STUDIES OF THE PLASMIN SYSTEM

I. MEASUREMENT OF HUMAN AND ANIMAL PLASMINOGEN. MEASUREMENT OF AN ACTIVATOR IN HUMAN SERUM

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The clot which forms in drawn blood occasionally lyses spontaneously, a phenomenon which Dastre explained in 1893 by the discovery of a proteolytic enzyme in serum which will digest the fibrin matrix of clots (1). Because plasmin (or fibrinolysin) is always present in a precursor form but is rarely active, the reasons for spontaneous appearance of activity have been sought. By use of the simple test of watching a blood clot for the occurrence of lysis, it has become evident that natural fibrinolytic activity occurs almost exclusively in pathological states (2). Use of more refined techniques in recent years has added little to the knowledge of fibrinolysis *in vivo*. Clot lysis or other evidence of proteolytic activity has been observed in shock (3), after traumatic injury or burns (4, 5), in acute febrile infection (6), in chronic liver disease (7, 8), sometimes with cancer (9), after injection of ACTH (4) or epinephrine (10), or with fever induced by pyrogens (11). Of normal persons only those who have performed severe physical exercise will show direct lytic activity (10). Fibrinolysis or proteolysis has been described during experimental anaphylaxis (12), but its importance is undecided. The possible function of the enzyme in the above states is entirely obscure, but in several other conditions it is postulated to be a direct cause of some of the manifestations of the disease.

Mothers with severe premature separation of the placenta occasionally have complete loss of clottable fibrinogen, presumably because of digestion by the circulating fibrinolysin that accompanies the condition, and the consequence is severe bleeding (13, 14). A similar hemorrhagic state occurring with widespread metastasis of cancer of the prostate has been correlated with the ability of prostatic tissue to produce a fibrinolysin (15). Streptococci produce a potent activator of the human enzyme, and the rapid spread of streptococcal cellulitis is thought to be due in part to the locally induced breakdown of enclosing fibrin deposits which are found at infected sites (16). The thin watery character of streptococcal pus is due also in part to activation of fibrin digestion by products of the bacteria (17).

Much evidence, therefore, suggests that fibrinolysis is a basic, physiologically important process, which requires a fuller understanding in order to complete

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our picture of several disease states. Despite widespread interest and many observations, however, elucidation of the role of fibrinolysis in any of the above conditions had not been forthcoming. This is due largely to the complicated nature of the lysing system, which needs quantitative observations of a number of components before a proper evaluation can be attempted. The natural process of activation can be seen only in the stress conditions cited, and activation in the test tube can be achieved solely by means that are essentially artificial. Such means, however, have resulted in fruitful studies of the enzyme's properties.

The nomenclature of Christensen gives the name plasminogen to the inactive enzyme precursor contained in the β-globulins of all plasmas (18). Certain activators convert it to an active protease called plasmin, which can hydrolyze a number of proteins and most particularly fibrin (16). Plasmin absorbs to fibrin and when so absorbed it is protected against inhibitory agents and thus seems more active against fibrin than against other proteins (19).

The process of conversion of plasminogen to plasmin is poorly understood. Activation with the streptococcal activator, now usually called streptokinase (18), has been studied the most thoroughly. Streptokinase is almost specific for human plasminogen, activating only traces of plasminogen from other species. However, when a small amount of human globulin is added to streptokinase, the resulting mixture activates the plasminogen of other species quite readily (20). The activating property of such a mixture is destroyed rapidly at acid pH (whereas plasmin is most stable at acid reaction), but after neutralization it can be restored by fresh addition of streptokinase (21). These facts suggest that activation by streptokinase is mediated by an activator present only in human plasma, so accounting for the ineffectiveness of streptokinase alone in non-human sera. It is interesting to note that this activator property is always found associated with human plasminogen and remains in constant ratio to plasminogen during Kline's procedure of purification (22); hence, its existence as an entity distinct from human plasminogen still needs proof.

Müllertz has reported the appearance of an activator in the blood of individuals undergoing electric shock therapy or sudden anoxemic death. This substance was found in the globulin fraction and was capable of activating either human or bovine plasminogen (23).

Other activating materials that are not species-specific have been discovered in urine (24), in a number of different tissues (25), and in cultures of staphylococci (26). A number of organic solvents such as chloroform and ether are activators also (16). Spontaneous activation of partially purified plasminogen has been observed to occur irregularly in solutions allowed to stand several days in the cold (25).

An inhibitory substance is found in normal blood which either destroys or neutralizes the hydrolytic property of active plasmin. This was once thought to be the same as the trypsin inhibitor of serum, but the studies of Shulman (27) and of Jacobsson (28) make this view unlikely. Inhibition of plasmin has variously been reported as a stoichiometric and immediate reaction (29) or else as slow and enzymatic (30), a conflict in observations which has needed elucidation but which suggests the existence of more than one inhibitory process.

In view of this complicated scheme of proenzyme, enzyme, a number of activators, and one or more inhibitors, it would seem that any attempt to evaluate the significance of the plasminogen system would require, at a given time, the separate measurement of each of the components in order to understand their interplay.

Methods of measurement which distinguish between activators and plasmin itself have been difficult to devise. Fibrin digestion is the favorite method for determination of plasmin and clots for this purpose are commonly formed from animal fibrinogen that is only partially purified. A number of workers have shown that these clots are contaminated with plasminogen from their animal source (20, 31). Consequently, lysis may be induced not only by the plasmin of the sample under test, but also by plasmin activated from the clot. Except when extreme care has been taken to obtain pure fibrin, work based on lysis of fibrin fails to distinguish between activator and plasmin and, therefore, has a limited value.

It seems obvious that the extent of activation of the plasminogen in the blood would be of physiological importance, yet the usual demonstration of clot lysis does not determine what proportion of the total plasminogen has become active. It should be pointed out that lysis of a clot *in vitro* can be accomplished by a minute proportion of the potential proteolytic activity of the blood, but that very small amounts of free plasmin in the circulation may be without much effect on the organism. Such considerations as these make quantitative measurements of both plasminogen and plasmin in a sample of blood of paramount importance.

Finally, the degree of plasmin inhibition has been studied only rarely in relation to effective proteolysis; even though it is evident that, since they are always present, inhibitors must modify the final significant activity.

As an approach to the complicated problem of simultaneous measurement of a number of factors, a quantitative and specific method for the stable precursor, plasminogen, has been developed and is presented in this paper. For this purpose, it was first necessary to remove inhibitors without loss of plasminogen, and then to accomplish complete conversion to plasmin, the only measurable form of the enzyme. Complete activation of human plasminogen has been achieved with streptokinase by use of appropriate conditions, but for animal plasminogen more complicated means were needed. It was found possible, however, to activate animal plasminogen completely with a combination of streptokinase and an activator in human globulin. This system was adapted to quantitative measurement of guinea pig plasminogen and was also found to be suitable for the assay of the activator in human serum. Once activated, plasmin was measured by its ability to hydrolyze a common protein, casein, and the activity is expressed in an arbitrary caseinolytic unit[1]. As the casein assay seems adaptable to the measurement of any of the factors of the lysing system, this unit can be used as a common measure for the activity of

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[1] A preliminary report of these studies appeared in *Fed. Proc.*, 1956, **15**, 606.

each component. The application of these methods to a study of the plasmin inhibitors of the blood will be presented in a later paper.

Materials

Casein.—Several lots of commercial casein proved unsatisfactory as delivered because of their content of substances that are soluble in trichloroacetic acid. The Hammarsten casein furnished by the Amend Chemical Co., New York, was found to be appropriate material for further purification. Fifty gm. of the dry powder was suspended in a liter of distilled water and stirred vigorously for an hour with a stirring device. The suspension was transferred to 250 ml. centrifuge bottles and centrifuged for 10 minutes at 1500 R.P.M. The slightly turbid supernatant was decanted and discarded; each bottle was refilled with distilled water and the precipitate resuspended by vigorous stirring. Centrifugation was repeated and the supernatant discarded. Two hundred ml. of 95 per cent alcohol was added to each bottle and the contents made into a slurry which was poured into a large Buchner funnel and the alcohol was filtered off. While in the funnel, the precipitate was washed twice more with 95 per cent alcohol and three times with anhydrous ether. The ether was allowed to evaporate thoroughly from the casein and the powder was ground in a mortar. The casein was considered to be satisfactory when 2.0 ml. of a 2 per cent solution, precipitated by 3.0 ml. of 10 per cent trichloroacetic acid, gave a supernatant fluid with an optical density at 280 mμ, not greater than 0.080. Because there may be some variation in the digestibility of various preparations of casein, several lots prepared as above were thoroughly drummed to make one large sample which was used for all the experiments described herein.

To make a 4 per cent casein solution buffered at pH 7.4, 4.0 gm. of casein was suspended in about 70 ml. of borate-saline buffer, pH 7.4, with vigorous stirring. 0.25 ml. N NaOH per gm. of casein was added to convert the casein to the sodium salt. Thirty minutes or more of vigorous stirring was required to complete solution. The solution was adjusted to pH 7.4 by glass electrode by slow addition of N NaOH. The volume was then brought to exactly 100 ml. by further addition of the borate-saline buffer. A small amount of insoluble material was removed by centrifugation. Casein solutions were stored in the refrigerator for about 1 week without preservative and then discarded.

Buffer.—Borate-saline buffer, pH 7.4, as described by Palitzsch (32), was used throughout. This was prepared by mixing 0.05 M sodium borate (19.108 gm. $Na_2B_4O_7 \cdot 10H_2O$ per liter of distilled water) with 0.2 M boric acid-salt solution (12.404 gm. H_3BO_3 and 2.925 gm. NaCl per liter of distilled water) until pH 7.4 was obtained by glass electrode. Approximately 1.2 parts borate solution to 8.8 parts boric acid–salt solution were required.

Streptokinase.—All experiments described here were performed with a single lot of varidase (No. 2200–10173), a lyophilized mixture of streptococcal enzymes consisting of streptokinase, streptodornase, other protein, and phosphate buffer, kindly furnished by the Lederle Laboratories Division, American Cyanamid Co. This material contained about 4000 Christensen units of streptokinase per mg.

Euglobulin Solution.—Plasminogen in human or guinea pig serum was separated from its inhibitors by precipitation in the euglobulin fraction. Serum was diluted 20-fold with distilled water, and the reaction was adjusted to pH 5.2 by glass electrode by addition of 0.5 per cent acetic acid with constant stirring. The resulting precipitate, after being allowed to ripen for 30 minutes, was transferred to a 50 ml. centrifuge tube and centrifuged 10 minutes at 1500 R.P.M. The supernatant was decanted and the tube was drained for several minutes. Any drops of fluid on the lip of the tube were wiped away with tissue. The precipitate was resuspended in borate-saline buffer to provide a concentration suitable for the particular testing that was desired (see text).

Purified Plasminogen.—Human plasma fraction III was used as the starting material[2] in preparation of plasminogen by Kline's method (33). The plasminogen powder was dried with alcohol and ether according to Cliffton and Cannamela (34). The dried powder dissolved readily in 0.0025 M HCl, in which solution it was stable and was found to be fully active after several weeks storage at 5°C.

Spectrophotometer.—Optical density readings at 280 mμ were obtained with a Beckman model DU spectrophotometer using a Beckman ultraviolet attachment and quartz cuvettes with a 1 cm. light path.

METHODS AND RESULTS

1. Human Plasminogen

A simple caseinolytic assay for trypsin has been described by Kunitz which measures the digestion products by their ability to absorb ultraviolet light after precipitation of undigested protein with trichloroacetic acid (35). As described by Remmert and Cohen (36), it was determined that plasmin digested casein sufficiently in a short time to use Kunitz' principle for plasmin assays. The next step was to determine an appropriate concentration of casein and digestion time which would provide a quantitative assay for plasmin in the amounts which may appear in the blood or other body fluids. The course of the digestion of casein in several concentrations over a period of 1 hour by the plasmin separated from an equal amount of serum is illustrated in Fig. 1. Up to a limit, increasing substrate concentration increases its digestion by a given concentration of an enzyme. Although a 2 per cent final concentration of casein during digestion is not at this limit for plasmin, it is sufficiently high to provide a sensitive assay when a 30 minute incubation period is used. The active enzyme is quite stable in the presence of casein for more than 30 minutes so that deterioration during the time of assay is not a problem. As a result of these experiments, digestion of 1.0 ml. 4.0 per cent casein by 1.0 ml. of enzyme solution for 30 minutes at 37°C. was adopted and used as a method throughout these studies.

Measurement of plasminogen or plasmin requires removal of the inhibiting or interfering substances of serum. This end has always been accomplished by precipitating the euglobulin from diluted serum by acidifying to pH 5.2 (37, 38). Reexamination of this method by the more refined means available to us showed, in several trials of the process, that the same amount of plasminogen was always precipitated with the euglobulin from a given serum. The precipitate was free of detectable inhibitor and by careful determination only a small amount of plasminogen, amounting to less than 1 per cent of the total, could be found in the supernatant fluid.

In order to measure the total plasmin precursor in the euglobulin, complete activation to plasmin becomes necessary without any loss of the newly active but rather labile enzyme during the process of activation. Trials of human globulin with several activators showed that the highest activities were obtained with streptokinase, because it can act within seconds and thus lead to complete activation before the unstable plasmin deteriorates greatly. Kline has pointed out that plasmin is quite stable in the
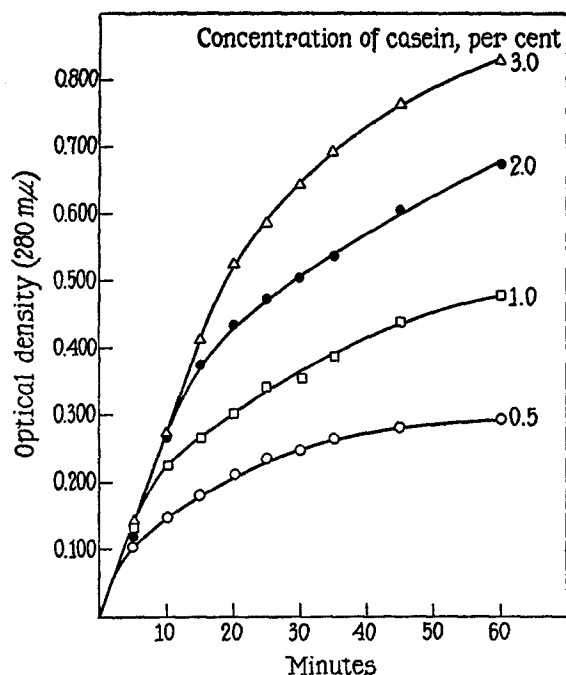
FIG. 1. Increase in digestion products with time and concentration of casein produced by a single concentration of human euglobulin activated with streptokinase. Incubated at 37°C. Final concentration of casein in digestion mixture indicated.

TABLE I

The Activating Effect of Streptokinase on the Proteolytic Precursor in the Euglobulin from 1.0 Ml. Normal Human Serum in the Presence of 1.0 Ml. 4.0 per cent Casein (Duplicate Determination)

Streptokinase	Optical density of digestion products (280 mμ)	
	1	2
μg.		
1.5	0.258	0.297
2	0.349	0.349
4	0.470	0.476
6	0.511	0.507
10	0.526	0.520
40	0.532	0.536
60	0.547	0.557
80	0.550	0.566
100	0.537	0.547
200	0.544	0.546
300	0.528	0.544
600	0.494	0.486
800	0.451	0.469
1000	0.451	0.421

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presence of casein (or other substrate) and that no enzyme can be lost when activation occurs with substrate present (39). Activation in the presence of casein was tested by the addition of varying amounts of streptokinase to a series of tubes containing human euglobulin already mixed with casein.

Table I shows the optical density of the digestion products which resulted from 30 minutes' incubation of each tube. Peak values were obtained with the use of 60 to 100 μg. of streptokinase and probably represent almost instantaneous activation, while the reduction in activity occurring with more streptokinase is due to inhibition by an excess. The optimal amount of streptokinase needed to secure all the potential activity of a given sample falls within rather narrow limits; and, until streptokinase becomes available as a pure substance, it will be necessary to redetermine the optimum with each new lot.

As an alternative method of activation, the streptokinase was allowed to act for several minutes on the euglobulin before the casein substrate was added. In this case, less streptokinase was needed, since such rapid activation was not required. For instance, if the varidase used for these experiments acted on the euglobulin for 5 minutes at 37°C. before the addition of casein, the activity from 10 to 20 μg. equalled that provided by 60 to 100 μg. in the previous experiment. The active enzyme was unstable at 37°; consequently, incubation for longer than 5 minutes before the addition of substrate resulted in detectable loss of activity. However, when purified materials such as the plasminogen preparation described by Kline (33) were tested, the active enzyme was so labile in neutral salt solutions in the absence of the stabilizing substrate that significant loss occurred even in 5 minutes at 37°. For this reason, activation in the presence of substrate with the larger amounts of streptokinase was adopted as a more general method.

In order to prepare a standard plasmin curve for reference in comparing unknowns, proteolytic determinations were done on a series of dilutions of a globulin suspension from normal serum. These were activated by the optimal amount of streptokinase in the presence of casein. Fig. 2 shows the increasing optical density readings obtained with increasing amounts of serum euglobulin. As defined by Kunitz (35), a proteolytic unit is the activity which gives rise, under the conditions of the test, to an increase of 1 unit of optical density at 280 mμ per minute of digestion. The specific activity of the serum globulin is obtained by drawing a straight line tangent to the first part of the curve as shown in Fig. 2. The slope divided by 30 minutes is the activity per ml. of serum, i.e.,

$$\frac{0.326}{0.4 \times 30} = 27.1 \times 10^{-3} \text{ units/ml. serum.}$$

A new curve is then plotted, the ordinates of which are the same as in Fig. 2, while the abscissae are expressed in plasmin units, 1 ml. serum being equal to 27.1×10^{-3} units. The data on the new curve can be employed as a general standard curve for determination of human plasmin activity, provided the same casein and conditions of assay are used. Fig. 3 shows a standard curve for human plasmin and a curve for guinea pig plasmin is superimposed. The difference between the two sets indicates that a new curve must be constructed for each species. The data in Fig. 2 indicate that
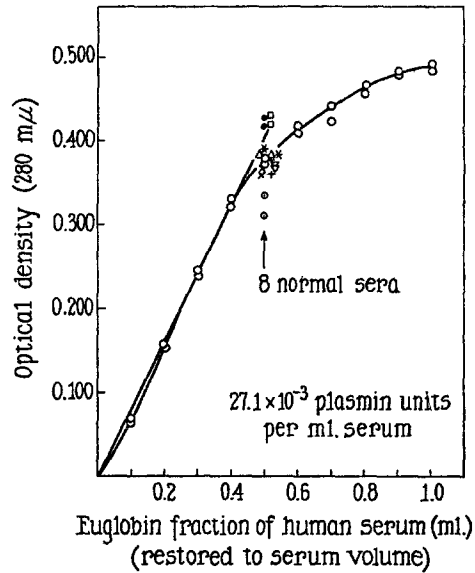
FIG. 2. Optical density of digestion products after incubating 1.0 ml. 4.0 per cent casein with an equal volume of euglobulin from normal human serum for 30 minutes at 37°C. Activation with streptokinase. Duplicate determinations on euglobulin from 0.5 ml. of 8 normal sera indicated by various symbols.
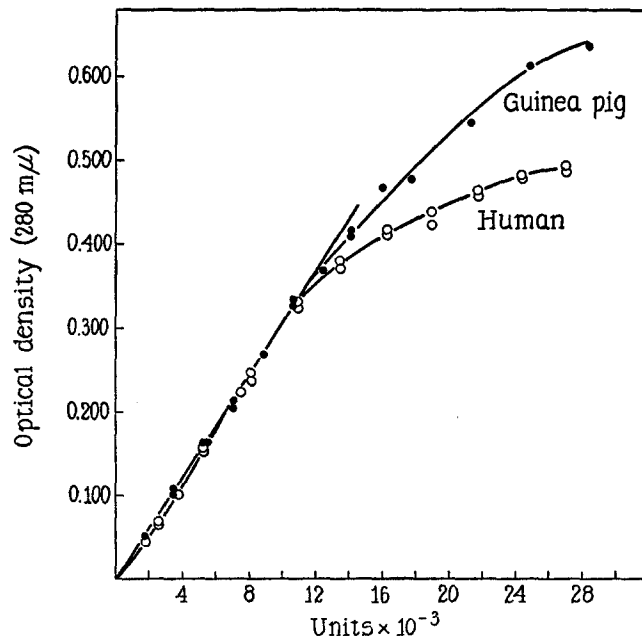

FIG. 3. Standard unit curve for human and guinea pig plasmin constructed as described in text. Human curve is from data in Fig. 2, guinea pig curve is from Fig. 4.

0.5 ml. of serum is an appropriate amount to use for routine assays; the optical densities found by duplicate determinations on 8 normal sera are indicated. From these data the following method is suggested for routine use:—

Measurement of Human Plasminogen.—2.0 ml. human serum is diluted to 40 ml. with distilled water and brought to pH 5.2 with 0.5 per cent acetic acid. After 30 minutes, the precipitate is separated by centrifugation for 10 minutes at 1500 R.P.M. and redissolved in 4.0 ml. borate-saline buffer. Purified plasminogen is weighed and dissolved directly in 0.0025 M HCl. Three aliquots of 1.0 ml. are pipetted into 16 × 100 mm. test tubes for duplicate determinations and one blank; 1.0 ml. of 4 per cent casein, pH 7.4, is added to each. A series of samples is placed in a 37° constant temperature bath and several minutes are allowed for equilibration. Then, serially, at ½ or 1 minute intervals 0.1 ml. of streptokinase solution of previously determined optimum concentration (see above) is added and mixed thoroughly. Each tube is allowed to incubate at 37°C. exactly 30 minutes and then 3.0 ml. of 10 per cent trichloroacetic acid is rapidly added to stop digestion and precipitate the undigested protein. A blank for each sample is made by adding trichloroacetic acid prior to the streptokinase.

A Cornwall syringe pipetting outfit is convenient for adding the trichloroacetic acid rapidly. At least 1 hour is allowed for completion of precipitation[3], then the precipitated protein is separated by centrifugation. The precipitate, being bulky and adherent, will not ordinarily separate cleanly with one centrifugation; therefore, it is convenient to centrifuge once, decant the supernatant into a second tube and centrifuge again. The optical density of the crystal-clear supernatant is read at 280 mμ in a spectrophotometer. After subtraction of the optical density of the blank, the activity of the sample in units is determined by reference to the unit curve already described.

2. Guinea Pig Plasminogen

Similar determinations of plasminogen in the serum of lower animals are less simple because there is no known *in vitro* activator that acts as rapidly on the serum of animal species as streptokinase does on human plasminogen. This fact has prevented the useful estimation of the plasminogen in animal sera and thus hampered study of the lysing system in experimental animals. With the appearance of the report that a mixture of human euglobulin and streptokinase results in a potent activator of certain animal sera (20), it seemed that its action might be sufficiently rapid to give complete activation of animal plasminogens.

The guinea pig was chosen as representative of those species whose plasminogen is not readily activated by streptokinase. Preliminary experiments showed that quite high activities resulted from guinea pig euglobulin if the globulin from only 0.05 ml. human serum was used as co-activator with streptokinase. Further experiments indicated that whole human serum gave results similar to separated globulin and, in this small volume, did not furnish enough inhibitor to change the result materially. Likewise, the purified human plasminogen was rich in the activating property.

Activation in the presence of substrate could not be used in this system, since amounts of human globulin sufficiently large to provide instantaneous activation would

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[3] At this point the precipitates may be stored overnight in the refrigerator.

also have had enough proteolytic activity to interfere seriously with the accuracy of the final determination. It seemed advisable, therefore, to allow activation to proceed for 5 minutes prior to addition of casein. Under these conditions, optimum activation was achieved with 10 to 20 μg. streptokinase and 0.05 ml. human euglobulin. The activity curve determined from a series of dilutions of guinea pig euglobulin is shown
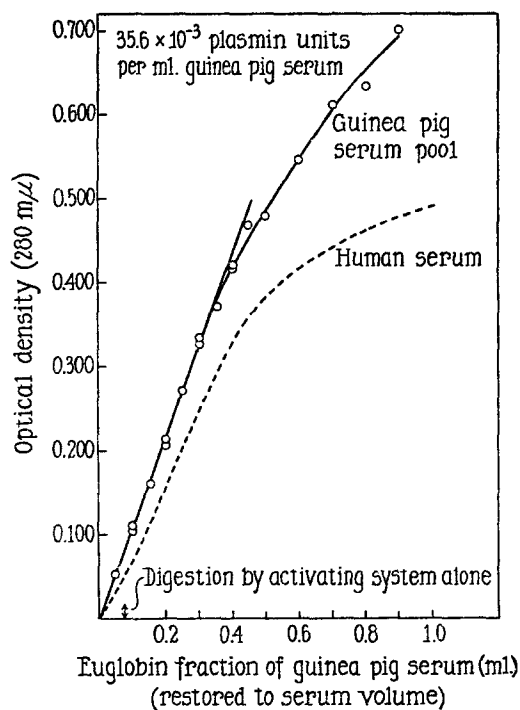

Fig. 4. Optical density of digestion products after incubating 1.0 ml. 4 per cent casein with euglobulin from normal guinea pig serum for 30 minutes at 37°C. Magnitude of correction applied for activating system indicated by arrow at lower left. Human curve from Fig. 2 superimposed in dotted lines.

in Fig. 4. The relatively small proteolytic activity inherent in the activating system is indicated. These data were used to construct the standard guinea pig curve already shown in Fig. 3. It is a general standard curve for the activity of guinea pig plasmin and may be used for conversion of optical density readings to plasmin units after the necessary correction for the activating system has been applied. Guinea pigs had, on the average, 35.6 $\times$ 10^{-3} units of plasmin per ml. serum. The procedure finally adopted is given in detail below.

Measurement of Guinea Pig Plasminogen.—The euglobulin precipitate from 2.0 ml. guinea pig serum is prepared and redissolved in 2.0 ml. buffer. 0.5 ml. aliquots are pipetted into each of three tubes to afford a set of duplicate determinations and one reagent blank. To each tube is added 0.5 ml. of human euglobulin in a 1-10 dilution from its concentration in

serum. It is essential for control purposes to determine the small amount of proteolytic activity possessed by the human globulin. For this, the guinea pig euglobulin is omitted from several tubes, the volume being made up with borate buffer. After equilibration in a 37° water bath, 0.1 ml. streptokinase solution of the previously determined optimal concentration is added to each tube. Exactly 5 minutes is allowed for activation, then 1.0 ml. warmed casein is added and proteolytic activity is determined as described for the human system. The reading of the appropriate reagent blank is subtracted from the optical density of each tube containing guinea pig euglobulin, as well as from each tube containing only the activating system. The latter value for the small proteolytic activity of the added human system is then subtracted from the optical density of the tubes containing guinea pig euglobulin. The resulting figure is a measure of the guinea pig plasmin alone. These optical densities are translated into plasmin units by reference to a standard curve for guinea pig plasmin as described above.

3. The Activator in Human Serum

The constituent in human serum that is needed for the activation of animal plasminogen by streptokinase has naturally attracted interest since its description (2). Further elucidation of its nature depends upon accurate measurement, so it seemed desirable to quantitate the amount present in serum samples. For this, it is possible to use the system described above for the measurement of guinea pig plasminogen, if one provides less than enough human serum for complete activation. In this case, the activity achieved is a measure of the human activating principle.

It was found that, with the optimal amount of streptokinase, 0.02 ml. of any of several human sera tested would activate about one-half the plasminogen in 0.5 ml. guinea pig euglobulin in 5 minutes at 37°. Accordingly, two human sera, one high and one low in activating ability, were examined in an experiment in which increasing amounts (up to 0.02 ml.) of serum were added to a constant amount of guinea pig euglobulin and streptokinase. The active protease was determined after 5 minutes at 37° and the results are illustrated in Fig. 5. A direct relationship is demonstrated between the concentration of human serum and the plasmin activated. It might be expected that globulins from different guinea pigs would vary in the rate at which they are activated. However, when globulins from several animals were tested simultaneously with a single human serum, the plasminogen activated in each was the same. A test was devised which uses 0.5 ml. of a 1–25 dilution of unknown serum with 0.5 ml. guinea pig globulin and 20 μg. of varidase. An arbitrary unit of activator was chosen and is defined as the amount of activator which will activate 1 unit of guinea pig plasmin in 5 minutes at 37°C. in the presence of streptokinase. The units of new plasmin activated multiplied by the dilution of serum equals the units activator per ml. serum. The experimental figures for some normal sera are shown in Fig. 5. These give calculated values of 525 to 780 $\times$ 10^{-3} units activator per ml. serum.

It should be stated that measurements of activator do not clarify its controversial status as a separate substance. Combination of streptokinase with either plasminogen itself or a second cofactor could explain our data on formation of activator. These experiments merely indicate an accurate means for estimating the activating property of human serum. There follows the method finally adopted for routine purposes.

Measurement of Human Activator.—Human serum is diluted 25-fold with borate-saline buffer, pH 7.4, and 0.5 ml. aliquots are pipetted into each of 3 tubes to provide duplicate determinations and one blank. To each is added 0.5 ml. of separated guinea pig euglobulin restored to serum concentration. The tubes are transferred to a 37°C. water bath and allowed to come to temperature. To each tube, 0.1 ml. of streptokinase of the previously determined optimal concentration is added. After exactly 5 minutes for activation, 1.0 ml. of previously warmed casein is added. After exactly 30 minutes of incubation, 3.0 ml. of 10 per cent trichloroacetic acid is added to each. Blanks are made by adding the trichloroacetic acid prior to adding the streptokinase. The corrected optical densities found on the clear supernatant fluids are
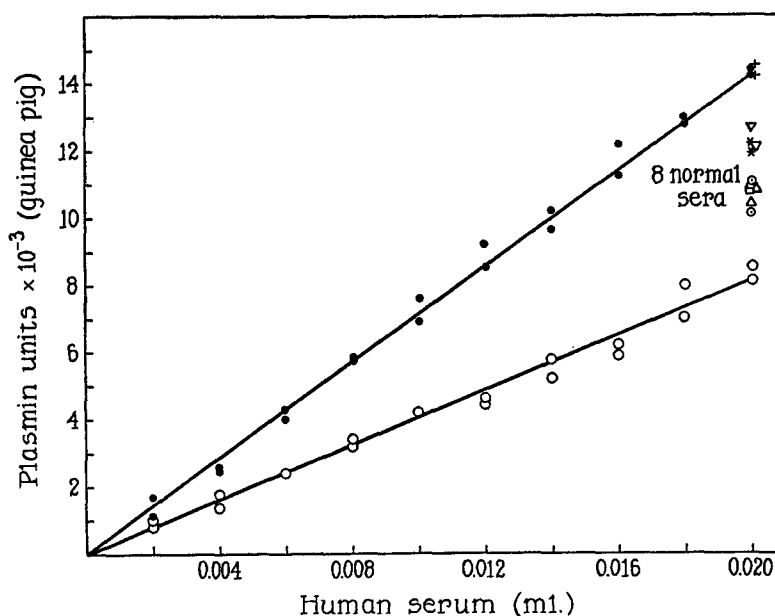

Fig. 5. Units of plasmin activity obtained from 0.5 ml. guinea pig euglobulin by varying amounts of two normal whole human sera after 5 minutes at 37°C. with streptokinase. Plasmin activated by 0.02 ml. portion of 8 normal human sera indicated at right.

translated into plasmin units by reference to a standard guinea pig plasmin curve. The unit activity per tube is multiplied by 50 to obtain the number of activator units per ml. of serum.

DISCUSSION

The casein method reported here offers the particular advantage that it can be used as a general method for determination of the several factors in the lysing system, expressing each of them in a common system of arbitrary units. Application of the same unit system to all the components should be of value in the quantitative description of their interactions. Extension of the caseinolytic assay to the estimation of inhibitor will be described in a subsequent paper.

Casein digestion for determination of plasmin has been used a number of times before. Christensen's work on the nature of streptokinase activation was so done (18), however, that his method would be unsuitable for routine determinations because it requires the euglobulin from about 10 ml. of serum for a single determination. Downie and Cliffton circumvented this difficulty by incubating the digestion mixture for 24 hours (40). Prolonged incubation incurs the risk of deterioration of the enzyme during digestion and introduces the necessity of preventing bacterial growth in a medium that favors multiplication. The assay of Remmert and Cohen uses activation by streptokinase in the presence of casein, although the reasons for so doing were not stated (36). The optimal amount of streptokinase, however, was not worked out by these authors. It has been the most satisfactory assay to date, but never has been adapted for measurement of total plasminogen of blood samples or assay of animal plasminogen. While this work was in progress, Müllertz reported an assay very much like ours which he used for work on the nature of activation by streptokinase. His paper, being concerned with other matters, did not deal with complete activation of human or animal plasminogen for purposes of assay in physiological conditions (41).

Since the only known action of plasmin that has physiological significance is the digestion of fibrin, assays using fibrin as a substrate theoretically would be the more desirable. In practice, preparation of fibrinogen free of contaminating plasminogen is quite difficult. Casein, on the other hand, is available cheaply in a well purified form free of enzymes and inhibitors. Reproducibility in a fibrin assay depends upon formation of clots that are uniform in size and density, a problem which has not been adequately solved. Furthermore, the usual method for measuring fibrin digestion is visual observation of clot lysis, and lysis time is too crude for a satisfactory quantitative test. Shulman has presented a clever approach to the last problem by tagging fibrin with I^{131} and measuring the radioactivity released by digestion (29). In this assay, also, contamination of the fibrin with plasminogen and the need for uniformity of clots are difficulties which have not been met.

Assays based on hydrolysis of synthetic esters of arginine and lysine (42, 43) have recently been developed and show great promise. They are rapid, easily done, and quantitative. At present, their specificity for plasmin is in doubt, because thrombin (44) and the first component of complement (45) have both been shown to digest the same esters. When crude biological fluids such as serum are used, these methods may not measure plasmin alone. At the present, plasmin is the only enzyme in blood that has been found to digest whole proteins at neutral pH.

The labile nature of plasmin and the presence of inhibitors in the blood make the accurate estimation of the active enzyme circulating in the blood at any particular time peculiarly difficult. The nature of inhibitor revealed by the

investigation to be reported in the subsequent paper indicates that progressive inhibition occurs in the test tube after a blood sample is obtained. Unfortunately, there are no means known to us for preserving the active enzyme either from its own instability or from the continuing action of inhibitors while the plasma or serum is being separated from the formed elements of the blood. On the other hand, if circulating activator were present at the time the blood was drawn, it is possible that activation of plasminogen could also continue in the test tube. The foregoing combination of facts indicates the error that is inherent in interpretation of direct proteolytic tests on blood plasma or serum. It seems that this difficulty will not be resolved readily.

CONCLUSIONS

A general assay method involving casein digestion is shown to be applicable for determination of several components of the plasmin system.

Total plasminogen of human serum can be measured by use of sufficient streptokinase for instantaneous activation, accompanied by protection of the active plasmin with casein.

Total plasminogen of guinea pig serum can be measured after complete activation with streptokinase in combination with human activator.

The activator principle in whole human serum can be measured by its ability to activate guinea pig plasminogen.

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