SUMMARY

1. Spectroscopic studies have been made on the *in vitro* conversion of haematin and some other haem derivatives into bile pigments through the action of ascorbic acid and oxygen.

2. The conversion of haematin into bile pigments is associated with increased optical density in the range 630–700 mμ. and evidence was obtained for the presence of verdohaematin and cholehaematin in the reaction products.

3. The yield of bile pigment derived from metmyoglobin was approximately eleven times that from oxyhaemoglobin under similar experimental conditions. Cholemyoglobin was observed spectroscopically.

4. Horse-radish peroxidase was converted into bile pigment in 2·9 % yield of its haem content, and the bile pigment haematin content of horse-liver catalase was increased from 27 to 39 %.

5. With the exception of cytochrome *c*, all haem derivatives in the series suffered, in varying degree, oxidative breakdown to bile pigment in the presence of ascorbic acid and oxygen.

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The Measurement of the Components of the Plasminogen–Plasmin System in Biological Fluids

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Streptokinase, a product of haemolytic streptococcal metabolism, is known to activate a serum enzyme precursor, plasminogen, into a proteolytic enzyme, plasmin; plasmin causes the lysis of fibrin and the liquefaction of fibrinous exudate. The action of plasmin can be inhibited by antiplasmin or antiplasmins (nomenclature of Christensen & MacLeod, 1945; Fig. 1). Mullertz & Lasen (1953) have suggested that the activation of plasminogen by streptokinase is not a direct reaction, but is mediated through an intermediary component.

Certain human infections result in the local production of fibrin and fibrinous exudates, which may prove harmful to the patient, and streptokinase has been employed therapeutically in man to disperse such exudates. Fletcher (1954) has shown that the

successful control of this type of therapy requires accurate assay methods for the components of the reaction in biological fluids, and a knowledge of the dynamics of streptokinase activation under physiological conditions. It is the purpose of this communication to describe a suitable method of plasmin assay, assess the accuracy of the method, and investigate some aspects of streptokinase activation by its use.

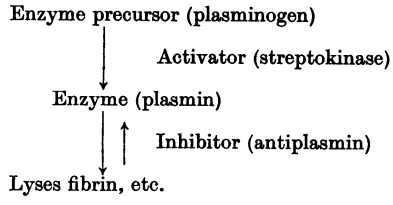

Fig. 1. The plasminogen–plasmin system.

As the aim of these experiments was the control of fibrin lysis in man, fibrin was chosen as the substrate for the measurement of enzymic potency. When the lysis of fibrin is used as an assay method, it is possible to determine either the lowest dilution of enzyme that will lyse a fixed amount of fibrin in a fixed time (Christensen, 1949), or to determine the time which the undiluted enzyme requires to lyse a fixed amount of fibrin (Lewis & Ferguson, 1950). These two methods produce concordant results when using purified reagents, but in biological fluids results are discordant.

The lysis of fibrin clots gives an indeterminate end point, but the method for plasmin assay described here practically eliminates observer error, since the end point is unequivocal.

EXPERIMENTAL

Materials

These were bovine fibrinogen (Armour Laboratories, Chicago), 40% of the total nitrogen being clottable by thrombin; human fibrinogen (Lister Institute, London), 97% of the total nitrogen being clottable by thrombin; thrombin, a single batch of 'Clotting Globulin' (Lederle Laboratories Division, New York); streptokinase–streptodornase (Burroughs Wellcome, Beckenham) and 'Varidase' (Lederle Laboratories Division, New York). Partially purified human plasminogen was prepared by the method of Remmert & Cohen (1949). This material, when freeze-dried and stored at 4°, eventually developed spontaneous plasmin activity. Two batches out of the twenty-three prepared exhibited powerful spontaneous activity, exceeding 15 Christensen units/mg. Chloroform-activated human plasmin was prepared by the method of Rocha e Silva & Rimington (1948). Chloroform-activated bovine plasmin was kindly supplied by Dr E. Loomis (Loomis, George & Ryder, 1947).

Sodium borate buffer, 0·25% (w/v), was used to maintain the pH of the assay reactions at 7·7.

Methods

Plasminogen was assayed by the method of Christensen (1949) with the modifications described by Christensen & Smith (1950).

Units of measurement. The units of streptokinase and plasminogen were those described by Christensen (1949). Plasminogen cannot be measured directly and has to be assayed after conversion into plasmin; this results in the Christensen unit of plasminogen being defined as the enzyme potency required to lyse a fibrin clot derived from a 0·2% (w/v) solution of bovine fibrinogen in a volume of 1 ml. at 35° in 30 min. Such a definition assumes, with the favourable experimental conditions laid down, complete conversion of plasminogen into plasmin to an extent of 1 unit/ml. It is not possible to obtain direct experimental proof of this assumption, and subsequent argument is based on the fact that, though owing to inefficient precursor conversion the Christensen method of plasminogen assay may give too low a reading for the plasminogen content, it will not give too high a reading.

It is, therefore, necessary to have a reference standard of enzyme activity, and this was provided by using a single batch of freeze-dried, chloroform-activated bovine plasmin (Loomis *et al.* 1947). The Loomis preparation assayed at 0·41 mg./ml. was equivalent to 1 Christensen unit of plasmin (mean of twenty determinations, S.D. 6·4%). The unit of plasmin activity is equivalent to the lysis of 0·5 mg. of pure human fibrin in a volume of 1 ml. at 35° in 30 min. The unit of antiplasmin is that amount of inhibitor neutralizing 1 unit of plasmin activity after 10 min. at 35°.

Plasmin assay. Plasmin was assayed as follows: stoppered tubes 7·5 × 1 cm. were placed in a rack inclined at an angle of 25° to the horizontal, immersed in a water bath, thermostatically controlled at 37°. A volume of 0·7 ml. of the solution to be assayed was placed in each tube and allowed to attain the temperature of the bath. Thrombin solution, 0·1 ml. (of sufficient strength to cause complete clotting of the added fibrinogen in 1 min.), was placed in each tube and the requisite quantity of fibrinogen added in a volume of 0·25 ml. The contents of the tubes were mixed by inversion, the time being taken at the instant of adding the fibrinogen. The tubes were replaced in the rack and when, after 1 min., clotting had occurred, a small glass bead (range 140–160 mg.) was placed on the top of the clot and the tube stoppered.

A shaking motor was then started, which rocked the tubes between angles of 25 and 5° from the horizontal 50 times/min. The time of lysis was taken to be when the ball reached the bottom of the tube, and this resulted in a very sharp end point. The shaking motion was required to prevent the ball adhering to the side of the tube. The reciprocal of the lysis time was compared with that produced by a standard solution of the enzyme, and the potency of the unknown solution was read off from the standard graph (Table 1). The reproducibility of the method was approximately ±10% over the range of potencies assayed.

The antiplasmin content of a fluid was ascertained by mixing the fluid with spontaneously active plasmin or chloroform-activated plasmin, incubating the mixture for 15 min. at 37°, and then assaying to determine the loss of activity. The time of incubation was chosen as 15 min., since experiments showed that 95% of the antiplasmin activity would be assayed at the end of this period.

Table 1. *Details of the results for the three fibrinogen concentrations employed for the plasmin assay*

Final fibrinogen concentration	0·125%	0·25%	0·5%
Formula of calculated line for reference preparations	$y=0{\cdot}0647x+0{\cdot}06$	$y=0{\cdot}0357x+0{\cdot}06$	$y=0{\cdot}0255x+0{\cdot}034$
Correlation coefficient	0·971	0·964	0·948
S.D. about regression line	0·0189	0·0192	0·0203
95% confidence limits of potency estimate at mid-point (6-point assay): $\frac{2s}{\bar{x}b\sqrt{n}}$ *	±10·6%	±9·7%	±8·7%
Range: mg. of Loomis enzyme/ml.	0·5–4	1·2–7·5	4–11
Range: Christensen units/ml.	1·25–10	3–19	10–28

Ordinate (y) $1/t$ (min.). Abscissae (x) mg. of Loomis enzyme/ml.
* Approximate formula for linear dose response lines. 6-Point assay refers to six assays of unknown substance. s = standard deviation about regression line; b = slope of line for reference preparation; $\bar{x}$ = mean value of determinations; n = number of determinations.

Accuracy of plasmin assay. In order to test the accuracy and reproducibility of the plasmin assay method, weighed quantities of the freeze-dried, chloroform-activated, bovine enzyme (Loomis preparation, 0·41 mg./ml. ≡ 1 Christensen unit) were assayed against the three batches of fibrinogen used over a period of 1 year. All determinations were combined in an attempt to find the maximum variation of the method during this period. Lysis-time readings were in all cases restricted to between 3 and 10 min. and if lysis times greater or less than this period were obtained, a shift was made to another fibrinogen concentration. The results, with the 95% confidence limits, are shown in Table 1.

It was concluded that this method of assay gave reproducible estimates of enzymic potency throughout the course of the investigation. It should be emphasized that the 95% confidence limits given in Table 1 are maximum errors, since with single batches of fibrinogen on a single day the variation about the assay line was less than half that indicated in Table 1. Since each individual experiment was performed under the latter conditions, the lesser error would apply, whilst for comparison between experiments the greater error would be the appropriate measure of accuracy.

To test the applicability of this method to human material the following experiments were performed. Chloroform-activated and spontaneously active human plasmin were diluted, and the diluted solutions assayed for enzyme potency. The enzyme potencies found were plotted against the concentration of the solution and the regression coefficient was calculated. Technical limitations rendered it impossible to use the 0·5% fibrinogen line, but when the regression coefficient was calculated for the chloroform-activated preparation using the other two lines, it was 0·9; the regression coefficient for the spontaneously active preparation was 0·93.

A purified streptokinase-activated plasmin was employed to cover all the assay lines in a similar fashion; the regression coefficient in this case was 0·9 and the standard deviation about the regression line was 12. Since the original concentration was, for convenience, taken as 100, this made the S.D. 12%. The reason for this greater degree of variation was that the assay lines do not superimpose at their junction; when the S.D. of the regression coefficient was calculated for the 0·25% line, it was 6%.

It was concluded that the method of assay was suitable for use with human plasmin solutions, which could be estimated with the same accuracy as bovine preparations.

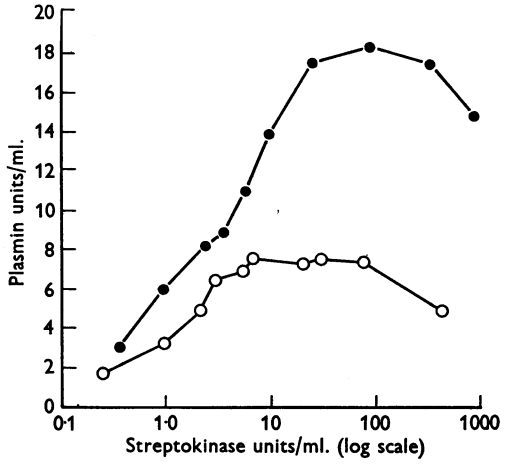

Fig. 2. Plasmin activity (units/ml.) found when differing concentrations of streptokinase are added to two concentrations of plasminogen. ●—●, 30 units plasminogen/ml.; ○—○, 10 units plasminogen/ml.

RESULTS

The dynamics of streptokinase activation

The use of partially purified human plasminogen (8000 units/mg. nitrogen), practically devoid of antiplasmin activity, has allowed the dynamics of plasminogen activation by streptokinase to be studied. Fig. 2 shows the amount of plasmin produced when varying concentrations of streptokinase are allowed to act upon fixed amounts of plasminogen. Small concentrations of streptokinase induce an increase in plasmin activity approximately related to the $\log_{10}$ of the streptokinase concentration employed. Eventually a more or less constant enzymic activity is reached, and no further increase of activity can be produced by increasing the streptokinase concentration as much as tenfold. Concentrations of streptokinase some 20–50 times those inducing maximal enzymic

activity cause a fall in enzymic activity. The explanation of this impairment of enzyme activity is not clear; neither the antiplasmin activity of the streptokinase nor the decay curves of enzyme activated with high streptokinase concentrations will account for this phenomenon. A wide range of streptokinase concentrations can be employed to induce the maximal enzyme activity of a solution, and titrations to ascertain this activity can be performed easily.

The activity produced by fixed concentrations of streptokinase acting upon varying amounts of plasminogen is shown in Fig. 3. Concentrations of streptokinase of 1, 10, and 100 units/ml. were employed in this experiment, the concentrations of plasminogen varied from 1 to 250 units/ml. It is seen that though, with a fixed streptokinase concentration, rising concentrations of precursor result at first in increasing levels of enzymic activity, they eventually fail to give this increase. Considerable conversion of precursor into enzyme is only achieved where the concentration of precursor is low.

An experiment of this nature bears on the validity of the Christensen method of plasminogen assay, which requires that plasminogen should be quantitatively converted into plasmin to the extent of 1 unit/ml. Table 2 shows the enzyme activity produced when low plasminogen concentrations are treated with 100 units of streptokinase/ml. (concentration used for plasminogen assay). It is seen that the enzymic activity produced is approximately proportional to the plasminogen concentration up to 4 units of plasminogen/ml. This finding suggests that the plasminogen assay gives results of good relative accuracy, even though the absolute accuracy remains uncertain.

In order to ascertain the degree of precursor conversion when the optimum rather than a fixed streptokinase concentration was used, the following experiment was performed. Diluted samples of a purified plasminogen solution were individually assayed for their plasminogen content. Each diluted solution was activated with several concentrations of streptokinase, and using the optimum streptokinase concentration ascertained by this procedure, the maximal enzyme concentration for each dilution was determined. This experiment clearly shows (Fig. 4) that it is not possible to activate all the precursor to form the enzyme with streptokinase except under conditions of very low precursor concentration. Streptokinase converts a high proportion of plasminogen into plasmin at low plasminogen concentrations but proves less effective at high plasminogen concentrations. This finding demonstrates the hazard of using any method of plasminogen assay unless the plasminogen concentration is controlled at a low level, as in the Christensen assay.

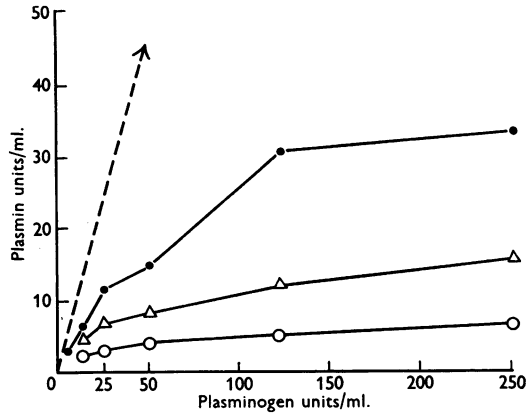

Fig. 3. Plasmin activity (units/ml.) found with differing plasminogen concentrations and three concentrations of streptokinase. ●—●, 100 units streptokinase/ml.; △—△, 10 units streptokinase/ml.; ○—○, 1 unit streptokinase/ml., – – –, theoretical line of 100% conversion of plasminogen into plasmin.

Table 2. *Enzymic activity produced when low concentrations of plasminogen are treated with* 100 *units of streptokinase/ml.*

	Amount of material (units/ml.)						
Plasminogen	0·5	1·0	1·5	2·5	4·0	8·0	12
Plasmin	0·5	1·0	1·5	2·2	3·6	4·5	5·4

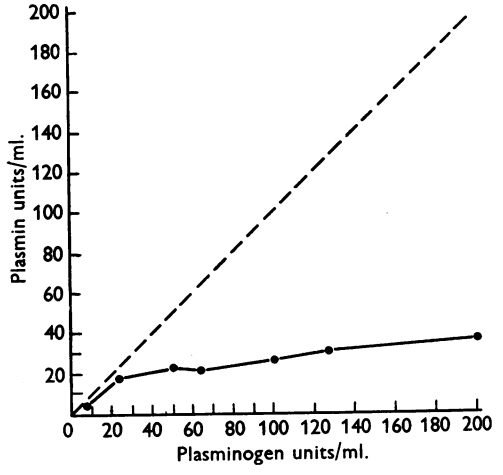

Fig. 4. Conversion of plasminogen into plasmin with 'optimum' streptokinase concentration. 'Optimum' streptokinase concentration is that concentration of streptokinase which will produce maximum fibrinolytic activity in a solution of plasminogen. – – –, theoretical line of 100% conversion of plasminogen into plasmin; ●—●, actual conversion of plasminogen into plasmin under the conditions of the experiment.

Antiplasmin and the properties of the enzyme–inhibitor complex

Plasmin mixed with serum, cerebrospinal or some other body fluids, shows a marked reduction in activity amounting in some cases to complete extinction of activity. This plasmin–antiplasmin complex has properties which influence both the therapeutic potentialities of the enzyme and the assay of enzymic potency in biological fluids.

The experiments of Schulman (1952), using radioactive fibrinogen as a substrate for the measurement of bovine plasmin activity, have shown convincingly that where serum dilutions of the order 1:1000 are used, plasmin forms a stoicheiometric and irreversibly bound compound with its inhibitor.

Fig. 5 shows that when human serum is assayed for antiplasmin activity, using spontaneously active human plasmin as a source of enzyme, the inhibitory effect of the serum is proportional to its concentration. Since the plasmin activity of spontaneously activated preparations of plasminogen is low, it has only been possible to investigate low serum concentrations by this method. The type of inhibition seen in this experiment differs from that described by Schulman in that the plasmin–antiplasmin complex can be partly dissociated by dilution and change in pH as is shown by the following experiment.

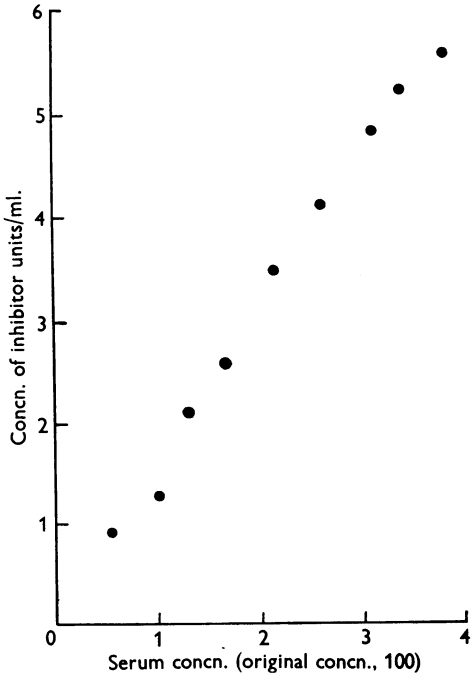

Fig. 5. Antiplasmin activity (inhibitor units/ml.) of varying concentrations of human serum.

Spontaneously active human plasmin (activity 23 units/ml.) was neutralized by a 1:10 dilution of human serum. The mixture was shown to be devoid of plasmin activity, the pH was altered to 5·3 by 1% (w/v) acetic acid, the mixture cooled, and the precipitate spun down. On being made up to the original volume, the precipitate contained 40% of the original enzymic potency.

Certain aspects of plasmin inhibition by solutions of high protein content cannot be accounted for by specific and irreversible combination of plasmin with an inhibitor. Macfarlane & Biggs (1946) demonstrated that the physiological fibrinolysis induced in man by fear or anxiety could only be demonstrated after considerable dilution of the plasma, and this observation has been repeatedly confirmed. Similarly, when solutions of spontaneously active human plasmin are mixed with sufficient serum to extinguish all fibrinolytic activity, some activity will be present on dilution of the solution. These findings are inconsistent with the theory of an irreversible and stoicheiometric combination of enzyme and inhibitor, and strongly suggest that a further mechanism is responsible for much of the plasmin inhibition shown by solutions of high protein content.

The fact that an easily dissociable, non-specific inhibition rather than a specific inhibition of plasmin is important at high protein concentrations may be seen from the following experiment. A solution of spontaneously active plasmin with a concentration of 10 mg./ml. was serially diluted with borate buffer and assays were performed on the dilutions. The effects of specific inhibition can be

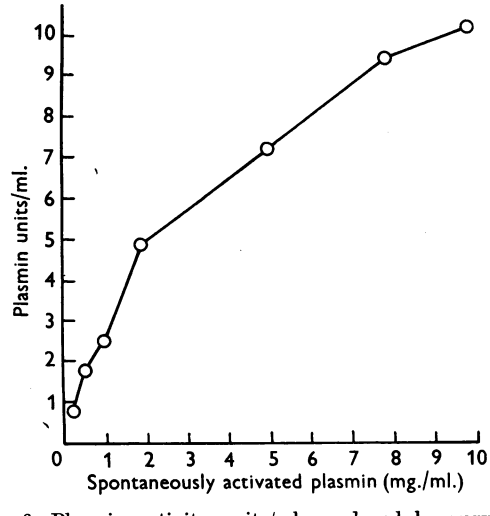

Fig. 6. Plasmin activity units/ml. produced by varying concentrations of 'spontaneously' activated human plasmin. The enzyme activity is not proportional to the concentration of dissolved material.

disregarded in such an experiment since, in view of the high excess of enzyme in all dilutions, the demand of a specific inhibitor would be satisfied. The results are shown in Fig. 6, where it is seen that the enzymic activity is not proportional to the concentration of dissolved plasmin, this activity being less than would be expected where the concentration of dissolved plasmin is high.

The finding that non-specific inhibition of plasmin is important in the inhibition of plasmin by body fluids means that the assay of active enzyme in biological fluids must be performed at the actual concentration in which the enzyme is present, otherwise the values obtained will be too high.

DISCUSSION

The experimental findings concerning the degree of conversion of precursor into enzyme by streptokinase at various precursor concentrations indicate the necessity of assaying plasminogen under conditions causing a high degree of conversion of precursor into enzyme. These conditions require a high dilution of precursor, such as is used in the Christensen method of assay. The difficulty of converting high plasminogen concentrations into equivalent amounts of enzyme with streptokinase has important implications with regard to the therapeutic uses of streptokinase.

The demonstration that non-specific inhibition of plasmin is important at high protein concentrations shows that the true level of fibrinolytic enzyme can only be ascertained when assays are conducted in biological fluids as near the original concentration as possible. This precaution is doubly necessary when assaying enzyme activity induced by streptokinase. Streptokinase-containing solutions will alter their content of free fibrinolytic enzyme on dilution, and errors from this source cannot be easily corrected.

The degree of availability for physiological purposes of enzyme that has undergone non-specific, rather than specific, inhibition in the body must be regarded as uncertain. The demonstration that pH changes may affect the dissociation of the enzyme–inhibitor complex suggests that minor local changes *in vivo* may cause the release of enzyme. *In vitro*, enzyme that has undergone non-specific inhibition is incapable of lysing fibrin. The time of lysis method of determination, as developed in this communication, measures only free fibrinolytic enzyme, and this method is therefore suitable for determining the enzymic potency of biological fluids during the course of therapy with fibrinolytic substances.

SUMMARY

1. A method of fibrin lysis with an objective end point, and suitable for the measurement of free-plasmin activity in biological fluids is presented. The accuracy of the method over the range 1–28 Christensen units/ml. is $\pm 10\%$.

2. Streptokinase converts a high proportion of plasminogen into plasmin at low plasminogen concentrations, but converts only a low proportion at high plasminogen concentrations.

3. The important role played by non-specific inhibition of plasmin as against specific inhibition of plasmin in biological fluids has been emphasized, and the possibility that such enzyme may be physiologically active has been suggested.

I am indebted to Prof. Sir Alexander Fleming and Prof. R. Cruickshank for much encouragement and advice. I wish to thank Dr E. Loomis of Parke Davis Laboratories for a generous gift of chloroform-activated bovine plasmin, and Dr P. Armitage of the Medical Research Council Statistical Unit for suggesting a method of analysing the results.

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