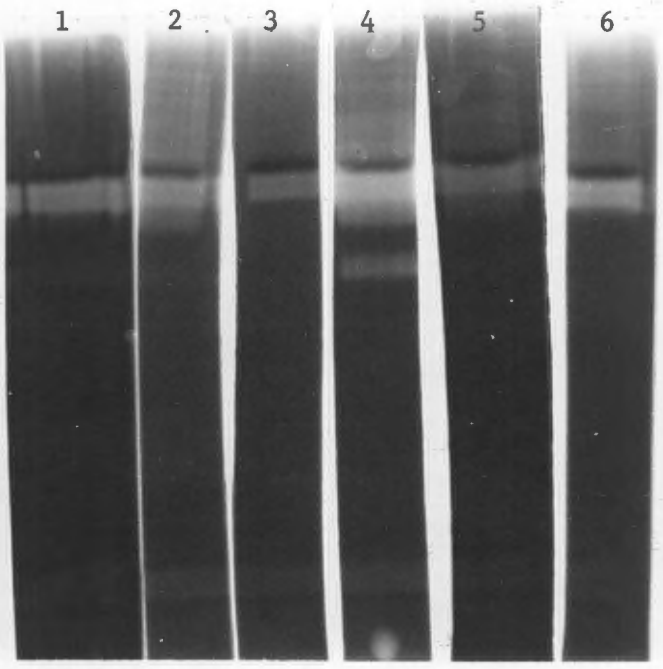
Molecular weight marker proteins were concentrated by electrophoresis into narrow zones that appeared as easily visible, intensely staining bands against the lighter blue colour of the stained gelatin background. Thus plasminogen activators and molecular weight marker proteins were visible in the same gel.

Figure 2.2

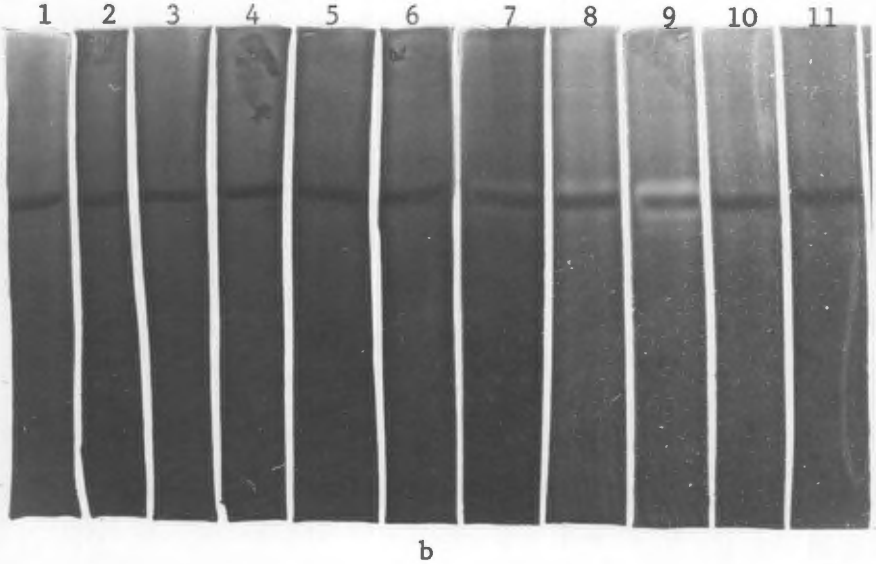
Figure 2.2 The effect of buffer composition and pH on the development of plasminogen dependent proteolytic bands in gelatin-gels.

a) Electrophoretic tracks containing harvest fluid of a cell line derived from a carcinoma of the bladder were incubated at 37°C in 10 ml of the following buffers: track no. 1. 0.1M Borate buffer pH 8.4; 2. 0.1M Tris-HCl pH 8.1; 3. PBS; 4. 0.1M Glycine-HCl pH 8.5; 5. 0.1M Carbonate buffer pH 8.7; 6. Distilled water.

b) Electrophoretic tracks containing harvest fluid of a cell line derived from a human melanoma (UCT-Mel 3) were incubated at 37°C in 10 ml of the following buffers:
Track No. 1. 0.1M Glcyine-HCl, pH 2.5; 2. 0.1M Glycine-HCl pH 3.5; 3. 4. and 5. 0.1M Sodium acetate-acetic acid pH's 3.5, 4.5 and 5.5; 6. 0.1M Tris HCl pH 7.5; 7. distilled water; 8. 0.1M Tris HCl pH 8.5; 9. 10. and 11. 0.1M Glycine-NaOH, pH's 8.8, 8.9 and 9.6.



a



b

As can be seen from Fig. 2.1 plasminogen activators released by Bowes melanoma cells appeared as a prominent, closely spaced doublet which migrated, in 11% polyacrylamide gels, to a position cathodal to albumin and a fainter third band with an electrophoretic mobility slightly greater than that of albumin. These corresponded to enzymes with apparent molecular weights of approximately 71 000, 69 000 and 62 000 respectively.

Urokinase and the enzymes released by cultured foreskin epithelial cells showed three major bands of plasminogen-dependent proteolysis. The two most prominent species were evident as a doublet that migrated to a position immediately anodal to albumin with approximate apparent molecular weights of 62 000 and 56 000. The third band had an electrophoretic mobility intermediate between that of ovalbumin and carbonic anhydrase corresponding to an enzyme with an approximate apparent molecular weight of 34 000. The epithelial enzyme track in Fig. 2.1 was somewhat overloaded for optimal resolution and higher molecular weight enzymes were visible. Since the technique relied upon preservation of enzyme activity the samples could not be heated with SDS before electrophoresis; it is probable, therefore, that the high molecular weight species observed in this sample represented aggregates of the lower molecular weight forms.

A summary of a survey of plasminogen activators released by a variety of cells cultured <u>in vitro</u> is presented in Table 2.1. The results agree well with those reported by Wilson et al (61), where plasminogen activators of human cells cultured <u>in vitro</u> were analysed by the Granelli-Piperno and Reich technique. Plasminogen activators with molecular weights in the 70 000 dalton range, could be detected in both melanoma cell lines, and in cell lines derived from a carcinoma of the renal pelvis, a carcinoma of the breast and a glioblastoma.

In the first analysis, copolymerization of gelatin and plasminogen into the resolving gel did not appear to interfere with the electrophoretic

TABLE 2.1

PLASMINOGEN ACTIVATORS SECRETED BY HUMAN CELLS CULTURED *in vitro*

SOURCE OF ENZYMES	MOLECULAR WEIGHT SPECIES[a)]			
	>95 000	70 000	60 000	35 000
Commercial urokinase			+++	+++
Normal bladder epithelium	++[b)]		+++	++
Normal kidney			+++	++
Normal thyroid			+++	++
8-wk embryo fibroblasts		++	+++	
Melanoma (Bowes I)		+++	+	
Melanoma 1 (UCT Mel 3)		+++	+	
Hypernephroma			+++	++
Hypernephroma	++		+++	++
Sarcoma of uterus	+	+	+++	
Carcinoma of bladder	+		+++	++
Carcinoma of renal pelvis	+	++	+++	++
Carcinoma of breast		+++	++	+
Lipoma			+++	+
Glioblastoma	+	++	+++	+

a) Molecular weights given are approximate. A molecular weight of "70 000" has been assigned to enzymes showing a doublet with apparent molecular weights of 69 000 and 71 000, and "60 000" has been assigned to the doublet corresponding to apparent molecular weights of 62 000 and 56 000. ">95 000" signified bands that migrated cathodally to phosphorylase b.

b) The relative abundance of each molecular species within a sample is based on a scale of + to +++.

behaviour of the marker proteins. In gels containing total polyacrylamide concentrations of 8, 10, 11 and 12%, marker proteins migrated distances that were inversely and linearly related to the logarithms of their molecular weights (Fig. 2.3).

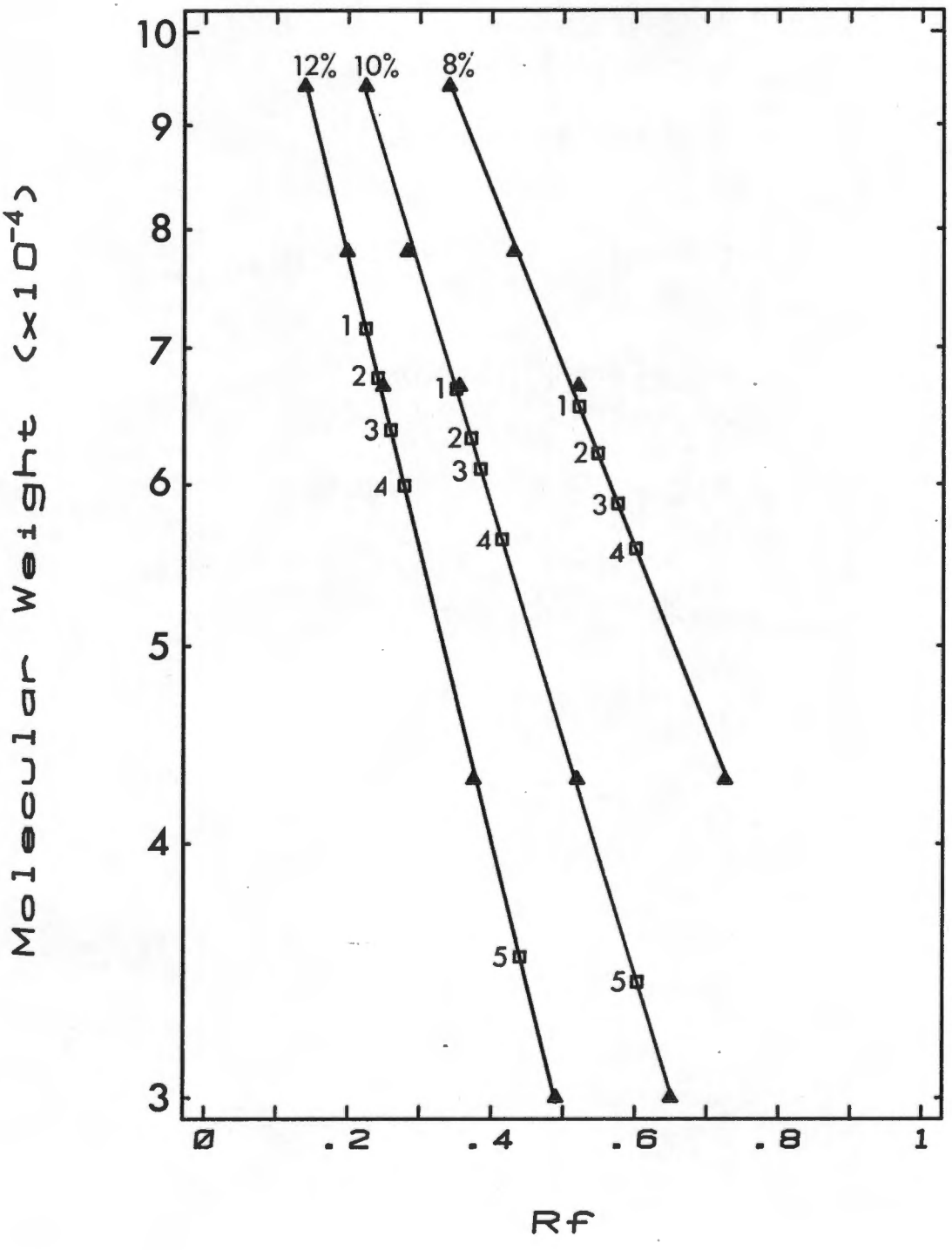
The electrophoretic behaviour of the plasminogen activators was, however, anomalous in that their mobilities relative to the reference proteins were critically dependent upon the total polyacrylamide concentration in the resolving gel. As is evident from Fig. 2.3, the apparent molecular weights that could be assigned to the enzymes by interpolation from the standard curves diminished with decreasing %T.

Since, in the technique I describe, protein substrates were co-polymerized into the resolving gel mixture, the concentration of SDS in the resolving gel and reservoir buffers may have been marginal for the abolition of specific noncovalent interactions between electrophoresed activators and their immobilized substrate, plasminogen. In this case, one might have expected specific affinity reactions to have retarded the electrophoretic mobility of the enzymes. To examine this possibility, two series of experiments were performed. In the first enzymes were electrophoresed in 11% gels containing amounts of co-polymerized plasminogen that varied from 10 to 30 µg/ml. In the second series, the concentration of plasminogen in the resolving gel was maintained constant at 10 µg/ml and the SDS concentration was varied from 0.1 to 1%. There appeared to be no effect of either plasminogen or SDS concentration on the mobility of the plasminogen activators relative to the marker proteins.

Figure 2.3

Figure 2.3 Effect of varying %T on electrophoretic mobility of plasminogen activators.

Reference proteins and plasminogen activators were electrophoresed on SDS-polyacrylamide gel slabs containing 8 , 10 or 12% T and copolymerized gelatin and plasminogen as described under methods and in the legend to Fig. 2.1. After electrophoresis and processing, the distances migrated relative to the phenol red tracking dye were measured (R_f) and plotted against the logarithms of the molecular weights of the reference proteins (▲). Straight lines were fitted for each %T series by the method of least squares. The numbered open squares represent points interpolated from R_fs of enzyme bands as follows: 1, 2 and 3, upper, middle and lower bands, respectively, of the melanoma cell activators: 3, 4 and 5, upper middle and lower bands, respectively, of urokinase. Note that the apparent molecular weights of the enzymes fell with decreasing %T.


Molecular Weight (x10⁻⁴)
10
9
8
7
6
5
4
3
12%
10%
8%
1
2
3
4
5
0
.2
.4
.6
.8
1
Rf

DISCUSSION

In this chapter I describe a technique in which samples containing plasminogen activators were electrophoresed in SDS-polyacrylamide gels containing co-polymerized plasminogen and gelatin as sequential substrates for detecting proteolytic bands. After electrophoresis the gels were incubated at room temperature in Triton X-100 to remove the SDS and so restore enzymatic activity. Subsequently, incubation at 37°C in glycine-NaOH, pH 8.3, buffer for 3 - 5 hr followed by fixation and staining revealed sharp clear zones of proteolysis against a blue background of undegraded substrate when as little as 1 mU of enzyme was electrophoresed. Plasminogen-independent proteases could be detected and analysed by omitting plasminogen from the polyacrylamide-gelatin matrix.

My procedure is a direct modification of the fibrin-agar underlay technique of Granelli-Piperno and Reich (62) with which it compares favourably in some respects and unfavourably in others. The advantages offered by the method I present are as follows: First, by incorporating substrate(s) directly into the resolving polyacrylamide gel the necessity for a second, indicator gel is obviated. Second, the relatively compact structure and smaller pore size of the polyacrylamide matrix limits lateral diffusion of proteases from concentrated zones, with the result that prolonged incubation at 37°C at the developing stage has a less deleterious effect upon the sharpness, and hence resolution of bands of enzymatic activity. This is particularly desirable when bands containing different amounts of enzyme are present in the same electrophoretic track and lengthy incubation periods are required to demonstrate minor components. The 1% agar matrix in the fibrin-agar underlay procedure imposes less of a constraint upon lateral diffusion so that zones of fibrinolysis spread with time, resolution is lost, and substrate clearing due to a major component may engulf that due to

adjacent minor bands. Third, gelatin is susceptible to hydrolysis by a wide variety of proteases, whereas fibrin is a relatively restricted substrate. Fourth, since polyacrylamide gels shrink with fixation and staining, absolute register between the molecular weight marker tracks in the resolving gels and fibrinolytic zones in the fibrin-agar indicator gels is lost. In the polyacrylamide-gelatin gels, marker proteins and proteolytic bands are visible in the same fixed and stained slab. Finally, gelatin is inexpensive and is readily available commercially in a form that is suitable for use without further purification. Fibrinogen does not have these two attributes.

On the other hand, the Granelli-Piperno and Reich technique, being a two-stage procedure, offers a degree of technical versatility that the polyacrylamide-gelatin gel technique does not have. When, for example, sequential reactions are being analyzed, the effects of inhibitors or experimental conditions on the two stages of the reaction can be studied separately. Furthermore, one is able to incorporate particulate substrates, such as erythrocytes, into the agar-underlay for the study of complement or other haemolytic macromolecules; this is not possible in the case of the polyacrylamide-substrate gels. In addition the polyacrylamide-gelatin gels are unsatisfactory for the analysis of crude samples when they contain proteases and proteins with very similar electrophoretic mobilities; the darkly staining protein bands then obscure or distort the clear proteolytic zones. Finally, with the fibrin-agar technique, the time course of the fibrinolytic reaction may be followed and recorded photographically under dark-ground illumination during the development stage. This confers the advantage that enzymatic activity of any band may be judged from the rate of its appearance. In our procedure proteolytic bands are only visible after the reaction has been terminated by fixation and staining. The

Granelli-Piperno and Reich procedure and the one that I describe should therefore be regarded as complementary to each other.

In a recent extensive survey (61) of human cells cultured in vitro, the Granelli-Piperno and Reich technique was used to show that melanoma cells characteristically release plasminogen activators that differ from the urokinase-type enzymes secreted by most normal cells and the majority of other tumours. The melanoma-type enzymes have, as the predominant electrophoretic components, a closely spaced doublet in the 70 000 M_r range whereas the major components of urokinase-type enzymes appear as a doublet in the 60 000 M_r region of the gel. As can be seen from Fig. 2.1, the procedure I describe readily distinguishes the melanoma- and urokinase-type enzymes.

Although the procedure has proved useful for discriminating between plasminogen activators of different types, it has been of less value for assigning precise molecular weights to the enzymes owing to the anomalous electrophoretic behaviour in gels of different total polyacrylamide concentrations. For reasons that are not entirely clear, I have been unable to resolve this difficulty by analysing the results obtained in gels of different %T by graphic procedures recommended by Chrambach and Rodbard (154) and Maizel (137) in which Ferguson plots (%T vs log electrophoretic mobility) are constructed for each of the reference proteins and enzyme bands. The slopes of these linear plots are measured to give retardation coefficients (K_R) for each protein species. It should, in theory, be possible to obtain a linear relationship between molecular weight and the square root of the retardation coefficient from which molecular weights of the enzymes could be assigned by interpolation. In the polyacrylamide-plasminogen-gelatin system, however, the Ferguson plots, although linear, did not extrapolate to the same ordinate intercept at zero %T for migration

in free solution and the relationship between $(K_R)^{\frac{1}{2}}$ and molecular weight for the reference proteins was not linear. Since the migration of enzyme bands was not affected by varying the concentration of plasminogen or SDS in the resolving gel I am unable to attribute the anomalous electrophoretic behaviour of the plasminogen activators to affinity interactions with the partially denatured specific substrate, plasminogen.

In summary, therefore, the technique is of value for distinguishing different plasminogen activators on the basis of their relative sizes, but it cannot, without further improvements, be used to assign precise molecular weights.

CHAPTER III

THE IMMUNOCHEMICAL LOCALISATION OF HUMAN AND ANIMAL PLASMINOGEN ACTIVATORS

It is now well established that plasminogen activators that are released by human cells cultured in vitro or that are detected in human tissues can be divided into at least two groups (61,51,52,107,111,115, 117,198).

The first group of enzymes are those that are inhibitable by antibodies to urokinase. These exist in several molecular weight forms. When, in any given specimen, multiple forms are present, those with molecular weights of 60 000 and 32 000 daltons usually predominate with lesser relative amounts of 46 000 and approximately 100 000 MW components (61).

The second group of plasminogen-dependent proteases are those that are not inhibitable by antibodies to urokinase. As with the previous group these, too, comprise enzymes with different molecular weights, but here a 70 000 dalton species predominates with lesser amounts of 100 000, 60 000 and 38 000 dalton enzymes (61). It is now known that melanoma cells characteristically release plasminogen activators representative of the second group (61, 115,117,130) and it has also been noted (198,56,55) that "tissue activator" is not inhibited by anti-urokinase antibodies but that it is inhibited by antibodies to melanoma activator (130).

At the time at which this work was started, an obvious deficiency of the classification of human plasminogen activators into "urokinase-like" and "non-urokinase-like" categories was the lack of a positive basis for establishing membership of the latter group or for establishing relationships between members of the "non-urokinase-like" enzymes. Could one, for example, consider "tissue activator" and "melanoma activator" as closely related or identical enzymes (as their molecular weights would

suggest) or were they entirely different molecules related neither to urokinase nor to each other?

Since I felt that an antibody directed towards any one of these non-urokinase-like activators would clearly be a useful reagent for establishing such relationships or class membership on the basis of immunochemical cross-reactivity, I prepared and characterised a rabbit antibody directed towards Mel-PA. This antibody has been used to confirm the distinction between urokinase and melanoma activator; it has been used to demonstrate cross-reactions between Mel-PA and plasminogen activators found in most tissues and body fluids; and it has been used to identify phylogenetic relationships between melanoma-type plasminogen activators that do not appear to exist for urokinase.

MATERIALS AND METHODS

Mel-PA released by Bowes I or Bowes II melanoma cells cultured *in vitro* was partially purified using affinity chromatography on benzamidine-agarose or zinc chelate-agarose and Con A-agarose as described in Chapter I. Human urokinase was obtained commercially (Leo Pharmaceutical Products, Ballerup, Denmark).

Serum-free harvest fluids containing plasminogen activators were obtained from a number of melanoma cell lines established in this laboratory. The tissue culture techniques used for establishment of these cell lines have been described in detail by Wilson and Dowdle (106) and Hoal (151).

Harvest fluids were prepared by washing the adherent monolayer cultures three times with DME or RPMI medium and incubating them for 24 hr under serum-free medium. The medium was then removed, centrifuged at 750g for 10 min and stored at -80^{o}C. Protease- and inhibitor-free bovine serum albumin (Appendix 1) was added to a final concentration of 0.4 mg/ml to stabilize the enzyme.

Urine samples were centrifuged at 750g for 10 min and the supernatants were stored at -20°C until use.

Human semen was obtained from normal, infertile and vasectomised males attending the Fertility Clinic at Groote Schuur Hospital. Samples were kept at room temperature for 30 min for liquefaction to take place. They were then centrifuged at 20 000g for 60 min. The clear seminal plasma was then stored at -20°C.

Saliva and tear samples were centrifuged at 20 000g for 15 min and used immediately.

Human breast milk was centrifuged at 20 000g for 60 min. The upper fatty layer was discarded and the supernatant was stored at -20°C before use.

Fresh samples of human tissue were obtained at surgery or from cadaver transplant donors. Animal tissues were procured freshly after slaughter. Tissue samples (usually approximately 200 mg) were rinsed in saline, fragmented with scissors and then homogenized in 1 ml of 0.5% Triton X-100 using a glass homogenizer. The homogenized tissue samples were extracted with 4 ml of 0.5% Triton X-100 in water for 2-4 hr at 4°C. The suspensions were then centrifuged at 1700g for 30 min to remove cellular debris and the clear supernatants were stored at -20°C until use.

Human plasminogen was purified from fresh frozen plasma by affinity chromatography on lysine-sepharose according to a modification of the method of Deutsch and Mertz (45) (Appendix 1.3). Human fibrinogen (A.B. Kabi) or bovine fibrinogen (Sigma) was further purified by salt precipitation according to the method of Laki (182) and freed of plasminogen according to the method of Mosessan (162) as described in detail in the Appendix (A1.1).

Rabbit antibodies to Mel-PA and urokinase.

Harvest fluids containing Mel-PA were partially purified by benzamidine-agarose chromatography or chromatography on Zn-chelate-agarose and Con A-agarose to give 10-30 ml of solution containing 10 000 to 15 000 urokinase units of enzyme.

Proteins in this sample were precipitated by adding TCA to a final concentration of 10%. The precipitate was collected by centrifugation (1700g; 30 min) and washed in acetone. It was then dissolved in 0.25 ml of 0.06M Tris HCl pH 6.8 containing 2.5% SDS, 10% glycerol and 0.004% phenol red. After boiling for 1 min, two samples of 0.125 ml volume each were electrophoresed in 11% polyacrylamide gel slabs (125 x 175 x 2 mm) containing 0.1% SDS (Appendix A2). After electrophoresis, the slabs were washed in 2.5% Triton X-100 for 1 hr at room temperature and in water for 30 min. Marker tracks (see below) were removed and the remainder of the gel was frozen at -20°C.

Molecular weight markers (Pharmacia) and a 100 µl sample of Mel-PA containing approximately 30 FU (80 UK units) of active enzyme were co-electrophoresed in adjacent tracks on the same gel. After electrophoresis the gel was washed in Triton X-100 and the track containing the separated molecular weight markers and that containing the electrophoresed enzyme were separated from the remainder of the gel. The active enzyme track was then cut horizontally into 1.7 mm slices and each slice was added to a well in a plate coated with ^{125}I-fibrin and containing 2 µg of plasminogen and 80 µg BSA in 200 µl of 0.1M Tris-HCl.

After incubation at 37°C for 30-60 min aliquots of 50 µl were taken from the wells and assayed for release of radioactive fibrin degradation products. By this method the exact position of Mel-PA in the slab gels could be determined. A typical profile of Mel-PA activity along the gel and the protein bands observed in tracks containing TCA precipitates

is presented in Fig. 3.1.

Sections of the frozen preparative gel containing separated TCA precipitated Mel-PA protein were excised and pulverised in a dismembranator (B. Braun, Melsungen AG, F.R.G., type 853062). The frozen powder was then suspended in 1 ml of PBS and homogenized with an equal volume of complete Freund's adjuvant. Each of two rabbits was then immunized by subcutaneous injection of 1 ml of this emulsion. After 2 to 3 weeks the rabbits were boosted with subcutaneous injections of 1 ml of emulsion prepared identically but with Incomplete Freund's adjuvant. The rabbits were bled at 2 to 4 weekly intervals thereafter. Further booster injections were given at approximately 2 monthly intervals.

Antibodies to crude commercial urokinase were prepared as described by Wilson et al (61).

Immune IgG was isolated from the rabbit sera by precipitation with 33% ammonium sulphate, dialysis into 0.015M sodium phosphate buffer pH 8.0 and ion exchange chromatography on DEAE-cellulose equilibrated in 0.015M sodium phosphate buffer pH 8.0 (199).

Active proteases were eliminated from the IgG preparation with 10 mM DFP at 45^{o}C for 1 hr and subsequent dialysis into PBS (61). The IgG was then stored frozen at -20^{o}C until required.

Detection of anti-Mel-PA and anti-UK antibody activity.

Two procedures were used for the detection and quantitation of antibodies to plasminogen activators. Both methods depended upon the specific inhibition of plasminogen-dependent fibrinolytic activity.

In the first procedure known amounts of the enzymes were incubated with serial dilutions of antibody and samples of the mixtures were assayed for residual enzymatic activity in the ^{125}I-fibrin assay.

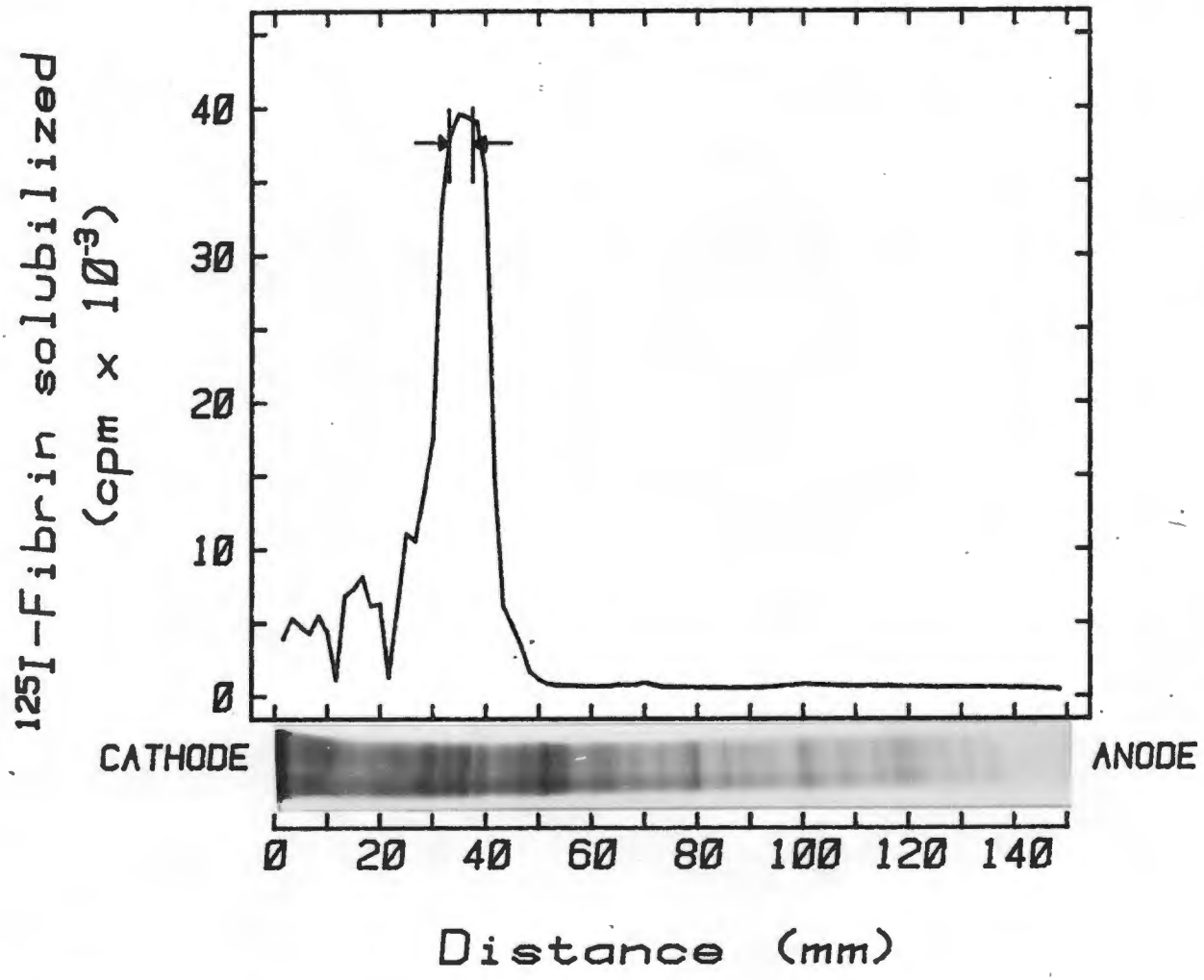


Figure 3.1 Electrophoresis of Mel-PA in polyacrylamide gel slab containing SDS.

Approximately 2 litres of serum-free harvest fluid was collected from cultures of Bowes I cells and subjected to benzamidine-agarose chromatography as described in Chapter I. Enzyme-containing fractions were pooled to give 30 ml of solution of which 15 ml was made 10% with respect to trichloracetic acid to precipitate enzyme and other proteins present. The precipitate was recovered by centrifugation (1700g; 30 min) and dissolved in 0.25 ml of 0.06M Tris-HCl pH 6.8 containing 2.5% SDS, 10% glycerol and 0.004% phenol red.

The photograph in the figure shows a fixed and stained 11% polyacrylamide gel slab track in which 15 µl of the TCA-precipitated Mel-PA sample had been electrophoresed.

A 100 µl sample of Mel-PA containing approximately 30 FU of active enzyme was co-electrophoresed in an adjacent track. This track was washed in 2.5% Triton X-100 after electrophoresis, frozen, and cut horizontally into 1.7 mm slices. The fibrinolytic activity in each slice was measured in the ^{125}I-fibrin assay.

The figure shows the profile of Mel-PA activity along the gel. The segment of the gel containing the TCA precipitated protein that was used for immunization of the rabbits is also indicated (⊣| |⊢).

In brief, Mel-PA or urokinase preparations were diluted with T-T(0.1) so that 10 µl of solution, when assayed in the standard ^{125}I-fibrin assay, would release approximately 30% of the total trypsinisable radioactivity in one hour. Fifty microlitre volumes of these solutions were added to tubes each of which contained an equal volume of purified IgG dissolved and diluted in PBS. The most concentrated antibody tube usually contained 1 mg/ml of IgG.

The tubes containing antibody and plasminogen activator were incubated at 4°C for 1 hr after which 20 µl samples were removed for assay of residual plasminogen activator activity in the ^{125}I-fibrin assay (Appendix A3.1). A typical antibody titration is shown in Fig. 3.3.

In the second procedure I used a combined immunochemical and electrophoretic approach that permitted identification of the molecular species of the inhibited plasminogen activator. This method was based on the electrophoretic and zymographic technique of Granelli-Piperno and Reich (62) as modified by Wilson et al (61).

Plasminogen activators were separated on a molecular size basis in 11% polyacrylamide gel slabs (60 x 70 x 1 mm) containing 0.1% SDS. The slabs were washed in 2.5% Triton X-100 for 1 hr at room temperature, rinsed briefly in water and then layered on indicator agar slabs containing 1.25% agar, 5 µg/ml human plasminogen, 0.06 units/ml thrombin and 2.5 mg/ml fibrinogen. The assemblies were then incubated at 37°C in a humid environment and examined periodically under darkground illumination for the presence of clear zones of fibrinolysis against an opaque background of undegraded fibrin.

To examine the susceptibility of electrophoresed plasminogen activator bands to inhibition by antibody, a narrow trough was cut into the fibrin-plasminogen-agar indicator slabs and filled with 100 µl of antibody solution containing 5 to 8 mg/ml IgG. The antibody was then

allowed to diffuse laterally into the gel by incubation at 4°C for approximately 5 hr. The polyacrylamide slab gels containing separated plasminogen activators were then carefully layered on the indicator gels in such a manner that one of a pair of adjacent electrophoretic tracks lay next to the trough and the other containing a duplicate sample lay one track width removed from the trough. The enzyme in the track close to the trough was thus exposed to the antibody while the duplicate was not. Typical examples of results obtained with this technique are shown in Fig. 3.5 and 3.8.

RESULTS

Immune response of rabbits to Mel-PA.

Rabbits were immunized with Mel-PA by repeated injections of enzyme and serum samples were obtained at 2-4 weekly intervals after the initial injection. The IgG fraction was isolated from each antiserum and assayed for its ability to inhibit Mel-PA using the ^{125}I-fibrin assay. The results obtained with 4 of the rabbits are shown in Fig. 3.2 where antibody activity is plotted as a function of time after the start of the immunization schedule. Antibody is expressed as the reciprocal of the concentration of antibody (in mg/ml) that was required to inhibit 50% of the activity of a standard Mel-PA preparation. Technical details are given in the Methods section and in the legend to Fig. 3.2.

As can be seen from the figure, antibody only became detectable after approximately 10 weeks and four injections of antigen had been given. Thereafter antibody titres increased. In two of the rabbits a plateau in serum antibody titre was reached after 4-5 months. At this stage 7 µg/ml of IgG in the incubation tube was capable of inhibiting 50% of the enzyme present. Antibody titres of the other two rabbits increased more

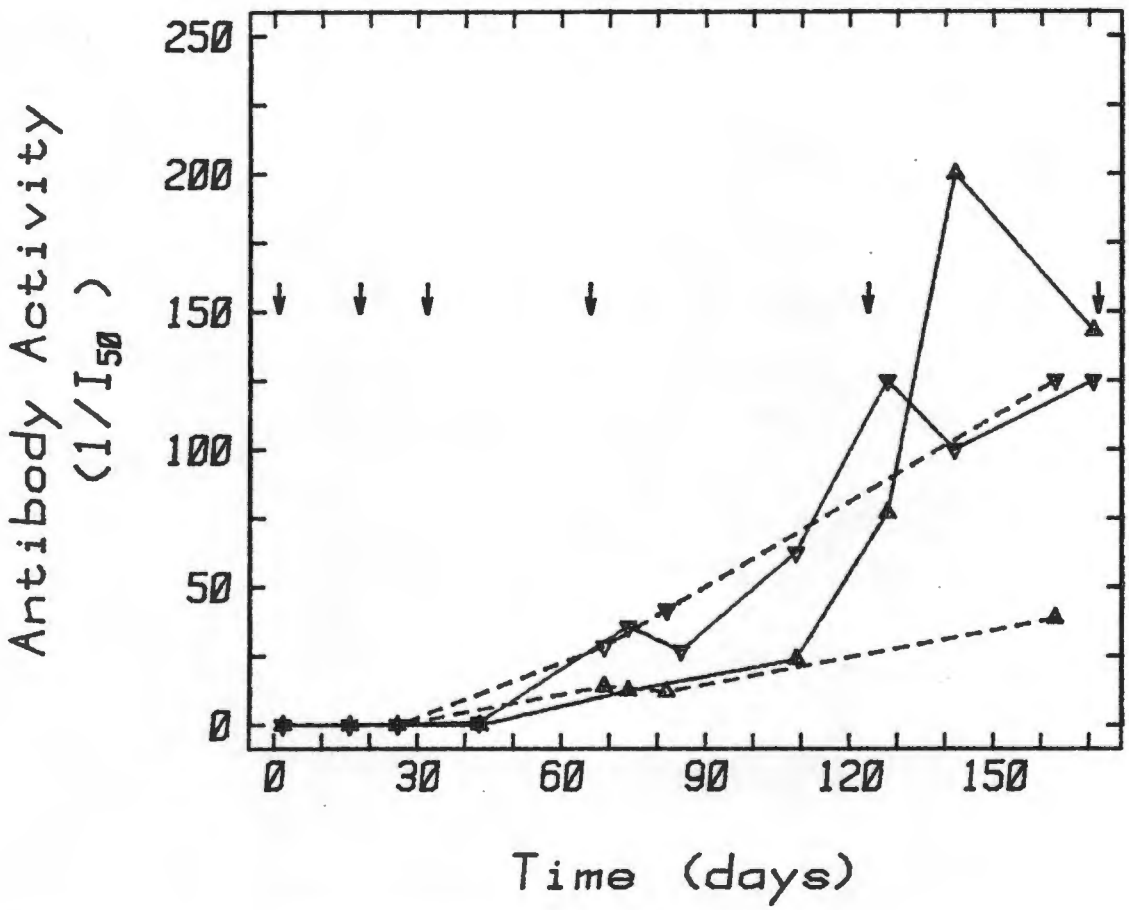


Figure 3.2 Immune response of rabbits to Mel-PA

Four rabbits were immunized by subcutaneous injection of 1 ml of an emulsion consisting of pulverized polyacrylamide gel segments containing 2000 - 3000 FU (approximately 0.1-0.2 mg; 5000-7500 UK u) of Mel-PA. The priming injection contained complete Freund's adjuvant; subsequent injections contained incomplete Freund's adjuvant.

The times at which the rabbits were injected are indicated in the Figure by arrows.

The rabbits were bled at various intervals after immunization and the IgG fractions were prepared from the antisera. Antibody activity of these fractions was determined by measuring the inhibition of Mel-PA in the ^{125}I-fibrin assay as described in the Methods section and in the legend to Fig. 3.3. Antibody activity in the IgG fraction is expressed as 1/I:50 or the reciprocal of the antibody concentration (in mg/ml) that inhibited the fibrinolytic activity of 0.1 FU of Mel-PA by 50% in the ^{125}I-fibrin assay.

Antibody titres started to increase after 2 months and after 3 injections of antigen had been given. Titres reached a maximum level after 4-5 months.

slowly and the maximum titre achieved was 8 µg/ml.

On the basis of these results a satisfactory immunization schedule was adopted in which rabbits were immunized and boosted 3 weeks later and at 2 monthly intervals thereafter. Serum samples were taken for antibody measurement 3-4 months after the initial injection. Only those antibody preparations that, at a concentration of 10 µg/ml or less, showed 50% inhibition of the enzyme activity were retained as satisfactory reagents. A typical antibody titration curve given by a useful antibody preparation is shown in Fig. 3.3.

Some idea of the affinity of such antibody for the enzyme is given by the fact that 7 µg/ml (i.e. $\simeq 5 \times 10^{-8}$M) IgG solution inhibited 50% of the Mel-PA activity when the enzyme was present at a concentration of 1×10^{-9}M (as calculated from active site titrations with ^{3}H-DFP; Chapter V).

It was important to eliminate all proteolytic activity from the IgG preparations and this was usually accomplished by treatment with DFP. On occasion this was not effective and such preparations were discarded.

Antibody specificity

Antibodies raised to Mel-PA or to urokinase were inhibitory only for the homologous enzyme and no cross reactions were observed either by assay for residual activity using the ^{125}I-fibrin technique (Fig. 3.4) or by the immunoelectrophoretic procedure (Fig. 3.5). Although urokinase and Mel-PA showed no apparent immunochemical relationship in inhibition assays, cross-reacting determinants that did not involve the active sites may still have existed on these two enzymes. To examine this possibility a binding assay was developed using solid-phase antibody.

The specific affinity reagent for this experiment was prepared

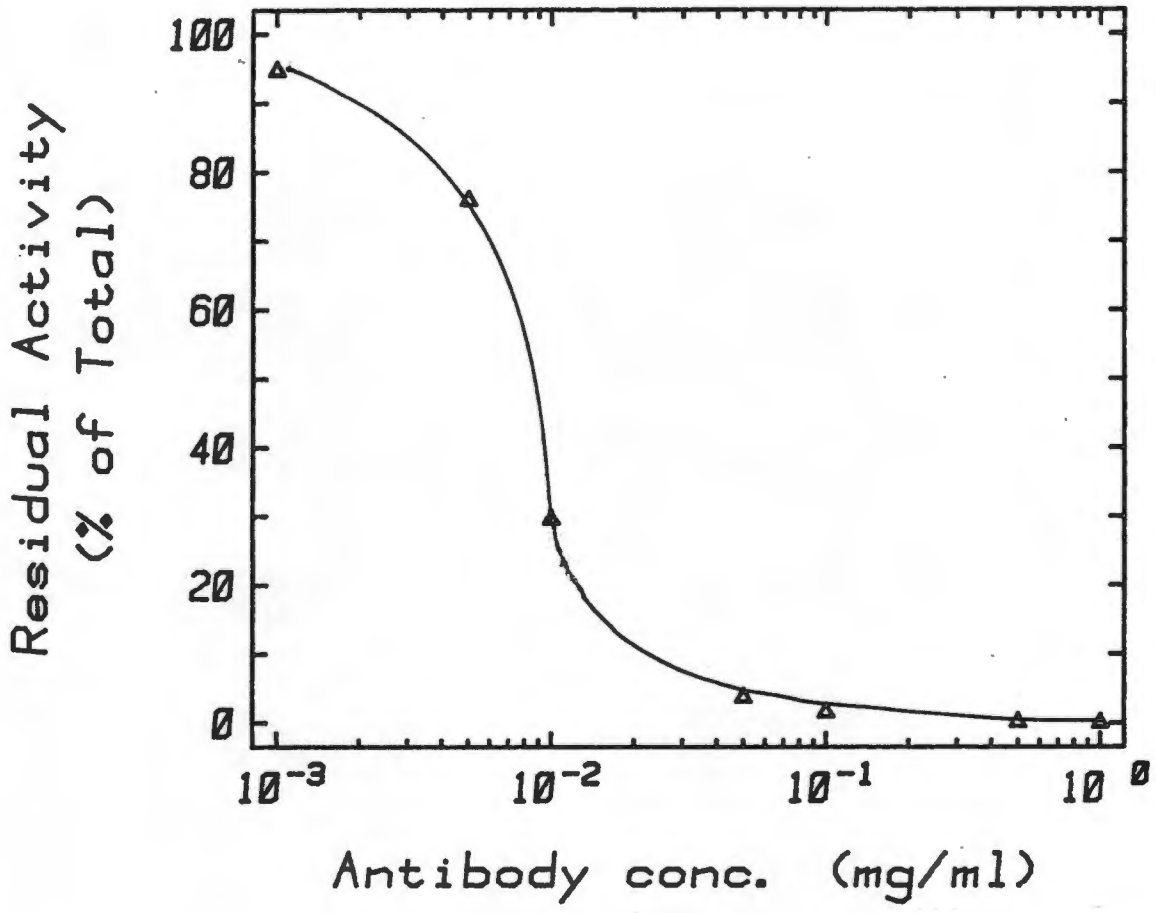


Figure 3.3 Inhibition-titration of Mel-PA with rabbit antibody to Mel-PA

Volumes of 50 µl of Mel-PA in TT (0.1) were added to tubes each of which contained an equal volume of purified anti-Mel-PA IgG diluted in PBS. The starting concentration of IgG was 1 mg/ml. The tubes were incubated at 4°C for 1 hr and 20 µl samples were then removed for assay of residual plasminogen activator activity in the ^{125}I-fibrin assay.

Mel-PA incubated in presence of an equal volume of PBS that did not contain antibody released 45% of the total radioactivity in one hour.

The antibody inhibited Mel-PA by 50% at a final concentration of 7 µg/ml in the tube.

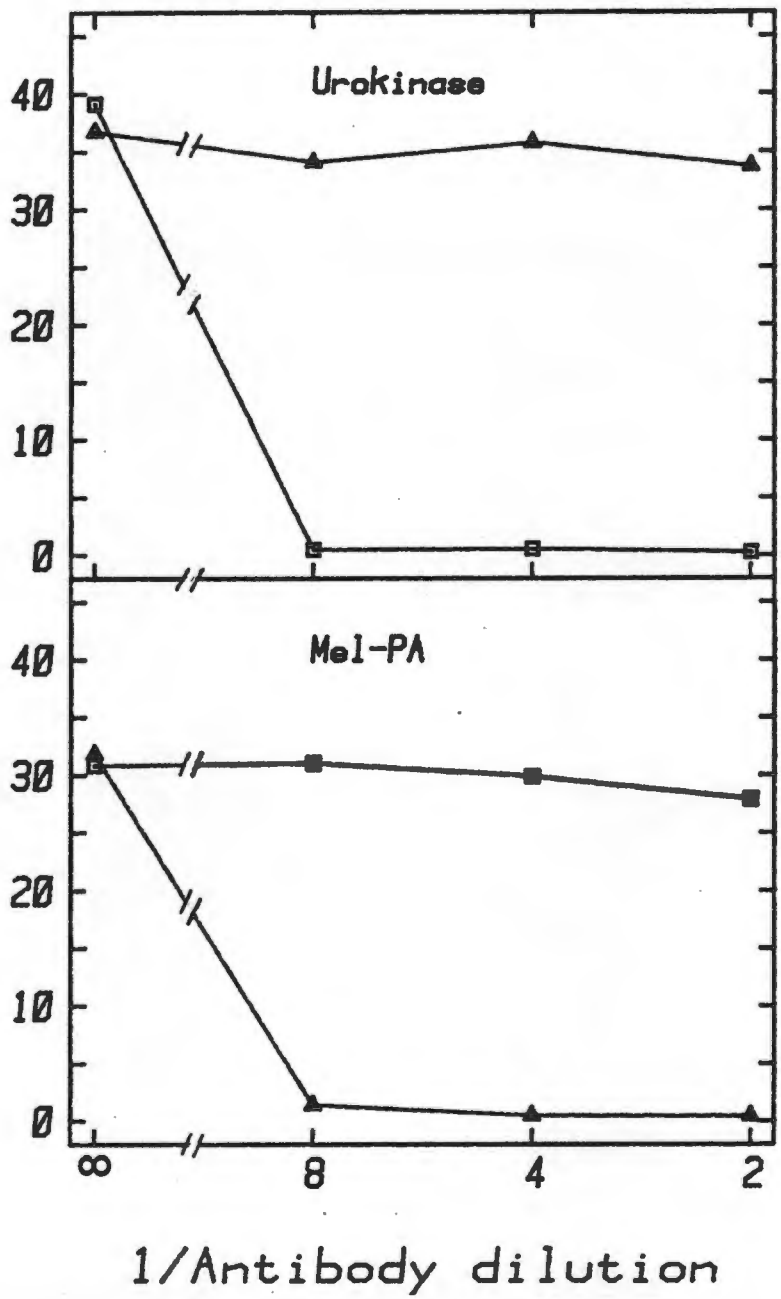


Figure 3.4 Specificity of anti-Mel-PA IgG or anti-urokinase IgG.

Mel-PA or urokinase in 25 µl 0.1MTris-HCl pH 8.1 containing 1.6 mg/ml BSA were incubated with 25 µl of serial 2-fold dilutions of antibody for 1 hr at 4°C. Residual plasminogen activator activity was then measured in the ^{125}I-fibrin assay.

Undiluted antibody solution contained 5 mg/ml of IgG. Control samples of Mel-PA released 30% of the total radioactivity in 1 hr and urokinase released 37% in 1 hr.

Antibody to Mel-PA (△—△) inhibited the activity of Mel-PA but not of urokinase and antibody to urokinase (□—□) inhibited the activity of urokinase but not of Mel-PA.

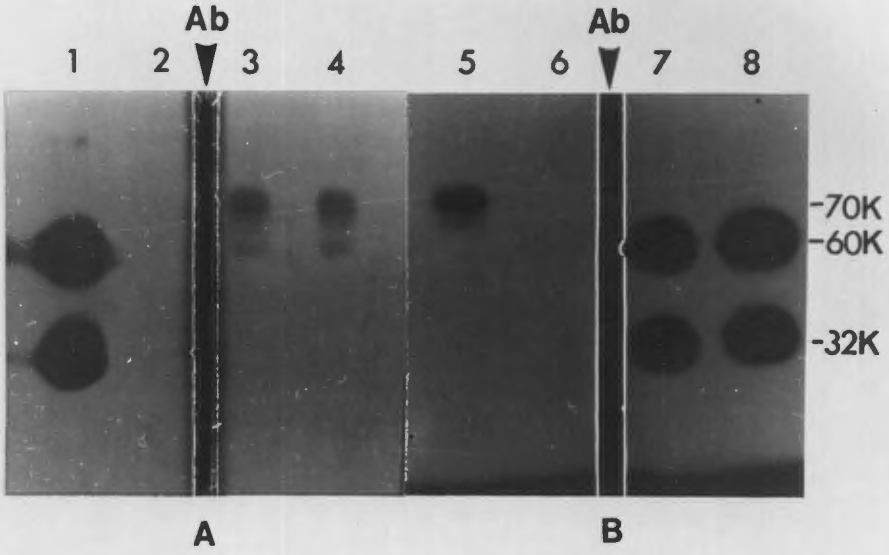


Figure 3.5 Combined immunoelectrophoretic and zymographic analysis of Mel-PA and urokinase.

Samples of urokinase (wells 1, 2, 7 and 8) or Mel-PA (wells 3, 4, 5 and 6) were electrophoresed in 11% polyacrylamide gel slabs containing 0.1% SDS. The slabs were washed in 2.5% Triton X-100 for 1 hr at room temperature, rinsed briefly in water, and layered on indicator-agar slabs containing fibrin and plasminogen. Narrow troughs (Ab ↓) had been cut into the indicator slabs and either anti-urokinase (Gel A) or anti-Mel-PA (Gel B) had been added and allowed to diffuse into the indicator slabs. The gel assemblies were incubated at 37°C in a humid environment. When distinct areas of fibrinolysis could be observed, the assemblies were photographed under dark-background illumination.

It can be seen that both bands of urokinase were inhibited by antibody to urokinase (Gel A) but not by antibody to Mel-PA (Gel B). Similarly, Mel-PA was inhibited by anti-Mel-PA (Gel B) but not by anti-urokinase (Gel A).

by linking 18 mg of anti Mel-PA antibody to 5 ml of cyanogen bromide-activated sepharose 4b (Appendix A.1).

The matrix was washed sequentially with 0.1M $NaHCO_3$ pH 8.3 containing 0.5M NaCl; 1 M ethanolamine pH 9.0; 0.1M Na acetate pH 4.0 containing 0.5M NaCl; and was then equilibrated in 0.1M Tris HCl pH 8.0 containing 0.1% Triton X-100 and 0.4M NaCl. Two 2 ml columns of the matrix were prepared and either 1 ml of urokinase (≃ 1.5 pmol) or 1 ml of Mel-PA (≃ 8 pmol) in RPMI containing 0.1% Triton X-100 were added. The columns were washed with the Tris buffer and adsorbed enzyme was eluted with 0.1M glycine-HCl pH 3.5 containing 0.15M NaCl and 0.1% Triton X-100. Fractions of 1 ml were collected and plasminogen activator activity was measured in each fraction in the ^{125}I-fibrin assay. The elution profiles shown in Fig. 3.6 demonstrate that all of the Mel-PA was adsorbed by the matrix whereas negligible amounts of urokinase bound. Approximately 75% of the total Mel-PA added could be recovered by elution at pH 3.5.

Inhibition of plasminogen activator by antibody in the fluorometric assay.

The anti-Mel-PA antibody readily interfered with the ability of Mel-PA to function as a plasminogen activator. It was therefore of interest to see whether or not it would also inhibit the direct amidolytic action of Mel-PA in a fluorometric assay. An experiment was accordingly performed in which 100 µl volumes of 5 serial two-fold dilutions of anti-Mel-PA antibody in PBS (undiluted concentration:- 5 mg/ml) were added to test tubes. An equal volume of Mel-PA (26 FU/ml) in T-T(0.02) was then added to each tube and these were incubated at room temperature for 1 hr. Samples of 50 µl of each mixture were then assayed for residual amidolytic activity in the fluorometric assay using the substrate Cbz-Gly-Gly-Arg-AMC (Appendix 3.2).

The amidolytic activity of Mel-PA was not inhibited by any of the concentrations of antibody tested. Instead, a slight increase in

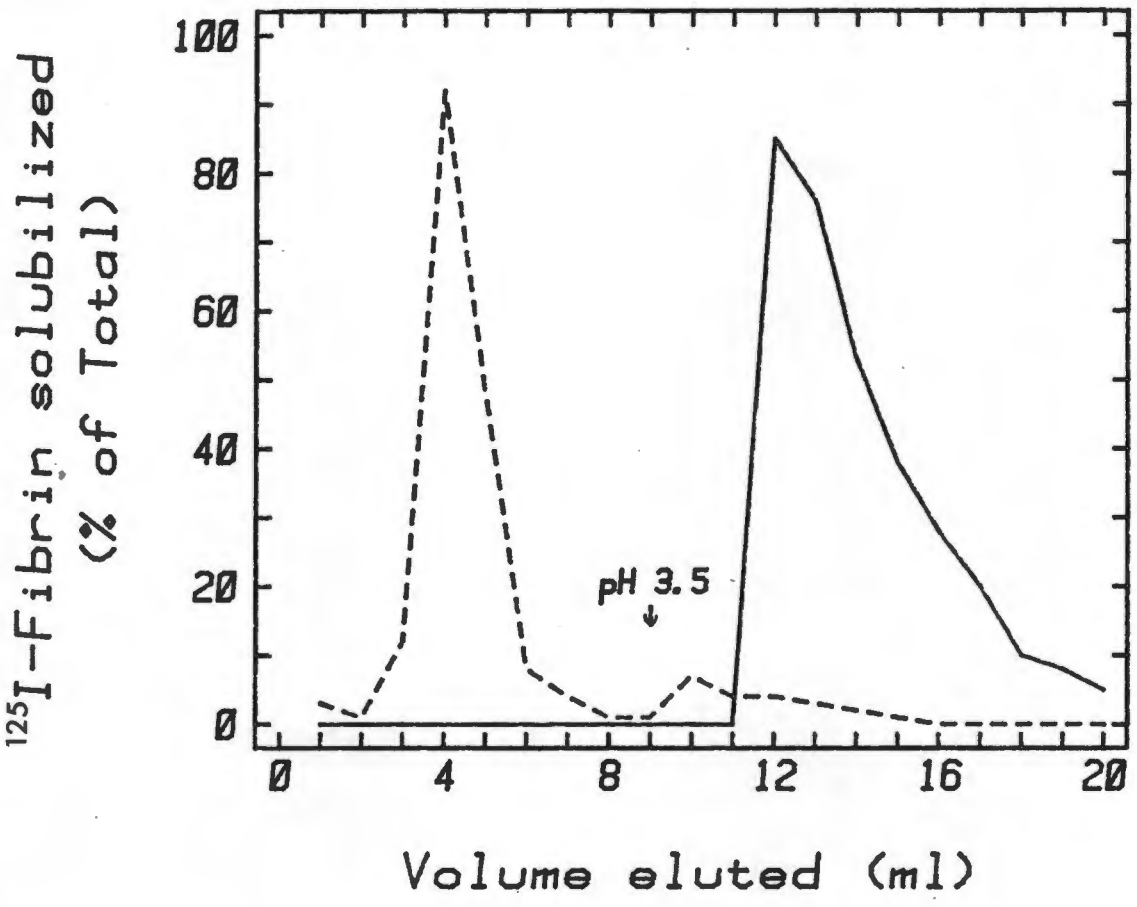


Figure 3.6 Immunoadsorption of Mel-PA or urokinase to solid-phase antibody.

Anti-Mel-PA IgG (18 mg) was coupled to 5 ml of sepharose 4b and the matrix was equilibrated with 0.1M Tris HCl pH 8.0 containing 0.1% Triton X-100 and 0.4M NaCl. Two 2 ml columns of the matrix were prepared and either 1 ml urokinase or 1 ml Mel-PA in RPMI containing 0.1% Triton X-100 were added. The columns were washed with Tris buffer and bound enzyme was eluted with 0.1M glycine-HCl pH 3.5 containing 0.15M NaCl and 0.1% Triton X-100. Fractions of 1 ml were collected and plasminogen activator activity was measured in each fraction in the ^{125}I-fibrin assay.

The elution profiles demonstrate that all of the Mel-PA (——) was adsorbed by the matrix whereas negligible amounts of urokinase (-----) bound. Approximately 75% of the total Mel-PA added could be recovered by elution at pH 3.5.

activity from 11.27 FU/ml to 13.19 FU/ml was seen.

Immunochemical relationships between Mel-PA and plasminogen activator released by other melanoma cell lines.

Mel-PA's released by Bowes I or Bowes II cells comprised predominantly a 70 000 MW doublet with insignificant amounts of 60 000 and 40 000 MW species and no evidence of higher molecular weight components. One would have expected therefore that antibodies to Mel-PA would have been directed largely against the 70 000 MW component particularly since polyacrylamide gel fragments containing this species were used to immunize the rabbits.

While other melanoma cell lines produce 70 000 MW plasminogen activators that do not cross-react with urokinase (61,115,117) the immunochemical identity of these enzymes to Mel-PA has not been formally established. Furthermore other melanoma lines frequently release plasminogen activators with molecular weights of approximately 110 000 daltons, 60 000 daltons and 40 000 daltons and the relationship of these plasminogen activators to each other and to the 70 000 MW species have not been clarified. It was therefore of interest to test harvest fluids from other melanoma cell lines for the presence of enzymes that would cross-react with Mel-PA. The results of these experiments are presented in Fig. 3.7 and Table 3.1. All plasminogen activators secreted by all melanoma lines were inhibited by anti-Mel-PA antibody. None were inhibited by urokinase.

Occurrence of Mel-PA and urokinase in tissues and physiological fluids of human origin.

Radioenzymatic and immunoelectrophoretic procedures were used to define molecular species of plasminogen activators present in various

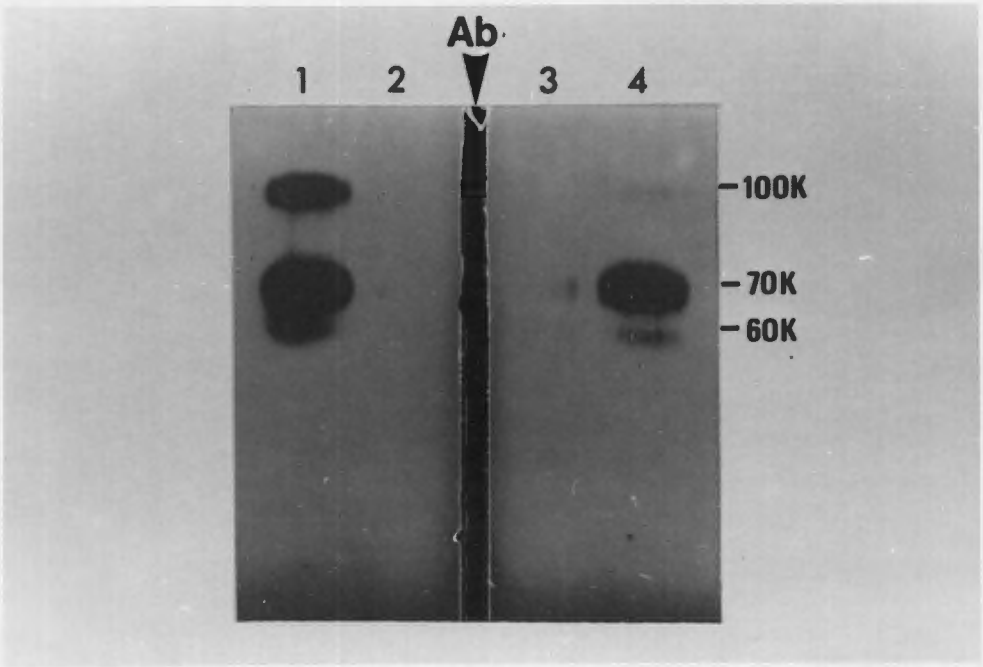


Figure 3.7 Electrophoretic and immunochemical analysis of plasminogen activators released by melanoma cells.

Samples of serum-free harvest fluids containing plasminogen activators released by UCT-Mel 5 (tracks 1 and 2) or UCT-Mel 1 (tracks 3 and 4) were analysed by the combined zymographic and immunochemical method. The trough contained antibody to Mel-PA.

All molecular species of plasminogen activators released by the two melanoma cell cultures were inhibited by antibody to Mel-PA.

Table 3.1 Plasminogen activators released by melanoma cells cultured in vitro.

Cell line	Plasminogen activator species (Molecular weight)			
	>100K	73K	60K	38K
Bowes I	-	++++	++	-
Bowes II	-	++++	++	-
UCT-Mel 1	++	++++	+++	+
UCT-Mel 2	++	++++	++	-
UCT-Mel 3	++	++++	+++	-
UCT-Mel 4	++	++++	+++	+
UCT-Mel 5	+++	++++	+++	+
UCT-Mel 7	++	++++	+++	+
M 127	++++	+++	-	-
M 170	++	++++	-	-

Plasminogen activators released by various melanoma cell lines were analysed by the radioenzymatic and immunoelectrophoretic procedures described in the Methods section. The entries in the table indicate the relative abundance of the different molecular weight species within a given sample and are scored on an arbitrary scale of - to ++++, where - indicates no enzyme.

All plasminogen activator species released by all melanoma cell cultures were inhibited by anti-Mel PA antibody.

normal body fluids and tissues of human origin. The samples were collected and analysed as described in the Methods section to give the results summarized in Table 3.2. Typical results are shown in Fig. 3.8 for the case of uterine tissue.

All tissues examined with the exception of liver, contained plasminogen dependent fibrinolysins. None contained measurable plasminogen independent fibrinolytic activity.

Most tissue homogenates and body fluids contained both Mel-PA and urokinase. Normal urine invariably contained only urokinase.

Plasminogen activators present in plasma and serum presented an extremely complex picture. The amounts present, their molecular weights and the readiness with which they could be detected were influenced by the anticoagulant used, by the conditions of storage of the samples and by clotting. I have therefore, omitted these data from the tables.

In all cases plasminogen activators present in tissues or body fluids that cross-reacted with Mel-PA had similar molecular weights to the enzyme released by melanoma cells in culture. As shown in Table 3.2 plasminogen activators of the Mel-PA type with the molecular weight of 70 000 daltons predominated with minor amounts of plasminogen activator with a molecular weight greater than 100 000 daltons. Urokinase type activators had a major component with a molecular weight of approximately 60 000 daltons with minor species of 32 000 daltons. In no single case have I observed a urokinase-like enzyme with a molecular weight of 70 000 daltons.

Table 3.2 Plasminogen activators released in human tissues and body fluids

Source	Species of plasminogen activator (Molecular weight; P.A.Type)				
	Mel-PA		Urokinase		
	100K	70K	100K	60K	32K
Urine				++++	++
Seminal plasma	+	+++		+++	+
Breast milk	++	+++		+++	
C.S.F.		+		+	
Tears	+	+		+++	
Saliva		+		+++	+
Uterus		++++		++	
Kidney		++		+++	
Liver		-		-	
Lung		+++		+++	
Brain (grey)	+	++		+++	
Brain (white)		++			
Thyroid		+++		++++	
Heart		+		++	
Aorta		++		+	
Vein		++		++	
Oesophagus		++		+	
Prostate		++		++++	++
Adrenal		+		+	

Plasminogen activators present in tissue extracts and body fluids of human origin were subjected to electrophoretic and immunochemical analysis to define their molecular weight and their relationship to Mel-PA or urokinase. The relative abundance of the different molecular species within a given sample was scored on an arbitrary scale of - to ++++ where "-" indicates no enzyme.

Human tissues and body fluids contained both immunochemical types of plasminogen activator, with the exception of urine, which only contained urokinase and liver, which contained no detectable enzyme.

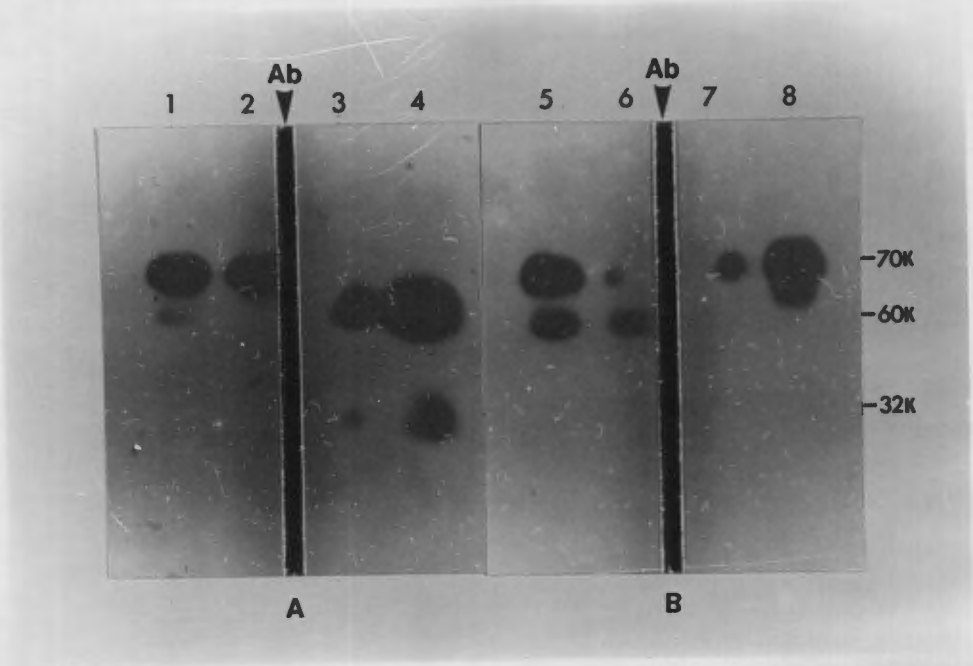


Figure 3.8 Electrophoretic and immunochemical analysis of plasminogen activators in uterine tissue extracts.

Samples of human uterine tissue extracts (wells 1, 2, 5 and 6), of urokinase (wells 3 and 4) or of Mel-PA (wells 7 and 8) were electrophoresed and analysed immunochemically as described in the Methods section and in the legend to Fig. 3.5.

The human uterine tissue extract contained two species of plasminogen activator. The species with a molecular weight of approximately 70 000 daltons was inhibited by antibody to Mel-PA (gel B) and the species with a molecular weight of approximately 60 000 was inhibited by antibody to urokinase (Gel A).

Relationship of plasminogen activators found in other species to human Mel-PA and urokinase.

In order to determine whether or not phylogenetic relationships exist between human plasminogen activators and those found in other species, a survey was undertaken in which enzymes from a number of animal sources were examined for their susceptibility to inhibition by antibody to Mel-PA or urokinase. The results of this survey are presented in Tables 3.3 to 3.6 and Fig. 3.9.

The different species of plasminogen activators present in tissue extracts and body fluids were first analysed by the electrophoretic and zymographic procedure to determine their molecular weight and the approximate values for these are given in the tables.

The immunochemical types of plasminogen activator present in a few representative samples of the tissue extracts or urines of the various animal species were then determined first by the radioenzyme assay and then by the combined electrophoretic and immunochemical procedures described in the Methods section.

The entries in tables 3.3 to 3.6 give the relative abundance of the different molecular weight species within a given sample scored on an arbitrary scale of - (no plasminogen activator activity) to +++. The results are not quantitative and do not provide a comparison between total amounts of plasminogen activators in the different tissues.

Most of the monkey tissue extracts tested contained plasminogen activators with a molecular weight of 60 000 daltons. Sometimes minor amounts of a 70 000 dalton and a 100 000 dalton species were encountered. This species could be inhibited by antibodies to Mel-PA but not by anti-urokinase antibodies. The 60 000 dalton species on the other hand could be inhibited by anti-urokinase antibody.

Monkey urine contained exclusively plasminogen activators of the

Table 3.3 Plasminogen activators in tissues and body fluids from the vervet monkey (Ceropithecus aethiopis)

Source	Species of plasminogen activator (Molecular weight)			
	100K[a)]	70K[a)]	60K[b)]	32K[b)]
Urine		-	+++	++++
Submaxilliary gland	+	+	++++	
Thyroid		-	+	
Pituitary		+	+	
Heart		+	++	
Bladder		+	+++	
Lung		-	+++	
Spleen[c)]		-	+++	
Brain (grey)		+	+	
Brain (white)		+	+	
Artery		+	+	
Seminal vesicle		+	++	
Liver		-	-	

See legend to Table 3.2

a) Enzymes in these columns were all inhibited by antibody to Mel-PA.

b) Enzymes in these columns were inhibited by antibody to urokinase.

c) Spleen contained plasminogen-independent fibrinolytic activity.

Table 3.4 Plasminogen activators in pig tissues and body fluids

Source	Species of plasminogen activator (Molecular weight)				
	100K[a)]	70K[a)]	100K[b)]	47K[b)]	25K[b)]
Urine	+	++		-	
Uterus		+		-	
Prostate	+	+++	+	++	
Bladder	+	++++		+++	
Seminiferous tube	+	+++		-	
Heart		++		-	
Lung		-		++	+
Brain (grey)		++		+	
Brain (white)		++		+	
Kidney	+	++	+	+++	+++
Adrenal	+	+		-	
Spleen c)	+	+		++	+++

See legend to Table 3.2

a) Enzymes in these columns were inhibited by antibodies to Mel-PA

b) Enzymes in these columns were not inhibited by antibodies to Mel-PA or urokinase.

c) This sample contained plasminogen-independent fibrinolytic activity.

Note: Pig urine contained a plasminogen activator immunochemically identical to Mel-PA.

Table 3.5 Plasminogen activators in rabbit and mouse tissues and body fluids

Source	Species	Species of plasminogen activator (Molecular weight)		
		70K[a)]	47K[b)]	25K[b)]
	Rabbit			
Urine		-	++++	+++
Uterus		++	-	
Bladder		+	-	
Kidney		-	++++	+
Lung		+	+++	
Ovary		-	-	
Heart		-	-	
Aorta		+++	+++	
Liver		-	-	
	Mouse			
Urine		-	++++	++
Seminal vesicle		-	+	
Bladder		-	+	+
Kidney		-	++	+
Lung		-	+	
Salivary gland[c)]		-	+++	++++
Heart		-	-	+
Brain		+	+	
Pituitary		+	+	
Liver		-	-	

See legend to Table 3.2

a) Enzymes in this column were inhibited by antibody to Mel-PA.

b) Enzymes in this column were not inhibited by antibody to Mel-PA or urokinase.

c) This sample contained plasminogen-independent fibrinolytic activity.

Table 3.6 Plasminogen activators in various animal tissues and body fluids

Species	Source	Species of plasminogen activator (Molecular weight)			
		70K[a)]	47K[b)]	43K[b)]	25K[b)]
Dog	Uterus	+	+++		
	Urine	-	+++		
Hamster	Uterus	+	+++		
	Urine	-	++++		+++
Guinea Pig	Uterus	+	+++		
	Urine	-	++++		
Goat	Urine	+	+	+	+
	Heart	-	-		

See legend to Table 3.2

a) Enzymes in this column were inhibited by antibody to Mel-PA.

b) Enzymes in these columns were not inhibited by antibody to Mel-PA or urokinase.

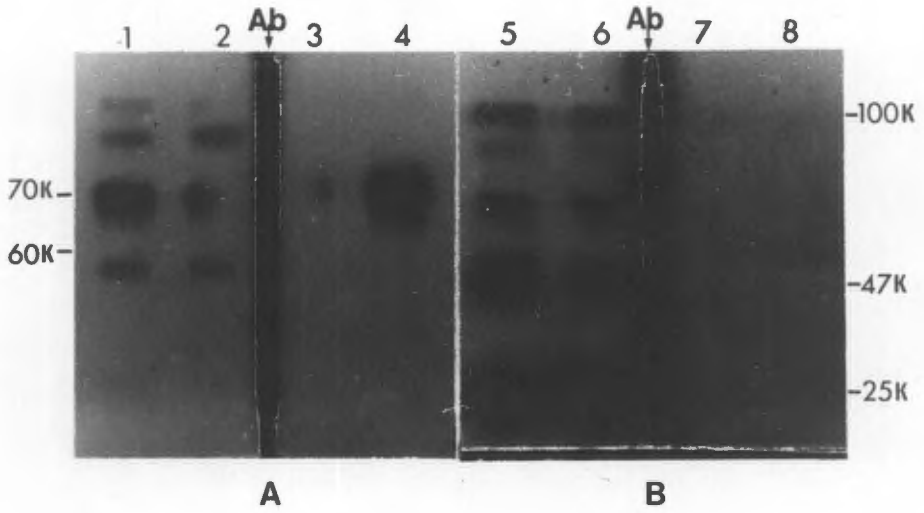


Figure 3.9 Electrophoretic and immunochemical analysis of plasminogen activators present in pig tissues.

Samples of extracts of pig prostate (wells 1 and 2) and pig kidney (wells 5 and 6) were compared to samples of Mel-PA (wells 3 and 4) or urokinase (wells 7 and 8) by the electrophoretic technique described in the Methods section and in the legend to Fig. 3.5. The troughs contained either anti-Mel-PA antibody (Gel A) or anti-urokinase antibody (Gel B).

Plasminogen activators with a molecular weight of 70 000 daltons and one with a molecular weight of > 100 000 daltons could be inhibited by antibodies to Mel-PA (Gel A) but not by antibodies to urokinase (Gel B). On the other hand, plasminogen activators with molecular weights of approximately 47 000 daltons and 25 000 daltons and one of > 100 000 daltons could not be inhibited by anti-urokinase or anti-Mel-PA.

urokinase-type. From these results it could be concluded that the distribution of plasminogen activators in monkey tissues and urine is similar to that found in the human. In addition both major species of plasminogen activator of monkey origin were immunochemically related to the equivalent human enzymes.

The analysis of the distribution of plasminogen activators present in tissues and body fluids of other animals such as pigs, rabbits, mice, dogs, hamsters and guinea pigs showed that they too contained two major species of enzyme. These had molecular weights of approximately 70 000 daltons and 47 000 daltons. Minor species with molecular weights of 100 000 daltons and 25 000 daltons were sometimes encountered. In the cases where immunochemical analyses were performed on the enzyme, the 70 000 dalton species in all of the animals tested could be inhibited by antibodies to human Mel-PA and not by antibodies to human urokinase.

The 47 000 dalton enzyme, on the other hand, was not inhibitable by antibodies directed to human urokinase or Mel-PA.

The distribution of the different species of plasminogen activators in the animals was frequently similar to that seen in man and the monkey in that tissues usually contained a mixture of Mel-PA-like enzyme and a lower molecular weight enzyme (usually 47 000 daltons) that was not Mel-PA. Urine from most animals contained exclusively the lower molecular weight species of plasminogen activator. The pig was quite unique in this respect in that it excreted exclusively a 70 000 dalton Mel-PA related plasminogen activator in the urine.

The goat was also found to be an exception to the general rule in that its urine contained 4 equally abundant enzymes with molecular weights of 70 000, 47 000, 43 000 and 25 000 daltons. Unfortunately no immunochemical analyses have been performed on urine samples from this species. An extract obtained from goat heart had no detectable plasminogen activator.

DISCUSSION

The experiments I report in this chapter have shown that human Mel-PA was immunogenic in rabbits and that the techniques used for raising antibodies to this enzyme proved successful. It was particularly useful to note that TCA precipitation of the enzyme did not compromise its immunogenicity and that the rabbits could be immunized with segments of polyacrylamide gel containing electrophoresed protein. Danø was able to raise satisfactory antibodies to murine plasminogen activator using a similar approach (200). The efficacy of this procedure enables one to exploit a very powerful method for achieving a considerable purification of the immunogen before injecting it, so avoiding the complications that result from heterodisperse antibody responses or from antigenic competition. It was also convenient that the urine and melanoma harvest fluid were sources of functionally pure plasminogen activators. Since the object was to achieve antibody to each enzyme that inhibited in a specific way, it did not matter if other proteins were present provided enzymatic purity could be relied upon.

In essence, the antibodies to Mel-PA and urokinase were required for purposes of classification of human plasminogen activators. The ability of immunoglobulins to discriminate between closely related antigenic determinants has made them extremely valuable reagents in other contexts and this particular case is no exception. I have been able to show with antibodies to Mel-PA and urokinase that two unequivocally distinct groups of plasminogen activator exist. Each of these groups comprises several molecular weight species and both of these are widely distributed in tissues and body fluids.

The antibody to Mel-PA clearly reacted with an antigenic determinant that was somehow involved in the catalytic function of the enzyme, although it did not combine directly with the active site. The

evidence for this is to be found in the fact that the antibody interfered with plasminogen activation but did not inhibit amidolytic activity in the fluorometric assay. I suspect that combination of antibody with the enzyme blocked binding of the plasminogen to Mel-PA at some point removed from the active site. Similar conclusions may be drawn from the results of Danø et al (200) who found that antibody to murine plasminogen activator did not inhibit binding of radioactive DFP to the active site; and from the results of Kucinski et al who found that the esterolytic action of urokinase was not blocked by antibody to this enzyme (56). It would have been of interest to have performed the symmetrical experiment to determine if binding of antibody to plasminogen activator could be blocked by plasminogen, but unfortunately I did not have sufficient enzyme to enable me to do this experiment in a conclusive way.

Lack of sufficent pure enzyme that could be appropriately labelled has also precluded attempts to develop a radioimmunoassay for Mel-PA. In any event this would have been difficult since the best antibodies I could raise only inhibited 50% of enzyme activity at a Mel-PA concentration of 1×10^{-9}M. They thus lacked the affinity for the enzyme that would have made them useful antibodies for clinical or physiological measurement where I suspect enzyme concentrations are lower by an order of magnitude.

The fact that antibodies to Mel-PA inhibit *all* plasminogen activators released by *all* melanoma cell lines merits comment in two respects. In the first place this observation confirms the general impression that melanoma cells cultured <u>in vitro</u> generally release plasminogen activators that are not the same as urokinase (61,115,117,202) and adds the positive observation that such enzymes are immunochemically identical. This would suggest that melanoma cells by virtue of their neuro-ectodermal origin are constrained by differentiation to produce only one family of plasminogen activators. Alternatively it may mean that

in vitro culture procedures select from a mixed melanoma cell population those clones that have the capacity to survive *in vitro* and that, fortuitously, make enzyme of the Mel-PA type. The observations by Markus et al (201) have a bearing on this question since they found that fragments of melanoma tissue removed from patients at the time of surgery invariably contained a mixture of urokinase and Mel-PA type enzymes. It is distinctly possible therefore that cellular synthesis and release of Mel-PA in melanoma cell populations correlated with *in vitro* proliferative potential.

Secondly, the fact that, irrespective of their molecular weights, all plasminogen activators that are released by melanoma cells are inhibited by anti Mel-PA provides good reasons to believe that these different species represent post-translational modification of a single gene product. A similar diversity of molecular size within the urokinase group of enzymes has been well documented (61,117,74,102). In view of studies I have performed on urinary plasminogen activators in health and disease and that I refer to in an appendix to this chapter, it should be noted that although it was not uncommon to find melanoma-type enzymes with a molecular weight of approximately 60 000 (corresponding to the predominant component of urokinase) I have not observed plasminogen activators inhibitable by urokinase antibody that have a molecular weight of 70 000. The fact that representatives of both Mel-PA and urokinase classes of enzyme may have similar molecular weights testified to the superiority of immunochemical criteria for classification over those based on molecular mass alone.

The analyses that I have performed have shown that most human tissues contain a mixture of urokinase and Mel-PA. They do not contain as the observations and conclusions of others would imply (198,75,76,85, 55) a single plasminogen activator that is distinct from urokinase and that justified the term "tissue plasminogen activator" to describe it.

Markus and co-workers (53,69,72), in comparing plasminogen activators extracted from neoplastic and normal tissues, obtained results essentially similar to mine in that they found that both contained mixtures of urokinase and Mel-PA. My results are at variance, however, with those of Rijken et al (198) who in their survey of extracts of human tissues, concluded that they lacked measurable urokinase and contained only plasminogen activators inhibitable by antibody to purified uterine tissue activator. Technical differences may explain the discrepancies between the results of these workers and those obtained by Markus et al (52) and myself. Firstly, they used a 1 M NaCl solution to extract their samples whereas I used 0.5% aqueous Triton X-100. Secondly, the fibrin-plate assay that they used may have been relatively more sensitive for the measurement of the Mel-PA type enzyme than it was for urokinase. Camiolo et al (53), for example, found that the apparent relative amounts of Mel-PA and urokinase present in the same sample differed according to whether a fibrinolytic or a caseinolytic assay was used. The electrophoretic and zymographic procedures that I employed not only separated urokinase and Mel-PA so that smaller amounts of the former might still be detectable in the presence of relatively large amounts of the latter, they also (as I show in Chapter VI) detected urokinase with greater sensitivity than they detected the Mel-PA type enzyme.

Whatever the reasons for these differences, analytical procedures have now advanced to the stage where it is clear that most tissues contain a mixture of Mel-PA and urokinase. The term "tissue plasminogen activator" is therefore a misnomer and should be abandoned.

The observations I have made on the immunochemical relationships between plasminogen activators present in the tissues and body fluids of different species confirm and extend those made by Kucinsky et al (56) who found that antibodies to human urokinase inhibited the enzyme present in

the urine of the baboon and the rhesus monkey but not those present in urine samples from dogs, pigs, rabbits or guinea pigs. Similarly, Danø et al (200) found that antibodies to the 48 000 dalton plasminogen activator isolated from MSV-infected mouse cells inhibited the enzyme present in urine from the mouse and the rat but had no effect on human urokinase or on the avian enzymes. Christman (107) prepared antibodies to the 50 000 dalton plasminogen activator released by SV-40 infected hamster cells and showed that this inhibited some hamster enzymes but not others; it did not inhibit plasminogen activator released by human cells in culture nor those released by murine melanoma cells or mammary carcinoma cells. Finally, Kok (55,66) has shown that pig tissue activator cross-reacts immunochemically with the enzyme isolated from human tissues.

The data that I have accumulated on the phylogeny of plasminogen activators justify the proposal that there is strong immunochemical homology between plasminogen activators of the Mel-PA type in all mammals. This would suggest that the Mel-PA type enzymes have an evolutionary significance that has preserved their identity throughout evolution and has safeguarded them against substantial mutation.

One may speculate that the basis for this selection is to be found in the need, shared by all warm blooded animals, for an enzymatic mechanism to preserve patency of the vascular tree. Observations are accumulating (195,209) to indicate that the Mel-PA type enzyme is particularly suited for this function since it combines readily with fibrin and hence focuses fibrinolytic activity on the blood clot without producing relatively unselective plasminogen activation.

The data also suggest that enzymes of the urokinase type, although present in all species, are immunochemically unique to certain orders. If, as seems probable, all serine proteases were derived from some single ancestral precursor, it is possible that the Mel-PA type enzyme represents a more ancient form from which urokinase was derived by

mutation at the time of evolutionary speciation. The fact that the pig has melanoma-type plasminogen activator in the urine whereas all other species have an enzyme corresponding to urokinase in the urine would be consistent with this notion. These speculations provide the motivation for a more thorough and systematic survey of molecular similarities between plasminogen activators in the hope that these will provide additional phylogenetic probes for the study of animal evolution.

ANALYSIS OF PLASMINOGEN ACTIVATORS PRESENT IN URINE SAMPLES COLLECTED FROM NORMAL INDIVIDUALS AND FROM SUBJECTS WITH RENAL DISEASE.

The survey that I have reported in this chapter of plasminogen activators present in normal tissues and body fluids has shown that urine collected from healthy subjects contained only the urokinase type enzyme whereas extracts of normal kidney tissue contained a mixture of urokinase and Mel-PA. These observations indicated that physiological or anatomical barriers exist that effectively confine Mel-PA to renal tissue and exclude it from the urine. If this were the case, damage to the kidneys might breach these barriers and so lead to the appearance of measurable amounts of Mel-PA in the urine of patients with renal disease. Since these suggestions have important diagnostic implications I have examined urine samples from 15 normal subjects and from 39 patients with one or other form of renal disease. In the paragraphs that follow I report the results of this study.

MATERIALS AND METHODS

Urine samples

Random urine samples were collected from 15 normal subjects and 39 patients with the renal diseases listed in Table 3.7. Freshly voided midstream or catheter urine specimens were transported to the laboratory on chipped ice. Samples for plasminogen activator analysis were taken from each specimen, centrifuged and made 0.1% with respect to Triton X-100. These were examined immediately or stored at $-20^{\circ}C$ for a maximum of one month without loss of activity. A second sample of each specimen was examined for bacterial contamination, for protein content and for the presence of cellular or other formed elements.

Electrophoretic detection of urinary Mel-PA.

The method used to detect Mel-PA in human urine depended upon the fact that this is the only plasminogen-dependent caseinolytic protease that migrates with a mobility that is immediately cathodal to albumin and corresponds to a molecular weight of approximately 70 000 daltons when it is electrophoresed in 11% polyacrylamide gel slabs containing 0.1% SDS.

Urine samples prepared as above were diluted with an equal volume of a buffer consisting of 0.1M Tris HCl pH 6.8, 2% SDS, 20% glycerol, 0.004% phenol red and 100 µg/ml of BSA that had been labelled with fluorescein isothiocyanate (Appendix 1). Samples (5 µl) of this mixture were then electrophoresed in 11% polyacrylamide gel slabs (70 x 115 x 0.5mm) containing 0.1% SDS. When the phenol red tracking dye had reached the bottom of the gel, electrophoresis was discontinued and the polyacrylamide gel slabs were washed in 2.5% Triton X-100 to remove the SDS.

The gels were then examined under ultraviolet light when the position of albumin could be identified without difficulty as a sharp green fluorescent band. The gel was divided with a sharp blade at this band so separating Mel-PA (cathodal to the band) from urokinase (immediately anodal to the albumin band). Both portions of the polyacrylamide gel slab were then layered on an indicator gel slab containing 2% powdered skim milk (Carnation), 1.25% agar and 5 µg/ml purified human plasminogen in 0.1M Tris HCl pH 8.1. Plasminogen was omitted from control indicator gel slabs to examine for plasminogen-independent caseinolysis. The assembly was then incubated at 37°C in a humid environment for detection of plasminogen activator activity as clear bands of caseinolysis against an opaque background. A typical result is shown in Figure 3.10.

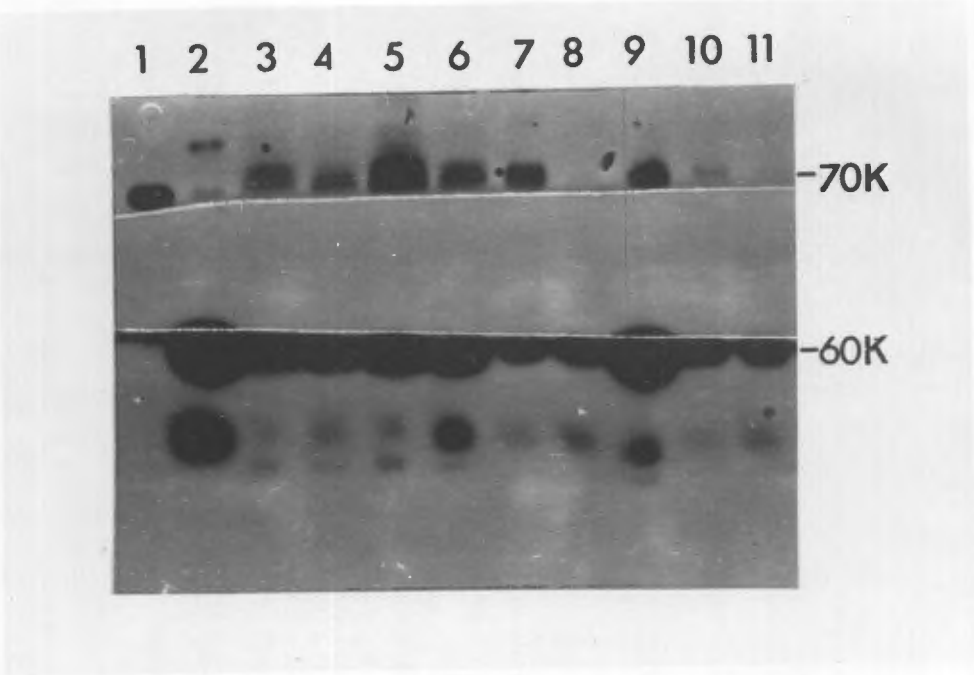


Figure 3.10 Electrophoretic analysis of urinary plasminogen activators.

Urine samples were diluted with an equal volume of 0.1M Tris HCl pH 6.8 containing 2% SDS, 20% glycerol, 0.004% phenol red and 100 µg/ml FITC-BSA. Samples (5 µl) of these were electrophoresed in an 11% polyacrylamide gel slab containing 0.1% SDS. After electrophoresis, the polyacrylamide gel slab was washed in 2.5% Triton X-100 and then examined under ultraviolet light for the position of the fluorescent albumin band. The gel was cut along this band and both portions were then layered on an indicator agar gel slab containing 2% powdered skim milk, 1.25% agar, 5 µg/ml human plasminogen in 0.1M Tris HCl pH 8.1. The assembly was then incubated at 37°C in a humid environment.

The photograph shows a typcial gel assembly after incubation for 24 hr. The tracks contained the following samples:
1) Mel-PA; 2) normal urine; 3) to 7) urine obtained after renal transplantation; 8) urine from patient with mesangio capillary GN; 9) - with IgA nephropathy; 10) - with membraneous proliferative GN; 11) - with chronic GN.

Reference samples consisting of normal urine and partially purified Mel-PA (approximately 1.5 UK u/track) were co-electrophoresed with each set of test samples. After 24 hr of incubation, the zones of caseinolysis corresponding to Mel-PA and urokinase were graded on a scale ranging from 0 (no plasminogen activator) to ++++ by visual comparison with the Mel-PA and urine reference standards which were assigned a value of ++++ in all cases.

Reagents

Plasminogen and protease- and inhibitor-free BSA were prepared as described in the Appendix (A1).

RESULTS

All electrophoresed urine samples tested contained plasminogen dependent caseinolytic activity that could confidently be identified as Mel-PA or urokinase in type by reference to the fluorescent albumin band and the co-electrophoresed enzyme markers. In no case was plasminogen independent caseinolysis observed with this protocol. In Table 3.7 I present the results of a survey in which these methods were applied to urine samples obtained from normal subjects and patients with different renal diseases. The urine samples from 15 normal individuals contained urokinase as the only caseinolytic agent whereas 13 of the 39 patients with renal disease had detectable Mel-PA in their urine in addition to urokinase.

Urine samples that contained Mel-PA levels came from patients with IgA nephropathy (4 out of 5), post infective glomerulonephritis (2/2), membrano-proliferative glomerulonephritis (2/3), renal tuberculosis (1/2), polycystic kidneys (2/3).

Table 3.7

Table 3.7

Samples of urine from patients with renal disease were analysed by the electrophoretic method described in the Methods section.

The presence or absence of Mel-PA (70K) or urokinase (60K) was graded on a scale ranging from 0 (= no plasminogen activator) to ++++ by visual comparison with the Mel-PA and urokinase reference standards which were assigned a value of ++++ in all cases.

Table 3.7 Electrophoretic analysis of plasminogen activators in urine.

Diagnosis	Protein conc. (mg/ml)	Plasminogen activator species (Molecular weight)	
		60K	70K
Mesangio capillary GN	13.5	++++	0
	52.5	++++	0
	ND	++++	0
	9.0	+	0
	ND	++++	0
Postinfective GN	2.9	++	+
	ND	++	+++
Membranous proliferative GN	0.14	++++	++
	5.20	++++	0
	3.85	+++	+
Chronic GN	ND	++	0
IgA nephropathy	9.15	++++	++
	0.39	++++	++
	0.70	+++	0
	2.20	+++	++
	0.07	++++	+++
Renal TB	0.20	+	0
Cortical necrosis	2.40	+++	0
Acute tubular necrosis	0.57	++	0
	ND	++++	0
	1.94	+++	0
Renal carcinoma	ND	++++	+
Reflux kidney	ND	++++	0
	0.05	+++	0
Polycystic kidney	0.07	++++	+++
	ND	+	+
	ND	0	0
Renal artery thrombosis	2.7	++	0
Preeclampsia toxaemia	1.45	+++	0
Renal artery-stenous	0.06	+++	0
Chronic pyelonephritis	ND	+++	0
	0.85	+++	0
Staghorn calcification	ND	+++	+
Diabetes mellitus	ND	+	0
Systemic lupus erythematosus	0.24	+++	0
	ND	+	0
	0.23	0	0
Haematuria	0.05	+	0

ND = not determined

I have also examined urine samples obtained on successive immediate post-transplant days in six renal allograft recipients. In all cases Mel-PA appeared transiently in the urine during the early post-operative period and disappeared as renal function improved. This temporal relationship was particularly well seen in the data depicted graphically in Fig. 3.11.

No correlation was observed between the presence of Mel-PA in the urine and the degree of proteinuria seen.

DISCUSSION

The results that I report in this brief survey provide reason to believe that the demonstration of Mel-PA in human urine by this technique is an abnormal finding that signifies the presence of renal disease. In making this suggestion I recognize that the absolute differences between normal and pathological urine specimens that I have documented may be quantitative rather than qualitative if my protocol were relatively insensitive to the detection of Mel-PA. It is significant to note in this regard that Rijken et al (198) using antibodies to urokinase or uterine "tissue activator" in conjunction with a fibrin plate assay, could detect small amounts of "tissue activator" in normal human urine. It is probable therefore that the fibrin plate assay is selectively more sensitive for the detection of Mel-PA.

It should be noted that only approximately one third of patients whom I examined showed the presence of Mel-PA using this particular protocol. The numbers involved are small and hence permit only tentative conclusions to be drawn. It is, nevertheless, worthy of comment that the five urine samples obtained from patients with mesangiocapillary glomerulonephritis and the three samples obtained from patients with acute tubular necrosis contained no Mel-PA whereas this enzyme was found in most other forms of diffuse parenchymal renal disease. It is possible, therefore, that

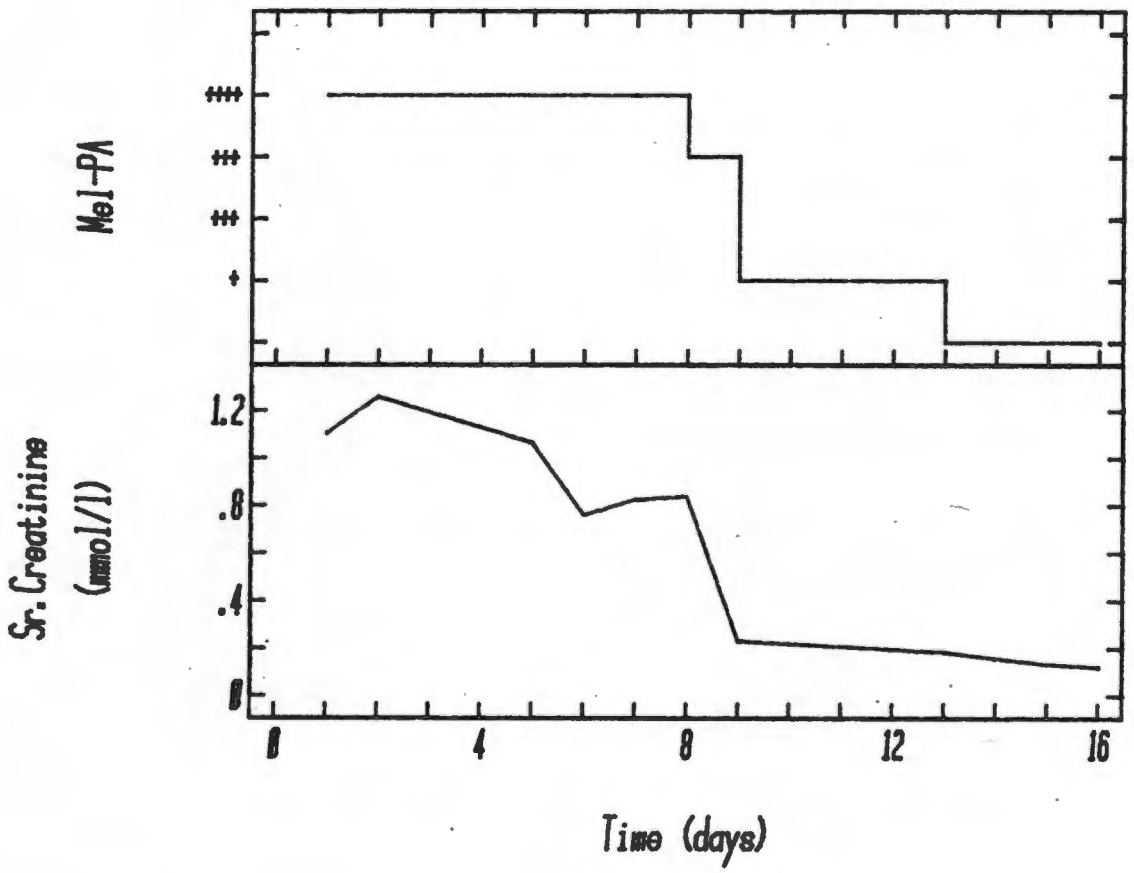


Figure 3.11 Analysis of plasminogen activators in urine of a renal allograft recipient.

Urine samples taken from a patient on successive days after renal transplantation was analysed for the presence of Mel-PA by the electrophoretic method described in the Methods section. Day 0 was the day of transplantation.

The relative amount of Mel-PA in the urines was plotted as a function of time after transplantation, and compared to the levels of creatinine in the serum of the patient.

Mel-PA was present in the urine during the early post-operative period when serum creatinine levels were also high. After 13 days both Mel-PA and creatinine levels had decreased to normal levels.

the urinary excretion of Mel-PA is found only with certain renal lesions and not with others.

In this survey I have not included patients with disease of the ureters or urinary bladder. Mel-PA may appear in the urine of patients with lesions in these areas, in which case its presence in the urine would not be a specific indicator of disease of the kidneys.

To the best of my knowledge these data provide the first record of an attempt to correlate the presence or absence of renal disease with the immunochemical characterization of plasminogen activators present in the urine. Several studies have been reported in which absolute levels of urinary plasminogen activator have been measured in patients with renal disease and generally speaking a diminution in urinary enzyme content has been observed (203-206). Since most reported studies were undertaken before techniques and reagents were available for the distinction between Mel-PA and urokinase, these results are not strictly comparable to those that I have obtained.

Although one may postulate that Mel-PA appears in the urine of patients with renal disease as a result of leakage across damaged cell membranes or other barriers that would normally contain the enzyme at some intracellular location within the kidney, there is as yet no entirely satisfactory justification for this assumption. It may be, for example, that Mel-PA is a constituent of the normal glomerular filtrate and is removed during traverse of the healthy collecting system. These removal mechanisms may be defective in patients with certain forms of renal disease. It is also possible that Mel-PA in diseased urine is derived from the plasma as a result of a selective filtration defect at the level of the glomerular basement membrane. Some support for this suggestion comes from the observations of Ekberg and Pandolfi (207) and McConnel et al (208) who have noted that plasminogen activators in normal human kidney are

localized to the collecting tubules and major blood vessels whereas in disease the enzyme is found in the glomeruli (207). These authors made no attempt to discriminate between urokinase or Mel-PA however and the appropriate studies using immunohistochemical procedures will be required to clarify this issue.

It was hoped that urinary Mel-PA concentrations could be used to monitor rejection after renal transplantation but this hope was not entirely realized. The enzyme was present as a transient phenomenon in all recipients of renal allografts and appeared to resolve with return of normal renal function in a manner that was not specifically related to other manifestations of immunological rejection or to immunosuppressive therapy. It would seem more likely that the transient release of Mel-PA into the urine of these patients is an indicator of the minimal degree of renal damage that invariably accompanies removal and engraftment of the donor kidney.

CHAPTER IV

CHARACTERIZATION OF AN INHIBITOR OF MEL-PA ISOLATED FROM THE SEEDS OF THE SOUTH AFRICAN LEGUME ERYTHRINA LATISSIMA.

Although plasminogen activators have been implicated in a number of physiological processes that appear to have a common requirement for regulated local proteolysis (60,81,211) the relative biological roles of urokinase and Mel-PA in these situations are uncertain.

As a general rule, studies of complex biological systems are facilitated by the availability of inhibitors of participating enzymatic components. I therefore felt that it would be useful to attempt to identify inhibitors of plasminogen activators that would distinguish between urokinase and Mel-PA. Such reagents might be of use for assigning specific functions to urokinase or Mel-PA and for the purification of these enzymes.

Dr. F. Joubert and his co-workers at the National Chemical Research Laboratories in Pretoria have a special interest in protease inhibitors derived from natural products. The close association that I enjoy with this laboratory enabled me to obtain samples from Dr. Joubert and to study these for their ability to function as specific and useful inhibitors of human plasminogen activators. A survey of compounds derived from seed extracts or snake venoms revealed that the seed of the legume Erythrina latissima contained a trypsin and plasmin inhibitor, DE-3, that strongly inhibited the action of Mel-PA but had no effect on urokinase. In this chapter I report the results of the experiments I performed to establish these facts.

It could also be shown that the inhibitor formed reversible complexes with Mel-PA but not with urokinase. This fact was exploited by coupling DE-3 to sepharose to provide an affinity reagent for the one-step purification of Mel-PA and pro-Mel-PA.

MATERIALS AND METHODS

DE-3 Inhibitor

Erythrina latissima seeds were collected and processed as described by Joubert et al (212). Briefly, the seeds were ground, defatted, and extracted at 10°C overnight with 0.5M sodium chloride solution. The extract was centrifuged and DE-3 inhibitor was recovered from the supernatant by ammonium sulphate precipitation followed by chromatography on Sephadex G50, DEAE-cellulose and DEAE-sepharose. The finally purified material migrated as a single band with an apparent molecular weight of 22 000 daltons when electrophoresed on a 15% polyacrylamide gel containing 0.1% SDS.

The purified DE-3 protein was labelled with ^{125}I by using the chloramine T method of Greenwood et al (213) as described in the Appendix (A1.9). Electrophoresis and autoradiography of the labelled material showed one radioactive band with an apparent molecular weight of 22 000 daltons.

Enzymes

Mel-PA was obtained as serum-free harvest fluid collected from Bowes I or Bowes II melanoma cells as described in Chapter I. As each harvest fluid was removed it was centrifuged to remove cellular debris, acidified to pH 5.5 with glacial acetic acid and made 0.1% with respect to Triton X-100. Harvest fluids treated in this way could be kept frozen at -20°C without apparent loss of enzyme activity for at least six months.

For certain experiments concentrated enzyme solutions were required. These were obtained by batch-adsorption of 2 litres of pooled serum-free harvest fluid with 40 ml of benzamidine-sepharose as described in Chapter I.

Active eluate fractions from the benzamidine-sepharose were pooled to give approximately 70 ml of solution containing 500 (Range 350 to 670) FU of active enzyme/ml. This was concentrated 5-10 fold by negative pressure ultrafiltration and dialysis in a 1.0 cm diameter Visking cellophane membrane immersed in 0.1M Tris HCl pH 8.1 containing 0.1% Triton X-100. Enzyme recovery to this point ranged from 60-80%. This solution was stored in portions at -20°C.

Urokinase was purchased as a lyophilized powder from Leo Pharmaceutical Products, Ballerup, Denmark. This material contained at least 7 different proteins as judged by SDS-gel electrophoresis and Coomassie-blue staining. It was free of plasminogen-independent fibrinolytic activity and contained no Mel-PA as judged by electrophoretic and immunochemical criteria (Chapter III).

Plasmin was generated from human plasminogen by incubation with insolubilized urokinase (Appendix A1.3). Protease- and inhibitor-free bovine serum albumin was added to give a final concentration of 1 mg/ml and the solution was kept at -20°C.

Trypsin was purchased from Worthington as thrice-crystallized bovine pancreatic trypsin and dissolved in 1 mM HCl to give a stock solution. The molarity of trypsin active sites in this solution was determined by titration with 4-methylumbelliferyl-p-guanidinobenzoate (MUGB) according to the method of Jameson et al (164) as described in Chapter V.

Plasminogen activator assay

The fluorometric assay of Zimmerman et al (133) was used in which the direct amidolytic action of urokinase and Mel-PA was measured fluorometrically by following the rate of increase in fluorescence at 455 nm that resulted from the amidolytic release of amino methyl coumarin (AMC) from the fluorogenic substrate Cbz-Gly-Gly-Arg-AMC. The reaction took

place at 25°C in a glass cuvette in the sample compartment of a fluorescence spectrophotometer (Perkin-Elmer; Model MPF-43A), and was initiated by the addition of 50 µl of enzyme solution so that the final reaction mixture consisted of 500 µl of 0.1M Tris-HCl pH 8.1 containing 4% DMSO and 5×10^{-4}M substrate. The excitation wavelength was 383 nm and the instrument was so set that a 4×10^{-7}M solution of AMC in the assay mixture gave a full scale recorder pen deflection. One fluorometric unit of enzyme was arbitrarily defined as that amount catalyzing the release of 10 pmol AMC/min. The activities of trypsin, plasmin and thrombin could also be measured with this assay.

The assay for the inactive precursor of Mel-PA, "pro-Mel-PA", depended upon the facts that (a) pro-Mel-PA is converted to Mel-PA by plasmin and (b) that plasmin is inhibited by bovine pancreatic trypsin inhibitor whereas Mel-PA is not. In a typical assay for pro-Mel-PA, 5 µl (2.5 µg) of plasmin solution was added to 295 µl of sample solution. After incubating for 60 min at 20°C, 50 µl of this solution was added to 10 µl of pancreatic trypsin inhibitor (10 KIU) to inhibit all plasmin activity and 50 µl of this mixture was assayed fluorometrically for its ability to hydrolyse Cbz-Gly-Gly-Arg-AMC. This result measured the sum of pro-Mel-PA and Mel-PA. Mel-PA content was measured on the sample treated identically but incubated with 5 µl of buffer instead of plasmin solution. Experimental results that justify these assumptions and procedures are given in Chapter V.

Radiometric standardization of Mel-PA

For the analysis of Mel-PA : DE-3 interactions in terms of molarities of the reactants it was necessary to define a relationship between fluorometric units of amidolytic activity and molarity of active

sites of the enzyme. This was achieved by active site labelling of a measured amount of Mel-PA with ^{3}H-DFP of known specific radioactivity as described in detail in Chapter V. Radioactivity in precipitated protein was measured and used to calculate a specific activity for Mel-PA of 0.99 FU/pmol of enzyme. In this approach I have assumed (a) that DFP forms a covalent bond with the active-site serine residue in Mel-PA, (b) that, under the conditions of the labelling procedure (i.e. a DFP : enzyme molar ratio of at least 1000 : 1) the enzyme was quantitatively labelled in a 1 : 1 stoichiometric ratio, and (c) that no protein other than Mel-PA was labelled. This latter assumption was verified by SDS gel electrophoresis of precipitated protein followed by autofluorography.

All other procedures and reagents were as described in the Appendix.

RESULTS

Inhibitory effect of DE-3

Functionally pure solutions of trypsin, plasmin, urokinase, thrombin and Mel-PA were diluted to contain approximately 30 FU/ml in 0.1M Tris HCl pH 8.1 containing 0.1% Triton X-100. To a sample of each solution (90 µl) I added 10 µl of a solution containing 20 mg/ml of purified DE-3 in 0.1M Tris HCl pH 8.1 to give a final concentration of inhibitor of approximately 10^{-4}M. After incubation for 30 min at room temperature the mixture was assayed for residual enzyme activity to give the results depicted in Figure 4.1. Trypsin, plasmin, and Mel-PA were completely inhibited whereas urokinase and thrombin were unaffected.

Kinetics of inhibition by DE-3

It is desirable, when describing the action of a proteinase inhibitor, to define the stoichiometry of the interaction between enzyme and inhibitor and the dissociation constant (Ki) governing that reaction. Estimates of stoichiometry are usually obtained by measuring the fractional activity (α) that remains when enzyme, at an initial concentration E_o, is exposed to different total concentrations (I_o) of inhibitor.

In such an experiment a plot of α as a function of I_o/E_o gives a graph with an initial negatively sloping portion. If favourable circumstances obtain and if I_o and E_o are in a suitable relationship to each other, this initial portion of the graph is linear and can be confidently extrapolated to intercept the abscissa at the molar ratio for the enzyme-inhibitor interaction in the limiting case of complete inhibition (i.e. $\alpha = 0$).

As Bieth (214) has shown, in a very useful treatment of the subject, stoichiometric titrations of this sort require an E_o : Ki ratio

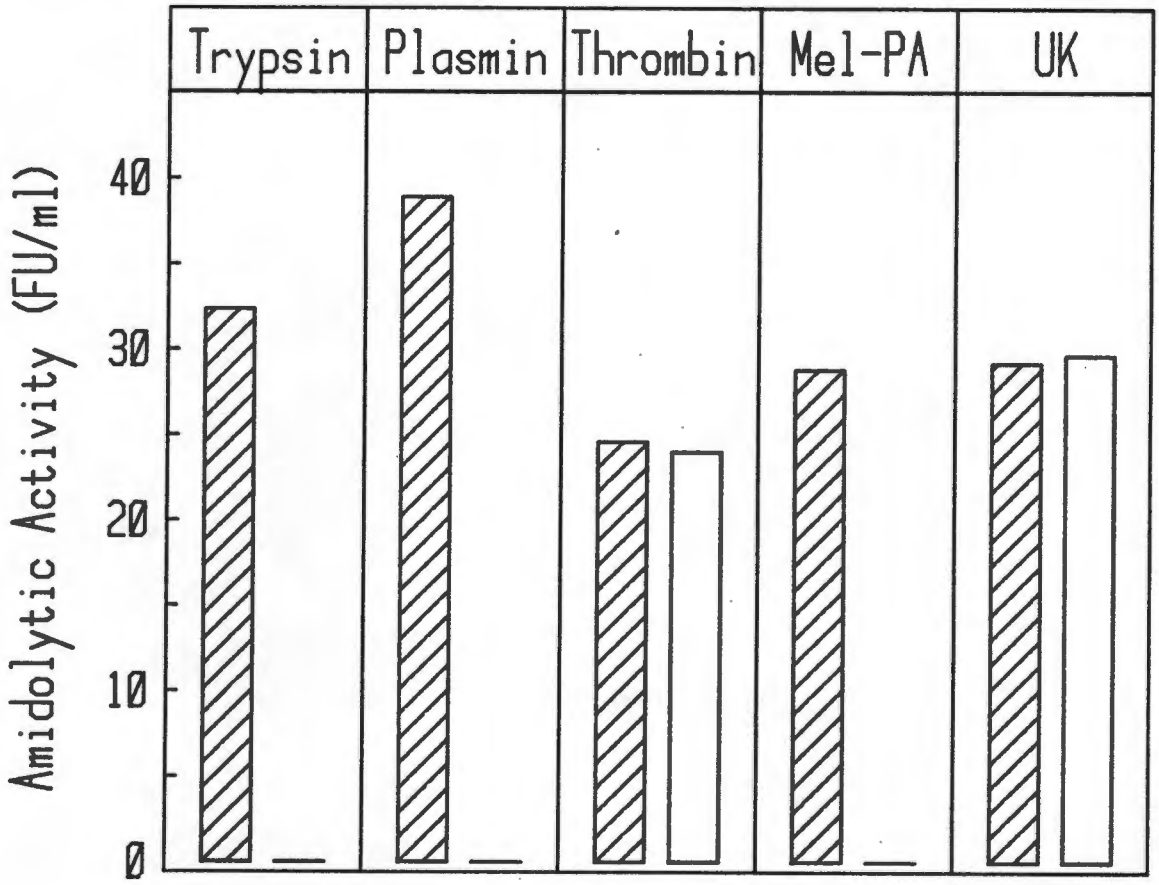


Figure 4.1 The effect of DE-3 on trypsin, plasmin, thrombin, Mel-PA and urokinase.

Solutions of trypsin, plasmin, thrombin, Mel-PA and urokinase were diluted to contain approximately 30 FU/ml in 0.1M Tris HCl pH 8.1 containing 0.1% Triton X-100.

To 90 µl of each solution was added 10 µl of a solution containing 1 x 10^{-3}M purified DE-3 in 0.1M Tris HCl pH 8.1 to give a final concentration of 1 x 10^{-4}M inhibitor. After incubation for 30 min at room temperature the mixtures (open bars) and control samples that had received 10 µl of solvent without inhibitor (shaded bars) were assayed for enzyme activity in the fluorometric assay.

Trypsin, plasmin and Mel-PA were completely inhibited by 1 x 10^{-4}M DE-3, whereas urokinase and thrombin were not affected.

of 1000 : 1 or greater. If enzyme supplies are ample or if the affinity of the inhibitor for the enzyme is sufficiently high such conditions are fairly easy to meet. Thus Joubert et al (212) were able to titrate trypsin with DE-3 and to demonstrate a 1 : 1 molar interaction between the proteinase and the inhibitor.

In the case of Mel-PA, supplies of enzyme were limited and the Ki proved to be relatively high. It was thus not feasible to perform a titration experiment which was amenable to "stoichiometric" interpretation. It was nevertheless possible to obtain data which could be used to estimate the Ki. These are presented graphically in Fig. 4.2 and come from an experiment in which 2.94×10^{-8}M of Mel-PA were titrated with DE-3 inhibitor in a final reaction volume of 0.1 ml. A simple interative computer programme was then used to define the value for Ki that would give the least sum of squared deviations of observed values for a from the calculated values derived from the theoretical relationship:

$$a = 1 - \frac{(E_o + I_o + Ki) - \sqrt{(E_o + I_o + Ki)^2 - 4.E_o.I_o}}{2E_o}$$

The value for Ki obtained in this way (214) was then used to construct the curve shown in Fig. 4.2.

The inhibitor-enzyme complex was not dissociated by substrate since addition of substrate to an equilibrium mixture of enzyme and inhibitor did not cause an exponential release of product nor could complete inhibition be reversed by substrate.

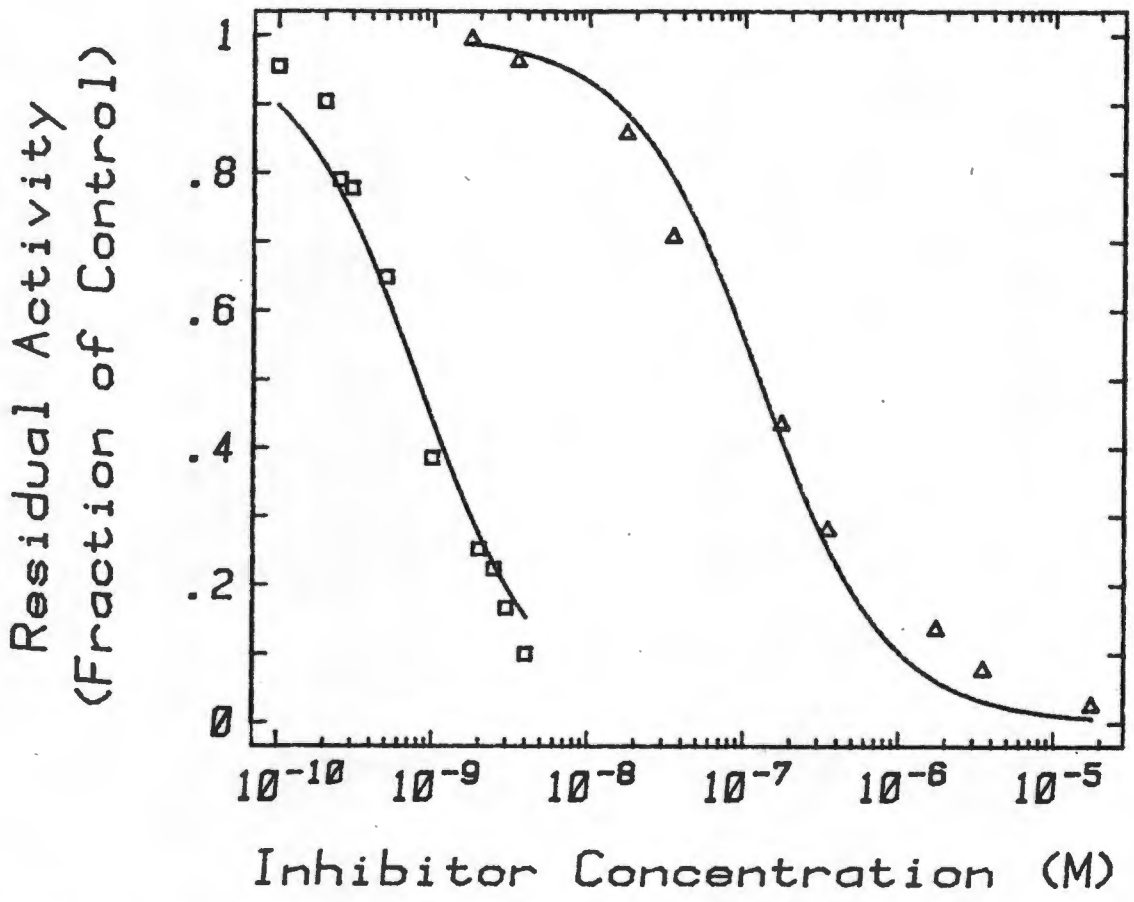


Figure 4.2 Inhibition of Mel-PA and trypsin by DE-3

Mel-PA (2.94×10^{-8}M; △—△) or trypsin (2.05×10^{-10}M; □—□) were incubated with DE-3 at the concentrations shown in 100 µl of 0.1M Tris HCl pH 8.1 containing 0.02% Triton X-100 for 1 hr at room temperature. Fifty microlitre aliquots of each mixture were then withdrawn for measurement of amidolytic activity in the fluorometric assay.

Each point represents the mean of duplicate values for the fractional residual enzyme activity plotted as a function of inhibitor concentration.

An interative computer programme was used to determine values for the inhibitor constants (Ki) for the two enzymes that gave the best least mean squares fit to the relationship

$$a = 1 - \frac{(Eo + Io + Ki) - \sqrt{(Eo + Io + Ki)^2 - 4.Eo.Io}}{2\,Eo}$$

where a = fractional residual enzyme activity; Eo = original enzyme concentration (2.94×10^{-8}M for Mel-PA; 2.05×10^{-10}M for trypsin) and Io = the concentration of inhibitor.

These Ki values (1.12×10^{-7}M for Mel-PA and 6.97×10^{-10}M for trypsin) were then substituted in the above equation to construct the curves.

Chromatographic analysis of DE-3-protease interactions

In order to determine whether or not DE-3 formed complexes with various proteases, mixtures of ^{125}I-labelled inhibitor with trypsin, Mel-PA or urokinase were analysed by molecular sieve chromatography on a calibrated 1.6 x 90 cm column of Sephadex G-75 fine equilibrated and eluted with 0.1M glycine NaOH pH 8.0 containing 0.15M NaCl and 0.1% Triton X-100.

The elution profiles of the various mixtures are shown in Fig.4.3. The DE-3 inhibitor on its own or together with urokinase showed one large peak and two smaller ones. The elution volume of the main peak corresponded to a molecular weight of approximately 19 000 daltons.

The mixture of Mel-PA and DE-3 showed 2 broad peaks. One of the peaks eluted with the void volume and presumably represented enzyme inhibitor complexes. The second peak corresponded to a molecular weight of approximately 19 000 daltons and represented free inhibitor.

The mixture of DE-3 and trypsin eluted at a single peak with an approximate molecular weight of 32 000 daltons.

Electrophoretic analysis of DE-3-protease interactions

The results obtained from molecular sieve chromatography indicated that Mel-PA and trypsin formed complexes with DE-3 whereas urokinase did not. To investigate the nature of the products of DE-3-protease interactions in more detail, enzyme-inhibitor mixtures were analysed by electrophoresis in polyacrylamide gel slabs under denaturing and non-denaturing and under reducing and non-reducing conditions.

Electrophoresis of mixtures of ^{125}I-DE-3 with trypsin, plasmin, urokinase, thrombin and Mel-PA in a 5-16% polyacrylamide gradient gel containing 0.1% SDS showed no evidence of complex formation with any of the proteases when the gel was fixed and studied by autoradiography (Fig.4.4).

Figure 4.3

Figure 4.3 Molecular sieve chromatography of inhibitor-protease mixtures

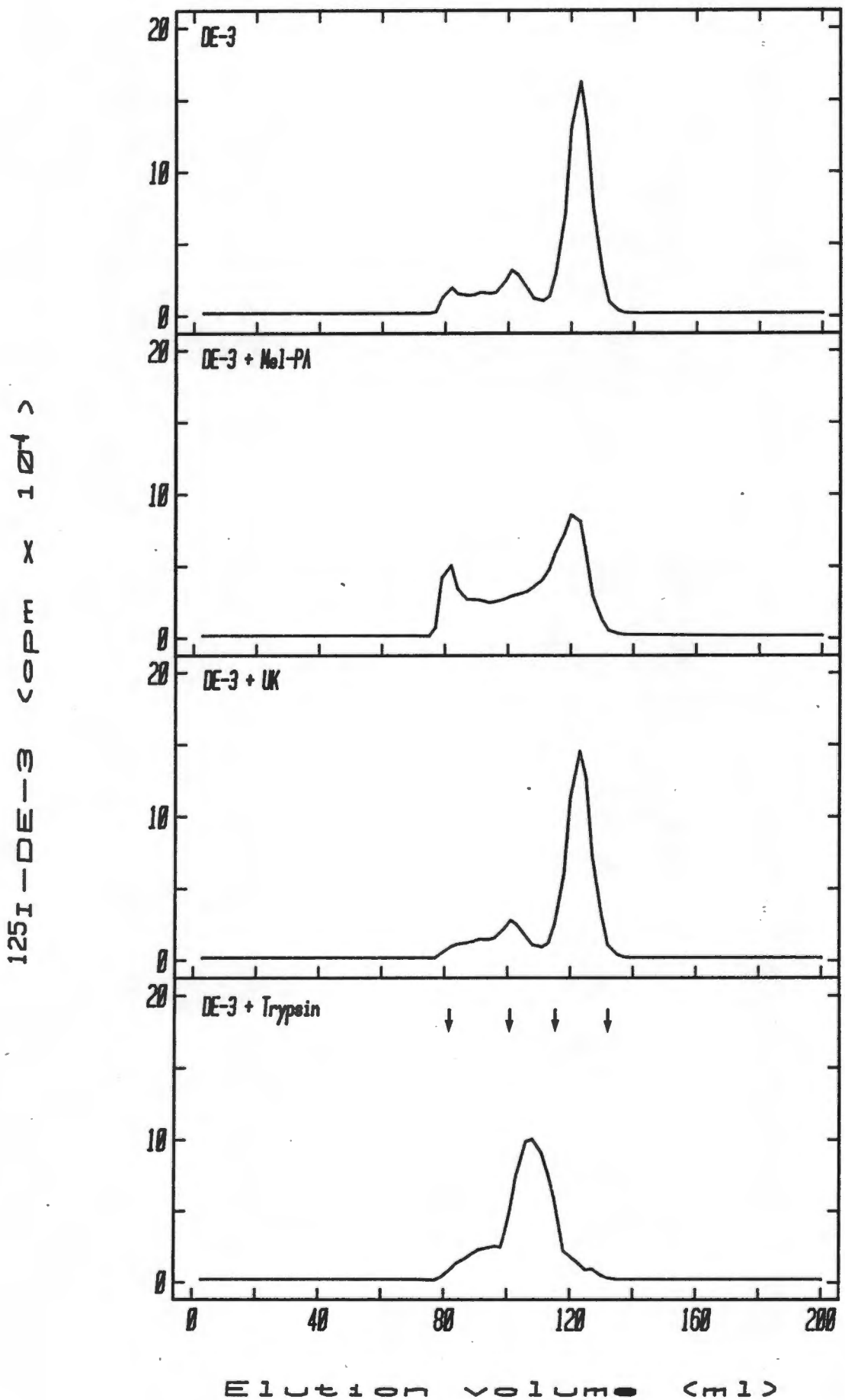
^{125}I-DE-3 (18.3 pmol; 1.5 x 10^6 cpm) was incubated for 10 min at 37°C with trypsin (27.0 pmol), Mel-PA (≃ 300 pmol) or urokinase (≃ 250 pmol), each in a total volume of 500 µl of 0.1M glycine NaOH pH 8.0 containing 0.15M NaCl and 0.1% Triton X-100.

The samples were then chromatographed on a Sephadex G75 fine column (1.6 x 90 cm) equilibrated and developed with 0.1M glycine NaOH pH 8.0 containing 0.15M NaCl and 0.1% Triton X-100. The flow rate was 5.4 ml/hr and 2.4 ml fractions were collected. The radioactivity in each fraction was determined by counting in a Beckman Autogamma Counter 4000.

The column was calibrated using ferritin (450 000 daltons) bovine serum albumin (67 000 daltons), ovalbumin (43 000 daltons), chymotrypsinogen (25 000 daltons) and cytochrome C (12 500 daltons). (Boehringer, Mannheim).

The elution profiles show that complex formation occurred between DE-3 and trypsin and between DE-3 and Mel-PA; whereas DE-3 treated with urokinase showed the same elution profile as the untreated inhibitor.

Untreated DE-3 eluted with an elution volume (Ve) corresponding to a molecular weight of 18 700 $\pm$ 1000; the DE-3-trypsin complex had a Ve corresponding to a molecular weight of 31 900. The DE-3-Mel-PA complex eluted with the void volume.


DE-3
DE-3 + Mel-PA
DE-3 + UK
DE-3 + Trypsin
20
10
0
0
40
80
120
160
200
125I-DE-3 (cpm x 10⁻⁴)
Elution volume (ml)

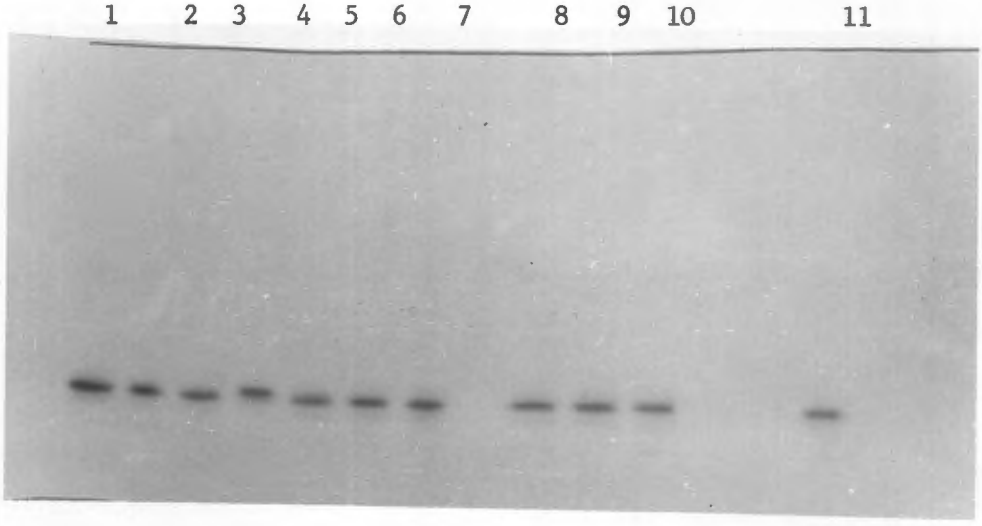


Figure 4.4 SDS-polyacrylamide gel electrophoresis of inhibitor-enzyme mixtures.

^{125}I-DE-3 (1.2×10^6 cpm) or DE-3 (1.35 nmol) were incubated with various proteases at room temperature for 30 min in 0.1M Tris HCl pH 8.0 containing 0.02% Triton X-100 in a total volume of 100 µl. SDS (10 µl of 25%) and glycerol (5 µl) were added and the samples boiled for 1 min. Aliquots of 5 µl were then electrophoresed in a 5-16% linear gradient of SDS polyacrylamide. Active trypsin concentration was 2 nmol/100 µl giving an Io/Eo ratio of 0.7. The other proteases were not pure and no estimates of molarity were made. The activities can be expressed in fluorometric units as follows:
Plasmin 80 FU/ml, 0.37 mg/ml; thrombin 2×10^4 FU/ml, 1 mg/ml; urokinase 2×10^5 FU/ml; Mel-PA 1.6×10^3 FU/ml; trypsin 1.4×10^8 FU/ml. After electrophoresis, the gel was fixed, stained and dried and autoradiographs were prepared.

The tracks contain from left to right: 1) DE-3; 2) and 3) Trypsin and DE-3; 4) and 5) Plasmin and DE-3; 6) and 7) Thrombin and DE-3; 8) and 9) Mel-PA and DE-3; 10) and 11) urokinase and DE-3.

No SDS-stable complex formation of the inhibitor with any of the enzymes was observed.

The inhibitor-protease complexes seen in the previous experiment were therefore not stable in the presence of SDS.

Complex formation between inhibitor and trypsin, however, could be clearly demonstrated by electrophoresis in the absence of SDS. This is illustrated in Fig. 4.5 which depicts an autoradiograph of an alkaline, non-denaturing gel containing electrophoresed mixtures of radioactive inhibitor with trypsin, urokinase or Mel-PA.

As is shown in the photograph, the radioactive inhibitor migrated as a single band with an Rf of approximately 0.6. When the inhibitor was pre-incubated with trypsin at a pH of 3.5 or 8.0 and then electrophoresed the free inhibitor band was no longer seen and most of the radioactivity was visible in a band with an approximate Rf of 0.1, indicating that the DE-3 had formed complexes with trypsin at both low and alkaline pH. An additional faint band with an Rf of approximately 0.7, just anodal to that of the free inhibitor band, could also be seen.

In the case of the Mel-PA-DE-3 mixture, the Rf 0.7 band was more prominent than in the DE-3-trypsin mixtures. Furthermore, a faint radioactive band at the stacking gel-resolving gel interface was just detectable. Presumably DE-3-Mel-PA complexes were too basic to enter the gel.

Urokinase and DE-3 did not form visible complexes in this experiment nor was the Rf 0.7 band visible when the inhibitor was incubated with this protease.

The Rf 0.7 band detected in the previous experiment where DE-3 was mixed with trypsin or Mel-PA suggested that these two proteases modified the inhibitor in a way that urokinase did not. To examine the nature of this modification an experiment was performed in which ^{125}I-DE-3 was incubated with trypsin, Mel-PA or urokinase. The mixtures were then

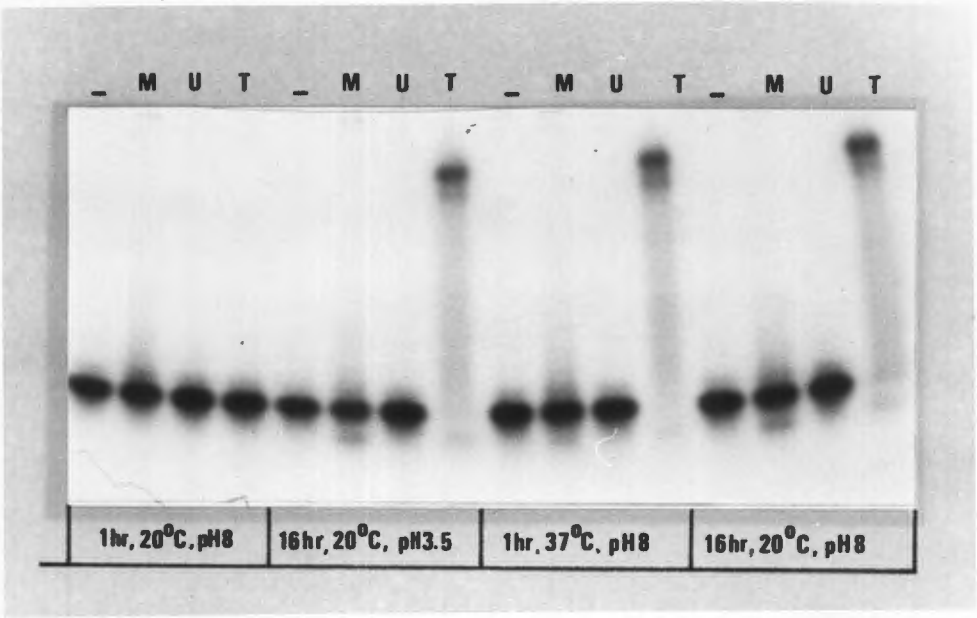


Figure 4.5 Alkaline polyacrylamide gel electrophoresis of inhibitor-enzyme mixtures.

Samples of ^{125}I-DE-3 (12.5 pmol; 1.2 x 10^6 cpm) were incubated under different conditions of time, temperature and pH with either Trypsin (10 pmol), urokinase (28 pmol), Mel-PA (30 pmol) or buffer in a total volume of 40 µl. After incubation 30 µl water, 10 µl of glycerol and 1 µl of 0.04% phenol red were added to each sample and 5 µl aliquots of each were electrophoresed in a 7% discontinuous polyacrylamide gel of pH 8.9.

After electrophoresis the gel was fixed and dried and an autoradiograph was prepared to give the results depicted above. The electrophoretic tracks in the photograph have been labelled to indicate (above) the nature of the enzyme and (below) the conditions under which enzyme and inhibitor were incubated before electrophoresis. Keys are as follows:

Enzyme: T = Trypsin; U = urokinase; M = Mel-PA; "-" = buffer alone.

Buffer: pH 8 :- 0.1M glycine - NaOH + 0.02% Triton X-100
pH 3.5:- 0.1M glycine - HCl + 0.02% Triton X-100.

reduced and alkylated and electrophoresed in 10-20% polyacrylamide gradient gels containing 0.1% SDS. The autoradiograph of the fixed and dried gel is shown in Fig. 4.6 from which it can be seen that the unreduced inhibitor migrated with a mobility corresponding to a molecular weight of approximately 22 000 daltons. Reduction and alkylation altered the electrophoretic mobility of the inhibitor so that it now migrated with an apparent molecular mass of approximately 23 000 daltons. As seen previously (Fig. 4.4) treatment of the ^{125}I-DE-3 with trypsin, Mel-PA or urokinase had no effect upon the mobility of the unreduced inhibitor in gels containing SDS. On the other hand, electrophoresis of enzyme-treated inhibitor after reduction and alkylation showed, in the case of Mel-PA an additional band with an apparent molecular mass of 15 000 daltons and, in the case of trypsin, two radioactive products - one with an apparent molecular mass of 15 000 daltons and a second with a molecular weight of 7000 daltons.

Figure 4.7 depicts the results of an experiment in which ^{125}I-DE-3 was treated with trypsin or Mel-PA. The mixtures were then electrophoresed in the first dimension in an alkaline gel under non-reducing and non-dissociating conditions and, in the second dimension, in a reducing gel containing 0.1% SDS. As can be seen, the Rf 0.7 band detected in the experiment depicted in Fig. 4.5 and the 15 000 dalton band shown in Fig. 4.6 could both be attributed to the same inhibitor fragment.

Effect of DE-3 on active site-labelling of Mel-PA and urokinase with ^{3}H-DFP.

In order to determine whether or not DE-3 interacted with Mel-PA at the active site of the enzyme, an experiment was performed in which Mel-PA and urokinase were incubated in the presence of DE-3 for 30 min at room temperature. Radioactive DFP was then added and the mixtures

Figure 4.6

Figure 4.6 SDS-polyacrylamide gel electrophoresis of inhibitor-enzyme mixtures under reducing and non-reducing conditions.

^{125}I-DE-3 (34.6 pmol, 4.5 x 10^6 cpm) was incubated with trypsin (100 pmol), Mel-PA (90 pmol), urokinase (60 pmol) or buffer in a total volume of 65 µl of PBS containing 0.02% Triton X-100 for 16 hr at room temperature.

After incubation the solutions were made 2% with respect to SDS; 0.5M with respect to Tris HCl pH 8.4; 0.002M with respect to EDTA and 8 M with respect to urea in a final volume of 100 µl. Proteins were reduced by addition of 1 M dithiothreitol to a final concentration of 0.01M and incubation at 37°C for 60 min under nitrogen.

Carboxymethylation was then achieved by the addition of 1M iodoacetamide to a final concentration of 0.022M. After incubation on ice for 30 min, 1M dithiothreitol was added to give a final concentration of 0.044 M and the solutions were then diluted ten-fold with SDS-sample buffer (0.06 M Tris HCl pH 6.8 containing 1% SDS and 10% glycerol). The total volume of each sample was approximately 1 ml at this stage.

Nonreduced samples were treated in a similar fashion with the exception that water was added instead of dithiothreitol and iodoacetamide.

Samples of 20 µl were electrophoresed on a 10-20% linear gradient of polyacrylamide, and autoradiographs were prepared.

The tracks of the gel shown in the figure contained radioactive inhibitor with buffer, Mel-PA, urokinase or trypsin. Tracks marked with an (a) contained nonreduced samples whereas tracks marked with a (b) contained reduced samples.

Mel-PA and trypsin had modified the inhibitor and a 15 000 dalton fragment was obtained. In the case of trypsin a faint band with a molecular weight of approximately 7000 dalton could be seen.

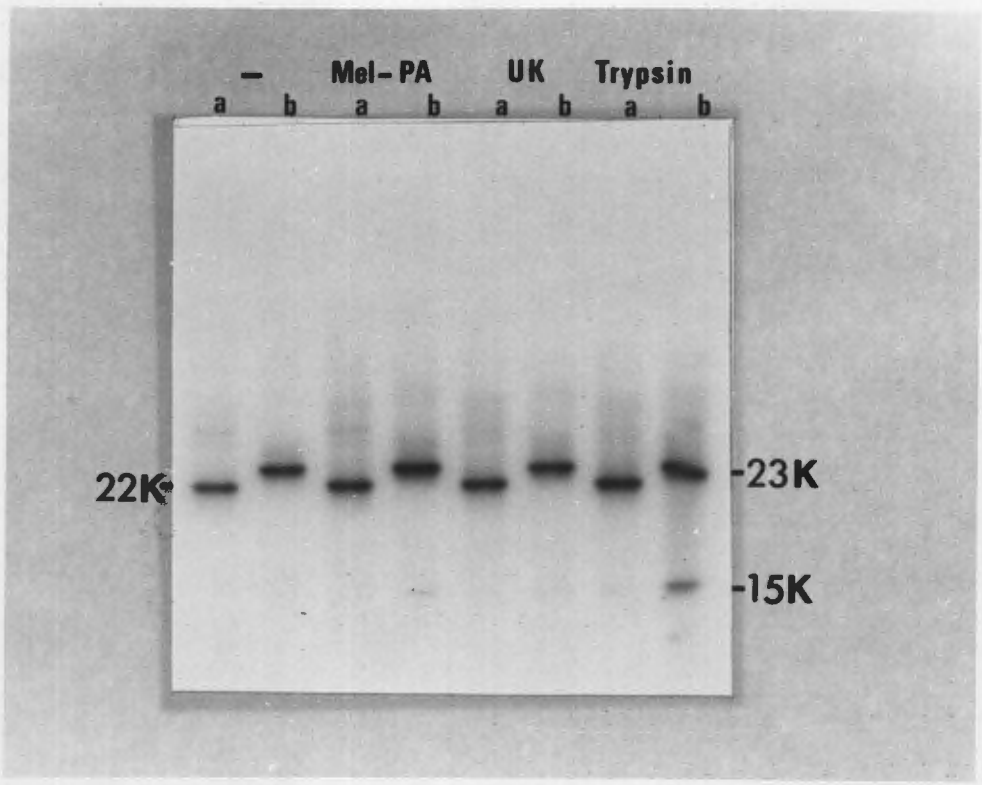

–
Mel-PA
UK
Trypsin
a
b
a
b
a
b
a
b
22K
23K
15K

Figure 4.7

Figure 4.7 Two-dimensional polyacrylamide gel electrophoresis of inhibitor-enzyme mixtures.

DE-3 (5.6 pmol, 5 x 10^5 cpm) was mixed with Mel-PA (approximately 70 pmol), or trypsin (50 pmol) in a total volume of 80 µl of 0.1 M Tris HCl pH 8.1 containing 0.02% Triton X-100. The samples were incubated for 16 hr at room temperature and 10 µl glycerol and 1 µl 0.04% phenol red were then added. Two samples of twenty µl of each mixture were then electrophoresed in a 3-15% linear gradient of polyacrylamide gel slab (120 x 120 x 0.75 mm) at pH 8.9.

After the phenol red tracking dye had migrated to a distance of 2 cm from the bottom of the gel, electrophoresis was stopped, and two tracks containing either DE-3-trypsin or DE-3-Mel-PA were separated from the remainder of the gel. The remainder was fixed, stained and dried and autoradiographs were prepared.

The two tracks containing DE-3-Mel-PA or DE-3-trypsin were incubated in 0.06 M Tris HCl, pH 6.8 containing 2.5% SDS, 5% β-mercaptoethanol and 10% glycerol at room temperature for 30 min.

After equilibration each track was layered across the top of a slab gel containing a linear gradient of 10-20% polyacrylamide and 0.1% SDS. Close contact between the tracks and the second dimension slab gels was ensured by embedding the tracks in 1% agarose in equilibration buffer. Molecular weight marker proteins were applied to one side of the gel in a well made in the agarose.

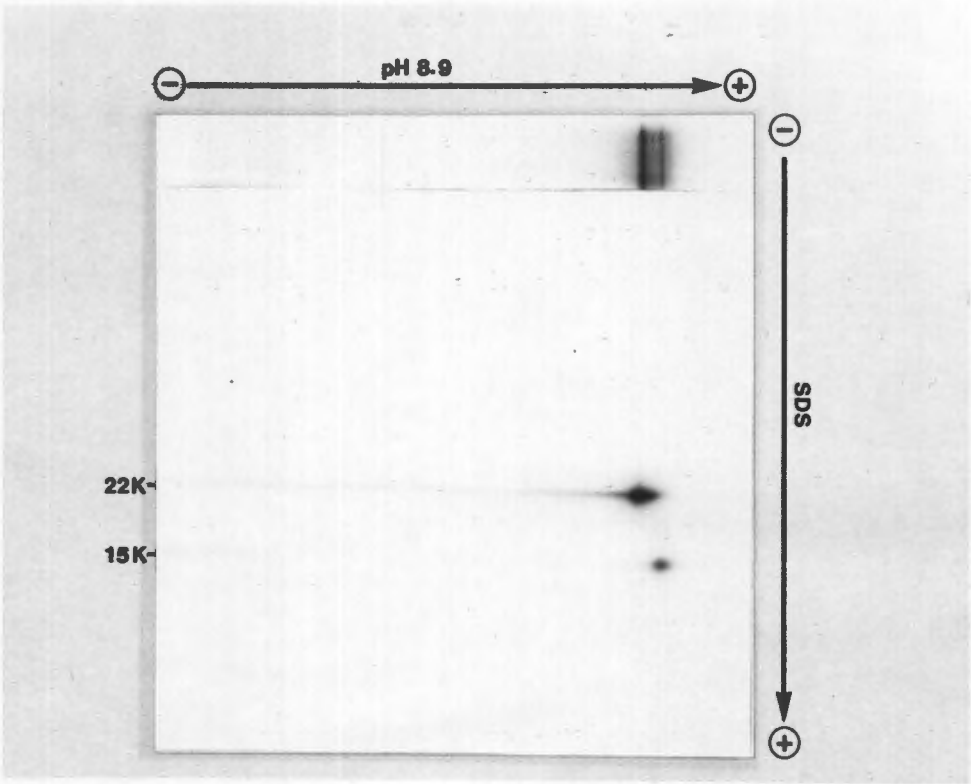
Electrophoresis was carried out at 20 mA per gel at 4°C until the red tracking dye of the marker proteins had reached the bottom of the gels. The gels were fixed, dried and autoradiographs were prepared.

The figure shows autoradiographs of the two SDS-slab gels with the corresponding tracks on top of each.

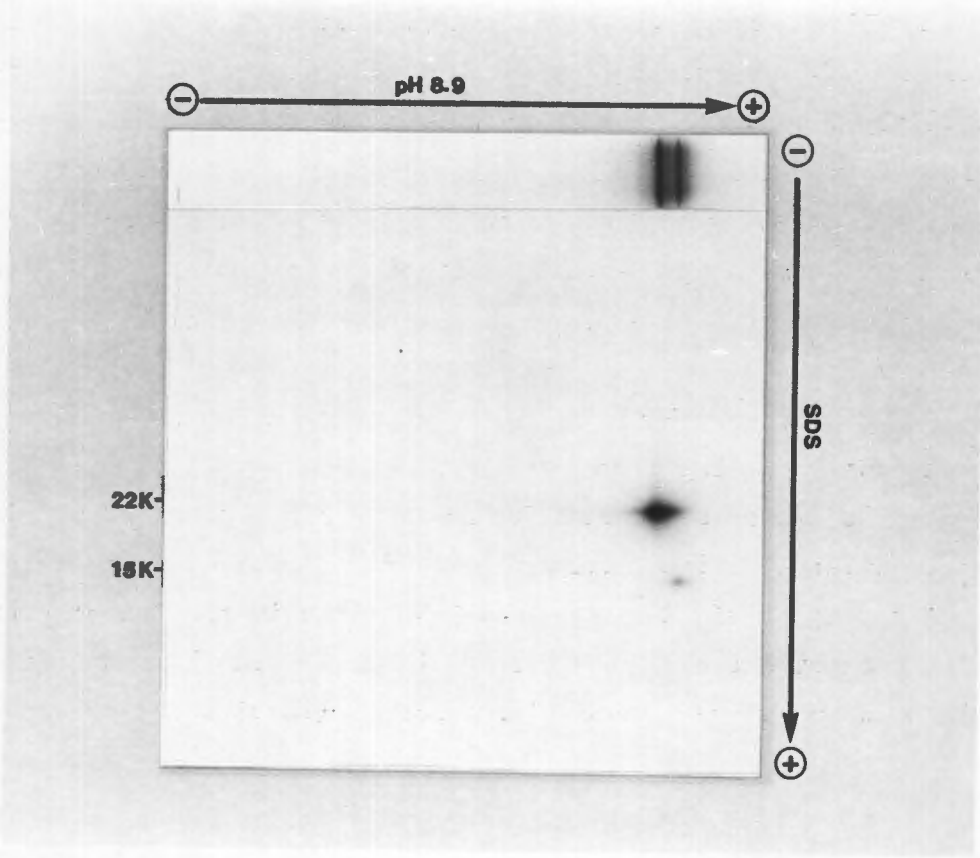
It can be seen that the two DE-3 bands obtained by electrophoresis under alkaline conditions could be resolved into two bands of molecular weights of 22 000 and 15 000 dalton in the SDS-polyacrylamide gel.

Treatment of DE-3 with urokinase (70 pmol) or buffer alone resulted in no change in the DE-3 molecule (Results not shown).

TRYPSIN



MEL-PA



were incubated for a further 30 min at 37°C. Proteins were then precipitated by addition of TCA to a final concentration of 6%. The precipitates were collected by centrifugation, washed and redissolved in a buffer containing SDS. Each enzyme-inhibitor solution was then divided into two portions. One of these was reduced by the addition of β-mercaptoethanol and the other was not. The samples were then electrophoresed in a 5-16% polyacrylamide gradient gel containing 0.1% SDS.

After electrophoresis the gel was fixed and dried and autofluorographs were prepared. The results are shown in Fig. 4.8.

In the absence of DE-3, ^{3}H-DFP-labelled Mel-PA migrated as a single band with a molecular weight of approximately 73 000 daltons. This was converted to a 35 000 dalton band by reduction. Both the 73 000 and the 35 000 molecular weight bands were reduced in intensity by pre-incubation of Mel-PA with DE-3.

Unreduced urokinase showed two radioactive bands with approximate molecular weights of 56 000 and 32 000 daltons. After reduction one band was seen with an apparent molecular weight of 32 000 daltons. The intensity of these bands was unaffected by DE-3.

These results show that the active site of Mel-PA was blocked by DE-3 and hence not available for covalent binding to DFP.

Purification of Mel-PA using affinity chromatography on DE-3-sepharose

The fact that DE-3 formed non-covalent complexes with Mel-PA but not with urokinase suggested that the inhibitor might be coupled to a solid phase support to provide an affinity reagent for the chromatographic separation of these two enzymes and for the purification of Mel-PA.

Purified DE-3 inhibitor was accordingly coupled to cyanogen bromide-activated sepharose 4b as described in the legend to Fig. 4.9.

Figure 4.8

Figure 4.8 Competition of DFP labelling of active sites with the inhibitor DE-3.

Samples of Mel-PA (180 pmol) or urokinase (420 pmol) were dissolved in 500 µl of 0.02% Triton X-100 in 0.1M Tris HCl pH 8.1 with (tracks labelled "b") or without (tracks labelled "a") 1 nmole of DE-3.

After incubation for 30 min at 20°C, ^{3}H -DFP (150 µCi; 23 nmol) was added and the mixtures were incubated for a further 30 min at 37°C.

Protein was then precipitated by the addition of SDS and TCA to final concentrations of 0.1% and 6% respectively. The precipitates were collected by centrifugation (1700g; 30 min) and each was dissolved in 40 µl of 0.06 M Tris HCl pH 6.8 containing 1% SDS and 10% glycerol. One sample (20 µl) of each enzyme-inhibitor mixture was reduced by addition of 2-mercaptoethanol to a final concentration of 5%. After boiling for 1 min, 10 µl samples were electrophoresed in an 5-16% linear gradient SDS polyacrylamide gel.

After electrophoresis, the gel was fixed and stained with Coomassie brilliant blue and autofluorographs were made as described in the Appendix.

When treated in this way, Mel-PA showed one radioactive band with a molecular weight of approximately 73 000 under nonreducing conditions (NR) and one with a molecular weight of 35 000 under reducing conditions (R). In the presence of DE-3 the intensity of these radioactive bands was diminished.

Nonreduced urokinase (NR) showed two bands with molecular weights of 56 000 and 32 000 daltons and one band of 32 000 daltons after reduction (R). The intensity of these radioactive bands was not reduced by the presence of DE-3.

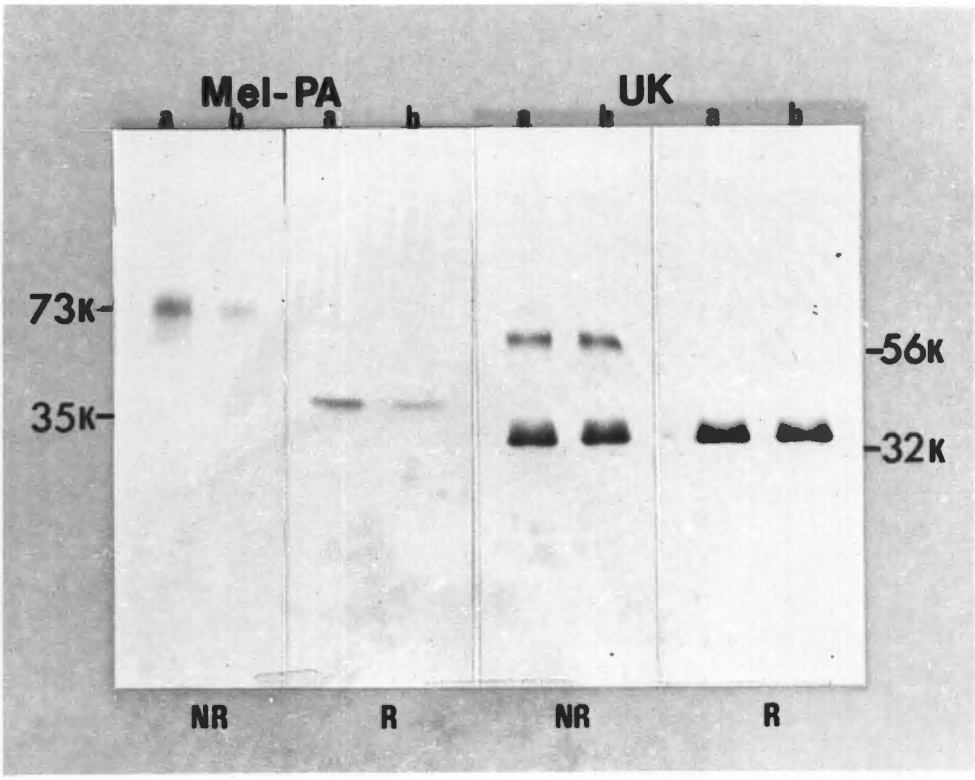

Mel-PA
UK
a
b
a
b
a
b
a
b
73K
35K
56K
32K
NR
R
NR
R

Figure 4.9

Figure 4.9 Chromatography on DE-3 sepharose 4b

Purified DE-3 (26 mg) was coupled to 5 ml of commercially available CNBr-activated sepharose 4b (Pharmacia) according to the manufacturers instructions.

The matrix was equilibrated with PBS pH 7.4 containing 0.4M NaCl, 0.1% Triton X-100 and 0.02% sodium azide. The matrix was then packed into a column.

Two litres of harvest fluid obtained from Bowes II cells was made 0.4 M with respect to NaCl and 0.1% with respect to Triton X-100 and filtered through a 0.45 μm membrane (Millipore).

The harvest fluid was then applied to the column at a flow rate of 45 ml/hr at room temperature and the effluent was collected into a beaker at 4°C. The effluent was monitored for the presence of plasminogen-dependent fibrinolytic activity in the ^{125}I-fibrin assay.

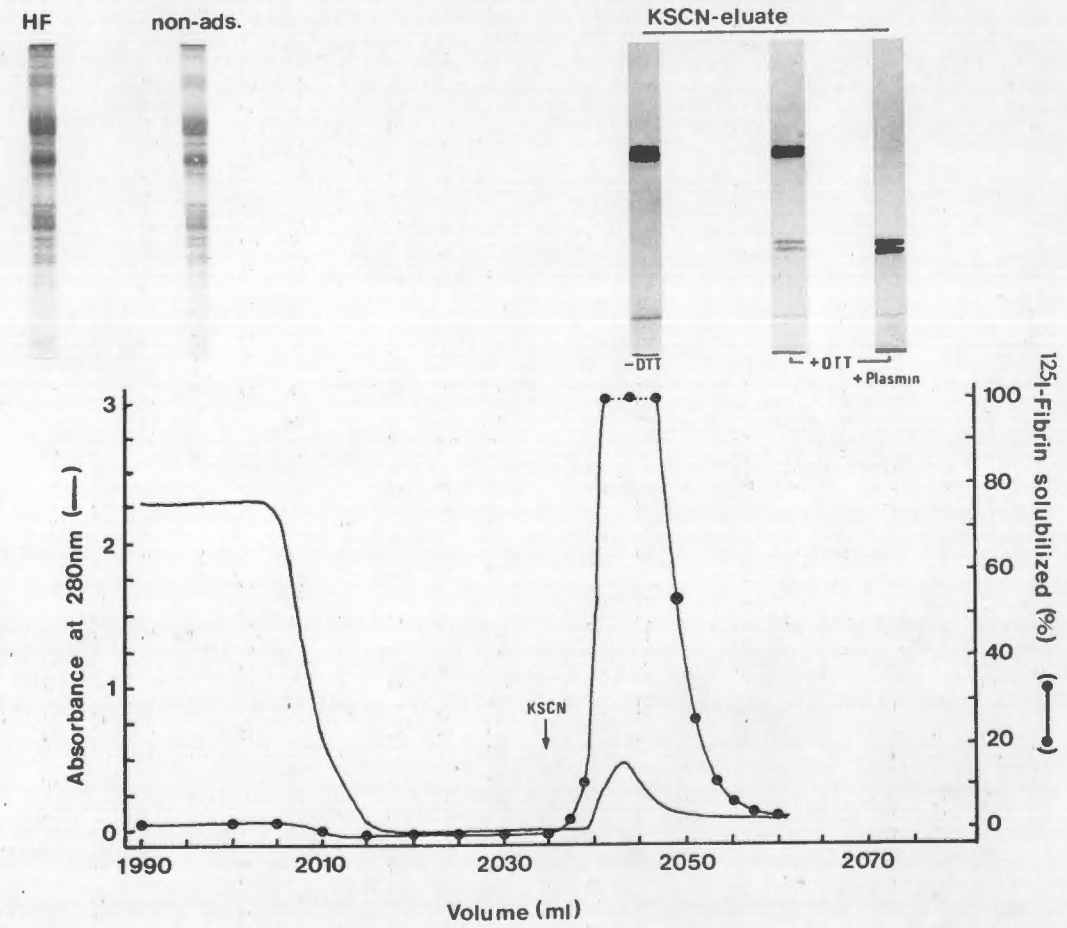
After the total volume of harvest fluid had passed through, the column was washed with 6 column volumes of PBS containing 0.4M NaCl and 0.1% Triton X-100. Five millilitre fractions were collected at this stage.

Adsorbed proteins were then eluted using PBS containing 1.6M KSCN, 0.4M NaCl and 0.1% Triton X-100 and 2 ml fractions were collected at 4°C.

The fibrinolytic activity in all fractions was determined in the ^{125}I-fibrin assay. Fractions containing the highest activity were pooled to give 6 to 8 ml of solution which was stored at -20°C. This usually represented 70-80% of the total activity applied to the column. Fractions containing lower activity were pooled separately. The total recovery of activity in both pools usually amounted to 90-100%.

The high OD_{280} values observed while the harvest fluid sample was being added to the column are attributable to the phenol red in the tissue culture medium.

The photographs above the elution profile depict SDS-polyacrylamide gel electrophoretograms of proteins precipitated with TCA from the corresponding subjacent fractions. Note that the KSCN eluate contained a single band of protein with a molecular weight of 72 000 daltons corresponding to electrophoretically pure Mel-PA.


HF
non-ads.
KSCN-eluate
-DTT
+DTT
+Plasmin
Absorbance at 280nm (—)
125I-Fibrin solubilized (%) (•—•)
KSCN
Volume (ml)
0
1
2
3
20
40
60
80
100
1990
2010
2030
2050
2070

The matrix was equilibrated in PBS pH 7.4 containing 0.4M sodium chloride, 0.1% Triton X-100 and 0.02% sodium azide. The matrix (5 ml settled volume) was packed into a column fashioned from a disposable syringe. Adsorption and elution of enzyme was done at room temperature and fractions were collected at 4°C. The data summarized graphically in Fig. 4.9 show that all of the Mel-PA present in 2 litres of Bowes II melanoma cell harvest fluid that had been adjusted to contain 0.1% Triton X-100 and 0.4M sodium chloride could be adsorbed to the column. Washing with 5 column volumes of equilibration buffer failed to displace Mel-PA. Elution of the enzyme could be satisfactorily achieved with 1.6M KSCN dissolved in a buffer consisting of 0.4M NaCl, 0.1% Triton X-100, 0.02% sodium azide and 0.01M sodium phosphate, pH 7.4.

In the early stages of its use the column behaved erratically inasmuch as incomplete recoveries of Mel-PA were obtained and fragments of bound inhibitor were eluted from the matrix with the KSCN solution. After 4 litres of Mel-PA harvest fluid had been processed on the column, however, reproducible results were obtained with 100% recovery of added enzyme. It could be shown by measurement of amidolytic activity before and after plasmin treatment that the affinity column adsorbed both Mel-PA and pro-Mel-PA. The inactive enzyme precursor was also recovered without loss.

Electrophoretic analysis of the active fractions eluted from the affinity column with potassium thiocyanate revealed a single band of protein with an apparent molecular weight of approximately 70 000 daltons corresponding to pure enzyme. No detectable contaminants were seen when the gel was stained with Coomassie brilliant blue or with a more sensitive silver stain described by Merril et al (215).

Urokinase did not adsorb to the affinity matrix and was completely removed with the washing buffer.

These data are summarized in tabular form in Table 4.1.

Table 4.1 Performance of a DE-3-affinity column for the purification of Mel-PA.

Run No. [i)]	Sample	Vol(ml)	Total Activity (FU)	% Recovery	Presence of DE-3	%Pro-Mel-PA
1	harvest fluid wash	300	2974			42.4%
	KSCN eluate	24	2022	68%	+	48.8%
2	harvest fluid wash	740	6845			N.D.
	KSCN eluate	47	6639	96.9%	+	N.D.
3	harvest fluid wash	1260	12839			45%
	KSCN eluate	40	11298	87.9%	-	39%
4	harvest fluid wash	2000	23740			52%
	KSCN eluate	51	24356	>100%	-	47%

i) Run numbers refer to sequential batches of Bowes II harvest fluids processed on 5 ml of DE-3-agarose.

Note
a) that enzyme recovery improved with successive use of the column,
b) that DE-3 leached from the column at the beginning but not with later runs,
c) that Pro-Mel-PA as well as Mel-PA could be purified.

Primary structure of DE-3: comparison with soybean trypsin inhibitor

In Fig. 4.10 I present the partial amino acid sequences of DE-3 and soybean trypsin inhibitor that had been aligned to show maximum homology. The results for DE-3 were obtained by Dr. F. Joubert in Pretoria and he has generously permitted me to report them in this thesis. The results for soybean trypsin inhibitor were obtained from the literature (219). The reactive site for both inhibitors is one Arg-X bond marked with an asterisk. It is evident that little homology exists for a number of residues on either side of this bond.

Figure 4.10 Primary structure of DE-3: comparison with soybean trypsin inhibitor.

DISCUSSION

The seeds of the legume Erythrina latissima contain a trypsin inhibitor of the Kunitz type (212) that has previously been designated DE-3. This protein has three unusual attributes. In the first instance, whereas soybean trypsin inhibitor and other known plant-derived Kunitz inhibitors have no effect on plasminogen activators (142,133) DE-3 is able to inhibit Mel-PA. This is of particular interest and may be related to the fact that sequence homology between the two inhibitors is poor close to the reactive site of the protein, despite excellent agreement in other regions. (Fig. 4.10).

Secondly, in its selective ability to inhibit Mel-PA without affecting the action of urokinase, DE-3 is able to distinguish between two important serine proteases, both of which have, as their major putative physiological function, the activation of plasminogen.

Thirdly, as is shown by the data presented in Fig. 4.5 to Fig. 4.7, DE-3 was cleaved by both trypsin and Mel-PA to give disulphide-linked fragments, one of which had a molecular mass of approximately 15 000 daltons. No such cleavage was seen when the inhibitor was treated with urokinase. One may conclude, therefore, that, as has been found with other Kunitz type inhibitors (216), the inhibitory action of DE-3 is related to its ability to serve as a substrate for the enzyme and to form relatively stable complexes with both inactive pro-Mel-PA as well as the active form of the enzyme. An association between trypsinogen and Kunitz inhibitors has previously been documented (220).

Considered collectively, these properties and the other data that I have accumulated support the proposal that the DE-3 molecule possesses at least two regions for interaction with serine proteases. The first is that site which is cleaved by the protease. It would appear

to be straddled by a disulphide bridge and to be situated 63 residues from the N-terminal end of the inhibitor molecule. The second is that region which binds to the specific binding site on trypsin, plasmin or Mel-PA but not to the analogous site on urokinase or thrombin. Attachment of the inhibitor to the active site of Mel-PA would not only block access of the fluorogenic substrate in the amidolytic assay, but would also block active site labelling with radioactive DFP (Fig. 4.8).

Immobilized DE-3 proved to be an affinity reagent that could be reused many times and yet remain effective despite the passage of an amount of enzyme that would have been expected to have converted all of the inhibitor into the cleaved form. It has been noted with other systems, that the same stable complex is formed between enzymes and either native or cleaved inhibitor (221,222). Presumably the same is true for the DE-3 : Mel-PA interaction.

The usefulness of DE-3 inhibitor for the purification of Mel-PA is apparent. It is to be hoped that further knowledge of its primary and tertiary structure in relation to other Kunitz type inhibitors and other serine proteases will provide useful insights into the mechanism of action of these enzymes and, in particular, into finer points of difference between the action of Mel-PA and urokinase which may shed light upon their different functional roles <u>in vivo</u>.

CHAPTER V

A COMPARISON BETWEEN CERTAIN PLASMINOGEN-INDEPENDENT ENZYMATIC FUNCTIONS OF UROKINASE AND THE MELANOMA PLASMINOGEN ACTIVATOR.

Distinctions between Mel-PA and urokinase have rested mainly upon electrophoretic and immunochemical criteria and upon differences in their provenance in the body. Relatively few reports have appeared in the literature to indicate that these enzymes also differ in such functional characteristics as the rates at which they hydrolyse synthetic substrates (155,156,157,158), the extent to which they bind to fibrin (57,58,130,169) and the extent to which their catalytic activity is enhanced by the presence of other macromolecules (53,57,159,160).

Since the functional differences that distinguish urokinase from Mel-PA have important implications for their possible physiological roles *in vivo*, and for their assay and for the study of their cellular synthesis *in vitro*, I felt it important to undertake a study of the catalytic activities of these two enzymes.

The availability of chemically defined substrates for urokinase and Mel-PA has made it possible to measure their amidolytic activities directly without considering the manner in which they function in more complex systems involving plasminogen, plasmin, plasmin substrates or other macromolecules. The separate study of the amidolytic and plasminogen-converting functions of Mel-PA and urokinase has enabled me to distinguish the effects of macromolecules and other substances that operate at both functional levels.

In this chapter I report the results of experiments in which I have confined myself to the study of the plasminogen-independent, amidolytic activities of urokinase and Mel-PA and the extent to which these functions

are influenced by various inhibitors. In the next chapter I consider urokinase and Mel-PA for their ability to activate plasminogen, for their role as fibrinolysins, and for the ways in which they differ in these respects.

MATERIALS AND METHODS

The melanoma plasminogen activator was obtained and isolated from Bowes melanoma cells cultured *in vitro* as described in Chapter I and Chapter IV. Human urokinase was obtained commercially (Leo Pharmaceutical Products, Ballerup, Denmark). Samples of Mel-PA and urokinase were functionally pure as judged by electrophoretic and immunochemical criteria and by the use of appropriate control assays under plasminogen-free conditions. Bovine fibrinogen (Sigma Chemical Company, St. Louis, Mo., U.S.A.; Fraction I, F 4000) and human fibrinogen (A.B. Kabi, Stockholm, Sweden) were freed of plasminogen by the method of Mosesson (162)(Appendix A1.1). Plasmin was generated from purified human plasminogen (Appendix A1.3) by incubation with insolubilized urokinase. Partially-purified thrombin was obtained from Parke-Davis Laboratories (Pty) Ltd., Isando, Tvl. South Africa, and was used for formation of fibrin clots from fibrinogen. Purified thrombin was obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. Bovine trypsin was obtained from Worthington Biochemical Corporation, Freehold, N.J., U.S.A. Other reagents and their commercial sources are listed in the Appendix (A5).

Amidolytic assays

The amidolytic activities of Mel-PA and urokinase were measured according to the method of Zimmerman et al (133) using two fluorescent

substrates, Cbz-glycyl-glycyl-arginyl-7-amino-4 methyl coumarin (Cbz-Gly-Gly-Arg-AMC) and Boc-valyl-glycyl-arginyl-7-amino-4 methyl coumarin (Boc-Val-Gly-Arg-AMC). Assays were carried out in glass cuvettes at 25°C. The reaction was initiated by addition of 50 µl of enzyme solution. The final reaction mixture consisted of 0.1M Tris HCl pH 8.1 containing 5×10^{-4}M substrate, 4% (v/v) DMSO, and enzyme in a total volume of 0.5 ml.

The initial rate of AMC release was monitored fluorometrically using a Perkin Elmer fluorescence spectrophotometer model MFP-43A equipped with a recorder. The excitation and emission wavelengths were set at 383 nm and 455 nm respectively. The instrument was standardized so that a 4×10^{-7}M solution of AMC in the assay buffer gave a full scale recorder pen deflection.

Activities of enzymes were expressed in fluorometric units/ml (FU/ml), where 1 FU represented that amount of enzyme which hydrolysed 10 pmol of substrate in one minute.

Labelling of active sites with radioactive DFP

Urokinase, Mel-PA and trypsin were labelled at the active-site serine by incubation with ^{3}H-DFP. After incubation the labelled enzymes were recovered and freed from unreacted radioactive DFP by precipitation and washing with TCA. The methods used were those recommended by Cohen et al (163) for serine proteases.

For experiments in which ^{3}H-DFP was used as a quantitative reagent for measurement of the concentration of plasminogen activator active sites in solution, it was necessary to know the specific activity of the radioactive inhibitor. This was calculated from the protein-bound radioactivity recovered after treatment of an accurately known

amount of trypsin with a 1000-fold molar excess of the radioactive DFP. In brief the procedures were as follows:

Purified trypsin was dissolved to an approximate concentration of 1 mM in 1 mM HCl. The concentration of active enzyme was then determined accurately by active site titration using methylumbelliferyl guanidinobenzoate (MUGB) according to the method of Jameson et al (164). This procedure was reproducible and reliable and gave data (Fig. 5.1) from which the molarity of the trypsin active sites could be confidently determined.

An ethylene glycol solution of radioactive DFP (6 Ci/mmol; 5 mCi/ml; Amersham International; Cat. No. TRK 207) was diluted with non-radioactive DFP (10 mM; Sigma Chemical Company) in isopropanol to give an approximate specific radioactivity of 0.3 Ci/mmol such that the final mixture of convenient volumes would provide statistically satisfactory amounts of protein-bound radioactivity in the treated enzymes and, at the same time, a molar excess of inhibitor over enzyme of at least 1000:1. Known amounts of trypsin and radioactive DFP were then mixed and incubated for 16 hours at 20°C in 0.1M Tris HCl pH 8.1 containing 0.1% Triton X-100. At the end of the incubation, radioactive protein was precipitated by the addition of TCA to give a final concentration of 6%. Protease- and inhibitor-free bovine serum albumin (PIF-BSA; Appendix A1.6) was added to a final concentration of 0.7 mg/ml to provide precipitate bulk and 0.2% SDS was added to maintain Triton X-100 in solution (165). The precipitate was washed sequentially with 6% aqueous TCA and acetone by suspension and centrifugation (1700g; 30 min) and dissolved in 1.0 ml 0.1M NaOH for radioassay. The solution was transferred to a scintillation vial containing 10 ml of Instagel (Packard Instrument Corp.) and the samples were counted in a Packard scintillation counter

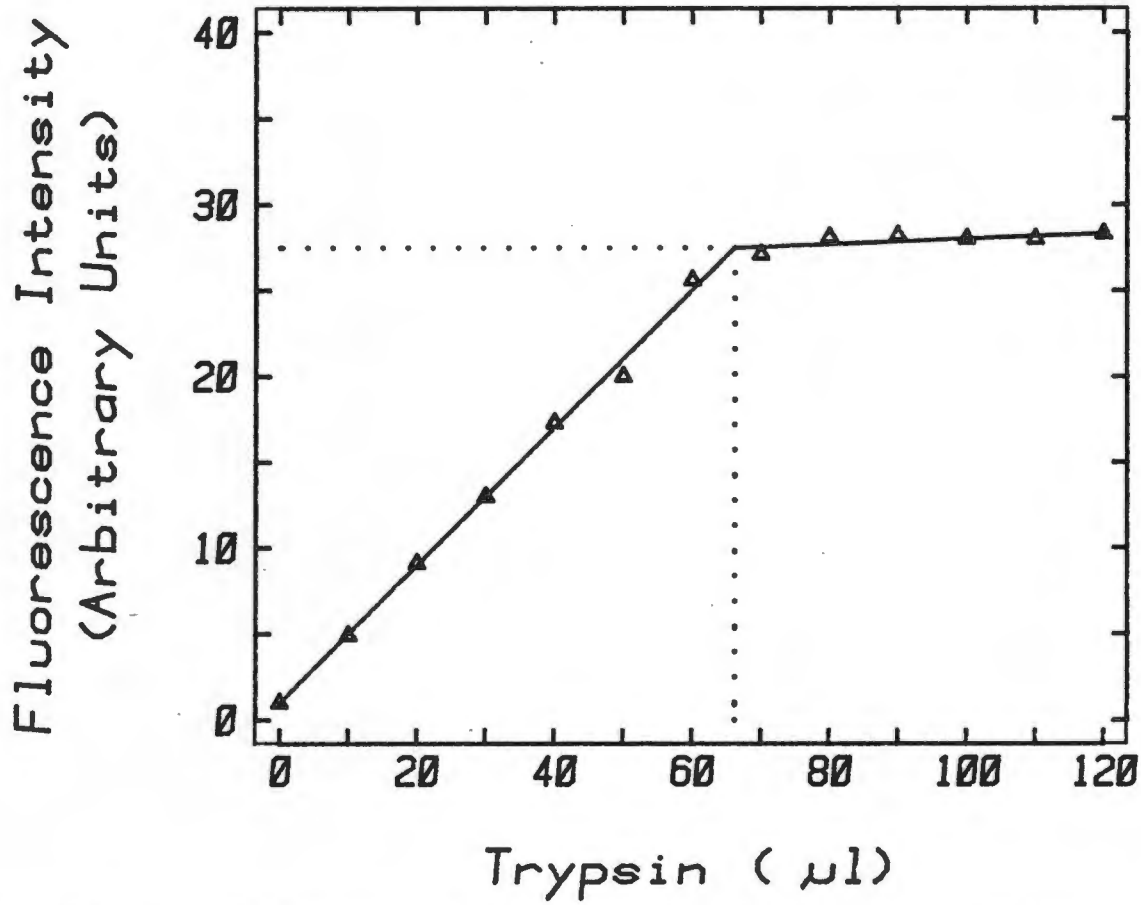


Figure 5.1 Determination of trypsin concentration by active site titration.

A stock solution of approximately 1 mM trypsin in 1 mM HCl was diluted 100-fold with 0.1M Na phosphate buffer, pH 6.5. Incremental volumes of 10 µl of this solution were added to a quartz cuvette containing 1.03 ml of 0.1M Na phosphate pH 6.5 and approximately 0.3 nmol of methyl umbelliferol guanidinobenzoate (MUGB).

Fluorescence was measured within 2 min of each addition of trypsin in a Perkin-Elmer fluorometer with excitation and emission wavelengths set at 323 nm and 446 nm respectively.

The plot of fluorescence intensity as a function of the cumulative volume of trypsin added comprised two distinct linear components - a steep portion followed by a plateau that was reached when all available MUGB had been hydrolysed. The intersection of the extrapolated regression lines for these two components was taken as the end point. As shown the coordinates for this point were 68 µl of trypsin solution and 28.5 fluorometric units. This latter value corresponded to a concentration of 0.254 µM methylumbelliferol as determined from a standard curve constructed by plotting fluorescence units as a function of methylumbelliferol concentration.

From these data the concentration of trypsin active sites in the stock solution was estimated to be 0.41 mM.

using the automatic external standard facility to convert cpm to dpm. Appropriate controls included (i) incubation of enzyme with solvent but without inhibitor to ensure that enzymatic activity was not lost spontaneously during the course of incubation and (ii) incubation of radioactive DFP solution without enzyme. With this second control the precipitation step was performed with albumin only to provide background counts for subtraction. Data obtained from a typical ^{3}H-DFP standardisation run are presented in Table 5.1.

SDS-polyacrylamide gel electrophoresis

The method for electrophoresis of proteins in polyacrylamide gels containing SDS and subsequent autoradiography of gels is described in detail in the Appendix A2.

RESULTS

Electrophoretic analysis of Mel-PA and urokinase: identification of active site subunits

Mel-PA and urokinase were labelled with radioactive DFP as described under Methods. The proteins were then electrophoresed in SDS-polyacrylamide slab gels under normal and reducing conditions. After electrophoresis the slab gels were processed for autoradiography either according to the method of Bonner and Laskey (139) or using En^{3}Hance (Appendix A2.5).

The results (Fig.5.2) showed that the urokinase preparation used contained DFP-binding proteins of MW 56 000 and 32 000. When this sample was electrophoresed under reducing conditions one labelled band was observed at a position corresponding to MW 32 000.

Mel-PA showed a radioactive band with a MW 73 000. When

Table 5.1

Table 5.1

Radioactive DFP (6 Ci/mmol; 5 mCi/ml) was diluted with non-radioactive DFP (10 mM) in isopropanol to give an approximate specific radioactivity of 0.3 Ci/mmol. Fifty four microlitres of this mixture was then added to 1 ml solutions containing either buffer, trypsin at the concentration shown, urokinase or Mel-PA. Mel-PA was dissolved in RPMI containing 0.1% Triton and urokinase was dissolved in 0.1M Tris HCl pH 8.1 containing 0.1% Triton.

The mixtures were incubated for 16 hr at room temperature. At the end of the incubation protease- and inhibitor-free BSA was added to a final concentration of 0.7 mg/ml and SDS was added to a final concentration of 0.2%. The proteins were collected by precipitation with 6% TCA and centrifugation at 1700g for 30 min. After the precipitates had been washed twice with 6% TCA and twice with acetone, they were dissolved in 0.1M NaOH. The solutions were quantitatively transferred in 1 ml volumes to scintillation vials containing 10 ml of Instagel and the samples were counted.

Analysis of data obtained from two typical titrations are shown in the table.

As can be seen, catalytic constants of 0.99 FU/pmol and 66.8 FU/pmol were calculated for Mel-PA and urokinase respectively.

Table 5.1 Active site titration of Mel-PA and urokinase with ^{3}H-DFP

Expt.	Sample	Amt. of enzyme pmol	Amt. of enzyme FU	dpm	dpm-bgd pmol	pmol calculated	FU/pmol
1	Buffer	-	-	2 485	-	-	-
	Trypsin	41.8		40 655	913.16		
		83.5		76 505	886.47		
		250.6		308 540	1221.29		
					1006.97		
	Mel-PA		44.43	50 170		47.36	0.938
			46.83	60 408		57.52	0.814
			274.30	249 655		254.46	1.078
			54.86	51 716		48.89	1.122
							0.988
2	Buffer	-	-	3 251	-	-	-
	Trypsin	41.8		52 988	1189.89		
		83.5		126 494	1475.96		
		125.3		180 869	1417.54		
					1361.13		
	Urokinase		11 619	230 111		172.86	67.22
			11 619	246 951			
			1 162	27 246		17.48	66.47
			1 162	26 846			
							66.84

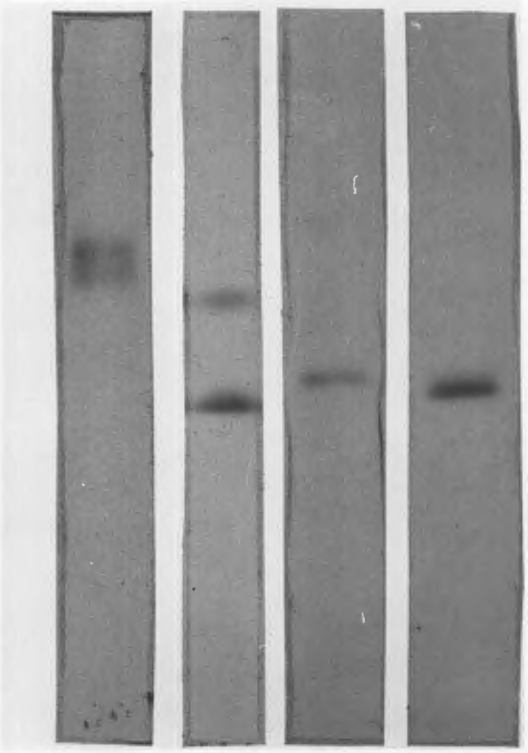


Figure 5.2 **Electrophoretic analysis of Mel-PA and urokinase labelled with ^{3}H-DFP.**

Fifteen microlitres of ^{3}H-DFP (6.5 Ci/mmol; 5 mCi/ml) were added to 500 µl T-T(0.02) containing either Mel-PA (≃ 250 pmol) or urokinase (≃ 30 pmol) and the mixtures were incubated at 37°C for 30 mins. A further 15 µl of ^{3}H-DFP was then added and incubation continued for 16 hours at room temperature. After incubation, protein was precipitated by the addition of 5 µl of 25% SDS and sufficient TCA to give a final concentration of 6%. The precipitates were collected by centrifugation (1700g; 30 min), washed in acetone and redissolved in 40 µl of 0.06M Tris-HCl pH 6.8 containing 1% SDS and 10% glycerol. Samples (20 µl) of the labelled Mel-PA and urokinase preparations were treated with 1 µl of 2-mercaptoethanol. All of the preparations were boiled for 1 min and 10 µl samples were electrophoresed in a 5-16% gradient polyacrylamide gel slab containing 0.1% SDS. After electrophoresis the slab was fixed and stained in Coomassie brilliant blue in 30% methanol:10% acetic acid, destained, impregnated with EN^{3}HANCE and dried. An autoradiograph was was by exposure of Osray RP X-ray film to the dried slab for 48 hr at -80°C.

The autoradiograph is presented in the figure. The wells contained (1) Mel-PA; (2) urokinase; (3) Mel-PA reduced and (4) urokinase reduced.

Note: Radioactive bands obtained with unreduced Mel-PA and urokinase electrophoresed with mobilities corresponding to molecular weights of 73 000 daltons and 56 000 daltons respectively. The urokinase sample contained a 32 000 dalton component. Reduction separated the active-site subunits of Mel-PA and urokinase into components with molecular weights of 35 000 daltons and 32 000 daltons respectively.

electrophoresed under reducing conditions, a single band of MW 35 000 was obtained. Staining of the gel with Coomassie brilliant blue showed that the band was converted by reduction into two subunits of approximate molecular weights of 35 000 and 38 000. The smaller of these subunits contained the active site.

Amidolytic activity of Mel-PA and urokinase

The amidolytic activity of the two enzymes was estimated fluorometrically using the substrate Cbz-Gly-Gly-Arg-AMC. Both Mel-PA and urokinase hydrolysed the arginine-AMC bond to liberate the AMC which could be measured fluorometrically. This assay was linear with enzyme concentration and with time for both enzymes (Fig. 5.3 and Fig. 5.4). Kinetic parameters for the reaction were calculated using the method of Cornish-Bowden and Eisenthal (167). These parameters were used to fit straight lines to double reciprocal plots of 1/V vs 1/S (Fig. 5.5). This method is preferred to Lineweaver-Burk plots (168) in which least squares regression analysis is applied directly.

Cbz-Gly-Gly-Arg-AMC was found to be a better substrate for urokinase (Km = 0.42 mM) than for Mel-PA (Km = 0.81 mM).

To define catalytic constants relating activity in Fluorometric Units to active site concentration, enzyme solutions of defined activity were reacted with ^{3}H-DFP of known specific radioactivity and protein-bound radioactivity was measured. From the results obtained it could be calculated that 1 pmol of urokinase active sites was equivalent to 66.8 FU and 1 pmol of Mel-PA active sites was equivalent to 0.99 FU (Table 5.1).

Results obtained when the amidolytic activities of the two enzymes were measured using Boc-Val-Gly-Arg-AMC are presented in Figure 5.5. This compound proved to be a slightly better substrate for Mel-PA (Km=0.48 mM)

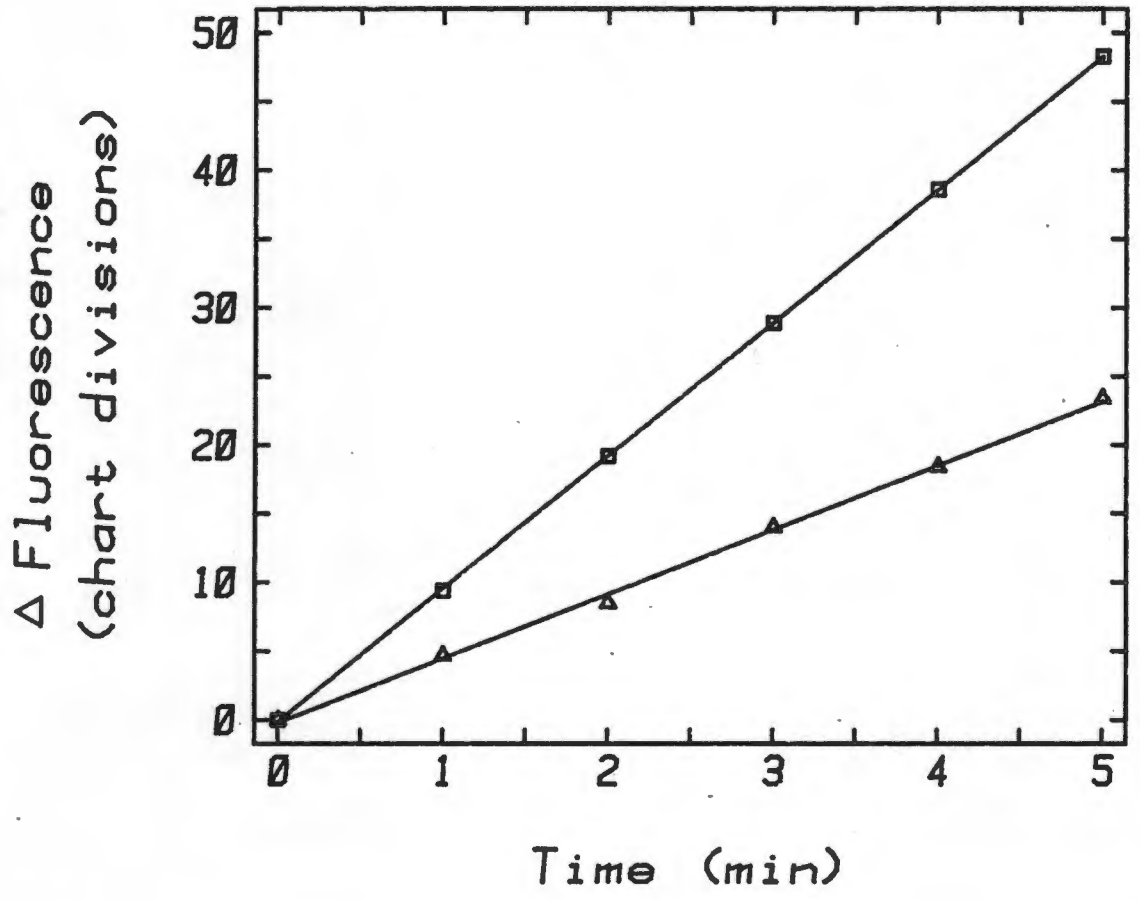


Figure 5.3 Hydrolysis of Cbz-Gly-Gly-Arg-AMC by Mel-PA and urokinase: kinetics of the reaction.

Reactions were carried out at 25°C in glass cuvettes containing final concentrations of 0.5 mM substrate and 4% DMSO in 0.1M Tris HCl pH 8.1.

Reactions were initiated by the addition of 50 µl of enzyme solution to give a final total volume of 0.5 ml.

The graphs in the figure show that fluorescence increase (Δ F) was linear with time for both urokinase (□—□) and Mel-PA (△—△).

For this experiment the instrument was standardized with a solution of AMC so that one division on the chart represented 2.3 pmol of AMC. The reaction cuvettes therefore contained 1.12 FU of Mel-PA and 2.3 FU of urokinase.

One fluorometric unit has been arbitrarily defined as that amount of enzyme hydrolysing 10 pmol of substrate in 1 min.

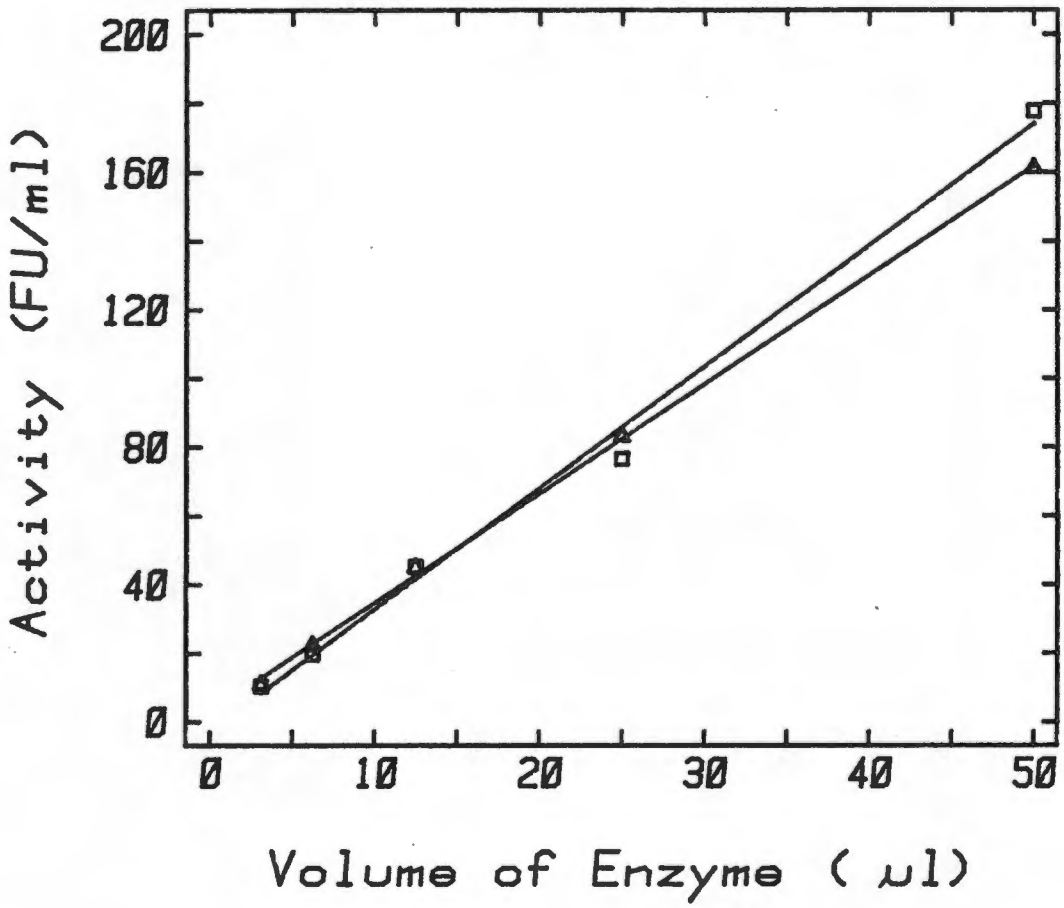


Figure 5.4 Hydrolysis of Cbz-Gly-Gly-Arg-AMC as a function of enzyme concentration.

The rate of hydrolysis of the substrate by different volumes of stock solutions of urokinase and Mel-PA was determined using the same experimental conditions as described in the legend to Fig. 5.3. Fluorometric units (FU) are as defined in the legend to Fig. 5.3. The assay was linear with enzyme concentration for both Mel-PA (Δ—Δ) and urokinase (□—□).

Figure 5.5

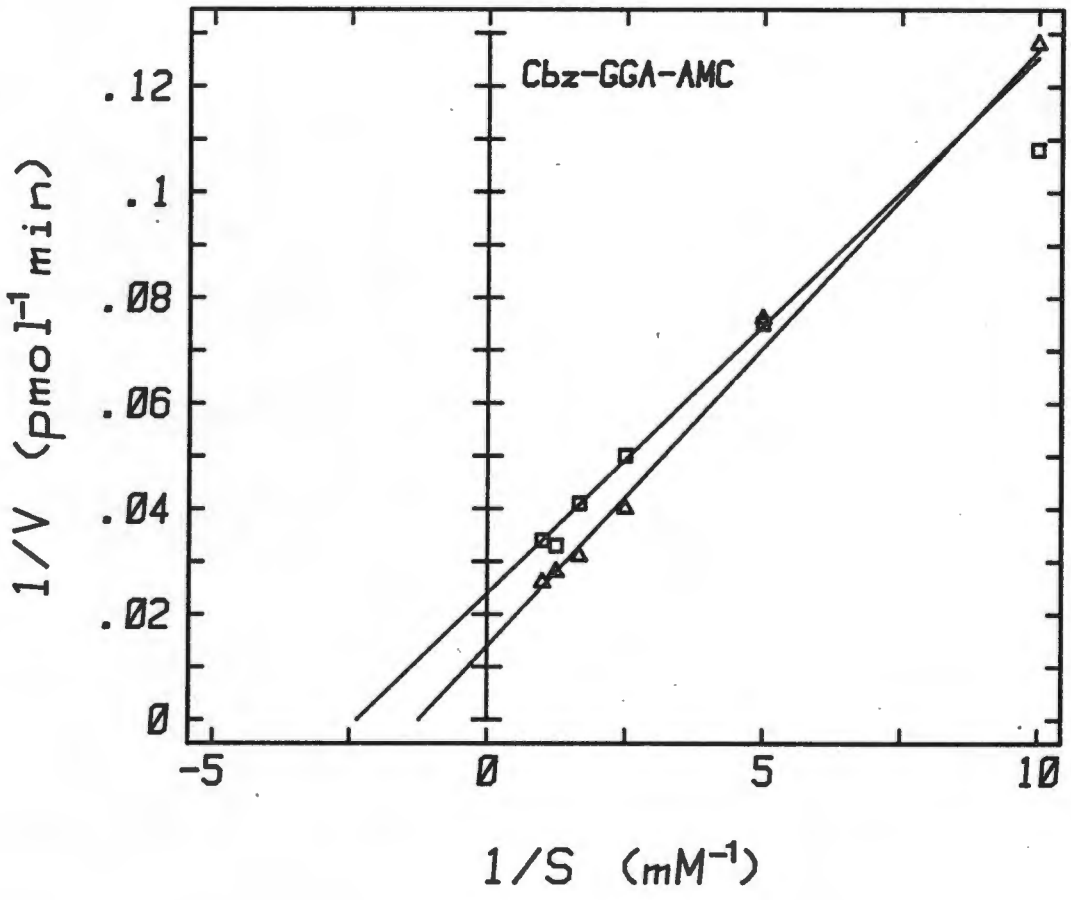
Figure 5.5 Lineweaver-Burk plots of the amidolytic activities of Mel-PA and urokinase.

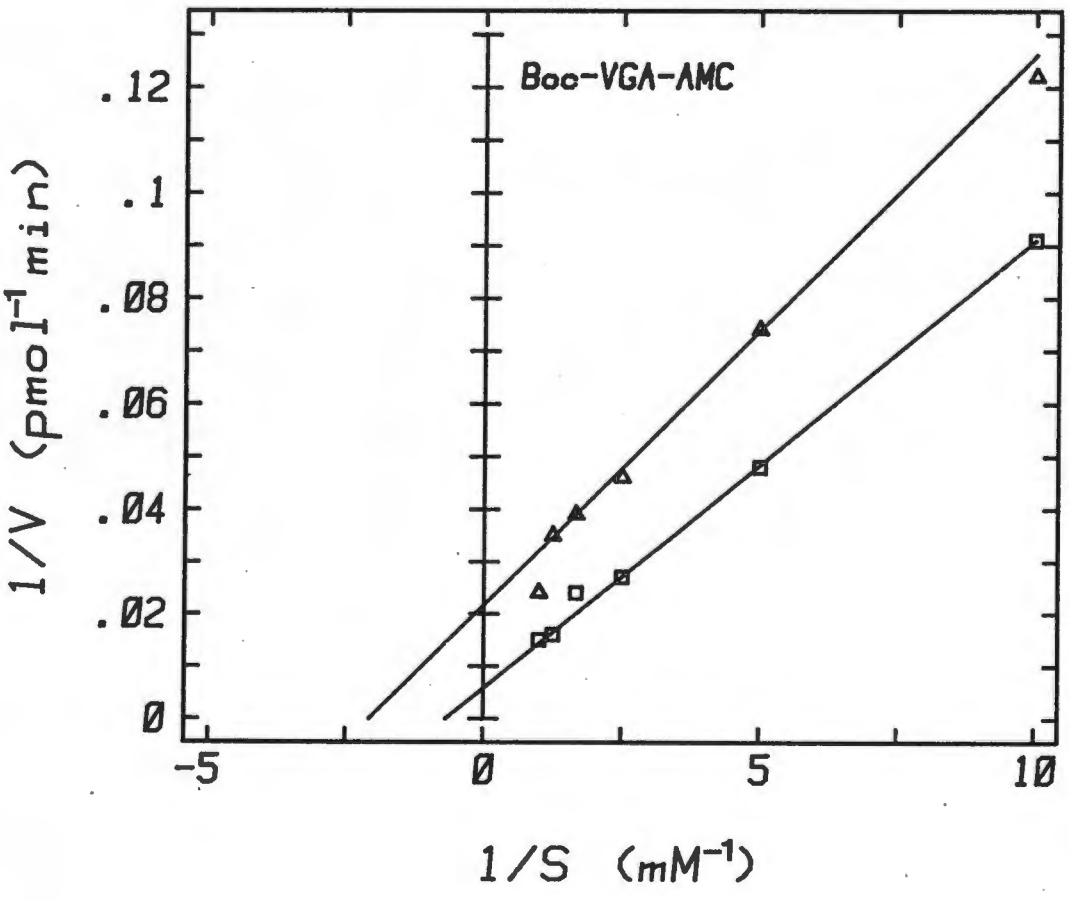
Reciprocals of initial reaction velocities with Mel-PA (△—△) or urokinase (□—□) were plotted as functions of the reciprocals of Cbz-Gly-Gly-Arg-AMC or BOC-Val-Gly-Arg-AMC concentrations.

Straight lines were fitted to the observed points using kinetic parameters calculated according to the method of Cornish-Bowden and Eisenthal (167).

The following results were calculated:-

	Km Values (mM)	
Substrate	Mel-PA	Urokinase
Cbz-Gly-Gly-Arg-AMC	0.81	0.42
BOC-Val-Gly-Arg-AMC	0.48	1.47


Cbz-GGA-AMC
1/V (pmol^{-1} min)
.12
.1
.08
.06
.04
.02
0
-5
0
5
10
1/S (mM^{-1})


Boc-VGA-AMC
1/V (pmol^{-1} min)
.12
.1
.08
.06
.04
.02
0
-5
0
5
10
1/S (mM^{-1})

than was Cbz-Gly-Gly-Arg-AMC and an inferior substrate for urokinase (Km 1.47 mM). Catalytic constants for Mel-PA and urokinase with this substrate were 1.12 FU/pmol and 17.88 FU/pmol respectively.

Amidolytic activity was determined as a function of pH for Mel-PA and urokinase using Cbz-Gly-Gly-Arg-AMC. The results (Fig. 5.6) indicated that both enzymes were neutral proteases with optima at pH 8.5.

Effect of inhibitors

Mel-PA and urokinase were tested for their susceptibility to inhibition by various proteinase inhibitors. The results obtained are summarised in Table 5.2, where the concentration of inhibitor necessary to achieve 50% reduction in enzyme activity (I_{50}) is presented.

From the results presented in the table it can be seen that neither Mel-PA nor urokinase were inhibited by Trasylol, soybean trypsin inhibitor, pancreatic trypsin inhibitor components (Kunitz) or ovomucoid. Iodoacetamide inhibited the enzymes slightly at relatively high concentrations.

Dithiothreitol, benzamidine, zinc chloride, DFP and NPGB inhibited urokinase more effectively than they inhibited Mel-PA. None of the inhibitors used, with the exception of the Erythrina inhibitor, (Chapter IV), were more effective against Mel-PA than they were against urokinase.

Effects of fibrinogen, fibrin and other proteins on the catalytic activity of urokinase and Mel-PA

Many authors have reported that "tissue activator" and urokinase differ in their binding to fibrin and in the extent to which their plasminogen activating activity is enhanced by fibrin (53,57,58,159,161).

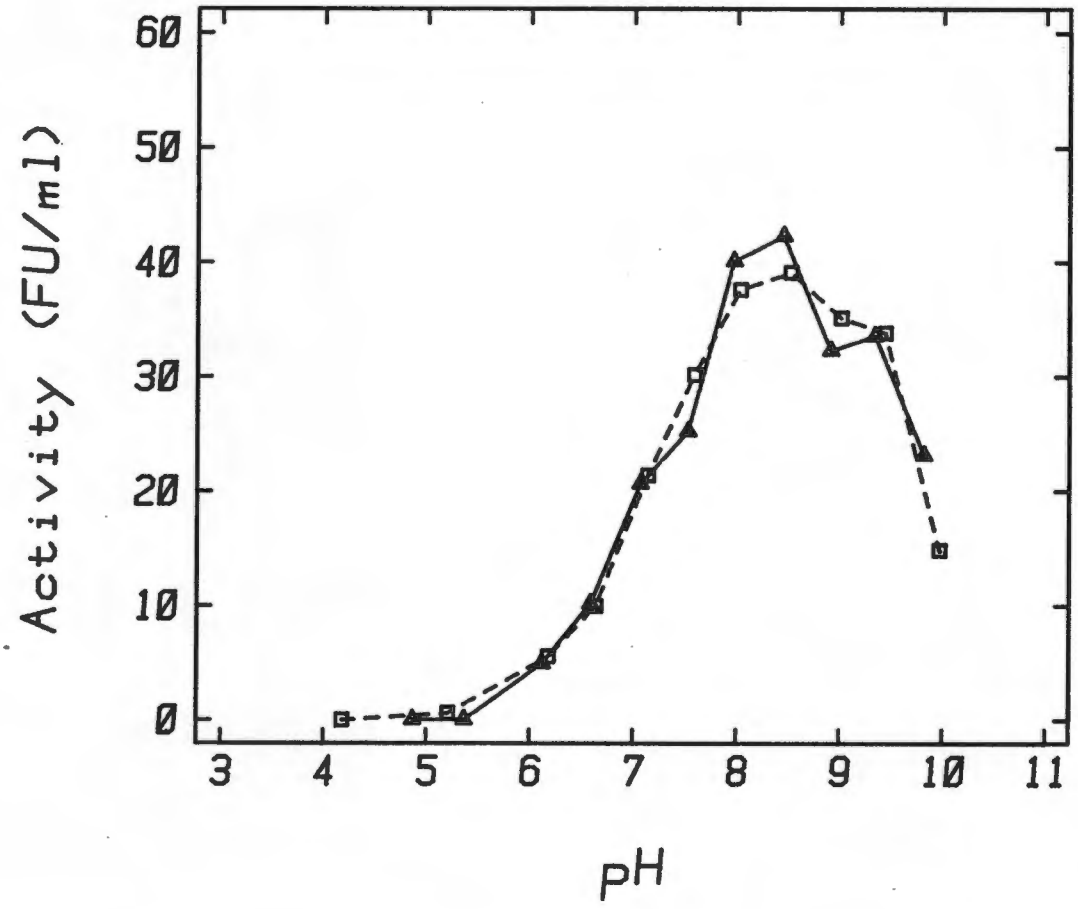


Figure 5.6 Determination of the optimum pH for hydrolysis of Cbz-Gly-Gly-Arg-AMC by Mel-PA and urokinase.

The amidolytic activity of Mel-PA or urokinase on Cbz-Gly-Gly-Arg-AMC was determined in a buffer consisting of 50 mM H_3PO_4, 50 mM Tris, 50 mM glycine adjusted to the desired pH's with HCl or NaOH. Conditions of the assay were otherwise the same as described in Fig. 5.3. The pH values used for plotting the data were determined by measuring the pH's of the final reaction mixtures.

The pH optima for Mel-PA (△—△) and urokinase (□—□) were both approximately pH 8.5.

Table 5.2 The effect of inhibitors on the amidolytic activity of Mel-PA and urokinase.

Inhibitor	I_{50} (M)	
	Mel-PA	Urokinase
Soybean trypsin inhibitor	$> 5 \times 10^{-5}$	$> 5 \times 10^{-5}$
Pancreatic trypsin inhibitor compound (Worthington)	$> 1 \times 10^{-4}$	$> 1 \times 10^{-4}$
Basic pancreatic trypsin inhibitor (Trasylol)	> 1000 KIU/ml	> 1000 KIU/ml
Ovomucoid	$> 5 \times 10^{-5}$	$> 5 \times 10^{-5}$
Benzamidine	7×10^{-3}	2.8×10^{-3}
Dithiothreitol	4.3×10^{-3}	1.2×10^{-3}
Iodoacetamide	$> 1.0 \times 10^{-1}$	0.9×10^{-1}
Zinc chloride	6.5×10^{-3}	4.4×10^{-4}
Nitrophenyl guanidino-benzoate (NPGB)	3×10^{-5}	5×10^{-9}
Diisopropyl fluorophosphate (DFP)	3×10^{-4}	3.5×10^{-5}
ε-aminocaproic acid (EACA)	$> 2 \times 10^{-1}$	2×10^{-1}

Mel-PA (≈ 20 FU/ml; ≈ 2×10^{-8}M) or urokinase (≈ 20 FU/ml; ≈ 3×10^{-10}M) in T-T (0.02) were mixed with various concentrations of inhibitors listed in the table in a total volume of 100 µl. After incubation for 60 min at 20°C, 50 µl were withdrawn from the mixture and assayed for amidolytic activity.

Although it has been reported (57) that neither fibrin nor fibrinogen affect the amidolytic actions of urokinase or "tissue activator" on synthetic substrates, I wished to satisfy myself that the same was true for Mel-PA. A number of experiments were therefore performed to examine this question and the results are presented in Figs 5.7 to 5.13. It is important to note that the fibrinogen used in these experiments was derived from different sources and purified by the method of Mosesson (162) to remove contaminating plasmin or plasminogen.

For the first experiment Mel-PA was purified by DE-3 affinity chromatography of Bowes II cell harvest fluid and samples were incubated either with solutions containing fibrinogen, a fibrin clot, casein, gelatin or buffer alone. Samples of urokinase were treated identically. After incubation at 20°C for 30 min samples were assayed for amidolytic activity with Cbz-Gly-Gly-Arg-AMC. The results (Fig. 5.7) were somewhat surprising in that they showed that fibrin and fibrinogen had increased the amidolytic activity of Mel-PA by approximately two-fold. Casein and gelatin had no effect on Mel-PA and none of the proteins tested enhanced the activity of urokinase.

A more detailed kinetic analysis of this phenomenon (Fig. 5.8a and b) showed that fibrinogen affected the Vmax of Mel-PA for the synthetic substrate but had no effect on the Km. Neither the Vmax nor the Km of urokinase for the synthetic substrate were altered significantly by fibrinogen.

During the course of these experiments it was noticed that the magnitude of the effect of fibrinogen on Mel-PA varied both with the Mel-PA and the fibrinogen preparations (Fig. 5.9 and Fig. 5.10). The activity of Mel-PA obtained from Bowes II cells cultured under serum-free conditions could be enhanced by as much as 10-fold by fibrinogen, whereas the same fibrinogen preparation enhanced the activity of other Mel-PA preparations

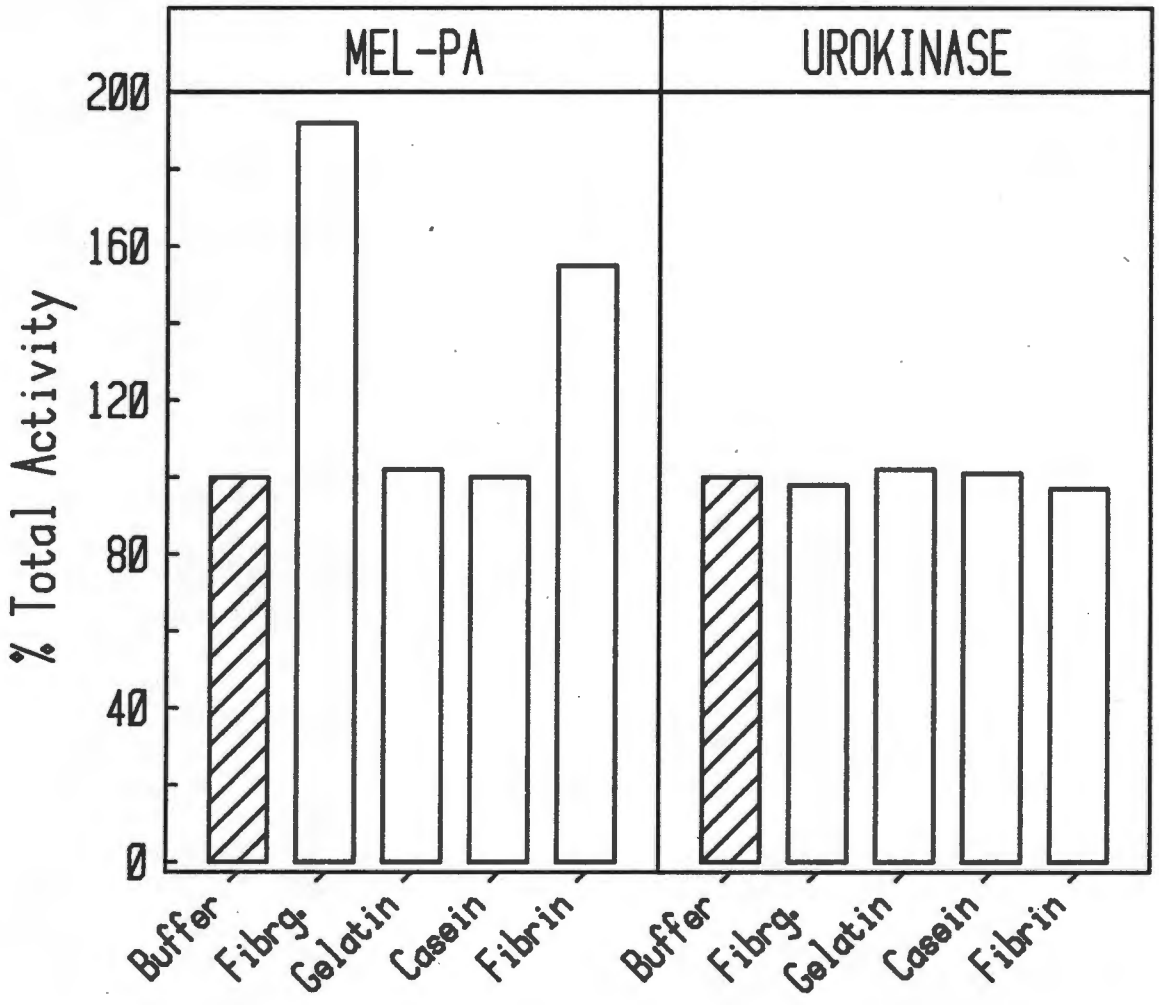


Figure 5.7 The effect of various proteins on the amidolytic activity of Mel-PA and urokinase.

Mel-PA (18 FU/ml) or urokinase (30 FU/ml) were incubated in a total volume of 100 µl of T-T (0.02) containing 0.1 mg fibrinogen, 0.1 mg gelatin, 0.1 mg casein or a washed fibrin clot. Fibrin clots were prepared by incubating 0.1 mg of fibrinogen with 0.1 units of thrombin in 100 µl PBS for 30 min at 37°C and then washed 3 times in T-T(0.1).

After incubation of the enzymes in the presence or absence of proteins for 30 min at room temperature, 50 µl samples were removed for determination of amidolytic activity using Cbz-Gly-Gly-Arg-AMC.

The bar graph in the figure shows that the amidolytic activity of Mel-PA was enhanced by fibrinogen and a fibrin clot, but not by gelatin or casein. None of the proteins had an effect on urokinase.

Figure 5.8

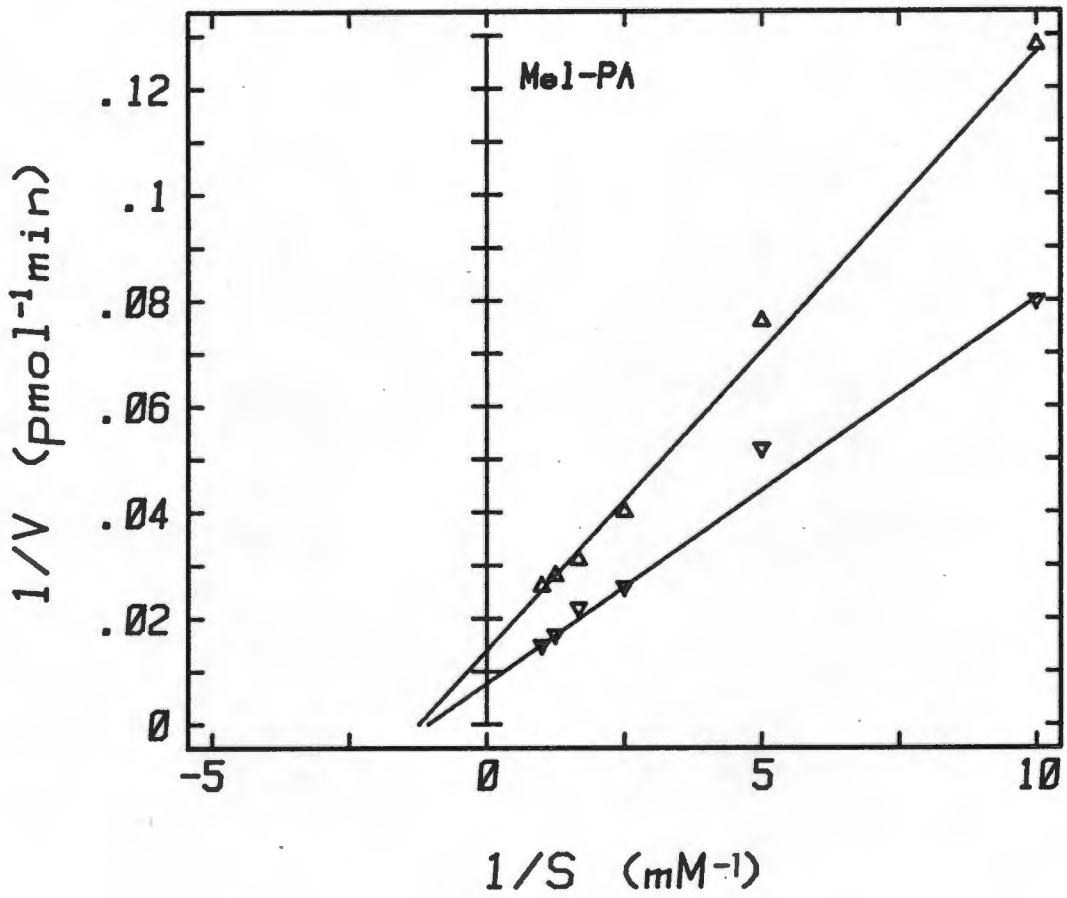
Figure 5.8 The effect of fibrinogen on the kinetic parameters of the amidolytic activities of Mel-PA and urokinase.

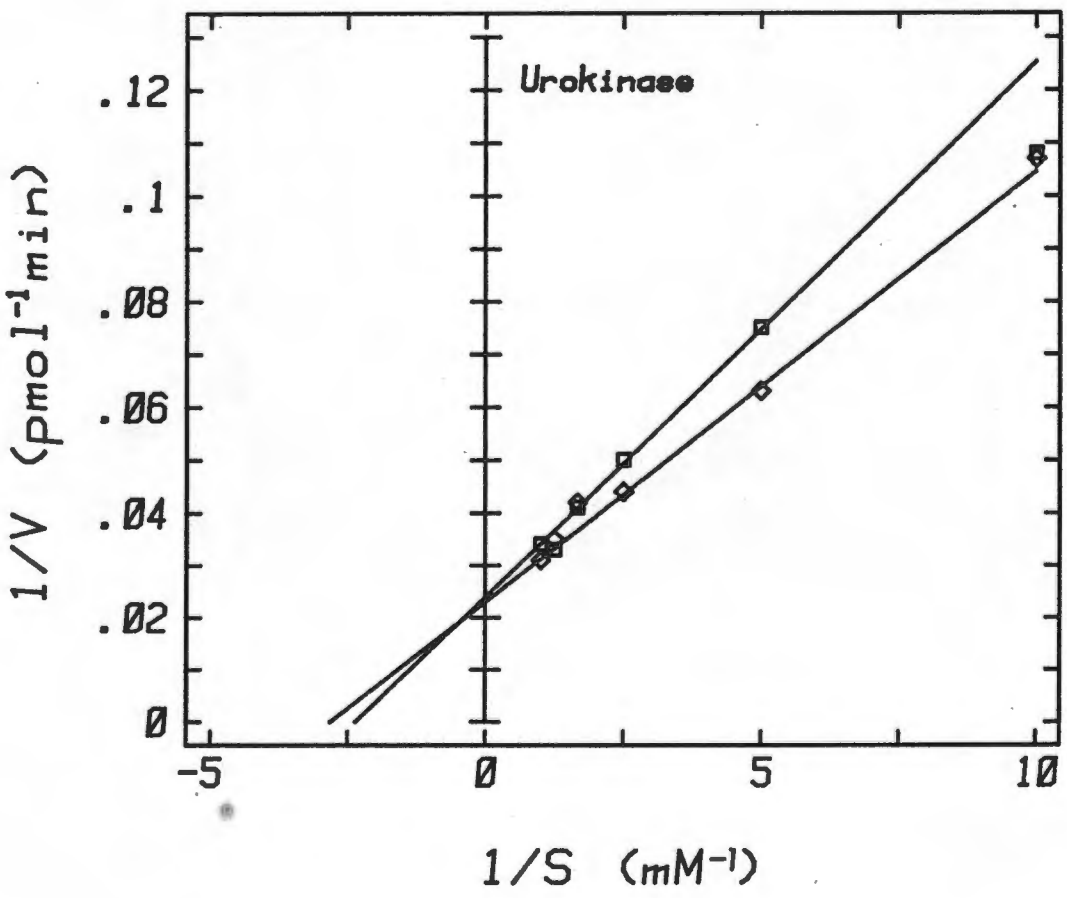
Mel-PA or urokinase were incubated at 20°C for 30 min in the absence or presence of 0.1 mg fibrinogen in a total volume of 1 ml of T-T(0.02). Amidolytic activities of the enzymes were then determined as a function of Cbz-Gly-Gly-Arg-AMC concentration by assay of 50 µl of the mixture under standard conditions.

The graphs show Lineweaver-Burk plots relating the amidolytic activity to substrate concentration. The kinetic parameters calculated according to the method of Cornish-Bowden and Eisenthal (167) were used to fit straight lines to the observed points.

The calculated results were as follows:-

		Parameters	
Enzyme	Fibrinogen	Km (mM)	Vmax (pmol/min)
Mel-PA	Absent △—△	0.81	71.9
	Present ▽—▽	0.94	129.7
Urokinase	Absent □—□	0.42	41.8
	Present ◇—◇	0.35	43.5


Mel-PA
1/V (pmol^{-1}min)
.12
.1
.08
.06
.04
.02
0
-5
0
5
10
1/S (mM^{-1})


Urokinase
1/V (pmol^{-1}min)
.12
.1
.08
.06
.04
.02
0
-5
0
5
10
1/S (mM^{-1})

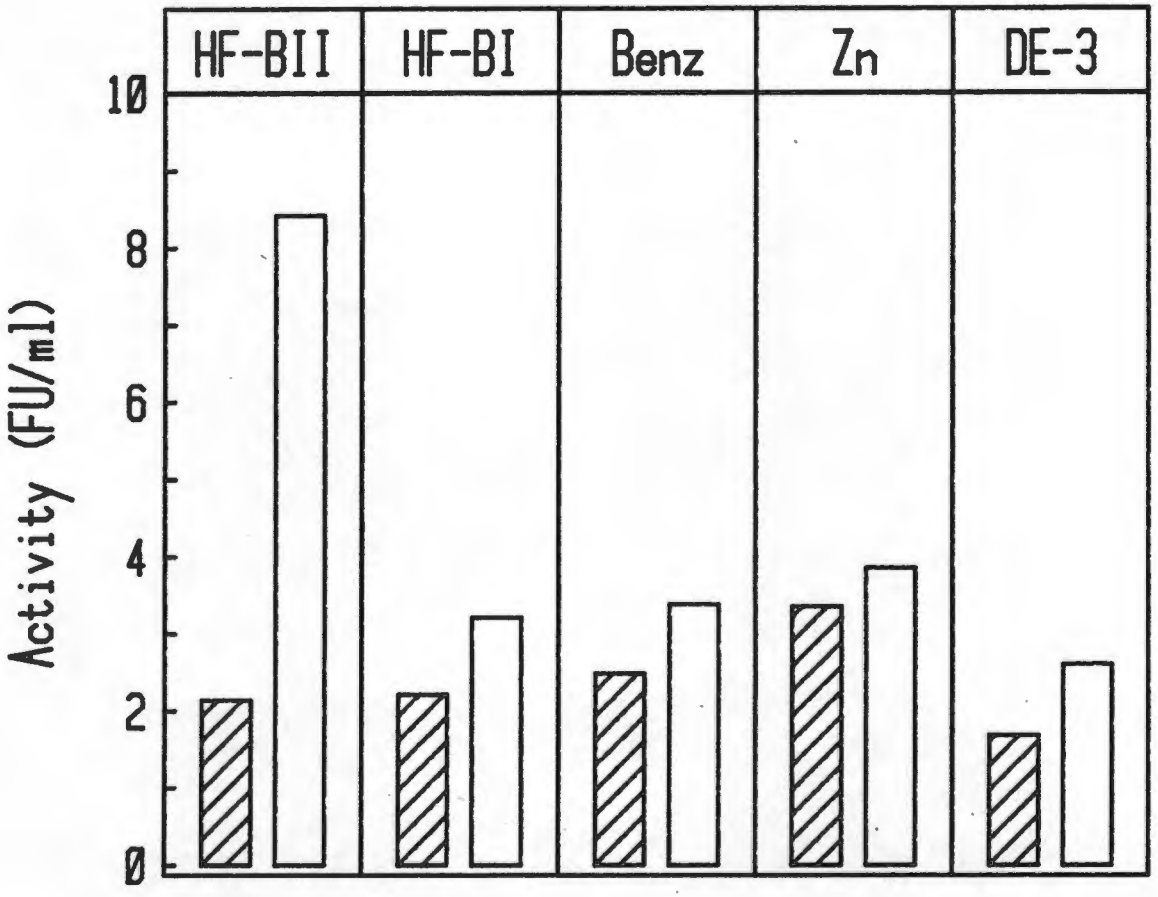


Figure 5.9 The effect of fibrinogen on different preparations of Mel-PA.

Five different Mel-PA preparations were diluted in T-T (0.02) to contain approximately 4-6 FU/ml.

Fifty microlitre samples of each of these solutions were mixed with either 50 µl of PBS (shaded bars) or 50 µl of PBS containing 0.2 mg of fibrinogen (open bars). After incubation for 10 min at 20°C, amidolytic activity of these mixtures was measured. The captions identify the Mel-PA preparations as follows:

(i) HF-BI : harvest fluid from Bowes I cells

(ii) HF-BII : harvest fluid from Bowes II cells

(iii) Benz : partially purified Mel-PA from Bowes I cells after benzamidine-agarose chromatography;

(iv) Zn : partially purified Mel-PA from Bowes I cells after Zn-chelate-agarose chromatography;

(v) DE-3 : purified Mel-PA from Bowes I cells after DE-3-agarose affinity chromatography.

Note that the amidolytic activities of different Mel-PA preparations were enhanced by the same fibrinogen preparation to different extents.

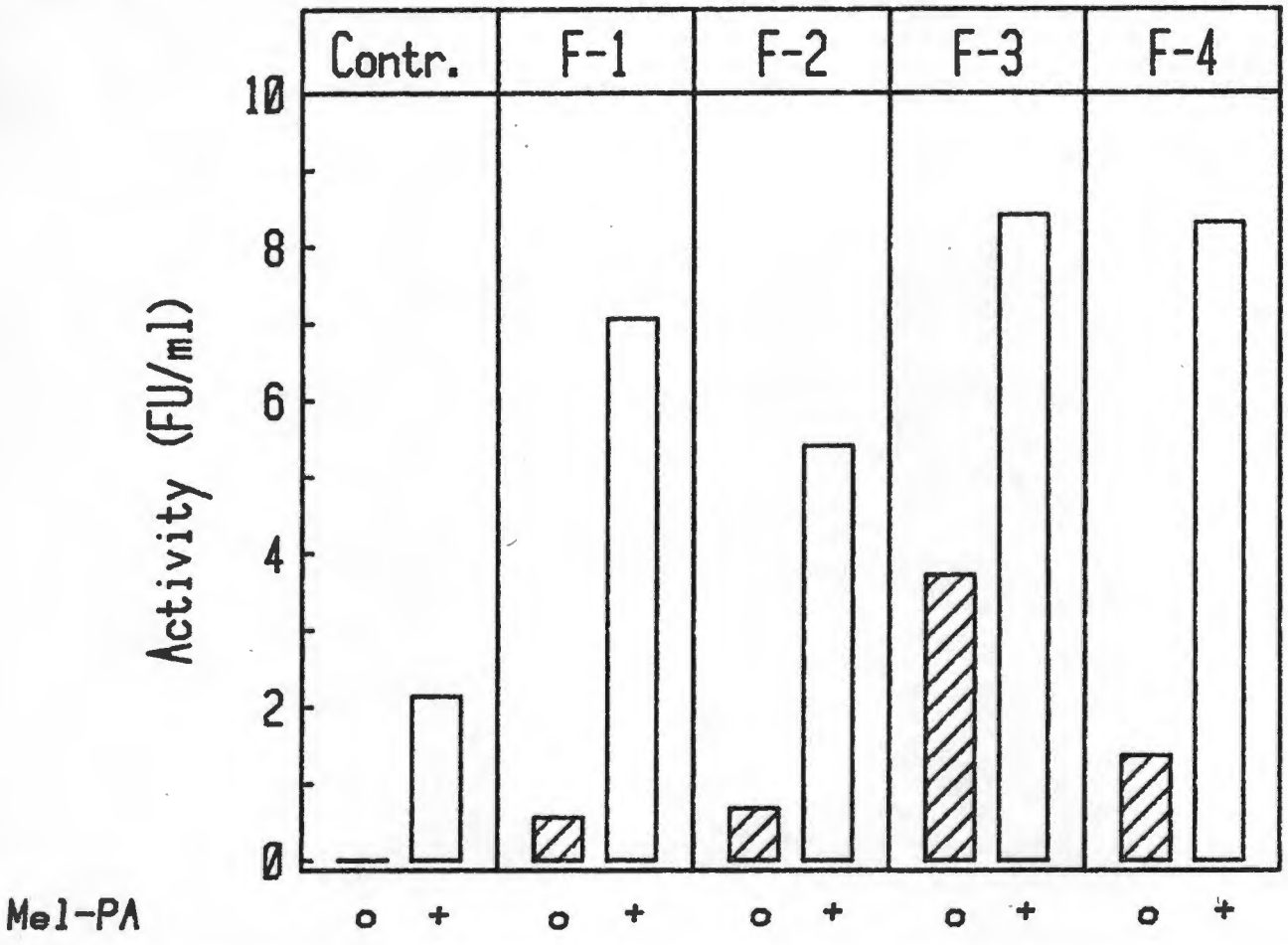


Figure 5.10 The effect of different preparations of fibrinogen on Mel-PA.

Two different preparations of human fibrinogen (F-1 and F-2) and t_{wo} of bovine fibrinogen (F-3 and F-4) were dissolved in T-T (0.02) buffer to give solutions containing 4 mg of protein /ml.

Samples of each of these solutions were diluted with equal volumes either of RPMI medium (shaded bars) or of Bowes II harvest fluid (open bars), and the amidolytic activity of each mixture was measured. Control samples were identical save for the absence of fibrinogen.

Mel-PA activity was enhanced to different extents by the different fibrinogen preparations.

All fibrinogen preparations had amidolytic activity with F-3 having the highest. This preparation also enhanced Mel-PA activity most effectively.

(notably those obtained from harvest fluids from Bowes I cells) only two-fold or not at all. This difference could not be ascribed to the presence of an inhibitor in the Bowes II cell harvest fluid, that was dissociated by fibrinogen, since Bowes II harvest fluid, that had been depleted of Mel-PA by passage over a DE-3 agarose affinity adsorbent (Chapter IV), had no effect on purified Mel-PA in the presence or absence of fibrinogen (Fig. 5.11).

When the different preparations of fibrinogen were tested for their effect on the same Mel-PA preparation (Fig. 5.10) they varied in the extent to which they were capable of enhancing the activity of Mel-PA. Furthermore it was noted that these fibrinogen preparations had amidolytic activity in the absence of Mel-PA, and that this activity correlated with the enhancement of Mel-PA activity.

The enhancement of Mel-PA activity by human fibrinogen occurred over a short period of time and reached a plateau after about 15 min of incubation (Fig. 5.12). The effect was also related to the fibrinogen concentration (Fig. 5.13). Enhancement of Mel-PA activity reached a plateau at a fibrinogen concentration of approximately 0.5 mg/ml (1.5 μM) or a fibrinogen:Mel-PA molar ratio of approximately 30:1. This rather large ratio and the fact that a plateau was reached indicated that the interaction between Mel-PA and fibrinogen was catalytic rather than stoichiometric in nature. This, taken together with the observations that fibrinogen affected the Vmax of the reaction but not the Km, and that the activities of different Mel-PA preparations were enhanced to different extents by the presence of fibrinogen, suggested that the fibrinogen contained an impurity that was capable of converting Mel-PA to a more catalytically active form without influencing the binding of the substrate to the enzyme. The observation that different fibrinogen preparations were

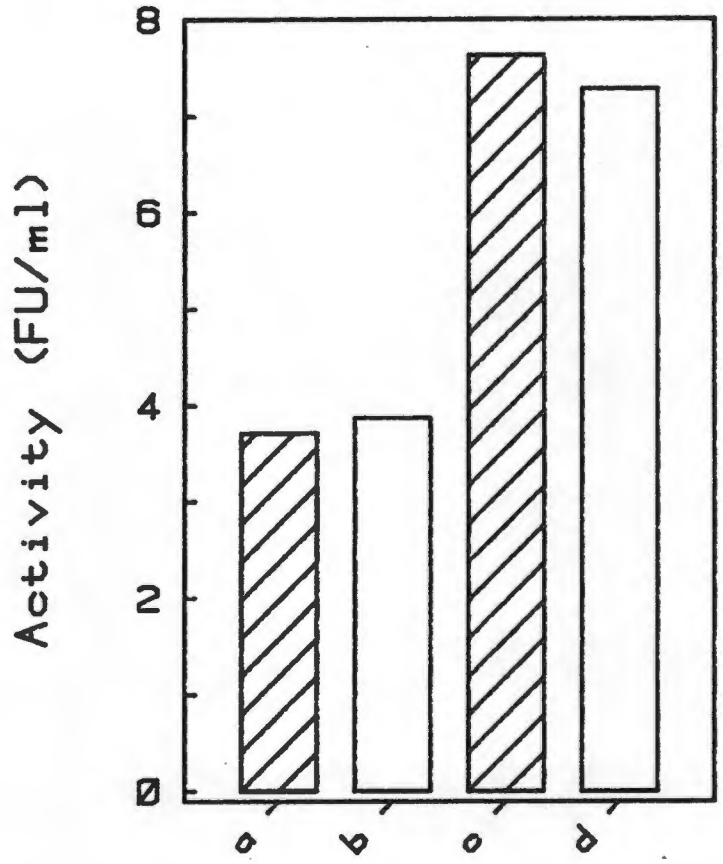


Figure 5.11 Effect of harvest fluid depleted of Mel-PA on the amidolytic activity of Mel-PA in presence and absence of fibrinogen.

Harvest fluid from Bowes II cells was depleted of Mel-PA by passage over a DE-3 agarose affinity adsorbent as described in Chapter IV. A purified Mel-PA preparation obtained by affinity chromatography on DE-3 agarose was diluted two hundred times either in medium containing 0.1% Triton X-100 or in harvest fluid depleted of Mel-PA.

Fifty microlitres of this dilution was mixed with 50 µl of PBS or 50 µl of PBS containing 4 mg/ml fibrinogen 3 (see Fig. 5.10). After incubation for 30 min at 20°C, the amidolytic activity in the samples was determined as described under Methods. Bar (a) represents the activity of Mel-PA diluted in medium and bar (c) represents the same in the presence of fibrinogen. Bar (b) and (d) represent the activity of Mel-PA diluted in harvest fluid depleted of Mel-PA in absence (b) and presence (d) of fibrinogen.

Harvest fluid depleted of Mel-PA had no effect on Mel-PA activity either in the presence or the absence of fibrinogen.

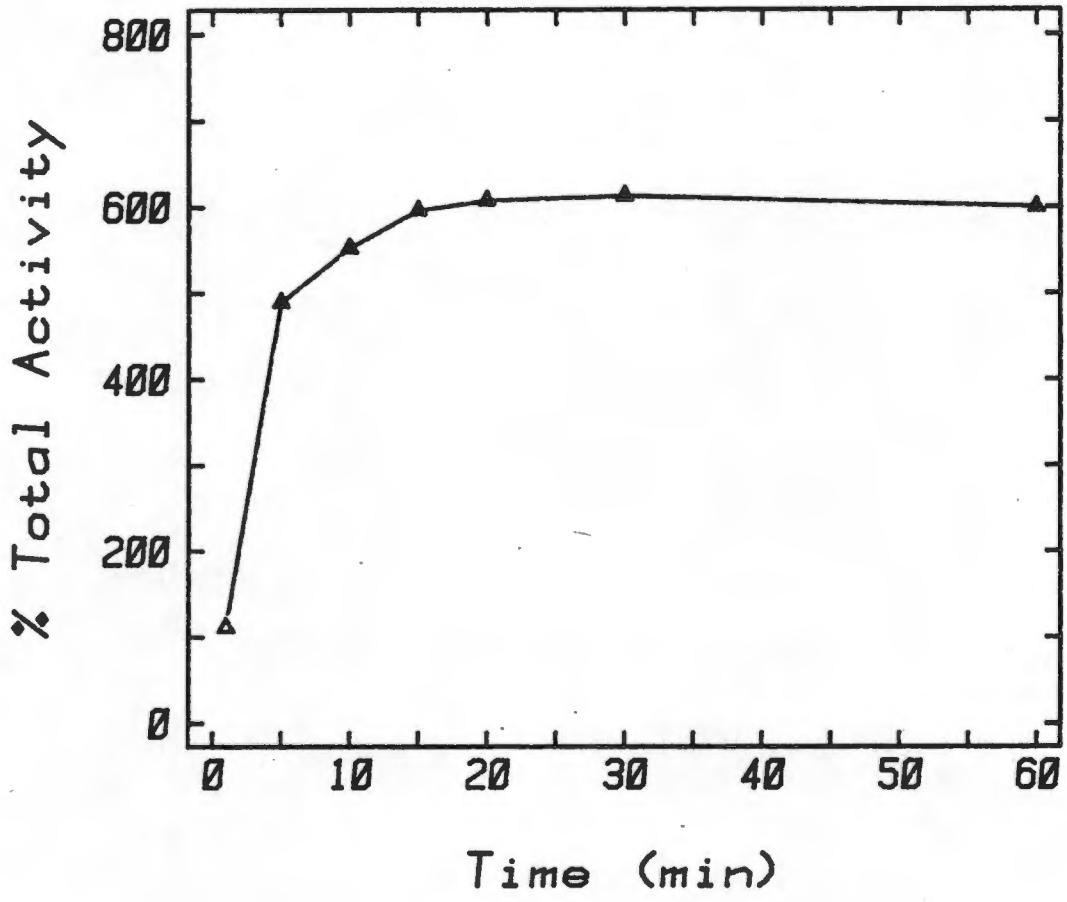


Figure 5.12 Time study of the effect of fibrinogen on Mel-PA.

Equal volumes of harvest fluid from Bowes II and fibrinogen 1 (F-1; Fig. 5.10) at 4 mg/ml were mixed and incubated at 20°C. At the times indicated, 50 µl samples were removed and assayed for amidolytic activity.

The results are expressed as the percentage of activity relative to a control sample containing no fibrinogen.

It can be seen that maximum enhancement was obtained after an incubation period of 15 min.

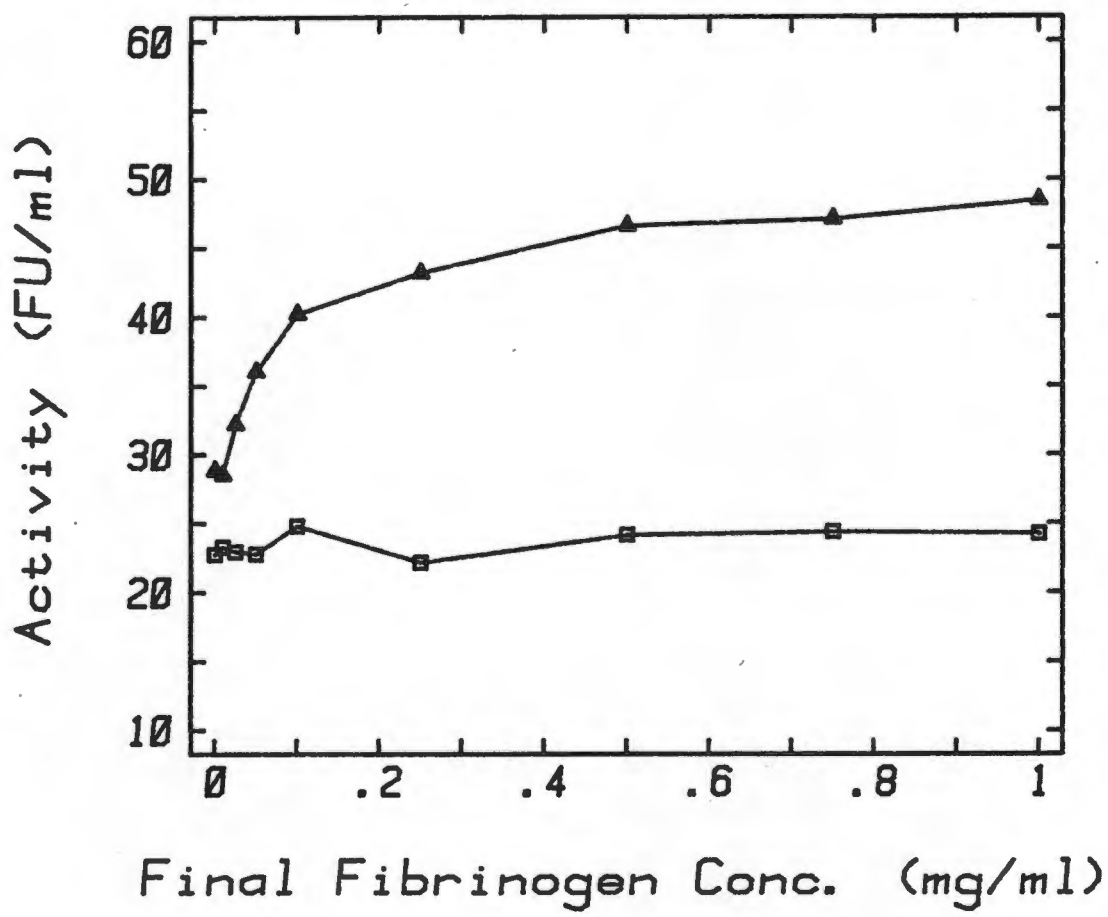


Figure 5.13 The effect of fibrinogen on Mel-PA and urokinase.

Ten microlitre volumes of solutions of fibrinogen 1 (F-1; Fig. 5.10) ranging in concentration from 0 to 10 mg/ml were added to a series of tubes containing 90 µl of a solution of either urokinase or Mel-PA in T-T (0.02). The Mel-PA was a purified preparation obtained by DE-3 agarose affinity chromatography (Chapter IV).

The tubes were incubated at 20°C for 60 min after which 50 µl samples were withdrawn for measurement of amidolytic activity.

The fibrinogen had no effect on urokinase (□—□) but enhanced the activity of Mel-PA (△—△) by approximately two-fold.

capable of hydrolysing the synthetic substrate in the absence of Mel-PA (Fig. 5.11) suggested that this impurity might be a protease.

The amidolytic activity of fibrinogen could be inhibited by NPGB, benzamidine, Trasylol and soybean trypsin inhibitor (Table 5.3). Attempts to inhibit the enhancing effect of fibrinogen on Mel-PA by the addition of trasylol or soybean trypsin inhibitor however failed, possibly because these compounds inhibited the fibrinogen-protease only weakly. Attempts to inhibit the protease irreversibly by treatment of the fibrinogen with DFP failed, because addition of isopropanol in which the DFP was dissolved resulted in precipitation of the protein. After treatment of the fibrinogen with 1 mM NPGB and dialysis, the amidolytic activity of fibrinogen was reduced by 33%, and the enhancing effect on Mel-PA was reduced by approximately 10% (Table 5.4).

The presence of DFP-binding proteins could be demonstrated in two fibrinogen preparations. This was achieved by the addition of 2 µl of 0.1M NPGB or 2 µl of DMSO to 50 µl of solutions containing 4 mg/ml of fibrinogen preparations 1, 3 (cf Fig. 5.10). The mixtures were then incubated for 1 hour at 37°C after which 2 µl ^{3}H-DFP (10 µCi; 6 Ci/mmol) was added and incubation was continued for 20 hours at room temperature. The proteins were mixed with an equal volume of 0.06M Tris-HCl pH 6.8 containing 2% SDS, 10% 2-mercaptoethanol and 20% glycerol, boiled and electrophoresed in a 5-15% polyacrylamide gradient slab gel, containing 0.1% SDS. Autoradiographs were made of the slab gel by exposure of an X-ray film for 5 weeks at -80°C. Faint bands of DFP binding proteins that were not present when the fibrinogen had been pretreated with NPGB were visible in the autoradiographs and corresponded to approximate molecular weights of 69 000, 67 000 and 65 000. A prominent band of MW 43 000 was visible in fibrinogen 3. Since the autoradiographic bands were too faint for photographic reproduction, I have included a diagram

Table 5.3 The effect of inhibitors on the amidolytic activity of fibrinogen

Inhibitor	conc. (mM)	% residual activity
NPGB	0.16	21.63
Benzamidine	167	18.39
EACA	167	100.00
BPTI	1667 KIU/ml	37.79
SBTI	0.16	37.79
DE-3	0.16	54.18
iodoacetamide	16.7	90.30
EDTA	16.7	91.63
EGTA	16.7	91.63

Thirty microlitres (120 µg) of fibrinogen 4 (F-4; Fig. 5.10) was added to 20 µl of T-T (0.1) and 10 µl of inhibitor solution. Control tubes were identical save for the fact that they received 10 µl of the relevant solvent without inhibitor.

The mixtures were incubated for 30 min at 20°C and 50 µl was withdrawn for assay of amidolytic activity. Residual activity obtained after incubation with inhibitor is expressed as the percentage of activity remaining relative to the inhibitor-free control.

Total activities were as follows:

fibrinogen + water = 3.24 FU/ml
fibrinogen + 10% DMSO = 3.19 FU/ml
fibrinogen + T-T(0.1) = 2.99 FU/ml

Benzamidine and NPGB inhibited the amidolytic activity most effectively.

Table 5.4 Effect of NPGB on the amidolytic and Mel-PA activating activities of fibrinogen.

Sample	Activity (FU/ml)	% of total activity
1. Fibrinogen	0.44	100
2. Fibrinogen (NPGB-treated)	0.29	66
3. Mel-PA	1.75	100
4. Mel-PA + Fibrinogen	7.96	430
5. Mel-PA + Fibrinogen (NPGB treated)	7.13	391

Twenty microlitres of 0.1M NPGB or DMSO alone were added to 2 ml of fibrinogen 1 (F-1; Fig. 5.10) containing 4 mg/ml protein, and incubated for 30 min at 37°C. The solutions were then dialysed for 20 hr at 20°C against PBS.

Thirty microlitres of harvest fluid from Bowes II cells were then incubated with 30 µl of NPGB-treated fibrinogen (5); 30 µl fibrinogen treated with DMSO (4); or 30 µl of PBS for 30 min at 20°C (3).

Thirty microlitres of NPGB-treated fibrinogen (2); or fibrinogen treated with DMSO (1) were incubated with 30 µl PBS. After incubation, the amidolytic activities of the samples were determined as described under Methods.

The results in the table show the amidolytic activity in each sample. It can be seen that NPGB reduced the amidolytic activity of fibrinogen by 33%. The activating effect of fibrinogen on Mel-PA was reduced by 10%.

to illustrate their electrophoretic mobilities relative to those of the marker proteins (Fig. 5.14).

The presence of caseinolytic proteases in the different fibrinogen preparations was also demonstrated by the zymographic method of Granelli-Piperno and Reich (62). Non-reduced samples of fibrinogen 1 or 3 were electrophoresed in 3-15% polyacrylamide gradient slab gels containing 0.1% SDS. After electrophoresis, the gels were washed in 2.5% Triton X-100 and overlaid on agar slabs containing casein or casein and plasminogen. After incubating at 37°C for 48 hr faint bands of plasminogen-dependent caseinolysis could be detected in all samples; fibrinogen preparation 3 showed several bands. The most prominent band in all samples had an approximate molecular weight of 63 000 (Fig. 5.15).

The association of proteolytic enzymes in the fibrinogen preparations with enhancement of Mel-PA activity suggested that susceptible Mel-PA preparations contained an inactive precursor enzyme that could be converted to active enzyme by limited proteolysis. This view was strongly supported by the experiments summarized in Fig. 5.16 to Fig. 5.18 and Table 5.5.

When amidolytic activities of different preparations of Mel-PA were measured before and after treatment with plasmin (8 µg/ml) for 60 min at 20°C varying degrees of enhancement were observed (Fig. 5.16). The time course and extent of this enhancement of Mel-PA activity with plasmin was similar to that observed when the same Mel-PA preparation was treated with fibrinogen (Fig. 5.17).

The presence of pro-plasminogen activator (Pro-Mel-PA) in some Mel-PA preparations could best be demonstrated by electrophoretic and enzymatic analysis of purified material obtained by DE-3 agarose affinity chromatography of harvest fluid from Bowes II cells (Chapter IV). The

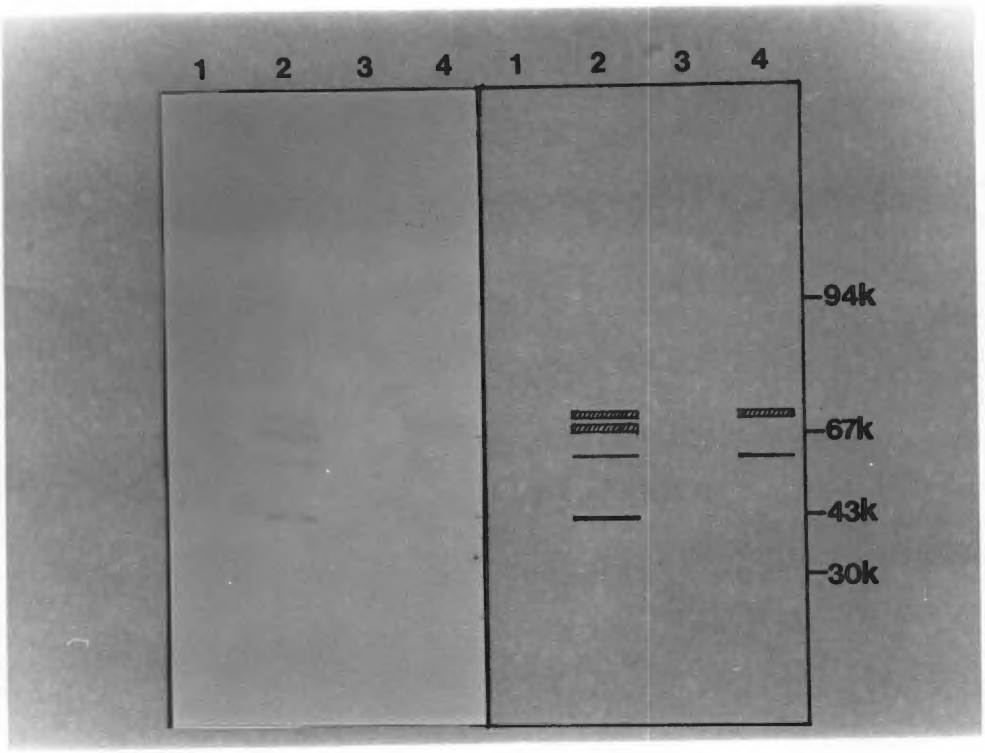


Figure 5.14 SDS-polyacrylamide gel electrophoresis of fibrinogen labelled with ^{3}H-DFP.

Two microlitres of 0.1M NPGB in DMSO or 2 µl of DMSO only were added to 50 µl of solutions containing 4 mg/ml of fibrinogen 1 or 3 (F-1 or F-3; Fig. 5.10). The mixtures were incubated for 1 hr at 37°C after which 2 µl of ^{3}H-DFP (6 Ci/mmol; 10 µCi) was added to each mixture and incubation was continued for 20 hr at 20°C. The protein solutions were then mixed with equal volumes of 0.06M Tris HCl pH 6.8 containing 2% SDS, 10% 2-mercaptoethanol and 20% glycerol. After boiling for 1 min the samples were electrophoresed in a 5-15% polyacrylamide gradient slab gel containing 0.1% SDS. Autoradiographs were made of the dried slab gel by exposure to an X-ray film for 5 weeks at -80°C (Appendix 2).

The figure shows a diagrammatic representation of the autoradiograph. Wells 1 and 3 contained samples of fibrinogen 1 and 3 that had been pretreated with NPGB. Wells 2 and 4 contained the same fibrinogens pretreated with DMSO only.

Faint bands of DFP binding proteins were visible and corresponded to molecular weights of 69 000, 67 000, 65 000 and 43 000 daltons. Pre-incubation with NPGB abolished DFP binding.

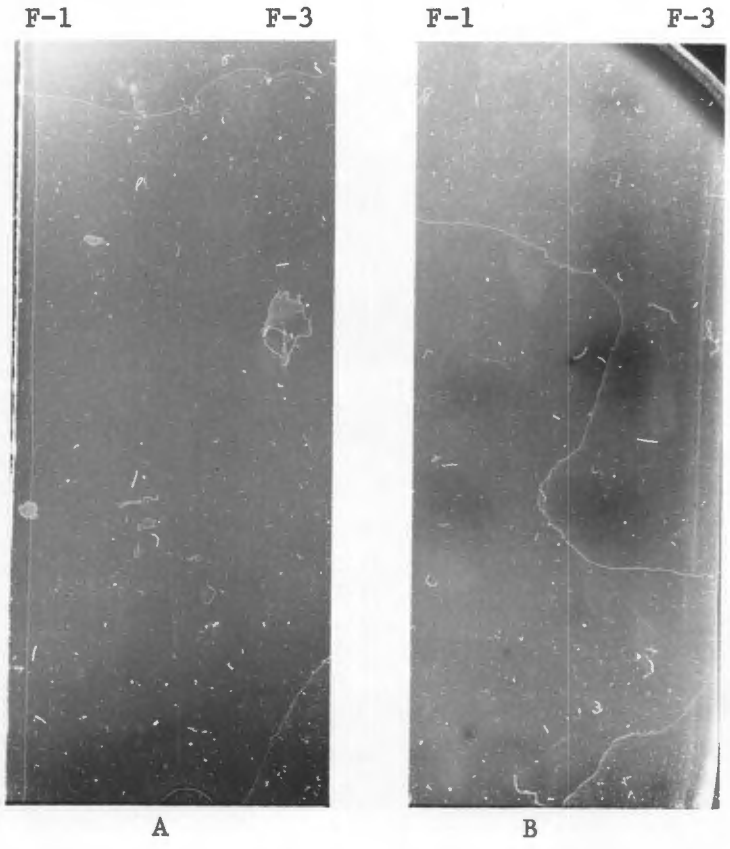


Figure 5.15 Analysis of fibrinogen preparations by electrophoresis and zymography.

Solutions of fibrinogen 1 and 3 (cf. Fig 5.10) containing 2 mg/ml were made 1% with respect to SDS and 10% with respect to glycerol and 15 µl samples were electrophoresed in a 3-15% polyacrylamide gradient slab gel containing 0.1% SDS.

After electrophoresis the slab gel was washed in 2.5% Triton X-100 in water for 1 hour and then in water.

The slab was then overlaid on agar slabs containing 1.25% agar and 20 mg/ml casein, with (Gel B) or without (Gel A) 5 µg/ml plasminogen. The assemblies were incubated at 37°C for 48 hr.

Faint bands of caseinolysis could be detected.

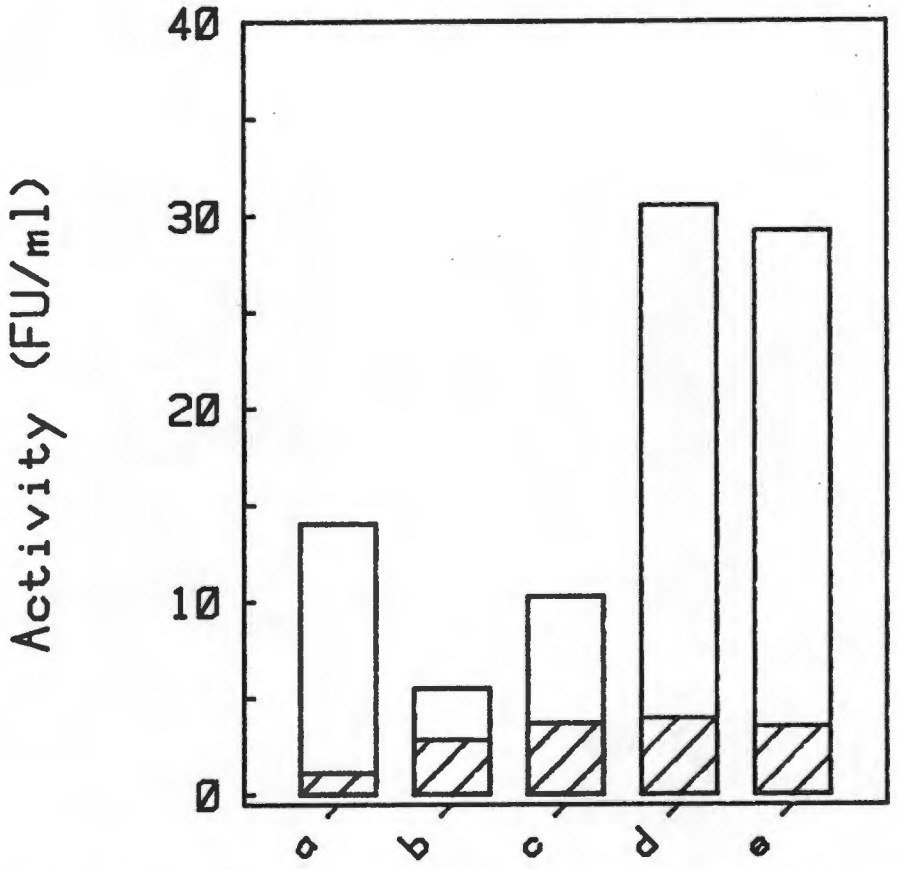


Figure 5.16 The effect of plasmin on various Mel-PA preparations

Various Mel-PA preparations were diluted to contain approximately 4 FU/ml in T-T (0.1), and 295 µl samples of these solutions were added to 5 µl of plasmin solution (0.5 mg/ml) or 5 µl PBS and incubated for 60 min at 20°C.

Fifty microlitres were then removed and added to 10 µl of a solution of BPTI (1000 KIU/ml). Fifty microlitres was then withdrawn from this mixture and the amidolytic activity was measured. The shaded bars represent amidolytic activity in the absence of plasmin and the open bars represent the activity in the presence of plasmin. Mel-PA samples included

a) harvest fluid from Bowes II cells;

b) harvest fluid from Bowes I cells;

c) Mel-PA from Bowes II cells purified on DE-3-agarose adsorbent

d) and e) Mel-PA from Bowes II cells on DE-3-agarose adsorbent in the presence of 0.1 KIU/ml BPTI.

Plasmin enhanced the activity of the various preparations to varying extents. By subtracting the amidolytic activity observed in absence of plasmin from that observed in presence of plasmin the amount of pro-Mel-PA in the samples could be calculated. It can be seen that harvest fluid from Bowes II cells grown in absence of serum contained the highest amount of pro-Mel-PA Purification of Mel-PA from this harvest fluid caused a certain degree of proactivator activation. This could be inhibited by BPTI.

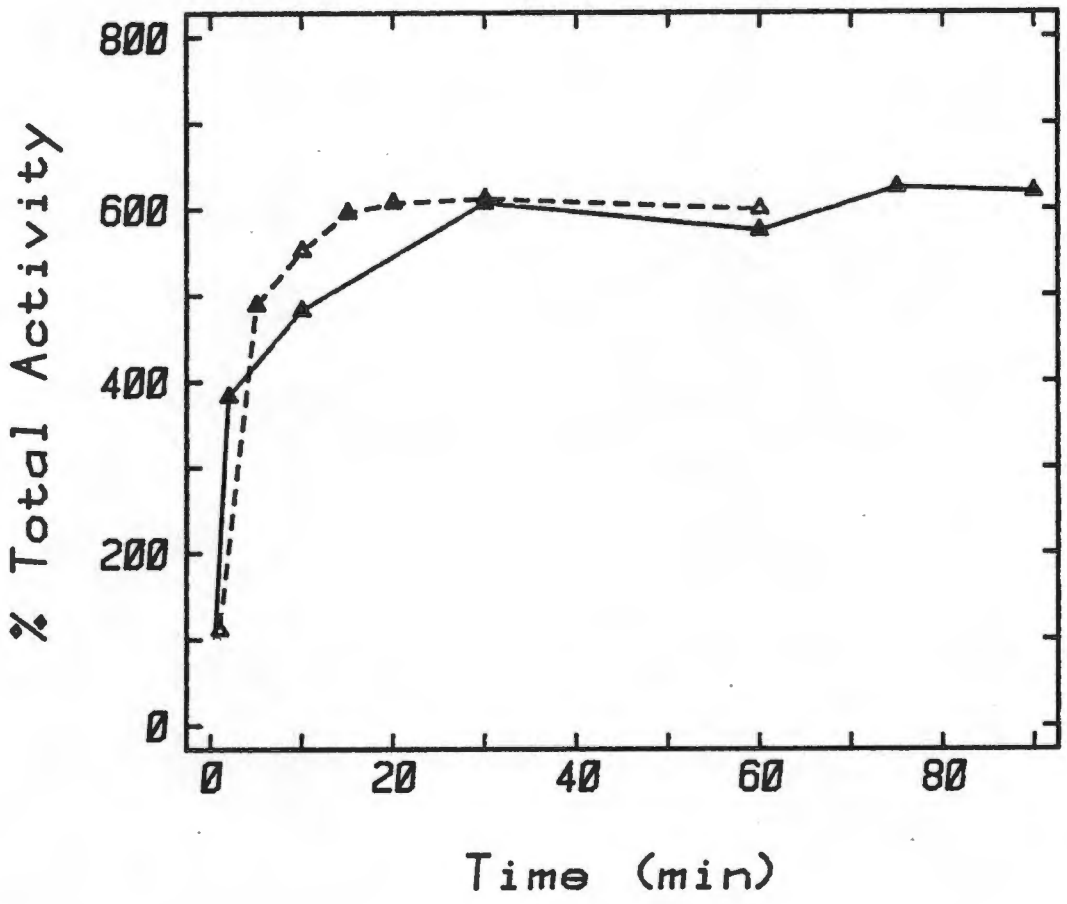


Figure 5.17 Time study of Mel-PA activation by plasmin.

Harvest fluid from Bowes II cells was adjusted to contain 8 µg/ml of plasmin and incubated at 20°C. At the times shown, 50 µl samples were removed and added to 10 µl of a solution of BPTI (1000 KIU/ml). This solution (50 µl) was then assayed for amidolytic activity (Δ—Δ). The results are expressed as a percentage activity observed relative to a control incubated in the absence of plasmin. Plasmin enhanced the activity of the Mel-PA to a maximum of 6-fold in approximately 30 min.

In the presence of fibrinogen (Δ--Δ; cf Fig. 5.12) the same Mel-PA preparation was also enhanced 6-fold.

results of a typical experiment are shown in Fig. 5.18. The preparation used in this experiment contained 80% of Mel-PA as pro-activator as judged by amidolytic assay before and after plasmin treatment. This sample contained only one protein band with a molecular weight of approximately 70 000 daltons after electrophoresis in SDS-polyacrylamide gels under non-reducing conditions. Under reducing conditions most of the protein also migrated in the 70 000 dalton region. Plasmin treatment converted the 70 000 dalton, single chain enzyme to the S-S-linked, two subunit form. Under reducing conditions this migrated as two bands with apparent molecular weights of 35 000 daltons and 38 000 daltons.

These experiments were still open to the interpretation that the one chain form of Mel-PA was not a true "pro-" enzyme in the sense that it possessed enzyme activity albeit with a low specific activity. To exclude this interpretation a preparation containing a mixture of active enzyme and putative pro-Mel-PA was incubated with 5 mM DFP to inhibit all detectable traces of amidolytic activity. The DFP was removed, the residual pro enzyme was treated with plasmin. After neutralization of the plasmin with BPTI, amidolytic activity was measured to give the results summarised in Table 5.5. These show that Mel-PA was indeed present in a form that was enzymatically inactive, impervious to the action of DFP, and activatable by plasmin to active Mel-PA.

Neither trypsin nor thrombin converted Pro-Mel-PA to Mel-PA when added at low concentrations (1×10^{-16}M trypsin; 1.6×10^{-6} units/ml thrombin) and trypsin at high concentration (1×10^{-9}M) caused a decrease of Mel-PA activity.

These experiments also showed that Mel-PA released by Bowes II melanoma cells cultured in the complete absence of serum was present mainly

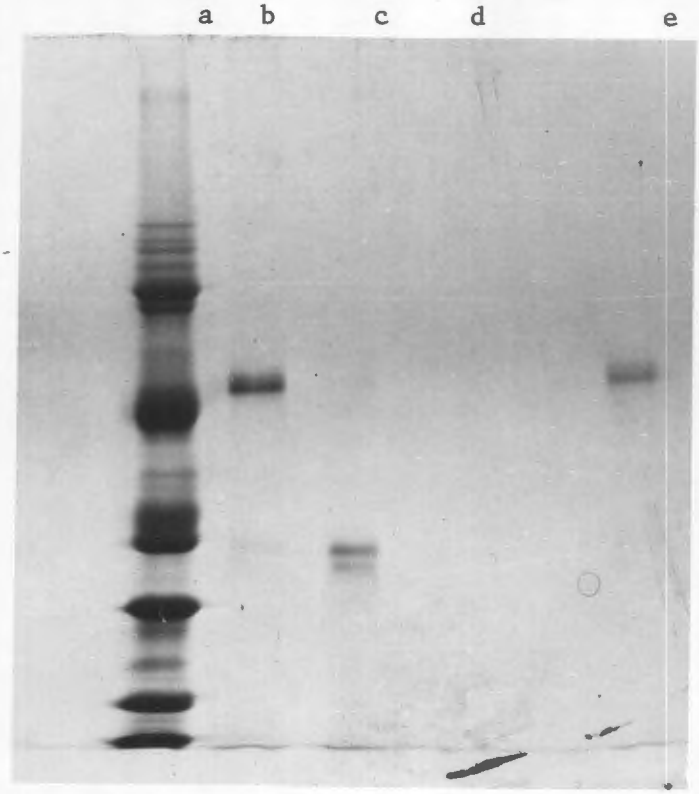


Figure 5.18 Limited proteolytic cleavage of pro-Mel-PA to Mel-PA by plasmin.

Mel-PA was purified by affinity chromatography on DE-3 agarose (Chapter IV) to give a solution containing 100 µg of protein/ml, and dialysed into T-T(0.1).

Samples of this solution were mixed with equal volumes of PBS or PBS containing 5 µg/ml of plasmin. After incubation for 16 hr at 20°C, SDS was added to a final concentration of 0.1% and the proteins were precipitated with 6% TCA. The precipitates from 200 µl samples of original enzyme solution were washed in acetone and redissolved in 20 µl of 0.06M Tris HCl pH 6.8 containing 1% SDS and 10% glycerol. Where necessary, these samples were reduced at this stage by the addition of 2 µl of 1 M DTT and incubation at 37°C for 30 min.

All samples were then boiled for 1 min, and 20 µl of each was electrophoresed in a 5-15% polyacrylamide gel slab containing 0.1% SDS. After electrophoresis the gel was stained with Coomassie brilliant blue and destained as described in the Appendix (A2). The tracks contain

(a) molecular weight markers;
(b) untreated Mel-PA, reduced;
(c) plasmin-treated Mel-PA, reduced;
(d) plasmin alone
(e) untreated Mel-PA, nonreduced

As can be seen from the photograph, plasmin treatment converted the one-chain form of Mel-PA into a two-chain form.

Table 5.5 Activation of DFP-treated, pro-Mel-PA with plasmin.

	Treatment		Activity (FU/ml)
	DFP	Plasmin	
a)	+	-	0.00
b)	+	+	8.23
c)	-	-	6.25
d)	-	+	17.48

Samples (500 µl) of a Mel-PA preparation containing approximately 20 FU/ml of total activator in T-T(0.1) were incubated in the presence (a and b) or the absence (c and d) of 5 mM DFP for 60 min at 20°C. Free DFP was removed according to the method of Penefsky (228) by centrifugation of 100 µl samples of the reaction mixtures through 1 ml columns containing Sephadex G25 fine equilibrated with T-T(0.1).

Volumes of 3 µl of 0.5 mg/ml plasmin (b and d) or 3 µl of PBS (a and c) were added to these samples and they were incubated for 30 min at 20°C. Fifty microlitres of each sample was then removed from the solution and added to 10 µl BPTI (1000KIU/ml) to inhibit plasmin activity. The amidolytic activity in each sample was then determined using the fluorometric assay.

Note that the sample had a total Mel-PA content of 17.48 FU/ml (d) of which 6.25 FU/ml was present in active enzyme form (c). The active enzyme was inhibited to undetectable levels by treatment with DFP (a). After treatment with DFP active enzyme could be generated by incubation with plasmin, showing that the precursor of Mel-PA was resistant to DFP treatment and lacked measurable enzyme activity. It is therefore reasonable to assume that pro-Mel-PA is a true pro-enzyme.

in the pro-activator form. On the other hand, Mel-PA obtained from Bowes I cells that required the intermittent presence of serum, was mainly present as the active enzyme. Treatment of Mel-PA in harvest fluid from Bowes II cells with low concentrations of foetal calf serum (0.01% to 2%) resulted in conversion of Pro-Mel-PA to Mel-PA (Table 5.6).

It was concluded from these results that Mel-PA is secreted by melanoma cells mainly as the inactive precursor enzyme. This becomes converted to the active enzyme by proteases present in foetal calf serum added to the medium. Purification of Mel-PA from harvest fluid of Bowes II cells usually yielded a mixture of Pro-Mel-PA and Mel-PA in roughly equal proportions. If basic pancreatic trypsin inhibitor (BPTI) (0.1 KIU/ml) was included during the purification procedure using DE-3 agarose, approximately 90% of the Mel-PA was obtained in Pro-Mel-PA form (Fig. 5.16).

Table 5.6 The effect of foetal calf serum on Mel-PA

FCS (% (v/v))	Activity of Mel-PA (FU/ml)
0	
.01%	5.85
.02%	5.76
.1%	5.74
.2%	6.45
1%	9.03
2%	9.11

Ninety microlitres of harvest fluid from Bowes II cells were mixed with 10 µl of foetal calf serum diluted in RPMI and incubated for 3 hr at 20°C. Amidolytic activity was determined in 50 µl samples withdrawn from the mixtures.

The values for activity given in the table represent activities obtained after subtraction of activities observed when foetal calf serum was incubated in medium only. This amounted to 1.15 FU/ml on average for all dilutions tested.

DISCUSSION

Immunochemical and electrophoretic studies reported in previous chapters have indicated that differences exist between urokinase and Mel-PA. It was therefore of importance to establish whether differences in these enzymes existed with respect to their enzymatic and functional properties. In this chapter I report the results of a series of experiments that were designed to analyse the catalytic properties of these enzymes on well defined synthetic substrates. In addition, the effects of various protease inhibitors and macromolecules, such as fibrinogen, on the catalytic activity of the two activators were examined.

In the first part of this chapter it was established that both urokinase and Mel-PA bind radioactive DFP, and that the DFP binding proteins had an approximate molecular weight of 73 000 daltons, in the case of Mel-PA, and 56 000 daltons and 32 000 daltons in the case of urokinase. Both activators were composed of two polypeptide chains joined by disulphide bridges. The subunit containing the active serine site of Mel-PA had a molecular weight of approximately 35 000 daltons and was larger than that of urokinase which had a molecular weight of approximately 32 000 daltons. The active site of Mel-PA was located on the smaller subunit of the two chain molecule. These results, that most plasminogen activators are composed of two polypeptide chains (68,130,134,170), correlate roughly with those reported in the literature. My results agree with those of Rijken et al (130) who found that the active site of Mel-PA was located on the smaller subunit of the two chain molecule.

The major part of this chapter was concerned with the differentiation of the two plasminogen activators with respect to their catalytic properties towards simple synthetic substrates. Well-defined synthetic

substrates have been widely used in the past to differentiate between enzymatic specificity of different enzymes. A number of synthetic substrates that are specifically useful for the analysis of plasminogen activators have already been synthesised. These include chromogenic substrates (158,171,172) and the more sensitive fluorogenic substrates (133,155,156,173,174). In both cases it has been shown that plasminogen activators from various sources are able to catalyse the hydrolysis of tripeptide substrates with arginine or lysine (only in the case of urokinase) at the C-terminal end adjacent to the leaving groups (155,156,157). "Tissue plasminogen activator" could be clearly differentiated from urokinase by its requirement for blocked N-terminal groups in these substrates (155,156).

In the experiments described in this chapter I have compared Mel-PA and urokinase for their ability to hydrolyse two fluorogenic substrates, namely Cbz-Gly-Gly-Arg-AMC and Boc-Val-Gly-Arg-AMC. The first of these substrates proved to be a better substrate for urokinase (Km = 0.42 mM) than for Mel-PA (Km = 0.81 mM) whereas the second substrate was worse for urokinase (Km = 1.47 mM) and better for Mel-PA (Km = 0.48 mM). The catalytic constant of urokinase for the first substrate was approximately 70 times larger than the corresponding value for Mel-PA, whereas in the second substrate it was only 16 times larger. Urokinase, therefore, seemed to be the more catalytically active enzyme as far as these two synthetic substrates were concerned.

Using Cbz-Gly-Gly-Arg-AMC for the measurement of amidolytic activity I have arbitrarily defined 1 fluorometric unit (1 FU) as that amount of enzyme that could hydrolyse 10 pmol of substrate in one minute at 25^{o}C in 0.1M Tris HCl pH 8.1 containing 5 x 10^{-4}M substrate and 4% (v/v) DMSO.

The assay of plasminogen activators using the synthetic substrate Cbz-Gly-Gly-Arg-AMC proved to be a useful standard assay for enzyme quantitation and formed the basis for comparison of the two activators in more complex assays involving plasminogen-dependent fibrinolysis. These experiments will be described in the next chapter.

Active site titration of the activator with ^{3}H-DFP of known specific activity enabled me to show that one fluorometric unit of Mel-PA represented 1.012 pmol of active enzyme and one fluorometric unit of urokinase represented 0.0149 pmol of enzyme. Having these catalytic constants has meant that enzyme samples could be standardised by fluorometric assays and their concentrations expressed in terms of pmol of active sites/ml. Other commonly used methods for active site titration of trypsin-like enzymes (164, 175, 181) could not be used for the titration of Mel-PA.

Apart from its usefulness for the direct quantitation of plasminogen activators, the fluorogenic substrate also proved useful for the analysis of the effects of inhibitors on Mel-PA and urokinase. The secondary inhibitory effects on plasmin that are encountered in the commonly used two-stage assays for plasminogen activators could thus be excluded.

Urokinase and Mel-PA appeared to differ in their susceptibility to a variety of inhibitors. The most pronounced difference was seen in the case of NPGB. Differences in the susceptibility to other inhibitors such as DFP and benzamidine could be merely a reflection of the different amounts of enzyme present in the assay. In these assays usually about 100 times more Mel-PA in terms of pmol of active enzyme than urokinase was present. From these studies it can be concluded that Mel-PA is a serine protease with trypsin-like specificity and is in this respect similar to plasminogen activators studied so far (68,74,80,109,116,118,130,176). It

is also similar to other activators in that it was not inhibited by any of the macromolecular trypsin inhibitors tested (133,142). So far, none of the commonly used inhibitors would be useful to clearly differentiate between urokinase and Mel-PA. The only useful inhibitor in this respect is the DE-3 inhibitor described in Chapter IV.

It has been reported in the literature by various investigators that urokinase and the "tissue activator" or the "vascular activator" differ in their interaction with fibrin. "Tissue activators" and "vascular activators" have been found to bind to fibrin (58,57,130,161,169) whereas urokinase does not (58,161,169), unless it is in a pro-activator form (177). In addition, plasminogen activation by tissue and vascular activators is greatly enhanced by the presence of fibrin (53,57,159,160), while urokinase is not affected (53,57). In this chapter I have reported unexpected results showing that Mel-PA activity was enhanced in the presence of fibrinogen and fibrin. At first this seemed to conflict with the results of other investigators. Usually "tissue activator" activity is not enhanced by fibrinogen (57), and neither fibrin nor fibrinogen are believed to have an effect on "tissue activator" activity if this is measured in amidolytic assays (57). I was able to show, however, that fibrinogen did have an effect upon Mel-PA that correlated with the presence of a contaminating protease. This protease, the precise nature of which I was not able to define, caused the conversion of a pro-enzyme form of Mel-PA (Pro-Mel-PA) to the active enzyme, Mel-PA, and thus increased the effective enzyme concentration in the assays. This was reflected by an increase in the Vmax of Mel-PA for the synthetic substrate, while the Km was not significantly affected. From these results I was able to explain the great variations in Mel-PA activities I observed when different Mel-PA preparations were measured in the presence of different fibrinogen preparations.

The presence of Pro-Mel-PA in various proportions in different

preparation of the enzyme could be demonstrated by the fact that plasmin treatment of these samples resulted in an increase in amidolytic activity. Furthermore, this increase was similar to that observed if the sample was treated with fibrinogen. Using a purified Mel-PA preparation it could be shown that plasmin caused the conversion of an inactive, DFP-resistant, one chain form of Mel-PA to an active two chain form with an active site serine residue available for covalent binding to DFP.

The existence of a one chain form of Mel-PA has also been demonstrated by Rijken et al (130,179) who showed that the yield of the one chain form could be greatly increased by inclusion of trasylol during cell culture and purification. Similar findings were reported by others working with urokinase (134,178). It can therefore be assumed that plasminogen activators are released in a pro-activator form from cells and tissues, and become converted to the active enzyme by proteases in the surrounding medium. The source of this protease in the case of melanoma cells cultured in vitro is presumably the foetal calf serum usually used to maintain growth of such cells. I was able to support this suggestion by the observations that a melanoma cell line that could be cultured in complete absence of serum (Bowes II cells; Chapter I) released Mel-PA mainly in the pro-activator form. Mel-PA present in harvest fluid from serum-dependent cells was mainly in the active two-chain form. Low concentration of foetal calf serum could be shown to increase Mel-PA activity in harvest fluid from Bowes II cells.

During the course of my studies I noticed at a fairly early stage that urokinase and Mel-PA differed markedly in their behaviour within and between various different assays where plasminogen alone, or plasminogen and a plasmin substrate, such as fibrin, were present. Such differences in behaviour of various plasminogen activators in different assay systems

was noticed by others (53,57,180) and has been ascribed mainly to the effect fibrin has on the "tissue" or "vascular" type activators. Since these functional differences between the activators have important implications for their possible physiological roles *in vivo* and for their quantitation *in vitro*, I have undertaken a study of these two enzymes with regard to their plasminogen activating potential in the presence and absence of other macromolecules. These experiments will be described in the following chapter and the results will be discussed in the light of conclusions reached from the experiments described in the present chapter.

CHAPTER VI

ACTIVATION OF PLASMINOGEN BY MEL-PA AND UROKINASE: COMPARISON OF ACTIVATION KINETICS AND EFFECT OF FIBRINOGEN, FIBRIN AND OTHER MOLECULES ON THE REACTION.

In the previous chapter I considered and compared urokinase and Mel-PA for their ability to function as plasminogen-independent amidolytic enzymes. In this chapter, I examine their contribution to the more complex phenomenon of plasminogen activation.

One might have predicted that the limited proteolytic action of Mel-PA and urokinase on their putative natural substrate, plasminogen, would have been essentially similar to their action as amidolytic enzymes when acting upon low-molecular-weight, synthetic substrates. While this prediction proved to be reasonably valid in the case of urokinase, it was far from accurate where Mel-PA was concerned.

In the case of Mel-PA I found that the ability of this protease to activate plasminogen was limited by factors other than the conventional limitations of substrate availability or of enzyme concentration. Furthermore, the catalytic behaviour of Mel-PA was unusual inasmuch as it was strongly influenced by the presence of fibrin or fibrinogen. Finally, Mel-PA showed a pronounced tendency to bind to fibrin that was less prominent with urokinase. The implications of these observations for the *in vivo* functions of these two enzymes and for their *in vitro* assay are considered.

MATERIALS AND METHODS

The melanoma plasminogen activator was obtained and isolated from Bowes I melanoma cells cultured *in vitro* as described in Chapter I and Chapter IV.

Partially purified human urokinase was obtained commercially from Leo Pharmaceutical Products, Ballerup, Denmark, as a white lyophilized powder which, in SDS-gel electrophoresis, was found to contain many impurities (Appendix A1.5). The contents of one vial (5000 Ploug units) were dissolved in a suitable volume (usually 2.5 ml) of 0.1M Tris HCl pH 8.1 containing 8 mg/ml bovine serum albumin or 0.1% Triton X-100. The solution was dispensed into 50 µl volumes and stored in liquid nitrogen.

Human fibrinogen (A.B. Kabi, Stockholm, Sweden) or bovine fibrinogen (Sigma Chemical Company, St. Louis, Mo., U.S.A. Fraction I, F-4000) was further purified by salt precipitation according to the method of Laki (182) and freed from plasminogen by the method of Mosesson (162). The latter method exploits the differential solubility of fibrinogen and plasminogen in the presence of lysine. The methods are described in detail in the Appendix A1.1. Electrophoretic analysis of the different purified preparations showed that they had been degraded to varying degrees (Appendix A1.1). They also varied widely in the extent to which they were contaminated with amidolytic activity (Chapter V).

Generally speaking, human fibrinogen preparations obtained from A.B. Kabi were superior to those obtained from other sources in that they showed a higher relative content of intact α chains and a lower amidolytic activity. These preparations were used for the preparation of ^{125}I-fibrinogen to be used in the ^{125}I-fibrin assay. Fibrinogen was labelled with Na^{125}I according to the method of Helmkamp et al as described in detail in the Appendix A1.2.

Human plasminogen was purified from outdated or fresh frozen human plasma by affinity chromatography on lysine-sepharose by a modification of the method of Deutsch and Mertz (45)(Appendix A1.3). Usually, both Lys- and Glu-plasminogen were obtained by this procedure and no attempt was made to separate these two species. Plasminogen was labelled with $Na^{125}I$ according to the iodine monochloride method of Helmkamp et al (183) (Appendix A1.4).

Other reagents and their commercial sources are listed in the Appendix (A5).

Brief outlines of the methods used for the various enzyme assays are given under the relevant "Results" section. Detailed descriptions are given in the Appendix (A3).

RESULTS

In the absence of fibrin, urokinase was a more efficient activator of plasminogen than was Mel-PA.

Two methods were used for the direct measurement of the rate of conversion of plasminogen to plasmin. The first method was that described by Danø and Reich (142) in which ^{125}I-plasminogen was incubated with plasminogen activators in the presence of bovine pancreatic trypsin inhibitor (BPTI). Plasmin heavy and light chains and unreacted plasminogen were then separated by electrophoresis in SDS-polyacrylamide gel slabs under reducing conditions. The relative amounts of each species were determined by counting slices of the gel. The results are expressed as the percentage of the plasminogen converted in a given period of time. Despite every care to isolate plasminogen free of plasmin, I was unable to obtain plasminogen that did not contain approximately 10% of the total radioactivity that migrated electrophoretically in the position of plasmin. This "background" or "spontaneous conversion" of plasminogen to plasmin remained a constant fraction of the total, even when incubated for prolonged times. In presenting my results, I have subtracted this background to give values for enzyme catalyzed conversion as "specific conversion".

The second assay used was that described by Mangel et al (181). Purified plasminogen and plasminogen activators were incubated together without BPTI. Timed aliquots were removed from the mixture for measurement of plasmin by active-site titration using the fluorescent compound di(p-guanidinobenzoyl)-fluorescyl-6-thioureido(p-benzoic acid)(FDE). The results are expressed as pmol of plasmin generated in a given period of time.

When plasminogen conversion was studied as a function of time, the results illustrated in Fig. 6.1 and Fig. 6.2 were obtained. By both assays

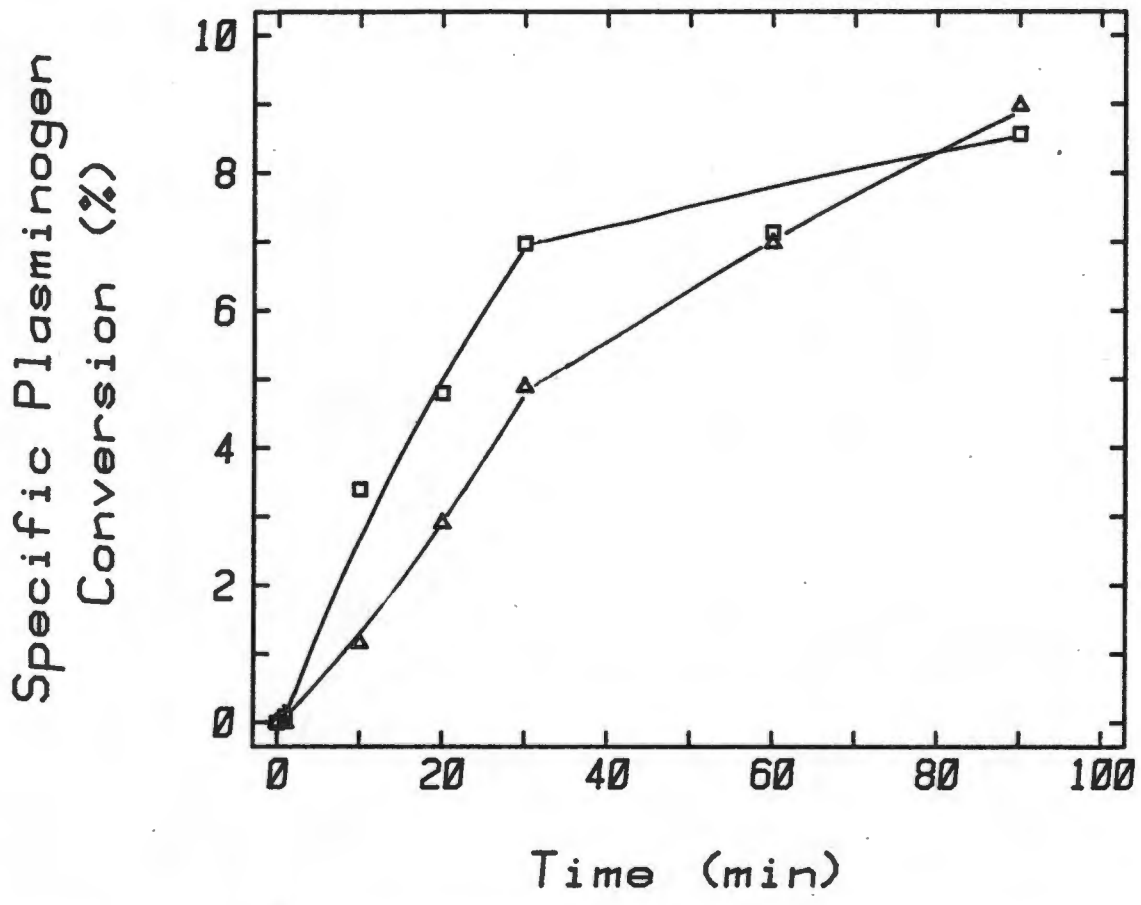


Figure 6.1 ^{125}I-plasminogen activation by urokinase and Mel-PA measured as a function of time.

Mel-PA (10 FU/ml; 10.12 pmol/ml)(△—△) or urokinase (6.67 FU/ml; 0.099 pmol/ml)(□—□) were incubated at 37°C in a total volume of 300 µl of T-T(.02) containing 29 µg/ml ^{125}I-plasminogen (4.6 x 10^7 cpm/ml) and 100 KIU/ml of BPTI. Control wells contained no plasminogen activator. At the indicated times 25 µl samples were removed from the solutions and added to tubes containing 5 µl 10% SDS, 5 µl glycerol and 2.5 µl 2-mercapto-ethanol. The samples were boiled and 5 µl aliquots of the mixtures were electrophoresed in 11% polyacrylamide-SDS gel slabs to separate plasmin and plasminogen. After autoradiographic identification, plasminogen and plasmin were recovered from the gels by excision. These were counted and % conversion was calculated as described in Appendix A3.3. Each point represents duplicate estimates of the % conversion after the percentage of spontaneous conversion (8.15%) had been subtracted.

Linearity of plasminogen conversion with time was observed until approximately 5% of the substrate had been converted.

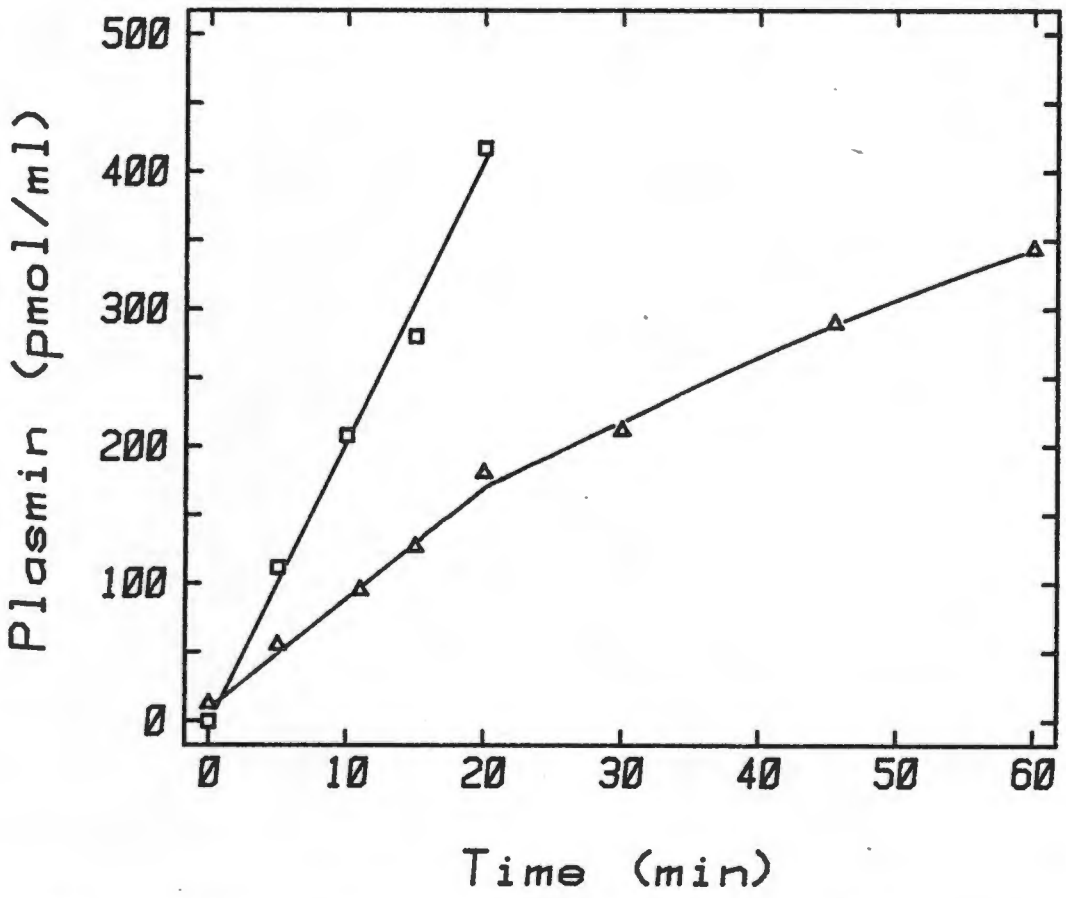


Figure 6.2 Plasminogen activation by urokinase and Mel-PA measured by active site titration of plasmin formed from plasminogen with time.

Mel-PA (40 FU/ml; 40.5 pmol/ml)(△—△) or urokinase (40 FU/ml; 0.6 pmol/ml)(□—□) were incubated at room temperature in a total volume of 500 µl of T-T(0.02) containing .756 mg/ml of plasminogen. At the indicated times 50 µl aliquots were removed and added to 950 µl PBS containing 0.79 µg/ml of FDE. After exactly 2 min incubation at room temperature, 20 µl of 0.1 mg/ml NPGB was added and the fluorescence was measured with excitation and emission wavelengths set at 491 nm and 514 nm respectively. The amount of plasmin generated was calculated from the fluorescence readings by reference to the fluorescence of a standard solution of completely hydrolysed FDE of known concentration. Blank values (i.e. fluorescence readings observed when samples of plasminogen without activators were assayed) were subtracted.

The rate of plasminogen activation by both urokinase and Mel-PA was linear with time for at least 20 min.

urokinase was more efficient as an activator of plasminogen than was Mel-PA. In the experiment shown in Fig. 6.1 the initial rate of conversion of urokinase and Mel-PA were roughly equivalent, yet the molar concentration of Mel-PA was 100-fold higher. In the experiment in which plasmin generated was measured by active site titration (Fig. 6.2) the rate of plasminogen conversion by Mel-PA was approximately one-half that by urokinase yet the molar concentration of Mel-PA was 67-fold higher than that of urokinase. It can also be seen that, in both assays, and with both urokinase and Mel-PA plasminogen conversion was linear with time for approximately 20 min, after which the time course of the reaction departed from linearity.

When plasminogen conversion was plotted as a function of the amount of plasminogen activator used in the assay it was once again evident that Mel-PA was very much less efficient than was urokinase. For the convenience of graphic presentation, the plasminogen activator concentration in these experiments (Fig. 6.3 and Fig. 6.4) is expressed in fluorometric units/ml (FU/ml). This enabled me to plot curves for the two enzymes with the same abscissal scale. It should be recalled (Chapter V) that 1 FU of Mel-PA represents 67 times more enzyme on a molar basis than does 1 FU of urokinase. It is apparent from the data presented in Fig. 6.3 that the rate of conversion of plasminogen was reasonably linear with plasminogen activator concentration until approximately 5% of the substrate had been converted after which the rate fell.

<u>The ability of Mel-PA to catalyse plasminogen conversion was enhanced by the presence of fibrinogen or fibrin.</u>

Numerous reports in the literature have indicated that fibrin serves as a "co-factor" for the conversion of plasminogen to plasmin by "tissue activator". The experiments performed to examine this phenomenon

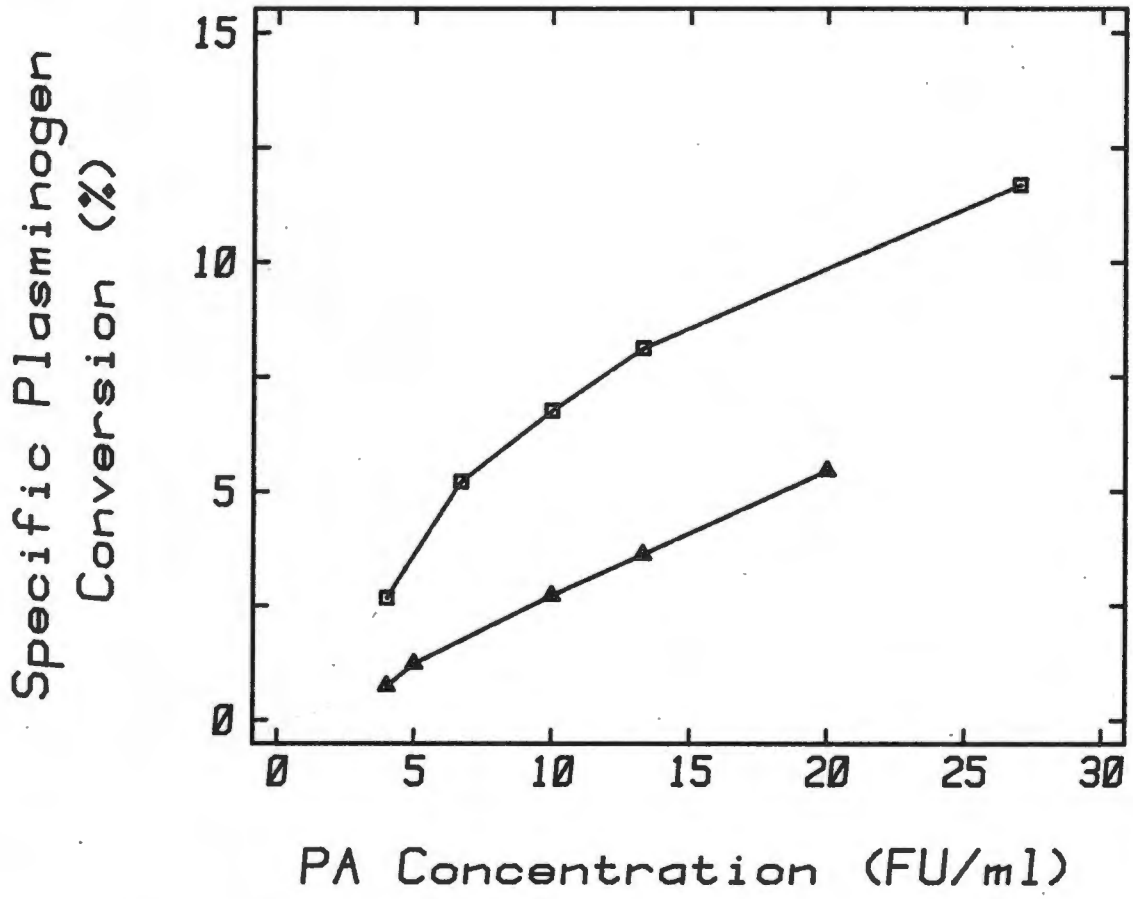


Figure 6.3 ^{125}I-plasminogen activation by urokinase and Mel-PA as a function of activator concentration.

Mel-PA (4-20 FU/ml; 4.05-20.24 pmol/ml) (△—△) or urokinase (4-27 FU/ml; 0.06-0.40 pmol/ml) (□—□) were incubated together with plasminogen (29 µg/ml; 4.6×10^7 cpm/ml) and BPTI (100 KIU/ml) at 37°C. After 20 min, plasminogen conversion to plasmin in each mixture was determined by electrophoresis and autoradiography as described in the legend to Fig. 6.1 and the Appendix (A3.3).

Each point represents the mean of duplicate estimates of the percentage conversion after subtraction of the percentage spontaneous conversion (9.50%). Linearity of plasminogen conversion by both plasminogen activators was observed until approximately 5% of the substrate had been converted.

Measured in terms of plasminogen activator conversion/mole of plasminogen activator, urokinase converted the plasminogen approximately 167 times more efficiently than did Mel-PA.

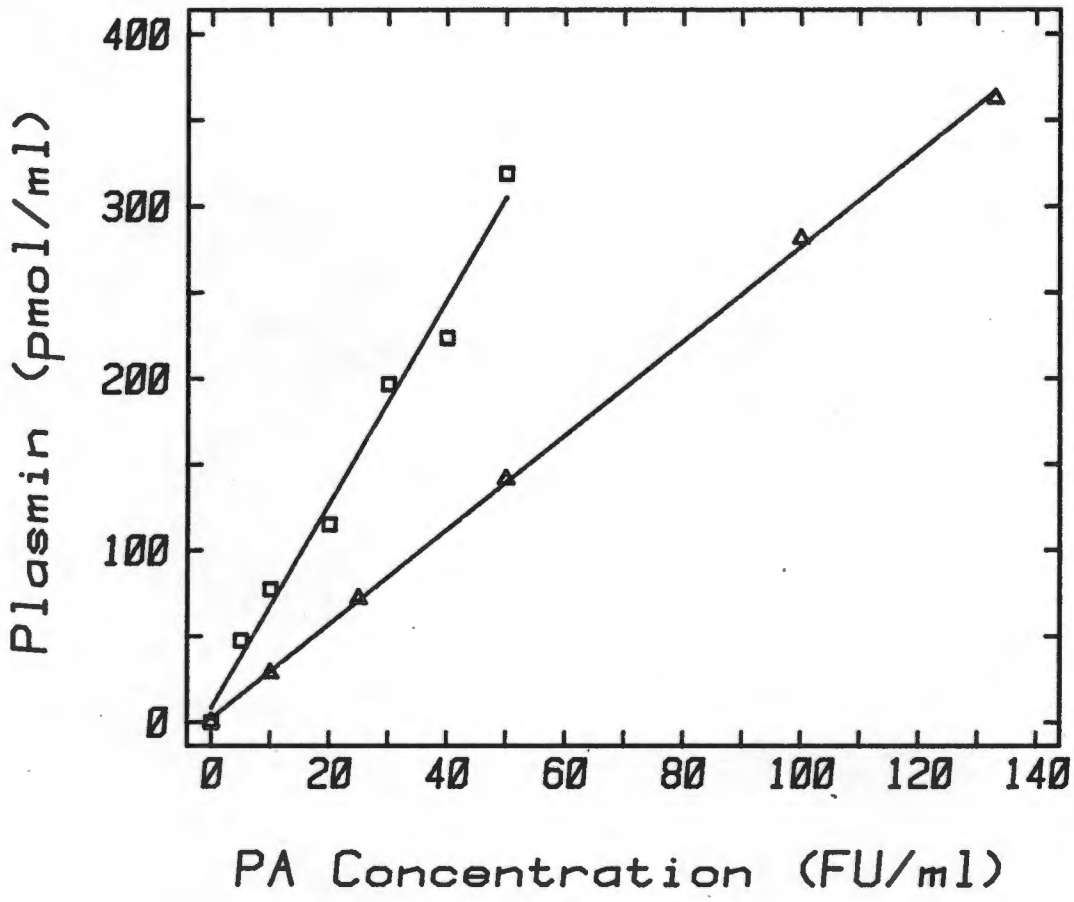


Figure 6.4 Plasminogen activation as a function of Mel-PA and urokinase concentration.

Mel-PA (10-133 FU/ml; 10.12 - 134.6 pmol/ml)(△—△) or urokinase (5 - 50 FU/ml; 0.07-0.75 pmol/ml)(□—□) were incubated at room temperature with 0.756 mg/ml plasminogen in a total volume of 200 µl. After 10 min plasmin generated was determined in 50 µl aliquots by active site titration using FDE as described in the Appendix A3.4).

In this assay urokinase was approximately 169 times more efficient than Mel-PA in converting plasminogen to plasmin.

Figure 6.5

Figure 6.5 The effect of fibrinogen or a fibrin clot on the conversion of ^{125}I-plasminogen to ^{125}I-plasmin by Mel-PA or urokinase.

Sixteen reaction tubes were prepared so that each received the same amount (8.7 µg; 1.4×10^7 cpm) of ^{125}I-plasminogen and 30 KIU of BPTI in a total final reaction volume of 300 µl of T-T(.02).

Six of the tubes contained 6 FU (6.06 pmol) of Mel-PA and six contained 6 FU (0.09 pmol) of urokinase. Duplicate tubes from each activator series contained either buffer alone, 20µg of fibrinogen, or a washed fibrin clot formed by the addition of 0.02 units of thrombin to 20 µg of fibrinogen.

Duplicate control tubes for the measurement of spontaneous plasminogen conversion contained buffer or fibrinogen but no plasminogen activator.

The tubes were incubated at 37°C for 20 min after which 50 µl aliquots were analysed for plasminogen conversion to plasmin by electrophoresis, autoradiographic identification and radioassay as described in the Appendix (A3.3).

Each bar represents the average of duplicate estimates of the percentage plasminogen converted after the percentage of spontaneous conversion (7.12% and 7.18%) had been subtracted.

Mel-PA activity was enhanced by the presence of fibrinogen and fibrin whereas urokinase activity was not.

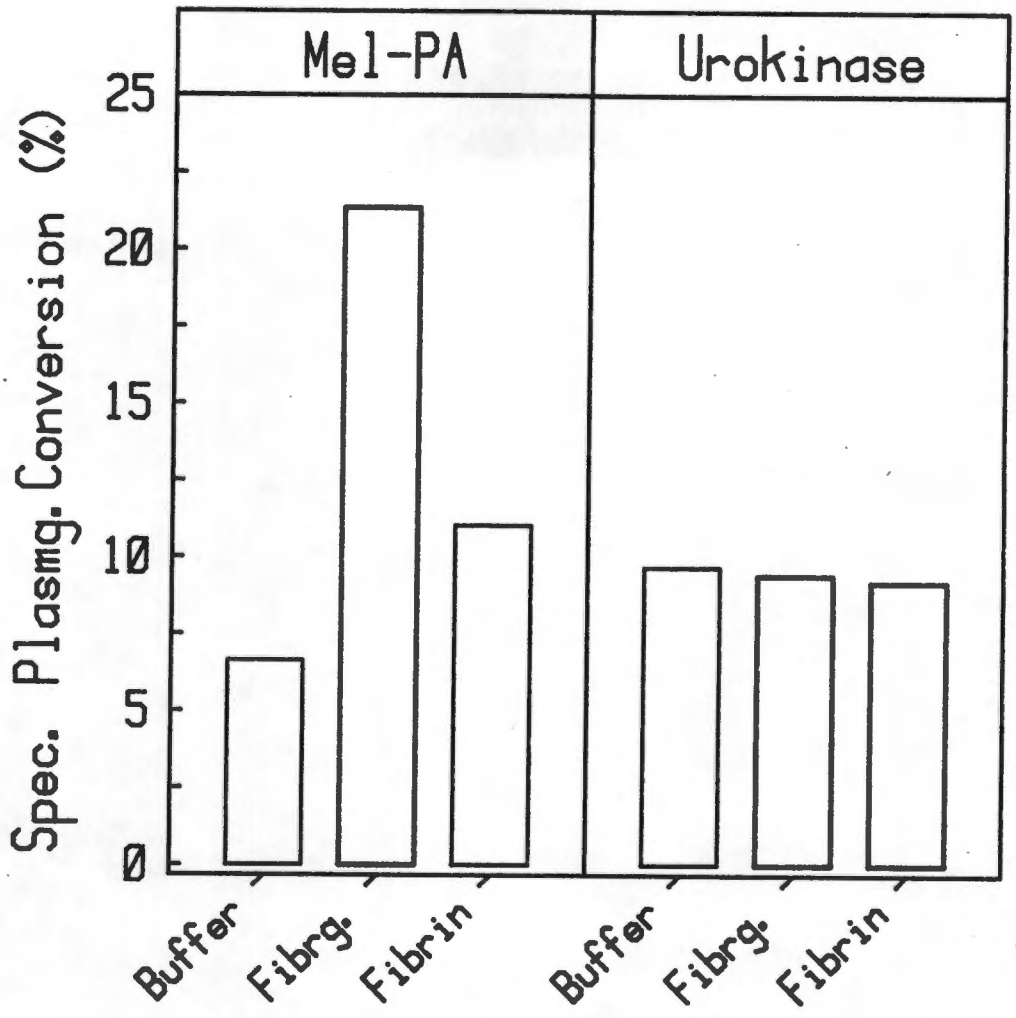

Mel-PA
Urokinase
Spec. Plasmg. Conversion (%)
25
20
15
10
5
0
Buffer
Fibrg.
Fibrin
Buffer
Fibrg.
Fibrin

Figure 6.6

Figure 6.6 ^{125}I-plasminogen conversion by Mel-PA and urokinase in presence and absence of insolubilized fibrinogen.

Each well of a Linbro multiwell tissue culture plate received 200 µl of 5 mM NaCl or of 5 mM NaCl containing 30 µg of fibrinogen.

The well contents were dried on to the plastic surface by incubating the plate at 45°C for 48 hr.

The wells were then washed with PBS and to each was added 300 µl of T-T(0.1) buffer containing 30 KIU of BPTI; 8.7 µg ^{125}I-plasminogen (1.4×10^7 cpm) and either 3 FU (3.04 pmol) Mel-PA or 2 FU (0.03 pmol) of urokinase.

The plates were incubated at 37°C and samples of the well contents were taken at the time points indicated for assay of plasminogen to plasmin conversion by electrophoresis, autoradiography and radioassay of the excised bands (Appendix A3.3). Each point represents the mean of duplicate analyses after subtraction of plasminogen that converted spontaneously (8.15%).

Key to symbols as follows:-

Mel-PA + fibrinogen ▽—▽

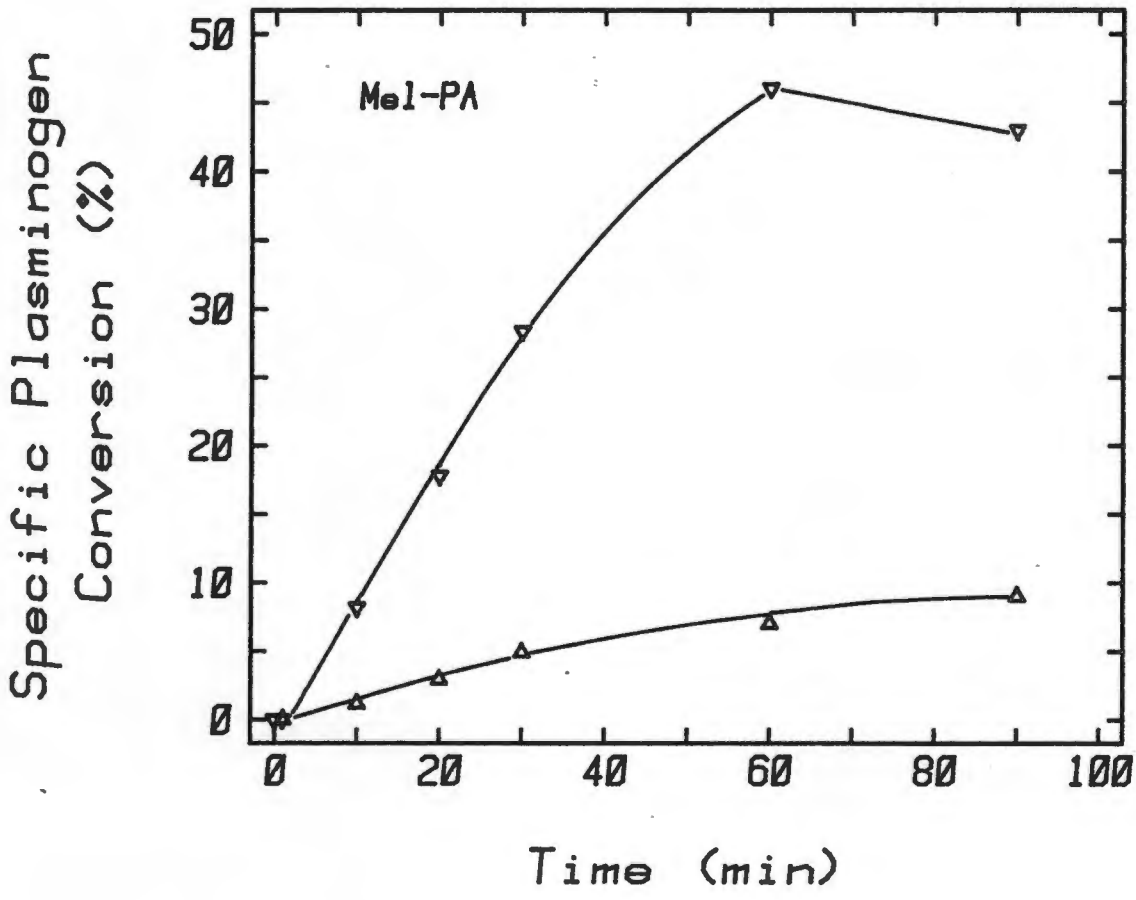
Mel-PA without fibrinogen △—△

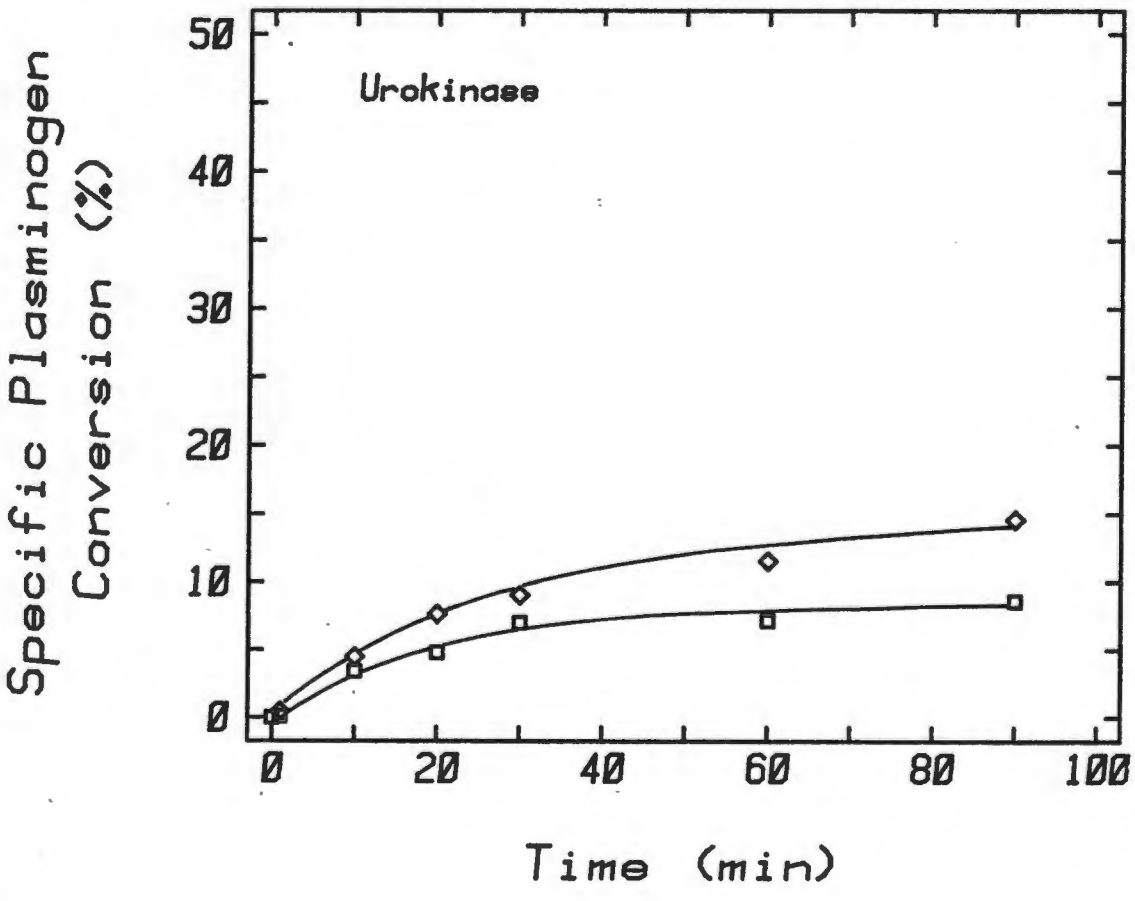
Urokinase + fibrinogen ◇—◇

Urokinase without fibrinogen □—□

Mel-PA and urokinase showed roughly comparable activities in the absence of fibrinogen in this experiment. It should, however, be noted that each well contained approximately 100x more Mel-PA than urokinase on a molar basis.

The activity of Mel-PA was enhanced by fibrinogen to a far greater extent than was the activity of urokinase.


Mel-PA
Specific Plasminogen Conversion (%)
50
40
30
20
10
0
0
20
40
60
80
100
Time (min)


Urokinase
Specific Plasminogen Conversion (%)
50
40
30
20
10
0
0
20
40
60
80
100
Time (min)

for its relevance to plasminogen conversion by Mel-PA are presented in Fig. 6.5 to Fig. 6.9. The results may be summarised as follows:

(i) *Soluble fibrinogen and a suspended fibrin clot enhanced Mel-PA activity but had no effect on the activity of urokinase.*

When ^{125}I-plasminogen conversion by Mel-PA or urokinase were examined in the presence of soluble fibrinogen or a suspended fibrin clot, it was observed that Mel-PA activity was enhanced approximately three-fold by fibrinogen and 1.7 fold by fibrin whereas urokinase activity was unaffected (Fig. 6.5). The "spontaneous conversion" of plasminogen to plasmin (approximately 7%) was unaffected by fibrinogen alone.

(ii) *Fibrinogen and fibrin adsorbed to plastic surfaces enhanced the activity of Mel-PA to a far greater extent than it enhanced the activity of urokinase.*

Thirty micrograms of purified fibrinogen in solution were added to each well of several Linbro tissue culture plates. The plates were then incubated at 45°C for 48 hr to dry the solutions and to secure adsorption of the fibrinogen onto the surfaces of the wells. Each well was then used as a reaction chamber for measuring the conversion of ^{125}I-plasminogen to plasmin by Mel-PA or urokinase. Control wells contained either no adsorbed fibrinogen or no plasminogen activator. The results of these experiments are shown in Fig. 6.6 where plasminogen conversion by Mel-PA or urokinase were measured as functions of time.

Mel-PA was more susceptible to the enhancing effect of the adsorbed fibrinogen than was urokinase. After 90 min in the absence of fibrinogen both Mel-PA and urokinase catalyzed the specific conversion of approximately 8% of the plasminogen to plasmin. In the case of urokinase the presence of fibrinogen increased this conversion by approximately 2-fold.

With Mel-PA plasminogen conversion was increased by approximately 6-fold.

To investigate the effect of fibrin rather than fibrinogen, a similar experiment was performed with wells to which 1 ml of 0.1 NIH units/ml of thrombin in PBS had been added. The results of this experiment are shown in Fig. 6.7 and are essentially similar to those obtained with fibrinogen. The activity of Mel-PA was enhanced whereas that of urokinase was not. As indicated in the Appendix (A3.1) I was unable to satisfy myself that thrombin treatment of surface-adsorbed fibrinogen effectively converted it to fibrin. I am therefore uncertain whether the effects I observed on enhancement of Mel-PA activity were attributable to fibrin or fibrinogen in this experiment.

In order to establish whether or not other proteins have a similar stimulatory effect on plasminogen activation an experiment was performed in which casein and gelatin were attached to the plastic surfaces of Linbro wells and compared with fibrinogen and fibrin in the ^{125}I-plasminogen conversion assay. The results are presented in Fig. 6.7 from which it can be seen that the activity of Mel-PA was enhanced by fibrinogen, casein and fibrin but not by gelatin. Casein caused a two-fold increase of activity of urokinase while other proteins had lesser stimulatory effects on the action of this enzyme. In the case of Mel-PA, fibrinogen was most active, casein and fibrin stimulated plasminogen activation to a lesser extent and gelatin was without effect.

Fig. 6.8 depicts the results of an experiment in which plasminogen conversion was studied as a function of Mel-PA or urokinase concentration in the presence of absence of fibrinogen. It is evident from the data obtained that fibrinogen enhanced the activity of Mel-PA at all concentrations of this enzyme to an extent that was greater than was observed with urokinase.

Figure 6.7

Figure 6.7 Effect of fibrinogen, casein, gelatin and fibrin on plasminogen activation by urokinase and Mel-PA.

Each well of a Linbro multiwell tissue culture plate received 200 µl of 5 mM NaCl or 200 µl of 5 mM NaCl containing 30 µg of the protein indicated. The plates were then incubated at 45°C for 48 hr to dry the well contents and to secure protein attachment to the bottom surfaces of the wells. The wells were then washed in PBS and to each was added 300 µl of T-T(0.1) buffer containing 8.7 µg of ^{125}I-plasminogen (1.4×10^7 cpm), 30 KIU of BPTI and either 2 FU (0.03 pmol) of urokinase or 3 FU (3.03 pmoles) of Mel-PA. This Mel-PA sample contained negligible amounts of pro-Mel-PA.

Control wells did not receive plasminogen activator.

The plates were incubated at 37°C for 20 min, after which plasminogen conversion to plasmin in each well was determined by electrophoresis, autoradiography and radioassay as described in Appendix A3.3.

Each bar represents the average of duplicate estimates of the ratio of plasminogen conversion in the presence of protein to that observed in the absence of protein. Spontaneous conversion of plasminogen to plasmin in the absence of plasminogen activator was approximately 12%. This value was subtracted from the experimental values before calculating the results.

Maximum specific conversion by urokinase in the presence of casein was 10%; conversion by Mel-PA in the presence of fibrinogen or casein was 19.5% or 13.1% respectively.

Note that Mel-PA activity was strikingly enhanced by fibrinogen, casein and fibrin. The activity of urokinase was slightly enhanced by casein.

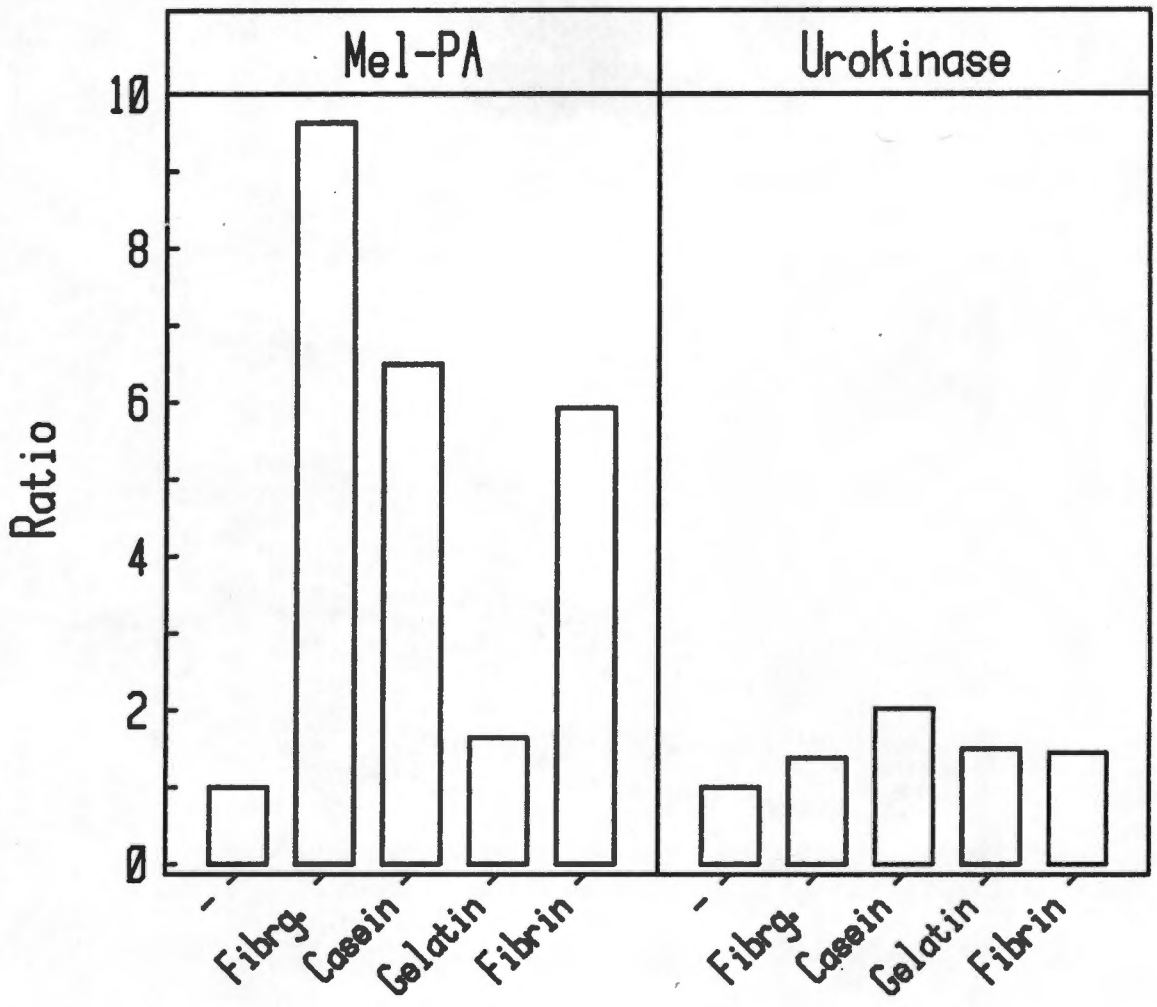

Mel-PA
Urokinase
Ratio
10
8
6
4
2
0
-
Fibrg
Casein
Gelatin
Fibrin
-
Fibrg
Casein
Gelatin
Fibrin

Figure 6.8

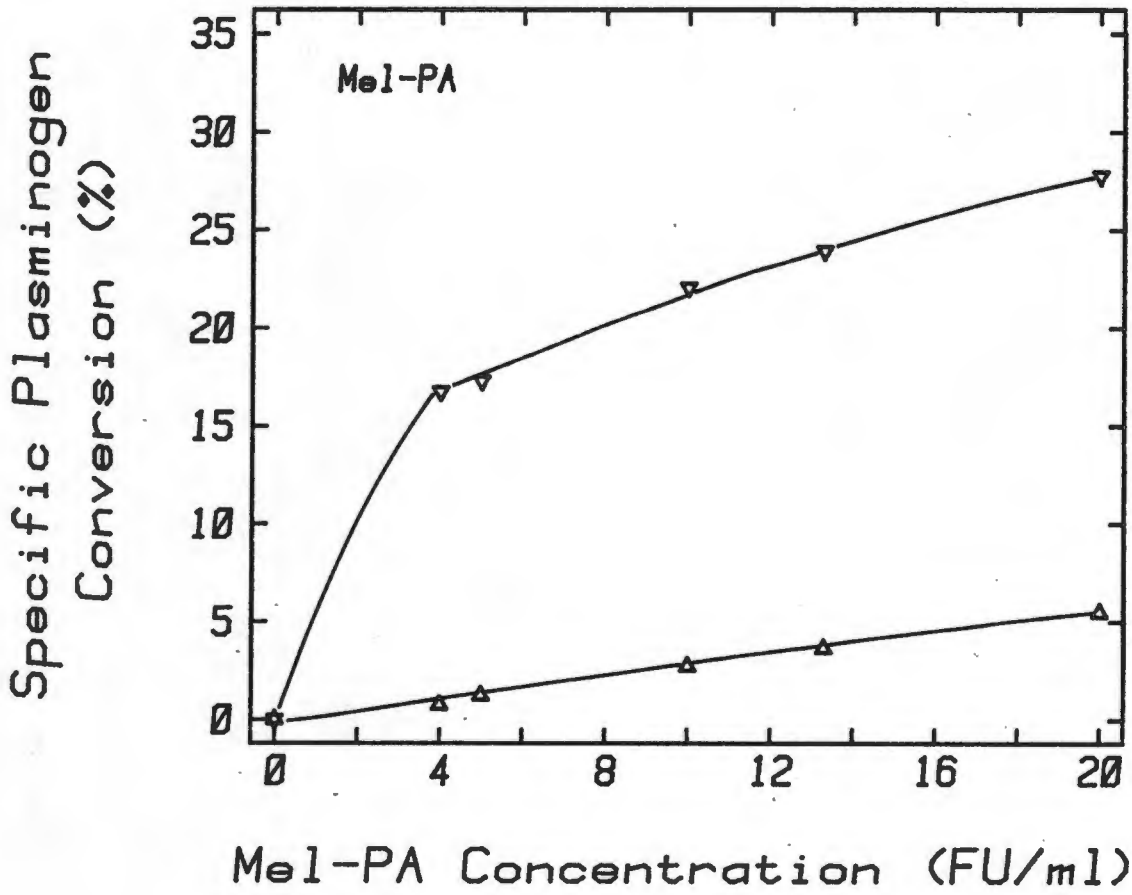
Figure 6.8 The effects of insolubilized fibrinogen and of plasminogen activator concentration on the conversion of ^{125}I-plasminogen to plasmin by Mel-PA and urokinase.

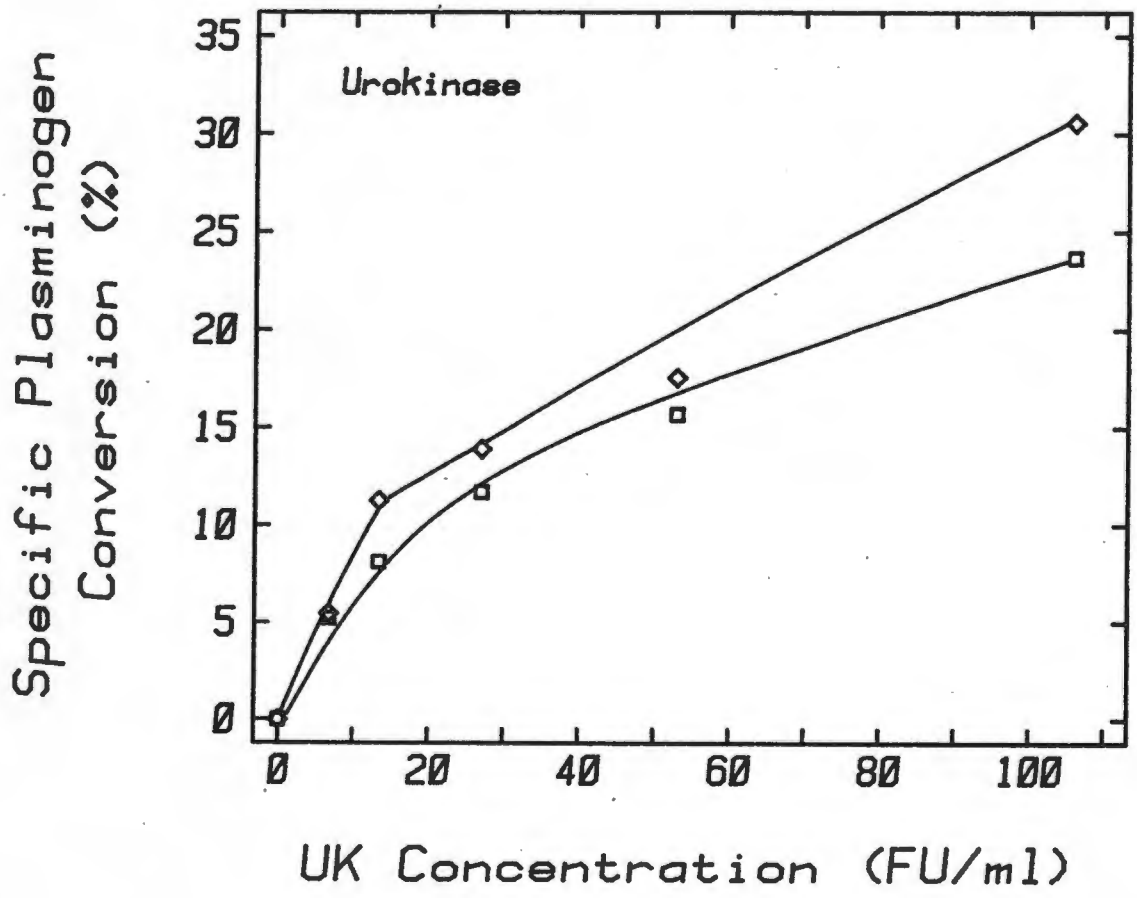
Wells of Linbro multiwell tissue culture plates were prepared with or without adsorbed fibrinogen (30 μg/well) and used to study ^{125}I-plasminogen conversion in 20 min by Mel-PA or urokinase at the concentrations shown.

^{125}I-plasminogen conversion was measured by electrophoresis, autoradiographic identification and radioassay as described in Appendix A3.3.

Mel-PA activity was greatly enhanced by fibrinogen (▽—▽ vs △—△) whereas urokinase activity was not (◇—◇ vs □—□).

In the absence of fibrinogen urokinase was more active than was Mel-PA.


Mel-PA
Specific Plasminogen Conversion (%)
0
5
10
15
20
25
30
35
0
4
8
12
16
20
Mel-PA Concentration (FU/ml)


Urokinase
Specific Plasminogen Conversion (%)
0
5
10
15
20
25
30
35
0
20
40
60
80
100
UK Concentration (FU/ml)

(iii) *The enhancement of Mel-PA activity increased with fibrinogen concentration.*

When plasminogen conversion by Mel-PA (2.02 pmol) or urokinase (0.04 pmol) was measured in wells coated with increasing amounts of fibrinogen the results illustrated in Fig. 6.9 were obtained. In the case of urokinase a slight increase of activity was observed when small amounts of fibrinogen were present in the wells. The enhancement was unaffected by increasing amounts of fibrinogen. Mel-PA activity on the other hand showed a marked and progressive increase with fibrinogen addition until approximately 0.2 mg (0.606 nmoles)/well was reached. Since each well only contained 2.02 pmol of plasminogen activator it is clear that the enhancement of plasminogen activation by fibrinogen was not stoichiometric with activator.

Fig. 6.9 also depicts data showing that Mel-PA bound to fibrinogen whereas urokinase did not. These data are considered in the next section.

Mel-PA binds to fibrin

A number of studies have shown that "tissue activator", "vascular activator" or Mel-PA can be adsorbed to fibrin, either during clot formation (58,130,169), or when the fibrin is bound to celite or to sepharose (90,57). To examine this phenomenon in more detail, I performed the following experiments.

In the first experiment Mel-PA or urokinase were incubated in Linbro wells that had been coated with fibrinogen. Nonadsorbed activators were then removed by washing in 0.1M Tris HCl pH 8.1 containing 0.02% Triton X-100. Radioactive plasminogen was added and, after 20 minutes of incubation, the contents of the wells were analysed by electrophoresis and autoradiography for plasminogen conversion. The results (Fig. 6.9) showed that a very small

Figure 6.9

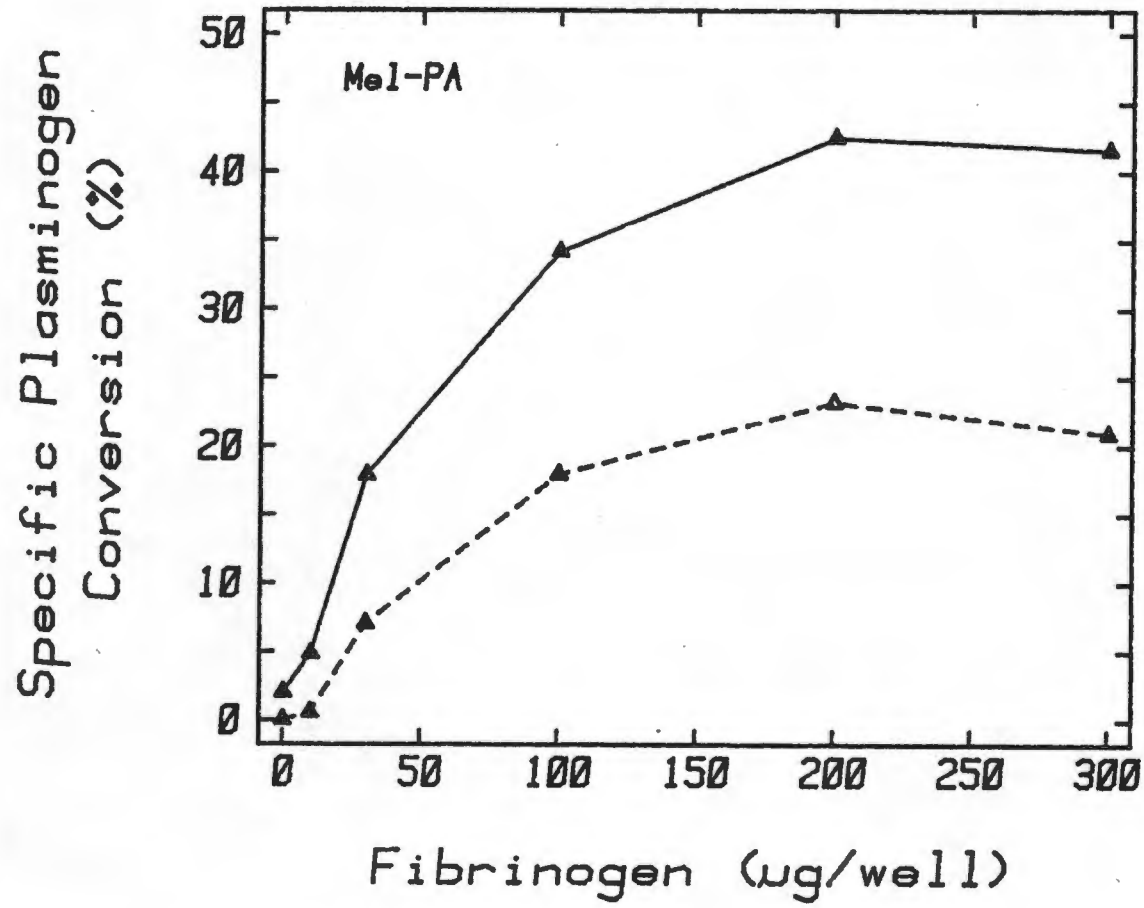
Figure 6.9 ^{125}I-plasminogen conversion by Mel-PA and urokinase as a function of fibrinogen concentration and binding of the activators to the insolubilized substrate.

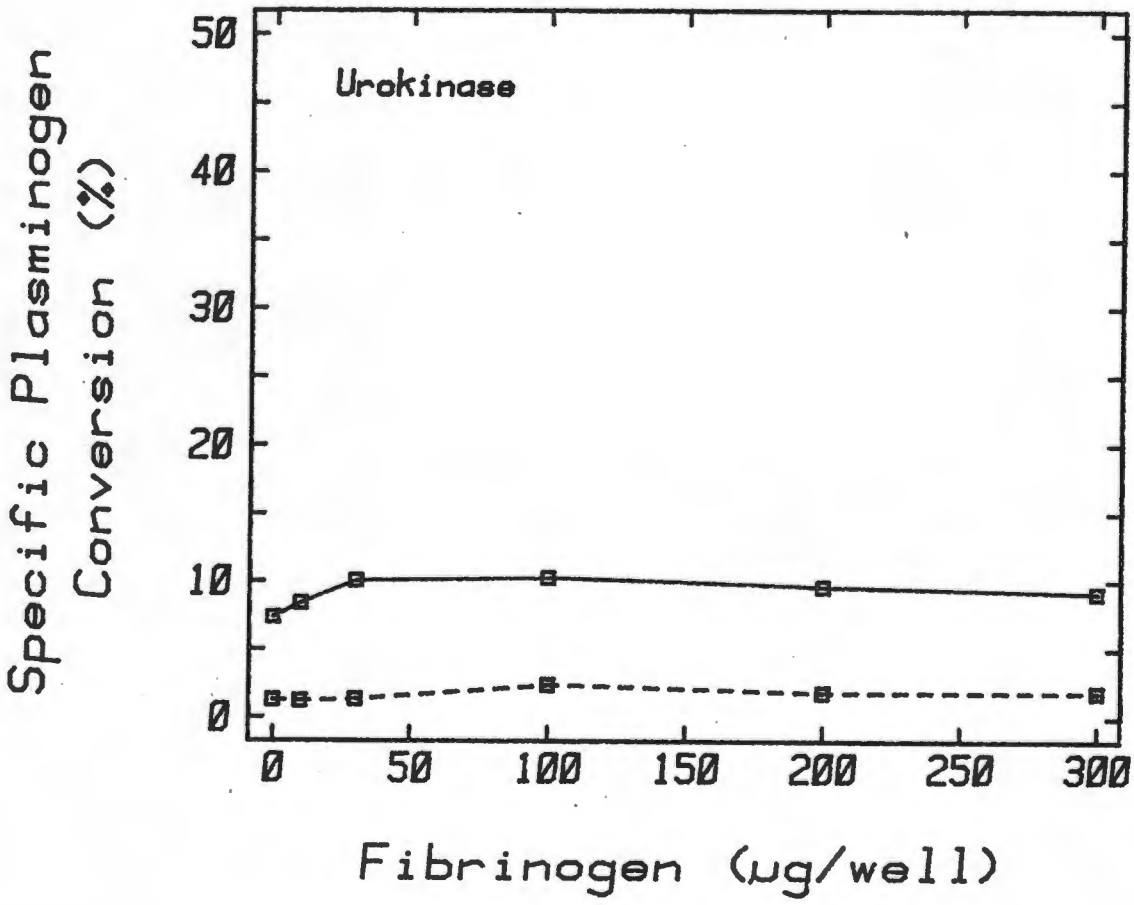
Individual wells of a Linbro plate were coated with the amounts of fibrinogen shown. These wells were then used to measure the conversion of ^{125}I-plasminogen to plasmin by 2 FU (2.02 pmol) of Mel-PA or by 3 FU (0.045 pmols) of urokinase in 20 min at 37°C (solid lines).

Mel-PA activity (△—△) increased with increasing amounts of fibrinogen until a plateau was reached at 200 µg/well of fibrinogen. Urokinase activity (□—□) was unaffected.

To examine the binding of plasminogen activators to insolubilized fibrinogen, Mel-PA (2 FU; 2.02 pmol) or urokinase (3 FU; 0.045 pmol) were incubated in the wells for 30 min at room temperature. Unbound plasminogen activators were then removed by washing the wells 3x with T-T(0.1). ^{125}I-plasminogen and BPTI were then added and conversion by bound activators was measured (interrupted lines). Each point on the graph represents the mean of duplicate estimates.

Mel-PA (△--△) was strongly adsorbed to the insoluble fibrinogen, whereas urokinase was not (□--□).


Mel-PA
Specific Plasminogen Conversion (%)
0
10
20
30
40
50
0
50
100
150
200
250
300
Fibrinogen (ug/well)


Urokinase
Specific Plasminogen Conversion (%)
0
10
20
30
40
50
0
50
100
150
200
250
300
Fibrinogen (ug/well)

amount of urokinase capable of activating plasminogen was adsorbed to the wells. This was unrelated to the amount of fibrinogen in the wells and was presumably non-specific.

In the case of Mel-PA, increasing amounts of the activator were adsorbed with increasing amounts of fibrinogen. The adsorption phenomenon reached a plateau at an approximate fibrinogen concentration of 0.2 mg (0.606 nmoles) per well. The same amount of fibrinogen gave maximum enhancement of Mel-PA activity (cf the previous section).

A similar experiment was carried out in which radioactive fibrinogen rather than radioactive plasminogen was used. Plasminogen activation was then determined by measuring plasminogen-dependent solubilization of the ^{125}I-fibrinogen substrate coated on to Linbro wells under conditions where the amount of activator present would have been rate-limiting. Urokinase or Mel-PA were added to ^{125}I-fibrinogen-coated Linbro wells and incubated at room temperature for 30 mins. Free activators were then removed by washing three times with Tris buffer containing either 0.3 mg/ml BSA or 0.02% Triton X-100. Plasminogen was then added and solubilization of the radioactive substrate was measured after incubating for 60 min at 37^{o}C. Conditions were chosen so that, at this time, linear kinetics for release of radioactive fibrin degradation product were still obtained. The results (Fig. 6.10) demonstrated that up to 50% of the available Mel-PA was adsorbed to the matrix whereas only 20% of the available urokinase was bound.

It was once again apparent that only a small fraction of the adsorbed fibrinogen was involved in the binding of Mel-PA since in this experiment fibrinogen and Mel-PA were present in a molar ratio of approximately 2000 to 1, yet only approximately one half of the available Mel-PA activity was bound.

Figure 6.10

Figure 6.10 The binding of Mel-PA and urokinase to insolubilized fibrinogen as measured in the ^{125}I-fibrin assay.

Linbro wells were coated with ^{125}I-fibrinogen (30 µg; 1 x 10^5cpm/well), treated with 10% plasminogen-free FCS in RPMI, and washed with PBS as described in the Appendix (A3.1).

To some of the wells Mel-PA was added; other wells received urokinase and others buffer alone. All additions were in 300 µl of 0.1M Tris-HCl pH 8.1 containing 270 µg/ml of BSA.

The plates were incubated for 30 min at room temperature after which the wells were washed with 0.1M Tris HCl pH 8.1 containing 0.3 mg/ml BSA (T-BSA) or with 0.1M Tris HCl pH 8.1 containing 0.02% Triton X-100 (T-T (0.02)).

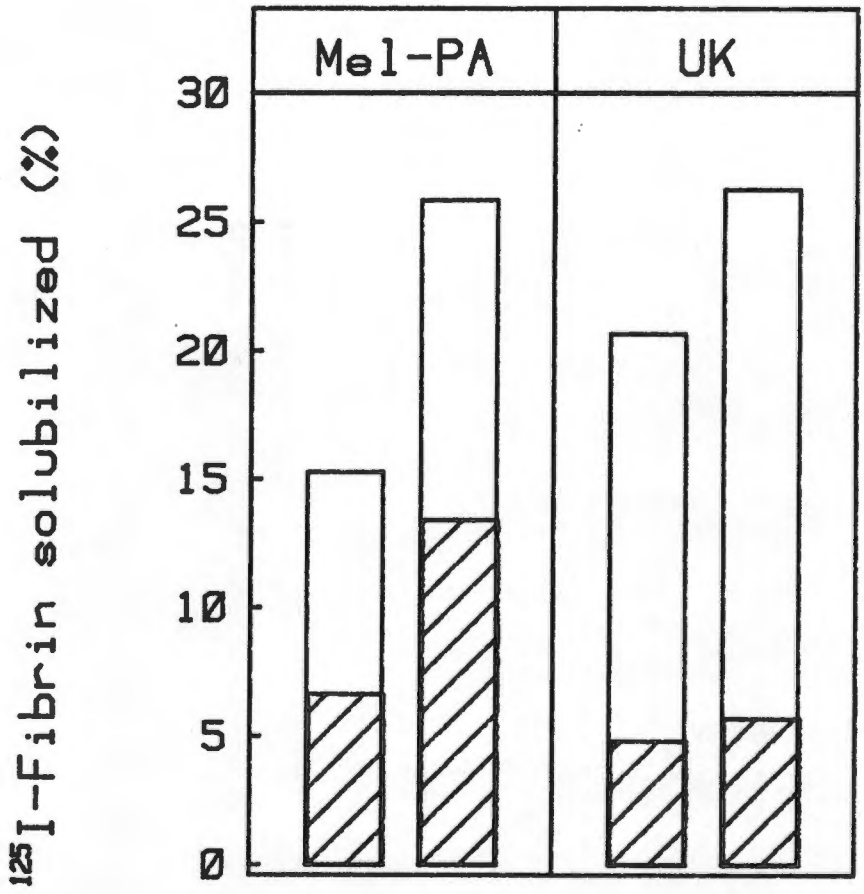
Wells previously exposed to urokinase or Mel-PA received, in a final volume of 300 µl, 2 µg of plasminogen and 80 µg of BSA in 0.1M Tris HCl pH 8.1.

Wells previously exposed to buffer alone received, in addition to BSA and plasminogen, either Mel-PA or urokinase in amounts equal to those added initially to the washed wells with which they were to be compared.

Total ^{125}I-fibrin solubilized was measured as a percentage of the total adsorbed radioactivity after 60 min at 37°C.

Open bars represent the means of triplicate measurements of total activity of plasminogen activator. Shaded bars represent corresponding values for the activators that had remained bound to the matrix after washing. It can be seen that up to 50% of the total Mel-PA added remained bound to the matrix, while only 20% of the urokinase added was bound.

Plasminogen activator activity was greater when Triton X-100 was present in the fluid used to wash the fibrin layers. The reason for this detergent effect is not known. It was not explored further.


Mel-PA
UK
30
25
20
15
10
5
0
^{125}I-Fibrin solubilized (%)

Mel-PA employed for this experiment was mainly in the active enzyme form. In another experiment (Table 6.1) I found that a larger percentage of total enzyme was bound when the Mel-PA preparation was in the proactivator form indicating that binding of plasminogen activators to fibrin was probably not only a function of the fibrinogen preparation used but it may also be related to the form of the enzyme.

Mel-PA is not degraded by plasmin

It has been suggested in the literature (142,181) that deviations from linearity in the kinetics of plasminogen-dependent fibrinolysis may be ascribed to degradation of plasminogen activator by plasmin generated during the course of the reaction. In order to test this suggestion, I performed an experiment in which urokinase (approximately 0.2 pmol) or Mel-PA (approximately 24 pmol) were incubated in the absence or presence of 0.25 mg/ml of plasmin at room temperature for up to 5½ hours. At different times during the course of incubation 50 µl samples were removed and the plasmin that they contained was immediately neutralized by the addition of 10 µl of BPTI (10 KIU). Fifty microlitres of this mixture was then measured for Mel-PA or urokinase activity in the fluorometric assay. As can be seen from Table 6.2, the activity of urokinase and Mel-PA remained constant in the presence of plasmin and showed no decrease relative to the activity in untreated samples. From this it could be concluded that plasmin did not degrade either Mel-PA or urokinase.

The kinetics of plasminogen-dependent fibrinolysis by Mel-PA and urokinase

One of the assays frequently used for the quantitation of plasminogen activators is that which measures the plasminogen-dependent degradation of insoluble ^{125}I-labelled fibrin. As commonly employed by ourselves (106)

Table 6.1 The binding of Mel-PA to insolubilized fibrin.

Mel-PA Sample[a]	% Pro-Mel-PA[b]	Fibrinolytic activity (% ^{125}I-fibrin solubilized in 60 min)		
		Total activity	Activity after wash	% bound
1	88.7	29.83	29.48	98.8
2	34.0	45.90	37.52	81.7
3	64.0	56.56	41.53	73.4
4	87.0	48.88	48.76	99.8
5	88.0	53.85	52.49	97.5

Five Mel-PA samples were obtained as described below and their contents of active Mel-PA and Pro-Mel-PA was determined from the results of fluorometric assays before and after plasmin treatment as described in Chapter V. These samples were then diluted in T-T(0.1) so that 0.2 ml would, in the presence of 2 µg of plasminogen, release approximately 30 to 50% of the ^{125}I-fibrin coated on the bottom of Linbro wells in 1 hr.

Aliquots (0.2 ml) of the samples were then added in quadruplicate to Linbro wells coated with ^{125}I-fibrin (30 µg; 100 000 cpm). After incubation for 1 hr at 0°C two wells of each quadruplicate set were washed three times with T-T(0.1) to remove unbound Mel-PA. Bound activity was measured as the amount of ^{125}I-fibrin solubilized in 1 hr after the addition of 0.3 ml of Tris HCl pH 8.1 containing 2 µg of plasminogen and 80 µg of BSA. Total Mel-PA activity added to the wells was measured as the amount of ^{125}I-fibrin solubilized in 1 hr after the addition of 2 µg of plasminogen and 80 g of BSA (0.3 ml of the same buffer) to wells that had not been washed.

Table 6.2 The effect of plasmin on Mel-PA and urokinase

Time of incubation (min)	Amidolytic activity (FU/ml)			
	Mel-PA + Plasmin	Mel-PA + PBS	Urokinase + Plasmin	Urokinase + PBS
2	42.24	33.37	22.81	21.21
30	42.03	34.86	22.79	23.21
120	44.08	36.60	22.14	20.27
300	42.33	36.11	21.88	20.02
330	44.20	37.95	21.72	20.62

Urokinase (approximately 0.20 pmol; 13.5 FU) or Mel-PA (approximately 24.3 pmol; 24 FU) were incubated with or without 0.125 mg plasmin in a final volume of 0.5 ml PBS at room temperature.

At the times give, 50μl aliquots were removed and added to tubes containing 10 µl (10 KIU) of BPTI. The amidolytic activity in 50 µl of this mixture was then determined in the fluorometric assay (Appendix 3.2) to give the values shown in the table. Plasmin controls, containing no plasminogen activator, showed no activity.

The activity of both Mel-PA and urokinase stayed constant over the 5.5 hr incubation period indicating that they were not degraded by plasmin.

and by others (118,120) this assay uses plasminogen concentrations that are rate-limiting. Since quantitative differences in plasminogen-dependence between Mel-PA and urokinase have important implications for the quantitation of these enzymes, I performed the experiment summarized in Fig. 6.11.

In this experiment, two concentrations of Mel-PA (0.47 and 0.24 pmol/ml) and of urokinase (0.4 and 0.2 pmol/ml) were tested for their ability to catalyse the degradation of solid-phase ^{125}I-fibrin in the presence of plasminogen concentration ranging from 0 to 106 µg/ml. It can be seen from the data shown that the rate of fibrinolysis (measured as the percentage of total ^{125}I-fibrin hydrolyzed/60 min) increased monotonically with plasminogen concentration.

Data for values less than 80% of total fibrinolysis (i.e. when availability of ^{125}I-fibrin was not rate-limiting) were analysed by the method of Cornish-Bowden and Eisenthal (167).These analyses gave apparent Km's for plasminogen for the two concentrations of Mel-PA of 16.2 µg/ml (0.18 µM) and 17.9 µg/ml (0.2 µM). The corresponding values for urokinase were 33.2 µg/ml (0.37 µM) and 37.3 µg/ml (0.42 µM).

The data illustrate that the apparent Km for plasminogen for Mel-PA was lower than that for urokinase.

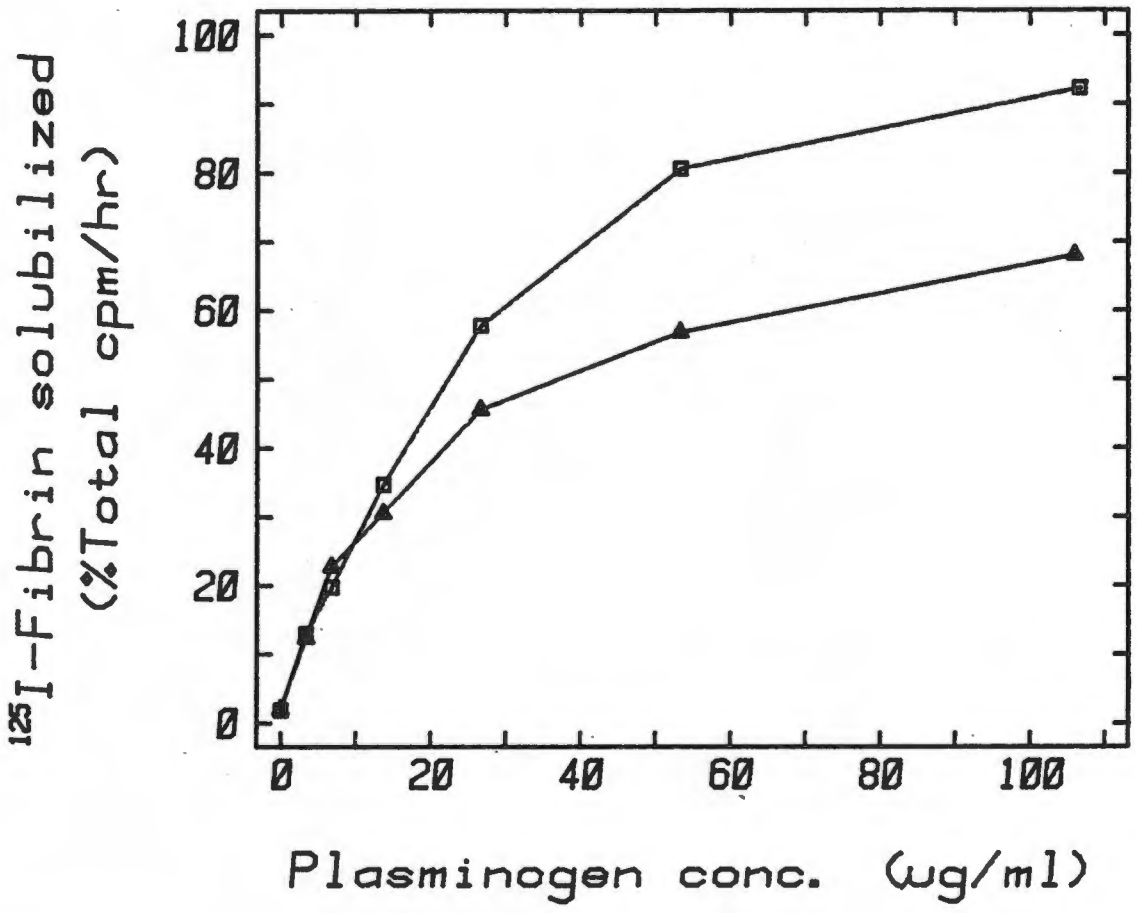


Figure 6.11 Effect of plasminogen concentration on fibrinolytic activity of Mel-PA and urokinase measured in the ^{125}I-fibrin assay.

The fibrinolytic activities of Mel-PA (approximately 0.47 pmol/ml)(△—△) and of urokinase (approximately 0.4 pmol/ml)(□—□) were measured in the ^{125}I-fibrin assay using the plasminogen concentrations shown (0-1.2 μM). ^{125}I-fibrin solubilization was measured after 60 min incubation at 37°C. The conditions of the ^{125}I-fibrin assay are described in detail in the Appendix (A3.1).

The rate of fibrinolysis increased with plasminogen concentration for both Mel-PA and urokinase. These data could be transformed into Lineweaver-Burk plots using Km and Vmax parameters calculated according to Cornish-Bowden and Eisenthal (167). These plots are shown in Fig. 6.12.

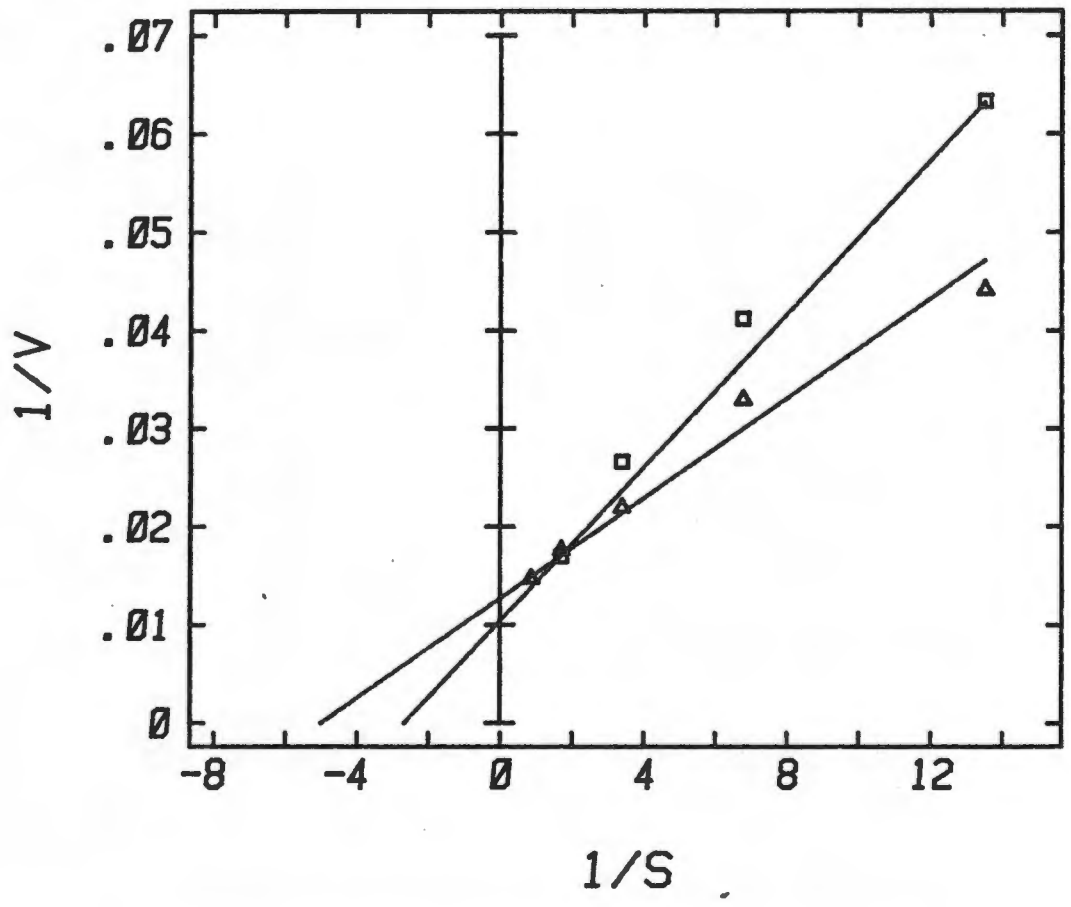


Figure 6.12 Lineweaver-Burk plots of the rate of fibrinolysis of Mel-PA and urokinase as a function of plasminogen concentration.

The data of Fig. 6.11 were used to construct Lineweaver-Burk plots of the reciprocals of the fibrinolytic activities of Mel-PA (△—△) or urokinase (□—□) as functions of the reciprocals of the plasminogen concentrations. Analysis of the data by the method of Cornish-Bowden and Eisenthal (167) yielded Km values of 0.2 μM for Mel-PA and 0.38 μM for urokinase, and Vmax values of 78.4%T/60 min for Mel-PA and 95.9%T/60 min for urokinase. These parameters were used to fit straight lines to the observed points.

DISCUSSION

The sites in plasminogen at which local proteolytic cleavage takes place to give rise to plasmin are well defined (17,19,36) and in this molecular sense the basis for the conversion of plasminogen to plasmin by urokinase or Mel-PA is well understood. When, however, one studies the kinetics of this conversion, the complexities become apparent, since little plasmin is generated if pure solutions of plasminogen activator and plasminogen are mixed. This is particularly true for Mel-PA.

The rate of plasminogen activation is profoundly influenced by the presence of certain amino acids (e.g. lysine, 6-aminohexanoic acid, trans-(amino-methyl) cyclohexane-carboxylic acid)(29,32,34,184), by the presence of fibrin (53,57,160) or certain denatured proteins (159), or by whether or not the plasminogen has lost N-terminal residues (34).

Any comprehensive account of plasminogen activation should, therefore, accommodate the following considerations.

1) <u>The rate of conversion of plasminogen to plasmin by plasminogen activators is accelerated by the presence of fibrin</u>.

There is general consensus about this fact. It has been documented on many occasions with various assays for the quantitation of plasminogen activation (53,57,159,160) and I have had no difficulty confirming it myself. This observation gives rise to a number of subsidiary questions which may be stated as follows:

a) Is the effect that is documented for fibrin also observed with fibrinogen?

A number of workers have addressed this question and their results are generally in agreement and allow the conclusion that fibrin but not

fibrinogen catalyses the activation of plasminogen (57,159,160).

My own observations in this regard are two-fold. Firstly, when studying the conversion of pro-Mel-PA to Mel-PA (Chapter V) I was able to obtain unequivocal evidence to show that both fibrinogen and fibrin may be contaminated by a protease which is able to perform this function. If, therefore, a plasminogen dependent fibrinolytic system contained large amounts of pro-Mel-PA, enhancement of overall fibrinolysis by fibrinogen could be ascribed in part by conversion of pro-Mel-PA to the active enzyme. Since, however, pro-Mel-PA is rapidly converted to Mel-PA by plasmin it is unlikely that much enhancement could be attributed to this mechanism.

Secondly, I have been able to document an accelerating effect of fibrinogen on the conversion of plasminogen to plasmin by Mel-PA but not by urokinase (Fig. 6.5). In fact, in the experiment referred to where plasminogen activation was measured directly the effect of fibrinogen was more marked than that of fibrin. I am at a loss to explain an effect of fibrinogen in this context when others (57,159,160) have not been able to document such an effect. It may be that the fibrinogen preparation that I used, although apparently free of denatured or polymerized material, did contain traces of fibrin, aggregates or other impurities that were responsible for this effect.

b) *Is the effect of fibrin on the enzymatic activation of plasminogen equally evident with urokinase and with Mel-PA?*

The evidence that is available in the literature (53,57,160) and the experiments that I have presented in this chapter (Fig. 6.5 to 6.9) would indicate that the catalytic action of Mel-PA is very much more susceptible to enhancement by fibrin than is the action of urokinase. It should be noted however, that plasminogen may exist in the Lys- or the Glu- forms. A failure to show an effect of fibrin on the activity of urokinase may have been due, in the particular experiments that addressed this problem,

to the fact that all of the plasminogen was in the wrong form for this effect to be demonstrable. This leads to the following question.

c) *Is the enhancement of plasminogen activation by fibrin in any way influenced by prior removal of the "preactivation peptide" from the N-terminal end of plasminogen?*

The literature does not give a satisfactory answer to this question. The appropriate experiment would require a factorial design and would examine for evidence of interactions between forms of plasminogen, type of plasminogen activators and the presence or absence of fibrin. It would seem that such experiments have not been done.

It is well known that urokinase activates Lys-plasminogen more efficiently than it does Glu-plasminogen (32,33,34,184), and it would seem that fibrin has no effect upon the action of urokinase. No one appears to have looked specifically for an interaction between the effects of fibrin and the effects of plasminogen modification. The same criticism may be levied at experiments that have examined the effects of fibrin on the action of Mel-PA. In this case, however, it is not enhancement by fibrin that is in question, since that has been very adequately demonstrated. Here one would ask whether or not synergism between the effects of fibrin and of plasminogen modification could be demonstrated.

2) Plasminogen binds to fibrin

In seeking an explanation for the enhancing effect of fibrin on plasminogen activation a number of workers have found direct evidence for a physical interaction between plasminogen and fibrin that could be detected by binding assays. Typical experimental approaches have been those of Rakoczi et al (185) and Cederholm-Williams (186,187) who measured incorporation of radioactive plasminogen into fibrin clots or Thorsen (39) who measured the partition of plasminogen between clot and supernatant solution

when a clot was formed in the presence of plasminogen. The experiments that relate to this phenomenon and that have been performed by others are generally convincing and the situation may be summarized as follows:

(a) Plasminogen that has been modified by limited proteolysis and so converted from glutamic acid-terminal to lysine- or methionine- or valine-terminals binds more efficiently to fibrin than does native plasminogen (39). The dissociation constant for the reversible binding of Lys-plasminogen to fibrin has been estimated to be approximately 6.3 nM (186). Values for the Kd of other forms of plasminogen do not appear to have been published yet.

(b) The binding of plasminogen to fibrin is mediated by lysine binding sites (40,42,188) that can be uncovered by the action of 6-aminohexanoic acid prior to removal of the preactivation peptide from Glu-plasminogen (189). Plasminogen contains one binding site with high affinity (Kd 9-16 μM) for 6-aminohexanoic acid (41,42) and four with low affinity (Kd ≃ 5 mM) (41). One of the binding sites is located on the first kringle structure (Kd = 16 μM) and another one on the fourth kringle structure (Kd = 35 μM)(42).

(c) The ease with which fibrin-bound plasminogen can be released by lysine or EACA (40) or the readiness with which these compounds inhibit binding of plasminogen to fibrin (39,187) testifies to the reversibility of the binding and to the role of the lysine binding site in its mediation.

(d) Although it is said that both fibrinogen and fibrin bind plasminogen, most of the studies of this phenomenon have been performed with fibrin in experiments in which plasminogen incorporation

into the fibrin meshwork during the clotting process has been measured (39,185,186). The data for the binding to fibrin are therefore sound. This is less so in the case of fibrinogen. It is, however, common laboratory experience that the best "plasminogen-free" preparations of fibrinogen are obtained by a method in which the protein is precipitated in presence of high concentrations of lysine (162) or by treatment of the fibrinogen preparation with lysine-sepharose (190).

Wiman and Wallén (40) have reported experiments showing that plasminogen was bound by fibrinogen coupled covalently to sepharose. It is of interest to note in this regard that Ball et al (191) using density gradient centrifugation were unable to show association between plasminogen and fibrinogen. Influenced, presumably, by the general belief that such binding should have been demonstrable, they attributed their failure to demonstrate it to the fact that the fibrinogen was contaminated with non-radioactive plasminogen to the extent that no further free plasminogen binding sites were available.

3) Plasminogen activators bind to fibrin

The results that I obtained (Fig. 6.9 and 6.10) showed quite clearly that Mel-PA bound to the fibrinogen or fibrin that was adsorbed to the bottom of a tissue culture well, and that it could not be displaced by subsequent washing. Urokinase, on the other hand, bound very little if at all. A number of other reports are available in the literature that confirm the fact that plasminogen activators bind to fibrin.

Husain et al (90) were able to isolate plasminogen activator from normal plasma with a celite-fibrin affinity column using arginine

to elute bound enzyme. It is of interest to note that the fibrin-celite column showed selectivity in that it adsorbed only certain of the plasminogen activators present in plasma and allowed others to run through. The authors did not identify which of the enzymes did not bind. It should be noted in this regard that plasma contains a complex mixture of plasminogen activators that include Mel-PA or "vascular activator" (64,66) a latent urokinase (63) and such compounds as kallikrein and Factor XII (192,193).

Several groups (58,130,169) have shown that plasminogen activators were incorporated into fibrin clots if the enzymes were added before the clotting process was initiated with thrombin. Adsorbed activator could subsequently be eluted from the clot demonstrating the reversibility of the process. Urokinase-type activators were not taken up into the clot whereas "tissue plasminogen activator", "vascular plasminogen activator" and "Mel-PA" were (58,130,169). In a recent report Allen and Pepper (169) have shown that vascular activator is incorporated into a fibrin clot more efficiently than Mel-PA suggesting a means for distinguishing these two plasminogen activators if, indeed, they are different. In a recent abstract Husain et al (1977) claim to have used fibrin-celite to purify the urokinase pro-activator from urine but the final publication of these results is not as yet available to me.

Relatively few attempts have been made to identify the molecular basis of the plasminogen activator-fibrin interaction. Allen and Pepper (169) have subjected plasmin-derived soluble fibrin polymers and plasminogen activators to co-chromatography on molecular exclusion gels and have shown co-acervate formation, suggesting that plasminogen activator binding sites in fibrin survive plasmin treatment. Unfortunately, however, the appropriate controls were not done to establish the specificity of these

interactions. It is by no means uncommon during biochemical studies of fibrinolysis to find diverse intermolecular interactions that are clearly trivial in nature and have little relevance to the coordinated function of this system.

To the best of my knowledge no systematic studies have been done to quantitate the interaction between plasminogen activator and fibrin, so that dissociation constants and molar stoichiometric ratios are not available. The data I have presented in Fig. 6.9 would indicate that the ability of adsorbed fibrinogen or fibrin to bind added plasminogen activator was saturated at a very low Mel-PA : fibrin ratio (approx. 1 : 300). This would suggest that it is neither fibrin nor fibrinogen that is binding but rather some contaminating component. Unfortunately I have no data to support or refute this supposition.

No reports are available to indicate whether binding of plasminogen activators to fibrinogen is in any way different from binding to fibrin. When I analysed the binding layer at the bottom of a Linbro well I found that much of the radioactivity had an electrophoretic mobility in SDS-gels that corresponded to unaltered fibrinogen (Appendix A3.1). I cannot, however, use that evidence to deduce the nature of the activator-binding molecule since the electrophoretic technique may well have failed to detect fibrin. Moreover, fibrinogen may have been denatured in the process of preparing the coated Linbro wells.

An abstract has appeared to indicate that soluble fibrinogen does not bind "tissue activator" whereas fibrinogen covalently coupled to solid surface does (194). This work promises to make an informative contribution when the final data are available in published form.

4) The enzymatic activation of plasminogen is an inherently limited phenomenon.

A number of studies of plasminogen activation have shown that the rate of conversion of plasminogen to plasmin follows an unusual kinetic course in which there is an initial rapid rate which progressively slows until a plateau is reached where no further plasminogen conversion is observed despite the abundant presence of residual plasminogen in non-rate limiting concentrations (159,181). Although reports on this intriguing phenomenon are somewhat conflicting it is worth presenting the following summary of those observations that appear to be valid.

(i) Radcliffe and Heinze (159) noted that the "plateau phenomenon" occurred after no more than a few percent of the available plasminogen had been converted. They were able to re-initiate plasminogen conversion by the addition of urokinase to the mixture. From these observations they reasonably concluded that a reduction in plasminogen concentration was not rate-limiting. By diluting "plateaued" reaction mixtures or by the addition of fresh components these authors were able to show that the phenomenon was not due to irreversible loss of plasminogen activator activity nor was it due to degradation of the "stimulator" molecule (e.g. fibrin or denatured protein) that was required as a co-factor. They attributed the phenomenon to "feedback" inhibition and indicated that they were engaged in its further study. They also made the important observation that the plateau was seen with Mel-PA but not with urokinase.

(ii) Danø and Reich (142), in a detailed kinetic study of plasminogen activation as a function of plasminogen activator concentration and time, found that the rate of the reaction levelled off after approximately five minutes in the absence of bovine pancreatic trypsin inhibitor whereas activation was linear for at least twenty minutes in the presence of

inhibitor. They ascribed this effect to the protection of the plasminogen activator against plasmin formed in the course of the reaction. Moreover, Mangel et al (181), in describing their assay for plasminogen activator, noted that the rate of the reaction slowed after a certain critical plasmin concentration had been reached. They indicated that the phenomenon was currently under investigation in the laboratory and suggested that it was the result of digestion by plasmin of itself, of plasminogen activator and of plasminogen. The experiment that I performed (Table 6.3) showed that neither urokinase nor Mel-PA were susceptible to digestion by plasmin.

Mel-PA appeared to have a lower Km for plasminogen in the presence of fibrin than did urokinase (Fig. 6.12). This would imply that Mel-PA is more efficient than urokinase as a plasminogen-mediated fibrinolysin despite the inferior action of Mel-PA as an amidolytic reagent (Chapter V) and the inferior conversion of plasminogen to plasmin in the presence of fibrinogen or fibrin in the direct assay (Fig. 6.8). Since the ^{125}I-fibrin assay involves sequential substrates, one of which is in an insoluble form, the kinetics are complex and are not linear with time. Furthermore, in the experiment I performed to study the effect of plasminogen concentration on the rate of fibrinolysis the initial reaction rates were not measured. The Km's given, therefore, have descriptive and comparative value only and should not be considered in the conventional rigorous sense. Values obtained by other workers are summarized in Table 6.3 and range from 0.2 μM to 40 μM depending, understandably, on the source of the activator and the nature of the experimental system.

Although I have no doubt that fibrin binds Mel-PA and plasminogen, and that it accelerates plasminogen activation by Mel-PA, I am uncertain of the mechanisms by which these phenomena are mediated.

In Chapter V I showed that fibrinogen and fibrin are contaminated

Table 6.3 Published Km values of plasminogen activators for plasminogen

P.A. type	Plasminogen	Assay	Km	Reference
Murine PA	human	^{125}I-plasminogen conversion	0.18μM	142
PA from rat cells (RT4-71-D2)	dog	active site titration of plasmin with FDE	7.56μM	181
Urokinase	human (lys-plasminogen)	hydrolysis of Bz-Arg-OEt by plasmin	40μM	196
Urokinase	human (glu-plasminogen)	hydrolysis of Bz-Arg-OEt by plasmin	32μM	197

by a protease that converts pro-plasminogen activator to plasminogen activator. These observations and stoichiometric considerations give reason to suspect that the other effects of fibrinogen and fibrin may similarly be mediated by impurities. Unfortunately I have no sound data that bear upon this suspicion, nor can I draw upon experimental results of my own or others that contribute to the intriguing suggestion by Radcliffe and Heinze (159) that fibrin is representative of other "denatured" proteins that may enhance plasminogen activation by a complex mechanism in which"negative feedback" is involved.

The unanswered questions and the uncertainties that exist reflect the complexity of this system and the great difficulty that one has in obtaining pure reagents. Plasminogen, plasminogen activators and fibrinogen are relatively unstable molecules that are notoriously liable to form coacervates with contaminating protein; they may exist in any one or more of several molecular forms reflecting degradation or complex formation, and they are all difficult to purify and to isolate. It is scarcely surprising, therefore, that results have been conflicting, that stoichiometric relationships between interacting components have been difficult to define and that there is as yet no entirely satisfactory account of the complex processes that are involved in plasminogen activation by Mel-PA.

As a speculative suggestion which might serve as a basis for further experimental study, I propose the following model for <u>in vitro</u> plasminogen activation by Mel-PA. The scheme is depicted diagrammatically in Fig. 6.13 and incorporates the following elements:

Firstly, it is assumed that plasminogen activation by Mel-PA requires a "third factor" that is provided by fibrin or, possibly, by other denatured proteins. This factor may provide a "catalytic surface" in much the same way that glass provides a surface for the activation of Hageman factor or antigen-antibody complexes supply the molecular substratum required for the assembly and activation of the first components of complement.

Plasminogen activator (reaction (1)) and plasminogen (reaction (2)) bind to the fibrin "surface". In Fig. 6.13 I have shown these reactions as reversible and sequential. There is good reason to believe that both of them are reversible; there is less reason to believe that they are sequential although there might well be advantages to plasminogen activator binding to fibrin having an effect upon the fibrin-plasminogen dissociation constant.

Bound plasminogen activator converts bound plasminogen to plasmin (this is depicted as reaction (3) in Fig. 6.13 and is shown as being irreversible). It is assumed that, for practical purposes, the binding of plasminogen activator and plasminogen to fibrin is essential for activation to take place.

The plasminogen activator-plasmin-fibrin complex may suffer either of two fates. Firstly (reaction (4)) the plasmin may dissociate to generate fresh binding sites for additional plasminogen to bind. This reaction has not to the best of my knowledge been documented. Secondly, the plasmin may digest the fibrin to which it is bound generating fibrin degradation of products to which plasminogen activator is attached (reaction (5)). It is known that the binding of plasminogen activator to fibrin survives plasmin treatment (169).

Plasminogen activator bound to fibrin degradation products may dissociate (reaction (6)) to release plasminogen activator to participate afresh in reaction (1) by binding to fibrin.

This schematic representation of plasminogen activation and the way it is influenced by fibrin has the advantage that it explains all of the well documented observations relating to this complex process and, in addition, provides an explanation for the "plateau effect" described by Radcliffe and Heinze (159). It is clear that the overall rate of fibrinolysis could be limited by the off-rate for plasmin from the plasminogen

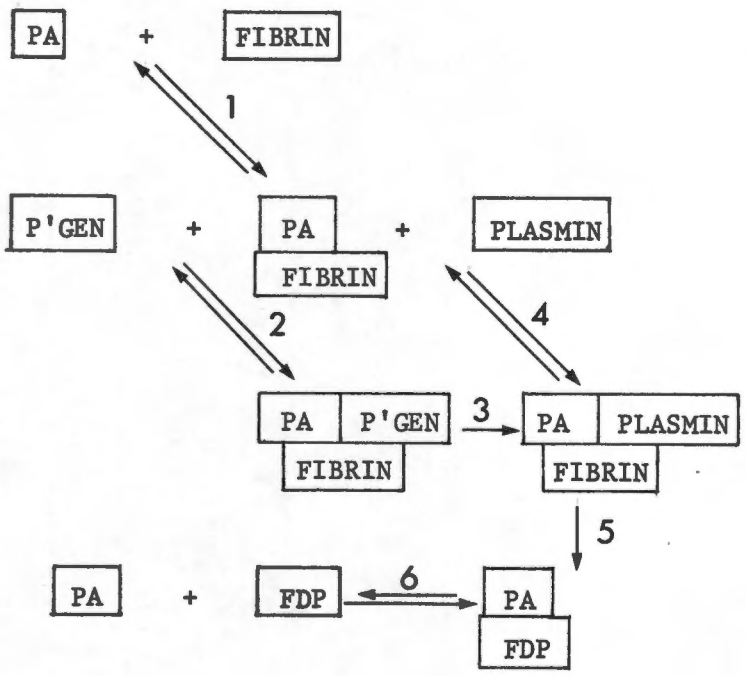


Figure 6.13 Schematic representation of plasminogen activation by Mel-PA in the presence of fibrin.

(For discussion see text)

activator-fibrin-plasmin complex (reaction (4)) and by the rate of dissociation of plasminogen activator from the fibrin degradation products (reaction (6)). An accumulation of plasmin or of FDP's would, by mass action effects, provide the "negative feedback" that Radcliffe and Heinze suggested.

All of the proposed reactions in this schematic representation are amenable to study and verification.

This model incorporates most of the suggestions set forward by Lijnen and Collen (195). It does not consider interactions with inhibitors that might be expected to influence reaction rates in vivo and which are fully dealt with in the informative and imaginative review that Lijnen and Collen have recently published.

IMPLICATIONS FOR THE ASSAY OF PLASMINOGEN ACTIVATORS

Whatever the steps involved in plasminogen activation may be, the role of fibrin and the distinct differences that exist between urokinase and Mel-PA have implications for the measurement of plasminogen activators.

I therefore compared the relative sensitivities of several different assays for plasminogen activators and recorded the extent to which they gave different results for Mel-PA and urokinase.

In documenting these comparisons, I have used, as standards of reference, solutions whose enzyme content was defined in terms of moles of active sites per litre. This was achieved by the fluorometric assay of amidolytic activity and active-site labelling with ^{3}H-DFP of known specific activity (Chapter V). The relative sensitivities of the different assays for urokinase and Mel-PA were then assessed using these standard solutions. The results are presented in Table 6.4.

All of the methods used are described in detail in the Appendix (A3) and I shall only draw attention to the salient features of each procedure.

To each assay I have assigned a value for "sensitivity" to provide some indication of the minimal amount of enzyme that would be reliably measured with that procedure. The estimate for sensitivity that I give, in each case, is unfortunately somewhat arbitrary since I have not gone to great lengths to establish this value in a rigorous way. This would have involved "blind" assays on coded samples and extensive statistical analyses that, at the time, I did not think were warranted. My estimates of "sensitivity" therefore are based upon the subjective judgements of myself and my associates in the laboratory. These judgements incorporate, not only the assessment of what constituted reliable reading above background, but also an evaluation of a reasonable time for the assay to proceed.

1) The fluorometric assay

The amidolytic activities of Mel-PA and urokinase were measured according to the method of Zimmerman et al (133)(Appendix A3.2).

Two fluorogenic substrates were used. These were Cbz-Gly-Gly-Arg-AMC and Boc-Val-Gly-Arg-AMC. Since straightforward, linear kinetics were observed in these assays (Chapter V) they were used in conjunction with ^{3}H-DFP labelling of active sites as a basis for the standardization of plasminogen activators. Using Cbz-Gly-Gly-Arg-AMC, it could be calculated that one fluorometric unit of urokinase corresponded to 0.015 pmol of urokinase and one fluorometric unit of Mel-PA corresponded to 1.01 pmol of Mel-PA.

One pmol of urokinase hydrolyzed 668 pmol of Cbz-Gly-Gly-Arg-AMC or 179 pmol of Boc-Val-Gly-Arg-AMC per min under the standard conditions used. One pmol of Mel-PA hydrolyzed 9.88 pmol of Cbz-Gly-Gly-Arg-AMC and

11.2 pmol of Boc-Val-Gly-Arg-AMC per min. These assays could measure 1.5×10^{-15} mol of urokinase and 1×10^{-13} mol of Mel-PA in 5 min.

2. *Conversion of ^{125}I-plasminogen to plasmin*

In this assay, described by Danø and Reich (142), purified plasminogen is labelled with radioactive iodine and incubated with plasminogen activator in the presence of the plasmin-inhibitor, BPTI. Following incubation, the plasmin and unreacted plasminogen are separated by SDS polyacrylamide gel electrophoresis under reducing conditions and relative amounts of each species are determined by radioassay of gel bands. Since the specific radioactivity of the plasminogen was known activity estimates in terms of pmol of plasminogen converted per min for urokinase or Mel-PA could be obtained. Under the standard conditions employed (Appendix 3.3) one pmol of urokinase catalysed the conversion of 8.1 pmol of plasminogen per min and one pmol of Mel-PA catalysed the conversion of 0.05 pmol of plasminogen per min. In this assay therefore, urokinase was approximately 160 times more efficient than Mel-PA. In the presence of fibrin or fibrinogen insolubilized on a plastic surface one pmol of Mel-PA catalyzed the conversion of 0.31 pmol of plasminogen per min. The same insolubilized substrate had no significant effect on urokinase.

3. *Active site titration of the plasmin formed from plasminogen*

This procedure was developed and described by Mangel et al (181) as a sensitive direct assay for plasminogen activators released by cells in culture. In this method, plasminogen activators and plasminogen are incubated together and the plasmin formed is measured fluorometrically using the plasmin active site titrant, FDE. Since the amount of fluorescent product of hydrolysis can be easily quantitated by reference to chemically

pure material, the amount of plasmin formed can be accurately determined. The assay is rigorous and yields results in terms of molar concentrations of plasmin active sites in the test solution.

Under the standard conditions of the assay described in Appendix 3.4, one pmol of urokinase catalyzed the conversion of 38.7 pmol of plasminogen per min and one pmol of Mel-PA catalysed the conversion of 0.25 pmol plasminogen per min. Urokinase was therefore once again, approximately 160 times more efficient than Mel-PA in the activation of plasminogen.

4. *The ^{125}I-fibrin assay*

In this procedure, a solid-phase substrate is provided by radioactive fibrinogen/fibrin deposited as a very thin layer on the surface of a plastic tissue culture well. Firm attachment is assured by drying for 48 hr at 45°C. Samples to be assayed are added to the wells as solutions and plasminogen activator activity is measured as the plasminogen-dependent solubilization of radioactivity as a function of time.

This technique was originally developed in Reich's laboratory (118). It was refined by Strickland and Beers (120) and subsequently by Wilson and Dowdle (106). Details of the assay are given in the Appendix (A3.1).

In this assay, one pmol of urokinase, might solubilize the radioactive fibrin with an initial reaction velocity of approximately 50% of the total radioactivity per min. The corresponding value for Mel-PA might be 35%. These values would depend very much upon the "age" of the fibrin plate used and on the quality and concentration of the plasminogen. In the table I give data obtained from three different assays. It can be seen that urokinase is approximately 1.5 times more efficient than Mel-PA in this assay. This fact should be borne in mind if urokinase standards are run in the assay simultaneously to serve for both enzymes. With this assay, it should also be remembered:

(a) - that the kinetics are not linear with time particularly at low enzyme concentration. This is illustrated in Fig. 3.1.1 in the Appendix (A3.1).

(b) - that, as usually used, plasminogen concentrations are rate limiting. If urokinase standards are used to measure Mel-PA, it is important that the concentration of plasminogen should be rate limiting, since, as discussed earlier in this Chapter (Fig. 6.12), urokinase and Mel-PA have different apparent Km values for plasminogen in this assay. Therefore, at a plasminogen concentration of 6 µg/ml the fibrinolytic activity of one pmol or urokinase would be approximately 1.5 times that of 1 pmol of Mel-PA. At 25 µg plasminogen/ml however, one pmol of urokinase would have a fibrinolytic activity that was approximately 2.5 times that of an equivalent amount of Mel-PA.

(c) - that the absolute fibrinolytic rate observed for a given amount of urokinase and Mel-PA is influenced by a number of other technical variables such as the salt concentration and the absence or presence of particular amino acids. Since most of these effects act upon plasmin in the second phase of the reaction they affect the fibrinolytic activity of Mel-PA and urokinase to the same extent and may be compensated for by the use of urokinase standards.

(d) - that the ^{125}I-fibrin assay measures both pro-Mel-PA and Mel-PA activator. The justification for this statement is provided in the data in Table 6.5.

Table 6.4

Table 6.4

The data in this table represent a compilation in summary form of the results obtained in this chapter and Chapter V.

In reading the table account should be given to the following considerations:-

(i) specific activity for Mel-PA and urokinase in the different assays were calculated from the results obtained when enzyme solutions that had been standardized with ^{3}H-DFP of known specific activity were assayed using the procedures listed in the first column.

(ii) The assay units are those that I have arbitrarily defined as indicated.

(iii) The ratios for specific activities of urokinase to specific activities of Mel-PA given in column 5 indicate the relative efficiency with which the two enzymes were measured with the different procedures listed.

(iv) The values given for "sensitivity" in columns 6 and 7 are intended to provide an approximate idea of the minimum amount of enzyme that could confidently be measured with each procedure. These values are somewhat arbitrary in that I have not attempted an extensive analysis of all the variables or criteria that would normally be considered in assigning a value of this sort. I have however taken into account the need to complete the assay in the reasonable period of time that I have given in parenthesis. The "reasonableness" of this period was judged in terms of convenience, linearity of the reaction, stability of the reagents, and tolerable "signal to noise" ratios.

(v) "Sensitivities" for the ^{125}I-fibrin assays take into account the fact that the reaction only became linear when approximately 20% of the radioactive fibrin had been solubilized. Owing to instability of the reagents under conditions of the assay and increasing background levels this assay could not be used to obtain reliable quantitative results after approximately 8 hr. It could however be prolonged for 24 to 48 hr if all that was required was an indication that enzyme was present.

(vi) The fibrin-plasmin-agarose assay has not been included in the above comparison since it was not possible to define a unit of activity and since the slopes of the lines relating the log of the enzyme concentration to the area of the lytic zone were not the same for Mel-PA and urokinase. The relative sensitivity of this assay for urokinase and Mel-PA and the amounts of these enzymes required to produce an 8 mm zone of fibrinolysis in 90 min are given in Table 6.6.

Table 6.4 Comparison of assays for quantitative measurement of Mel-PA and urokinase.

Assay	Units of assay	Specific Activity (units/pmol)			Sensitivity (pmol)	
		Mel-PA	UK	UK/Mel-PA	Mel-PA	UK
Fluorometric assay with Cbz-Gly-Gly-Arg-AMC	1FU = 10pmolAMC min^{-1}	0.988	66.8	67	0.10 (5 min)	0.0015
- with BoC-Val-Gly-Arg-AMC	1FU = 10pmol AMC min^{-1}	1.119	17.88	16	0.09 (5 min)	0.006
^{125}I-plasminogen conversion	1 U=pmol ^{125}I-plasmin formed min^{-1}	0.049	8.14	166	2 (20 min)	0.015
Plasmin active site titration	1 U=pmol plasmin min^{-1}	0.245	38.66	158	2 (10 min)	0.015
^{125}I-Fibrin assay	1 U= 1% total cpm released min^{-1}	36.6	56.4	1.5	0.009 (20% in 60min)	0.006
		9.0	14.6	1.6		
		38.5	53.8	1.4	0.00013 (20% in 9 hr)	0.00009

Table 6.5 The ^{125}I-fibrin assay of different Mel-PA preparations

Mel-PA[a] preparation	Amidolytic Activity[b]			Fibrinolytic activity[c] (UK u/ml)	Fibrinolytic Activity / Amidolytic Activity	
	Active Enzyme (FU/ml)	Total Enzyme (FU/ml)	Pro-Mel-PA (% of total)		Active	Total
1	20.39	23.31	12.5	55.2	2.72	2.4
2	23.93	25.98	7.9	69.1	2.9	2.7
3	27.21	32.43	16.1	74.5	2.7	2.3
4	1.61	13.03	87.6	31.4	19.5	2.4
5	1.56	12.55	87.6	30.4	19.5	2.4
6	1.33	12.20	89.0	24.4	18.3	2.0

(a) Mel-PA preparations consisted of serum-free harvest fluids collected from Bowes I cells (1,2 and 3) or serum-free harvest fluids collected from Bowes II cells (4,5 and 6) as described in Chapter I.

(b) Amidolytic activity of the samples was measured in the fluorometric assay as described in the Appendix A3.2. Active enzyme was determined without plasmin treatment of the Mel-PA preparation. Total enzyme was measured after incubation of the samples with plasmin. Pro-Mel-PA was taken as the difference between these two values.

(c) The fibrinolytic activity of the samples was measured in the ^{125}I-fibrin assay as described in the Appendix A3.1.

Note that the ratio of fibrinolytic activity to total amidolytic activity was constant at approximately 2.4 for all Mel-PA preparations whereas the ratio of fibrinolytic activity to active enzyme in the amidolytic assay was affected by the Pro-Mel-PA content. These results indicate that the ^{125}I-fibrin assay measures total Mel-PA. This is not surprising since plasmin generation during the course of this assay clearly converts all of the Pro-Mel-PA to active enzyme.

The fibrin-/casein-agarose assay

One of the assays most commonly used by leading European and Scandinavian workers (217) involves the formation of a plasminogen-rich fibrin clot that is prepared as a thin film. Plasminogen activator samples are applied as drops of uniform size to the clot. These are allowed to diffuse and to digest the fibrin over a 17 hr period at 37°C. The diameter of the lysis zones given by a standard set of urokinase dilutions is used to determine the amount of plasminogen activator present in an unknown sample by interpolation. The relationship that is obtained between the diameter of the fibrinolytic zone and the amount of enzyme applied is not linear and it has not been rigorously established that "tissue activator" and urokinase behave similarly in this assay. Nevertheless, this is one of the original assays for plasminogen activators and credit for its introduction is due largely to Astrup and his co-workers (218).

Although this procedure is theoretically straightforward and, for the most part, technically an easy assay to perform, I found it difficult to cast the clot, apply the sample and incubate the assembly in such a way that symmetrical, radial diffusion took place to give zones of fibrinolysis that were circular, concentric with the point of application of the sample and easy to read.

Furthermore, I wished to compare casein and fibrin as possible plasmin substrates in assays of this kind and I also wished to define conditions for the optimal zymographic detection of plasminogen activators using the fibrin-/casein-agarose underlay procedures developed by Granelli-Piperno and Reich (62).

I therefore developed a technique in which the radial diffusion principle of the fibrin plate assay was maintained but in which a more stable

medium for diffusion was provided by incorporating the substrate in an agarose matrix. This also had the convenient advantage of allowing me to substitute casein for fibrin as a final substrate.

In these assays, slabs of agarose containing purified plasminogen and fibrin or casein are cast in suitable moulds and allowed to set. Wells are then punched in the substrate layers into which samples of plasminogen activator solution are introduced. After diffusion and incubation under suitable conditions the visible zones of fibrinolysis or caseinolysis are measured and related to plasminogen activator activity. These assays are described in detail in the Appendix (A3.5).

It soon became apparent that the assay depended upon two distinct processes. In the first, plasminogen activator diffused radially from the point of application in the well to a distance that was a direct function of the concentration of the enzyme in the well. The second process involved the activation of plasminogen and the digestion of the second substrate, casein or fibrin, to produce the zone of clearing whose dimensions were to be related to enzyme concentration. It was clearly possible that during this second phase, plasmin generated might also diffuse with the result that the radius of the zone of ultimate lysis would be a function of the distance moved by plasminogen activator and plasmin and might be influenced by any interactions between plasminogen, plasminogen activator, substrate and matrix that might result from plasminogen activation.

To separate these processes I resorted to the strategy of allowing the first phase of plasminogen activator diffusion to take place at $4^{\circ}C$ for 16 hr. At this temperature activation of plasminogen did not occur. The second phase of the assay, which led to the development of the zone of fibrinolysis or caseinolysis, was accomplished by incubating the gels at $37^{\circ}C$ for 1.5 hr. The results of assays of this kind are presented in Fig. 6.14 and 6.15, from which it can be seen that sharp circular zones of

Figure 6.14

Figure 6.14 The fibrin-/casein-agarose assay.

The photographs show typical appearances of (A) a fibrin-agarose plate and (B) a casein-agarose plate.

Fibrin- or casein-agarose plates were prepared as described in the Appendix (A3.5) by pipetting a 45°C solution of 1.25% agarose in 0.1M Tris HCl pH 8.1 containing 50 µg/ml of plasminogen and either 2% casein or .25% fibrin, into a mould fashioned from two glass plates separated by 1 mm diameter strands of wire. When the agarose had set, the top siliconized plate was removed and 2.5 mm diameter holes were punched into the agarose layers.

Samples (3 µl) of Mel-PA or urokinase were then inoculated into the wells. The plates were incubated at 4°C for 16 hr and then at 37°C for 90 min, after which time the diameters of the clear rings of lysis were measured.

From left to right the wells contained serial two-fold dilutions starting with a concentration of 250 FU/ml (253 pmol/ml) of Mel-PA (rows a & b) and 250 FU/ml (3.7 pmol/ml) of urokinase (rows c & d).

Note: (i) that the rings of lysis were much sharper in the casein-agarose plates.

(ii) the rings of lysis produced by urokinase were larger than those produced by equivalent amounts of Mel-PA.

(iii) equivalent amounts of Mel-PA gave larger rings in the casein plates than in the fibrin plates. The reverse was true for urokinase.

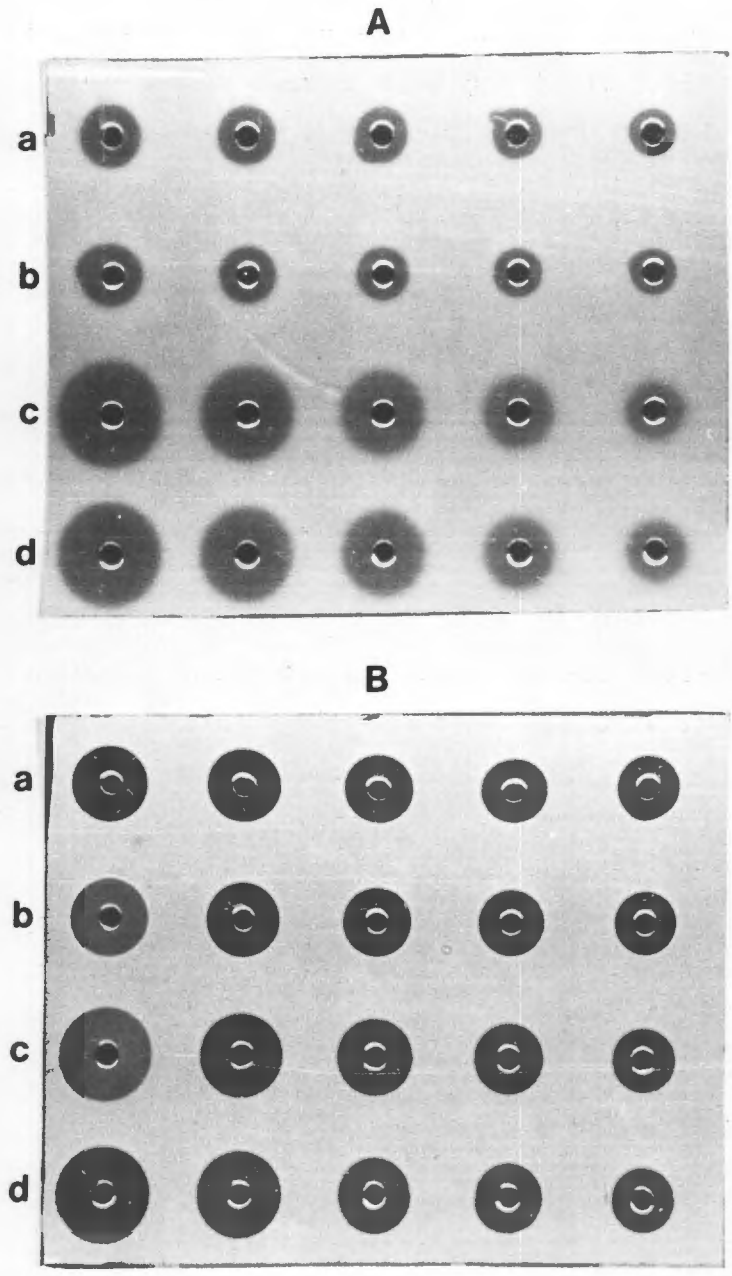

A
a
b
c
d
B
a
b
c
d

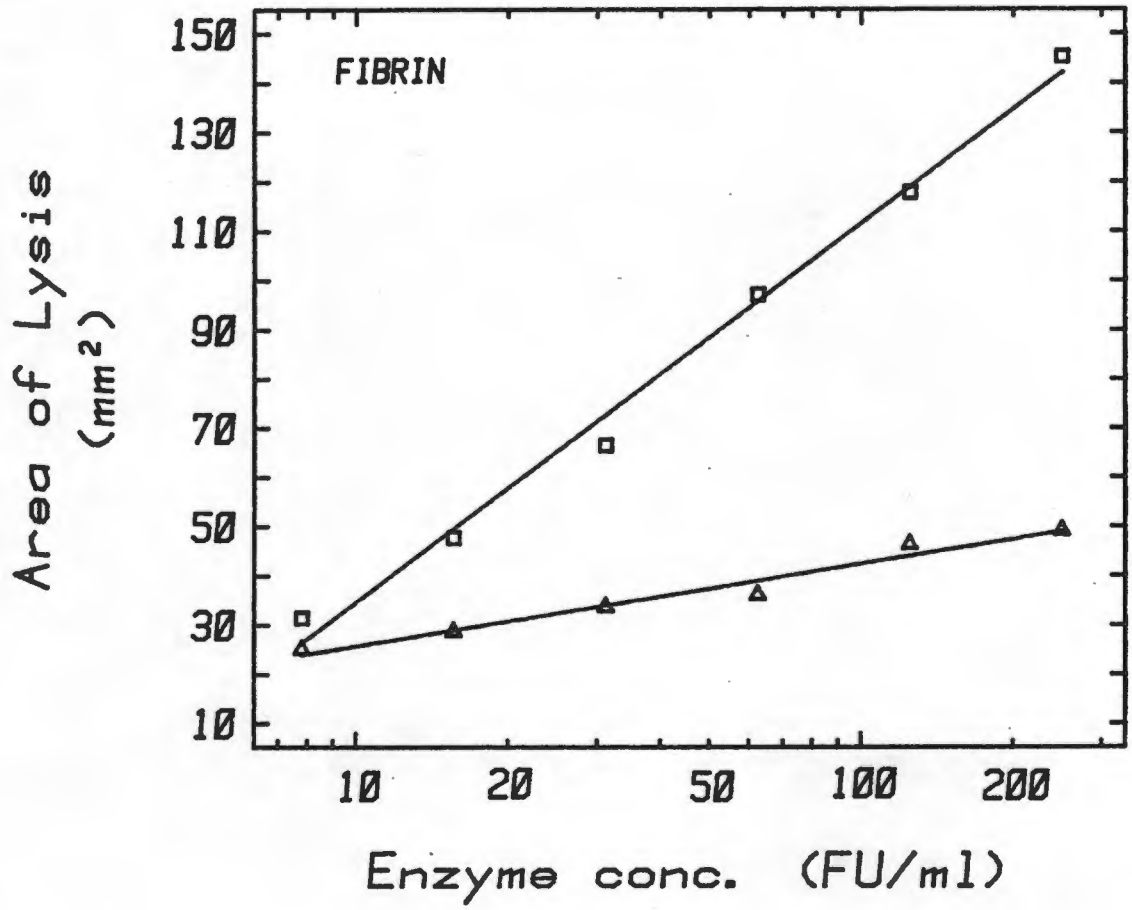
Figure 6.15

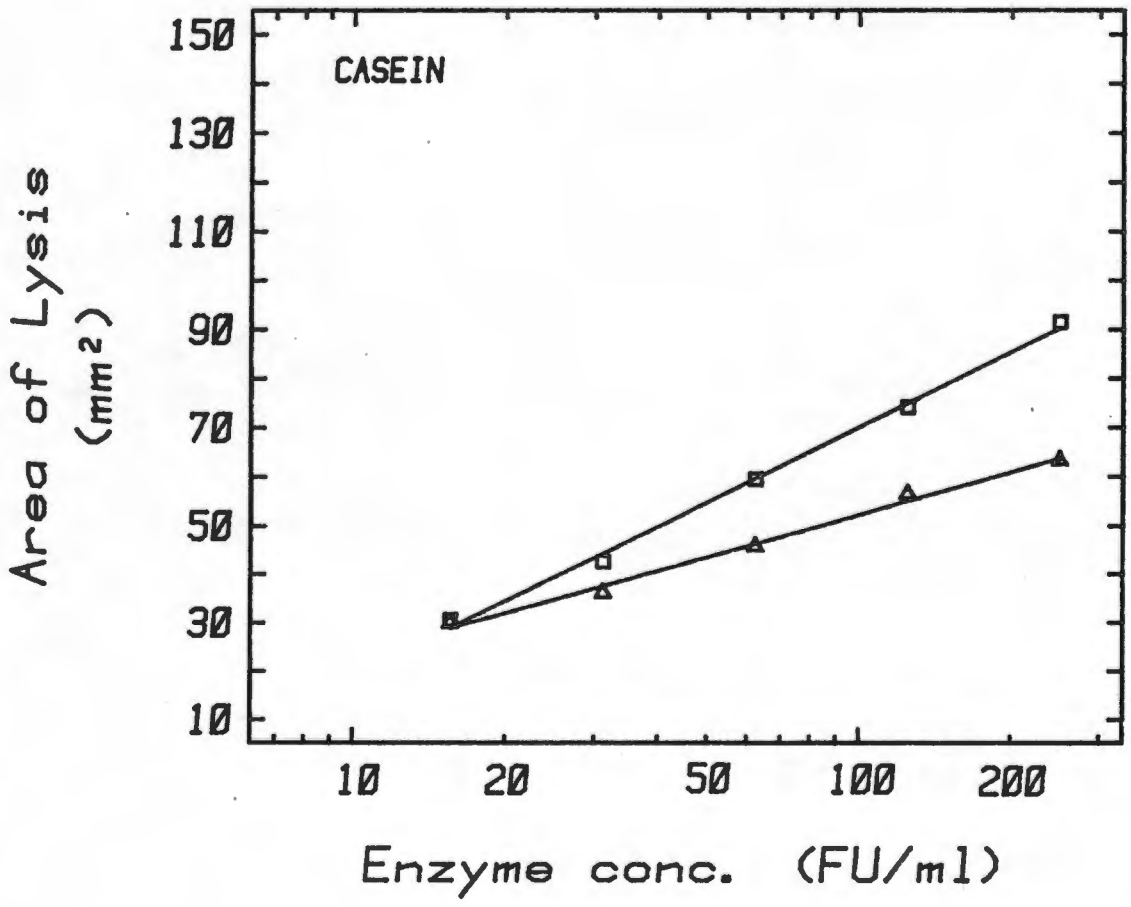
Figure 6.15 The fibrin-/casein-agarose assays.

The figure shows the linear relationship between the areas of zones of proteolysis, as observed in Fig. 6.14, and the logarithm of the concentrations of plasminogen activators. Two-fold dilutions of Mel-PA (△—△) and urokinase (□—□) starting with a concentration of 250 FU/ml were analysed.

Note: (i) that in both assays urokinase gave larger areas of lysis than equivalent amounts of Mel-PA.

(ii) that the slopes of the regression lines for Mel-PA and urokinase differed.


FIBRIN
Area of Lysis (mm²)
150
130
110
90
70
50
30
10
10
20
50
100
200
Enzyme conc. (FU/ml)


CASEIN
Area of Lysis (mm²)
150
130
110
90
70
50
30
10
10
20
50
100
200
Enzyme conc. (FU/ml)

substrate clearing were visible, that could easily be measured. The casein proved to be a more satisfactory substrate in terms of the clarity of the edge of the lytic zone and the ease with which the edge could be measured.

It was noted that a linear relationship could be defined between the area of the zone of proteolysis and the logarithm of the concentration of the enzyme in the sample introduced into the well. This is shown in Fig. 6.15a where fibrin was used as a substrate and in Fig. 6.15b where casein was digested. In these figures the concentration of the enzyme in the samples introduced into the wells is given in terms of fluorometric units/ml.

The results illustrate two striking facts. Firstly, this assay is very much less sensitive for Mel-PA than it is for urokinase. This is shown by the fact that the slope of the line relating the log of Mel-PA concentration to the area of the zone of lysis was considerably less for Mel-PA than it was for urokinase. As a result, a given amount of Mel-PA in fluorometric units produced a very much smaller zone of lysis than did an equivalent amount of urokinase measured in fluorometric units. If one takes into account the fact that one fluorometric unit of Mel-PA represents, in molar terms, approximately 67 times the amount of urokinase giving one fluorometric unit of activity, it is apparent that this assay is indeed very much less sensitive for Mel-PA than it is for urokinase.

In the second place, it can be seen that the discrepancy between urokinase and Mel-PA was less marked with casein than it was with fibrin. The results are summarized in Table 6.6 where the relevant calculations are given to show that, with fibrin as the substrate approximately 5000 times less urokinase than Mel-PA was needed to give a lysis zone of 8 mm diameter. With casein as the substrate approximately 260 times less urokinase than Mel-PA was required.

I presume, without complete justification for doing so, that the

Table 6.6 Fibrin/casein agarose assay.

	Fibrin		Casein	
	Urokinase	Mel-PA	Urokinase	Mel-PA
Sensitivity (m)	76.9	16.6	45.0	26.6
Relative sensitivities	4.6		1.7	
pmol for 8mm diam. zone of proteolysis	0.4	2070	1.1	283

This table was compiled from the parameters obtained when the data presented in Fig.6.14 and 6.15 were fitted by linear least squares regression analysis to the equation:

$$y = m \log x + c$$

where y = square of the diameter (in mm^2) of the zone of proteolysis and x = the concentration, in FU/ml, of enzyme introduced into the wells.

In each case the slope of the line (or the parameter m) indicates the increase in area one would expect for a 10-fold increase in enzyme concentration. This is taken to be a measure of the sensitivity of the assay. Relative sensitivities for the assays are calculated as ratios of the relevant slopes.

To provide a more intuitive idea of the relative efficiency with which urokinase and Mel-PA were detected in the different assays I have also given the amounts of each enzyme that would have been required to give zones of lysis 8 mm in diameter. This diameter was chosen as one that could be conveniently measured with reasonable accuracy.

poor performance of Mel-PA in this assay can be attributed firstly to its tendency to bind to fibrin so that its diffusion through the matrix was impaired and secondly to the fact that agarose, with or without casein, failed to provide an entirely satisfactory environment for Mel-PA to diffuse and to function as an activator of plasminogen.

Conclusions

The experiments that I have done to compare various assay systems for plasminogen activators have been limited both in their variety and in their design and I am thus able to draw only limited conclusions regarding the best assays to use or the factors that influence them. I nevertheless feel justified in making the following statements:

(i) Provided sufficiently pure enzyme is available the fluorometric assay using synthetic substrates gives most satisfactory results in the sense that activity can be defined in terms of pmoles of active sites per unit volume.

(ii) Despite the shortcomings associated with lack of linearity at low enzyme concentrations and the need for simultaneous assay of reference standards, the ^{125}I-fibrin assay provides a sensitive and convenient procedure for the assay of plasminogen activators.

Provided the correct concentration of plasminogen is chosen and provided measurements are confined to that part of the time curve before which the radioactive substrate becomes rate limiting, the assay gives approximately equivalent rates of fibrinolysis for equimolar amounts of urokinase and Mel-PA. An additional advantage of this assay is that it measures both Mel-PA and pro-Mel-PA.

(iii) The assays based on the radial diffusion of PA through agarose containing plasminogen and plasmin substrates are best used to measure

urokinase with casein as the second substrate. They have the advantage of ease of performance but they lack sensitivity. It is of interest to note in this regard that Camiolo et al (53) found that the zone of lysis on a fibrin plate given by Mel-PA was considerably larger than that given by equivalent amounts of urokinase as measured in the caseinolytic assay.

The assay is not quantitative in the sense that enzyme activity cannot be expressed in useful units. For this reason dilutions of a standard plasminogen activator solution whose activity is known would have to be included as described for the ^{125}I-fibrin-assay. Since the assay measures urokinase and Mel-PA with different sensitivities urokinase standard solutions cannot be used for the quantitation of Mel-PA and vice versa. The activity of each type of plasminogen activator would have to be measured against a standard of the same type of enzyme.

(iv) The assay of plasminogen activator based on the direct measurement of conversion of radioactive plasminogen to plasmin is laborious and has little practical value with regard to the routine measurement of plasminogen activator. It has, however, proved very useful in the analysis of mechanisms underlying plasminogen activation and the effects of various macromolecules on this process.

(v) The measurement of plasminogen conversion based upon active site titration of generated plasmin has more practical value than the one involving radioactive plasminogen because it is comparatively easy to perform and because it gives a more quantitative measure of plasmin generation. In addition, as described by Mangel et al (181) the technique is sufficiently sensitive to allow measurement of plasminogen activator activity secreted by cells cultured in vitro. Drawbacks of

this assay would be its relative insufficiency for detecting Mel-PA type plasminogen activators and the large amounts of purified plasminogen that are required.

APPENDIX

A.1 MATERIALS

A1.1 Fibrinogen

Bovine fibrinogen (Fraction I, F-4000, Sigma Chemical Company) or human fibrinogen (A.B. Kabi) were obtained commercially and further purified by precipitation as described by Laki (182). They were then freed of plasminogen by the method of Mosesson (162) which exploits the differential solubility of fibrinogen and plasminogen in the presence of lysine. The details are as follows:-

A solution of 2g of fibrinogen in 100 ml of 0.1M potassium phosphate buffer, pH 6.4, was adjusted to 200 ml with distilled water. After standing overnight at 4°C the solution was centrifuged at 1000g for 15 min and the pellet was discarded.

The fibrinogen was precipitated by the addition of ammonium sulphate to a final concentration of 25%, collected by centrifugation and redissolved in 50 ml of 0.6M NaCl.

After adjusting the pH to 7.4 with dilute NH_4OH, the fibrinogen solution was dialysed against three changes of 0.6M NaCl and adjusted with 0.6M NaCl to contain 10 mg/ml of fibrinogen. Lysine HCl (0.12M in 0.005M sodium phosphate buffer, pH 7.0) was then added to a final lysine concentration of 0.02M.

The solution was brought to 0°C in a salt/ice bath and any precipitate that formed was discarded. The fibrinogen was then precipitated by addition of ice-cold ethanol to a final concentration of 7%. The precipitate was collected by centrifugation, redissolved in 0.6M NaCl and adjusted to a protein concentration of 10 mg/ml. Ethanol precipitation in the presence of lysine was repeated once. The final precipitate was dissolved in 0.6M NaCl and dialysed against PBS. The protein concentration

was adjusted to 10 mg/ml and the final solution was stored frozen in 2 ml aliquots at -20°C.

Electrophoretic analysis of different preparations of human or bovine fibrinogen in SDS-polyacrylamide gels under reducing conditions showed that they varied in subunit chain composition. The electrophoretic patterns of 2 different preparations of bovine and of human fibrinogen are shown in Fig. A1.1.1. All bovine fibrinogen preparations showed degradation. The human fibrinogen preparations were the best in that they still contained intact α chains.

A1.2 Preparation of ^{125}I-fibrinogen

The purified fibrinogen was labelled with ^{125}I using the iodine monochloride method of Helmkamp et al (183).

Carrier free Na^{125}I (10 mCi; 17.5 mCi/μg) was diluted to 1 ml with 0.4M borate buffer, pH 7.65 containing 0.32M NaCl. To this was added 0.3 ml of 0.6% Na_2SO_3 in water to destroy any peroxides present. After standing at room temperature for 15 min, the pyrex tube containing the solution was placed in a boiling water bath and aerated for 15 min. After cooling to room temperature 0.8 ml 2M NaCl containing 242 nmoles of ICl was rapidly added and the mixture was squirted into a tube containing 4 ml of 20 mg fibrinogen in borate buffer. The reaction mixture was then passed through a 2 ml column of Dowex AG-1X8 that had previously been treated with nonradioactive fibrinogen to block nonspecific binding sites. The ^{125}I-fibrinogen was collected as the non-adsorbed radioactive fraction and was dialysed against three changes of PBS containing antibiotics. It was then passed through a 0.45μ Millipore filter and stored at 4°C.

Specific activities of 6-9 x 10^4 cpm/μg protein were regularly achieved.

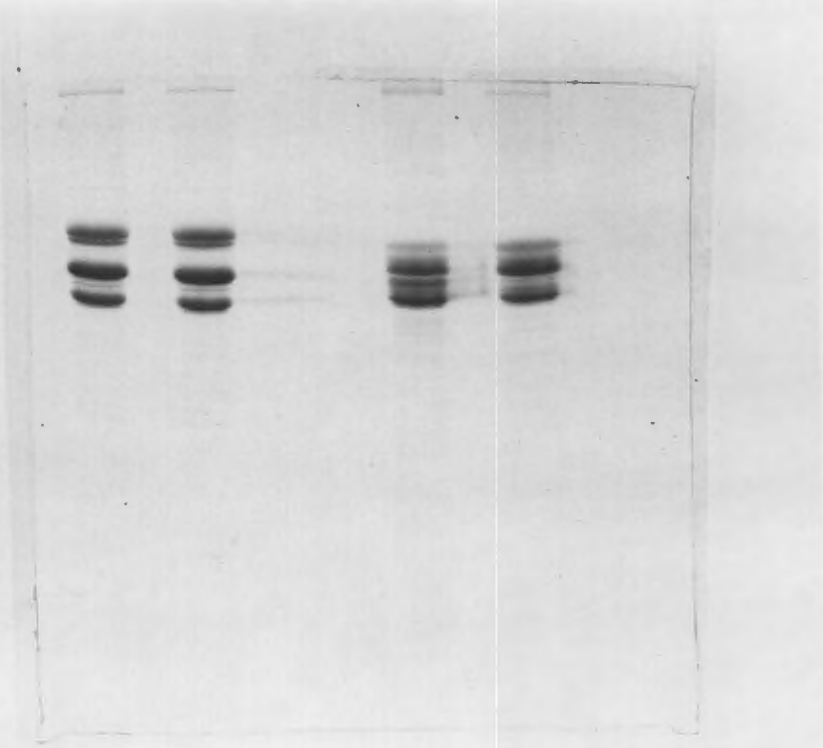


Figure A1.1.1 Electrophoretic analysis of different fibrinogen preparations

Solutions of either human or bovine fibrinogen (2mg/ml) were mixed with an equal volume of 0.1M Tris HCl pH 6.8 containing 2% SDS, 20% glycerol, 10% β-mercaptoethanol and 0.004% phenol red. They were boiled for 1 min and 5 µl samples were then electrophoresed in a 6-15% gradient of polyacrylamide containing 0.1% SDS. After electrophoresis the slab gel was stained with 0.1% Coomassie brilliant blue in 30% methanol and 10% acetic acid and then destained.

The photograph shows the electrophoretic patterns of 2 human fibrinogen preparations (tracks 1 and 2) and 2 bovine fibrinogen preparations (tracks 3 and 4).

Note that the bovine fibrinogen preparation in track 3 was heavily degraded. The other bovine fibrinogen preparation was considered good enough for use in fibrin agar indicator gels (see Appendix 3.5).

Human fibrinogen preparations, which generally showed the least degradation were used for iodination and for the preparation of fibrin coated plastic wells to be used for the ^{125}I-fibrin assay.

A1.3 Purification of plasminogen and preparation of plasmin.

Plasminogen was isolated from human plasma according to a modification of the method of Deutsch and Mertz (45) using affinity chromatography on lysine-sepharose.

Fresh or expired blood bank human plasma collected in acid citrate dextrose was centrifuged at 1700g for 30 min and the pH was adjusted to pH 7.4. A 200 to 300 ml sample was then passed, at room temperature, through a lysine-sepharose 4b column (2.5 x 20 cm) that had been equilibrated with PBS containing 0.5 mg/ml of streptomycin and 0.5 mg/ml of penicillin.

The column was then washed with 0.18M potassium phosphate buffer pH 7.4 containing 0.6M NaCl and 40% ethylene glycol. This step eluted a large opalescent peak of lipo-proteinaceous material that contained no plasminogen. Adsorbed plasminogen was eluted with 0.1M potassium phosphate, pH 7.4, containing 0.2M EACA.

EACA was removed from the eluate by dialysis against PBS at $4^{o}C$ after which traces of contaminating plasmin were inactivated by treatment with 10 mM DFP for 2 hr at $40^{o}C$. Unreacted DFP was removed by dialysis against PBS. The final solution was dispensed in 1-2 ml volumes and stored at $-20^{o}C$.

When analysed by SDS-PAGE, the plasminogen preparations showed two protein bands in roughly equal proportions with approximate molecular weights of 90 000 and 86 000 daltons. These presumably corresponded to partially degraded lysine-plasminogen and native glutamic-acid plasminogen.

Human plasmin was obtained by activation of plasminogen with urokinase-coupled sepharose. Commercial urokinase (Leo; 5 000 Ploug units) was dissolved in 1 ml of 0.1M $NaHCO_3$ pH 8.9 and coupled to 1 ml of CNBr-activated sepharose 4b. One ml of plasminogen at 5 mg/ml in

0.1M Tris HCl pH 8.1 containing 0.1M lysine was mixed with 1 ml of urokinase-sepharose and incubated at room temperature for 3 hr. The urokinase-sepharose was recovered by filtration and the filtrate was stored at -20°C.

A1.4 Radio-iodination of plasminogen

Human plasminogen was iodinated by the iodine-monochloride method of Helmkamp et al (183) using a five-fold molar excess of ICl over plasminogen and 5 mCi of $Na^{125}I$ (15.7 mCi/µg; Amersham).

Carrier-free $Na^{125}I$ (5 mCi) was diluted to 2 ml with 0.2M borate buffer pH 7.65 containing 0.16M NaCl and 100 µl of 5×10^{-4}M ICl was added. Two millilitres of plasminogen (0.9 mg) in PBS was then added and the mixture was incubated at room temperature for 10 min. Free iodine was removed by passage of the mixture over a 2 ml column of Biorad AG1-X8 ion exchange resin that had been equilibrated in saline. Nonspecific binding sites on the column and tubes used in the procedure were blocked by pre-exposure to non-radioactive plasminogen.

The radioactive plasminogen was passed through a 45 µ Millipore filter, dispensed in volumes of 200 µl and stored at 4°C.

The specific radioactivity of the preparation was 90.6 Ci/mmol of plasminogen (0.04 atoms ^{125}I/mol). This preparation was used as a substrate in the ^{125}I-plasminogen conversion assay (Appendix 3.3).

A1.5 Urokinase

Partially purified urokinase was obtained from Leo Laboratories Ltd., Hayes, Middlesex. The contents of 1 vial (5000 Ploug units) were dissolved in a suitable volume (usually 2.5 ml) of 0.1M Tris HCl pH 8.1 containing 8 mg/ml bovine serum albumin or 0.1% Triton X-100. The solution was dispensed into 50 µl volumes and stored in liquid nitrogen.

Electrophoretic analysis of 15 µl of a typical urokinase preparation in an 11% polyacrylamide gel containing SDS showed that it contained many impurities (Fig. A1.5.1).

A1.6 Bovine serum albumin

Protease and inhibitor-free bovine serum albumin was prepared according to Wilson et al (106).

Bovine serum albumin (BDH) was dissolved in water to a concentration of approximately 10 mg/ml and acid-labile protease inhibitors were removed by adjusting the pH of the solution to pH 3.0 with 0.1M HCl and incubating at room temperature for 2 hr. The solution was then neutralized with 0.1M NaOH and protease activity was removed by incubation with 10 mM DFP at 40°C for 2 hr. After extensive dialysis against 0.1M Tris HCl pH 8.1, the solution was adjusted to 8 mg/ml and was stored at -20°C.

Fluorescein-labelled bovine serum albumin was prepared by mixing a 10 mg/ml solution of the protein with 15 µg fluorescein (FITC)/mg of protein in 0.05M sodium carbonate buffer pH 9.0. The solution was stirred for 2 hr at 4°C and then dialysed against PBS.

This preparation was used for the electrophoretic analysis of urine samples as described in Chapter III.

A1.7 Benzamidine sepharose

Meta-aminobenzamidine was prepared from m-nitro benzamidine by catalytic hydrogenation according to the method of Hixson and Nishikawa (135).

Meta-nitrobenzamidine HCl (25 g) together with 150 ml methanol was introduced into a 500 ml Parr glass hydrogenation vessel. The vessel was purged of oxygen with a stream of nitrogen and stoppered. Palladium

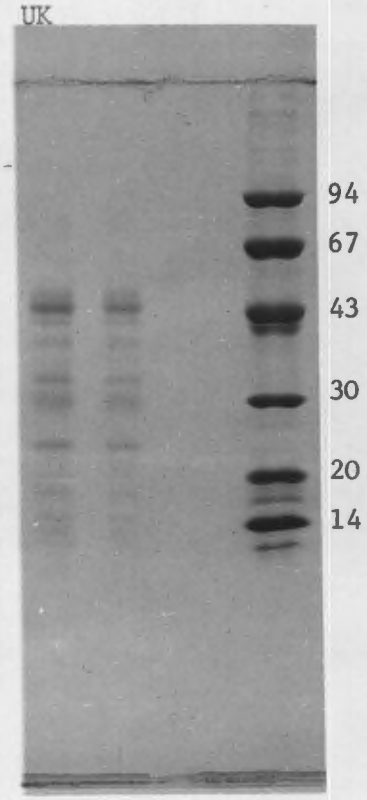


Figure A1.5.1 Electrophoretic analysis of commercial urokinase.

Fifteen microliters of a commercial urokinase solution (2500 Ploug units/ml) in 0.05M Tris HCl pH 8.1 containing 1% SDS and 10% glycerol were electrophoresed in a gradient of 6-15% polyacrylamide containing 0.1% SDS. After electrophoresis the gel was fixed and stained with Coomassie brilliant blue.

The urokinase preparation contained many impurities.

(10% on charcoal; 300 mg) was introduced rapidly and the vessel was re-stoppered. The vessel was then placed into a screen cage and connected to a Parr pressure reaction apparatus equipped with a mechanical shaker. The vessel was purged with hydrogen, and hydrogenation proceeded at an initial pressure of 50 psi for 2 hours.

At the end of the reaction the catalyst was removed from the solution by filtration on a bed of diatomaceous earth. Anhydrous diethyl ether (100 ml) and concentrated HCl (0.125 moles) were then added and the solution was placed at -15°C for 16 hours. The fine white needles of meta-aminobenzamidine were collected by filtration, washed with ice-cold tetra-hydrofuran and dried. The yield was 22.13 g.

Sepharose CL-4b was activated with CNBr according to the method of Nishikawa and Bailon (136).

Cyanogen bromide was dissolved in N-methyl pyrrollidone to give a 12.5% solution. Forty millilitres of the solution were then added dropwise with stirring to 100 ml of wet, settled sepharose CL-4b suspended in 50 ml of water. The reaction was carried out at 15°C for 10-15 minutes while a constant pH of 10.8 was maintained by the automatic addition of 2M NaOH with a Radiometer Autoburette assembly.

After thorough washing in water and 0.1M $NaHCO_3$ pH 8.9, 13.1 g of ε-amino-caproic acid dissolved in 30 ml of 0.1M $NaHCO_3$, pH 8.9 was added. After reaction at 4°C for 24 hr with tumbling, the matrix was washed with 0.2M NaCl. Titration of the carboxyl-groups indicated that 22 μmoles of EACA had been linked/ml of gel.

Aminobenzamidine was linked to the CH-sepharose by carbodiimide coupling according to the method of Hixson and Nishikawa (135).

Meta-aminobenzamidine (8g) was dissolved in 40 ml 0.2M NaCl pH 4.75 and 5 g of 1-ethyl-3-(3'-dimethyl amino-propyl) carbodiimide HCl

(EDC) was dissolved in 10 ml of water. This solution was added to 100 ml of settled CH-sepharose and the pH was maintained at 4.75 with 2M HCl.

The reaction was continued at 4°C for 24 hr after which the matrix was washed exhaustively and sequentially with 0.01M HCl in 0.5M NaCl; 0.01M NaOH in 0.5M NaCl and 0.2M NaCl.

A1.8 Insolubilized inhibitor (DE-3) or IgG

Proteins were linked directly to sepharose that had been activated with cyanogen bromide (210).

Sepharose CL-4b (Pharmacia) was activated with CNBr according to the method of Nishikawa and Bailon (136) as described in Appendix A1.7. Alternatively, commercially available CNBr-activated sepharose 4b (Pharmacia) was used.

For the coupling of proteins to the matrix, 3-10 mg of the protein per ml of settled matrix was used. Coupling was performed in 0.1M $NaHCO_3$ pH 8.9 in all cases and the suspensions were mixed by tumbling at 4°C for approximately 16 hrs.

The matrices were then washed with at least 5 volumes of coupling buffer and incubated in the presence of 1M ethanolamine pH 8.5 for 2 hr at room temperature to block residual binding sites. The matrices were then washed with 0.1M sodium acetate buffer pH 4.5 containing 0.5M NaCl followed by coupling buffer and then they were equilibrated in the buffer used for chromatography.

A1.9 Radio-iodination of DE-3

DE-3 was iodinated with $Na^{125}I$ according to the method of Greenwood et al (43) using chloramine T.

DE-3 (100 µg) in 0.5 ml of 0.05M phosphate buffer, pH 7.5 and 0.5 ml of a 1 mg/ml solution of chloramine T in water were rapidly added to 10 µl of a solution of carrier-free $Na^{125}I$ (1 mCi; 100 mCi/ml) obtained from Amersham. The mixture was stirred at room temperature for 5 min and 0.5 ml of 1 mg/ml Na_2SO_5 was added.

The mixture was then immediately removed and loaded on to a Sephadex G25 fine column (1 x 20 cm). Free iodine was separated from the labelled protein by elution with 0.05M phosphate buffer pH 7.5 containing 0.02% NaN_3. Fractions containing radioactivity that eluted in the void volume were pooled to give 3 to 4 ml of radioactive solution.

The specific radioactivity of DE-3 obtained was between 0.9 and 1.25×10^5 cpm/pmol (60 - 80 µCi/nmol).

A.2 POLYACRYLAMIDE GEL ELECTROPHORESIS

A2.1 Sodium-dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

The discontinuous system of Laemmli (223) as described by Maizel (137) for electrophoresis in polyacrylamide slab gels containing SDS was used. The upper stacking gel contained 3% polyacrylamide: 0.08% bisacrylamide in 0.125M Tris HCl pH 6.8 containing 0.1% SDS, 0.1% TEMED and 0.02% ammonium persulphate. The lower resolving gel contained either a uniform concentration or a linear gradient of polyacrylamide : bisacrylamide (30:0.8) in 0.38M Tris HCl pH 8.8 containing 0.1% SDS, 0.1% TEMED and 0.02% ammonium persulphate. Reservoir buffer consisted of 0.025M Tris, 0.192M glycine pH 8.5 containing 0.1% SDS.

Electrophoresis was carried out at 4°C at a constant current ranging from 8 mA to 20 mA depending upon gel size and cooling efficiency. After electrophoresis the gels were prepared for analysis as required for each experiment.

Samples to be electrophoresed were generally made 2.5% or 1% with respect to SDS and 10% with respect to glycerol; 4 µg/ml of phenol red was added to serve as a tracking dye. The samples were heated for 1 min in a boiling water bath before loading.

For reduction of disulphide bonds the samples were made 5% with respect to β-mercaptoethanol in addition to the SDS and glycerol.

Usually 5-10 µg of total protein was loaded per well on small gels (60 x 70 x 1 mm) and 20 - 100 µg were loaded per well on large gels (150 x 120 x 2mm). After electrophoresis the gels were fixed and stained with 0.1% Coomassie brilliant blue R250 in 10% acetic acid : 30% methanol.

For the detection of bands containing very small amounts of protein the silver stain method of Merril et al (215) as modified by

Dr. D. Belin of the Rockefeller University (personal communication) was used. After electrophoresis, the gels were immersed in fixing solution consisting of 30% methanol : 10% acetic acid in water for a minimum of 1 hr. The gels were then expanded in water for 30 min. The water was changed every 10 min. A freshly made solution of 10% glutaraldehyde in water was then added and the gels were incubated for 30 min. The glutaraldehyde was then removed by washing the gel in 3 x 1 litre volumes of H_2O over 30 min.

A silver stock solution was made by dissolving 20g $AgNO_3$ in 100 ml of water. Immediately before use, 1.6 ml of the 20% solution of $AgNO_3$ was added slowly to 8.4 ml of 0.09M NaOH and 0.56 ml of 28% NH_4OH. After all the $AgNO_3$ had dissolved the volume was made up to 40 ml with 20% ethanol in H_2O. The gel was incubated in this mixture for 5 min and then washed twice (2 min) in water. Bands were developed by immersion of the gel in an aqueous mixture containing 0.005% citric acid, 0.0185% formaldehyde and 10% ethanol. After the desired intensity of staining had been achieved, the gels were washed in 2 x 1 litre volumes of water and dried.

Proteins at low concentration were also prepared for electrophoresis by precipitation with trichloracetic acid (TCA) in the presence of 0.1% Triton X-100 according to the method of Retz and Steel (165).

Samples containing 0.1% Triton were made 0.1% with respect to SDS and the proteins were precipitated by the addition of TCA to 6% final concentration. The proteins were pelleted by centrifugation at 3000 rpm for 30 min in round bottomed tubes. The precipitates were washed in 1 ml of acetone, recentrifuged and dissolved in a minimum volume of 0.06M Tris HCl pH 6.8 containing 2.5% SDS, 5% glycerol and 4 µg/ml phenol red. The samples were heated for 1 min in a boiling waterbath before loading on the gels.

A2.2 Alkaline discontinuous polyacrylamide gel electrophoresis

This was carried out according to the method of Maizel (137). Upper stacking slab gels consisted of 3% polyacrylamide : 0.08% bis-acrylamide in 0.0625M Tris HCl pH 6.7. Lower resolving slab gels consisted of 7% polyacrylamide : 0.19% bisacrylamide in 0.38M Tris HCl pH 8.9. TEMED at 0.1% and ammonium persulphate at 0.02% were used as catalyst and initiator respectively. The reservoir buffer was 0.005M Tris, 0.038M glycine, pH 8.5.

Samples for electrophoresis were diluted with 0.01M Tris HCl pH 6.7 containing 5% glycerol and 4 µg/ml phenol red. Before electrophoresis the samples were boiled for 1 min.

A2.3 Polyacrylamide gel electrophoresis and zymography on fibrin agar gels

The procedures followed were as described by Granelli-Piperno and Reich (62). Enzymes were separated by SDS-polyacrylamide gel electrophoresis and active enzyme bands were localized by layering the polyacrylamide slab on an agar gel layer containing plasminogen and fibrin.

After electrophoresis SDS was removed from the gels by incubation with gentle agitation, in 2.5% aqueous Triton X-100 for 1 hr at room temperature. The gel was then rinsed in water, drained and layered on the fibrin-plasminogen indicator gel.

The indicator gel was cast in a mould to give a slab that was 0.8 mm in thickness and that contained 1.25% agar, 5 µg/ml of purified human plasminogen, 2 mg/ml of bovine fibrinogen and 0.06 units/ml of thrombin in 0.05M Tris HCl pH 8.1. "Casein" gel contained 20 mg/ml of skimmed milk powder instead of fibrinogen and thrombin.

The whole assembly was incubated at 37°C in a humid chamber.

Bands of protease activity could be seen as clear lysis zones in the opaque fibrin layer under dark background illumination. After the desired size of lysis bands had been achieved the fibrin gel could be preserved by staining for 10 min in a solution of 0.1% amido black in 70% methanol and 10% acetic acid and destaining in 70% methanol and 10% acetic acid.

Fibrin gels without plasminogen were employed for the zymography of plasminogen-independent proteases and to provide a control indicator system for the definitive identification of plasminogen activators.

Molecular weight marker proteins could be coelectrophoresed in the same gels. After electrophoresis the tracks containing the markers were separated and stained in 0.1% Coomassie brilliant blue in 30% methanol : 10% acetic acid.

A2.4 Zymography in gelatin-SDS polyacrylamide gels

This technique for the electrophoretic analysis of proteases in SDS-polyacrylamide gels containing copolymerized plasminogen and gelatin has been described in detail in Chapter II.

A2.5 Autoradiography of polyacrylamide gel slabs containing electrophoresed radioactively labelled compounds.

For the autoradiography of slab gels containing proteins labelled with ^{125}I the gels were first stained and fixed in 0.1% Coomassie brilliant blue in 30% methanol : 10% acetic acid. After destaining in 30% methanol : 10% acetic acid, the gels were shaken for 30 min in 200 ml water to which had been added approximately 2 ml of glycerol. The gels were then dried between cellophane sheets under vacuum. The dried gels were placed in contact with Osray RP1 X-ray film (Agfa-Gevaert) for various times before development and fixing of the films.

For the detection of β-emitters, such as ^{3}H, the fluorographic method of Bonner and Laskey (139, 166) was used. Gel slabs were soaked twice for 30 min in 20 times their volume of DMSO. They were then immersed in 4 times their volume of 20% 2,5 diphenyloxazole (PPO) in DMSO for 3 hr. The scintillant was then precipitated in the gel by immersion in water for 1 hr, and the gels were then dried between cellophane sheets. Osray RP1 X-ray film was exposed to the gel at -80°C for varying lengths of time.

Alternatively, Coomassie blue stained gels were impregnated with "En^{3}Hance" (Packard Instrument Company, Inc. Ill. U.S.A.) according to the manufacturers instructions.

A2.6 Molecular weight determination in SDS polyacrylamide gels

The method of Weber and Osborne (224) was used for the determination of molecular weights using co-electrophoresed marker proteins obtained from Pharmacia Fine Chemicals (phosphorylase b - 94 000 daltons; bovine serum albumin 67 000 daltons; ovalbumin - 43 000 daltons; carbonic anhydrase 30 000 daltons; trypsin inhibitor - 20 100 daltons; and α -lactalbumin - 14 400 daltons).

Molecular weights of unknown proteins were obtained by interpolation from a curve constructed by plotting the logarithm of the molecular weights of the marker proteins as a function of their electrophoretic mobility relative to phenol red.

A3 ASSAYS FOR PLASMINOGEN ACTIVATOR

A3.1 The ^{125}I-fibrin assay

In this procedure a solid-phase substrate was provided by radioactive fibrinogen/fibrin deposited as a thin layer on the bottom inside surface of plastic tissue culture wells. Samples to be assayed were added to the wells as solutions and plasminogen activator activity was measured as plasminogen-dependent solubilization of radioactivity as a function of time. This technique was originally developed in Reich's laboratory (118). It was refined by Strickland and Beers (120) and subsequently by Wilson and Dowdle (106).

The preparation of ^{125}I-fibrinogen has been described (A1.2). Fibrinogen preparations from different sources as well as from different batches within the same source varied widely in subunit integrity and composition (A1.2) and in intrinsic amidolytic activity (Chapter V). Only fibrinogen preparations that yielded low background values in the ^{125}I-fibrin assay were used.

For the preparation of insolubilized substrate plates 200 µl of a 5 mM NaCl solution containing 30 µg (100 000 to 130 000 cpm) of ^{125}I-fibrinogen were added to each well of Linbro multiwell tissue culture plates. The plates were then incubated at 45°C for 48 hr to dry the solutions and to assure firm attachment of the fibrinogen to the surface of the wells. Before each assay, 1 ml of PBS was added to each well and the plates were incubated at 37°C for 30 min. Unbound radioactivity, usually amounting to 20 - 30 000 cpm, was removed by washing each well twice with PBS and once with 0.1M Tris HCl pH 8.1. Residual liquid in the wells was removed with suction.

For the assay 280 µl of 0.1M Tris HCl pH 8.1 containing 2 µg of human plasminogen and 80 µg of protease and inhibitor-free bovine serum

albumin (Appendix 1.6) were added to each well at 0°C.

Dilutions of plasminogen activators in suitable buffers (usually 0.1M Tris HCl pH 8.1 containing 0.1% Triton X-100) were added to the wells in 20 µl volumes and the plates were incubated at 37°C. At various time points the plates were set on chipped ice and 50 µl aliquots were removed from each well for radioassay in a Packard Gamma counter. Control wells contained plasminogen without plasminogen activator for the determination of fibrinolysis by the plasminogen on its own.

Samples were also assayed in the absence of plasminogen to measure plasminogen-independent proteolytic activity.

The total radioactivity that could be solubilized from a well was determined by incubation of the substrate in 300 µl medium containing 2.5 mg/ml trypsin.

The cumulative radioactivity released into each well was calculated and, after subtraction of the cumulative radioactivity released by plasminogen alone, the percentage of radioactivity released relative to the total radioactivity released by trypsin could be calculated. The results were then expressed as the percentage of total radioactivity solubilized in a given time (e.g. %T/30 min).

Since the release of radioactive fibrin degradation products measures effects upon two sequential substrates and since the second of these substrates is present in an insoluble form, the kinetics are understandably complex. Thus one usually finds that the release of radioactivity is not linear with time but follows rather a sigmoid curve characterized by an initial "lag period" followed by a fairly linear period of cumulative release with a plateau when available radioactive fibrin becomes rate-limiting. These kinetic features are illustrated in Fig. A3.1.1 which illustrates two additional factors that are worthy of comment. Firstly, as can be seen

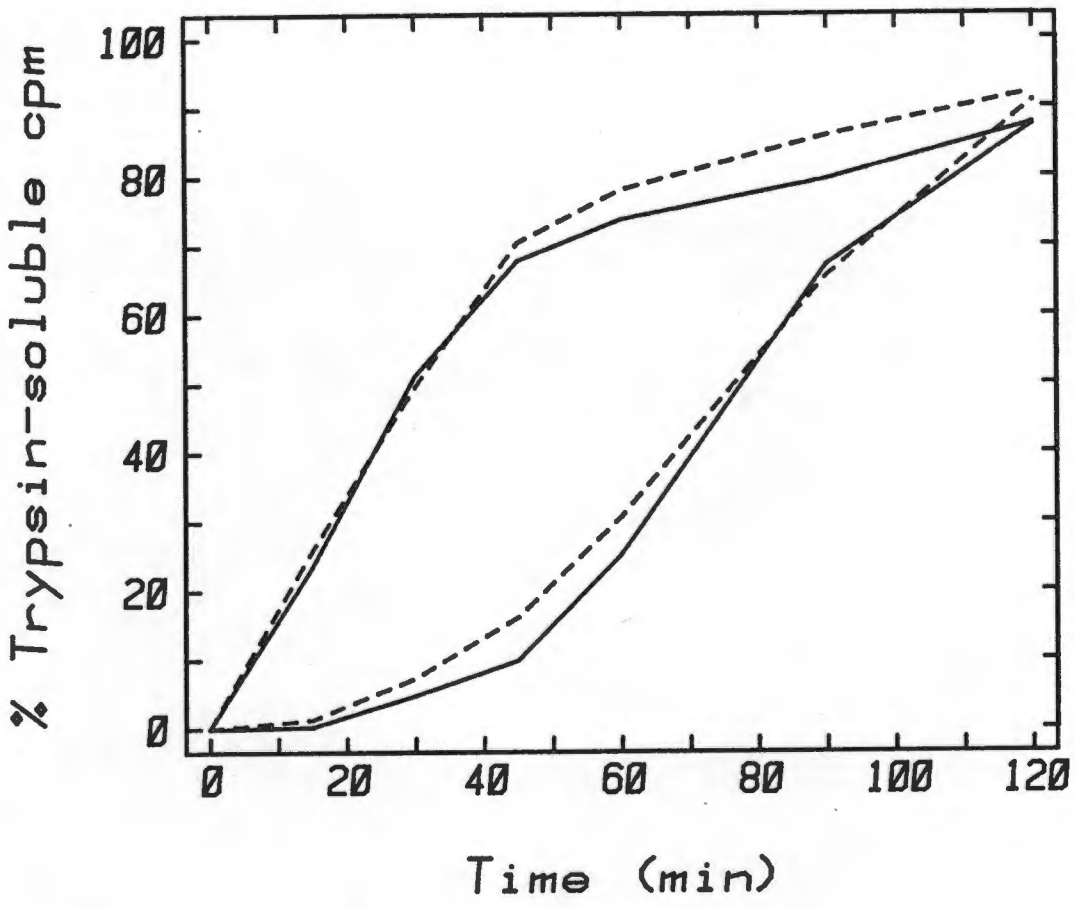


Figure 3.1.1 Kinetics of plasminogen activator activity in the ^{125}I-fibrin assay.

Twenty microlitres of Mel-PA or urokinase were added to 280 µl of 0.1M Tris HCl pH 8.1 containing 2 µg of plasminogen and 80 µg bovine serum albumin in a plastic well coated with ^{125}I-fibrinogen. The plate was incubated at 37°C and 25 µl aliquots were removed at different times. The radioactivity was counted in each sample and the percentage of radioactivity relative to the total radioactivity released by 300 µl of 2.5 mg/ml trypsin was calculated. The figure depicts the increase in the percentage of total radioactivity released with time by two different dilutions each of Mel-PA (————) or urokinase (– – – – –).

It can be seen that relatively linear kinetics were obtained for both urokinase and Mel-PA at the higher concentrations. At the lower concentrations both enzymes showed a lag phase. This was more pronounced with Mel-PA than it was with urokinase.

from the figure the deviation from linearity of the cumulative time curve was most pronounced when low concentration of enzyme were being assayed i.e. amounts of plasminogen activator that released 20% or less of the trypsinizable radioactivity in the first hour.

Secondly, it can be seen that Mel-PA and urokinase behaved in this assay with essentially similar kinetics with the only difference being that at low enzyme concentrations the initial lag with Mel-PA was more evident than it was with urokinase.

I presume that this initial lag with low enzyme concentrations reflects the fact that both plasminogen activator and plasminogen were rate limiting and that a steady rate of fibrinolysis only comes about when an adequate concentration of plasmin has been attained.

When any comparative estimates of fibrinolytic activity of plasminogen activator preparations were required (e.g. in chromatography fractions), different dilutions of the preparations were assayed and only those samples capable of releasing at least 20% of the total radioactivity in 1 hr were used for the calculation of enzyme activity. Such activity estimates varied widely between assays and depended largely on the "age" of the insolubilized substrate and the plasminogen preparation used in the assay.

This inconsistency between assays could be accommodated by including, with each run, serial doubling dilutions of a standard urokinase preparation, usually starting at a concentration of 0.2 Ploug units per well. As can be seen in Fig. A3.1.2, a useful linear relationship between amount of enzyme and percentage of total trypsinisable radioactivity released over a limited range of concentration at any given time could be observed for both Mel-PA and urokinase. Fibrinolytic activity of unknown samples could thus be estimated in terms of urokinase units/ml (UK u/ml) by comparison to the urokinase standard curve at any given time.

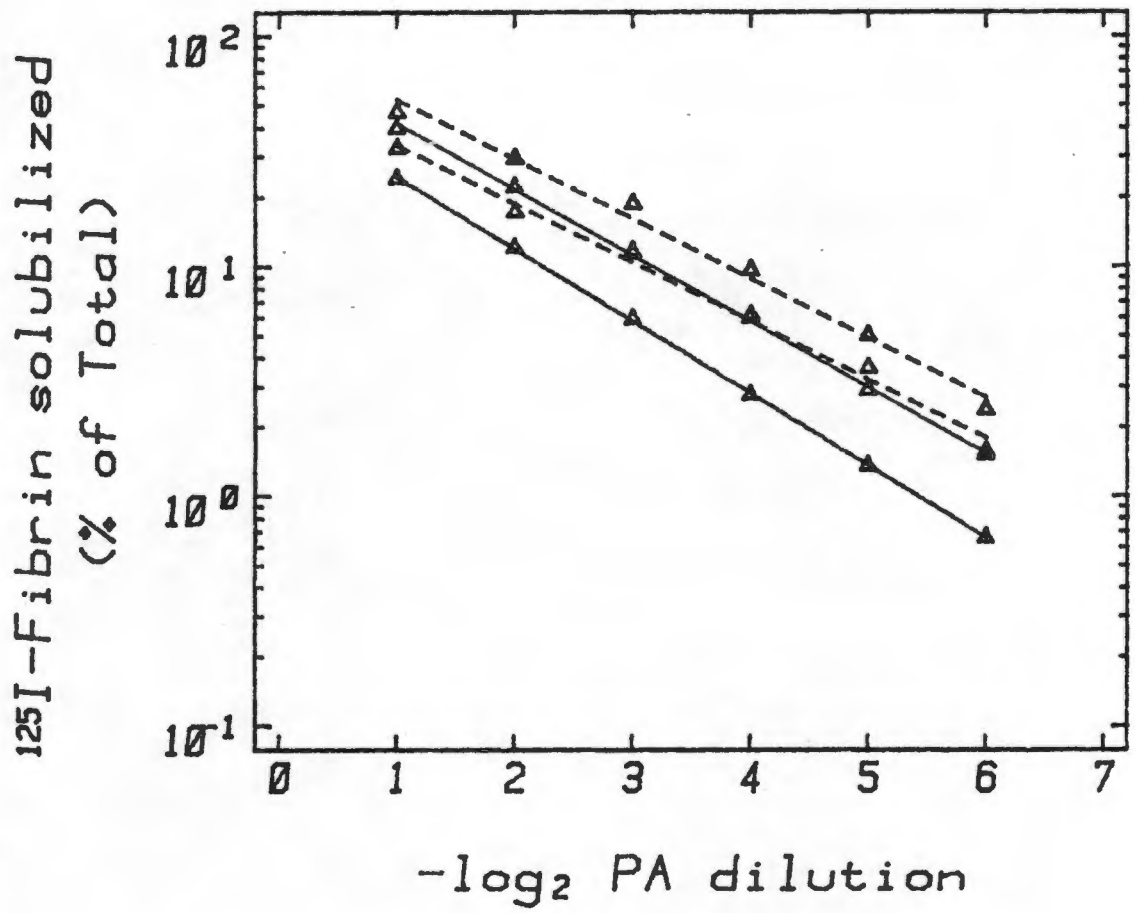


Figure A3.1.2 Relationship of enzyme concentration and cumulative release of radioactive fibrinogen degradation products in the ^{125}I-fibrin assay.

The cumulative release of radioactive fibrinogen degradation products by six doubling dilutions of urokinase (Δ—Δ) or Mel-PA (Δ--Δ) was measured in the ^{125}I-fibrin assay after 45 min and 60 min. The highest concentration of urokinase in the experiment was 0.2 Ploug units/well.

A double logarithmic plot of the data gave a useful linear relationship of enzyme concentration and cumulate release of radioactivity at the times tested.

As originally described (120) the ^{125}I-fibrin assay required conversion of fibrinogen to fibrin by thrombin that was believed to be present in serum. The authors gave as evidence for the fact that conversion to fibrin had taken place data to indicate that approximately 13% of the radioactivity was solubilized with thrombin treatment. This is the amount one would have expected as the result of thrombin action.

Since I was uncertain both of the need to convert adsorbed fibrinogen to fibrin by exposure to thrombin present in serum and of the efficacy of this procedure; and since I felt that inhibitors present in serum might interfere with the assay, I performed an experiment in which I "activated" Linbro plates with 10% plasminogen-free foetal calf serum in RPMI as is usually done by others or with 0.1 NIH units/ml thrombin in PBS containing 1 mM $CaCl_2$. Test plates were treated with PBS alone.

After washing, the ^{125}I-fibrinogen/fibrin layers were examined for their ability to serve as substrates for the assay of Mel-PA and urokinase. As can be seen from the data summarised in Fig. A3.1.3 it made no difference either to the total counts remaining adsorbed to the dish or to the amount of radioactivity released by equivalent amounts of Mel-PA or equivalent amounts of urokinase whether the layers were treated with PBS alone, PBS and thrombin or dilute foetal calf serum.

After treatment of the radioactive substrate with 100 µl of 0.065M Tris HCl pH 6.5 containing 1.0% SDS, 10% glycerol and 5% 2-mercaptoethanol at 60°C for 10 min, approximately 25% of the total radioactivity could be released. Analysis of this material obtained from wells treated with FCS, thrombin or PBS by SDS-polyacrylamide gel electrophoresis, showed that the material released from the wells was the same in all cases and was similar to the material originally put on the wells. I was not able to conclusively establish whether conversion of fibrinogen to fibrin took place in incubation of the substrate with either thrombin on foetal calf serum.

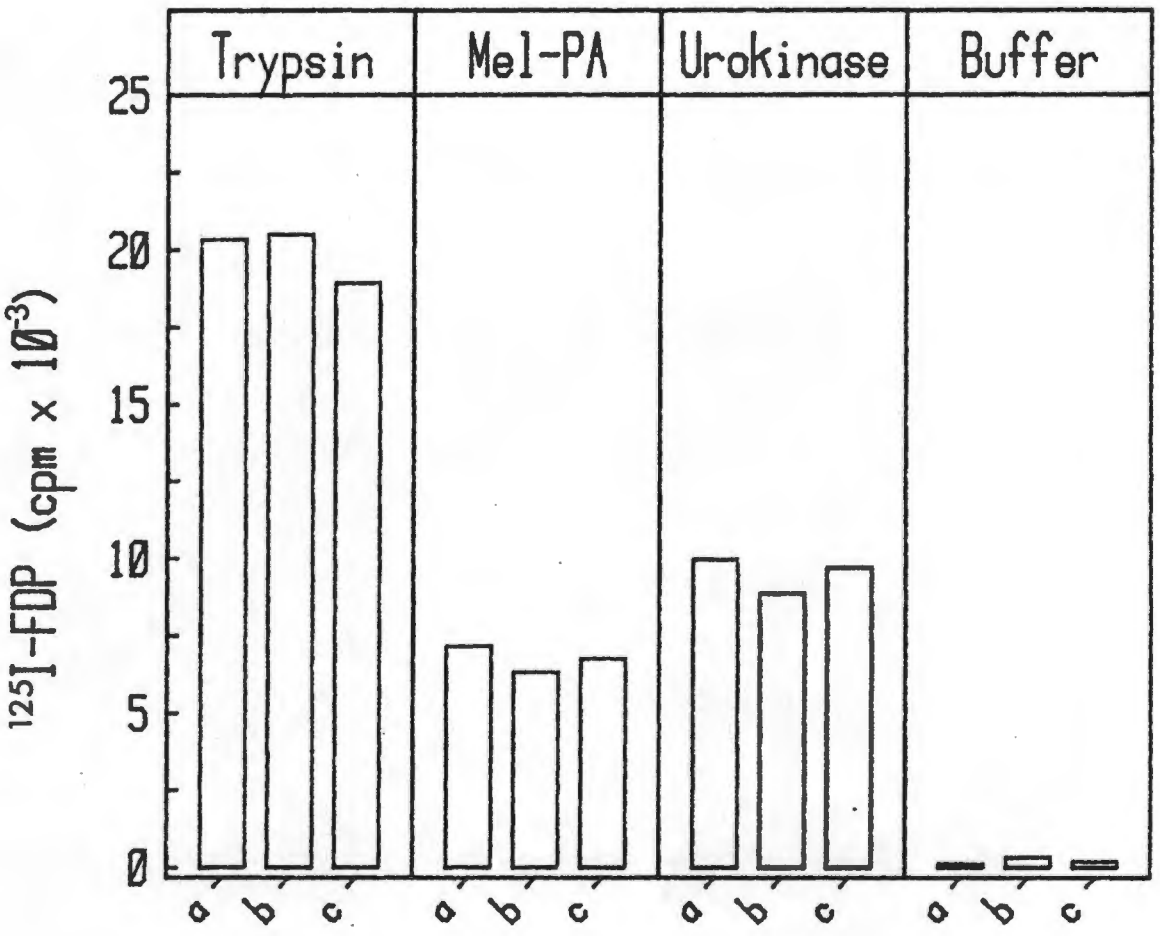


Figure 3.1.3 Effect of pretreatment of ^{125}I-fibrinogen coated Linbro plate wells.

To wells containing insolubilized ^{125}I-fibrinogen (30 µg; 100-120 000 cpm) were added 1 ml of either RPMI containing 10% plasminogen-free foetal calf serum (a); 0.1 NIH units/ml thrombin in PBS containing 1 mM $CaCl_2$(b); or PBS (c). The plate was incubated at 37°C for 2 hr and each well was then washed twice with PBS and once with 0.1M Tris HCl pH 8.1.

The release of radioactivity from the wells by trypsin, Mel-PA, urokinase or buffer alone was then measured, and the results are shown in the bargraph.

As can be seen from the figure, pretreatment of the insolubilized ^{125}I-fibrinogen with the various reagents did not make any difference to the radioactivity released by trypsin, Mel-PA, urokinase and buffer alone.

A 30 min preincubation with PBS to remove loosely bound counts is, therefore, all that is needed to prepare plates for the assay. It is not necessary to prepare plasminogen-free serum or dilute thrombin.

A3.2 Fluorometric assay

The fluorescent substrates, N-Cbz-glycyl-glycyl-arginyl-7-amino-4 methyl coumarin (Cbz-Gly-Gly-Arg-AMC) and Boc-Val-Gly-Arg-AMC were used to assay amidolytic activity of proteases according to the method of Zimmerman et al (133) and Morita et al (173).

Assays were carried out either in glass cuvettes or in quartz cuvettes at 25°C. A volume of 0.05 ml of enzyme at appropriate dilution was added to 0.45 ml of 0.1M Tris HCl pH 8.1 containing 4% DMSO, and 5×10^{-4}M of substrate. The initial rate of AMC release and concomitant increase in fluorescence was monitored using a Perkin Elmer fluorescence spectrophotometer, model MFP-43A, equipped with a recorder. The excitation and emission wavelengths were set at 383 nm and 455 nm respectively. The instrument was standardized every day such that a 4×10^{-7} solution of AMC in the assay mixture gave full scale recorder pen deflection (= 100 relative fluorescence units).

Activities of enzymes are expressed as fluorometric units/ml (FU/ml), where 1 FU represents that amount of enzyme capable of hydrolysing 10 pmol of substrate in one minute under the assay conditions.

A3.3 Assay for measurement of the conversion of ^{125}I-plasminogen to plasmin by plasminogen activators

In this assay the conversion of the single chain of plasminogen to the two chains of plasmin by plasminogen activators was measured. The method of Danø and Reich (142) was employed.

Purified human plasminogen was radiolabelled with ^{125}Iodine according to the method of Helmkamp et al (183)(Appendix A1). The specific radioactivity of the plasminogen was 90.6 Ci/mmol.

In the assay 60 µl of radioactive plasminogen (2.3 x 10^8 cpm/ml; 0.147 mg/ml), 30 µl of basic pancreatic trypsin inhibitor (BPTI)(1000 KIU/ml) and 20 µl urokinase or Mel-PA at suitable concentrations were incubated at 37°C in a total volume of 300 µl of 0.1M Tris HCl pH 8.1 containing 0.02% Triton.

After 20 min incubation, 50 µl aliquots were removed from the mixture and added to 10 µl of 10% SDS containing 0.004% phenol red, 10 µl glycerol and 5 µl 2-mercapto-ethanol. The samples were heated for 1 min in a boiling water bath and 5 or 10 µl were loaded on 6-15% gradient polyacrylamide gels (120 x 65 x 1 mm). All samples and gels were prepared in duplicate. After electrophoresis, the gels were fixed in 10% acetic acid 30% methanol and dried. Autoradiographs were made by exposure of Osray RP-1 Xray films (Agfa-Gevaert) to the dried gel for 24 hr at -20°C.

The bands corresponding to plasminogen, and the two chains of plasmin were excised from the dried gel and the radioactivity determined. The percentage of radioactivity recovered after background subtraction in the bands of plasmin heavy and light chains relative to the total radioactivity recovered in each track indicated the extent of conversion. Background counts for subtraction were obtained in corresponding areas of tracks in which non converted plasminogen had migrated.

The radioactive plasminogen in the gel consisted of a closely spaced doublet, presumably corresponding to the Lys- and Glu-forms of

the plasminogen molecule (18). The two forms could also be observed in the plasmin heavy chains, but not in the light chains. Furthermore, it could be clearly seen that cleavage of plasminogen by both Mel-PA and urokinase resulted in the same product plasmin chains, indicating that both activators activate plasminogen via the same mechanism, i.e. by cleavage of a susceptible Arg-Val bond of plasminogen.

A3.4 Plasminogen conversion assay using active site titrant for plasmin (FDE-assay)

In this procedure described by Mangel et al (181) plasminogen activators are assayed by measuring the rate of generation of plasmin from plasminogen. Plasmin is measured by active site titration.

The fluorescent active site titrant, di-(p-guanidino-benzoyl) fluorescyl-6-thioureido-(p-benzoic acid), (abbreviated FDE) used in these studies was synthesized in this laboratory according to the method of Mangel et al[1] (181).

Plasminogen at a final concentration of 0.756 mg/ml (8.4 µM) was incubated at room temperature with various concentrations of Mel-PA and urokinase, ranging from 50 - 2000 FU/ml in 0.1M Tris HCl buffer pH 8.1 containing 0.02% Triton. After 10 min incubation 50 µl aliquots were removed and added to 950 µl PBS containing FDE to give a final concentration of 0.75 µg/ml in the assay tube. After exactly 2 min incubation at room temperature, 20 µl of a 1 mg/ml solution of NPGB was added and the fluorescence was measured immediately in a Perkin Elmer fluorometer, with excitation and emission wavelengths set at 491 nm and 514 nm respectively. The amount of plasmin in pmol was determined by reference to the intensity of fluorescence given by a standard solution of hydrolysed substrate.

[1] I am grateful to Dr. M. Bailey for supplies of this material.

A3.5 The fibrin agarose plate assay

In this assay the plasminogen dependent lysis of fibrin in an agar gel by urokinase and Mel-PA was compared.

For the preparation of the fibrin agarose plates, the same purified human fibrinogen and human plasminogen as used in the ^{125}I-fibrin assay were used. The plates were prepared according to the method of Granelli-Piperno and Reich (62) where they were used as indicator gels for localizing plasminogen activator activity in SDS-polyacrylamide gels. The slabs (110 x 90 x 1 mm) were cast between two glass plates held apart by thin wires 1 mm in diameter. Mixtures of 1.25% agarose, 2.5 mg/ml plasminogen-free human fibrinogen, 0.05 mg/ml purified human plasminogen and 0.06 NIH units/ml thrombin in 0.1M Tris HCl pH 8.1 were poured between the plates at 45°C, and allowed to solidify at room temperature.

For casein agarose plates the fibrinogen and thrombin were replaced by incorporating 20 mg/ml skim milk powder (Protea) into the agarose.

Holes of 2 mm diameter were punched into the slabs after removal of one of the glass plates. Samples were inoculated into the holes in 3 µl volumes. The plates were first incubated at 4°C for 16 hours to allow diffusion of the plasminogen activators and then at 37°C for 90 min for plasminogen dependent lysis to occur.

The diameters of the clear lysis zones were then measured and related to plasminogen activator activity.

A.4 PROTEIN DETERMINATION

Different methods were used to determine the protein concentrations in solutions, depending on the concentration of protein to be measured as well as the buffer system in which the proteins were dissolved.

A4.1 Determination by UV absorption

This method was adapted from Layne (225) and was used to give approximate estimates of proteins at high concentrations. The absorbance of a protein solution was measured at wavelengths of 280 nm and 260 nm in a Unicam Spectrophotometer, and the protein concentration was calculated according to the formula.

$$\text{Protein concentration in mg/ml} = 1.55\ OD_{280} - 0.76\ OD_{260}$$

A4.2 Determination by the Lowry method

This method of Lowry et al (226) was used for protein solutions in the concentration range of 50 - 200 µg/ml and with buffers that did not contain substances such as Triton X-100, that interfered in the assay system.

Solutions of BSA were used to construct a standard curve. Since this method does not give a linear relationship between concentration and absorbence, the standard curve of OD_{750} vs µg BSA was constructed by fitting a second degree polynomial equation to the observed points. The concentrations of unknown samples could then be compared by interpolation using the parameters of the quadratic equation.

A4.3 The Coomassie blue dye binding assay

This assay of Bradford et al (227) which employs the quantitative binding of Coomassie brilliant blue to proteins in an acid environment, was used to measure proteins in dilute solution in the presence of substances that interfered in the other assays. Although BSA gives a higher reading

than most other proteins, it was still employed to construct a standard curve, since it was the major contaminant encountered in samples to be measured. Standards ranging from 1 - 15 µg and samples in 100 µl volumes were mixed with 1 ml of the Coomassie blue G250/phosphoric acid reagent and the absorbance was read at 595 nm. Standard curves were constructed as in the other assays.

Since this assay can be performed rapidly it was mainly used for the protein concentration determinations in fractions obtained from column chromatography.

REAGENTS

Special reagents or laboratory ware were obtained from the following sources:

Cell culture media and foetal bovine serum, Grand Island Biological Co., Grand Island, N.Y.; Standard disposable plastic tissue culture ware, Falcon Plastics, Oxnard, Calif., C.A. Greiner und Söhne GMBH, Nürtingen, W. Germany; Corning Ltd., Stone, Staffordshire, England; Linbro Multiwell plates, Flow Laboratories Ltd., Irvine, Scotland; Cbz-glycyl-glycyl-arginyl-AMC and Boc-glycyl-valyl-arginyl-AMC, Bachem Feinchemikalien AG, Bubendorf, Switzerland; urokinase, Leo Pharmaceutical Products, Ballerup, Denmark; human fibrinogen, streptokinase, A.B. Kabi, Stockholm, Sweden; thrombin, Parke-Davis Laboratories (Pty) Ltd., Isando, Tvl, South Africa; ^{3}H-leucine, ^{3}H-DFP (diisopropylfluorophosphate), Na^{125}I, Amersham International plc, Amersham, Buckinghamshire, England; bovine trypsin, soybean trypsin inhibitor, pancreatic trypsin inhibitor compound; ovomucoid, Worthington Biochemical Corporation, Freehold, N.J.; iminodiacetic acid, nitro-benzamidine, Aldrich Chemical Company Inc., Milwaukee, Wisc.; ECD, The Ott Chemical Company, Muskegon, Mich.; DE-52 cellulose, Whatman Ltd., Springfield Mill, Maidstone, Kent, England; ε-aminocaproic acid, cyanogen bromide, tetrahydrofuran, Fluka AG, Buchs SG, Switzerland; acrylamide, bis-acrylamide, Eastman Kodak Company, Rochester, N.Y.; agar, trypsin for tissue culture, Difco Laboratories, Detroit, Michigan; X-ray RP-1 film, Agfa Gevaert, Belgium; 2,5 diphenyloxazole, Dimilume, Instagel, Soluene, Packard Instruments Company Inc., Downer's Grove, Ill.; En^{3}Hance, New England Nuclear, Boston, Mass.; molecular weight marker protein mixture, Sepharose 4b, Sepharose Cl-4b, CnBr-activated sepharose 4b, Sephadex G75, Sephadex G25, Cytodex, Pharmacia Fine Chemicals, Uppsala, Sweden; Coomassie brilliant blue G250, Serva Feinbiochemica,

Heidelberg, W. Germany; Streptomycin sulphate, penicillin, Glaxo-Allenburys (SA)(Pty) Ltd., Wadeville, Tvl, South Africa; lysine-HCl, bovine serum albumin, Triton X-100, sodium dodecyl sulphate, N,N,N',N'-tetra methylethylene diamine (TEMED), β-mercaptoethanol, Coomassie brilliant blue R250, chloramine T, N-methyl pyrrolidone, BDH Chemicals Ltd., Poole, England; diisopropylfluorophosphate (DFP), 1,4 butane diol diglycidyl ether, phenol red, thrombin, bovine fibrinogen, 4-methylumbelliferyl p-guanidinobenzoate, 4-methylumbelliferone, benzamidine, HEPES, Sigma Chemical Company, St. Louis, M.O.; ethylene glycol, sodium borohydride, glutaraldehyde, silver nitrate, formaldehyde, trichloracetic acid, amido black, dimethyl sulphoxide, gelatin, arginine, potassium thiocyanate, E. Merck, Darmstadt, W. Germany.

All other reagents were analytical grade.

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