Übersicht — Review — Revue Générale

Studies on the Fibrinolytic Enzyme of Human Plasma*)

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Despite the general acceptance of the presence of a fibrinolytic enzyme in human plasma, knowledge concerning the biochemical aspects of this enzyme system is still extremely superficial. Nevertheless considerable information concerning the various components of the plasma fibrinolytic enzyme system has accumulated in recent years. It is the purpose of this article to briefly review some of the current knowledge in this field; to describe some of our recent studies; and to comment on the areas requiring further investigation and clarification.

Historical note

Historically, the knowledge of the fibrinolysin of human blood stems from three sources of information previously considered to be independent of each other. The major developments along each of these lines (i. e., a streptococcal enzyme capable of inciting fibrinolysis of human blood clots, the known fibrinolytic activity of blood, and the known proteolytic activity of blood), as well as their subsequent integration into a unified concept, are reviewed in Fig. 1.

Streptococcal fibrinolysis. In 1933 Tillett and Garner (1) demonstrated that filtrates of broth cultures of certain strains of hemolytic streptococci contained a substance capable of inciting rapid fibrinolysis of human plasma clots. This substance was termed streptococcal fibrinolysin. In 1941, Milstone (2) demonstrated that clots made with highly purified human fibrinogen and thrombin were not lysed by streptococcal fibrinolysin. However, if a small amount of a euglobulin from human serum was added to the mixture, rapid lysis of the clot resulted. The serum euglobulin could be readily isolated in a partially purified form by isoelectric precipitation at pH 5.2. Milstone named this substance the plasma lysing factor, and believed that it interacted with streptococcal fibrinolysin to form an active lysing system. From 1943 to 1945, Kaplan (3) and Christensen (4), working independently, demonstrated that the plasma euglobulin described by Milstone, was, in reality, an inactive precursor of a proteolytic (and fibrinolytic) enzyme which could be rapidly activated by streptococcal fibrinolysin. Because the streptococcal product was an activator rather than a fibrinolysin, Christensen suggested that it be renamed streptokinase.

Spontaneous fibrinolysis of clotted blood. The knowledge that human blood contained fibrinolytic activity is very old. Denis, and then Zimmerman, well over a hundred years ago, observed that the fibrin of human blood obtained from wet cupping dissolved in 12—24

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hours (5). Dastre, in the late 19th century, noted that the fibrin yield of dog blood decreased with the rate and magnitude of the phlebotomy (6). Although his observations may have been due to hemodilution, Dastre postulated that the decreased yield was due to actual destruction or lysis of the fibrin and defined the phenomenon as „fibrinolysis". Nolf (7), one of the great pioneers in our concepts of blood coagulation, believed that the coagulation process was a proteolytic one, and that fibrinolysis of blood clots simply represented the end stages of the process by which clotting originally took place. It is of interest that the action of thrombin as a proteolytic enzyme has recently been firmly established (8). It is, however, generally agreed that the processes involved in clotting are distinct from those involved in fibrinolyis. Nevertheless it is to Nolf's credit that he introduced the view that fibrinolysis was due to a proteolytic enzyme in plasma.

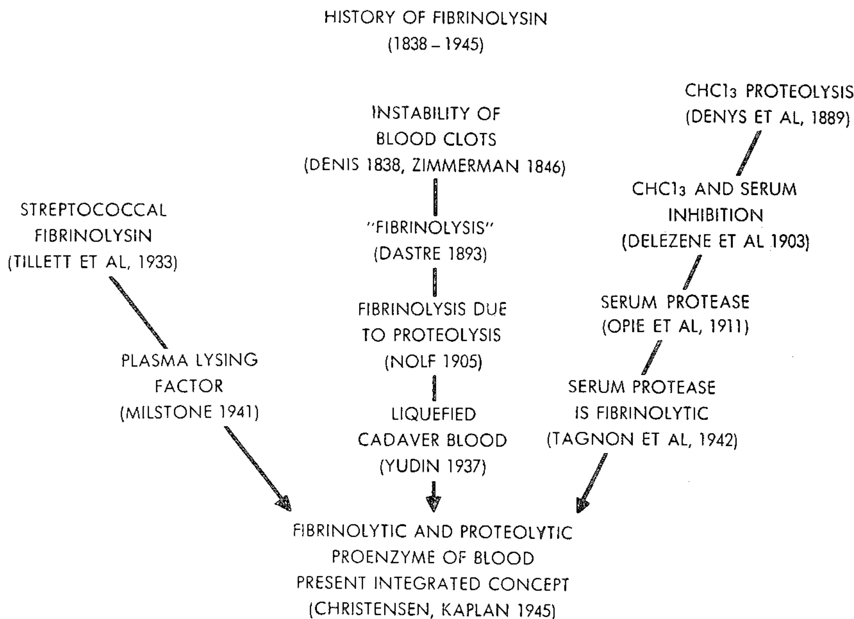

Fig. 1: Historical development of knowledge concerning fibrinolysin. Note the three channels of information and their present integration into one unified concept.

Interest in the spontaneous fibrinolysis of human blood clots was reawakened by Yudin (5). He demonstrated the possibility of utilizing cadaver blood without anticoagulants for transfusion purposes. Yudin used blood from fresh corpses, selecting donors who were the victims of accidental or sudden death rather than those who had died from a chronic illness. Following phlebotomy, the blood clotted, but subsequently reliquefied in several hours and remained in a fluid state thereafter. The fairly rapid activation of fibrinolytic activity in some patients with severe shocking episodes is now well recognized.

Proteolytic activity in blood. A third channel of information concerning fibrinolysis relates to observations on a proteolytic enzyme of serum with many similarities to trypsin. In 1889, Denys and Marbaix (9) found that a thermo-labile proteolytic agent developed in serum previously treated with chloroform. Delezene and Pozerski (10), some years later, demonstrated that chloroform treatment of serum removes the inhibition to proteolytic enzymes, and is followed by the appearance of an active enzyme in the serum. In 1911, Opie et al (11) demonstrated that the proteolytic enzyme, which appears in serum following chloroform treatment, had many of the characteristics of trypsin (pH optima, and ability to digest casein and

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gelatin). Tagnon et al, in 1942 (12) noted that the globulin fraction of chloroform treated serum was strongly fibrinolytic.

Thus the stage was set for the studies of Christensen (4) and of Kaplan (3), who pointed out that phenomenon of fibrinolysis was due to the same proteolytic enzyme which had been observed in the chloroform treated serum; that this existed in the natural state as an inactive precursor; and that it could be rapidly activated by streptokinase. This view, which now appears well established, is summarized in Fig. 2.

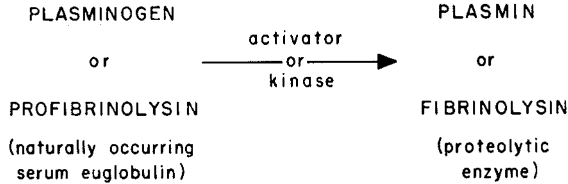

Fig. 2: Simplified schema of plasminogen-plasmin system.

As shown in Fig. 2, the naturally occurring precursor of the proteolytic and fibrinolytic enzyme of serum is referred to as plasminogen, although the term profibrinolysin remains a favorite among many "coagulationists". In the presence of an activator or kinase, this serum globulin is rapidly converted to an active proteolytic enzyme termed plasmin or fibrinolysin. Although human plasma, tissue and urinary kinases have been described, streptokinase is still the best known of the activators, and has been used in most laboratories for studies on the system under consideration. Although not shown in Fig. 2, serum (13, 14) and platelets (15) contain an antiplasmin or antifibrinolysin which can rapidly inactivate plasmin. When the antiplasmin is removed by chloroform or acetone or other similar physical agents (16), slow but progressive spontaneous activation of the plasminogen occurs.

Plasmin, although it digests fibrin into several soluble incoagulable fragments, is not restricted in its action to fibrin. It is a proteolytic enzyme resembling trypsin in its pH optima (17) and the types of links it splits (18). For this reason the terminology plasminogen and plasmin appears to be preferable, and is in keeping with the terminology of trypsinogen and trypsin. In this report reference will be made to plasmin and fibrinolysin interchangeably.

Characteristics of plasminogen (profibrinolysin) and plasmin (fibrinolysin)

In vivo, plasminogen occurs in close relationship to fibrinogen and fibrin. In the process of clotting, significant amounts of plasminogen are adsorbed onto the fibrin clot. In areas of exudation, plasminogen appears simultaneously with fibrinogen and fibrin. In addition, studies of transudates and exudates reveal a good correlation between the concentrations of fibrinogen and plasminogen (19). Fibrinogen, free of all traces of plasminogen, is extremely difficult to prepare, even after repeated precipitations of the fibrinogen (20). However major separation of plasminogen from fibrinogen is simple, since plasminogen has the characteristics of a globulin (2). In the alcohol fractionation procedure of Cohn, the bulk of the plasminogen is in Fraction III, whereas the fibrinogen appears in Fraction I. Reliable studies on the characteristics of human plasminogen have been hampered by the lack of techniques for its purification. Recently Kline (21) has developed a method for plasminogen purification which consistently yields preparations several hundred fold purified as compared

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to serum. Physiochemical studies by Shulman (22) on plasminogen preparations prepared in our laboratories by the Kline technique (21) indicate that these preparations are moderately ultracentrifugally homogeneous and may be used for further characterization studies. Plasmin preparations of comparable purity have been prepared by allowing the "Kline" plasminogen preparations to undergo spontaneous and complete activation without significant loss of enzyme (23). Table 1 cites some of Shulman's observations on the physicochemical characteristics of these plasminogen and plasmin preparations.

PHYSICOCHEMICAL STUDIES ON HUMAN PLASMINOGEN AND PLASMIN

(S. Shulman, N. Alkjaersig, and S. Sherry: To be published)

DATA	PLASMINOGEN	PLASMIN
SEDIMENTATION CONSTANT	4.28	3.56
DIFFUSION CONSTANT (Cm^2 Sec^{-1})	2.96×10^{-7}	–
MOLECULAR WEIGHT	141,000	107,000 (?)
INTRINSIC VISCOSITY	0.070	–
ABSORPTION SPECTRUM – MAXIMUM	2800 A°	2800 A°
TYROSINE	5.1%	6.4%
TRYPTOPHANE	2.7%	3.6%
ELECTROPHORETIC MOBILITY (pH 2.1)	8.2 and 7.3×10^{-5}	8.9×10^{-5}
ISOELECTRIC POINT	6.0	7.1

Table 1

It will be noted from the data in Table 1 that plasminogen and plasmin have sedimentation constants of 4.28 and 3.56 respectively. On the basis of a diffusion constant of 2.96×10^{-7} cm^2 sec^{-1}, and the sedimentation data, a molecular weight of 141 000 has been calculated for plasminogen. Assuming plasminogen and plasmin to have similar molecular configurations, the molecular weight of plasmin may be estimated as 107 000. Viscosity studies reveal an intrinsic viscosity for plasminogen of 0.070. Providing the plasminogen molecule is an anhydrous elongated ellipsoid of revolution, the viscosity data suggests that plasminogen has a 7 : 1 length to thickness ratio. Ultraviolet absorption studies reveal single absorption maxima at 2800 A° for both plasminogen and plasmin. On the basis of the absorption data, plasminogen appears to contain 5.1% tyrosine and 2.7% tryptophan. Plasmin contains 6.4% tyrosine and 3.6% tryptophan.

On electrophoretic study at pH 2.1 (glycine buffer, 0.10 ionic strength) plasmin has a single peak with a mobility of 8.9×10^{-5}. In contrast, plasminogen preparations appear to be composed of two peaks of approximately equal size with mobilities of 8.2 and 7.3×10^{-5}. At higher pHs both plasminogen and plasmin have single peaks. It is still unclear whether the double peak seen with plasminogen preparations at low pH represents two substances or may be associated with plasminogen itself. We have been unable to chemically separate

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the plasminogen preparations into two components. Since purified preparations of plasminogen and plasmin are poorly soluble at neutral pH, extrapolation of the mobility data at various pHs has been used for isoelectric point determination. On this basis, isoelectric points of 6.0 and 7.1 have been determined for plasminogen and plasmin respectively.

Purified preparations of plasminogen contain 12% nitrogen and significant, though not large, amounts of carbohydrate. Whether the carbohydrate is an integral part of the molecule remains to be determined.

The Action of Plasmin

Plasmin is a proteolytic enzyme and is not restricted in its action to fibrin. It has been shown to act upon fibrinogen, casein, gelatin, beta lactaglobulin, azocoll hide powder (19), Ac globulin (24, 25), at least two components of serum complement (26), and perhaps prothrombin (27). In purified systems, plasmin attacks fibrinogen and fibrin at approximately equal rates showing no unusual preference for the latter (4, 28). Per unit of proteolytic activity (casein digestion), the action of plasmin on fibrinogen and fibrin is similar to that observed for trypsin and chymotrypsin (20).

Although the original observations of Holmberg (29) suggested that the action of plasmin on fibrin or fibrinogen is limited to a single split into two large fragments, our studies indicate that these substrates are more extensively hydrolysed by plasmin. The results of an experiment describing the products of plasmin's action on fibrin are shown in Fig. 3.

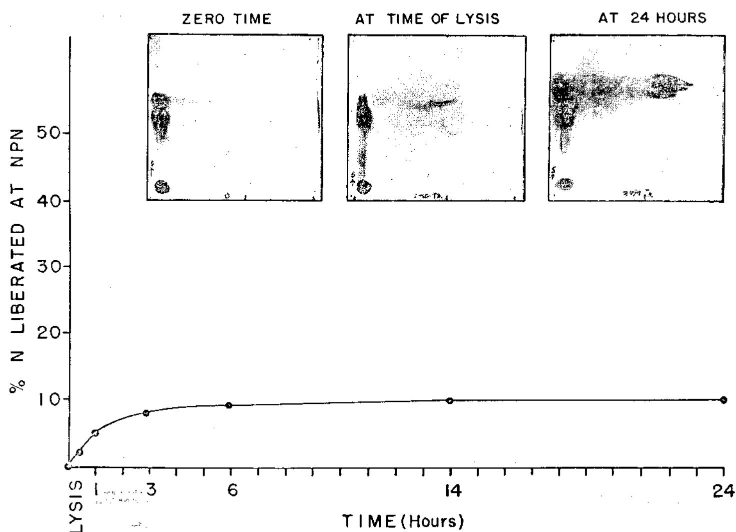

Fig. 3: Proteolysis of fibrin by plasmin. See text for details.

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In this experiment, plasmin was added to fibrinogen and the mixture clotted with thrombin. At serial intervals, aliquots were assayed for the release of acid soluble nitrogen. It will be noted that at the time of lysis of the fibrin clot, which occurred in 2 minutes, 2% of the available fibrinogen nitrogen was rendered trichloracetic acid soluble. The release of acid soluble nitrogen subsequently rose to 10%, and then leveled off. At the top of Fig. 3 are shown the results of 2 phase paper chromatography as originally developed by Franklin and Quastel for proteins and polypeptides (30). This technique was applied, in this experiment, to the incubation mixture at zero time; at the time of clot lysis; and at the end of 24 hours. At the upper left of Fig. 3 are shown the results of the chromatography at zero time prior to the addition of thrombin. The movement of fibrinogen is confined to a single phase. At the time of clot lysis, shown in the upper center, one large peptide fragment has moved out into the second phase; and at 24 hours, shown in the upper right, at least 4 components are evident in the second phase. These large peptide fragments were not acid soluble. The results shown on the slide suggest that fibrin, under the action of plasmin, eventually is hydrolyzed into several large polypeptides, along with some smaller acid soluble fragments. More recently, Shulman has shown electrophoretically that fibrin is broken down into four or more different components by the action of plasmin (31).

The observation of Troll, Sherry and Wachman (18) that plasmin hydrolyzes esters of arginine and lysine has given valuable information concerning the sites of action of plasmin, and has made available new quantitative techniques for plasmin assay. As a background for these studies with synthetic substrates, Fig. 4 depicts in diagrammatic fashion, the essential and modifying factors in determining synthetic substrates for such proteolytic enzymes as trypsin and chymotrypsin.

SYNTHETIC SUBSTRATES FOR PROTEOLYTIC ENZYMES

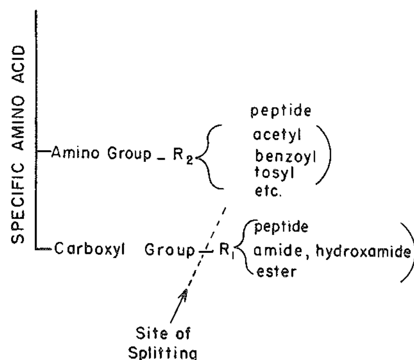

Fig. 4: Schematic representation of essential and modifying factors determining synthetic substrates for proteolytic enzymes. See text.

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The specific amino acid residue to which the amino and carboxyl group are attached, is the most important single factor in determining whether the compound will be a substrate or not. Chymotrypsin prefers aromatic amino acid compounds, and trypsin attacks only basic amino acid compounds. A second consideration is the nature of the link at the carboxyl group. This is the site of enzymatic splitting. Note that several types of links may be split, e. g., the peptide, amide, hydroxamide and ester. In Neurath's studies of trypsin and chymotrypsin (32), the ester link was by far the most sensitive. This type of ester link, i. e., with the carboxyl group of an amino acid, is not attacked by the known blood esterases. Finally the nature of the substitution on the amino group also modifies the sensitivity of the substrate. The unsubstituted amino group is the least sensitive. The presence of a peptide, acetyl, benzoyl, tosyl etc., increases the sensitivity of the substrate.

Although proteolytic enzymes are capable of rapidly hydrolyzing certain amino acid ester links, this activity should not be construed as evidence for the existence of ester links in protein molecules. On the other hand, it has been demonstrated that the action of a number of proteolytic enzymes on proteins are at amino acid sites indicated by their esterase activity (33).

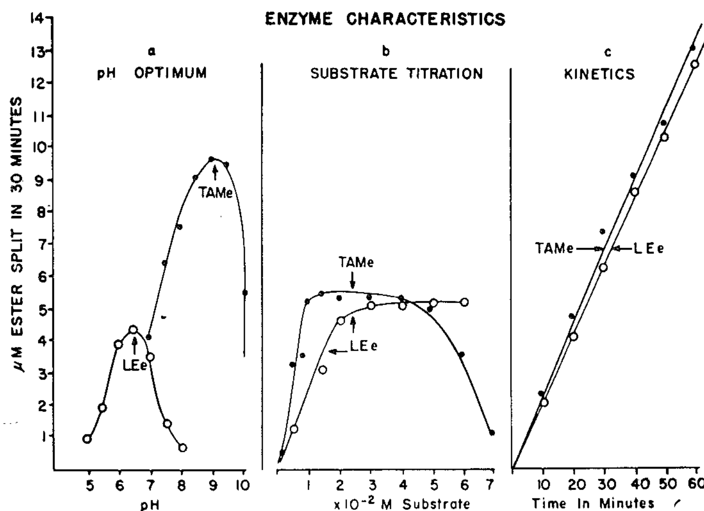

Fig. 5: Enzyme characteristics. (a) pH optimum curves with a plasminogen preparation obtained by isoelectric precipitation of Fraction III containing 62.4 γ of nitrogen per ml. of digestion mixture activated with 1000 units of streptokinase. The substrates, TAMe and LEe, were 0.02 M; 0.25 M Tris buffers were used for the former and 0.1 M imidazole buffers for the latter. (b) Substrate titration curves; a human globulin preparation prepared according to Milstone containing 126 γ of nitrogen per ml. of digestion mixture activated by 1000 units of streptokinase was used as the enzyme source. TAMe was incubated at pH, 9, 0.25 M Tris buffer, and LEe at pH 6.5, 0.1 M imidazole buffer. (c) Kinetics of ester digestion of 0.02 M TAMe at pH 9, 0.25 M Tris buffer, and 0.04 M LEe at pH 6.5, 0.1 M imidazole buffer with 0.1 ml. of plasma (889 γ of nitrogen) activated with 1000 units of streptokinase as the source of enzyme. All experiments were carried out at 37°. Esterase activity was determined by the method described in the text. (Reprinted from Troll, W., S. Sherry, and J. Wachman, 1954. J. Biol. Chem. *208*:85).

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Some of the characteristics of action of plasmin on tosyl arginine methyl ester (referred to as TAMe) and lysine ethyl ester (referred to as LEe) are shown in Figure 5.

Section A describes the pH optimum curves, and section B, the substrate titration curves. In section C are shown the zero order kinetics observed with these substrates up to 70% substrate digestion. Calculation of the Michaelis constant for these reactions revealed a very high degree of affinity of the enzyme for the synthetic substrates.

Although a large number of esters of other amino acids were also studied, the activity of plasmin appears to be limited to the hydrolysis of arginine and lysine esters. The observation of the esterase action of plasmin on these amino acid esters should not be considered as an unusual activity of plasmin. Trypsin attacks the same esters as plasmin.

In Table 2 is shown a comparison of several activities of a number of plasmin preparations.

COMPARISON OF VARIOUS PLASMIN PREPARATIONS TO TRYPSIN

ENZYME	CASEIN UNITS/Mg N	ESTERASE ACTIVITY		FIBRINOLYTIC ACTIVITY UNITS/Mg N	RATIO FIB. U. / CAS. U.
		TAMe UNITS/Mg N	LEe UNITS/Mg N		
BOV. FIBRINOLYSIN (Loomis)	1.0	25	10	8	8.0
PLASMIN, HUMAN (Fr. III3)	4.3	92	63	32	7.4
PLASMIN, HUMAN (Sp. Act.)	58	658	450	438	7.6
PLASMINOGEN, HUMAN (SK Act.)	63	748	551	-	-
TRYPSIN	2,015	107,000	5,000	14,900	7.4

Table 2

The proteolytic activity is expressed as casein units per mg N. One casein unit represents the release of 450 gamma of acid soluble tyrosine from a casein substrate under the conditions of the test (34). The esterase activity is expressed as micromoles of ester hydrolyzed in 30 minutes at 37° C per mg N. TAMe and LEe were used as synthetic substrates. The fibrinolytic activity is expressed as units per mg N, with one unit representing that amount of enzyme which will produce lysis of a standard bovine fibrinogen — thrombin clot in 30 minutes at 37° C (35). In the last column is the ratio of fibrinolytic to proteolytic activity observed with these various preparations.

The bovine fibrinolysin was a chloroform activated plasmin (36) obtained through the courtesy of Dr. Loomis. The Cohn fraction III3 was obtained from Dr. Surgenor of Harvard University, and had undergone complete spontaneous activation. The spontaneously activated human plasmin was derived by spontaneous activation of a Kline purified plasminogen preparation. The human plasminogen preparation was also prepared by the Kline method (21) and activated by streptokinase. The results obtained with trypsin are shown at the bottom of the Table for comparison.

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From the data in Table 2, it will be noted that the human plasmin preparations under investigation at present are about 60 times more purified than the chloroform activated bovine plasmin, and about 15 times more purified than the plasmin obtained by the alcohol fractionation procedure. On the other hand, the most purified plasminogen or plasmin preparations presently available have only about 1/35th the proteolytic activity of trypsin.

It will also be noted that all the preparations have esterase activity and fibrinolytic activity which correlate well with their proteolytic activity. The fibrinolytic activity per unit of casein proteolysis as shown in the last column of Table 2 is similar for all the preparations and for trypsin as well. Trypsin does differ from plasmin in that its attack on TAMe ist 20× greater than on LEe, whereas the ratio of attack of plasmin on these two substrates is considerably smaller.

The question as to whether plasmin and trypsin are identical has been raised on many occasions. Although trypsin and plasmin have many similarities, e. g., activity against a number of similar protein substrates at the same pH optimum, and ability to act upon the same synthetic substrates, the evidence for their dissimilarity is overwhelming. Trypsinogen and plasminogen require different activators (37), the enzymes in purified systems have different ratios of attack against synthetic substrates (18), and their inhibition by crystalline soybean inhibitor is quite different (17).

Methods for Assay of Plasmin

Plasmin is commonly assayed in most laboratories by one or more of the following methods; fibrinolysis (35, 38) casein proteolysis (34) and the hydrolysis of synthetic substrates (18).

Fibrinolytic assays consist of either measuring the area of lysis produced by plasmin solutions placed on the surface of a fibrin plate (fibrin plate method) (38) or measuring the rate of lysis of a standard bovine fibrinogen — thrombin clot containing the plasmin solution to be assayed (clot lysis method) (35). The direct relationship observed between the reciprocal of the lysis time and the plasmin concentration, when the clot lysis method is used, is illustrated in Fig. 6.

At very high concentrations of plasmin, the digestion of fibrinogen is so rapid that the addition of thrombin does not result in the formation of a clot.

Also shown in Fig. 6 is the linear relationship observed when casein proteolysis, as measured by acid soluble tyrosine release, is used as a measure of plasmin activity. The digestion of casein by plasmin (as measured by the release of acid soluble tyrosine, nitrogen, or U.V. absorbing material) can readily be utilized as a quantitative assay for plasmin activity, and in many laboratories is used in preference to fibrinolysis. Assays, other than fibrinolysis (excepting

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the heated fibrin plate technique [39]), are particularly important when plasmin is measured in the presence of activators, for almost all animal fibrinogen preparations used in fibrinolysis studies are contaminated with significant amounts of plasminogen (40, 20).

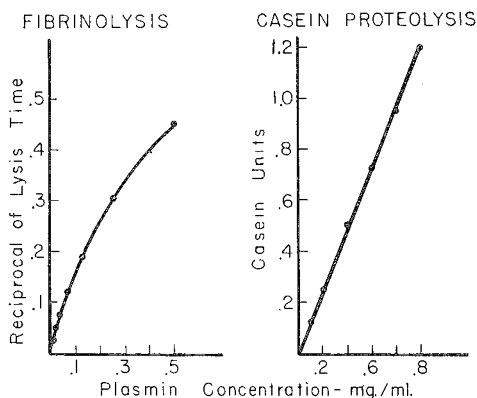

Fig. 6: Fibrinolysis and casein proteolysis as a function of plasmin concentration. The plasmin was prepared by spontaneous activation of plasminogen in 50% glycerol. Casein proteolysis is expressed as units according to the method of Remmert and Cohen (34).

The demonstration that esters of arginine and lysine are sensitive substrates for plasmin, has made available newer methods for plasmin assay. As arginine ester substrates we have found TAMe or BAMe (benzoyl arginine methyl ester) to be particularly suitable. LEe or LMe (lysine methyl ester) have served as suitable lysine ester substrates. The hydrolysis of the amino acid esters may be measured by the disappearance of ester (Hestrin colorimetric method for esters [18]) or by the appearance of a free carboxyl group (titration procedures [18] or by CO_2 liberation in a Warburg apparatus [41, 42]). The use of synthetic substrates has proven particularly useful in studying plasmin activity in the presence of inhibitors (e.g., plasma) since the synthetic substrates can effectively compete with most inhibitors for the enzyme.

It is beyond the scope of this report to discuss the relative advantages of one type of method as compared to another. Suffice to say that each method has its advantages and disadvantages, and the choice of method is best related to the particular circumstance in which plasmin is being studied. In our own studies we have uniformly employed all three types of methods simultaneously.

Studies on the activation of plasminogen

A) Spontaneous activation. Recently we have noted that plasminogen, purified by the Kline technique will spontaneously activate to completion in the

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presence of stabilizing agents (23, 43). Glycerin proved to be a most satisfactory stabilizing agent for plasminogen and plasmin. The plasminogen preparations were adjusted to appropriate pH and ionic strength, and dissolved in 50% glycerin. The activation velocity was dependent on the temperature, ionic strength and pH. An incubation temperature of 30° C was found to be most suitable, for at this temperature little to no loss of plasminogen or plasmin occurred, while at higher temperatures significant loss of activity was observed. At 30° C the activation was complete in approximately a week.

Fig. 7 describes the spontaneous activation of a purified human plasminogen preparation in a solution containing 0.05 M phosphate buffer, pH 7.6, and glycerin 50% by volume, incubated at 30° C.

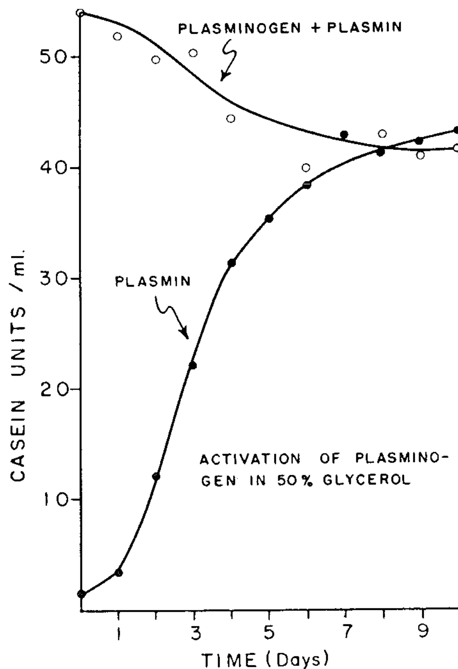

Fig. 7: Spontaneous activation of human plasminogen in 50% glycerol. Human plasminogen prepared by method of Kline (21). See text for details.

During the activation approximately 30% of the total available tyrosine of plasminogen became trichloracetic acid soluble. Although only the results of the casein assays are described in Figure 7, similar observations were made when fibrinolysis or the hydrolysis of synthetic substrates were used as assay methods.

When the reaction mixture undergoing spontaneous activation was seeded with plasmin at the beginning of the experiment, the activation was observed to be more rapid, usually being completed by the fourth day. On the other hand, when the reaction mixture was seeded with a plasmin substrate, the

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activation rate was delayed. These studies suggest that the spontaneous activation is autocatalytic in nature.

Plasmin obtained by this technique, i.e., spontaneous activation of plasminogen in the presence of glycerin, is stable for months at room temperature in the glycerin solution. If desired, the solution may be dialyzed against 0.01 N HCl for removal of glycerin and salts, and then lyophilized from the acid solution. By this method one may consistently obtain preparations of human plasmin, free of activators and of comparable purity to the starting "Kline" plasminogen preparation.

B) Plasminogen activators.

1. Trypsin. Fig. 8A describes studies on the activation of human plasminogen at different trypsin concentrations.

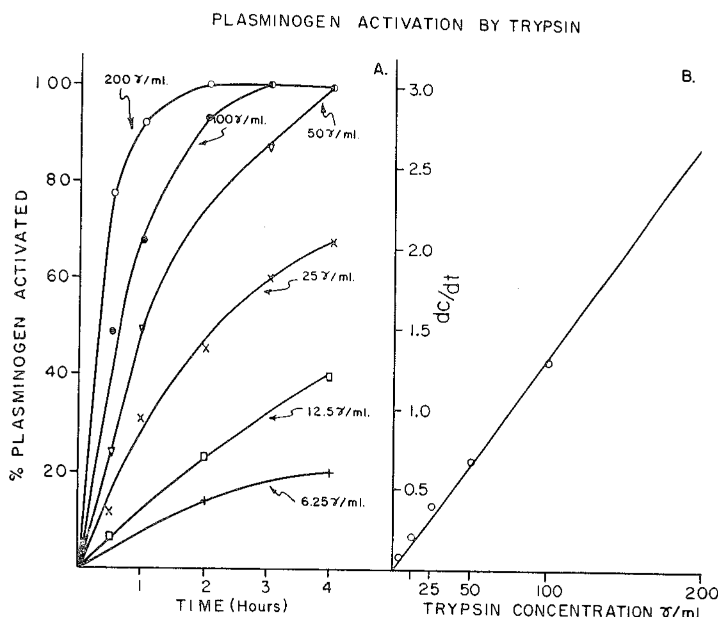

Fig. 8: Activation of human plasminogen by trypsin. Plasminogen prepared according to Kline (21). Plasmin assayed by casein proteolysis (34). Incubation carried out in 50% glycerol at 37° C. Section A. Activation curves obtained with each of the trypsin concentrations employed. Section B. Initial reaction velocity (dc/dt) plotted as a function of the trypsin concentration. See text for other details.

Small amounts of trypsin were utilized in these studies so that the proteolytic activity of the trypsin would be negligible as compared to that of the plasmin formed. In addition, the plasmin was separated from the trypsin by quantitatively precipitating the former at pH 2.0 with 1 M NaCl. Since the activation is relatively slow, the studies shown in Fig. 8A were carried out in the presence of 50% glycerin of the latter's stabilizing effects. When the initial reaction velocity for each of the trypsin concentrations studied was plotted as a function of the trypsin concentration, as shown in Fig. 8B, a linear relationship was observed. These observations are consistent with an enzymatic conversion of plasminogen to plasmin by trypsin. The addition of soybean and other trypsin inhibitors to the activation mixture will, as expected, inhibit the activation of plasminogen by trypsin. This latter observation becomes important when we consider a biological system rather than a purified system. In a biological system, the

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level of free plasmin represents the balance between the rate of activation of plasminogen and the rate of inactivation by antiplasmins. Thus in serum, which normally contains considerable amounts of inhibition to both plasmin and trypsin, a slow activator like trypsin will not be expected to liberate significant amounts of free plasmin. It is not surprising that one cannot demonstrate plasminogen activation by trypsin in serum (25).

2. *Urokinase* Fig. 9A describes studies on the activation of human plasminogen by urokinase at several urokinase concentrations.

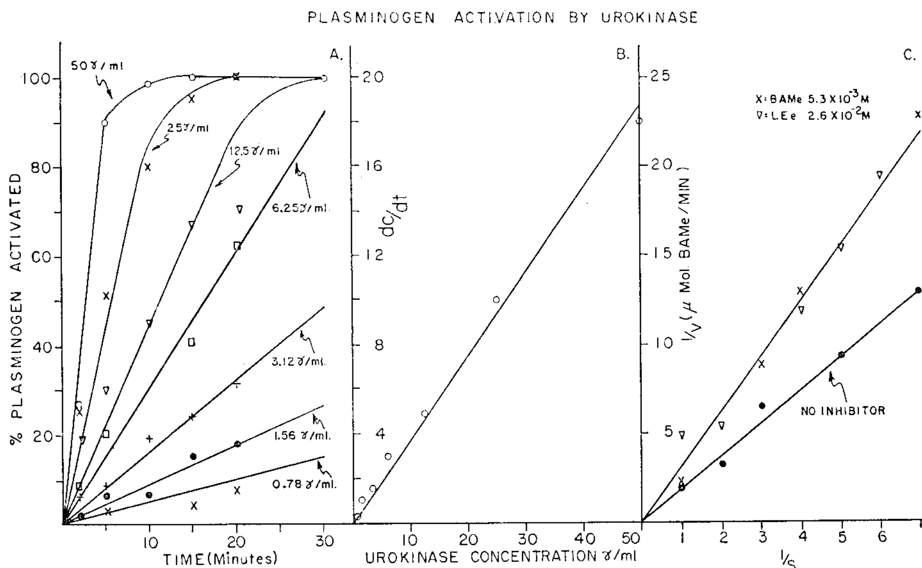

Fig. 9: Activation of plasminogen by urokinase. Human plasminogen prepared according to Kline (21). Plasmin assayed by casein proteolysis (34). Urokinase preparation obtained through the courtesy of Ploug (44). Incubation temperature 25 ° C. Section A. Activation curves obtained with each of the urokinase concentrations employed. Section B. Initial reaction velocity (dc/dt) plotted as a function of the urokinase concentration. Section B. Lineweaver-Burk plot (45) of data obtained from experiments in which plasminogen concentration (S) was varied at a fixed urokinase concentration in the presence and absence of BAMe and LEe. For these latter experiments plasmin activity was assayed by amino acid esterase methods (18).

The urokinase used in this experiment was obtained through the courtesy of Dr. Ploug. This preparation prepared from human urine and purified by chromatographic techniques (44) proved to be approximately 50 times more purified than previous preparations of urokinase studied in our laboratory. Per unit of nitrogen, the preparation of urokinase prepared by Ploug, is approximately one-twentieth as active as the most purified streptokinase preparations presently available.

When the initial reaction velocities described in Fig. 9A were plotted as a function of urokinase concentration, as shown in Fig. 9B, a linear relationship was observed. Data obtained in experiments where the plasminogen concentration was varied at a fixed urokinase concentration fulfilled the criteria of a Lineweaver-Burk plot (45) for an enzymatic or catalytic reaction.

In high concentrations, the urokinase preparation splits esters of arginine and lysine and is proteolytic for casein. However the esterase activity per unit of casein proteolysis is about 5 times greater than observed for plasmin. The esterase activity of the urokinase appears to be intimately associated with its activating function, since synthetic arginine and lysine esters act as competitive inhibitors in the activation of plasminogen by urokinase (Fig. 9C).

The physiological significance of urokinase remains obscure. It is still unknown whether it arises in the genito-urinary tract or represents an excretory product. Significant amounts are

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present in normal human and other animal urines. In preliminary screening of a variety of human diseases, a consistent reduction in urokinase excretion has been observed only in patients with chronic nephritis.

3. Streptokinase activation. Streptokinase is at present the best known activator of human plasminogen. The most purified preparations of streptokinase made available to us by the Lederle Laboratories *) contain approximately 600 units of streptokinase activity per gamma of nitrogen. A few milligammas of streptokinase can activate significant amounts of plasminogen in a matter of minutes. Since the activation of plasminogen by streptokinase is very rapid, and continues while plasmin is being assayed, the activated plasmin must be separated from streptokinase in order to study the true kinetics of conversion of plasminogen to plasmin. As shown by Troll and Sherry (46), the plasmin may be quantitatively precipitated free of streptokinase by 1 M NaCl at pH 2.0.

In Fig. 10A is shown a study of the kinetics of plasminogen activation by streptokinase at different streptokinase concentrations.

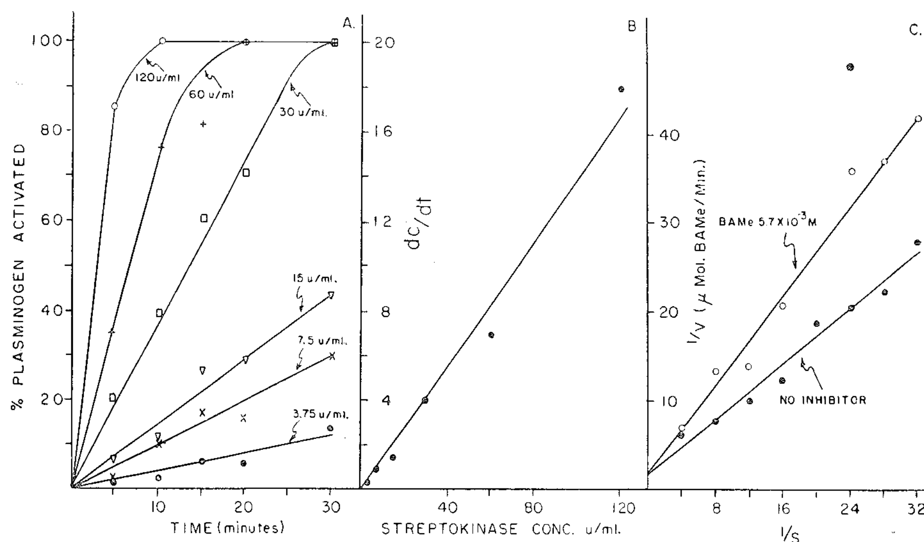

Fig. 10: Activation of plasminogen by streptokinase. Human plasminogen prepared according to Kline (21). Plasmin assayed by casein proteolysis (34). Streptokinase obtained through courtesy of Lederle Laboratories. Incubation temperature 25 ° C. Results expressed as in Fig. 9.

A linear relationship is noted between initial reaction velocity and streptokinase concentration (Fig. 10B). Data obtained from studies in which streptokinase concentration was kept constant, and plasminogen concentration varied also fulfilled the criteria of a Lineweaver-Burk plot for an enzymatic reaction (Fig. 10C).

Streptokinase preparations unlike trypsin and urokinase preparations have never been shown to have amino acid esterase or proteolytic activity. However, in recent years, as a result of the studies of Geiger (47), Mullertz and Lassen (48), and Troll and Sherry (46), considerable evidence has been obtained suggesting that the activation of plasminogen by streptokinase is not direct but occurs in a two step process. In the first step, streptokinase reacts immediately, stoichiometrically, and perhaps reversibly with a plasma factor to form an activator. The activator then catalytically converts plasminogen to plasmin. Although the original studies of Troll and Sherry (46) demonstrated that the activator hydrolyzes

*) Through the courtesy of Mr. F. Ablondi and Drs. B. Hutchings and J. Ruegsegger.

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lysine esters only, our more recent studies suggest that the activator, formed by the interaction of streptokinase and the plasma factor, splits arginine, as well as lysine esters, and may even be mildly proteolytic for casein. The observation that BAMe acts as a competitive inhibitor in the activation of plasminogen by streptokinase (Fig. 10C) supplies additional evidence in support of the view that the activator splits arginine as well as lysine esters.

The identity of the plasma factor which interacts with streptokinase to form an activator is still obscure. It is purified along with plasminogen or plasmin by present procedures and has, as yet, not been separated from these substances. Although Astrup and his group believe that the plasma factor is separate and distinct from plasminogen or plasmin itself (40), several laboratories (49,50) including our own (20, 46), have suggested that activator may be formed by an interaction between streptokinase and human plasmin or plasminogen without invoking a separate or new factor. Our suggestion that the activator may represent a streptokinase-plasmin complex is based on several types of evidence: 1. The activator properties of human plasminogen or plasmin preparations are reversibly dependent on the presence of streptokinase (20, 46); 2. In the presence of streptokinase, the activator properties of different human plasminogen or plasmin preparations, in various stages of purification, appear to be directly related to the plasminogen or plasmin concentration (20, 49): 3. The activator has some of the characteristics of plasmin, i. e., it is a proteolytic enzyme capable of hydrolyzing lysine and arginine esters; and 4. Other properties of human plasmin (but not bovine plasmin) are reversibly altered by the presence of streptokinase (Fig. 11).

More definitive evidence is necessary before reaching a final decision on the nature of the human plasma factor which interacts with streptokinase to form a plasminogen activator.

4. Other plasminogen activations. The tissue kinases, originally described by Astrup and Permin (51) and confirmed by others (52, 53, 54), have not been sufficiently purified for characterization studies. They apparently occur in association with inhibitory substances, and their physiological significance remains to be determined. Although staphylococci also produce a plasminogen activator (staphylokinase) (55) relatively little is known about this latter substance.

Several investigators have suggested the presence of a serum kinase which may normally be present as an inactive precursor (56, 57). In the presence of severe physiological stress or sudden death due to trauma, the kinase becomes activated, catalyzing the conversion of plasminogen to plasmin. Although this may be an attractive hypothesis, additional evidence supporting this view is required.

Mechanism of activation of plasminogen

In each of the types of activation studied, i.e., spontaneous, trypsin, urokinase and streptokinase, it can be shown that from 25—30% of the plasminogen nitrogen becomes acid soluble during the process of activation. These observations are in fair agreement with the estimated 24% reduction in the molecular size of plasmin as compared to plasminogen. The liberation of large amounts of acid soluble nitrogen suggest that proteolysis is involved in the activation of plasminogen. Furthermore, in each of the instances studied, the activation appears to be carried out by an enzyme capable of hydrolyzing arginine and lysine esters, as well as casein. This latter observation supports the enzymatic proteolytic nature of the activation of plasminogen. It is noteworthy that the rapid activators (urokinase and streptokinase) in contrast to the slow activators (trypsin, plasmin) have a much higher esterase to proteolytic (casein) ratio, consistent with their greater specificity.

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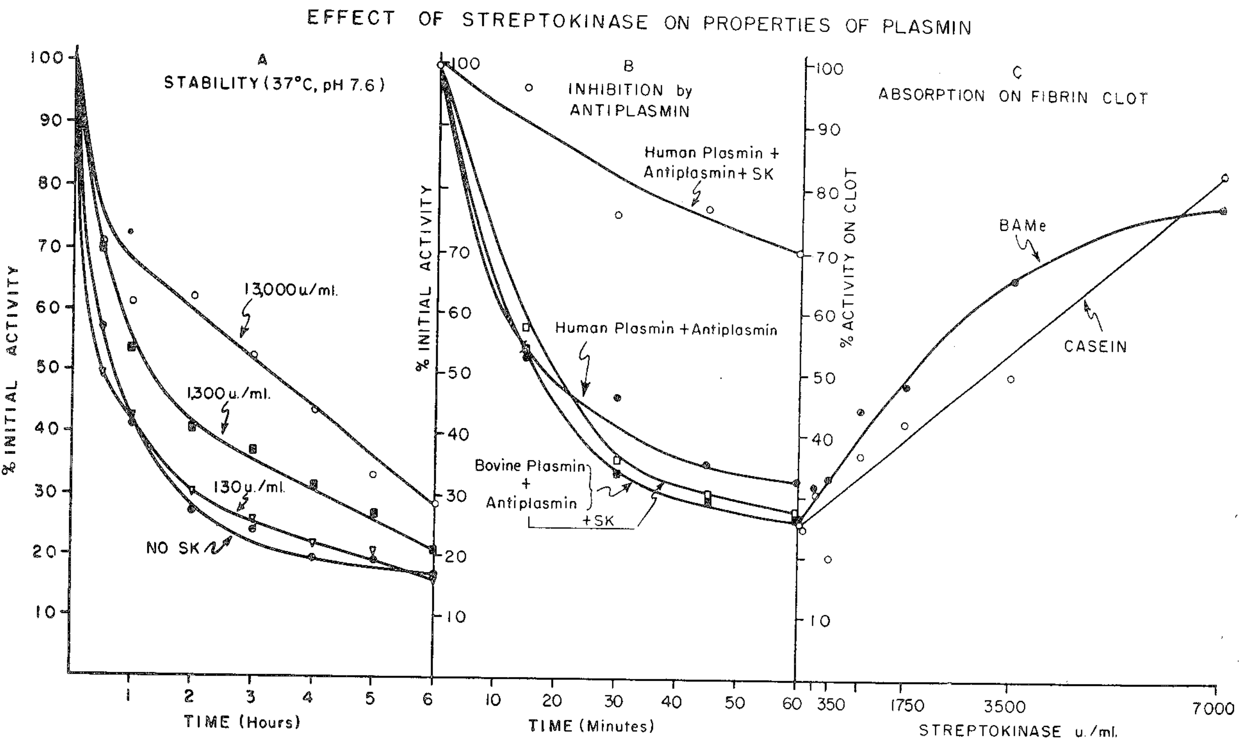

Fig. 11: Effect of streptokinase on properties of plasmin. Section A. Effect of varying concentrations of streptokinase on the rate of thermal inactivation of a spontaneously activated human plasmin preparation. 37 ° C. pH 7.6. Section B. Effect of streptokinase on the rate of inactivation of plasmin by bovine antiplasmin. Bovine plasmin and antiplasmin obtained through courtesy of Loomis (36, 58). Human plasmin was a spontaneously activated preparation. Section C. % plasmin adsorbed on a bovine fibrinogen-thrombin clot as a function of streptokinase concentration.

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Antiplasmins of blood

As is so common in nature with its systems of checks and balances, there is present in serum considerable inhibitory activity to plasmin. Whether this inhibitory activity resides in one or more substances still remains to be determined. However, Loomis, Ryder, and George have isolated a protein from beef serum which accounts for 65% of the total serum inhibition (58). Although this material usually is found, by salting procedures, in the albumin fraction, it is distinct from albumin and is probably a globulin. The reaction of the serum inhibitor with plasmin has been studied by Shulman utilizing a method of studying proteolysis with I^{131} tagged fibrinogen (59, 60). On the basis of his observations, Shulman concluded that the reaction of antiplasmin with plasmin is stoichiometric and irreversible.

More recently Johnson and Schneider (15) have demonstrated the presence of antiplasmin in platelets. Although it has been suggested that serum antiplasmin may be concentrated on the surface of the platelet (61), our studies indicate that the platelet antifibrinolysin is distinct from the serum inhibitor. Shown in Fig. 12 are observations on the inhibition of human plasmin by partially purified preparations of serum antifibrinolysin and platelet antifibrinolysin.

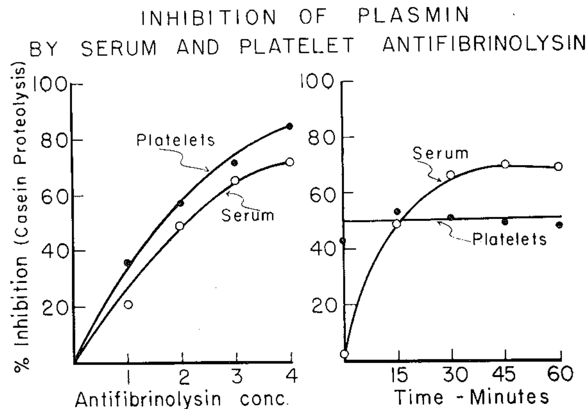

Fig. 12: Inhibition of a spontaneously activated plasmin preparation by partially purified preparations of serum and platelet antifibrinolysin. Serum antifibrinolysin was of bovine origin and obtained through the courtesy of Loomis (58), platelet antifibrinolysin was prepared from dried bovine platelets. See text.

Fig. 12A describes the relation of inhibition to concentration, and Fig. 12B describes the relation of inhibition to time at a fixed antiplasmin concentration. Note that the relation of inhibition to concentration of inhibitor, shown in relative rather than absolute amounts, is qualitatively similar for platelet and serum antifibrinolysin. However the serum inhibitor reacts with plasmin in a relatively slow and progressive manner as compared to the platelet

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inhibitor, whose reaction with plasmin appears to be almost immediate. In addition, studies with synthetic substrates indicate that the platelet antiplasmin forms a tighter complex with plasmin than does the serum antifibrinolysin. These observations suggest that at least two distinct mechanisms may exist for the protection of thrombi from the action of fibrinolysin.

Some physiological and clinical comments

Considerable confusion exists in the physiological and clinical areas of fibrinolysis. The presence of a fibrinolytic enzyme in the plasma has obviously suggested a mechanism by which thrombi and fibrinous exudates may spontaneously disappear rather than organize. Although one may be tempted to relate the rapid resolution of a pneumonic exudate to the activation of plasminogen, plasmin has neither been isolated nor identified as such, in pneumonic areas undergoing resolution. It is just as likely that leucoprotease and other tissue cathepsins may be responsible for this latter phenomenon.

It has been postulated that plasmin may actively participate in the hypersensitivity reaction either directly (62, 63) or by releasing histamine (64). The observation that plasmin activation, in vivo, by streptokinase administration (65, 66, 43) is not associated with any damage to tissues or the occurrence of hypersensitivity reactions, raises serious doubts as to the validity of these views.

The appearance of proteolytic or fibrinolytic activity in the blood has been reported in a wide variety of clinical and pathological conditions, e.g., cases of sudden death (5); liver disease (67); shock secondary to trauma, surgery, or cardiac asystole (68); the hemorrhagic diathesis following total body irradiation (69); electroshock therapy (70); fear (71); exercise (72); injections of adrenalin (72), and amniotic fluid emboli (73). It has been suggested in each of these conditions, that plasmin has been activated. Although it is possible that plasmin activation has occurred by a similar mechanism in all the situations just enumerated, it should be borne in mind that, in vitro, several different types of activation are possible including the spontaneous activation of plasmin in the absence of antiplasmin.

From a biochemical standpoint, and depending upon the amount of antifibrinolysin in the blood, one might expect that the presence of a circulating fibrinolysin in vivo would be associated with varying alterations in other plasma constituents. In *in vivo* studies in experimental animals, it has been possible to activate plasminogen either in the presence of normal amounts of antiplasmin or in association with the simultaneous removal of antiplasmin (25). Two distinct biochemical patterns are observed with these two types of activation. When plasminogen is activated in the *absence of antiplasmin,* digestion of all the serum proteins which have been shown to be in vitro substrates for

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plasmin (fibrinogen, ACglobulin, serum complement) occurs. As a result, the blood is rendered incoagulable or clots poorly and slowly, and then lyses rapidly. In addition the one state prothrombin time is delayed. Increased amounts of proteolytic activity in the plasma or serum are readily demonstrated. With these biochemical abnormalities one may well expect a bleeding tendency. The cases of "fibrinolytic purpura" which have been described during the course of severe liver disease, or following amniotic fluid emboli, $CHCl_3$ poisoning, phosphorus poisoning, and perhaps extensive traumatic insults, may well be on this basis, i.e., related to the disappearance of plasma inhibition and simultaneous or subsequent activation of plasmin, rather than to the liberation of activators alone. Furthermore, this type of laboratory pattern (an incoagulable or poorly clotting blood with evidence of an active fibrinolysin) may be produced by proteolytic enzymes other than plasmin. Increased proteolytic and fibrinolytic activity may appear in the blood of patients with extensive metastatic carcinoma of the prostate (74), but the evidence indicates that the increased circulating proteolytic activity in this instance is due to the liberation of prostatic fibrinolysin rather than plasminogen activation. Recently, in examining the blood of a leukemic patient with a hemorrhagic tendency said to be due to a circulating fibrinolysin, it was surprising to find an increase in circulating proteolytic activity but no evidence of hydrolysis of the synthetic substrates upon which plasmin is known to act. These observations suggested the presence of a different type of proteolytic enzyme, other than plasmin, in the blood of this particular patient.

In contrast, when activation of plasminogen is accomplished, in the presence of *normal serum inhibition,* by the injection of activators alone, an entirely different biochemical pattern is observed (25). The plasmin formed from plasminogen activation is fairly rapidly inactivated by antiplasmin, relatively minor changes are observed in plasma constituents, and there is a normal clotting time. However when clotting occurs during the period of plasminogen activation, plasmin is adsorbed onto the clot and produces rapid fibrinolysis. It is unlikely that a diffuse hemorrhagic tendency would be observed in the presence of a relatively normal coagulation mechanism, and observations in man (66, 43) and in experimental animals (25, 75) following the injection of plasminogen activators alone are consistent with these views. It is of interest however, that bleeding may occur at sites of recent trauma where hemostasis has been effected by intravascular thrombi (43, 25, 75).

When "fibrinolytic purpura" occurs clinically, three types of therapeutic agents have been usually employed: 1) infusions of serum albumin, since the latter is contaminated with antiplasmin; 2) fibrinogen, to provide the necessary clotting substrate; and 3) whole blood transfusions, to combat shock, provide inhibitors, fibrinogen, and other deficient factors. In most instances the above

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agents have served to control the bleeding tendency. In other instances bleeding has continued to a fatal termination. These latter instances have served to emphasize the need for developing additional therapeutic agents, e.g., purified or concentrated preparations of serum or platelet antiplasmin, and perhaps, inhibitors of the natural activators.

Concluding remarks

In this report, some of the biochemical characteristics of the various components of the fibrinolytic enzyme of human plasma have been presented. It is apparent that our knowledge concerning the plasma fibrinolytic enzyme is still superficial and with many areas of confusion, nevertheless, a firm foundation for future development is slowly emerging.

Résumé

Aprés une introduction historique les auteurs indiquent les propriétés physico-chimiques actuellement connues du plasminogène et de la plasmine. La plasmine attaque différents substrats et scinde à partir de la fibrine 2—10% d'azote soluble dans l'acide trichloro-acétique, à côté de fragments plus gros. Trypsine et plasmine attaquent les mêmes substrats synthétiques, la trypsine est toutefois 35 fois plus active que les préparations de plasmine les plus actives. Le rapport activité fibrinolytique/scission de la caséine est semblable pour les deux ferments. La trypsine et la plasmine sont certainement deux enzymes différents puisque ils sont activés à partir de leur précurseurs par différents activateurs, qu'ils sont touchés différemment par les inhibiteurs et que les rapports de leurs activités à l'encontre des esters de l'arginine et de la lysine sont différents. Les méthodes de dosage sont brièvement discutées.

Le plasminogène le plus pur est activé en une semaine par autocatalyse spontanée dans la glycérine 50%, à 30° C et au pH 7,6. Après l'activation on trouve environ 30% de la tyrosine totale soluble dans l'acide trichloro-acétique. La trypsine active le plasminogène en deux à quatre heures suivant la concentration, l'urokinase en 10 à 30 min., la streptokinase encore plus rapidement. Il existe une relation linéaire entre la concentration de ces activateurs et la vitesse de réaction. Tous ces activateurs attaquent les esters de l'arginine et de la lysine ainsi que la caséine directement. Apparemment leur action sur le plasminogène est aussi une action protéolytique. Pour agir la streptokinase a besoin d'un facteur plasmatique qui n'a pu être séparé du plasminogène. Les auteurs pensent qu'il pourrait s'agir là d'un produit de réaction entre la streptokinase et la plasmine, respectivement le plasminogène.

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Il n'existe pas encore d'information importante sur l'action de la kinase tissulaire et des staphylokinases.

L'antiplasmine est une globuline qui réagit stochiométriquement avec la plasmine et qui l'inactive irréversiblement. Une autre antiplasmine se trouve dans les thrombocytes. Alors que l'action de l'antiplasmine sérique progresse lentement, celle de l'antiplasmine des thrombocytes est instantanée.

On ne devrait pas attribuer une action certaine du système fibrinolytique sur la dissolution des infiltrats pulmonaires ou sur les réactions d'hypersensibilité. Par contre le système est activé lors de mort soudaine, de maladies de foie, de choc, d'arrêt du coeur, d'irradiation du corps entier, de peur, d'effort, d'injection, d'adrénaline et d'embolie due au liquide amniotique. L'activation peut être due soit à la disparition de l'antiplasmine soit à l'apparition d'activateurs. Dans le premier cas le danger d'hémorragie est beaucoup plus grand parce que le fibrinogène, le facteur V et le complément sérique sont détruits, que le sang coagule très mal ou lentement et que le caillot se dissout. Si au contraire il n'y a qu'apparition d'activateurs avec une antiplasmine normale, le temps de coagulation est normal, l'action fibrinolytique est brève et il n'y a hémorragie qu'au niveau des plaies.

Zusammenfassung

Nach einem geschichtlichen Überblick führen die Autoren die bisher bekannten physikalisch-chemischen Konstanten für Plasminogen und Plasmin an. Plasmin greift verschiedene Substrate an und spaltet aus Fibrin neben großen Bruchstücken in 2- bis 10%iger Trichloressigsäure löslichen Stickstoff ab. Trypsin und Plasmin greifen dieselben synthetischen Substrate an, doch ist Trypsin 35mal aktiver als die aktivsten Plasminpräparate. Das Verhältnis fibrinolytische Aktivität zu caseinspaltender Aktivität ist für beide Fermente gleich. Dennoch sind Trypsin und Plasmin sicher verschiedene Fermente, da sie durch verschiedene Aktivatoren aus ihren Vorstufen aktiviert werden, von Hemmstoffen verschieden beeinflußt werden und das Verhältnis ihrer Aktivitäten gegenüber Arginin und Lysinestern verschieden ist. Die verschiedenen Bestimmungsmethoden werden kurz besprochen.

Reinstes Plasminogen in 50% Glyzerin wird innerhalb einer Woche autokatalytisch bei 30 Grad und einem pH von 7,6 spontan aktiviert. Hierbei entsteht 30% säurelösliches Tyrosin. Trypsin aktiviert Plasminogen in Abhängigkeit von der Konzentration in 2 bis 4 Stunden, Urokinase in 10 bis 30 Minuten, Streptokinase noch schneller. Bei diesen Aktivatoren besteht eine lineare Beziehung zwischen Konzentration der Aktivatoren und Reaktionsgeschwindigkeit. Sämtliche Aktivatoren greifen Lysin- und Argininester sowie Casein direkt an und wirken offenbar selbst proteolytisch auf Plasminogen ein. Für die Wirkung

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der Streptokinase ist ein Plasmafaktor erforderlich, der bisher nicht vom Plasminogen abgetrennt werden konnte, so daß die Autoren annehmen, daß es sich bei diesem Faktor um ein Reaktionsprodukt zwischen Streptokinase und Plasmin bzw. Plasminogen handeln könnte.

Über die Wirkung der Gewebe-Kinasen und der Staphylokinasen liegen noch keine ausreichenden Informationen vor.

Das Antiplasmin ist ein Globulin, welches mit Plasmin stöchiometrisch reagiert und dieses irreversibel inaktiviert. Ein anderes Antiplasmin findet sich in den Thrombozyten. Während die Wirkung des Serum-Antiplasmin langsam progredient ist, ist die des Thrombozyten-Antiplasmin schlagartig.

Dem fibrinolytischen System dürfte keine sichere Wirkung bei der Lösung eines pneumonischen Infiltrates oder bei Überempfindlichkeitsreaktionen zukommen. Hingegen wird es aktiviert bei plötzlichem Tod, Leberkrankheiten, Schock, Herzstillstand, Ganzkörperbestrahlung, Furcht, Anstrengung, Adrenalininjektionen, Fruchtwasserembolie. Diese Aktivierung kann durch Verschwinden des Antiplasmin oder durch Auftreten von Aktivatoren zustandekommen. Im ersteren Fall ist die Gefahr des Auftretens einer hämorrhagischen Diathese viel größer, weil Fibrinogen, Faktor V und Serumkomplement zerstört werden, das Blut nicht oder nur langsam und schlecht gerinnt und das Gerinnsel sich sogleich auflöst. Tritt jedoch nur ein Aktivator bei vorhandenem Antiplasmin auf, so bleibt die Gerinnungszeit normal, die fibrinolytische Aktivität ist nur kurze Zeit nachweisbar und es kommt nur zu Blutungen aus Wundstellen.

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