magnesium at 0° which occurs in the salt-sucrose mixture. The most effective concentration was 1.5 mM. Incubation of mitochondria at 24° in 0.1 M-potassium chloride, 0.05 M-sucrose and 1.5 mM-manganese chloride after they had lost endogenous magnesium at 0°, resulted in an uptake of most of the magnesium lost.

9. The loss of mitochondrial phosphate that occurred in 0.1 M-potassium chloride plus 0.05 M-sucrose at 0° was abolished by the addition of 1.5 mM-manganese chloride to the medium. Incubation at 24°, in the manganese-containing medium, of mitochondria which had already lost phosphate resulted in a restoration of the phosphate content.

10. In sucrose or saline media at 0° or at 25° the presence of mM-manganese chloride resulted in a lower water content of mitochondria than occurred in its absence.

11. The mode of action of manganese in the stabilization of mitochondria is discussed.

We wish to thank Professor H. A. Krebs, F.R.S., for his interest in the work, Dr W. C. Werkheiser for helpful discussion and Miss B. M. Notton for technical assistance. The work was done during the tenure of a Christopher Welch Scholarship by one of us (J.E.A.), who also thanks the Ministry of Education for a personal grant. The work was aided by a grant from the Rockefeller Foundation.

REFERENCES

- Amoore, J. E. & Bartley, W. (1958). *Biochem. J.* **69**, 223.
 Amoore, J. E., Parsons, D. S. & Werkheiser, W. C. (1958). *Biochem. J.* **69**, 236.
 Bartley, W. (1953). *Biochem. J.* **54**, 677.
 Bartley, W., Notton, B. M. & Werkheiser, W. C. (1957). *Biochem. J.* **67**, 291.
 Beaufay, H., Hers, H. G., Berthet, J. & de Duve, C. (1954). *Bull. Soc. Chim. biol., Paris*, **36**, 1539.
 Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.
 Fonnesu, A. & Davies, R. E. (1956). *Biochem. J.* **64**, 769.
 Garner, R. J. (1946). *Biochem. J.* **40**, 828.
 Hanes, C. S. & Isherwood, F. A. (1949). *Nature, Lond.*, **164**, 1107.
 Lardy, H. A. & Wellman, H. (1953). *J. biol. Chem.* **201**, 357.
 Lindberg, O. & Ernster, L. (1954). *Nature, Lond.*, **173**, 1038.
 Mann, C. K. & Yoe, J. H. (1956). *Analyt. Chem.* **28**, 202.
 Maynard, L. S. & Cotzias, G. C. (1955). *J. biol. Chem.* **214**, 489.
 Orange, M. & Rhein, H. C. (1951). *J. biol. Chem.* **189**, 379.
 Sanderson, P. H. (1952). *Biochem. J.* **52**, 502.
 Siekevitz, P. & Potter, V. R. (1955). *J. biol. Chem.* **215**, 221.
 Slater, E. C. & Cleland, K. W. (1953). *Biochem. J.* **55**, 566.
 Stanbury, S. W. & Mudge, G. H. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 675.
 Tapley, D. F. (1956). *J. biol. Chem.* **222**, 325.
 Werkheiser, W. C. & Bartley, W. (1957). *Biochem. J.* **66**, 79.

The Esterase Activity of the Fibrinolytic System

By M. LASSEN

Biological Institute of the Carlsberg Foundation, and University Institute of Physical Chemistry, Copenhagen, Denmark

(Received 1 November 1957)

The solubilization of fibrin clots in mammalian blood is caused by a proteolytic enzyme termed plasmin. This enzyme is not present in significant concentrations in normal plasma, but it can be produced from its precursor, plasminogen, through the interaction of appropriate activating substances.

The most potent and commonly used activator of human plasminogen preparations is streptokinase. This substance is found in the culture filtrate from certain strains of β -haemolytic streptococci (Tillett & Garner, 1933). Investigations by Kaplan (1944) and Christensen (1945) revealed the activating properties of the streptococcal product. The conclusion that the conversion of plasminogen into plasmin is catalysed by streptokinase was reached by Christensen & McLeod (1945), and although the reaction has later proved to be more complicated (Geiger, 1952; Müllertz &

Lassen, 1953; Sherry, 1954; Asahina & Oka, 1954), it is still probable that the last step in the reaction sequence, the conversion of plasminogen into plasmin, is enzymic.

Müllertz & Lassen (1953) suggested that streptokinase is unable to activate pure human plasminogen, but that an activator of plasminogen is formed through a reaction between streptokinase and a substance, termed proactivator, which is present in large amounts in human plasma. This concept was accepted by Troll & Sherry (1955), but was later rejected by Sherry, who now presumes identity between plasminogen and proactivator and supposes the activator to be a complex of streptokinase with plasminogen or plasmin (Sherry & Alkjaersig, 1956).

It seems to be commonly accepted that the formation of activator proceeds through a stoicheiometric reversible reaction between streptokinase

and 'proactivator'. This was clearly shown in qualitative experiments by Troll & Sherry (1955), and in entirely different quantitative experiments by Müllertz (1955).

Experiments performed by Müllertz (1954) and M. Lassen (unpublished work) indicated that the mechanism of the streptokinase activation was even more complicated than was suggested by the above-mentioned investigators. However, the necessary condition of an experimental approach to these complicated problems is the availability of more accurate methods for determining the components in question. Troll, Sherry & Wachman (1954) found that streptokinase-activated human globulin resembles trypsin in being able to catalyse the hydrolysis of L-lysine esters and esters of L-arginine with a bound α -amino group. Later experiments performed by Troll & Sherry (1955) relate most of the lysine esterase activity to the activator, and the arginine esterase activity plus the remaining lysine esterase activity to plasmin. The following scheme summarizes the suggestions proposed:

Troll & Sherry found arginine esters well suited for the estimation of plasmin since, as compared with the protein substrates generally used, inhibitors had little effect on their hydrolysis. Accordingly, benzoyl-L-arginine ethyl ester was used as a plasmin substrate in the present investigations. The hydrolysis of this and of L-lysine ethyl ester was followed in the Warburg apparatus by measuring the carbon dioxide produced from bicarbonate. This procedure is highly accurate and allows estimations of the amounts of substrate split without interrupting the reaction.

MATERIALS AND METHODS

Benzoyl-L-arginine ethyl ester. (BAEe.) The hydrochloride of this compound was prepared according to the method of Bergman, Fruton & Pollok (1939) as modified by Schwert & Eisenberg (1949). The melting point was 130° (uncorrected), as found by Schwert & Eisenberg. This ester showed no spontaneous hydrolysis under the experimental conditions used here.

L-Lysine ethyl ester. (LEe.) The hydrochloride of this compound was prepared according to the method of Werbin & Palm (1951). The ester showed little or no spontaneous hydrolysis under the test conditions.

Streptokinase. Varidase was kindly placed at my disposal by the Lederle Laboratories Division, American Cyanamid Co. The units are those stated on the packages according to Christensen (1949).

Human plasminogen and proactivator. Plasma (1 vol.) was mixed with 4 vol. of acetone at 0°, and the precipitate washed with acetone and ether. The dried powder obtained

was easily soluble and no deterioration of active compounds (except of plasmin inhibitors: T. Astrup, paper in preparation) had occurred. The yield was 60–80 mg./ml. of plasma.

Bovine plasmin. Bovine plasminogen was activated with a mixture of streptokinase and human proactivator. Owing to the lability of purified bovine plasmin at neutral pH (Müllertz, 1955), a solution containing 1% of bovine fibrinogen, prepared according to Astrup & Müllertz (1952), was used as a source of plasminogen. Proactivator was supplied as human globulin prepared according to Milstone (1941) and redissolved to the original serum volume in diethylbarbiturate buffer, pH 7.8, ionic strength 0.15. The activation was performed at room temperature by adding 5 ml. of globulin solution and 10 000 units of streptokinase to 50 ml. of fibrinogen solution. A qualitative impression of the reaction's course was obtained by following the increasing fibrinolytic effect of the mixture. Samples of the mixture (0.1 ml.) were incubated for 3 min. at 37° with 2 ml. of diethylbarbiturate buffer, pH 7.8, containing 10 National Institute of Health units of thrombin. Then 1 ml. of 0.3% bovine fibrinogen was added, and lysis time was measured as the time which elapsed between this addition and the solubilization of the clot formed. After incubation of the streptokinase-globulin-fibrinogen mixture for 90–120 min. constant lysis times were obtained. The reaction was therefore stopped at 120 min. by the addition of 240 ml. of acetone, and the precipitate was dried with acetone and ether.

This preparation was easily soluble, and the plasmin was stable for 3 hr. at 25° and pH 7.34. The inevitable contamination by human plasmin constituted less than 5% of the total plasmin activity.

Manometric technique. The technique is that generally used (Parks & Plaut, 1953; Lundquist, Thorsteinsson & Buus, 1955). Acid liberated by hydrolysis of the substrate releases CO₂ from a CO₂-NaHCO₃ buffer. The CO₂ produced is measured manometrically in the Warburg apparatus. The assay was performed at 25° in 15 ml. Warburg vessels, with a liquid volume of 0.2 ml. in the side arm and 2 ml. in the flask.

Unless otherwise stated the following concentrations of esters and of the buffer constituents were used for determining the esterase activities. BAEe esterase (plasmin; the amounts are given in the legends to the figures): 25 mg. of BAEe giving a concentration of 0.033 M; 0.25 M-NaHCO₃ in the liquid phase and 50% (v/v) of CO₂ in the gas phase giving pH 7.34. Under these conditions the decrease in pH during the reaction was calculated as 0.03 when the whole manometer scale was utilized (equivalent to about 380 μ l. of CO₂). LEe esterase (activator and plasmin; the amounts are given in the legends to the figures): 20 mg. of LEe giving a concentration of 0.037 M; 0.033 M-NaHCO₃ and 0.13 M-NaCl were present in the liquid phase and 100% of CO₂ in the gas phase. This gives pH 6.16 and an ionic strength of 0.27. The decrease in pH during the reaction was calculated at 0.12 when the whole manometer scale was utilized.

Calculation for the carbon dioxide-bicarbonate buffer

Solubility of carbon dioxide. This must be known for the calculation of the flask constants. The value 0.756 l./l. atm. for the solubility in pure water at 25° (*International Critical*

Tables, 1928) was used. However, since the solubility of CO_2 varies with the concentration of bicarbonate, this value is not correct, so the error in the flask constants caused by the use of the incorrect value was calculated. It amounted to about one-fifth of the error in the solubility coefficient for CO_2 . The relative error was insignificant, and the value was therefore applicable.

Calculation of pH. The apparent first ionization constant (K_1) of carbonic acid is given by the expression

$$K_1 = \frac{(H^+)(HCO_3^-)}{(CO_2)},$$

in which the parentheses represent activities of the constituents enclosed.

At concentrations of bicarbonate higher than infinite dilution, the solubility of CO_2 is altered. However, the activity of CO_2 is proportional to the partial pressure of CO_2 , and is therefore equal to the concentration in pure water at the same pressure.

The activity coefficient of HCO_3^- ion decreases when the concentration is increased, and K_1' , as defined by

$$K_1' = \frac{(H^+)[HCO_3^-]}{(CO_2)},$$

will therefore increase with increasing concentration. (The parentheses represent activities and the brackets concentration of the constituents enclosed.) The corresponding decrease in pK_1' was calculated from determinations of pH in solutions containing different concentrations of NaHCO_3 and saturated with CO_2 at 25° . The partial pressure of CO_2 was equal to the barometric pressure corrected for the vapour pressure of water at 25° . The pH was measured potentiometrically with a glass electrode (type b from Radiometer, Copenhagen, which has no significant Na^+ ion error below pH 11–12) and a calomel reference electrode (Fig. 1). The experimental error was about ± 0.01 , which is a lower accuracy than usual in such investigations, but is satisfactory for practical work with the Warburg apparatus.

Influence of carbonate. All CO_2 – NaHCO_3 buffers contain a certain amount of carbonate due to the reaction

Most of the acid produced by hydrolysis of the substrate is consumed by the reaction

Fig. 1. Relationship between pK_1' (defined in the text) and the concentration of sodium bicarbonate. The value at infinite dilution is that found by Harned & Davis (1943).

But a certain amount of the acid escapes estimation, since it is consumed by carbonate:

In order to calculate the concentration of carbonate in a certain buffer, it is necessary to know the second ionization constant K_2' of carbonic acid, defined by

$$K_2' = \frac{(H^+)[CO_3^{2-}]}{[HCO_3^-]}.$$

(The parentheses represent activity and the brackets the concentrations of the constituents enclosed.)

The relation between pK_2' and the ionic strength (Fig. 2) was calculated from determinations of the pH in different concentrations of a Na_2CO_3 – NaHCO_3 buffer at 25° . The pH was measured as above.

The concentration of carbonate in the initial state can now be calculated from the equation

$$\frac{K_2'}{K_1'} = \frac{(H^+)[CO_3^{2-}]}{[HCO_3^-]} \bigg/ \frac{(H^+)[HCO_3^-]}{(CO_2)} = \frac{[CO_3^{2-}](CO_2)}{[HCO_3^-]^2}.$$

In the final state the increase in (CO_2) can be calculated from the increase in the pressure measured in the Warburg apparatus. Exact calculations of the decrease in the concentration of bicarbonate are laborious, but tests have shown that below pH 8 the amount of bicarbonate arising from the decrease in carbonate may be neglected and the change in bicarbonate concentration may be calculated from the output of CO_2 alone.

Values corrected as described were used in the experiments on the relation between pH and BAEe esterase activity. The corrections were unnecessary in the other experiments, where comparisons have been made only between results obtained at the same pH.

RESULTS AND DISCUSSION

Benzoyl-L-arginine ethyl ester esterase (plasmin)

With the concentration of BAEe used (0.033M) the amount of substrate split is proportional to time and to the concentration of bovine plasmin

Fig. 2. Relationship between pK_2' (defined in the text) and the ionic strength. The value at infinite dilution is taken from *Handbook of Chemistry and Physics* (1948).

(Fig. 3). Similar results were obtained with human plasmin and with trypsin.

The relationship between reaction rate and substrate concentration is shown in Fig. 4, the maximum rate being reached at about $0.015M$ -substrate concentration when 0.5 mg. of bovine plasmin was the enzyme source.

The reaction rate does not alter much between pH 7 and 8 (Fig. 5), but optimum activity seems to be at about pH 7.9 for both human and bovine plasmin. Troll *et al.* (1954) found the optimum at pH 9 for human plasmin with the same substrate. The difference may be due to the 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer used in their experiments.

Fig. 3. Hydrolysis of BAEe with bovine plasmin. Temperature 25° ; 50% CO_2 in the gas phase, giving pH 7.34, was used. In the main compartment of the flasks: 2.5 (A), 5 (B), 10 (C) or 20 (D) mg. of bovine plasmin dissolved in 2 ml. of $0.25M$ - $NaHCO_3$. In the side arms: 25 mg. of BAEe dissolved in 0.2 ml. of $0.25M$ - $NaHCO_3$.

Fig. 4. Relationship between rate of hydrolysis and concentration of BAEe. Temperature 25° ; 25% CO_2 in the gas phase, giving pH 7.64, was used. In the main compartment of the flasks: 30, 24, 18, 12 or 6 mg. of BAEe dissolved in 2 ml. of $0.25M$ - $NaHCO_3$. In the side arms: 5 mg. of bovine plasmin dissolved in 0.2 ml. of $0.25M$ - $NaHCO_3$.

Fig. 5. Effect of pH on the rate of hydrolysis of BAEe. Temperature 25° . $0.25M$ - $NaHCO_3$ in the liquid phase and 100, 50, 25 or 12.5% CO_2 in the gas phase, giving the pH values 7.04, 7.34, 7.64 or 7.94 respectively was used. Curve 1: bovine plasmin; ordinate = μ l. of CO_2 /min. In the main compartment of the flasks: 10 mg. of bovine plasmin dissolved in 2 ml. In the side arms: 25 mg. of BAEe dissolved in 0.2 ml. Curve 2: human plasmin; ordinate = $10 \times \mu$ l. of CO_2 /min. In the main compartment of the flasks: 25 mg. of BAEe dissolved in 2 ml. In the side arms: 5 mg. of plasma acetone-dried powder + 1000 units of streptokinase dissolved in 0.2 ml. The observed values were corrected for the amounts of acid consumed by the conversion of carbonate into bicarbonate.

Determination of plasminogen in human plasma

Such determinations are complicated by the high content of plasmin inhibitors in plasma as well as in the preparation of streptokinase used for the activation. A simple estimation of plasmin activity in the activated sample will give no information of the amount of plasmin formed, since the amount of plasmin bound by inhibitors is quite unknown. The method described below is based upon the following two facts:

(1) The inhibitors present in the streptokinase preparation are ineffective in the present method. This is evident from the identical plasmin activities obtained when plasminogen in plasma is activated by a large excess of streptokinase and by twice this amount of streptokinase (Fig. 6, broken line).

(2) A preparation of human plasmin in which no activator and probably no inhibitors are present can easily be made by heating human globulin activated by streptokinase to 100° at pH 2 (Müllertz, 1955).

A determination of plasminogen in plasma can now be performed as follows: plasminogen is converted into plasmin by adding an excess of streptokinase, and the resulting plasmin activity measured (Fig. 6, broken line). Increasing amounts of the above-mentioned preparation of human plasmin are added to plasma, and the resulting activities measured (Fig. 6, curve *B*). The mixture of plasma and plasmin which has the same activity as plasma activated by streptokinase also contains the same amount of plasmin. Therefore a final determination of plasmin present in the heated preparation (Fig. 6, curve *A*) permits a calculation of the concentration of plasminogen in plasma in terms of $\mu\text{l. of CO}_2/\text{min./ml. of plasma}$ (here 44.5). Further, it seems probable that a calculation of initial plasmin concentration and of inhibitor should be possible through more detailed analysis of such curves.

Lysine esterases (activator and plasminogen)

With the concentration of LEE used, the amount of substrate split is proportional to time and to the

Fig. 6. Estimation of plasminogen in human plasma. Temperature 25° ; 50% CO_2 in the gas phase, giving pH 7.34, was used. In the side arms: 25 mg. of BAE dissolved in 0.2 ml. of 0.25 M- NaHCO_3 . In the main compartment of the flasks: broken line, 100 $\mu\text{l.}$ of human plasma + 1000 units of streptokinase + 1900 $\mu\text{l.}$ of 0.25 M- NaHCO_3 . Esterase activity: 3.05 $\mu\text{l. of CO}_2/\text{min.}$ With 2000 units of streptokinase instead of 1000 units the esterase activity was 3.08 $\mu\text{l. of CO}_2/\text{min.}$ Curve *A*: 2 ml. of plasmin solution of the concentration denoted on the abscissa. The solution of plasmin was prepared as described by Müllertz (1955) except that the last precipitate was dissolved in 0.25 M- NaHCO_3 . Before use 1 vol. of this solution was diluted with 2 vol. of 0.25 M- NaHCO_3 . Curve *B*: 2 ml. of 0.25 M- NaHCO_3 containing the same concentration of plasma as that used for the broken line and the same concentrations of plasmin as those used for curve *A*.

concentration of lysine esterase (solutions of streptokinase and plasma acetone-dried powder; Fig. 7).

Optimum activity was found at pH 6.1 (Fig. 8). With the selected buffer the pH decreases during the reaction from 6.16 to 6.04 when the whole manometer scale was utilized (corresponding to about 380 $\mu\text{l. of CO}_2$). The figure shows that only slight alterations in esterase activity take place during this shift of pH.

The straight lines obtained in Fig. 7 show the lysine esterase to be stable at pH 6.1 in the presence of LEE, but in the absence of LEE, lysine esterase was found to be labile, which is in accordance with the investigations on the stability of activator performed by Müllertz (1955). Since the manometric technique involves equilibration for 30 min. with the gas phase, the method can hardly be used for the estimation of preformed lysine esterase. To determine the amount of lysine esterase formed from given amounts of the precursor preparations the reaction must be started by adding one precursor, e.g. streptokinase, to a mixture of LEE and the other precursor. The slope of the linear part of the curve then gives the lysine esterase activity. This method was used to determine the amount of lysine esterase formed from constant amounts of acetone-dried powder of plasma and varying

Fig. 7. Hydrolysis of LEE with solutions of streptokinase and acetone-dried powder of plasma. Temperature 25° ; 100% CO_2 in the gas phase. In the side arms: 20 mg. of LEE dissolved in the solution described. In the main compartment of the flasks: 0.25 (*A*), 0.5 (*B*), 1.0 (*C*) and 2.0 (*D*) ml. of a solution of 100 mg. of plasma acetone-dried powder + 10 000 units of streptokinase in 10 ml. of the solution described, plus the same solution to a total volume of 2 ml. The readings were started 5 min. after mixing.

amounts of streptokinase (Fig. 9). Assuming that the point of intersection between the ordinate and the extrapolated curve represents the plasmin activity of all samples, the relationship between the

Fig. 8. Relationship between rate of hydrolysis of LEe and pH. Temperature 25°. For pH values above 6: liquid phase as described and 100, 50, 25 or 12.5% CO₂ in the gas phase, giving pH values 6.04, 6.34, 6.64 or 6.44 respectively was used. For pH 5.74: as for pH 6.04 except that NaCl was substituted for half of the NaHCO₃. In the main compartment of the flasks: 20 mg. of LEe + 5 mg. of plasma acetone-dried powder dissolved in 2 ml. In the side arms: 500 units of streptokinase dissolved in 0.2 ml. The points represent the slopes of the linear part of the curves.

Fig. 9. Relationship between the concentration of lysine esterase and of streptokinase. Temperature 25°; 100% CO₂ in the gas phase. In the main compartment of the flasks: 20 mg. of LEe + 16 mg. of plasma acetone-dried powder dissolved in 2 ml. of the solution described. In the side arms: The amounts of streptokinase denoted on the abscissa dissolved in 0.2 ml. of the solution described. Points represent the slopes of the linear part of the curves. (The curve with 100 units of streptokinase is shown in Fig. 10.) Broken curve: for explanation, see text.

concentration of activator and streptokinase should be obtained by a displacement of the zero point of the co-ordinate system to this intersection point. The curve obtained like this resembles very closely the curve obtained by Müllertz (1955) showing the relation between the concentration of activator and of streptokinase in solutions containing a constant concentration of proactivator. Still keeping the above assumption the results are furthermore in excellent agreement with the interpretation that both plasmin and activator can hydrolyse LEe (Troll & Sherry, 1955), that the formation of activator proceeds as a stoichiometric reaction (Troll & Sherry, 1955, Müllertz, 1955) and the formation of plasmin is a catalysed reaction (Christensen & McLeod, 1945). However, the assumption does not agree with the suggestion of Sherry & Alkjaersig (1956) that proactivator and plasminogen are identical, which means that plasminogen is the precursor to both activator and plasmin. Under these conditions the formation of increasing concentrations of activator with increasing concentrations of streptokinase should involve a simultaneous decrease in the concentration of plasmin, as indicated by the broken curve in Fig. 9. Assuming that this is correct, the results are still in accordance with the results obtained by the above-mentioned investigators. On the other hand, this assumption can hardly be reconciled with the fact that increasing concentrations of

Fig. 10. Formation of lysine esterase in the presence of LEe. Temperature 25°; 100% CO₂ in the gas phase. In the main compartment of the flask: 20 mg. of LEe + 16 mg. of plasma acetone-dried powder dissolved in 2 ml. of the solution described. In the side arm: 100 units of streptokinase dissolved in 0.2 ml. of the solution described.

streptokinase do not reduce the BAEe esterase activity of human plasmin (legend to Fig. 6).

Fig. 10 shows the curve used to measure the amount of lysine esterase obtained with the smallest concentration of streptokinase in Fig. 9. It is obvious that the formation of lysine esterase was finished about 60 min. after the start. This interval of time was shorter in the mixtures used to obtain the remaining points of the curve in Fig. 9. Since the formation of activator is known to proceed very rapidly (Müllertz, 1955), and since LEe is known to inhibit the formation of plasmin but not the formation of activator (Troll & Sherry, 1955), it is most probable that the initial positive acceleration of the curve is due mainly to the slow transformation of plasminogen into plasmin. Apparently the lysine esterase is not quite stable in this mixture. This lack of stability was also found in all mixtures containing an excess of proactivator, whereas lysine esterase made with excess of streptokinase was found to be stable in the presence of LEe (Fig. 7).

*Activation of plasminogen in the presence of
benzoyl-L-arginine ethyl ester*

The manometric technique has been used to follow the conversion of plasminogen into plasmin. Activator, bovine plasminogen and BAEe were mixed in the Warburg flask at zero time, and the hydrolysis of substrate was followed with time. [Experimental conditions were as follows. In the side arms: 2–8 mg. of plasma acetone-dried powder + 250–2000 units of streptokinase dissolved in 0.2 ml. of 0.25 M-NaHCO₃. In the main compartment of the flasks: 25 mg. of BAEe + 4–10 mg. of bovine plasminogen (prepared according to Müllertz, 1953) dissolved in 2 ml. of 0.25 M-NaHCO₃. A temperature of 25° and 100 % carbon dioxide in the gas phase, giving pH 7.04, were used.] The hydrolysis caused by the plasmin present in the activator preparation was estimated in separate experiments and subtracted from the values obtained. When these corrected results were plotted against time squared, straight lines were obtained. However, it was found that BAEe inhibited the activation rather strongly (contrary to the findings of Troll & Sherry, 1955). Since the concentration of BAEe decreased during the experiments the activator activity increased, and therefore it was impossible to interpret the experimental results.

SUMMARY

1. The esterase activity of the fibrinolytic system was measured with benzoyl-L-arginine ethyl ester or L-lysine ethyl ester as substrate. The acid formed was estimated by measuring the carbon dioxide liberated from bicarbonate solutions.

2. The relation of the first and second ionization constants of carbonic acid to the ionic strength was determined so as to make possible calculations on the carbon dioxide-bicarbonate buffer in the assay method.

3. A method of determining human plasminogen in the presence of plasmin inhibitors is described.

4. The relationship between the concentration of lysine esterase and of streptokinase is established, and the results are discussed in connexion with those obtained by other investigators.

This investigation was made possible by a grant from Statens almindelige Videnskabsfond, Denmark. It forms part of the investigations on fibrinolysis for which Dr Tage Astrup receives support from The Josiah Macy Jr. Foundation, New York, and the National Danish Association against Rheumatic Diseases.

REFERENCES

- Asahina, M. & Oka, M. (1954). *Bull. nat. Hyg. Lab., Tokyo*, **72**, 117. Cited in *Chem. Abstr.* (1955), **49**, 6351.
- Astrup, T. & Müllertz, S. (1952). *Arch. Biochem. Biophys.* **40**, 346.
- Bergman, M., Fruton, J. S. & Pollok, H. (1939). *J. biol. Chem.* **127**, 643.
- Christensen, L. R. (1945). *J. gen. Physiol.* **28**, 363.
- Christensen, L. R. (1949). *J. clin. Invest.* **28**, 163.
- Christensen, L. R. & McLeod, C. M. (1945). *J. gen. Physiol.* **28**, 559.
- Geiger, B. W. (1952). *J. Immunol.* **69**, 597.
- Handbook of Chemistry and Physics* (1948). 37th ed. Ed. by Hodgman, C. D. Cleveland, Ohio: Chemical Rubber Publishing Co.
- Harned, H. S. & Davis, R. J. (1943). *J. Amer. chem. Soc.* **65**, 2030.
- International Critical Tables* (1928). 1st ed., vol. 3, p. 279. Ed. by Washburn, E. W. (for National Research Council, U.S.A.). New York: McGraw-Hill Book Co. Inc.
- Kaplan, M. H. (1944). *Proc. Soc. exp. Biol., N.Y.*, **57**, 40.
- Lundquist, F., Thorsteinsson, T. & Buus, O. (1955). *Biochem. J.* **59**, 69.
- Millstone, H. (1941). *J. Immunol.* **42**, 109.
- Müllertz, S. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 291.
- Müllertz, S. (1954). *1st Int. Conf. Thrombosis and Embolism*, p. 75.
- Müllertz, S. (1955). *Biochem. J.* **61**, 424.
- Müllertz, S. & Lassen, M. (1953). *Proc. Soc. exp. Biol., N.Y.*, **82**, 264.
- Parks, R. E. & Plaut, G. W. E. (1953). *J. biol. Chem.* **203**, 755.
- Schwert, G. W. & Eisenberg, M. A. (1949). *J. biol. Chem.* **179**, 665.
- Sherry, S. (1954). *J. clin. Invest.* **33**, 1054.
- Sherry, S. & Alkjaersig, N. (1956). *J. clin. Invest.* **35**, 735.
- Tillett, W. S. & Garner, R. L. (1933). *J. exp. Med.* **58**, 485.
- Troll, W. & Sherry, S. (1955). *J. biol. Chem.* **213**, 881.
- Troll, W., Sherry, S. & Wachman, J. (1954). *J. biol. Chem.* **208**, 85.
- Werbin, H. & Palm, A. (1951). *J. Amer. chem. Soc.* **73**, 1382.