5. Following growth in unsupplemented basal medium, washed-cell suspensions of *S. fragilis* can acquire the ability to ferment raffinose, lactose and galactose, the rate of adaptation decreasing in that order. Adaptation does not occur in presence of sodium azide. The initial rate of glucose and fructose fermentation by these suspensions is very low and maximum rate is not reached for 2 hr., the increase in rate being prevented by sodium azide.

6. Failure to ferment raffinose following growth

in unsupplemented basal medium is not due to lack of hydrolytic activity towards raffinose, but appears to be due to a deficiency in the glycolytic system.

7. Evidence is presented which suggests that glucose and fructose strongly inhibit invertase synthesis by *S. fragilis*.

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Fibrinolysins of Human Plasma

A COMPARISON OF FIBRINOLYTIC PLASMA FROM NORMAL SUBJECTS AND FROM CADAVER BLOOD WITH PLASMIN PREPARED BY ACTIVATION WITH CHLOROFORM

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This paper is concerned with the nature of the fibrinolytic activity which develops in human blood in a variety of circumstances. The centre of interest is the spontaneous fibrinolysis produced in the normal subject by physical or emotional disturbances such as severe exercise or fear or by injection of adrenaline (Macfarlane, 1937; Macfarlane & Pilling, 1946; Biggs, Macfarlane & Pilling, 1947). Fibrinolytic activity can also be produced *in vitro* by treating plasma with chloroform or certain

bacterial filtrates, and this has been shown to be due to the conversion of a zymogen ('plasminogen') present in normal plasma to its active, proteolytic form ('plasmin') (Christensen, 1945, 1946; Christensen & MacLeod, 1945). Normal plasma and serum were found to contain a powerful inhibitor to plasmin. Intense fibrinolytic activity sometimes occurs in blood taken post mortem, particularly in cases of sudden or violent death (Mole, 1948).

The question arises whether these phenomena are due to the same active agent. Macfarlane & Pilling (1946) considered that spontaneous fibrinolysis in living subjects was due to activation of the enzyme plasmin characterized by Christensen, and to a decrease in antiplasmin. Mole (1948) pointed out differences between post-mortem fibrinolysin ('cadaver lysin') and plasmin, and Bidwell & Macfarlane (1951) found that the fibrinolytic activity produced in living subjects by exercise also differed from plasmin, so that further identification of the factors concerned is clearly required.

Recent work has emphasized the lability of plasma fibrinolysin (Fearnley, Revill & Tweed, 1952; Truelove, 1953; Fearnley & Tweed, 1953), a finding which may explain discrepancies in previous observations. Using the test devised by Macfarlane (1937) and modified by Macfarlane & Biggs (1946), it was shown that, if the interval between collection of the specimen and setting up the test is sufficiently brief (about 15 min.), most normal subjects give a positive result; so that fibrinolysis observed after exercise, and even in cadaver blood (Mole, 1948), may arise from a quantitative rather than a qualitative change in the state of the blood.

The following paper presents some further observations on the inter-relationship of the fibrinolytic phenomena produced respectively by exercise, by sudden death and by the action of chloroform, and on the technical difficulties of testing. Some of the results have been the subject of a preliminary communication (Bidwell & Macfarlane, 1951).

METHODS

Materials

Plasma. Citrated human plasma, used as the source of fibrinogen, thrombin and plasmin, was obtained from the hospital 'blood bank'.

Fibrinogen. The phosphate precipitation method of Jaques (1943) was modified as described by Biggs & Macfarlane (1953). The dried material contained salts (about 60%, w/v); the protein was about 85% clottable; it was contaminated with plasminogen (i.e. clots were lysed by streptokinase).

Thrombin. Solutions of the dried material (prepared as described by Biggs & Macfarlane, 1953) were made fresh for each experiment in a concentration of 20 units/ml. (using Thrombin Topical, Parke, Davis and Co., as standard).

Fibrinolysin preparations. 'Exercise plasma.' The exercise entailed running up and down a spiral, stone staircase, 39 ft. in vertical height, some 10-14 times until the subject felt exhausted. Blood was taken by venepuncture and mixed with one-ninth its volume of 3.8% (w/v) trisodium citrate; centrifuged for 10 min. at 2000 rev./min., and the plasma separated. In a few experiments as specified the anticoagulant was NaCl.

'Normal plasma.' This was prepared in the same way from blood withdrawn from normal subjects before exercise.

'Cadaver lysin.' When blood was found to be fluid at

routine post-mortem examinations (see Mole, 1948) as much as possible was withdrawn from the femoral vein. In the majority of cases the blood remained fluid after collection without anticoagulant and could not be clotted by thrombin. Since therefore fibrinogen was shown to be absent it could be concluded that fibrinolysis had taken place in the vessels before collection of the sample. The fluid obtained was thus a mixture of blood cells, serum and the products of fibrinolysis; the cells were removed by centrifuging. In two cases the blood clotted after collection; in these samples no fibrinolytic activity could be demonstrated. It was found that the fibrinolytic activity of cadaver blood was very much greater than that of specimens from living subjects but the volume of blood obtainable after death was usually very small.

Plasmin. Chloroform-activated plasmin was prepared by the method of Rocha e Silva & Rimington (1948). It was found that such preparations would clot plasma, but that equally active preparations which did not clot plasma could be made if the starting material was first treated with alumina as described for fibrinogen by Biggs & Macfarlane (1953). The clotting properties appeared to be due to contamination by thrombin and not to be a function of plasmin (Nikolic, 1951).

Determination of comparative amounts of fibrin in partially lysed and whole clots

Reagents. All reagents were of analytical quality where available. **Buffered saline.** 5.0 ml. sodium diethylbarbiturate:sodium acetate buffer base (Michaelis, 1931) + 5.0 ml. 0.1 N-HCl + 90 ml. 0.85% (w/v) NaCl (pH 7.4 measured with a glass electrode). This buffer was used for making up fibrinogen solutions and as a diluent. **Sodium hydroxide.** 0.1 N-NaOH, filtered before use. **Sodium carbonate.** 12.5% (w/v) Na_2CO_3 , filtered before use. **Copper sulphate.** 0.01 M- CuSO_4 . **Phenol reagent.** Folin & Ciocalteu (1927) phenol reagent (from British Drug Houses Ltd.). One vol. of the stock reagent was diluted with two vol. of distilled water just before use.

Determination of fibrin. Clots were collected by winding on to a glass rod, roughened over the lower part, which had been left *in situ* after use as a stirring rod for mixing the reagents before clotting. The rod, with the adhering clot, was transferred to another tube containing about 10 ml. of ice-cold 0.85% (w/v) NaCl in an ice-water bath. The tubes of this second series had a capacity of about 12 ml. and were graduated at 8.0 ml. The clot, still attached to the rod, was washed with two more changes of ice-cold saline (about 30 ml. in all) and then dissolved by adding 2.0 ml. NaOH solution down the rod and immersing the tube in a boiling-water bath for 2-3 min. After cooling, the volume was made up to 8.0 ml. with Na_2CO_3 solution. The mixture could then be left until it was convenient to develop the colour.

CuSO_4 solution (1.0 ml.) was added and mixed, followed by 1.0 ml. of diluted phenol reagent (added from a glass syringe with rapid stirring). The colours were read after not less than 0.5 hr. using a photoelectric colorimeter and an Ilford red filter; the blank was prepared from the reagents. The colour was recorded in terms of the amount of tyrosine required to produce the same colour under the same experimental conditions.

It was found that this procedure is preferable to other modifications of the Folin & Ciocalteu method; it is well recognized that the colour is due to other factors in addition to tyrosine.

Measurement of plasmin activity

(1) *The method of Rocha e Silva & Rimington (1948).* 0.5 ml. enzyme or enzyme-inhibitor mixture + 0.30 ml. fibrinogen (1.3 mg. dried preparation/ml.) + 1 drop thrombin, incubated at 37°. The lysis time was recorded when the bubbles in the clot rose to the surface. One unit of enzyme activity was the amount required to effect lysis in 10 min. This method was used in following the stages of purification of plasmin and for a few experiments with cadaver lysin; it was insufficiently sensitive for work with exercise plasma. It has the theoretical disadvantage of requiring complete destruction of the substrate.

(2) *The method of partial lysis.* The method was much more sensitive than (1) and is theoretically more sound, but in practice is laborious. A solution of fibrinogen was prepared of such concentration that when 4.0 ml. of fibrinogen solution + 1.0 ml. buffered saline was clotted with 0.2 ml. thrombin, the amount of clot, as determined by the method given above, was equivalent to 144 μ g. tyrosine. In the determination of enzyme activity, a mixture of 4.0 ml. fibrinogen solution + 1.0 ml. enzyme (or enzyme-inhibitor mixture prepared previously) was placed in a water bath at 37°. After 5 min. incubation, to allow the mixtures to reach 37°, 0.2 ml. thrombin was added and thoroughly and rapidly mixed using a glass rod roughened over the lower part. The rod was left in the tube. Exactly 45 min. after the addition of thrombin the amount of clot remaining was determined by the method given above for the determination of fibrin. One unit of enzyme by this method was defined as the amount required to lyse 25% of the clot in 45 min. at 37°, the amount of fibrinogen and thrombin and the final volume being fixed as described (approx. 2.5 units by this method = 1 unit by method (1)).

Demonstration of fibrinolysis in exercise plasma

'Dilution test' (Macfarlane, 1937; Macfarlane & Biggs, 1946). 0.4 ml. of exercise plasma was added to 6.0 ml. of buffered saline to give a dilution of 1/16. Two sets of three tubes were prepared as follows: (a) 1.6 ml. diluted plasma. (b) 0.8 ml. diluted plasma + 0.8 ml. buffered saline. (c) 0.4 ml. diluted plasma + 1.2 ml. buffered saline. To each tube of one set was added one drop of thrombin and to each of the second set 0.1 ml. of 0.05 M CaCl_2 . The contents of the tubes were mixed by inverting. The six tubes were placed in a water bath at 37°, together with a similar set of tubes prepared from normal plasma. The test should be made at a standard time after withdrawing the blood because the fibrinolytic effect may disappear during storage. The tubes were observed to ensure that coagulation occurred and examined again after 24 hr. for lysis of the clot. When possible the tubes were observed more frequently as the lysis time gives an approximate measure of the fibrinolytic activity.

Method of partial lysis. This method is similar to the method of partial lysis described for plasmin. The differences are (a) the source of fibrinogen in the plasma itself, (b) the incubation times are necessarily much longer. Buffered saline (4.8 ml.) was placed in a series of tubes; 0.2 ml. exercise plasma was added, mixed with a rod and placed in a water bath at 37°. The mixtures were allowed 5 min. to reach 37° before starting the experiments. Thrombin (0.2 ml.) was added at this time unless otherwise stated. Clots were collected after the desired incubation time and the amount of fibrin determined as described above.

Demonstration of fibrinolysis by cadaver lysin

The techniques for plasmin and exercise plasma can be modified to show the high fibrinolytic activity of cadaver lysin. Fibrinogen is not present in cadaver lysin so must be added either in the form of a solution of purified fibrinogen or as normal plasma. Mole (1948) used plasma from the hospital blood bank; we have used 0.2 ml. normal plasma or 0.3 ml. 'bank' plasma in a final volume of 5.0 ml.

RESULTS

Difference between 'thrombin clots' and 'calcium clots'

In the preliminary work on exercise plasma it was necessary to decide whether to clot the citrated plasma by the addition of thrombin or by recalcification. Fig. 1 shows that the speed of lysis when

Fig. 1. The difference between 'thrombin clots' and 'calcium clots'. Each tube contained 0.2 ml. exercise plasma and 5.5 ml. buffered saline. Clotting produced by: 0.2 ml. 1.1% (w/v) CaCl_2 , x—x; 0.2 ml. 1.1% (w/v) CaCl_2 + 0.2 ml. thrombin, O—O; 0.2 ml. thrombin, ●—●. Mixtures were incubated in water bath at 37°. Similar sets of clots from plasma taken before exercise had given no evidence of lysis at the end of the experiment.

calcium ions and thrombin were both added to the plasma was the same as for calcium ions alone and was slower than that of the clots formed with thrombin alone in the absence of added calcium. The effect therefore cannot be due to plasmin in the thrombin nor to activation of the intrinsic clotting system of the plasma.

It was possible that such a result might arise from activation by excess citrate. The experiment was repeated using sodium chloride as an anticoagulant (Richardson, 1958) instead of sodium citrate.

Blood obtained by venepuncture was mixed with an equal volume of 10% (w/v) sodium chloride. It was diluted for use with 9 vol. of (a) 0.167%, w/v, sodium chloride; (b) 0.167 (w/v) sodium chloride containing 0.0005M-calcium chloride; (c) 0.167% (w/v) sodium chloride containing 0.0025M-calcium chloride. The mixtures of series (a) were clotted with thrombin; those of series (b) and (c) were allowed to clot naturally. The final concentration of calcium in mixture (b) was approximately that of ionized calcium and in mixture (c) of total calcium in plasma. The final concentration of sodium chloride was physiological and the mixtures contained no foreign ions. The lysis of the clots followed the same course as in Fig. 1, namely series (b) and (c) lysed at the same rate, despite a fivefold difference in added calcium, and series (a) lysed more rapidly. The difference is therefore not due to citrate nor probably to direct inhibition by calcium ions. It was decided that subsequent experimental work would be more conveniently done using thrombin.

Fig. 2. Time lag in lysis of clot in exercise plasma. Plasma before exercise, $\times-\times$; plasma after exercise, $O-O$.

Time lag in digestion of clots in exercise plasma

The course of lysis of the clots in Fig. 1 suggested that there was not a steady decrease in the amount of clot collected but rather that the amount remained constant for a certain time and then began to diminish rapidly. This was confirmed in the experiment shown in Fig. 2 in which no decrease in the amount of clot was demonstrable during the first 3 hr. of incubation, yet within the next $1\frac{1}{2}$ hr. all the clot had been digested.

Inhibition of plasmin by exercise plasma

A suggestion that fibrinolytic plasma obtained after exercise might contain less plasmin inhibitor than normal plasma (Macfarlane & Pilling, 1946)

was tested with the result shown in Fig. 3. It was found to be inhibitory to the same extent as normal plasma in the reaction between plasmin and fibrin. It was also found that the plasmin inhibitor of plasma was effective in this system only if the mixture of enzyme and inhibitor was made first and afterwards added to the substrate (fibrinogen).

It seemed therefore that the other suggestion of Macfarlane & Pilling (1946), namely that fibrinolytic plasmas contain an increased amount of plasmin, might still be true, since this plasmin would be formed in the presence of the substrate, fibrinogen, and therefore might be unaffected by plasmin inhibitor. This supposition was tested in two ways (i) by the preparation of 'artificial fibrinolytic plasma' (a mixture of plasmin and normal plasma),

Fig. 3. Inhibition of plasmin by plasma samples taken before and after exercise. Two ml. enzyme-inhibitor mixture contained: 0.004 ml. plasma before exercise, $\times-\times$; 0.004 ml. plasma after exercise, $O-O$; no inhibitor, $\bullet-\bullet$. Mixtures stood 0.5 hr. at room temperature, 1.0 ml. added to 4.0 ml. fibrinogen at 37° . After 5 min., thrombin (0.2 ml.) was added. Clots were collected after 45 min. at 37° and estimated by the standard method.

and (ii) by investigating the properties of mixtures of fibrinolytic and normal plasma.

(i) In attempts to prepare artificial fibrinolytic plasma by adding plasmin to normal plasma it was found that the addition of a relatively large amount of plasmin might produce a mixture which would lyse rapidly, e.g. in 15–20 min., while if only a little less plasmin were used the clot would not lyse at all (see Table 1). In these experiments enzyme and

inhibitor were 100 times as concentrated as when purified fibrinogen was used as the substrate (see Fig. 3) and the plasmin inhibitor appeared to be effective in the presence of fibrinogen.

Table 1. *Lysis times of plasmin-plasma mixtures*

(Each mixture contained 0.2 ml. plasma and varying amounts of plasmin added to sufficient buffered saline to bring the volume to 5.0 ml. The mixtures were clotted with thrombin and incubated at 37°.)

Plasmin (ml.)	Lysis time
0.8	No proper clot formed
0.7	7-12 min.
0.6	13 min.
0.5	> 15 < 35 min.
0.4	No lysis after 24 hr.
0.3	No lysis after 24 hr.
0	No lysis after 24 hr.

Fig. 4. Effect of mixing exercise plasma and normal plasma. Blood was taken into equal volume of 10% (w/v) NaCl, before and after exercise. Diluent: 1 vol. Michaelis buffer base + 1 vol. 0.1N-HCl + 8 vol. distilled water. Plasma (0.5 ml.) + 4.5 ml. diluent placed in water bath at 37°. Thrombin (0.2 ml.) was added after 5 min. Clots were collected after incubation times as shown (abscissa).

Mixtures of plasma		
Before exercise (%)	After exercise (%)	
0	100	+ + +
20	80	○ — ○
40	60	△ — △
55	45	● — ●
100	0	x — x

The results in Table 1 are in marked contrast to those shown for exercise plasma in Fig. 2, and it was impossible to imitate this type of lysis; another point of difference was that the artificial lytic clots fragmented so that it was impossible to collect them

quantitatively on to stirring rods as was done with the fibrinolytic clots made from exercise plasma.

(ii) The activity of mixtures of normal and exercise plasmas was found to depend only on the proportion of exercise plasma in the mixture (Fig. 4). This difference between exercise plasma and an artificial mixture of plasmin and plasma was confirmed by preparing such an artificial fibrinolytic plasma and using it in mixtures with normal plasma in exactly the same way as in the previous experiment. Even a small admixture of normal plasma was sufficient to prevent fibrinolysis.

Table 2. *Lysis of plasma clots by dilutions of cadaver lysin*

(Each mixture contained 0.3 ml. 'bank' plasma and varying amounts of lysin added to sufficient buffered saline to bring the final volume to 5.0 ml. The mixtures were clotted with thrombin and incubated at 37°.)

Cadaver lysin (ml.)	Lysis time
0.15	< 30 min.*
0.075	48 min.
0.0375	75 min.
0	No lysis overnight

* After lysis had occurred a second amount of 'bank' plasma (0.30 ml.) was added to this tube (thereby doubling the plasmin inhibitor); lysis again took place in 20-30 min.

Table 3. *Inhibition of plasmin by fibrinolytic cadaver 'serum'*

(Method of measurement of activity as for plasmin by method (1). 0.25 ml. Plasmin was mixed with 0.25 ml. inhibitor in (a) and (b).)

Inhibitor	Lysis time min. sec.	
(a) Fresh normal plasma		
1/50	60	0
1/100	14	15
1/200	11	0
Physiological saline	5	35
(b) Cadaver lysin		
1/12.5	18	0
1/25	17	0
1/50	11	55
1/100	7	35
1/200	6	55
Physiological saline	5	25
(c) 0.25 ml. physiological saline + 0.25 ml. cadaver lysin 1/12.5	16-19	

Cadaver lysin behaves in this respect like exercise plasma, since dilutions of lysin in mixtures containing a constant amount of plasma seem to be unaffected by any inhibitor in the plasma (see Table 2 and contrast with Table 1). Crude cadaver lysin itself contains an inhibitor to plasmin (Table 3).

Comparison of the fibrinolytic activity of cadaver lysin and plasmin tested against clots made from (a) crude plasma, (b) purified fibrinogen

Exercise plasma could not be used in this experiment because it is insufficiently active. The results in Table 4 show that the cadaver lysin was much less active than the plasmin in dissolving a clot made from purified fibrinogen but more active in dissolving a clot made from crude plasma.

Table 4. *Comparison of the fibrinolytic activity of cadaver lysin and plasmin tested against clots made from (a) crude plasma, (b) purified fibrinogen*

((a) A modification of the method of Mole (1948) described in Table 2. (b) Method as for plasmin by method (1).)

Method	Enzyme (ml.)	Lysis times	
		Cadaver lysin (min. sec.)	Plasmin (10 mg./ml. min. sec.)
(a) (Crude plasma)	0.2	45 0	No lysis
	0.1	75 0	No lysis
	0.05	100 0	No lysis
(b) (Purified fibrinogen)	0.125	17 0	2 30
	0.0625	14 0	3 55
	0.0313	15 30	6 30
	0.0156	19 0	13 30

Fig. 5. The effect of exercise plasma on fibrinogen. Blood was taken before and after exercise into an equal volume of 10% (w/v) NaCl. Plasma (0.50 ml.) + 4.50 ml. 0.167% NaCl in water bath at 37°. Zero time for reaction 5 min. after placing in bath. Thrombin (0.2 ml.) was added at times indicated on graph (a) immediately, $\times-\times$; (b) after 2½ hr., $O-O$; (c) after 5 hr., $\bullet-\bullet$. In the controls from plasma taken before exercise the clots were still intact next day.

The effect of exercise and cadaver fibrinolysins on fibrinogen

Fig. 5 shows that fibrinogen did not appear to be attacked at all in exercise plasma; the same result was obtained with cadaver lysin (Fig. 6). These experiments were made with plasma clots; Fig. 7

Fig. 6. Effect of cadaver lysin on fibrinogen in plasma. Each tube contained 4.5 ml. buffered saline, 0.30 ml. 'bank' plasma and 0.20 ml. cadaver lysin. Zero time for reaction was taken as 5 min. after placing in water bath at 37°. Thrombin (0.2 ml.) added (a) immediately, $\times-\times$; (b) after 60 min., $O-O$. In the controls without lysin, the amount of clot did not diminish during the experiment.

Fig. 7. Effect of cadaver lysin on purified fibrinogen. Each tube contained 4.0 ml. fibrinogen solution and 1.0 ml. of cadaver lysin diluted 1 in 12.5. Zero time for reaction was 5 min. after placing in water bath at 37°. Thrombin added (a) immediately, $\times-\times$; (b) after 45 min., $O-O$. In the controls without lysin the amount of clot did not diminish during the experiment.

shows the action of a very active cadaver lysin on fibrin made from purified fibrinogen. The clots were lysed completely 15–20 min. after adding the thrombin, but the amount of clot obtained and the time required for lysis were the same after a preliminary incubation of the mixture of fibrinogen and lysin for 45 min. before adding thrombin.

Fig. 8. Comparison of rates of fibrinolysis and fibrinogenolysis by plasmin. Each tube contained 4.5 ml. fibrinogen solution, and 0.5 ml. plasmin, and was placed in a water bath at 37°. Thrombin was added (a) after 5 min., $\times$ — $\times$; (b) after 30 min., $\bigcirc$ — $\bigcirc$; (c) after 60 min., Δ — Δ . Extrapolation of each curve through the values obtained for fibrin to the time of the corresponding addition of thrombin gave an estimate of the amount of unchanged fibrinogen present at that moment and enabled the rate of fibrinogenolysis to be determined. The amount of clot recovered from the control mixtures (without plasmin) did not diminish during the experiment.

In view of these results the action of plasmin on fibrinogen and fibrin was re-investigated by the method of partial lysis. The rate of destruction of fibrin was found to be approximately 2.5 times as fast as the rate of destruction of fibrinogen (see Fig. 8).

Comparison of the properties of cadaver lysin and plasmin

pH optimum. The method of determination of enzymic activity was that of Rocha e Silva & Rimington (1948) as described above for plasmin (method 1); it was chosen since the whole determination is completed in about 15 min., thus

minimizing the effects of variation in enzyme stability at different pH values (Table 5).

Neither plasmin nor cadaver lysin show a well defined optimum; the most that can be said is that, over the range of pH values 6.5–8.5, plasmin is more active at the acid end of the range and cadaver lysin at the alkaline end. In view of the greater ease in observing the clots in the range pH 7.0–7.4, the pH used in all other determinations of activity was 7.2–7.4.

pH of optimum stability. Plasmin was heated to 37° for 30 min. in veronal:acetate:HCl buffers (Michaelis, 1931) at varying pH. After neutralization the solutions were tested by the method of Rocha e Silva & Rimington (1948). It was found that plasmin was most stable over the range pH 7.0–8.0 in good accord with Christensen & MacLeod (1945) who found pH 7.0–7.4. The amounts of cadaver lysin available were sufficient for only a much smaller experiment and attention was therefore concentrated on Mole's (1948) statement that cadaver lysin had the following properties: 'at pH 3–4 little activity was lost in 1 min. at 90°, while at pH 7.4 there was partial loss of activity at 60° for 10 min. and complete inactivation at 70°.'

The comparative stability of cadaver lysin at acid pH values was not confirmed. The results in Table 6 indicate that the loss in activity of cadaver lysin, crude or partially purified, was probably at least as great as that of plasmin (about 90 %).

As the operation of heating to 90° for 1 min. is so difficult to control, heating in a water bath at 45° for 30 min. was tried. Since the results in the previous experiment had differed from those of Mole (1948), it was considered that the test for activity should be the same as that used by Mole, namely lysis of plasma clots. The same conclusion was drawn from this experiment (see Table 7) as the previous one, namely that cadaver lysin was much less stable at pH 3–4 than at pH 7.0.

Fractionation of cadaver lysin. Mole (1948) stated that cadaver lysin differed from plasmin in that it was not found in the euglobulin fraction. The present experiments have not supported this statement since good recovery was found after either dilution with 20 vol. of distilled water and saturation with carbon dioxide or precipitation with ammonium sulphate at pH 7.0 (33 or 50 % saturation).

Is fibrinolytic activity due to a kinase?

Since Astrup has described a fibrinokinase present in tissue (Astrup & Permin, 1947; Astrup, 1951; Astrup & Stage, 1952), the effect of the presence of haemolysed red cells in a fibrin clot was investigated with the idea in mind that haemolysis might liberate a kinase. The result was completely

Table 5. pH of optimum activity of plasmin and cadaver lysin

(Buffer: sodium veronal:acetate:HCl (Michaelis, 1931). Fibrinogen (5.2 mg./ml.) in saline; samples were diluted with 3 vol. of the appropriate buffer and the pH values determined by glass electrode. The enzyme was diluted in the same buffer. The enzymic activity was determined by the method of Rocha e Silva & Rimington (1948) as described for plasmin method 1.)

		Dilution of enzyme														
		2		4		8		16		32		64		128		
		Lysis times														
Enzyme	Expt. no.	pH	min.	sec.	min.	sec.	min.	sec.	min.	sec.	min.	sec.	min.	sec.		
Plasmin	1	6.8	1	30	2	10	3	45	7	05	14	10	—	—		
		7.3	1	30	2	15	3	55	6	55	13	15	—	—		
		8.0	1	25	2	30	3	35	6	45	13	10	—	—		
		8.9	Clots all difficult to see and lysis times could not be determined													
	2	6.6*	—	—	1	5	3	5	7	20	17	30	—	—		
		7.3	—	—	2	40	4	20	9	50	19	50	—	—		
		8.4	2	20	3	15	5	25	11	15	—	—	—	—		
Cadaver lysin	1	6.6	—	—	No clot at 12 min.				(11 35)		15	35	25	0		
		7.1	—	—	(7 0)		5	10	6	5	9	5	14	50	21	30
		8.3	—	—	No clot seen				(5 20)		12	10	10	5	15	0
	2	6.7	—	—	(11 10)		9	10	10	5	13	25	18	30	30	0
		7.0	—	—	8	50	9	35	8	55	10	45	17	50	24	0
		7.2	—	—	8	30	6	25	7	5	10	0	13	10	21	45
		7.7	—	—	No clot at 5 min.				7	5	8	45	12	50	21	45
		8.0	—	—	No clot at 5 min.				7-8	—	17	0	(13 0)	21	0	

* At lower pH values the clot was unsatisfactory.

Table 6. Effect of heating acidified solutions of cadaver lysin and plasmin to 90° for 1 min. (Mole, 1948)

(The enzyme preparations used were (i) cadaver lysin, (ii) the euglobulin fraction of cadaver lysin and (iii) plasmin. Glass tubes 6 cm. long, 1.5 cm. diameter, walls 1 mm. thick, were used; 2.0 ml. solution in these tubes reached a temperature of 83° after plunging into a water bath at 90° for 1 min. 2 ml. amounts of enzyme solution were adjusted to pH 3-4 (glass electrode) by the addition of 0.1 N-HCl, heated as described above, cooled rapidly and the pH values readjusted to 7.2-7.4. Volumes were adjusted to 4.0 ml. and the enzymic activities determined by the method of Rocha e Silva & Rimington (1948) as described for plasmin (method 1).)

Enzyme	Dilution	Lysis times	
		Acidified and heated solutions	Unheated controls (min.)
Cadaver lysin	1	No lysis after 1 hr.	32-35
	2		Approx. 25
	4		Approx. 25
Euglobulin fraction of cadaver lysin	1	No lysis after 1 hr.	7-10
	2		9-11
	4		12-14
Plasmin		min.	min. sec.
	1	19	—
	2	24	2 5
	4	(30)	3 25
	8	(60)	6 35
	16	—	13 30

negative. The presence or absence of fresh platelets had no influence on the lysis time of plasma clots by cadaver lysin. Attempts were made to prepare artificial fibrinolytic plasma by adding streptokinase to fresh normal plasma, but the large variation in antistreptokinase in different specimens made the method impracticable. In the course of

these experiments it was found that small amounts of streptokinase appear to be without effect on fibrinogen until clotting has taken place, while large amounts of streptokinase could activate the plasminogen before clotting. These observations were made possible by the use of refined concentrated antistreptococcal serum.

Table 7. *Effect of heating acidified cadaver lysins*

(The treated and control solutions were tested by the method of Mole (1948) as described in Table 2.)

Treatment	Volume of lysin used in test (ml.)	Lysis time
Lysin (2.0 ml.) acidified to pH 3-4, heated at 45° for 30 min. and neutralized. Vol. adjusted to 6.0 ml.	0.6	> 3.75 < 6 hr.
Lysin (2.0 ml.) at own pH heated to 45° for 30 min. and neutralized. Vol. adjusted to 6.0 ml.	0.6	70 min.
Lysin control, untreated	0.2	65 min.
Saline control	—	No lysis (24 hr.)

DISCUSSION

Difference between 'calcium clots' and 'thrombin clots'

Macfarlane & Biggs (1946) and Bidwell & Macfarlane (1951) reported that plasma clotted by calcium ions lysed more slowly than plasma clotted by thrombin. It has been shown that this difference is not due to contamination of the thrombin by plasmin, nor, probably, to direct inhibition by calcium ions. Fearnley & Tweed (1953), who have also remarked on this difference, attributed it to the interaction of calcium and 'another factor'. Lorand (1948, 1950, 1951) has provided good evidence for his suggestion that plasma clots made in the presence of calcium contain bonds which are not found in plasma clots made in the absence of calcium; such bonds would explain the greater resistance of 'plasma calcium clots' to fibrinolysis, and may provide the second factor suggested by Fearnley and Tweed. The fibrinolytic activity of purified thrombin described by Guest & Ware (1950) was manifested only by such highly concentrated thrombin (of the order of 10000 units/ml.) that it is improbable that it could have had any effect in these experiments.

Difficulties in measuring the degree of fibrinolytic activity

Fearnley and co-workers (1952, 1953) and True-love (1953) have commented on the lability of the fibrinolytic phenomenon in plasma. Their method of assessing the degree of fibrinolytic activity is based on the work of Macfarlane (1937) and Macfarlane & Biggs (1946), and depends on the number of mixtures which lyse when a series of plasma dilutions are clotted by thrombin or calcium. Thus the mixtures differ not only in concentration of the active agent but also in all other constituents of the plasma, including the substrate and possible inhibitors. It

is desirable to take a fixed dilution of plasma so as to control these factors. Unfortunately the most obvious method of determining enzymic activity, i.e. the determination of the amount of substrate changed after a given time, is precluded by the observation that digestion of clot is preceded by a variable time lag (Fig. 2). The total lysis time is probably some inverse function of the fibrinolytic activity and as yet provides the best method of measurement despite the disadvantage of requiring complete solution of the substrate. At present it appears that any method of comparing activity in specimens from different individuals can be only approximate as there will undoubtedly be differences in substrate concentration and possibly other relevant factors influencing lysis time.

Comparison of fibrinolysis by plasmin, exercise plasma and cadaver lysin

Normal plasma did not inhibit fibrinolysis by natural or cadaver lysin but was strongly inhibitory to plasmin. The ratios of activities of given quantities of cadaver lysin and plasmin on fibrinogen and plasma clots were different. Several authors have stated that plasmin attacks fibrinogen as readily as it attacks fibrin (e.g. Christensen, 1945; Permin, 1949; Westphal, Kocholaty, McDonald, King & Jensen, 1950), although Fantl & Simon (1948), investigating fibrinolysis following electrically induced convulsions, found that the fibrinogen was apparently unattacked. Experiments described above have shown that natural lysin and cadaver lysin fail to digest fibrinogen, while plasmin digests fibrinogen although at a slower rate than it digests fibrin. These results are not in conflict with those of Christensen (1946). He found identical rates of action of plasmin on fibrinogen and lysed clot, while here it is the rates of action of fibrinogen and of unlysed fibrin which are compared.

Mole (1948) found that cadaver lysin differed from plasmin as described in the literature by Christensen (1945, 1946) and others. Some of the properties of plasmin and cadaver lysin have now been compared under the same experimental conditions. Although confirmation has not been obtained of some of the most striking differences between cadaver lysin and plasmin reported by Mole (1948), his conclusion that the two are different entities is probably correct.

It appeared from the experiments reported above that the fibrinolysin produced *in vivo* is probably the same as that in post-mortem blood, although the activity is very much greater in the latter; neither system behaves as if the effect were due to an excess of plasmin preformed in the plasma, although the possibility that the actual lysis might be due to plasmin produced by an activator during or following clotting is not excluded.

Attempts to establish the existence of a kinase in fibrinolytic plasma have as yet been inconclusive. It may be relevant to note that under certain conditions the streptokinase-plasminogen-fibrinogen system can behave like plasma fibrinolysin in becoming demonstrable only after the fibrinogen has clotted, and that Williams (1951) suggested that the fibrinolytic activity of urine noted by Macfarlane & Pilling (1947) might be due to a kinase capable of activating plasminogen present as a contaminant in the substrate.

SUMMARY

1. Fibrinolytic plasma clots made in the presence of free calcium lyse less rapidly than those made in its absence. The effect is not due to activation by excess citrate nor, probably, to a direct inhibition by calcium ions.

2. Fibrin clots made from exercise plasma remain constant in amount for a variable time and then lyse completely within a comparatively short time.

3. In contrast to plasmin prepared by chloroform

activation, samples of lysin from living subjects and from cadavers were not inhibited by normal plasma and did not digest fibrinogen.

4. The ratios of activities of given quantities of cadaver lysin and plasmin on fibrinogen and plasma clots were different.

5. Cadaver lysin and plasmin could not be differentiated with regard to optimum pH of activity and stability; both were found in the euglobulin fraction.

6. Fibrinolysin in plasma from living subjects and cadaver lysin may differ only quantitatively; neither is identical with plasmin. Attempts to demonstrate a kinase have been inconclusive.

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