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The Isolation and Purification of a Bovine-Plasma Protein which is a Substrate for the Coagulant Fraction of Russell's-Viper Venom

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In the preceding paper Williams & Esnouf (1962) showed that the coagulant protein of Russell's-viper venom was an enzyme. Peden & Peacock (1958) found that, during the incubation of Russell's-viper venom with a substance present in bovine serum, coagulant activity developed. Macfarlane (1961) extended these observations and showed that this substance was adsorbed by barium sulphate from the serum and could be recovered by elution with 0·2M-sodium citrate. The purpose of this paper is to describe the isolation and purification of the substrate for the venom protein from bovine plasma by chromatography on diethylaminoethylcellulose columns. The physical properties of the protein have been determined and its interaction with purified coagulant protein from Russell's-viper venom has been investigated.

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MATERIALS AND METHODS

Abbreviations. DEAE-cellulose, diethylaminoethylcellulose; TAMe, toluene-*p*-sulphonylarginine methyl ester.

Preparation of the barium sulphate eluate

Ox blood for the preparation of plasma was collected in plastic buckets containing 0·1 vol. of 1·34% (w/v) sodium oxalate. About 10 l. of blood was obtained from each of 4–5 animals immediately after slaughter. The plasma (usually about 20 l.) was collected after centrifuging the blood at 1000*g* for 45 min. at 4°, and stirred with citrate-washed barium sulphate, 100 g./l. of plasma (Biggs & Macfarlane, 1957), for 15–20 min. at room temperature. The barium sulphate was recovered by centrifuging at 1000*g* for 45 min. at 10°, and then washed twice with 0·15M-sodium chloride and once with water, the volume for each wash being one-quarter that of the original plasma. The barium sulphate was then stirred at room temperature for 10 min. with 0·2M-sodium citrate–citric acid buffer, pH 6·8, the volume being one-tenth that of the original plasma. The supernatant solution after centrifuging was dialysed against 0·02M-sodium phosphate buffer, pH 7·2,

at 4°. Dialysis was carried out in ⅛ in. tubing (Visking) for 24 hr. against four changes of buffer, the volume of which was at least eight times that of the protein solution.

For the preparation of serum 200 ml. of blood was collected in glass bottles and allowed to clot at room temperature. The clotted blood obtained from each animal was incubated at 37° for 4 hr., by which time most of the prothrombin had been converted into thrombin, after which the serum was obtained by centrifuging. Sera obtained from all the animals used in the preparation of the plasma were mixed and the mixture was used to determine the amount of venom substrate which was present in the plasma.

Chromatography of the barium sulphate eluate

Columns of DEAE-cellulose (Whatman DE 50) were prepared as described by Williams & Esnouf (1962). The DEAE-cellulose was washed with 2M-sodium chloride in 0·05M-sodium phosphate buffer, pH 6·0, and then equilibrated with 0·02M-sodium phosphate, pH 7·0. The dialysed plasma-protein fraction was placed on the column after centrifuging to remove insoluble protein, and the fractionation was carried out with 0·02M-sodium phosphate buffer containing various concentrations of sodium chloride. The fractionation was carried out at 4°, and details of individual experiments are given in the text. The elution of the protein from the column was followed by measuring the extinction of the fractions at 280 mμ in 1 cm. cells.

Coagulation tests

Plasma. Human blood obtained from two or three persons was collected in cellulose acetate tubes containing 0·02 vol. of 19% (w/v) trisodium citrate. The blood was centrifuged at 3000*g* for 30 min., and the supernatant plasma was centrifuged at 12 000*g* for 20 min. to remove most of the platelets. The plasma was decanted into cellulose acetate tubes and stored at −15°.

Factor V. This was prepared from human plasma (Biggs & Macfarlane, 1957), and was stored in 2 ml. quantities at −15°.

Buffers. Solutions of tris adjusted to pH 7·3 with N-hydrochloric acid were made 0·01M with respect to tris.

Calcium chloride solution. This was prepared by dissolving calcium carbonate in the theoretical amount of hydrochloric acid. The solution was boiled to remove carbon dioxide. Tris (0·1M; 0·1 vol.) was added and the pH was adjusted to 7·3 with hydrochloric acid. The solution was 0·025M with respect to Ca^{2+} ions.

Brain phospholipid. A crude mixed-phospholipid preparation was obtained from human brain and emulsified in tris–hydrochloric acid buffer, pH 7·3 (Williams & Esnouf, 1962).

Venom-substrate assay. The venom substrate was assayed by determining the coagulant activity generated when the venom substrate was incubated with the purified coagulant protein obtained from Russell's-viper venom. The coagulant activity was estimated from the clotting time of recalcified normal citrated plasma after the addition of activated venom substrate. A portion (0·1 ml.) of venom-substrate solution in 0·15M-sodium chloride buffered with 0·01M-tris–hydrochloric acid buffer, pH 7·3 ('tris–saline'), and 0·1 ml. of the purified coagulant protein (2 μg./ml.) in tris–saline were incubated at 37° with 0·1 ml. of the calcium chloride solution. After 5 min., 0·1 ml. of the mixture was diluted with 2·4 ml. of tris–saline and mixed for 10 sec. A portion (0·1 ml.) of this solution was added with 0·1 ml. of calcium chloride solution to a mixture containing 0·1 ml. of factor V solution and 0·1 ml. of the phospholipid emulsion (0·5 mg. of phospholipid/ml.), and incubated. After 3 min., 0·1 ml. of this mixture was added together with 0·1 ml. of calcium chloride solution to 0·1 ml. of plasma and the clotting time determined. The solutions of factor V, venom substrate, purified coagulant protein and the phospholipid emulsion were kept at 0° and were warmed to 37° immediately before use. The calcium chloride solution was kept at 37°. All incubations were carried out at 37° in a water bath.

For the studies on the kinetics of the reaction between the coagulant protein and the venom substrate, a simplified system was used for the assay of the coagulant activity. As these studies were designed to determine the initial rate of reaction a rapid test system was required, and thus the incubation for 3 min. with factor V, phospholipid and calcium chloride was omitted. The assay system was modified as follows. The venom substrate, venom protein and calcium chloride solutions were incubated as above. The concentrations of the reactants were varied as required for each particular experiment. After 1 min., 0·1 ml. of the reaction mixture was added to 0·4 ml. of the phospholipid emulsion (0·25 mg. of phospholipid/ml.) and mixed for 10 sec. Then 0·1 ml. of this mixture was added at the same time as 0·1 ml. of calcium chloride solution to 0·1 ml. of plasma and the clotting time determined. With both assay systems the clotting times obtained were related to units of activity by reference to standard curves prepared by incubating 0·9 ml. of the venom-substrate solution with 0·9 ml. of a solution of purified coagulant protein (2 μg./ml.) and 0·9 ml. of the calcium chloride solution for 5 min. at 37°. In this time the venom substrate was completely activated. The mixture was then chilled to 0° and dilutions were prepared in ice-cold tris–saline containing 0·67 μg. of the coagulant protein/ml. This was done to maintain a constant amount of venom protein at each dilution of the activated venom substrate. Then 0·1 ml. of each diluted solution was added to the appropriate amount of tris–saline buffer and the coagulant activity was assayed by either procedure. For the assay in the presence of added factor V 0·1 ml. of each of the diluted venom-substrate solutions was added to 2·5 ml. of tris–saline, and for the simplified system 0·1 ml. of each venom-substrate solution was added to 1 ml. of tris–saline. These dilutions were necessary particularly in the simplified test system because in the kinetic experiments only a small proportion (10%) of the venom substrate was activated in the first minute, and the venom substrate used to calibrate the test system was fully activated. The units of activity obtained from the clotting times in the kinetic experiments were divided by 10 to compensate for this dilution during the preparation of the standard curve. The clotting times were plotted on log–log co-ordinates against the units of the venom substrate present in each of the dilutions and a straight line was obtained.

Protein concentrations. These were determined as described by Williams & Esnouf (1962).

Amino acid-esterase activity. This was measured as described by Williams & Esnouf (1962) with TAMe from L. Light and Co. Ltd.

Measurements of physical properties of the venom substrate. Electrophoresis and the experiments to determine the partial specific volumes, sedimentation coefficient, diffusion coefficient and the molecular weight of the venom substrate were carried out by the techniques described by Williams & Esnouf (1962), except that the protein was dissolved in 0·1 M-sodium chloride buffered with 0·01 M-tris–hydrochloric acid, pH 7·3. For the determinations of the molecular weight by the approach-to-equilibrium method, the experiments were carried out at a rotor speed of 15 220 rev./min.

Analysis of the N*-terminal end groups.* The protein solutions were allowed to react with fluoro-2,4-dinitrobenzene in an ethanol–water solution of sodium hydrogen carbonate (Sanger, 1945). The DNP-proteins were hydrolysed in 6 N-hydrochloric acid in sealed tubes for 9 hr. at 110° in a hot-air oven, and the ether-soluble DNP-amino acids were separated by paper chromatography (Blackburn & Lowther, 1951).

RESULTS

Assay of venom substrate

The validity of the venom-substrate assay with the complete system was tested as follows. Various quantities of venom substrate were added to reaction mixtures containing the same amounts of purified coagulant protein of Russell's-viper venom and calcium chloride, and the coagulant activity formed in each reaction mixture was determined in the standard assay system. Also, large reaction mixtures were prepared and the activity of various dilutions was determined as outlined in the Materials and Methods section for preparation of a standard curve. The results of these experiments are presented in Fig. 1. In both experiments a log–log plot of the clotting times against the amount of venom substrate in the reaction mixture, or dilutions of the reaction mixture, gave a straight line. In the experiment in which there were different concentrations of venom substrate in the reaction mixture the amount of coagulant activity formed was dependent on the venom-substrate concentration.

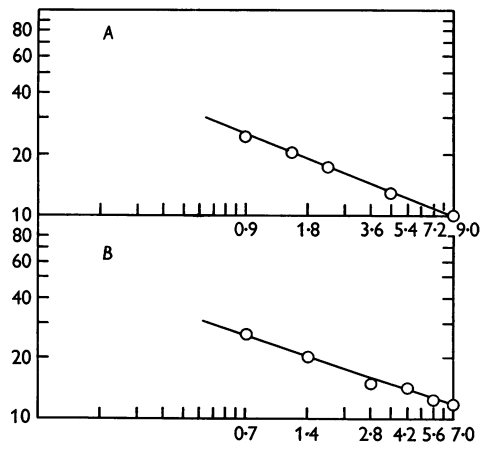

Fig. 1. Assay of venom substrate. In *A* the amount of venom substrate in the reaction mixture was varied, and in *B* the venom substrate was fully activated in the reaction mixture and diluted solutions of this were assayed for activity. The results were plotted on log–log co-ordinates.

The effect of the omission of each of the components from the test system was studied (Table 1). Omission of any of the components from the system resulted in marked lengthening of the clotting time of the plasma. In further control experiments the ability of the reaction mixture after incubation with phospholipid and factor V to clot fibrinogen was determined. Reaction mixtures, diluted 1:25 and incubated with phospholipid and factor V for 3 min., clotted recalcified plasma in 10 sec., but failed to clot 0·5 % fibrinogen solution in 30 min. Reaction mixtures added to the phospholipid–factor V mixture without dilution clotted plasma in 5 sec., and clotted 0·5 % fibrinogen solution in 6½ min. These experiments indicate that only a very small amount of thrombin was formed in the assay system.

Chromatography of plasma-protein fraction on diethylaminoethylcellulose

The fractionation of the plasma-protein fraction obtained from 1 l. of plasma is given in Fig. 2. Four fractions were obtained, the second of which was arbitrarily divided into three parts for testing. The distribution of the venom substrate among the protein fractions is shown in Table 2: it was concentrated in fraction 3. The yield of venom substrate in this experiment was only 44 % of that in the original plasma. The fractionation was carried out to obtain information on the distribution of venom substrate among the various fractions, and only portions of the peaks which contained the highest protein concentration were included in the material taken for testing.

Table 1. *Effect of omission of each of the components of the standard assay system on the clotting time*

The assay system is described in the text.

Stage	Omission	Clotting time (sec.)
1	None	12
1	Venom protein	84
1	Venom substrate	36
2	Factor V	23
2	Phospholipid	115
2	Ca^{2+} ions	23
2	Stage 1	83
3	Ca^{2+} ions	55
3	Stages 1 and 2	240

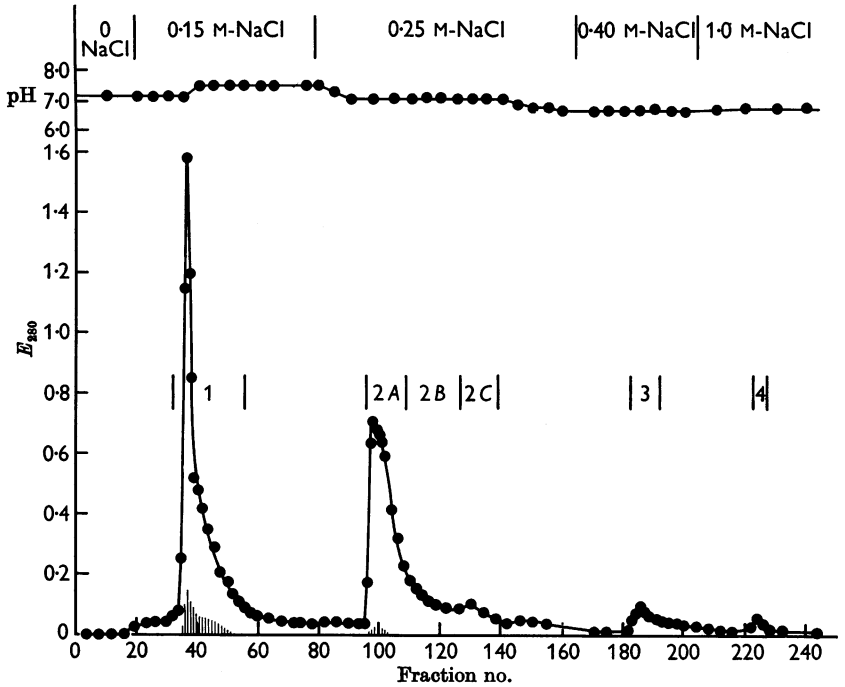

Fig. 2. Fractionation on DEAE-cellulose of the proteins adsorbed by barium sulphate from 1 l. of plasma. The chromatogram was developed with 0·02 M-sodium phosphate buffer, pH 7·0, containing sodium chloride at the concentrations shown. Fractions (20 ml.) were collected at a flow rate of 130 ml./hr. The protein concentration in the fractions was obtained from the changes in E_{280}. The hatched areas represent extinction at 420 mμ due to yellow pigment. The numbers above the peaks indicate which fractions were combined for testing.

Table 2. *Distribution of venom substrate in the fractions obtained from the diethylaminoethyl-cellulose column*

For details of the column and fractions see Fig. 2.

Fraction no.	Protein* (% recovered)	Sp. activity (units/μg.)	Total activity (units)	Activity (% recovered)	Purification
1	39	0·01	2 100	2	7
2*A*	28	0·04	3 600	4	28
2*B*	10	0·04	2 200	2	28
2*C*	4	0·08	1 300	1	56
3	3	4·00	44 000	44	2 860
4	1	0·13	400	0·4	93

* Total recovery of protein from column was 98%. The difference between this and the sum of the protein in each fraction is due to protein not included in the fractions.

Because it was not possible to determine the amount of the venom substrate in the original plasma owing to the presence of prothrombin, an estimate was made by assaying the venom substrate present in the serum prepared from the same blood as the plasma. The coagulant activity generated in 1 ml. of this serum during the incubation with venom protein was called 100 units, and the venom-substrate content of the fractions is expressed in terms of these arbitrary units. Assays of the plasma after adsorption on barium sulphate, which also removed the prothrombin, indicated that 90% of the venom substrate had been removed, but because of the prothrombin in the eluate no assays of the venom-substrate content of these preparations were carried out.

These experiments demonstrated that the amount of the venom substrate in plasma was small, and therefore larger columns were used for preparative work. Columns (9 cm. × 28 cm.) containing 180 g. of DEAE-cellulose were used. On each column was placed 2 l. of the barium sulphate

eluate, washed on with 200 ml. of 0·02 M-sodium phosphate buffer, pH 7·0. Then 4·8 l. of 0·02 M-sodium phosphate buffer, pH 7·0, containing 0·15 M-sodium chloride was then run through the column, followed by 6 l. of the buffer containing 0·25 M-sodium chloride. The flow rate was 500 ml./hr. The effluent from these two solvents, collected in bulk, contained 96 % of the protein added to the column. The column was next treated with 0·02 M-sodium phosphate buffer, pH 7·0, containing 0·4 M-sodium chloride. The flow rate was reduced to 350 ml./hr. and the effluent was collected in 50 ml. fractions. After 1·3 l. of this solvent had passed through the column, a protein fraction which accounted for 3 % of the protein placed on the column appeared in the next 1 l. of effluent. As expected from the previous experiments this fraction contained the venom substrate.

In preliminary experiments it was found that dialysis against water before freeze-drying caused a loss in the ability of the substrate protein to be activated by the venom protein, and therefore other means of concentrating the venom substrate were necessary. For this purpose advantage was taken of the fact that the venom substrate was readily adsorbed by barium sulphate. The following procedure was found to be satisfactory for the concentration of venom substrate obtained from the DEAE-cellulose columns.

The effluent fractions containing 0·4 M-sodium chloride were diluted with an equal volume of water and stirred at room temperature for 15 min. with 50 g. of barium sulphate/l. The barium sulphate was collected by centrifuging. Measurement of E_{280} of the supernatant solution indicated that 96 % of the protein had been adsorbed. The barium sulphate was next stirred at room temperature for 7 min. with 0·2 M-sodium citrate buffer, pH 6·8 (volume one-twentieth that of the diluted effluent). After centrifuging to remove the barium sulphate, the supernatant was found to contain 68 % of the adsorbed protein. The elution was repeated with sodium citrate solution (volume one-fortieth of the original volume of column effluent), and a further 25 % of the adsorbed protein was recovered. The protein solutions obtained by eluting the barium sulphate were combined and dialysed at 4° for a total of 15 hr. against two changes of 30 vol. of 0·15 M-sodium chloride. A sample was taken for testing and the remainder was freeze-dried. The residue was dissolved in 6 ml. of water and dialysed at 4° for 14 hr. against 400 vol. of 0·1 M-sodium chloride buffered with 0·01 M-tris–hydrochloric acid, pH 7·3. The yield of venom substrate was about 150 mg. from 20 l. of plasma.

On electrophoresis of this material only one component was seen, both at pH 6·5 in 0·1 M-phosphate buffer and at pH 7·3 in 0·1 M-sodium chloride buffered with 0·01 M-tris–hydrochloric acid. In both buffers the protein was negatively charged. However, when the protein was examined in the ultracentrifuge, a major component, with S_{20} 4·2, and a minor component, with S_{20} 10, were seen. These two components were resolved by centrifuging in a preparative ultracentrifuge. A portion (2 ml.) of the protein solution dissolved in 0·1 M-sodium chloride buffered with 0·01 M-tris–hydrochloric acid, pH 7·3, was layered on 2·5 ml. of the buffered salt solution containing 10 % (w/v) of sucrose in a centrifuge tube. The tube was centrifuged at 40 000 rev./min. for 6 hr. at 2° in a S.W. 39 L. rotor (Spinco model L ultracentrifuge). After the rotor had come to rest without braking, the contents of the tube were fractionated by piercing the bottom of the tube with a pin and collecting five 0·9 ml. fractions. Then 25 μl. of each of these was added to 2·5 ml. of 0·9 % sodium chloride and E_{280} was measured to locate the light component (venom substrate). The heavy component was packed at the bottom of the centrifuge tube. Most (70 %) of the light component was collected in the third sample at the interface between the two layers. To remove the sucrose, the protein solution was dialysed against 0·1 M-sodium chloride buffered with 0·01 M-tris–hydrochloric acid, pH 7·3. This fraction was homogeneous when examined in the ultracentrifuge.

The purification of the venom substrate during the various stages is given in Table 3. With the larger columns material of greater specific activity (5·6 units/μg. of protein) was obtained than that (4 units/μg. of protein) with the smaller columns.

Physical properties of the venom substrate

The physical properties of the venom substrate were determined on preparations obtained after preparative ultracentrifuging. The value for the partial specific volume, $\bar{v}$, of the venom substrate calculated from the density measurements was 0·738. The sedimentation coefficient, S_{20}, of the plasma-venom substrate was 4·23. The diffusion coefficient, D_{20}, was $4{\cdot}57 \times 10^{-7}$ cm.^{2}/sec. Determinations of the molecular weight by the approach-to-equilibrium method gave a value of 84 800 ± 700 (S.E.M.) from five determinations, and by combination of sedimentation and diffusion data a value of 87 000 was obtained.

In preliminary experiments when the venom substrate was rechromatographed and dialysed against water before freeze-drying, sedimentation coefficients ranging from 3·5 to 3·8 were obtained. Since in these preparations the specific activity of the venom substrate was lower (2–3 units/μg. of protein), the low sedimentation coefficients may indicate a partial denaturation of the protein. In three experiments the venom substrate was iso-

Table 3. *Purification and yield of venom substrate at each of the stages of purification*

For experimental details see text.

Stage	Vol. (ml.)	Total protein (mg.)	Protein yield (%)	Total activity (units)	Yield of activity (%)	Sp. activity (units/μg.)	Purification
Starting plasma (estimated from serum)	21 000	1 500 000	100	2 100 000	100	0·0014	1
$BaSO_4$ eluate prepared from plasma*	2 040	8 300	0·5	—	—	—	—
DEAE-cellulose column†: fraction 3	1 000	265	0·018	1 439 000	68	5·4	3 860
$BaSO_4$ eluate prepared from fraction 3	170	237	0·016	1 210 000	58	5·1	3 640
Protein obtained after preparative ultracentrifuging	24	168	0·011	940 000	45	5·6	4 000

* The venom substrate could not be assayed at this stage owing to the presence of prothrombin.
† Column used contained 180 g. of DEAE-cellulose (for details see text).

lated from serum, and sedimentation coefficients of 2·5, 2·8 and 2·2 were obtained. The latter preparation was found to have become partially autoactivated. For the preparation with S_{20} 2·8, assuming the value of $\bar{v}$ to be the same as for the plasma-venom substrate, a value for D_{20} of $6{\cdot}2 \times 10^{-7}$ cm.²/sec. was found, and the molecular weight was calculated to be 36 000.

Activation of the venom substrate

To determine whether any physical change occurred when the venom substrate was activated by the purified coagulant protein, 15·4 mg. of venom substrate (of specific activity 5·4 units/μg.), dissolved in 2·2 ml. of 0·1 M-sodium chloride buffered with 0·01 M-tris–hydrochloric acid, pH 7·3, was mixed with 0·1 ml. of a solution containing 1 mg. of purified coagulant protein/ml. and 0·2 ml. of 0·1 M-calcium chloride in 0·01 M-tris–hydrochloric acid buffer, pH 7·3. This was compared with a control sample in which 0·1 ml. of buffer was added in place of the purified coagulant protein. Both samples were incubated at 37° for 20 min. and then chilled in melting ice. The reaction mixture containing the purified coagulant protein became grossly cloudy during the incubation but the control sample remained clear. A sample of each was taken for assay of coagulant activity: the complete reaction mixture contained 92 % and the control 0·5 % of maximum activity. The precipitate accounted for 10 % of the protein and redissolved on the addition of further buffer.

Both the control and reaction mixtures were then analysed in the ultracentrifuge at 1°. No change in the sedimentation coefficient of the control sample was found (S_{20} 4·20), though in the reaction mixture two components were seen, one with S_{20} 3·72 and the other with S_{20} 1·9. When the samples were subjected to electrophoresis the protein in the control sample moved as a single component but two components were found in the reaction mixture. From these observations it was concluded that a part of the venom-substrate molecule had been split off during the incubation with the venom protein. This conclusion was confirmed by preliminary experiments in which the *N*-terminal end groups present in the control and reaction mixture were analysed. In the control samples both DNP-alanine and DNP-glycine were present, but in the reaction mixture an additional compound was found which was either DNP-leucine or DNP-isoleucine.

Kinetics of the activation of the venom substrate

Williams & Esnouf (1962) showed that TAMe would act as a competitive inhibitor during the activation of the venom substrate by the purified coagulant protein. This inhibition was almost complete at a final concentration of TAMe of 0·05 M. It was also found in preliminary experiments that during the activation of the venom substrate a TAMe-esterase activity developed, and advantage was taken of this to study the kinetics of the activation of the venom substrate. In these experiments different samples of the venom protein and the venom substrate were incubated together with Ca^{2+} ions in 0·1 M-potassium chloride at 37°. The pH of the reaction mixture was kept constant by the addition of 0·05 N-sodium hydroxide. At various times TAMe was added to the reaction mixture and the esterase activity was determined from the amount of 0·05 N-sodium hydroxide added to maintain the pH of the reaction mixture constant at 8.0.

The results of such an experiment are given in Fig. 3. The venom protein and venom substrate were incubated with calcium chloride at pH 8·0, and TAMe which had been previously adjusted to pH 8·0 was added at various times. The broken line shows the result of adding the TAMe to the reaction

mixture before the addition of the calcium chloride: in this instance no esterase activity was generated. The curves do not go through zero because the TAMe itself undergoes spontaneous hydrolysis at pH 8·0 and also in some preparations of the venom substrate there was slight TAMe-esterase activity.

The kinetics of the activation of the venom substrate were also studied from measurements of the coagulant activity. Williams & Esnouf (1962) showed that the purified coagulant protein of Russell's-viper venom acts as an enzyme in activating a coagulation factor, presumably the venom substrate, in bovine serum. Kinetic studies were accordingly carried out to determine whether purified venom substrate behaved similarly. In these experiments the assay system without factor V was used, to avoid the relatively long incubation period necessary when this factor was present. Standard curves gave a straight-line relationship between clotting time and the venom-substrate concentration, but 5–10 times as much venom substrate was required to give the same clotting times as were found in the complete system.

The time-course of the appearance of coagulant activity in reaction mixtures containing the same concentrations of the venom substrate incubated with three different concentrations of purified coagulant protein is given in Fig. 4. The reaction appeared to follow first-order kinetics even with the lowest concentration of purified coagulant protein. Subsequent experiments were done with the coagulant activity formed in the first minute of incubation taken as the initial rate.

The effect on the initial rate of formation of coagulant activity was directly proportional to the concentration of purified coagulant protein over a tenfold range, suggesting that the purified coagulant protein was acting as an enzyme.

Fig. 5 shows the effect of the venom-substrate concentration on the amount of coagulant activity

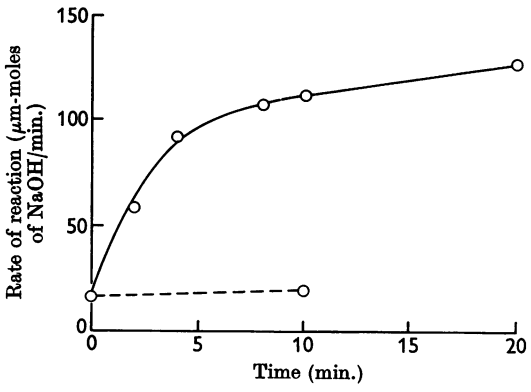

Fig. 3. Appearance of TAMe-esterase activity during the incubation of 100 μg. of venom substrate with 0·25 μg. of venom protein and 0·01 M-calcium chloride. The reactants were dissolved in 0·1 M-potassium chloride, made to 1·5 ml. with 0·1 M-potassium chloride and adjusted to pH 8·0. The incubation was carried out at 37°. At the times shown, 0·5 ml. of 0·2 M-TAMe in 0·1 M-potassium chloride, pH 8·0, was added, and the rate of reaction was determined from the amount of 0·05 N-sodium hydroxide required to maintain the pH of the mixture at 8·0. The broken line represents an experiment in which TAMe was added before Ca^{2+} ions.

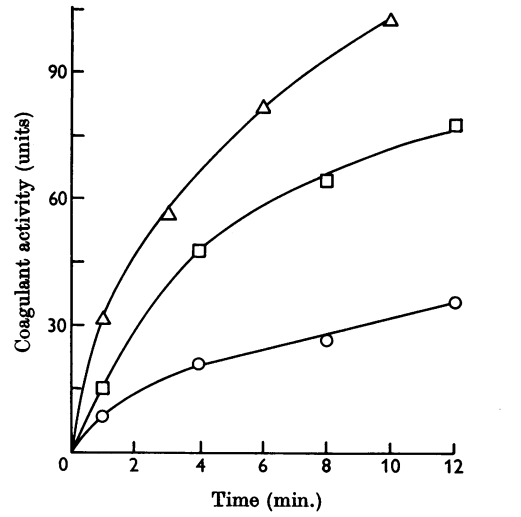

Fig. 4. Appearance of coagulant activity during incubation of 0·1 ml. of a solution containing 100 μg. (560 units) of venom substrate/ml. of tris–saline, 0·1 ml. of purified coagulant protein dissolved in tris–saline to give the concentrations (μg./ml.) 0·003 (○), 0·006 (□) and 0·017 (△), and 0·1 ml. of 0·025 M-calcium chloride in 0·01 M-tris–hydrochloric acid, pH 7·3. The incubations were done at 37°, and at the times shown 0·1 ml. of the mixture was assayed for coagulant activity by the simplified assay procedure (see text).

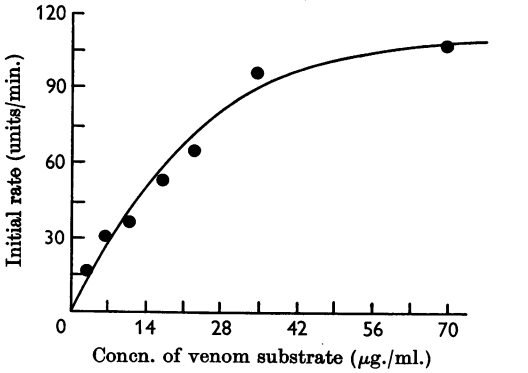

Fig. 5. Effect of the concentration of venom substrate on the initial rate of formation of coagulant activity. For details see Fig. 4. The concentration of the venom protein used in these experiments was 0·07 μg./ml.

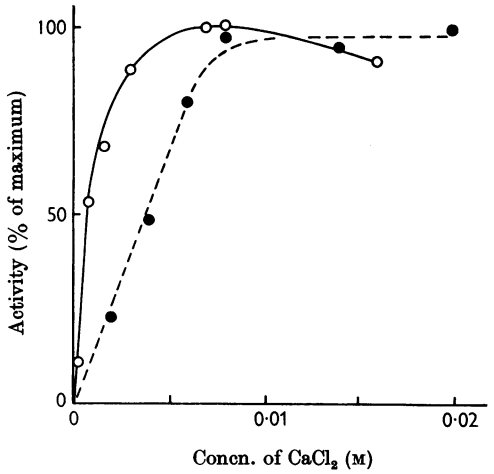

Fig. 6. Effect of concentration of Ca^{2+} ions on the initial rate of formation of coagulant activity (○). The reaction mixture contained 0·007 μg. of venom protein and 6·7 μg. of venom substrate and various concentrations of calcium chloride as shown. The effect of concentration of Ca^{2+} ions on the appearance of esterase activity (●) was determined as follows: 100 μg. of venom substrate and 0·25 μg. of venom protein were incubated at 37° with Ca^{2+} ions at the concentration shown; the reactants were dissolved in 0·1 M-potassium chloride, made to 1·5 ml. with 0·1 M-potassium chloride and adjusted to pH 8·0; after 2 min., 0·5 ml. of 0·05 M-TAMe in 0·1 M-potassium chloride, pH 8·0, was added and the esterase activity determined, activity being expressed as a percentage of the activity obtained at the optimum concentration of Ca^{2+} ions.

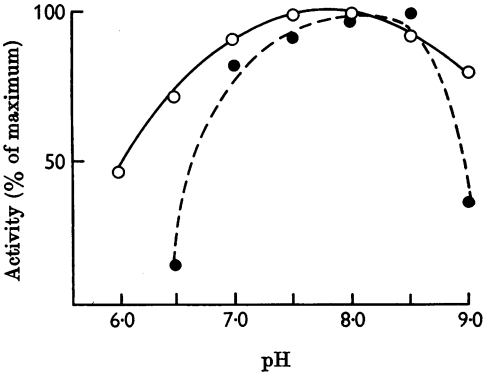

Fig. 7. Effect of pH on the initial rate of formation of coagulant activity (○). Venom protein (0·007 μg.) and venom substrate (6·7 μg.) were incubated with 8 mM-calcium chloride at the pH values shown. The activity was assayed at pH 7·4; this was done by emulsifying the phospholipid (0·5 mg./ml.) in 0·1 M-tris–hydrochloric acid, pH 7·4. The effect of pH on the appearance of esterase activity (●) was determined as follows. Venom substrate (100 μg.), venom protein (0·25 μg.) and calcium chloride. (final concentration 0·01 M) were used. The reactants were dissolved in 0·1 M-potassium chloride and made to 1·5 ml. The pH was adjusted to the values shown by the addition of sodium hydroxide. After 2 min., 0·5 ml. of 0·2 M-TAMe in 0·1 M-potassium chloride at pH 8·0 was added and the esterase activity determined at pH 8·0. Activity was expressed as a percentage of the activity obtained at the optimum pH.

formed in 1 min. Here the plot has the shape of a rectangular hyperbola, as would be expected if the venom substrate were acting as a substrate. The experiments used to validate the venom-substrate-assay system are also consistent with this interpretation of the role of the venom substrate in this system.

The effect of Ca^{2+} ions on the activation of the venom substrate was investigated (Fig. 6). In these experiments the effect of Ca^{2+} ions was determined by the esterase technique and by the assay for coagulant activity. The optimum concentration of Ca^{2+} ions was 7 mM.

The activation of the venom substrate was investigated over a range of pH values with both assay procedures, and Fig. 7 shows that the activation of the venom substrate was optimum over a wide pH range.

The esterase activity of the activated venom substrate was investigated further. For this 10 μl. of the venom protein (176 μg./ml.) was incubated with 0·15 ml. of venom substrate (4 mg./ml.) and 0·2 ml. of 0·1 M-calcium chloride. All the reagents were dissolved in 0·1 M-potassium chloride and adjusted to pH 8·0. The mixture was incubated at 37° for 20 min. and then diluted to 6 ml. with 0·1 M-potassium chloride; 0·5 ml. samples of the mixture were taken to investigate the esterase activity of the activated venom substrate.

The rate of hydrolysis of TAMe was found to be independent of Ca^{2+} ions and was inhibited 60 % by soya-bean trypsin inhibitor (100 μg./ml.). The pH optimum for the hydrolysis of TAMe by the activated venom substrate (Fig. 8) was 8·0. The effect of TAMe concentration on its rate of hydrolysis by activated venom substrate was studied (Fig. 9), and the Michaelis constant was calculated to be $2·5 \times 10^{-2}$ M. When lysine ethyl ester (0·02 M) was used as substrate no hydrolysis was observed over the pH range 6·5–8·0.

DISCUSSION

The identification of venom substrate, obtained from bovine plasma, with one of the human coagulation factors cannot be made with any degree of certainty from the chemical point of view. However, the venom substrate has a biological

activity which is similar to that ascribed to 'factor X' (Stuart–Prower factor) (Hougie, Barrow & Graham, 1957; Denson, 1958), because the venom substrate was found to correct the coagulation defect found in a case of congenital factor X deficiency. Macfarlane (1961) concluded that the substance which reacted with the venom in ox-serum eluate was factor X; it behaved in coagulation tests in a similar way to a preparation of human factor X. As the venom substrate is the purified protein fraction made from such an eluate, it is likely that this purified protein is bovine factor X.

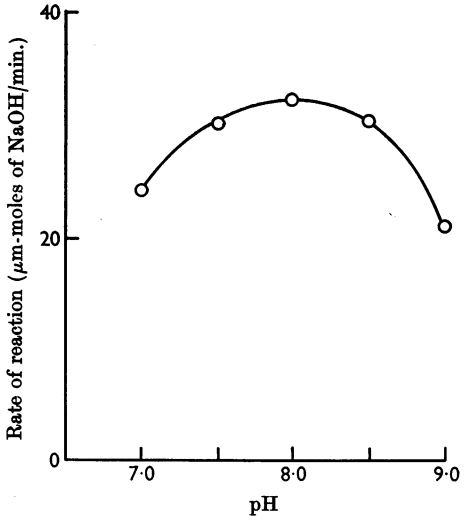

Fig. 8. Effect of pH on the TAMe-esterase activity of the activated venom substrate. A sample (0·5 ml.) of activated substrate (see text) and 0·015 M-TAMe in 0·1 M-potassium chloride (2·0 ml. final vol.) were incubated for 20 min. at 37° at the pH values shown, which were maintained by the addition of 0·05 N-sodium hydroxide.

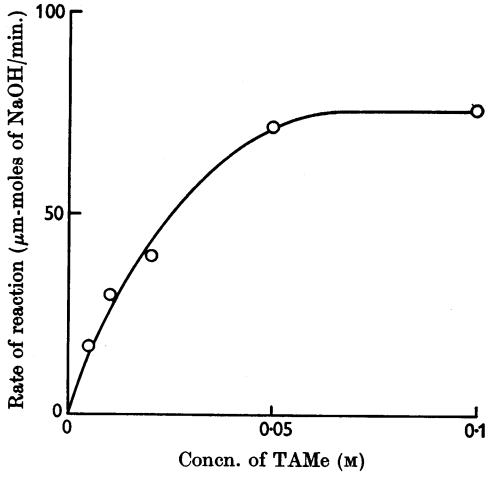

Fig. 9. Effect of TAMe concentration on its rate of hydrolysis by the activated venom substrate. Activated substrate (0·5 ml.) was incubated with TAMe at the concentrations shown in 0·1 M-potassium chloride (20 ml. final vol.) at 37° for 20 min. at pH 8·0, which was maintained by the addition of 0·05 N-sodium hydroxide.

The venom substrate was obtained as a protein fraction which, by the criteria applied in this paper, is homogeneous. This, however, does not necessarily mean that the protein fraction has functional purity: it may be contaminated with small amounts of other coagulation factors of high specific activity.

The difference between the molecular weights of the preparations made from plasma and serum suggests that in the serum the venom substrate has been hydrolysed by some proteolytic enzyme, such as thrombin, formed during coagulation of the plasma (Lorand, 1954). As preparations of the venom substrate obtained from serum are capable of activation by the venom protein, it must be supposed that venom protein hydrolyses the substrate at a different site to give an active fragment. The suggestion that venom coagulant protein acts as a proteolytic enzyme is supported by the appearance of a new *N*-terminal amino acid as the result of incubation of the venom substrate with the venom protein.

Activated venom substrate, thrombin (Sherry & Troll, 1954) and the venom coagulant protein (Williams & Esnouf, 1962) all have TAMe-esterase activity, and appear to act specifically on different substrates in the plasma. It cannot be concluded on the evidence available so far that both the venom protein and venom substrate behave as esterases or as proteolytic enzymes similar to trypsin, which also possess esterase activity (Schwert, Neurath, Kaufman & Snoke, 1948).

The assay system shows that the activated substrate interacts with factor V in the presence of phospholipid and Ca^{2+} ions. The product of this interaction would appear to have the property of converting prothrombin into thrombin. This interpretation, however, may not apply to the coagulation of whole blood by the venom *in vivo*.

SUMMARY

1. The substrate for the coagulant protein of Russell's-viper venom was isolated from bovine plasma and some of its physical properties were determined.

2. The activation of the venom substrate appears to be the result of some hydrolytic activity of the venom protein.

3. The kinetics of the activation process were investigated.

4. The activated venom substrate has amino acid-esterase activity as well as a powerful coagulant activity.

We are grateful to Mr J. R. P. O'Brien, in whose Department this work, and that described in the preceding paper, was carried out, for the interest he has shown in this problem. We wish to thank Dr R. G. Macfarlane, F.R.S., for many useful discussions. We should also like to thank Dr M. M. Pickles for the loan of the electrophoresis apparatus, and Dr W. E. Van Heyningen for the use of the Spinco model E ultracentrifuge. The authors wish to thank Mr P. J. Tosh for the construction of various items of apparatus. One of us (M.P.E.) acknowledges a grant from the Medical Research Council during the early part of this work.

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The Conversion of ^{14}C-Labelled 'Glucose Cycloacetoacetate' into L-Ascorbic Acid in Albino Rats

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The first isotopic investigation into the biosynthesis of ascorbic acid was that of Jackel, Mosbach, Burns & King (1950). They used uniformly (U) labelled [U-^{14}C]glucose, which gave rise to [U-^{14}C]-ascorbic acid in chloretone-stimulated rats. The work of Horowitz, Doerschuk & King (1952) and Horowitz & King (1953) supported the contention that in animals D-glucose is converted into L-ascorbic acid without fragmentation but with inversion of its carbon skeleton. A general scheme linking D-glucose and D-galactose with L-ascorbic acid was proposed by Isherwood, Chen & Mapson (1954), in which the postulated intermediates are:

D-Glucose → D-glucuronic acid → L-gulonic acid → L-ascorbic acid

D-Galactose → D-galacturonic acid → L-galactonic acid → L-ascorbic acid

This scheme is supported by work on animals, though not by the work of Loewus, Jang & Seegmiller (1956) and Loewus & Jang (1957) with strawberries and cress seedlings, in which glucose was converted into L-ascorbic acid without fragmentation, but, in contrast with rats, there was no inversion of the molecule. Galactose also passes through an intermediate triose pool, which symmetrically distributes the label between C-1 and C-6. This resembles the route taken by L-[6-^{14}C]-sorbose in chloretone-treated rats (Burns, Mosbach, Schulenberg & Reichenthal, 1955).

Work with *Phaseolus radiatus* by Nath, Chitale & Belavady (1952) also suggests that the scheme originally proposed by Isherwood *et al.* (1954) is not the only pathway leading to ascorbic acid formation from glucose. Although acetoacetate injection decreased the ascorbic acid content in animals (Nath, Sahu & Chitale, 1953*b*), it induced a slight increase when administered simultaneously with glucose (Nath, Belavady, Sahu & Chitale, 1953*a*). The condensation product of glucose and acetoacetate 'glucose cycloacetoacetate' gave a significant increase in ascorbic acid content of plasma of rats and germinated mung beans, and was postulated to be a precursor of ascorbic acid. The conversion of glucose cycloacetoacetate into ascorbic acid was confirmed by chromatography (Nath & Belkhode, 1959) and by biological studies with scorbutic guinea pigs (Nath & Belkhode, 1960). Radiochemical evidence indicated that [^{14}C]glucose cycloacetoacetate gave rise to labelled ascorbic acid in germinating *Phaseolus radiatus* (Thangamani & Sarma, 1956). That glucose cycloacetoacetate is a constituent of normal blood has been reported by Nath, Behki & Sahu (1958). Work with [1-^{14}C]- and [6-^{14}C]-glucose has suggested that in the conversion of glucose cycloacetoacetate into L-