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Comparison of Certain Properties of Human Plasminogen and "Proactivator". (23165)

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Mammalian plasma contains an enzyme precursor termed plasminogen (profibrinolysin)(1) which can be activated by various agents *in vitro* to form plasmin (fibrinolysin)(1), an enzyme capable of dissolving fibrin clots. Streptokinase,* a commonly employed activator of human plasminogen, is incapable of activating some animal plasminogens(2). Geiger(3) first reported that more than one factor was involved in the activation of certain animal plasminogens by SK. Later, Müllertz and Lassen(4) showed that although bovine plasminogen was refractory to activation by SK, the addition of human globulin to the system resulted in the activation of the bovine plasminogen. As a result of their investigations, Müllertz and Lassen postulated that SK reacts with a proactivator (human factor) to form a plasminogen activator which converts plasminogen (bovine) to bovine plasmin. The studies of Troll *et al.*(5) and Troll and Sherry(6) indicated that this 2-stage activation process also applied to the SK activation of human plasminogen, the first step being a stoichiometric combination of SK with proactivator to form activator; the second stage, the enzymatic conversion of plasminogen to plasmin by this activator. Employing synthetic substrates, they concluded that both the plasminogen activator and plasmin hydrolyze lysine esters, whereas plasmin hydrolyzes arginine esters as well.

It is the purpose of this paper to present data which indicate that the proactivator described by Müllertz and Lassen may be human plasminogen. This conclusion is based on evidence obtained from a comparison of the solubility, stability and purification characteristics of proactivator and human plasminogen. Further evidence supporting this conclusion is provided through studies of the autocatalytic conversion of human plasminogen to human plasmin.

Preparations. Human plasminogen. Frac-

* Streptokinase—abbreviated SK.

tion† III, Human Plasma (Baxter Laboratories) and placental blood Fraction III_2 were used. Fraction III and placental blood Fraction III_2 were purified according to Kline (7), Christensen(8) or by methods described in the text. *Bovine Plasminogen.* Fifty-two liters of fresh citrated bovine plasma were warmed to 25° and with continuous stirring, 520 ml of 20% calcium chloride were added slowly. The fibrin which formed was removed by filtration through cheese cloth and 500 ml of 1% sodium ethyl mercurithiosalicylate were added to the serum filtrate. The serum was adjusted to pH 5.2, added to 10 volumes of cold distilled water and the precipitate allowed to settle at 5° for 24 hours. Most of the supernatant fluid was then siphoned off and the precipitate collected by Sharples centrifugation. Twenty grams of sodium chloride were added to the precipitate to aid in obtaining a homogeneous suspension. It was suspended in 4 liters of distilled water, adjusted to pH 6.4, and lyophilyzed. *Streptokinase*—Varidase® (Lederle) was dialyzed, adjusted to pH 6.4 and lyophilyzed. *Substrates*—Synthetic substrates were prepared as follows: L-lysine ethyl ester dihydrochloride (LEE) according to Werbin and Palm (9), and p-toluene sulfonyl arginine methyl ester monohydrochloride (TAME) according to Troll *et al.*(5). All compounds met the usual criteria for analytical purity. They were stored over phosphorus pentoxide. *Buffers*—pH 6.5 and pH 7.15 – 0.1 M phosphate buffers were used in the assays.

Methods. Lysine ethyl esterase assay. This assay provided a measure of the total activator and plasmin activity of SK activated plasminogen, since both enzymes hydrolyze LEE(6). When this assay is carried out in the absence of SK, it provides a measure of plasmin only. SK and varying dilutions of human plasminogen are prepared in pH 6.5 buffer. LEE is dissolved in pH 7.15 buffer at a concentration of 0.168 M immediately before use to minimize spontaneous hydrolysis(10). SK, plasminogen dilutions and buffer are warmed to 37° and then added to a series of conical centrifuge tubes in the following order: 0.5 ml pH 6.5 buffer, 0.5 ml of varying plasminogen concentrations, 0.5 ml LEE and 0.5 ml SK. Enzyme blanks for each reaction mixture are included by substitution of pH 6.5 buffer for LEE. A substrate control is composed of 0.5 ml LEE and 1.5 ml pH 6.5 buffer. The pH of all mixtures is 6.4-6.5. The reaction is started by addition of SK and after one hour at 37°, the mixtures are analyzed by the copper method(10). Values of enzyme blanks and substrate control are subtracted from values of the reaction mixtures and the difference is converted to % hydrolysis of LEE. A plot of plasminogen concentration versus % hydrolysis yields a straight line to at least 30% hydrolysis. Since the extent of hydrolysis is dependent upon the SK concentration employed for activation (Fig. 1a), the optimal SK concentration is determined by titration. The optimal SK concentration is used for assay. A typical assay plot is shown in Fig. 1a. One unit of LEE esterase is defined as that quantity of SK activated plasminogen which effects a 1% hydrolysis of LEE under the described conditions. *Activator assay.* Although an assay has been described(11), the following

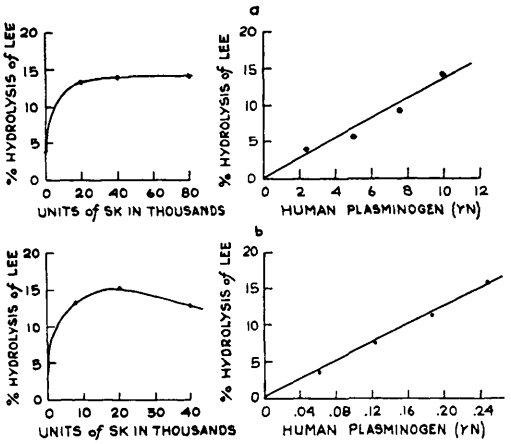

FIG. 1. (a) SK titration curve (10 γ human plasminogen) and LEE assay of a human plasminogen.‡ The fraction was assayed using 40,000 u SK and had a calculated potency of 1.4 u/γ N. (b) SK titration curve (.25 γ human plasminogen) and activator assay of a human plasminogen.‡ The fraction was assayed using 20,000 u SK and had a calculated potency of 60 u/γ N.

† Obtained from Dr. T. D. Gerlough of E. R. Squibb and Sons through the courtesy of Dr. J. N. Ashworth of American Red Cross.

TABLE I. Comparison of Human Plasma and Purified Plasminogen for Activator, TAME Esterase and LEE Esterase.

Enzyme source	Units/γ N			Ratio of activities		
	LEE esterase	TAME esterase	Activator	LEE esterase	TAME esterase	Activator
Fresh human plasma* (soluble)	.07	.08	2.5	1	1.1	35
Kline plasminogen(6); further purified (soluble)	1.4	1.2	60	1	.9	43

* Obtained through the courtesy of Mr. Frank Clarke of these laboratories.

procedure provides a more quantitative measurement of the activator of bovine plasminogen. This assay reflects the "proactivator" content of human plasminogen. Assays are carried out as described under LEE esterase assay except that bovine plasminogen, prepared in pH 6.5 buffer at a concentration of 4% is added in place of the 0.5 ml of pH 6.5 buffer. Since the incorporation of bovine plasminogen in the test system permits the use of lower concentrations of human plasminogen, hydrolysis of LEE resulting from plasminogen activator or human plasmin is negligible(11). The optimal SK concentration for maximal activation is determined for each human plasminogen fraction (Fig. 1b). It should be pointed out that in the system used for the assay of activator, there is approximately 10 times the amount of protein that is present in the LEE esterase assay. In the establishment of the optimal SK concentration (Fig. 1b) it has not been determined whether the indicated inhibition occurring at high SK concentrations is real or artifact. A plot of plasminogen versus % hydrolysis (Fig. 1b) is linear when the extent of hydrolysis does not exceed approximately 15%. A unit of activator is that quantity of SK activated human plasminogen, which results in 1% hydrolysis of LEE, under the conditions of the assay. *Tosyl arginine methyl esterase assay.* The method described by Troll *et al.*(5), employing formaldehyde in the titration of tosyl arginine was used to determine plasmin activity. However, a pH meter was employed for measuring the endpoint of the titration. One unit of plasmin is defined as that quantity of plasmin which results in 1% hydrolysis of TAME after 30 minutes, under the conditions of the assay.

Results. Comparison of human plasma and purified human plasminogen by activator, LEE esterase and TAME esterase assay. Preliminary purification experiments(7) were carried out using plasminogen of placental and blood origin, in an attempt to separate proactivator from plasminogen. It became apparent that an increase in purity of proactivator resulted in a corresponding increase in purity of plasminogen. Accordingly, plasminogen prepared by Kline's procedure(7) was further purified‡ to yield a fraction soluble between pH 2-11. This fraction was compared with a sample of fresh human plasma for activator, LEE and TAME esterase activity (Table I). The ratio of LEE esterase:TAME esterase:activator was almost identical for both fractions.

Comparison of LEE esterase and activator activity during autocatalytic conversion of plasminogen to plasmin. A fraction of human plasminogen‡ was dissolved in pH 7.6—0.1 M phosphate buffer at a concentration of 0.71 mg N/ml, preserved with merthiolate, mixed with an equal volume of glycerine§ and placed in a 37° water bath. During a 2 week incubation period, aliquots were assayed by activator and LEE esterase assay. In addition, aliquots were tested by LEE esterase and activator assay with SK omitted, so that autocatalytic plasmin and the effect of this plasmin on bovine plasminogen might be determined.

When assayed with SK, the total LEE esterase activity (plasmin + activator) was relatively constant, while proactivator, as reflected by activator assay, diminished rapidly. In contrast to the decrease in proactivator, autocatalytic plasmin (LEE esterase assay—without SK) activity increased, sug-

‡ Unpublished data.

§ Personal communication from Dr. S. J. Sherry.

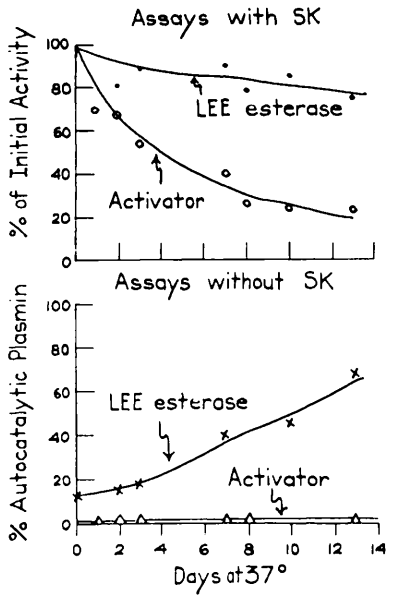

FIG. 2. Comparison of LEE esterase and activator activity during the autocatalytic conversion of plasminogen ——→ plasmin. ● Lee esterase assay (SK) represents total LEE activity (plasmin + activator); ○ Activator assay (SK) reflects proactivator content; × LEE esterase assay (without SK) represents autocatalytic plasmin only; △ Activator assay (without SK) reflects activator content in absence of SK.

gesting that either plasmin brings about the destruction of proactivator or plasmin is formed from proactivator.

Under the conditions of this experiment, plasmin did not activate bovine plasminogen (activator assay—without SK).

Stability of proactivator and plasminogen. Troll *et al.*(5,6) demonstrated a marked difference in stability of proactivator and plasminogen to heat as measured by TAME esterase and LEE esterase assays.

In view of the solubility characteristics of plasminogen, it became apparent to us that the stability of plasminogen and the instability of proactivator to heat might be related to solubility. Plasminogen preparations are generally more soluble at pH 9, the pH of the TAME esterase assay, than at pH 6.4, the pH of the LEE esterase and activator assays.

Fraction III was suspended in 0.85% sodium chloride at 6.25 mg N/ml stirred for ½ hour, adjusted to pH 7 and centrifuged. The opalescent supernatant fluid was incubated at 50° and at intervals aliquots were removed for LEE esterase, TAME esterase and activator assays. The results (Fig. 3a) show the same stability of proactivator and plasminogen by activator and LEE esterase assay; however, a greater stability of plasminogen was apparent by TAME esterase assay. This would be expected if plasminogen is more soluble in the TAME esterase assay than in the other assays.

Stability of proactivator and plasminogen in a soluble plasminogen fraction. Since the above experiment suggested a relationship between solubility and apparent stability of plasminogen, a soluble fraction was prepared. To a sulfuric acid extract(8) of Fraction III was added 3% sodium chloride. The pH was adjusted to 6.4 and the precipitate that formed was removed by centrifugation. Fifty ml of this soluble fraction, containing 0.27 mg N/ml was incubated at 50°. The solution became increasingly turbid during the incubation period. At intervals, aliquots were removed and added to an equal volume of pH 6.5 buffer. Before carrying out TAME esterase and activator assays each aliquot was filtered through a No. 2 Whatman paper to give a clear solution.

The data (Fig. 3b) show the stability of the precursor of the tosyl arginine esterase and the precursor of the activator to be identical.

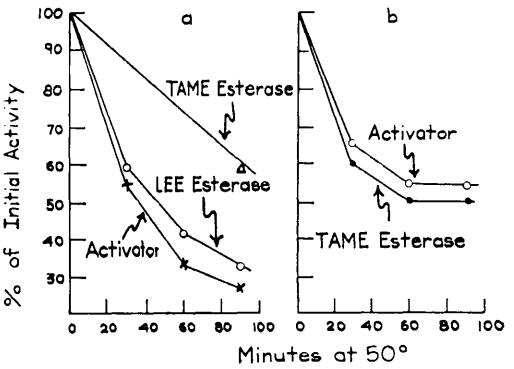

FIG. 3. (a) Stability of plasminogen at pH 7 as measured by synthetic substrate assay systems. △ TAME esterase assay, pH 9; ○ LEE esterase assay; × Activator assay. (b) Stability of plasminogen at pH 6.4 as measured by TAME esterase and activator assays in which all solutions assayed were soluble.

TABLE II. TAME and LEE Assays of Spontaneous Plasmin.*†

	TAME assays			LEE assays		
	u/ml	Mean value	% plasmin	u/ml	Mean value	% plasmin
SK	180			114		
	165	174		126	121	
	177			123		
			94			61
No SK	156			72		
	180	164		75	74	
	156			75		

* Plasmin prepared from Placental Fr III_2 by Kline's method(7).

† Prepared through the courtesy of Mr. Merle Querry of Lederle Laboratories Division, Amer. Cyanamid Co.

Evidence that activator does not hydrolyze TAME. We have confirmed the observation (6) that activator does not significantly hydrolyze TAME at pH 9. A fraction of plasminogen containing large quantities of spontaneously active plasmin (Table II) was assayed on TAME and LEE in the presence and absence of SK. TAME assays were almost identical with or without SK, but LEE assays were higher with SK. This would indicate that the activator does not significantly hydrolyze TAME at pH 9, but manifests a specificity for LEE.

Discussion. The data show that proactivator and human plasminogen have the same heat stability and purification properties. Moreover, when the plasminogen content is reduced through its autocatalytic conversion to plasmin there is also a reduction in proactivator content. These observations indicate that either proactivator and human plasminogen are closely related or they are a single substance. Purification studies are in progress in an attempt to resolve this question.

Assuming proactivator to be human plasminogen, the following mechanism for the activation of bovine plasminogen is indicated:

$$(1)\quad SK + \text{human plasminogen} \xrightarrow{\text{(proactivator)}} SKX \quad \text{(activator)}$$

Rapid $$(2)\quad SKX \ (\text{activator}) + \text{bovine plasminogen} \xrightarrow{\text{enzymatic bovine plasmin}}$$

Slow $$(3)\quad \text{Plasmin} + \text{bovine plasminogen} \xrightarrow{\text{enzymatic bovine plasmin (12)}}$$

Evidence that plasmin is not a proactivator was provided by the finding that during the autocatalytic conversion of plasminogen to plasmin the proactivator content decreases (Fig. 2) the opposite of what would be expected if plasmin were a proactivator. It has been demonstrated(12) that, under the proper conditions, plasmin and trypsin activate bovine plasminogen; however, this activation is very slow in relation to the activation by SK plus human plasminogen. Furthermore, it is possible that the activator and plasmin might synergistically activate bovine plasminogen.

Müllertz and Lassen(4) have reported that the inability of SK to activate bovine plasminogen is due to the low proactivator content of bovine plasminogen. In view of the present evidence, the following possibilities must also be considered: (a) bovine plasminogen, because of its molecular structure, does not readily combine with SK to form activator; (b) bovine plasminogen, although molecularly the same as human plasminogen, is bound at critical centers preventing its combining with SK; (c) bovine plasminogen preparations contain inhibitors which prevent SK from combining with bovine plasminogen but do not inhibit activation by the SK human plasminogen mixture; or, (d) SK combines with bovine plasminogen to form an SKX complex which is incapable of activating bovine plasminogen or does so at a very slow rate not observable under our test conditions.

Summary. Human plasma and highly purified soluble human plasminogen contain the same proactivator:plasminogen ratio. The difference in apparent stability of plasminogen and proactivator was traced to the difference in solubility as effected by the pH of the respective assays. During the autocatalytic conversion of plasminogen to plasmin, proactivator content diminishes as plasmin is formed. A discussion of these observations is presented, wherein it is proposed that human plasminogen and the proactivator of bovine plasminogen in human plasminogen are the same factor.

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Sensitivity of Pertussis-Inoculated Mice to Serotonin.* (23166)

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It is well known that pertussis vaccine increases susceptibility of mice to histamine(1) and to active(2) and passive(3,4) anaphylaxis. It hardly seems likely that the concomitant sensitivity of pertussis-inoculated mice to histamine and to anaphylaxis is coincidental. However, some evidence has been presented against the importance of histamine in mouse anaphylaxis(5). Recently, Fink demonstrated that the anaphylactic contraction of isolated mouse uterus is inhibited by lysergic acid diethylamide (LSD) and reserpine(5). It was also shown that the egg-white sensitized and normal mouse uterus is 1000 times more sensitive to serotonin than to histamine. In view of the aforementioned experiments, the sensitivity of pertussis-inoculated mice to serotonin was determined.

Materials and methods. Male 20 to 27 g Tumblebrook Farm (TF) Swiss Webster mice and male 17 to 21 g Swiss Webster mice from the National Laboratory Animals Co. (NLA) were inoculated i.p. with 0.1 ml of *Hemophilus pertussis* vaccine (48.8 billion organisms/ml). Four days later (when the peak of histamine sensitivity is known to occur) pertussis-inoculated mice as well as uninoculated control animals were challenged i.p. with various doses of histamine dihydrochloride or serotonin creatinine sulfate. In the experiment involving pertussis-inoculated and uninoculated NLA mice 5 dosage groups containing 8 mice per group were employed in determining the LD_{50} of either histamine or serotonin. An exception to this procedure occurred in the uninoculated mice challenged with histamine, where only 1 group of 8 mice was challenged with a single dose of histamine. Since all animals survived, the LD_{50} was expressed as $>$ the dose injected. In the experiment involving pertussis-inoculated and uninoculated TF mice 3 dosage groups consisting of 6 mice per group were utilized in each instance in determining the LD_{50} of each drug. Total number of animals used in both experiments was 200. Deaths were noted for a period of 24 hours. Median lethal doses were calculated by the method of Litchfield and Wilcoxon(6) and are expressed in terms of mg/kg of base.

Results. The data in Table I demonstrate that administration of pertussis vaccine greatly increases susceptibility of the mouse to the lethal effects of both serotonin and histamine. The LD_{50} and 19/20 confidence limits of serotonin were 7.9 (5.4 – 11.4) mg/kg in pertussis-inoculated TF mice and 364 (251 – 528) mg/kg in uninoculated TF ani-

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