The Activation of Staphylococcal Free Coagulase by Plasma Constituents and the Hydrolysis of N^{α} -Toluene-*p*-Sulphonyl-L-Arginine Methyl Ester (TAME) by Activated Coagulase

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Staphylococcal coagulase, first described by Loeb (1903), was shown by Smith & Hale (1944) to bring about the coagulation of fibrinogen only in the presence of a third factor, found in the plasma of certain animals (Duthie & Lorenz, 1952). This third factor was originally called coagulase activator by Smith & Hale, but Tager (1948) objected to the use of this term on the grounds that the word activator implied an unproven kinase-like action on coagulase, and called the substance coagulase-reacting factor. Miale (1949) referred to the same substance as coagulase globulin and called the active product coagulase thrombin. The actual identity of the factor and its relation to the known components of the physiological blood-clotting mechanism has remained in doubt since it was first described.

All preparations of human prothrombin so far examined have contained coagulase activator and it has been suggested that the two may be identical (Tager, 1956*a*; Duthie & Lorenz, 1952). However, Tager (1956*a*) has obtained from human plasma a preparation of coagulase activator which was free from prothrombin activity and which had electrophoretic and sedimentation properties different from those of purified prothrombin.

Little is known of the nature of the reaction between coagulase and coagulase activator which produces an active fibrinogen-clotting material. It has been reported that when the two materials are incubated together, there is a progressive increase in the clotting activity of the mixture (Smith & Hale, 1944; Duthie & Lorenz, 1952), but the reaction has not been studied in detail. It is the purpose of this paper to describe an investigation of the kinetics of this reaction, and to demonstrate that the active fibrinogen-clotting product (here called activated coagulase) resembles human thrombin in certain respects.

Sherry & Troll (1954) have shown that the synthetic substrate N^{α} -toluene-*p*-sulphonyl-L-arginine methyl ester is hydrolysed by human thrombin. They stated that the compound competitively inhibited the clotting of fibrinogen by thrombin, and from this they deduced that the

same active site was involved in both reactions. The present studies show that activated coagulase reacts in a similar manner.

MATERIALS AND METHODS

Coagulase. An electrophoretically homogeneous sample prepared from cultures of *Staphylococcus aureus* strain Newman (N.C.T.C. 8178), which had a specific activity of 430 units/mg. of protein, was used in the clotting experiments. This preparation, at a concentration of 75 μ g./ml., clotted human citrated plasma in 24 hr. at 37° (Duthie & Haughton, 1958). A second preparation having a specific activity of 220 units/mg. of protein was used in the N^{α} -toluene-*p*-sulphonyl-L-arginine methyl ester (TAME) hydrolysis experiments.

Coagulase activator. Prothrombin prepared from human citrated plasma by the method described by Biggs & Macfarlane (1957) was a good source of coagulase activator and was used as such. This preparation contained at least four electrophoretic components when examined in the Perkins-Elmer Tiselius apparatus at pH 6.8 in Na_2HPO_4 -citric acid buffer, *I* 0.1.

Bovine fibrinogen. Bovine plasma fraction I, supplied by Armour Ltd., Hampden Park, Eastbourne, Sussex, was dissolved in 0.9% NaCl soln. and used at a concentration of 0.4%.

Human fibrinogen. Freeze-dried preparations prepared by ether-fractionation of time-expired human plasma (Kekwick & Mackay, 1954) were obtained from the Blood Products Laboratory, Lister Institute, Elstree, Herts.

Plasminogen. This was prepared by the method of Milstone (1941) from human citrated plasma.

Streptokinase. This was supplied by the Lederle Laboratories Division of the American Cyanamid Co., New York, U.S.A.

Soya-bean trypsin inhibitor. This was obtained from the Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.

N^{α} -toluene-*p*-sulphonyl-L-arginine methyl ester. TAME was prepared by Dr I. Campbell of the Department of Chemistry, Southampton University after the method given by Bergmann, Fruton & Pollok (1939) and Troll, Sherry & Wachman (1954).

Buffers. All dilutions of coagulase and coagulase activator were made in 0.07M- Na_2HPO_4 - KH_2PO_4 buffer, pH 6.8 (Clark, 1928), in the clotting experiments. 2-Amino-2-hydroxymethylpropane-1.3-diol (tris; 0.02M, pH 8.5) was used in the TAME hydrolysis experiments.

Coagulase assay. Coagulase was assayed by the method already described (Duthie & Haughton, 1958).

Coagulase activator assay. Coagulase activator was measured as follows: 0.2 ml. of human fibrinogen (0.3%), 0.1 ml. of suitable dilutions of the coagulase activator and one drop of 6% (w/v) Filter-Cel (Johns Manville, Artillery Row, London) were placed in small tubes (5 cm. $\times$ 0.8 cm.) and warmed to 37°. Coagulase (0.2 ml. containing 2 units/ml.) was added to each tube in turn and the tube was tapped slowly, while viewed against a dark background, until clumping of the particles was seen. The clotting time was measured and referred to the curve given by dilutions of fresh normal human plasma, which was assigned the arbitrary value of 200 units/ml.

Activated coagulase assay. A mixture consisting of 2 ml. of coagulase (24 units/ml.) and 2 ml. of coagulase activator (40 units/ml.) was incubated at 37° for 30 min., after which the clotting activity remained relatively stable (see Results section). Dilutions of this mixture were made in 0.07M-phosphate buffer, pH 6.8, and 0.2 ml. amounts immediately added to equal volumes of 0.4% bovine fibrinogen previously warmed to 37°. The straight line obtained when the reciprocal of the concentration was plotted against the clotting time is shown in Fig. 1 and corresponds to the equation $T - 6 = K/x$, where T is the clotting time in seconds, x is the concentration of activated coagulase and K is a constant. One unit of activated coagulase was defined as the amount present in 1 ml. of 0.07M-phosphate, pH 6.8, which when added to an equal volume of 0.4% bovine fibrinogen in 0.9% NaCl soln. at 37° caused a clot in 20 sec. The equation now becomes $T - 6 = 14/x^*$, where x^* is the activated coagulase concentration in units/ml.

Measurement of hydrolysis of N α -toluene-p-sulphonyl-L-arginine methyl ester. TAME hydrolysis was measured by an electrometric formol-titration method. In all experiments, except when otherwise stated, the following conditions were used. Tubes containing 2 ml. of 0.025M-TAME buffered with tris (0.02M, pH 8.5) were brought to 37°.

Fig. 1. Dilution curve of activated coagulase. Activated coagulase (0.2 ml.) was added to 0.2 ml. of 0.4% bovine fibrinogen previously warmed to 37° and the clotting time was measured.

Enzyme (0.5 ml.) was added, and samples (1 ml.) were taken immediately and after the required incubation period and added to an equal volume of 40% (w/v) formaldehyde soln. in tubes measuring 5.0 cm. $\times$ 1.8 cm. The formalized samples were titrated against 0.01N-NaOH, a Pye Master pH meter being used to follow the titration and the mixture being stirred continuously by a slow stream of oxygen. Results were expressed as μ moles of acid liberated/ml. of mixture/min., by comparison of the initial and final titration curves.

RESULTS

Nature of the reaction between coagulase and coagulase activator

Preliminary experiments showed that when activator was added to free coagulase and incubated at 37°, the clotting activity of the mixture at first increased and then declined. This indicated that the active clotting material formed (here called activated coagulase) was unstable at this temperature. The reaction was studied by incubating mixtures containing varying proportions of coagulase and activator in 0.07M-phosphate buffer, pH 6.8, and periodically measuring the concentration of activated coagulase present in the mixtures. At intervals three samples were taken from each tube; an equal volume of buffer or coagulase or activator was added, the sample mixtures were incubated for a further 10–15 min. and the activated coagulase present was measured. This test was designed to provide information on the relative concentrations of coagulase and activator present in the mixture at a given time. (i) If coagulase were present in great excess, the addition of further coagulase would not be expected to lead to an immediate increase in the concentration of activated coagulase present and the effect of adding free coagulase would then be similar to that of adding buffer. The addition of activator to a mixture containing an excess of free coagulase, however, would be expected to lead to a rapid increase in the concentration of activated coagulase. (ii) If a large excess of activator were present, the results expected would be the reverse of the above.

Measurements made during the course of one typical experiment are presented in Table 1. It is clear that after incubation for 6 hr. the reaction mixture contained an excess of free coagulase, since the addition of more activator resulted in a sharp increase in the concentration of activated coagulase present, whereas addition of more free coagulase resulted in a decrease. After incubation for 70 hr., however, the addition of activator to the mixture resulted in a decrease in the concentration of activated coagulase. This decrease was similar to the fall seen when the mixture was diluted with an equal volume of buffer. When free coagulase was

added at this stage, there was a sharp rise in the concentration of activated coagulase present. Thus one may conclude that there was progressive destruction of free coagulase in the incubation mixture, and that some activator was still present in the mixture after all the free coagulase had been destroyed. This destruction was not due to plasmin present in the activator preparation, since it was found that the course of the reaction was not modified by the addition of soya-bean trypsin inhibitor to a final concentration of 200 $\mu\text{g./ml.}$ This material is a potent inhibitor of plasmin (Grob, 1949).

Two series of experiments were then performed. In the first (Table 2), falling concentrations of

coagulase were incubated at 37° with constant amounts of activator. In the second series (Table 3), falling concentrations of activator were incubated with constant amounts of coagulase. It can be seen that in all cases there was an initial increase in the concentration of activated coagulase in the mixtures, followed by a decrease. The time taken before the beginning of the decrease is a measure of the time taken for completion of the reaction, and it was found that this was prolonged as either the initial activator concentration decreased, or the initial coagulase concentration increased.

After incubation for 70 hr., the mixtures represented in Table 2 were divided into three equal parts. To one was added an equal volume of

Table 1. *Concentration of activated coagulase in a mixture*

The mixture contained free coagulase (50 units/ml.) and coagulase activator (40 units/ml.) in 0.07 M- Na_2HPO_4 - KH_2PO_4 buffer, pH 6.8 (Clark, 1928), as a function of incubation time at 37°.

Incubation time of mixture (hr.)	Concn. of activated coagulase in mixture (units/ml.)	Concn. of activated coagulase (units/ml.) 10 min. after the addition of a sample of the mixture to an equal volume of		
		Coagulase (50 units/ml.)	Activator (80 units/ml.)	Phosphate buffer, pH 6.8
6.0	7.0	3.5*	11.5*	3.5*
70.0	0.3	3.5	0.2	0.2

* Activated coagulase concentrations in these three mixtures after a further incubation for 15.5 hr. at 37° were 3.5, 1.3 and 3.3 units/ml. (columns 3-5 respectively).

Table 2. *Formation and destruction of activated coagulase in mixtures containing a constant amount of activator and varying amounts of free coagulase*

Values given are for the concentration of activated coagulase (units/ml.).

Incubation time of mixture	Concn. of coagulase (units/ml.) added to an equal volume of activator (80 units/ml.)						
	96	48	24	12	6	3	1.5
20 sec.	7	3.5	2.3	1.5	0.8	0.46	0.28
5 min.	7	7	7	2.3	0.9	0.51	0.35
15 min.	7	7	7	2.3	0.9	0.54	0.34
2.5 hr.	7	7	5	2	0.8	0.48	0.28
21 hr.	7	2.8	0.6	0.25	0.1	0.07	0.04
31.5 hr.	2	1.0	0.35	0.17	0.05	0.04	0.02
46.5 hr.	0.67	0.4	0.2	—	—	—	—
70 hr.	0.3	0.2	—	—	—	—	—

Table 3. *Formation and destruction of activated coagulase in mixtures containing a constant amount of free coagulase and varying amounts of activator*

Values given are for concentration of activated coagulase (units/ml.).

Time of incubation of mixture	Concn. of activator (units/ml.) added to an equal volume of coagulase (24 units/ml.)							
	80	40	27	20	13.5	10	6.7	5.0
20 sec.	4.7	1.6	1.2	0.9	0.7	0.54	0.47	0.38
5 min.	7	2.3	1.4	0.9	0.64	0.52	0.42	0.28
20 min.	7	2.8	1.7	1.1	0.67	0.56	0.42	0.33
2.5 hr.	7	2.8	1.6	1.2	0.80	0.54	0.42	0.32
18 hr.	1.1	1.6	2.0	1.6	0.85	0.52	0.38	0.28
28.5 hr.	0.6	0.8	1.3	1.7	1.2	0.60	0.44	0.32
43.5 hr.	0.31	0.33	0.47	0.67	1.3	0.9	0.50	0.37
67 hr.	0.12	0.14	0.17	0.2	0.33	0.56	0.70	0.50

buffer, to the second an equal volume of activator (80 units/ml.) and to the third an equal volume of coagulase (24 units/ml.). The mixtures were incubated at 37° for 10 min. and the activated coagulase concentration was measured. The results obtained are given in Table 4.

It was found that the addition of activator or buffer caused no increase in activated coagulase concentration, whereas a sharp rise occurred when coagulase was added; the same amount of activity developed in each tube. It should be noted that 64 times as much coagulase had been destroyed in the first tube of the series as in the last. Furthermore, when an equal volume of coagulase (24 units/ml.) was added to a fresh sample of activator (40 units/ml.) it was found that the same concentration of activated coagulase was formed in this mixture as in each of the other tubes. This experiment was repeated with three different batches of coagulase and three different preparations of activator. A similar result was obtained in each case. It was concluded that when coagulase and activator are incubated together the coagulase is destroyed, whereas the activator is present at the end of the reaction in unreduced concentration.

Samples were also taken from the mixtures represented in Table 3 after incubation for 30 and 69 hr. Equal volumes of coagulase (24 units/ml.), activator (80 units/ml.) or buffer were added and the tubes incubated for a further 10 min. at 37°. Activated coagulase was then assayed in each. The results, which are given in Table 5, provide further evidence of progressive destruction of free coagulase during the reaction. After incubation for 30 hr., the first four tubes of the series contained

excess of activator and the last four contained excess of free coagulase. However, after incubation for 69 hr. only the last two tubes contained an excess of free coagulase and all the others contained excess of activator.

Effect of pH on the activation of free coagulase

Solutions of free coagulase (1 unit/ml.) and activator (8 units/ml.), in 0.07 M-KH₂PO₄-Na₂HPO₄ buffer, were adjusted to the required pH with 0.2 N-NaOH or -HCl and mixed in equal volumes. The mixtures were incubated at 37° for 30 min. and

Fig. 2. Effect of pH on the activation of free coagulase in 0.07 M-KH₂PO₄-Na₂HPO₄ buffer. The conditions were as described in the text.

Table 4. Activated coagulase formed in the mixtures detailed in Table 2 when free coagulase or activator is added after 70 hr. at 37°

Substance added to an equal vol. of the mixture after incubation for 70 hr.	Concn. of coagulase (units/ml.) initially added to an equal volume of activator (80 units/ml.) (see Table 2)							Control: fresh activator (40 units/ml.)
	96	48	24	12	6	3	1.5	
Buffer	0.19	0.13	0.06	0.03	0.03	0.03	0.03	0.03
Activator (80 units/ml.)	0.20	0.14	0.06	0.03	0.03	0.03	0.03	0.03
Coagulase (24 units/ml.)	3.5	2.8	2.8	2.8	2.8	2.8	2.8	2.8

Table 5. Activated coagulase formed in the mixtures detailed in Table 3 when free coagulase or activator is added to samples after 30 and 69 hr. at 37°

Incubation time of mixture (hr.)		Substance added to an equal vol. of the mixture	Concn. of activator (units/ml.) initially added to an equal volume of coagulase (24 units/ml.) (see Table 3)							
			80	40	27	20	13.5	10	6.7	5.0
30		Coagulase (24 units/ml.)	4.7	2.0	1.6	1.3	1.0	0.8	0.47	0.33
		Activator (80 units/ml.)	0.28	0.28	0.47	0.8	1.7	2.0	2.0	2.3
69		Coagulase (24 units/ml.)	2.3	1.3	0.9	0.72	0.60	0.56	0.48	0.48
		Activator (80 units/ml.)	0.08	0.11	0.11	0.12	0.17	0.29	0.82	1.1
		Buffer	0.08	0.10	0.10	0.11	0.14	0.28	0.35	0.28

the pH was adjusted to 6.8. Activated coagulase was then measured and the results were plotted against initial pH (Fig. 2). It was found that the rate of formation of activated coagulase from free coagulase and activator in 0.07 M-phosphate buffer showed a sharp optimum at pH 6.8.

Demonstration of esterase activity in activated coagulase

Volumes (2 ml.) of 0.025 M-TAME buffered to pH 8.5 with tris were warmed to 37°. To three tubes were added: (1) 0.5 ml. of coagulase (12.5 units/ml.); (2) 0.5 ml. of coagulase activator (20 units/ml.) and (3) 0.5 ml. of activated coagulase prepared by incubating a mixture of equal volumes of coagulase (25 units/ml.) and activator (40 units/ml.) for 30 min. at 37°. It was found (Table 6) that hydrolysis occurred only in the presence of activated coagulase.

In order to eliminate the possibility that the hydrolysis observed might be due to the activation of plasminogen in the admittedly impure activator preparation, a potent preparation of plasminogen (Milstone, 1941) was activated with streptokinase. Hydrolysis of TAME by this preparation was found to be 25% inhibited by 40 µg. of soya-bean trypsin inhibitor/ml. and 75% inhibited by 400 µg./ml. Activated coagulase, in contrast, was not detectably inhibited by 400 µg. of soya-bean trypsin inhibitor/ml. (Table 6).

Influence of pH on the hydrolysis of N^α-toluene-p-sulphonyl-L-arginine methyl ester by activated coagulase

Because of the low buffering capacity of tris below pH 7.5 it was necessary to use a mixed buffer for this experiment. The buffer used contained 0.03 M-tris and 0.01 M-Na₂HPO₄-KH₂PO₄ (Clark, 1928). Mixtures containing 3 ml. of this buffer and 1 ml. of 0.1 M-TAME were adjusted to the required pH, and divided into two equal parts and warmed to 37°. To one set of tubes was added 0.5 ml. of water and to the other set 0.5 ml. of activated coagulase. Samples (1 ml.) were taken

immediately from each tube and, after incubation for 30 min., were added to equal volumes of 40% (w/v) formaldehyde soln. All samples were titrated against 0.01 N-NaOH and the spontaneous hydrolysis occurring in the control tube was subtracted from that occurring in the presence of the enzyme. The results obtained over the range pH 5.5–10.0 are shown in Fig. 3. The graph shows a sharp optimum at pH 8.5.

Determination of K_m for the hydrolysis of N^α-toluene-p-sulphonyl-L-arginine methyl ester by activated coagulase

Reaction velocities were measured over a 50-fold range of substrate concentrations. For this series of experiments the reaction time (normally 30 min.)

Fig. 3. Effect of pH on the rate of hydrolysis of TAME by activated coagulase. Experimental conditions were as described in the text.

Table 6. *Hydrolysis of N^α-toluene-p-sulphonyl-L-arginine methyl ester by activated coagulase and by plasmin, and the effect of soya-bean trypsin inhibitor*

Standard conditions were used as described in the text; results are expressed as µmole of acid liberated/min./ml. of mixture. Plasmin consisted of a mixture of equal volumes of streptokinase, 100 000 units/ml., and plasminogen. Activated coagulase was a mixture of equal volumes of coagulase (25 units/ml.) and activator (40 units/ml.) maintained at 37° for 30 min. before use.

Test substance	Soya-bean trypsin inhibitor (µg./ml.) present in final mixture		
	Nil	40 µg./ml.	400 µg./ml.
Coagulase (25 units/ml.)	0.01	—	—
Activator (40 units/ml.)	0.01	—	—
Activated coagulase	0.37	0.37	0.38
Plasmin	0.17	0.13	0.05

was reduced to 5 min. This shortening of the reaction time had three advantages: (i) The amount of substrate hydrolysed was small compared with the initial concentration. (ii) Errors due to spontaneous hydrolysis of TAME were reduced. (iii) Errors due to instability of activated coagulase were reduced.

The results obtained are presented in Fig. 4 by the method of Lineweaver & Burk (1934), and correspond to a K_m of $3.3 \times 10^{-3} M$. Conditions used were: 0.015 M-tris, pH 8.5 ± 0.05 ; activated coagulase concentration 3.5 units/ml. (measured at pH 6.8); temperature 37° ; reaction time 5 min.

Inhibition by N^α -toluene-p-sulphonyl-L-arginine methyl ester of the clotting of human plasma by coagulase

TAME was found to be a powerful inhibitor of clotting of plasma by coagulase down to a concentration $10^{-4} M$. In the experiment shown in Fig. 5, 0.2 ml. amounts of coagulase (0.5 unit/ml.) were added to 0.2 ml. amounts of oxalated human plasma diluted 1:4 and containing increasing concentrations of TAME; 0.02 ml. of 6% (w/v) Filter-Cel was added before mixing and the clotting time measured as in the coagulase-assay test (Duthie & Haughton, 1958). By comparing the clotting time with the standard coagulase-assay curve, the percentage inhibition of coagulase by TAME at each concentration was measured. The percentage inhibition of coagulase, when plotted against the negative logarithm of the TAME concentration (Fig. 5), gave a straight line. This observation is consistent with the view that TAME was acting as a competitive inhibitor.

The converse experiment, i.e. the inhibition of TAME hydrolysis by fibrinogen, was not possible owing to coprecipitation of TAME and fibrinogen at the relatively high TAME concentration needed.

DISCUSSION

Nature of coagulase activation

The present study is concerned with the interaction of coagulase and the coagulase activator present in human plasma, and not with the exact identity of the latter. When Smith & Hale (1944) first described coagulase activator they suggested that it was a kinase converting coagulase into an active material which in turn reacted with fibrinogen. In a later study (Smith, Hale & Smith, 1947) they proposed the alternative hypothesis, i.e. that coagulase is a kinase converting activator into the clotting material. Tager (1956b) went somewhat further by suggesting that coagulase acts on prothrombin in a manner similar to thrombokinas. No real evidence has been advanced for any of these views.

Fig. 4. Double reciprocal plot of substrate concentration against reaction velocity for the hydrolysis of TAME by activated coagulase. Standard conditions were used as described in the text. The ordinate values ($1/v$) are the reciprocals of μ moles of acid liberated/min./ml. of reaction mixture. The abscissa values ($10^{-2}/S$) are the reciprocals of TAME molarity in the mixtures.

Fig. 5. Inhibition by TAME of the clotting of human plasma by free coagulase at 37° . Free coagulase (0.2 ml.; 0.5 unit/ml.) was added to 0.2 ml. of human plasma diluted 1:4 in 0.9% NaCl soln. and containing 0.02 ml. of 6% (w/v) Filter-Cel and TAME at twice the required final concentration. The clotting time was measured and inhibition assessed by reference to a standard coagulase-dilution curve.

We have found that when purified staphylococcal free coagulase was mixed with coagulase activator (Tables 2 and 3), the fibrinogen-clotting activity of the mixtures (i.e. the concentration of activated coagulase present) increased to a maximum and then fell. The rate at which this maximum was reached was then a measure of the time taken to exhaust one of the reagents. When the initial coagulase concentration was kept constant and the activator was varied (Table 3), it was found that the maximum clotting activity was reached more rapidly the higher the initial activator concentration. Conversely when a constant amount of activator was incubated with varying amounts of coagulase (Table 2), maximum clotting activity was reached more rapidly the lower the initial concentration of coagulase. Finally, when the mixtures had been incubated for about 3 days (Tables 4 and 5), those in which the clotting activity had passed its maximum, i.e. mixtures in which one of the reagents had been exhausted, always contained coagulase activator but no coagulase. This was demonstrated by a marked increase in activity when more coagulase was added, whereas the addition of more activator gave no increase. Furthermore, when the same amount of coagulase was added to each the activated coagulase produced reached the same concentrations in all tubes (Table 4), showing that the same amount of activator was still present in all of them, although 64 times as much coagulase had been consumed in the first tube as in the last.

These results are consistent with the hypothesis that coagulase as liberated by staphylococci is enzymically altered by coagulase activator, and that during this reaction an active fibrinogen-clotting material is formed. Our results do not, however, provide conclusive evidence that the active clotting material obtained is a breakdown product of coagulase. There are two possible interpretations which may be represented as:

where A is activator, C is coagulase, C' is an inactive breakdown product of coagulase and the coagulase-activator complex (AC) is the active clotting material;

where C'' is the unstable active clotting material derived solely from coagulase and D is an inactive breakdown product of it.

These two alternatives are kinetically indistinguishable at present and a final answer to this question depends on isolation of the active clotting material. It is certain, however, that coagulase activator is not permanently altered in the reaction and that coagulase is destroyed. It is therefore justifiable to regard the reaction as being

of an enzymic nature, coagulase being the substrate and activator the enzyme. Since the activator preparation used was not homogeneous, coagulase may have been destroyed by some factor other than activator. Plasmin, however, was not involved since destruction of coagulase occurred in the presence of soya-bean inhibitor.

Hydrolysis of N α -toluene-p-sulphonyl-L-arginine methyl ester by activated coagulase

Sherry & Troll (1954) have shown that the synthetic amino acid ester TAME is hydrolysed by both plasmin and thrombin. In the present study it has been shown that a similar hydrolysis is brought about by activated coagulase, but not by coagulase which has not been activated, a finding previously noted by Tager (1956b). In addition, clotting by both thrombin and activated coagulase are inhibited by low concentrations of TAME, all of which suggests that they act on fibrinogen in a similar manner. More definite evidence, which yet remains to be obtained, would depend on showing that identical fibrino-peptides (Lorand, 1954) are liberated by both coagulants.

Another similarity between the hydrolysis of TAME by thrombin and by activated coagulase is the pH optimum. Thrombin was reported as having a pH optimum of 8.0 by Ronwin (1957) and of 9.0 by Sherry & Troll (1954). We have found that TAME is most rapidly hydrolysed by activated coagulase at pH 8.5. The Michaelis constants for both reactions are also very similar. TAME is shown to inhibit the clotting of plasma by free coagulase down to a concentration of 10^{-4} M, which may suggest that the same active site is involved in both the clotting of fibrinogen and in the hydrolysis of TAME.

SUMMARY

1. From a study made of the interaction between staphylococcal-free coagulase and the activator found in human plasma it appears that coagulase is enzymically degraded by coagulase activator.

2. The active clotting material produced is either the enzyme-substrate complex or a degradation product of coagulase.

3. In 0.07 M- Na_2HPO_4 - KH_2PO_4 at 37° , activation of coagulase proceeds most rapidly at pH 6.8.

4. Activated coagulase has been shown to hydrolyse the synthetic substrate N α -toluene-p-sulphonyl-L-arginine methyl ester.

5. Hydrolysis of N α -toluene-p-sulphonyl-L-arginine methyl ester occurs most rapidly at pH 8.5, and a Michaelis constant of 3.3×10^{-3} M has been calculated.

6. Concentrations of N α -toluene-p-sulphonyl-L-arginine methyl ester down to 10^{-4} M have been shown to inhibit the clotting of human plasma by free coagulase.

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Tissue Components of the Domestic Fowl

3. THE NON-PROTEIN NITROGEN OF PLASMA AND ERYTHROCYTES*

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Peters & Van Slyke (1946) define non-protein nitrogen as that nitrogen which is not removed from biological fluids by the usual deproteinizing agents. The same authors (1932) state (obviously with respect to human blood) that 'for non-protein nitrogen tungstic acid and trichloroacetic acid filtrates yield practically identical results'. We have not found satisfactory confirmation of this statement in the literature (cf. Folin, 1922). Greenwald (1915) showed with sheep, ox and pig bloods that both precipitation by 9 vol. of trichloroacetic acid (2.5–22.0%) and direct dialysis gave identical values for non-protein nitrogen. Folin & Wu (1919, cf. p. 86, table 1) compared the non-protein nitrogen of human and fowl bloods after deproteinization by trichloroacetic and tungstic acids; tungstate filtrates contained appreciably less, the difference being especially marked with fowl blood. Hiller & Van Slyke (1922) studied the non-protein nitrogen of slaughter-house bovine blood (previously treated with urease) after deproteinization by a variety of precipitants; the values obtained were by no means identical. Hunter (1949) has indicated the variability of batches of sodium

tungstate in their capacity to precipitate blood ergothioneine (cf. Benedict & Newton, 1929). Puech & Cristol (1929) and Godfried (1939) considered that more amino acid nitrogen is present in blood filtrates when made with trichloroacetic acid than with phosphotungstate. Hunter (1957), in a study of the deproteinization of human blood, plasma, erythrocytes and tissue fluids by heat after appropriate pH adjustment, found that parallel treatments with tungstic acid gave filtrates with lower non-protein nitrogen values in each instance. Christensen & Lynch (1946), comparing trichloroacetic acid and tungstate filtrates of human plasma and erythrocytes, found higher amino acid levels in the former.

Workers on non-protein nitrogen of fowl blood since Folin & Wu (1919) (cf. collated data in Shimer, 1937; Herrmann, 1946a, b; Charkey, Wilgus, Patton & Gassner, 1950; Hsu & Combs, 1952; Hazelwood & Lorenz, 1957) appear, with one exception, to have examined only tungstate filtrates of whole blood; in single instances serum and plasma were analysed. The values found cover a wide range. Despite the observations of Benedict (1915) that non-uric acid chromogens are present in fowl blood, uncertainty exists with regard to

* Part 2: Bell (1957c).