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Formation and Properties of the Activator of Plasminogen and of Human and Bovine Plasmin

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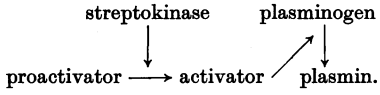
Plasminogen is the precursor of plasmin, a proteolytic and fibrinolytic enzyme of blood. Streptokinase, an agent produced by cerlain streptococci (Garner & Tillett, 1934), effects the conversion of human plasminogen into plasmin (Kaplan, 1944; Christensen, 1945), presumably by a direct enzymic effect (Christensen & MacLeod, 1945). Bovine plasminogen was activated only very slightly by streptokinase, but large amounts of plasmin were produced in bovine plasminogen by a tissue activator (Astrup & Permin, 1948), probably by a stoicheiometric reaction (Astrup, 1951). Geiger (1952) demonstrated an increased proteolytic activity in mixtures of globulin preparations from man and other species after activation by streptokinase. He suggested that a complement-like factor was involved in the activation of plasminogen by streptokinase. Müllertz & Lassen (1953)

showed that large quantities of bovine plasmin were formed on the addition of a mixture of streptokinase and human globulin to bovine plasminogen, and that large amounts of an activator of plasminogen were formed by the addition of streptokinase to human globulin. The demonstration of an activator of plasminogen in fibrinolytic human blood (Müllertz, 1953) indicated that all components necessary for formation of the activator were present in blood. It was suggested (Müllertz, 1952; Müllertz & Lassen, 1953) that a proactivator–activator system was involved in the activation of plasminogen in blood. In the following scheme the interaction of the different components is shown, but the type of the reactions is not denoted:



As bovine plasminogen preparations contain large amounts of plasminogen, and little or no proactivator, bovine plasmin is not formed on addition of streptokinase. Human blood contains large amounts of proactivator and relatively small amounts of plasminogen. By addition of streptokinase large amounts of activator and small amounts of plasmin are formed.

In the present paper methods for the estimation of activator and of plasmin are described, and the formation of activator by the interaction of streptokinase and proactivator is studied. By means of the activator a complete activation of bovine plasminogen is obtained. Properties of the activator and of partially purified, completely activated and activator-free preparations of bovine and human plasmin are described and compared.

MATERIALS AMD METHODS

Human globulin. Serum was obtained from out-dated, citrated, pooled bank blood by recalcification. The euglobulin fraction was precipitated by diluting the serum twenty times with distilled water and adding 1% acetic acid until a pH value of 5·3 was obtained. The precipitate was dissolved in 0·9% NaCl (adding 0·1N-NaOH until a pH value of 7·6 was reached) or phosphate buffer (pH 7·4 or 7·6) to a volume equal to 20% of the original serum volume. The solution was stored at −20°. A single stock solution of globulin was used in the experiments on the activator, and contained 2·9 mg. nitrogen/ml. (micro-Kjeldahl). The concentration of human globulin was expressed as the volume of this stock solution (in ml.) contained in 1 ml. of the final solutions (=ml./ml.).

Streptokinase. Varidase (kindly supplied by Lederle Laboratories Division) was used. It was a lyophilized preparation containing about 3000 units/mg. A stock solution containing 10 mg./ml. distilled water was stored at −20°.

Bovine plasminogen. The preparation and properties were as described by Müllertz (1953) and Müllertz & Lassen (1953). No significant activity on casein was present or could be produced by streptokinase in solutions of this preparation.

Plasminogen activator. Human globulin (4 vol.) and streptokinase (10 mg./ml., 1 vol.) were incubated at 37° for 10 min., cooled and diluted with ice-cold phosphate buffer (pH 7·4 or 7·6). A maximal conversion of proactivator into activator was obtained. The final concentration of activator was expressed as per cent (v/v) of the undiluted solution prepared fresh each time.

Bovine plasmin. A solution of plasminogen (5 mg./ml.) containing 1% (v/v) activator was left for 4 hr. at 22° and pH 7·4 to obtain a complete activation. The solution was adjusted to pH 2·9 by addition of 0·15N-HCl, left at 22° for 30 min. and brought to pH 7·6 by addition of 0·15N-NaOH. This treatment was found to destroy activator in the presence of plasminogen with only a small loss of plasmin. The final concentration was equivalent to 2·5 mg. of the plasminogen preparation/ml. The activity of the human plasmin added with the activator solution was about 3% of the total activity. The bovine plasmin had approximately the same activity as the human plasmin and contained about half as much nitrogen per ml. (0·13 mg. N/ml., micro-Kjeldahl).

Human plasmin. Human globulin (100 ml.) was mixed with 100 ml. of 0·9% NaCl and 50 ml. of streptokinase (0·2 mg./ml.) and kept at 37° for 15 min. A maximal formation of plasmin, as estimated by the heated fibrin plate method, occurred under these conditions. The solution was adjusted to pH 1·8 with 0·15N-HCl, immersed in a boiling-water bath for 30 min., cooled and brought back to pH 7·6 and a volume of 500 ml. with 0·15N-NaOH and phosphate buffer (pH 7·6). The destruction of inhibitory substances, of streptokinase and of activator by this treatment was established in separate experiments. Denatured protein was removed by centrifuging and the supernatant was dialysed against distilled water at 5° for 48 hr. and adjusted to pH 5·3 by addition of 1% acetic acid. After centrifuging, the precipitate was dissolved in 100 ml. of 0·9% NaCl, 0·15N-NaOH being added to give a pH 7·4 or 7·6. The solution was stirred for 30 min. at 40° to achieve as complete a dissolution as possible, and undissolved protein was removed by centrifuging. The loss in activity could not be accurately determined because inhibitors were destroyed simultaneously with plasmin. The final solution contained 0·24 mg. N/ml. (micro-Kjeldahl) and was about seven times more active (per mg. N) than the original solution (heated fibrin plate method).

Buffers. Sodium diethylbarbiturate buffer (Michaelis) 0·1M, pH 7·8, was used in the preparation of the fibrin plates. Phosphate buffer (Sørensen), 0·1M, pH 7·6 (or 7·4), was used in the casein experiments. In the experiments on pH of optimum stability and optimum activity the following buffers were used: lactate buffer, 0·15M, pH 2·9; acetate buffer, 0·15M, pH 4·0–5·5; phosphate buffer (Sørensen) 0·1M, pH 6·0–7·6; borate buffer (Sørensen), 0·1M, pH 8·0–10·3. To the buffer solutions were added NaCl or distilled water so as to give an ionic strength of 0·15.

Casein. 'Hammarsten' casein (Merck) (3 g.) was dissolved in 100 ml. of distilled water by addition of 0·15N-NaOH to pH 8·0, heated at 100° for 15 min. and cooled (Kunitz, 1947). As this preparation yielded high and varying blank values (at 275 mμ., after deproteinization), the casein was precipitated with perchloric acid. The amount

of low-molecular compounds adhering to the precipitate was reduced by precipitating at the acid side of the isoelectric point and by repeated washing. A boiled solution (100 ml.) of casein was acidified by the dropwise addition of 2–3 ml. of N-NCl, with constant stirring. At the onset of precipitation 4 ml. of N-HCl were added quickly, bringing the pH to 2·0–2·2. In this way a massive isoelectric precipitation of slowly soluble casein was avoided. The clear solution (100 ml.) was precipitated by the quick addition of 150 ml. of 0·17 M perchloric acid and left suspended in the acid overnight at room temp. It was washed three times with distilled water, redissolved in phosphate buffer and 0·15 N-NaOH and made up to the original volume, a pH of 7·4 or 7·6 and an ionic strength of 0·15. The solution, designated as 3% (w/v) casein, was stored in small bottles (10–20 ml.) at −20°. This solution produced blank values below 0·100 (at 275 mμ., after deproteinization, at 1·5%, w/v) and was completely stable for 20 min. at 37°.

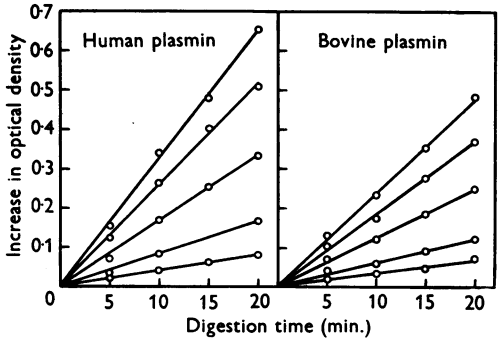


Fig. 1. Relationship between increase in optical density and digestion time for mixtures of equal volumes of casein (3·0%, w/v) and plasmin (100, 75, 50, 25, and 12·5%, v/v) (37°, pH 7·6).

Estimation of plasmin by casein digestion. Proteolytic splitting of casein was determined by the increase in optical density at 275 mμ. of the acid-deproteinized reaction mixtures (Kunitz, 1947). The absorption in the range 270–280 mμ. was found to be considerable for trichloroacetic acid, but insignificant for perchloric acid, which was preferred. A maximal precipitation of protein in 1·5% (w/v) casein solutions was obtained at final concentrations above 0·25 M perchloric acid. A final concentration of M perchloric acid was used and yielded reproducible and low readings with blank mixtures of casein and human and bovine plasmin.

The casein solution (3%, w/v) and the enzyme solution were placed in a water bath (37°) until temperature equilibrium was reached (5 min.) and equal volumes were then mixed in a test tube. Samples of 2 ml. were removed immediately after mixing and after 5, 10, 15 and 20 min. at 37°, and pipetted into test tubes containing 3 ml. of 1·7 M perchloric acid. After 1 hr. at room temp. the precipitates were removed by centrifuging. The optical densities of the supernatants were read against a water blank and the increase in optical density was calculated, 2 cm. cuvettes being used. Deproteinized solutions of casein digested by bovine plasmin showed an absorption maximum at 275 mμ. and all readings were done at this wavelength. A Hilger Uvispek spectrophotometer was used.

With human and bovine plasmin proportionality between increase in optical density E and digestion time t at a constant enzyme concentration was observed (Fig. 1). The velocity constant k, calculated as the increase in optical density per minute of digestion time, denotes the slope of the curves. Proportionality was also found between values of k and enzyme concentration (Fig. 2), indicating that k could be used as a correct expression of the enzyme concentration. Under the applied conditions the splitting of casein followed a zero-order reaction, indicating that the casein substrate was present in excess.

The standard deviation on k was found to be 1·9% (mean value, 0·0176; n, number of determinations, 14). In some experiments the increase in optical density per 20 min., $E_0 - E_{20}$, was determined by removing samples at 0 and 20 min. only. The standard deviation on $E_0 - E_{20}$ at

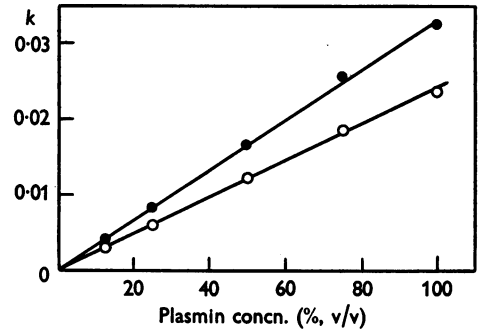


Fig. 2. Relationship between enzyme concentration and activity; k = increase in optical density/min. digestion time. ●, Human plasmin; ○, bovine plasmin (37°, pH 7·6).

three different enzyme concentrations was 2·7% (mean, 0·244), 4·7% (mean, 0·171) and 9·0% (mean, 0·078) ($n = 14$).

Estimation of plasmin by fibrinolysis. The heated fibrin plate method described by Lassen (1952) was used. The estimations were performed as described by Astrup & Müllertz (1952). The standard deviation on an estimation of human plasmin was determined to 5%.

Estimation of activator on a bovine-fibrin substrate containing plasminogen. Astrup & Permin (1947) originally demonstrated that purified bovine fibrinogen contains large amounts of plasminogen, and this observation has repeatedly been confirmed (Lewis & Ferguson, 1950; Lassen, 1952; Müllertz & Lassen, 1953; Sherry, 1954). 'Armour' bovine fraction I and bovine fibrinogen prepared as described by Ware, Guest & Seegers (1947) or Jaques (1943) all contain large quantities of plasminogen (Astrup, personal communication). Human globulin, activated by streptokinase, contains large amounts of activator and relatively small amounts of plasmin, and the activator was estimated from the lysis produced by activation of the plasminogen contained in the bovine-fibrin substrate of the standard fibrin plate method (Astrup & Müllertz, 1952). The standard deviation on a single estimation was 5%. The plasmin present in the highly diluted samples used for the

assay of the activator by this method produced no lysis of a plasminogen-free fibrin substrate (the heated fibrin plate method, Lassen, 1952). The susceptibility of unheated and heated fibrin to plasmin was determined on six different samples of activator-free human and bovine plasmin. Unheated fibrin was two to four times more susceptible to plasmin than heated fibrin, but this difference must be considered maximal, as even minute amounts of activator in the plasmin samples will cause increased lysis in the unheated fibrin substrate. Consequently, the lysis of diluted activator solutions in clotted bovine fibrinogen is caused by activation of plasminogen in the substrate, and the effect of the plasmin present in the samples is insignificant.

Estimation of activator by means of casein containing added bovine plasminogen. Activator was determined from its effect on an excess of bovine plasminogen in the presence of an excess of casein, as follows. In one test tube 1·00 ml. of a solution of bovine plasminogen (5 mg./ml.), 2·50 ml. of a solution of casein (3%, w/v), and 0·50 ml. of phosphate buffer (pH 7·6) were mixed, and 1·00 ml. of a solution of activator was added to another test tube. The tubes were incubated until temperature equilibrium was reached (5 min.) and the contents were mixed by pouring from one tube to the other several times. Samples of 2·00 ml. were removed immediately after mixing and after 20 min. at 37° and pipetted into test tubes containing 3·00 ml. of 1·7 M perchloric acid. After centrifuging, the optical density at 275 mμ. of the supernatants was determined and the increase per 20 min. ($E_0 - E_{20}$) was calculated.

A reference curve for activator concentration was obtained as follows: Solutions were prepared containing human globulin (0·40 ml./ml.) and 8·0. 1·6, and 0·8 mg. of streptokinase/ml. respectively. At 8·0 and 0·8 mg. of streptokinase/ml. a complete conversion of proactivator or of streptokinase respectively was obtained, and a concentration of streptokinase of 1·6 mg./ml. was found to be equivalent to the globulin concentration used. Appropriate dilutions of these solutions were prepared and the activator activity was determined as described. By plotting $E_0 - E_{20}$ against activator concentration, curves of identical shape were obtained with all preparations (Fig. 3). By means of this curve the activator content of a given solution can be expressed as a per cent of a standard solution or in arbitrary activator units. Controls of (1) casein + buffer, (2) casein + plasminogen, (3) casein + streptokinase and (4) casein + plasminogen + streptokinase showed no or negligible increases in optical density per 20 min. at 37°. The activity of the human plasmin added with the solutions was determined in a control of (5) casein + activator, and amounted to 1–2% of the total activity observed in the mixture of casein, plasminogen and activator. The accuracy of the method was determined in a series of experiments. In the experiments described in section (ii) single batches of casein and plasminogen, stored in solution at −20°, were used, and the standard deviation on a single estimation of activator was found to be 2·0% ($n = 10$) and 1·6% ($n = 10$). In the remaining experiments two lyophilized preparations of plasminogen and two deep-frozen batches of casein were used, and the standard deviation was found to be 5·6% ($n = 16$) and 4·9% ($n = 10$).

Method used to study the formation of activator. The components were allowed to react in relatively concentrated solutions, and the amount of activator formed was determined in highly diluted samples withdrawn from the activation mixtures. In the experiments described in section (ii), 25 μl. samples were removed and added to the assay mixture of casein and plasminogen, a final dilution of 1:200 being obtained. In the remaining experiments, the reaction mixtures were still more concentrated, and 25 μl. samples were removed and mixed with 3·00 ml. of phosphate buffer (37°), from which 1·00 ml. was immediately transferred to the assay mixture, a final dilution of 1:605 being obtained. A necessary precaution for a study of the formation of activator under varying conditions was that the reaction was not significantly influenced by the assay procedure. Information on this point was supplied by an estimation of the maximum amount of activator that might form during the assay. Solutions of globulin and of streptokinase were diluted separately, and added at the same time to a mixture of casein and plasminogen in concentrations equal to those used for the determinations described above. The amount of activator formed during the assay was expressed as a per cent of the amount which was formed by the same quantities of streptokinase and globulin when the reaction was allowed to run to completion in concentrated solutions (2 hr., 22°) before addition to the assay mixture. For concentrated solutions of human globulin (0·40 ml./ml.) and streptokinase (8 mg./ml.), and of human globulin (0·15 ml./ml.) and streptokinase (0·60 mg./ml.), 4 and 2% respectively were formed, and even smaller values were observed for other solutions. Thus the rate of reaction in diluted solutions was so slow that no significant change was likely to take place during

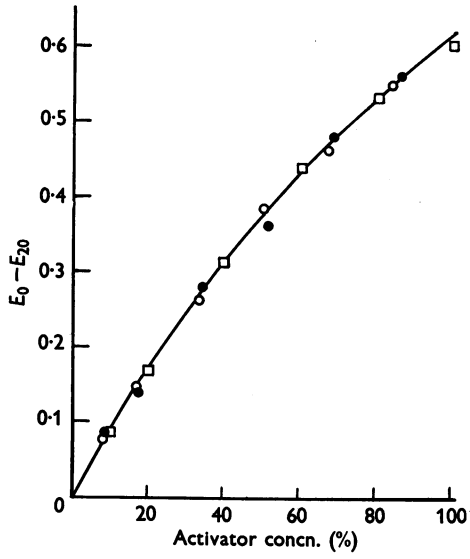


Fig. 3. A reference curve for activator concentration. Solutions containing: human globulin (0·40 ml./ml.); □, 8·0 mg.; ○, 1·6 mg.; ●, 0·8 mg. of streptokinase/ml.; were left at 22° for 4 hr. to complete the reactions and diluted in an ice bath to obtain stock solutions of equal activator activities. Abscissa: concentration of activator in per cent of the stock solutions. Ordinate: increase in optical density at 275 mμ. (20 min., 37°, pH 7·6, casein method).

the assay. Further, the standard reference curve should be a correct expression of the relation between $E_0 - E_{20}$ and activator concentration.

RESULTS

Formation and properties of the activator of plasminogen in human blood

(i) *Formation and deterioration of activator at different concentrations of streptokinase.* By presupposing an enzymic effect of streptokinase on the proactivator, it should be possible to obtain a maximum formation of activator at low streptokinase concentrations, by increasing the reaction time at conditions under which the different components were stable. Preliminary experiments showed that the deterioration of activator was high at 37°, moderate at 22° and slow at 5°, and

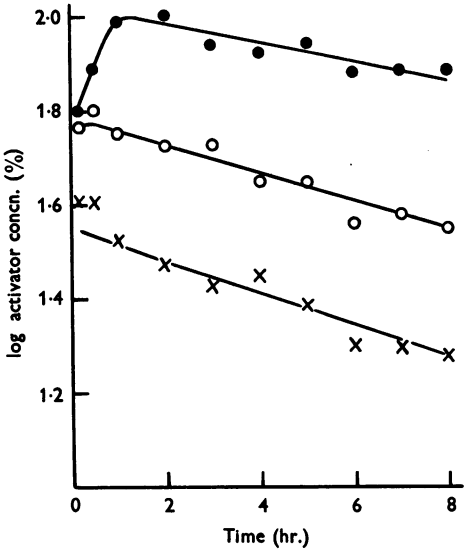


Fig. 4. The formation and deterioration of activator with time (pH 7·6, 22°) in solutions containing: human globulin (0·40 ml./ml.); ●, 8·0 mg.; ○, 1·6 mg.; ×, 0·8 mg. of streptokinase/ml. The activator concentrations were calculated as a per cent of the maximum concentration.

proactivator and streptokinase, kept separately, did not deteriorate significantly in 6 hr. at 22°. The formation and deterioration of activator at 22° were followed in solutions of human globulin containing an excess and suboptimum amounts of streptokinase (Fig. 4). After an initial increase, a slow decrease in activator concentration was observed in all instances. At the two lower streptokinase concentrations only a partial conversion of proactivator occurred. The logarithms of the activator concentration decreased proportionately to the reaction time, indicating that the loss of the activator was due to a simple protein denaturation, proceeding as a first-order reaction. The results contradicted a catalytic effect of streptokinase if activatable proactivator remained in the solutions containing suboptimum amounts of streptokinase. This assumption was verified by the increase in activator concentration produced by addition of more streptokinase to a solution of human globulin containing a small amount of streptokinase (Fig. 5). The results indicated a stoicheiometric reaction between streptokinase and proactivator.

The possibility remained that streptokinase might be bound progressively by antistreptokinase present in the globulin, whereby a catalytic process might appear as a stoicheiometric reaction. Experiments showed, however, that identical amounts of activator were formed at a suboptimum concentration of streptokinase (0·8 mg./ml.) in solutions containing 0·40 and 0·80 ml. of human globulin per ml., and thus significant amounts of antistreptokinase were probably not present in the globulin solution.

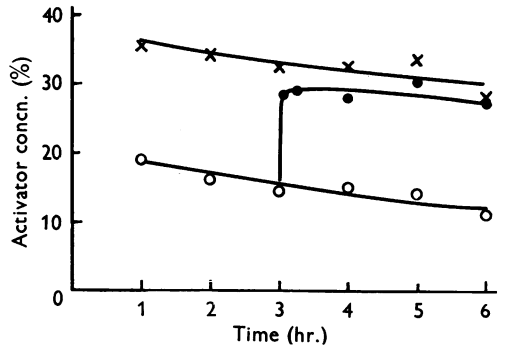


Fig. 5. Increase in activator concentration on the addition of streptokinase to a solution of human globulin containing a suboptimum amount of streptokinase. The solution contained human globulin (0·40 ml./ml.) and the following concentrations of streptokinase: ×, 1·6 mg./ml. for 6 hr.; ○, 0·8 mg./ml. for 6 hr.; ●, 0·8 mg./ml. for the first 3 hr. and 1·6 mg./ml. for the last 3 hr. (22°, pH 7·6, casein method).

(ii) *Mode of reaction between streptokinase and proactivator.* A study was made of the amount of activator formed at different proportions of streptokinase and proactivator. Two experiments were performed. In the first the globulin concentration was kept constant and the streptokinase concentration was varied; in the second the opposite was done. The amount of activator formed was expressed in arbitrary activator units, designating the maximum amount formed as 100 units. In the presence of an excess of one component, the

amount of activator formed was found to be proportionate to the concentration of the other component. It was therefore possible to express the concentration of streptokinase and globulin in activator units calculated from the number of units formed by one component in the presence of an excess of the other component. In the first experiment (streptokinase varied, globulin kept constant) a concentration of 0·60 mg. (98 units) of streptokinase/ml. was found to be equivalent to the concentration of the globulin used in all solutions (0·15 ml./ml. = 98 units/ml.). In the second experiment this streptokinase concentration was used in all solutions and the globulin concentration was varied above and below the calculated equivalent concentration. By plotting the amount of activator formed against the concentration of the varied component, the results of the two experiments conformed to a common curve (Fig. 6). In two

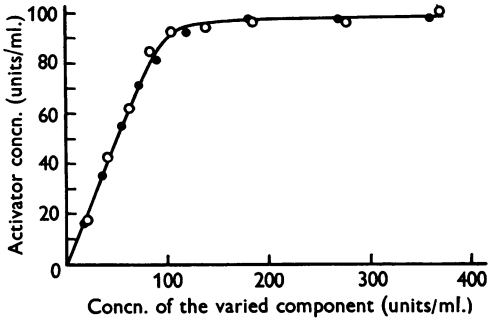


Fig. 6. Relation between the concentration of activator and of streptokinase in solutions containing a concentration of proactivator of 98 units/ml. (=0·15 ml. of human globulin/ml.), ●; and between the concentration of activator and of proactivator in solutions containing a concentration of streptokinase of 98 units/ml. (=0·60 mg./ml.), ○. The solutions were left for 2 hr. at 22° and pH 7·6 to complete the reaction before the determination of activator was performed by the casein method (pH 7·6, 37°).

similar experiments, with a different stock solution of streptokinase and about twice as concentrated solutions, 0·27 ml. of human globulin/ml. was found to be equivalent to 1·35 mg. of streptokinase/ml., and a curve of a similar shape was obtained. The experiments indicated a reaction proceeding to equilibrium (see Discussion).

On the assumption that two components, streptokinase and proactivator, combine in a reversible reaction to form a single component, activator, the concentration of activator in any mixture in equilibrium should be reduced by dilution. If two components, streptokinase and proactivator, react to form two components, activator and an inactive component, the equilibrium should not be influenced by dilution. The limit of accuracy of the estimations did not permit a consideration of more complicated reactions. The effect of dilution was studied in two different ways. In the first part of the experiment streptokinase was added to proactivator in equivalent concentrations and the reaction was allowed to proceed to equilibrium in a concentrated solution and in a solution diluted 1:10. In the second part of the experiment the concentrated solution containing the components in equilibrium was diluted 1:10 and kept for sufficient time to allow any changes in the equilibrium to take place. The activator contents of the concentrated and the two diluted solutions were then determined. The reduction in concentration of the reacting components due to the dilution was corrected for by performing the determinations on the diluted solutions by means of samples ten times larger than those from the concentrated solution. Identical final concentrations of the reacting components in the assay mixture were thereby obtained, and identical amounts of activator should be found in all instances, if the equilibrium had not been affected (Table 1). The equilibrium was not influenced by

Table 1. *Effect of dilution on the activator content of solutions containing equivalent amounts of streptokinase and proactivator*

A solution (*A*) containing human globulin (0·10 ml./ml.) and streptokinase (0·40 mg./ml.) was prepared. Samples were withdrawn and diluted 1:10 at once (*B*) and after 145 min. (*C*). After 265 min. the amounts of activator in samples of 25 μl. from *A* and of 250 μl. from *B* and *C* were determined and expressed as a per cent of the amount present in *A* (mean = 100%). Incubation: 22°, pH 7·6; determinations: casein method.

	A	*B*	*C*
Mean ± S.E.	100·0 ± 0·51	97·9 ± 0·49	100·4 ± 0·63
No. of results	(10)	(10)	(10)
Range	98·0–102·3	94·4–99·8	99·3–105·2

diluting a solution containing the components in equilibrium, but slightly smaller amounts of activator were formed in a diluted than in a concentrated solution. In control experiments the concentration of activator was determined at short intervals of time in the concentrated and the two diluted solutions. A somewhat lower rate of reaction between streptokinase and proactivator in the diluted solution was observed, but the difference was too small to effect a significantly greater loss of activator in the concentrated than the diluted solution during the experiment. No significantly different stability was observed.

(iii) *Stability of the plasminogen activator.* A solution containing human globulin (0·40 ml./ml.) and streptokinase (8 mg./ml.) was left at 22° for 3 hr., to obtain a complete conversion of pro-activator into activator, and then diluted 1:40. Samples were adjusted to different pH values, in an ice bath, with 0·15N-NaOH or 0·15N-HCl and 1/16 volume of an appropriate buffer, diluted to equal volumes, heated at 22°, 37° and 50° for 30 min., cooled, and brought back to pH 7·6 by addition of 0·15N-NaOH or 0·15N-HCl and phosphate buffer and readjusted to equal volumes. The residual amount of activator was estimated by the casein method. The results (Fig. 7) showed the activator to be very labile below pH 5 or above pH 9–10 with an optimum stability between pH 6·5 and 8·0. Identical results were obtained with a different preparation of activator (casein method) and with concentrated and diluted solutions of another activator preparation (standard fibrin plate method). Activator was also found to be destroyed at pH 2·9 in the presence of its substrate, plasminogen. Solutions of bovine plasminogen and activator were mixed and the pH was immediately brought to pH 2·9. After neutralization, only insignificant amounts of plasmin were formed in this mixture, as compared with control solutions (Table 2). The stability of the activator differed considerably from that of human plasminogen (Christensen & Smith, 1950; Kline, 1953), streptokinase (Christensen, 1947*b*) and human plasmin (see below), especially at acid reactions. This difference in stability made it possible to prepare human plasmin free from activator, and shows that the effect on bovine plasminogen of solutions of human globulin and streptokinase is caused by a substance different from previously known components of the proteolytic-enzyme system of blood.

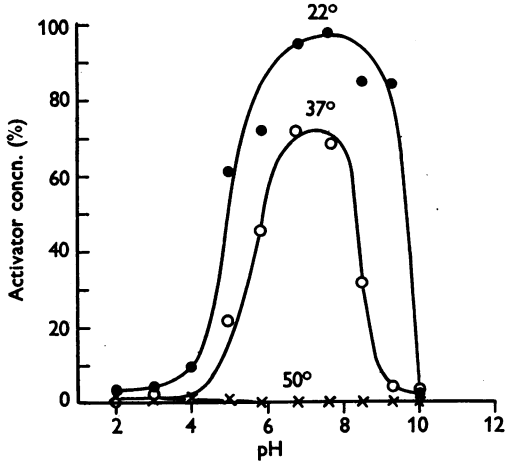


Fig. 7. Residual concentration of activator in solutions heated at different pH values and temperatures for 30 min. The concentration of activator is expressed as per cent of that of an untreated sample kept at pH 7·6 and 0° during the experiment (casein method). The values were corrected for the effect of the plasmin contained in the samples, as determined on casein without added plasminogen.

(iv) *Activation of bovine plasminogen by the activator.* Experiments were performed to determine the conditions under which a complete activation of bovine plasminogen by the activator occurred. The smallest amount of activator that produced maximum activation of plasminogen in 1 hr. at 22° was established and the formation of plasmin at this activator concentration, pH 7·4 and 22°, was followed. In the incompletely activated samples removed within the first 60 min. more plasmin was formed during the assay, resulting in concave digestion curves, and the plasmin activity, k, was determined by means of tangents drawn to the initial part of these curves. No further activation of plasminogen during the assay occurred in the samples removed subsequently. The activation curve (Fig. 8) indicated a complete initial activation, followed by a slow deterioration of the plasmin formed. Addition of an excess of activator to the mixture after activation for 4 hr. did not increase the activity significantly.

Table 2. *Deterioration of activator at acid reaction in the presence of plasminogen*

Plasminogen: bovine, 10 mg./ml.; activator: human, 2% (v/v); buffer: phosphate buffer, pH 7·4; the activation mixtures contained 0·25 ml. of each component. Each mixture was finally diluted to 2·5 ml., added to an equal volume of casein and the plasmin activity ($E_0 - E_{20}$) was determined.

Activation mixture	Procedure (1)	$E_0 - E_{20}$	Procedure (2)	$E_0 - E_{20}$
1. Plasminogen + activator	Heated at pH 2·9, 22° for 30 min.; adjusted to pH 7·4 and added to casein	0·008	Heated at pH 2·9, 22° for 30 min.; adjusted to pH 7·4; kept at 22° for 30 min. and added to casein	0·033
2. Plasminogen + buffer		0·002		0·000
3. Activator + buffer		0·005		0·010
4. Plasminogen + activator	Kept at pH 7·4, 22° for 30 min. and added to casein	0·349	Kept at pH 7·4, 22° for 60 min. and added to casein	0·385

The formation of plasmin at different activator concentrations was studied in an experiment at 22° (4 hr.) and at 5° (48 hr.). In all instances a progressive activation of plasminogen at small activator concentrations and a slow deterioration of plasmin resulted in approximately identical final plasmin activities. The results suggested that the activation of plasminogen by activator was a catalytic reaction, and the preliminary results (Müllertz & Lassen, 1953), which indicated a stoicheiometric reaction, were probably caused by the lability of plasmin under the applied conditions (37°). However, the deterioration of plasmin and the continuous formation of plasmin during the assay make it difficult to obtain conclusive evidence as to the mode of reaction by these methods.

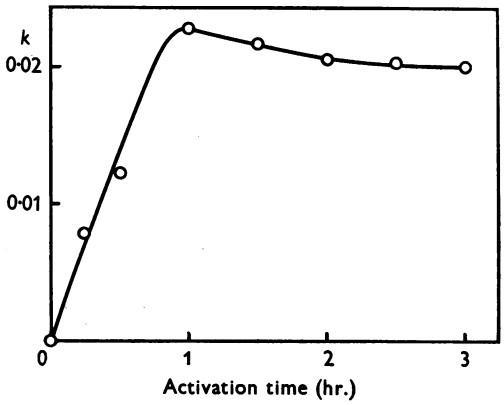


Fig. 8. Formation and deterioration of plasmin in a solution of bovine plasminogen (5 mg./ml.) containing 1% (v/v) activator (22°, pH 7·4). Samples were removed and diluted 1:5 with phosphate buffer and the plasmin activity was determined by the casein method (37°, pH 7·6). Control: activator + casein: 0·0001.

Properties of human and bovine plasmin

(i) pH *of optimum activity.* Samples of a 6% (w/v) casein solution were adjusted to different pH values with 0·15N-HCl and 0·15N-NaOH and diluted with appropriate buffer solutions to a final concentration of 3% (w/v). Casein and plasmin solutions were left at 37° until temperature equilibrium was reached (5 min.), and then equal volumes were mixed. The pH was controlled before and after the experiment by a glass electrode. Direct relationships between digestion time and increase in optical density were observed in all cases, indicating that no significant deterioration of plasmin occurred during the experiment. The pH activity curves for human and bovine plasmin were almost identical (Fig. 9), with a maximum of activity between pH 7·5 and 8·3 and a rapid decrease in activity above and below this range. Similar results were obtained with a bovine plasmin activated by chloroform as estimated on gelatin (Kaplan, Tagnon, Davidson & Taylor, 1942) and with human plasmin activated by streptokinase or chloroform as estimated on casein (Ratnoff, 1948; Remmert & Cohen, 1949) or gelatin (Christensen & MacLeod, 1945).

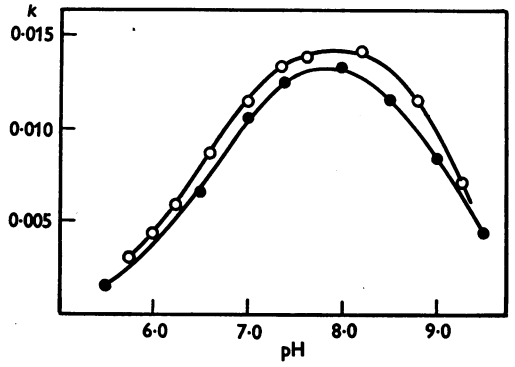


Fig. 9. pH of optimum activity of: ○, bovine plasmin; ●, human plasmin (casein method, 37°).

(ii) *Stability.* Samples of human and bovine plasmin were placed in an ice bath and adjusted to different pH values with 0·15N-HCl and 0·15N-NaOH and 0·1 vol. of an appropriate buffer. The samples were heated at different temperatures for 30 min., cooled, brought back to pH 7·6 and diluted with phosphate buffer (pH 7·6) to twice the original volume. The pH was controlled before and after heating by a glass electrode. The residual activity was determined by the casein and the heated fibrin plate method. The results of the casein experiments (Figs. 10, 11) were confirmed by the fibrin plate experiments. The high stability of human plasmin at pH 1·8 and 100° was established in experiments with three different plasmin preparations and three crude samples of globulin activated by streptokinase, with both casein and heated fibrin as substrates. The high stability of bovine plasmin at pH 3 was confirmed in experiments with the same plasmin, and a plasmin prepared from another batch of plasminogen (casein method). At pH 6·0–8·0 human plasmin was fairly stable, but bovine plasmin was very labile. Human and bovine plasmin were completely stable for 20 min. at 37° in the presence of casein (Fig. 1).

(iii) *Effect of human plasmin on bovine and human plasminogen.* The formation of plasmin in serum or globulin solutions during storage or after chloroform treatment has been ascribed to an autocatalytic reaction (Christensen, 1947*a*; Rocha e Silva & Rimington, 1948; Kocholaty, Ellis & Jensen, 1952) and the effect of streptokinase and human globulin

on bovine plasminogen might be explained by a catalytic effect of human plasmin on bovine plasminogen. The plasminogen activator, present in high concentrations in the mixture of streptokinase and human globulin, was destroyed by the treatment at pH 1·8 and 100° for 30 min. used in the preparation of human plasmin. This plasmin produced no activation of bovine plasminogen, but human plasminogen activator activated the plasminogen in the presence as well as in the absence of human plasmin (Table 3). Similar results were obtained with human plasmin and plasminogen. No activation of bovine plasminogen was produced by a bovine plasmin activated spontaneously (Astrup, 1951) or by tissue activator (Astrup & Sterndorff, 1952).

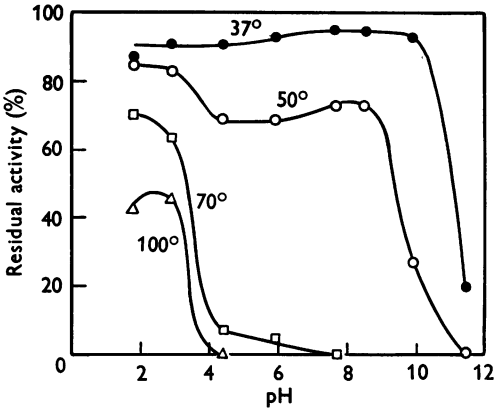


Fig. 10. Stability of human plasmin heated at different temp. and pH for 30 min. The residual activity (*k*, casein, 37°, pH 7·6) was expressed as a per cent of the plasmin content of a control kept at 0° and pH 7·6 during the experiment.

DISCUSSION

The experiments indicate that streptokinase and a component in human blood react to form an activator of plasminogen by a reaction proceeding to an equilibrium. The following two possible reactions will be considered:

(1) streptokinase + proactivator ⇌ activator,

(2) streptokinase + proactivator ⇌ activator + inactive component.

At the equilibria we have:

$$\frac{[A]}{([SK]-[A])\,([PA]-[A])} = K_1 \qquad (1)$$

and

$$\frac{[A]\,[B]}{([SK]-[A])\,([PA]-[A])} = K_2, \qquad (2)$$

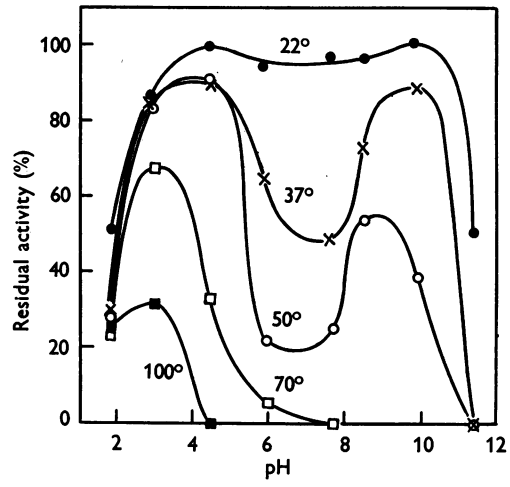


Fig. 11. Stability of bovine plasmin (see Fig. 10).

where [SK] and [PA] represent the concentrations of streptokinase and proactivator added, [A] the activator concentration, [SK]−[A] and [PA]−[A] the free concentrations of streptokinase and proactivator respectively in the solution at equilibrium, and [B] the concentration of the inactive component. In the experiments considered here [B]=[A]. All concentrations are calculated in activator units/ml. In Fig. 4 the relation between [A] and [SK] (for [PA]=98) and between [A] and [PA] (for [SK]=98) was recorded. The points

Table 3. *Effect of human plasmin on bovine plasminogen*

Human plasmin: diluted 1:2 (Expt. 1) and 1:10 (Expt. 2), 1·0 ml.; bovine plasminogen: 2·5 mg./ml., 1·0 ml.; activator: human activator, 1% (v/v) 0·5 ml. All mixtures were diluted to 2·5 ml. with phosphate buffer (pH 7·4), kept at 22° for 60 min. and the plasmin activity ($E_0 - E_{20}$) was determined (casein). Controls of (1) bovine plasminogen and (2) activator yielded activities below 0·002.

Activation mixtures	$E_0 - E_{20}$ Expt. 1	$E_0 - E_{20}$ Expt. 2
1. Human plasmin	0·128	0·032
2. Human plasmin + bovine plasminogen	0·103	0·015
3. Human plasmin + bovine plasminogen + activator	0·429	0·382
4. Human plasmin + activator	0·106	0·029
5. Bovine plasminogen + activator	0·350	0·350

represent the experimentally determined values, and the curve is calculated from eqn. 1 for $K_1 = 1$. The curve is almost coincident with another curve calculated from eqn. 2 for $K_2 = 87$, and the accuracy of the determinations did not allow further conclusions about the mode of reaction. For equivalent concentrations of streptokinase and proactivator we have [SK] = [PA] = [C] and according to (1):

$$\frac{[A]}{([C]-[A])\,([C]-[A])} = K_1,$$

or

$$[C] = [A] + \sqrt{\left(\frac{[A]}{K_1}\right)}.$$

By inserting the highest value of K_1 that will fit the experimental data of Fig. 4 ($K_1 = 2$) the minimum theoretical effect of dilution on the equilibrium (1) could be calculated. Tenfold dilution should reduce the concentration of activator to 8·2 % ($K_1 = 1$) or 8·6 % ($K_1 = 2$) of the concentration in the undiluted solution. The activator concentration of a solution in equilibrium was not reduced below the 10 % that would be expected if equilibrium (2) were valid. However, a slight reduction to 9·8 % was observed if streptokinase and globulin were mixed and allowed to react completely in undiluted and diluted solution. The latter observation cannot be explained from the evidence available here. Nevertheless the results do not agree with a reaction such as (1), but suggest that equilibrium (2) is valid.

Previous studies have led to different concepts of the mechanism of formation of human plasmin by streptokinase. Christensen & MacLeod (1945) found that the transformation of human plasminogen into plasmin was a first-order reaction whose rate was proportional to the concentration of streptokinase within certain limits. They concluded that the reaction was catalysed by streptokinase. Remmert & Cohen (1949) confirmed this observation. Ratnoff (1948) observed a catalytic effect of streptokinase on a heated human plasminogen, and streptokinase appeared to react stoicheiometrically with untreated plasminogen. Wasserman (1952) found that the reaction between streptokinase and human plasminogen was stoicheiometric, when assayed by means of a substrate of clotted bovine fibrinogen. However, his results obtained with this plasminogen-containing substrate are probably pertinent to the formation of activator by the interaction of streptokinase and proactivator, whereas the formation of plasmin was followed by Christensen & MacLeod by means of gelatin, and by Remmert & Cohen and Ratnoff by means of casein. In the present paper, streptokinase and proactivator were found to interact stoicheiometrically and it was found most probable that the activator catalysed the transformation of plasminogen into plasmin. If these concepts are correct, the formation of plasmin on the addition of streptokinase to preparations containing both proactivator and plasminogen will appear as a catalysed reaction.

The presence of large amounts of plasminogen-activating substances in different fibrinolytic preparations from human blood has not been previously realized, and may explain a number of discrepancies in previous studies. The high lytic activity produced in a bovine-fibrin substrate by human globulin, activated by streptokinase, is due primarily to activation of the plasminogen in the substrate (cf. Müllertz & Lassen, 1953; Sherry, 1954). Previous studies on the interaction of streptokinase, human plasminogen and plasmin, performed by means of a bovine-fibrin substrate, are therefore primarily pertinent to the proactivator–activator system and only to a very slight degree to plasminogen and plasmin. This also applies to fibrinolytic methods generally used for assay (Astrup & Müllertz, 1952; Christensen, 1949; Fletcher, 1954). Discrepancies observed in studies on fibrinolysins occurring in blood in living and dead organisms (for references see Bidwell, 1953) are probably also caused by activation of plasminogen in the fibrin substrates by an activator in the lytic samples (Müllertz, 1953).

Reports on the stability of plasmin have not been concordant. In the present paper completely activated preparations of human and bovine plasmin free from inhibitory and activating components have been made. Changes in activity of incompletely activated enzyme preparations which contain activating substances and inhibitors may depend on destruction or enhancement of these factors and cannot be evaluated with certainty. Loss of activity during the preparation has therefore been considered of minor importance. In the present work, human and bovine plasmin showed a remarkable high stability at acid reaction. Sherry (1954) also noted a high stability of human plasmin at pH 2. Christensen & MacLeod (1945) found a maximum stability of human plasmin at pH 7·0–7·4, with a rapid loss above and below these values in the pH range 3·5–11. An incomplete activation or loss of plasmin by adsorption on protein denatured by heating at acid reaction and removed before the assay by viscosimetry on gelatin may be considered as an explanation of this discrepancy. At neutral reaction human plasmin was found to be rather labile, the stability being increased considerably by heating and chloroform treatment (Ratnoff, 1948) or by addition of casein or esterified basic amino acids (Kline, 1954). It was also found by Christensen (1945) to be fairly stable. Bovine plasmin was found to be very labile at neutral reaction and the stability curve in the pH range 5·0–9·5 was approximately the inverse of

the pH/activity curve. Bovine plasmin was stabilized in the presence of casein. Lewis & Ferguson (1951) found a similar stability of a dog-blood lysin in the pH range 5·0–9·7. The results indicate that both human and bovine plasmin are autodigestive. Thus the rate of autodigestion should be high in purified preparations, whereas a competitive inhibition occurs in crude preparations in the presence of added protein or after heat or chloroform treatment, which make contaminating serum globulins more susceptible to plasmin.

SUMMARY

1. An activator of plasminogen is formed by the interaction of streptokinase and a component, a proactivator, in human blood.

2. At suboptimum concentrations of streptokinase, only a partial conversion of proactivator into activator was obtained, followed by a slow deterioration of the activator formed. The amount of activator formed in solutions containing varying proportions of streptokinase and of proactivator indicated a stoicheiometric reaction proceeding to an equilibrium. Experiments on the effect of dilution suggested that streptokinase and proactivator interact to form two components, an activator and an inactive component.

3. The activator was very labile at acid and alkaline reactions, with an optimum of stability between pH 6·5 and 8·0, differing in this respect from other known components of the proteolytic–enzyme system in blood.

4. A complete conversion of bovine plasminogen into plasmin was accomplished by addition of the activator. The preparations of human and bovine plasmin were purified by a treatment at acid reaction, which produced a destruction of streptokinase, activator and inhibitory substances.

5. Human plasmin did not catalyse the conversion of human and bovine plasminogen into plasmin. Human and bovine plasmin showed nearly identical relationships between pH and activity on casein, with an optimum between pH 7·5 and 8·3. They showed a remarkably high stability at acid reaction. At neutral reaction human plasmin was fairly stable, while bovine plasmin was rather labile. Plasmin was stabilized by casein.

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