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The Fractionation of Russell's-Viper (*Vipera russellii*) Venom with Special Reference to the Coagulant Protein

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Russell's-viper venom accelerates the coagulation of blood, even at very high dilutions, and requires Ca^{2+} ions (Macfarlane & Barnett, 1934) and phospholipid (Macfarlane, Trevan & Attwood, 1941) for its maximum activity. Biggs, Douglas & Macfarlane (1953) showed that preincubation of venom with serum and platelets resulted in a progressive increase in coagulant activity. Peden & Peacock (1958) found that ox serum was particularly active in potentiating the coagulant activity of the venom, and suggested that the venom acted enzymically on a substrate contained in the serum. The serum factor can be partially purified by adsorption on barium sulphate and elution by 0·2M-citrate (Macfarlane, 1961). By using a specific antiserum to inhibit the venom, Macfarlane (1961) obtained further evidence suggesting that the venom acted enzymically on a serum component to form an actively coagulant product.

The crude venom used in these experiments contains several enzymes (Roy, 1938; Ghosh, 1940; Zeller, 1948) which may be implicated in its effect on blood coagulation. Grasset & Schwartz (1955) attempted to fractionate the venom by electrophoresis on paper strips, and recovered a fraction which contained 80% of the coagulant activity of the crude venom. This fraction hydrolysed gelatin.

The purpose of the work described here was to obtain from Russell's-viper venom, by chromatography on diethylaminoethylcellulose columns, the fraction that has coagulant activity, and to purify this fraction further so that its physical properties could be determined. The biochemical properties of this fraction have also been investigated in an attempt to determine the mechanism by which the venom coagulates the blood.

MATERIALS AND METHODS

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

Russell's-viper venom. Dried venom was generously provided by the Wellcome Physiological Research Laboratories.

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Plasma. This was prepared by the method of Born & Esnouf (1959) with sodium citrate as anticoagulant. Plasma prepared from blood collected from two or three donors was pooled, dispensed in 3 ml. volumes in cellulose acetate tubes and stored at −15°. The plasma was not used if it had been stored for more than 7 days.

Phospholipid emulsion. A dry powder of the acetone-insoluble fraction of human brain was extracted with 15 vol. of chloroform–methanol (1:1, v/v). The extract was washed twice with water and the solvent removed in a rotary evaporator at 30°. The residue was dissolved in benzene and stored at −20°. For preparation of emulsions the benzene was removed from samples of the stock solution, and the residue was dissolved in peroxide-free ether. The ether solution was then added to buffer and the ether was removed by bubbling nitrogen through the mixture for 30 min. at room temperature.

Bovine-serum fraction. Blood (200 ml.) was collected from each of 4–6 cows at the slaughter-house, allowed to clot in glass bottles, and incubated at 37° until most of the prothrombin had been converted into thrombin. This usually took 3–4 hr. The serum was obtained by centrifuging, pooled, and stored at −15°. The bovine-serum fraction was prepared by stirring the serum with citrate-washed barium sulphate (Biggs & Macfarlane, 1957), 1 g./10 ml. of serum, at room temperature for 10 min. The barium sulphate was washed twice with 0·15M-sodium chloride and twice with water. The proteins adsorbed by the barium sulphate were eluted by mixing with 0·2M-sodium citrate buffer, pH 6·8, at room temperature for 5 min. The volume of sodium citrate solution was 0·2–0·1 that of the original serum. The mixture was centrifuged and the supernatant was dialysed overnight against 0·15M-sodium chloride and stored at −15°.

Buffers. Tris was dissolved in water to give the molarity indicated, and the pH of this solution was adjusted with N-hydrochloric acid to give pH 7·3. This buffer is referred to as 'tris–hydrochloric acid'. The tris–hydrochloric acid buffer made up in 0·15M-sodium chloride is referred to as 'tris–saline'.

Calcium chloride solutions. These were prepared by adding a known amount of calcium carbonate to an equivalent amount of hydrochloric acid in water. After the calcium carbonate had dissolved the solution was boiled to remove dissolved carbon dioxide. Sufficient tris was then added to give a final concentration of 0·01M, and the pH was adjusted to the desired value with hydrochloric acid. The solution was then made up to final volume with water. The solution is referred to as 'buffered calcium chloride'.

Assay of coagulant activity. In all experiments coagulant activity was estimated from the ability to accelerate the

coagulation of normal citrated platelet-poor human plasma. The coagulant activity of the various protein fractions obtained from the column was assayed after dialysis against 0·01 M-tris–saline buffer, pH 7·3. Fresh plasma (0·1 ml.) was incubated with 0·1 ml. of the protein fraction in 0·01 M-tris–saline buffer, pH 7·3, and 0·1 ml. of a phospholipid emulsion in 0·01 M-tris–hydrochloric acid buffer, pH 7·3. The phospholipid emulsion contained 0·50 mg. of phospholipid/ml. To the incubation mixture at 37° was added 0·1 ml. of buffered 0·05 M-calcium chloride and the clotting time of the plasma was recorded. Duplicate determinations agreed within 1 sec. Calibration curves for a range of venom concentrations were constructed.

In the studies on the activation of bovine-serum fraction by purified venom protein, these two components were permitted to react in the presence of Ca^{2+} ions, and then the ability of the mixture to accelerate the coagulation of recalcified plasma in the presence of added phospholipid was determined. In these experiments 0·1 ml. of bovine-serum fraction and 0·1 ml. of purified venom protein were mixed and brought to 37°. Buffered 0·025 M-calcium chloride (0·1 ml.) at 37° was added and the reaction was timed from this point. After the appropriate interval a sample of the reaction mixture was added to 0·4 ml. of a brain-phospholipid emulsion in 0·01 M-tris–hydrochloric acid buffer, pH 7·3, containing 0·25 mg. of phospholipid/ml. After 15 sec., 0·1 ml. of the phospholipid mixture was added simultaneously with 0·1 ml. of buffered 0·025 M-calcium chloride, pH 7·3, to 0·1 ml. of plasma, and the clotting time was recorded. The plasma, buffered calcium chloride and phospholipid emulsion were previously warmed to 37°. Any variations from this procedure are noted below.

Optimum concentrations of phospholipid and calcium were determined by preliminary experiments and were used for all assays. The concentration of the purified venom protein was such that in the absence of the bovine-serum fraction the clotting time of the plasma exceeded 30 sec. Reaction mixtures containing bovine-serum fraction, buffer and calcium chloride gave coagulation times of 65–95 sec. The recalcification time of the indicator plasma containing phospholipid in the concentrations used in the assay systems ranged from 63 to 95 sec. That thrombin was not generated in the system was demonstrated by experiments in which complete reaction mixtures added to phospholipid emulsions as outlined above clotted recalcified plasma in 9 sec., but did not clot 0·5% fibrinogen in 10 min. at 37°.

The coagulant activity generated in the reaction mixture was determined from the coagulation time of the plasma in the assay system by reference to a standard curve. Such curves were prepared from experiments in which reaction mixtures containing purified venom protein, bovine-serum fraction and calcium chloride were incubated for sufficient periods to permit development of maximum coagulant activity. The reaction mixture was then placed in melting ice and dilutions were prepared in ice-cold 0·01 M-tris–saline buffer, pH 7·3, containing purified venom protein in the same concentration as was present in the reaction mixture. The venom protein was included in the diluting buffer to ensure a constant venom-protein effect on the assay system. Solutions containing 10, 20, 40, 60, 80 and 100% of the reaction mixture were tested for coagulant activity in the same assay system as was used for the particular experiment. The clotting times obtained were plotted against the concentration of reaction mixture with log–log co-ordinates and a straight line was obtained.

Fractionation of crude venom. Crude Russell's-viper venom was fractionated on columns of DEAE-cellulose (Whatman DE 50). The columns were eluted with 0·01 M-tris solutions whose pH was adjusted with phosphoric acid to the values required. DEAE cellulose powder (25 g.) was suspended in 2 M-sodium chloride dissolved in 0·01 M-tris–phosphate buffer, pH 6·0. The slurry was packed in five equal portions into a glass column 3·4 cm. in diameter. Each portion was packed under pressure from a nitrogen cylinder. The first portion was packed under a pressure of 50 mm. Hg and for each subsequent portion the pressure was increased by 50 mm. Hg. The height of the packed column was 28 cm. The column was washed with 2 l. of the buffer used for preparing the slurry and then was equilibrated with 0·01 M-tris–phosphate buffer, pH 8·5. Crude Russell's-viper venom was dissolved in 0·01 M-tris–phosphate buffer, pH 8·5, to give a concentration of 7–10 mg./ml. A small amount of insoluble material was removed by centrifuging, and the clear solution was applied to the column. Quantities of venom ranging from 4 to 12 mg. of protein/g. of DEAE-cellulose were used in five different experiments with similar results. In all experiments the fractionation was carried out with 0·01 M-tris–phosphate buffer at, successively, pH 8·5, 7·0 and 6·0. After 500 ml. of buffer at pH 8·5 had passed through the column, the buffer was changed to one at pH 7·0 and this was used until the pH of the effluent had reached 7·0. Buffer at pH 6·0 was then substituted and used until the column had become equilibrated at pH 6·0. The column was then eluted successively with 0·15 M-, 0·5 M- and 1·0 M-sodium chloride dissolved in 0·01 M-tris–phosphate buffer, pH 6·0. Fractions (10 ml.) were collected and the protein concentration in each was estimated from measurements of the extinction of 280 mμ in a 1 cm. cell.

The protein fractions eluted from the column were combined, dialysed against water at 4° and freeze-dried. The dried protein was dissolved in 0·15 M-sodium chloride and stored at −20°.

Determination of protein concentration. Protein concentration was estimated as a routine from the extinction of the solutions at 280 mμ in a 1 cm. cell. The factor necessary for conversion of E_{280} into mg. of protein was determined for each fraction investigated. The concentrations of reference solutions of each protein (3–10 mg. of protein/ml.) were determined in a differential refractometer (Cecil & Ogston, 1951). The refractive-index increment for the protein was taken as $1{\cdot}84 \times 10^{-2}$; this was confirmed from measurements made with serum albumin and trypsin solutions of known concentrations. After measuring the protein concentration in the refractometer the reference solutions were diluted and the E values were determined.

Assay of the enzyme activities of the protein fraction. The protein fractions after the chromatography of the crude venom were tested for the enzymic activities known to be present in the crude venom. L-Amino acid oxidase was assayed manometrically with L-leucine as substrate (Zeller, 1948). Lecithinase activity was measured in ethereal solutions (Long & Penny, 1957); the substrate was ovolecithin which had been purified by chromatography on a silicic acid column (Hanahan, Dittmer & Warashina, 1957). Phosphodiesterase was assayed by the method of

Butler (1955), with dialysed yeast ribonucleic acid core as substrate, which was kindly provided by Dr K. Burton. Phosphomonoesterase was determined by the method of Burton & Petersen (1960), with *p*-nitrophenyl phosphate as substrate. 5′-Nucleotidase was assayed by the method of Heppel & Hilmoe (1955); the release of 1 μg. of inorganic phosphorus/μg. of protein during incubation for 10 min. was taken as a unit of enzymic activity. Adenosine triphosphatase was measured at pH 7·2 in 0·08 M-tris–acetate buffer with mM-ATP as substrate; incubations were carried out at 37° for 15 min., after which 0·2 vol. of 10% (w/v) trichloroacetic acid was added and the inorganic phosphorus released was estimated (Fiske & Subbarow, 1925), the release of 1 μg. of inorganic phosphorus/μg. of protein during incubation for 15 min. being taken as a unit of enzymic activity. Proteinase activity was determined by a potentiometric technique (Waley & Watson, 1953). Solutions (2%, w/v) of bovine serum albumin (fraction V), bovine serum globulin (fraction II) (Armour and Co.) and gelatin were used as substrates. The substrates and the purified venom protein were dissolved in 0·1 M-potassium chloride. A portion (1 ml.) of the substrate was mixed with the venom protein and made up to 2 ml. with 0·1 M-potassium chloride. In some experiments calcium chloride was added (final concentration 0·1 M). Proteolysis was measured by determining the amount of 0·05 N-sodium hydroxide required to maintain the pH of the reaction mixture at the predetermined value.

Assay of amino acid-esterase activity. This was carried out with TAMe (Schwert, Neurath, Kaufman & Snoke, 1948), obtained from L. Light and Co. Ltd., and lysine ethyl ester (Werbin & Palm, 1951), obtained from the Mann Research Laboratories Inc., by the method described for the proteinase assay. All results were corrected for spontaneous hydrolysis. Soya-bean trypsin inhibitor used in some experiments was dissolved in 0·1 M-potassium chloride and used at a final concentration of 100 μg./ml.

Electrophoresis. This was carried out in an Antweiler micro-electrophoresis apparatus (Shandon and Co. Ltd.). The protein solutions were equilibrated with the various buffers before electrophoresis.

Sedimentation-velocity experiments. These were done in a Spinco ultracentrifuge (model E) at 59 780 rev./min. with the AN-D rotor at 20°. The protein solution was equilibrated with the buffer before the experiments.

Viscosity determinations were made in an Ostwald viscometer at 25°. Measurements of the densities of the buffer and protein solutions were made at 25° with a linear density gradient made from mixtures of bromobenzene and kerosene (Esso 'blue' paraffin) which had been previously saturated with 0·2 M-potassium bromide. The gradient was calibrated with standard solutions of potassium chloride. The value of the partial specific volume, $\bar{v}$, for the protein was obtained from the density measurements (Schachman, 1957).

The homogeneity of the protein preparations was determined by an analysis of the sedimenting boundary obtained during the sedimentation-velocity experiments (Schachman, 1957).

Molecular-weight measurements. The weight-average molecular weight (M_w) was measured by the Archibald (1947) technique under the following conditions: rotor speed 11 573 and 20 410 rev./min. in the AN-D rotor at 20°. The protein concentrations used were 0·3–0·6% (w/v).

The protein concentration at the meniscus (c_m) was evaluated by measuring the areas under the schlieren pattern of enlarged tracings of the photographs. The initial protein concentration at the meniscus was determined in a second experiment with a synthetic boundary cell. The molecular weight was calculated from the following relation:

$$M = \frac{(\delta c/\delta x)_{x=x_m}}{c_m x_m} \cdot \frac{RT}{(1-\bar{v}\rho)\omega^2} \quad (1)$$

where x_m is the distance of the meniscus from the axis of rotation. The other symbols have their usual significance.

The molecular weight was also calculated from the classical Svedberg equation

$$M = \frac{RTS}{D(1-\bar{v}\rho)} \quad (2)$$

where S is the sedimentation coefficient ($S_{20,w}$) and D the diffusion coefficient ($D_{20,w}$).

Diffusion-coefficient measurement. This was done in the centrifuge with a synthetic boundary cell rotating at 10 589 rev./min. in the AN-D rotor at 20°. Photographs were taken at intervals of 16 min. for 2 hr. The apparent diffusion coefficient, $D_{app.}$, was obtained from the expression:

$$D_{app} = \frac{1}{4\pi t} \cdot \left(\frac{A}{H_{max.}}\right)^2 \cdot (1-\omega^2 St) \quad (3)$$

Measurements of the area under the boundary, A, and the maximum height of the boundary, $H_{max.}$, were made from enlarged tracings of photographs. S is the sedimentation coefficient ($S_{20,w}$) and t the time (sec.); the other symbols have the usual significance. The diffusion coefficient, $D_{20,w}$, was obtained from a plot of $D_{app.}$ against $1/t$ (Schachman, 1957). The diffusion coefficient was determined on a sample of the same protein solution as had been used in the sedimentation-velocity experiments.

RESULTS

Purification of the coagulation protein. The elution diagram obtained when Russell's-viper venom was fractionated on DEAE-cellulose is given in Fig. 1. In this experiment the yield of ultraviolet-absorbing material from the columns was 98% and the yield of coagulant activity was 93%. The coagulant activity of the fractions obtained from the column was compared with that of crude venom. The activities of each of the protein fractions relative to that of crude venom are given in Table 1. Nearly all of the coagulant activity was recovered in fraction 7.

Only one component was found when the fraction containing the coagulant activity (fraction 7) was examined in the electrophoresis apparatus in 0·1 M-sodium acetate buffers at pH 5·5 and 6·0, and in 0·1 M-sodium phosphate buffers at pH 6·5 and 7·0. This fraction appeared to be isoelectric between pH 5·5 and 6·0; however, it was not possible to measure its electrophoretic mobility with the apparatus available.

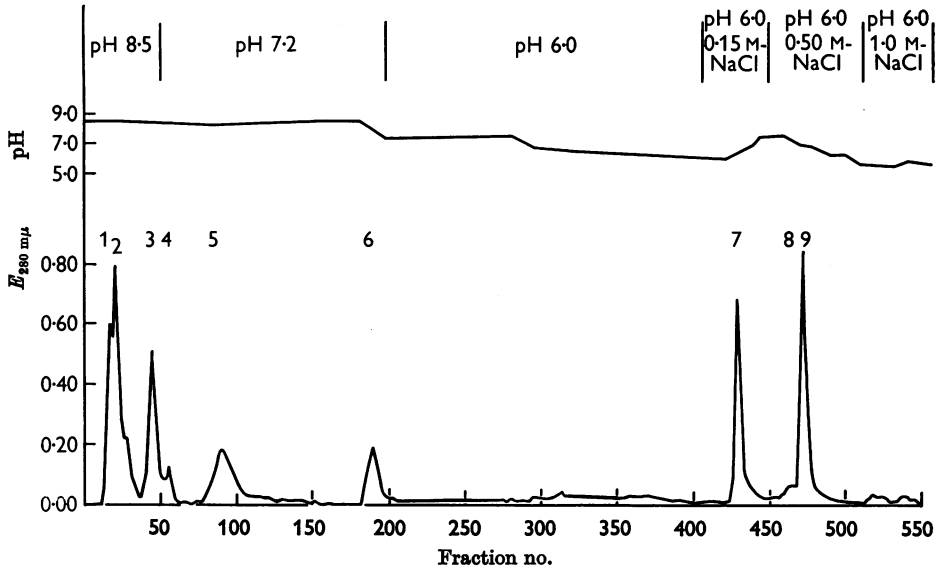

Fig. 1. Fractionation of crude Russell's-viper venom on DEAE-cellulose. A solution of 298 mg. of crude venom in 45 ml. of 0·01 M-tris–phosphate, pH 8·5, was fractionated at room temperature as outlined in the text. The solvents used are indicated at the top of the Figure. The pH indicated is that of the effluent solution. The flow rate was 75 ml./hr. Fractions of 10 ml. were collected. The peaks are numbered for reference to Tables 1 and 2.

Table 1. *Distribution of coagulant activity in the fractions obtained from the column*

The percentage of total protein in each fraction was estimated from the extinction at 280 mμ. The remainder of the protein eluted from the column can be accounted for by the protein not included in the fractions. Coagulant activity was determined as outlined in the text. Coagulant activity relative to crude venom was determined by estimating the amount of crude venom required to give the same coagulation time as the samples from the peak by reference to a standard curve prepared as in Fig. 4 and dividing the crude-venom concentration by the protein concentration in the fraction. Activity recovered in each fraction was estimated by assigning an arbitrary unit to the activity of the crude venom (1 unit equals the coagulant activity of a solution containing 1 μg. of crude venom/ml.) and determining the total units recovered in each peak.

Fraction	Percentage of total protein applied to column	Coagulant activity relative to crude venom	Activity recovered in peak (%)
1	8	0·001	<0·1
2	13	0·002	<0·1
3	9	<0·001	<0·1
4	2	<0·001	<0·1
5	7	<0·001	<0·1
6	5	0·2	0·7
7	10	12	92
8	1	0·3	0·3
9	13	0·007	0·1

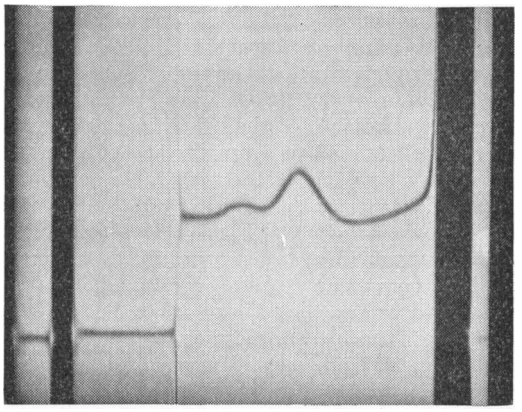

Fig. 2. Ultracentrifuge pattern of fraction 7 as recovered from the DEAE-cellulose column. The protein concentration was 0·5% (w/v) in 0·1 M-sodium phosphate buffer, pH 6·5. Sedimentation was from left to right. The centrifuge was run at 59 780 rev./min. for 84 min. at 6°.

When examined in the ultracentrifuge fraction 7 was found to contain two components (Fig. 2), which had S_{20} values of 4·7 and 2·8 s at pH 7·0 in 0·1 M-sodium phosphate buffer. Attempts to resolve these two components by rechromato-

graphy on DEAE-cellulose columns were unsuccessful, but it was possible to separate them by ultracentrifuging into a sucrose density gradient. For this a Spinco model L preparative ultracentrifuge was used with a swinging bucket rotor no. S.W. 39.

The sucrose gradient was prepared with an apparatus in which a reservoir was connected to a mixing vessel in a closed system so arranged that the solution in the reservoir replaced the solution removed from the mixing vessel while the volume in the mixing vessel remained constant. The reservoir contained 4·2 ml. of 0·1 M-sodium phosphate buffer, pH 7·0, and the mixing vessel contained 4·2 ml. of a 20 % (w/v) sucrose solution in the same buffer. After filling the reservoir and mixing vessel, the tubing connecting them was opened and the solution in the mixing vessel was rapidly stirred with a magnetic stirrer. The solution (4·2 ml.) was then run from the mixing vessel slowly down the side of the centrifuge tube without disturbance of the gradient. The concentration gradient prepared in this way was from 20 to 7 % (w/v) sucrose. After this, 0·6 ml. of a solution containing not more than 30 mg./ml. of the coagulant-protein fraction from the column (fraction 7) in 0·1 M-sodium phosphate buffer, pH 7·0, was carefully layered on top of the gradient. The centrifuge tubes were placed in the rotor, previously cooled to 0°, and were centrifuged at 36 000 rev./min. After 40 hr. the rotor was allowed to come to rest without braking to minimize disturbance of the gradient. The rotor temperature at the end of the run was 3°. The contents of the tubes were fractionated by piercing the bottom of the centrifuge tube with a pin and collecting two drops in each of a series of test tubes. To each of the test tubes was added 2·5 ml. of 0·15 M-sodium chloride and the E_{280} of the fractions was measured (Fig. 3). Two components were resolved. Fractions containing the faster-moving component were combined, dialysed against water and then freeze-dried. The fractions containing the slower-moving component were treated in the same way. The residues were redissolved in 0·01 M-sodium phosphate buffer, pH 7·0, for analysis in the ultracentrifuge. A sample of each component was diluted and dialysed against 0·01 M-tris–saline buffer, pH 7·3, for assay of the coagulant activity. Table 2 shows that the faster-moving component had marked coagulant activity and that the slower-moving component was less active by a factor of 10. Possibly the activity observed in this fraction was due to contamination with the faster-moving component.

There was a decrease in the specific activity of the purified protein when compared with the fraction before centrifuging. This loss in activity was probably due to partial inactivation of the protein during the ultracentrifuging and subsequent manipulations. The effect on the coagulation time of plasma of different concentrations of crude venom, of the coagulant protein obtained from the column (fraction 7), and of the final purified coagulant protein, is given in Fig. 4. For the three preparations almost parallel straight lines were obtained, when the results were plotted on logarithmic co-ordinates, showing that the coagulation time of the plasma could be used as an assay system for

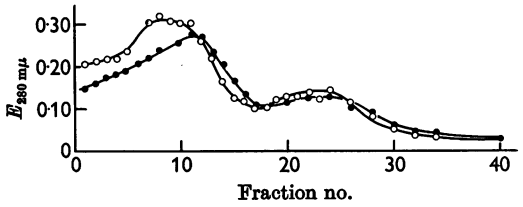

Fig. 3. Separation of the heavy and light components of fraction 7 by centrifuging into a sucrose gradient (see text). The sedimentation was from right to left at 36 000 rev./min. The two curves were derived from duplicate samples centrifuged simultaneously.

Table 2. *Purification of the coagulant protein*

The coagulant activity of the various components was determined as outlined in the text.

Component	Yield of protein (%)	Sp. activity (units/μg. of protein)
Crude venom	—	1
Fraction 7 (from column)	10·4	12
Heavy component (S_{20} 4·8)	4·1	8
Light component (S_{20} 2·8)	1·3	0·9

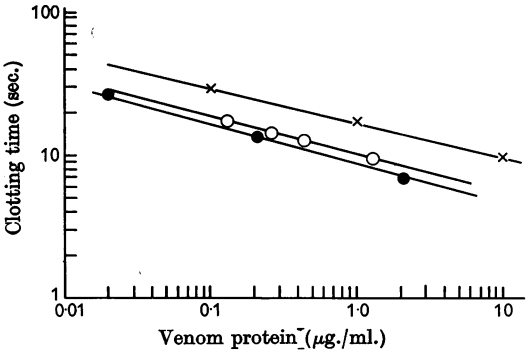

Fig. 4. Coagulant activity of purified coagulant protein obtained after centrifuging (○) compared with that of fraction 7 from the column (●) and with that of crude Russell's-viper venom (×). The data are plotted on log–log co-ordinates to obtain a straight line and facilitate comparison of the activities of the three preparations. Coagulant activity was determined as described in the text.

different preparations of the coagulant protein with various specific activities.

The purified venom protein had no coagulant action on fibrinogen. When 0·1 ml. of purified venom-protein solution containing up to 100 μg./ml. was incubated at 37° with 0·1 ml. of 1·0% (w/v) bovine fibrinogen in 0·01 M-tris–saline buffer, pH 7·3, no clot was found after 45 min., even in the presence of buffered 0·05 M-calcium chloride. The addition of thrombin at this point caused the mixture to clot in 10–15 sec.

Physical properties of the coagulant protein. The results of these measurements are summarized in Table 3. There was a variation in the value of S_{20} with pH; however, insufficient material was available to study the phenomenon in detail.

The constant value (± S.E.M.) obtained for the $D_{app.}$ ($4{\cdot}66 \pm 0{\cdot}06 \times 10^{-7}$ cm.^{2}/sec.), and the presence of only a single component during the electrophoresis experiments, have been taken as a valid indication of physical homogeneity.

Enzymic activities of the protein fractions. Experiments were carried out to determine whether purified coagulant protein contained any of the several enzymic activities known to exist in crude Russell's-viper venom, both as a test of functional purity and to determine whether any enzymic activity was present which could be related to the coagulant activity of this fraction.

Examination of the distribution among the separate fractions of the various enzymic activities tested (Table 4) shows that none of them was present in the purified coagulant-protein fraction. In addition L-amino acid-oxidase activity was tested. Though this activity was readily demonstrated in the crude venom (335 μl. of oxygen/hr./mg. of dry venom), no activity was found in any of the fractions alone or in the presence of μM-flavin–adenine dinucleotide. The reason for the disappearance of this enzymic activity is not clear.

The coagulant fraction of Russell's-viper venom has been shown to have a gelatinase activity

Table 3. *Physical properties of the coagulant protein*

Determinations were carried out as described in the text.

Prep. no.	Protein concn. (mg./ml.)	pH	$\bar{v}$	S_{20}	D_{20}	Mol.wt.
1	5·4	5·8	—	5·80	—	—
2	2·2	5·8	—	5·88	—	—
3	3·0	5·8	—	5·75	—	—
4	5·0	6·45	—	5·53	4·66† ±0·06	106 700
5	7·0	6·45	—	5·52	—	—
6	11·0	7·0	—	4·85	—	105 300* ±1 400
7	5·0	7·0	—	4·80	—	—
8	2·9	7·0	0·720	5·05‡	4·20‡	104 700

* By Archibald's (1947) method; other mol.wt. values were obtained from sedimentation and diffusion data.
† $D_{app.}$ obtained from boundary analysis during the sedimentation-velocity run.
‡ $S_{20,w}$; $D_{20,w}$.

Table 4. *Enzymic activities of the protein fractions obtained after chromatography of Russell's-viper venom on diethylaminoethylcellulose*

Determinations were carried out as described in the text. The units of enzyme activity for the last two columns are given in the Materials and Methods section.

Peak no.	Amount of protein* (μg.)	Activity of lecithinase† (% of lecithin hydrolysed in 60 min.)	Activity of diesterase (ΔE_{260}/30 min./μg. of protein)	Activity of monoesterase (ΔE_{260}/90 min./μg. of protein)	Activity of 5′-nucleotidase (units/μg. of protein)	Activity of adenosine triphosphatase (units/μg. of protein)
1	90	0	0·08	0·08	25·8	4·8
2	86	0	0·27	0·40	26·0	3·5
3	16	0	0·50	0·38	25·0	11·2
4	8	0	0·37	0·75	0·0	0·0
5	28	0	0·00	0·00	11·0	4·6
6	76	95	2·70	0·00	29·0	7·1
7*	100	0	0·00	0·00	0·0	0·0
8	20	0	0·10	0·35	0·0	14·5
9	46	0	0·04	0·00	4·1	10·2

* Material obtained after high-speed centrifuging (coagulant protein).
† The amount of protein used for this assay was one-half that shown in the previous column.

(Grasset & Schwartz, 1955). When the proteinase activity of the coagulant protein was assayed with bovine serum albumin and γ-globulin no activity was detected between pH 7·0 and 8·5. With gelatin as substrate slight activity was obtained at pH 8·0 in the presence of Ca^{2+} ions. The coagulant protein was also tested against synthetic proteinase substrates. The purified venom protein had esterase activity towards TAMe. The formation of toluene-*p*-sulphonylarginine during this reaction was demonstrated by paper chromatography. The optimum pH for the enzymic hydrolysis of TAMe was 8·1 (Fig. 5), and with 0·05 M-TAMe the reaction rate was proportional to enzyme concentration over a fourfold range.

The effect of substrate concentration on the reaction rate is shown in Fig. 6. From these data a Michaelis constant of $5·9 \times 10^{-4}$ M and a maximum reaction velocity of 77 μm-moles/min. were calculated (Lineweaver & Burk, 1934). Neither calcium chloride at a final concentration of 0·025 M nor soya-bean trypsin inhibitor at a final concentration of 100 μg./ml. had any effect on the esterase activity. The coagulant protein also hydrolysed lysine ethyl ester (0·02 M) but at a much slower rate (6 μm-moles/min./50 μg. of protein) than was obtained with TAMe. The optimum pH for the hydrolysis of lysine ethyl ester was 7·0.

Coagulant activity of the purified venom protein. Macfarlane (1961) suggested that crude Russell's-viper venom acts as an enzyme during the activation of a coagulation factor present in bovine serum, and experiments were carried out to test whether the purified venom protein behaves similarly to the crude venom.

To interpret the data obtained from these experiments the coagulant activity found when the bovine-serum fraction was incubated with purified venom protein has been expressed in arbitrary units. Thus the coagulant activity which can be derived from 0·1 ml. of each undiluted preparation of the bovine-serum fraction is defined as 100 units, and hence 1:10 diluted serum preparation would contain 10 units of potential activity, which after complete activation by the purified venom protein would yield 10 units of coagulant activity.

The units of activity were determined from the clotting time of the plasma by reference to standard curves prepared as described in the Materials and Methods section. The maximum number of units in each experiment was determined from the dilution of bovine-serum fraction used in the experiment.

The validity of the system used to assay coagulant activity was demonstrated by an experiment in which a standard curve was constructed as outlined above, and then the activity which would be developed in reaction mixtures containing various concentrations of bovine-serum fraction was determined. The coagulation times were converted into units by reference to the standard curve. The coagulant activity was directly proportional to the concentration of bovine-serum fraction in the reaction mixture.

The yield of coagulant activity was found to be independent of the concentration of purified venom protein. When 0·1 ml. of the bovine-serum fraction was incubated with 0·1 ml. of purified venom pro-

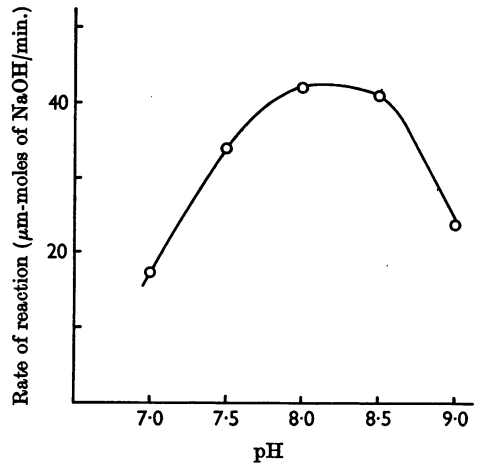

Fig. 5. Effect of pH on the TAMe-esterase activity of the coagulant protein. The reaction mixture contained 50 μmoles of TAMe and 45 μg. of purified venom protein in a final volume of 2·0 ml. of 0·1 M-potassium chloride. The rate of reaction was measured by the rate of addition of 0·05 N-sodium hydroxide necessary to maintain the original pH.

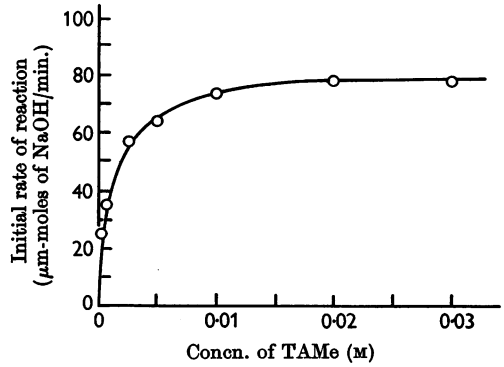

Fig. 6. Effect of concentration of TAMe on the rate of hydrolysis by the venom protein. The reaction mixture was that used in Fig. 5, except that the purified venom-protein concentration was 50 μg./ml. and the TAMe concentrations were those shown in the Figure. The reaction mixture was adjusted to pH 8·0 and the rate of reaction was determined as before.

tein at different concentrations (2, 5, 10 and 20 μg./ml.) and 0·1 ml. of buffered 0·025 M-calcium chloride, pH 7·3, the resultant coagulant activity formed was the same for each of the venom concentrations used after incubation for 5 and 10 min. at 37°. The control coagulation times in the absence of the serum fraction caused by the highest concentration of the venom (20 μg./ml.) were 19 sec., equivalent to 8% of the activity found in the complete system. At the lower venom concentrations clotting times of 30 sec. or greater were obtained.

The coagulant activities developed during the first minute of incubation of different amounts of bovine-serum fraction with the same amount of purified venom protein and calcium chloride are shown in Fig. 7. The plot of the coagulant activity formed against the concentration of bovine-serum fraction has the form of a rectangular hyperbola, which would be expected if the bovine-serum fraction were functioning as substrate in the system.

The rate of formation of coagulant activity at three concentrations of venom protein is shown in Fig. 8. The rate is linear at the lowest concentration of purified venom protein used but deviated from linearity at high concentrations. The coagulant activity formed during the first minute was directly proportional to the concentration of venom protein over a tenfold range, as would be expected if the venom were acting as an enzyme.

The activation of the bovine-serum fraction is dependent on the Ca^{2+} ion concentration (Fig. 9). The optimum rate of activation was obtained with

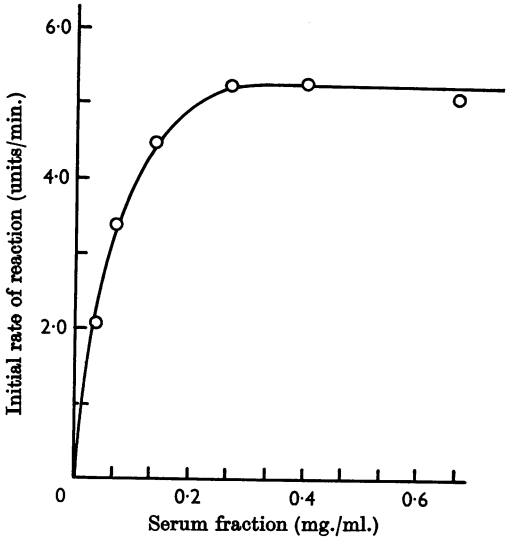

Fig. 7. Effect of bovine-serum-fraction concentration on the rate of formation of coagulant activity. A portion (0·1 ml.) of the purified venom-protein solution (0·21 μg./ml. in 0·01 M-tris–saline, pH 7·3), 0·1 ml. of buffered 25 mM-calcium chloride solution, pH 7·3, and 0·1 ml. of bovine-serum fraction were incubated at 37° for 1 min. after the addition of Ca^{2+} ions, then 0·1 ml. of the reaction mixture was assayed for coagulant activity without dilution in buffer. The final bovine-serum-fraction concentrations were those noted in the Figure. The highest concentration of bovine serum used contained 100 units of potential activity in 0·1 ml.

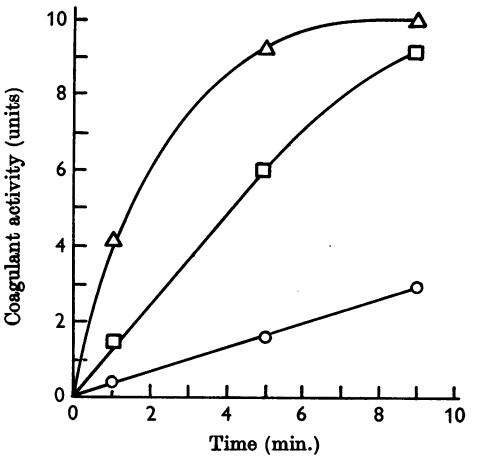

Fig. 8. Rate of appearance of coagulant activity on incubation of purified venom protein with bovine-serum fraction. The reaction mixtures were prepared as in Fig. 7, except that the bovine-serum fraction contained 10 units of potential activity in 0·1 ml. and the purified venom-protein solutions contained sufficient protein to give final concentrations (μg./ml.) of 0·03 (○), 0·10 (□) and 0·33 (△). At the various intervals, noted after the addition of calcium, 0·1 ml. of the reaction mixture was assayed for coagulant activity without dilution in tris–saline.

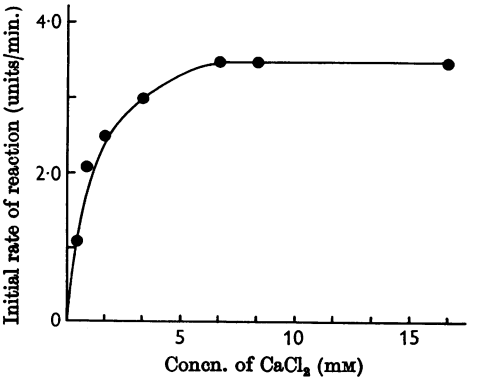

Fig. 9. Effect of calcium concentration on the rate of formation of coagulant activity. The reaction mixture was prepared as in Fig. 7 except that the bovine-serum fraction contained 50 units of potential activity/0·1 ml. and the buffered calcium chloride solutions contained sufficient Ca^{2+} ions to give the final concentrations noted in the Figure.

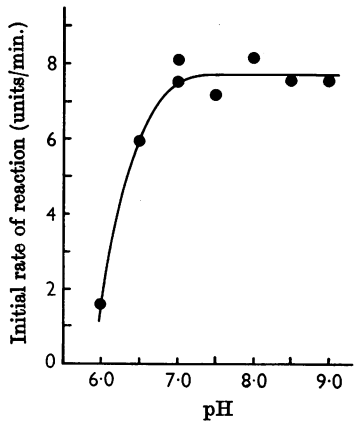

Fig. 10. Effect of pH on the formation of coagulant activity. The reaction mixtures were prepared as in Fig. 7 except that the purified venom-protein solution contained 0·1 μg./ml.; the bovine-serum fraction contained 20 units of potential activity/ml.; and the pH of the purified venom protein, bovine-serum fraction and calcium chloride solutions were adjusted to the values given in the Figure before they were mixed. At 1 min. after adding calcium chloride, 0·1 ml. of the reaction mixture was assayed for coagulant activity as outlined in the text, except that the phospholipid emulsion contained 0·5 mg. of brain phospholipid/ml. in 0·1 M-tris–chloride buffer, pH 7·3.

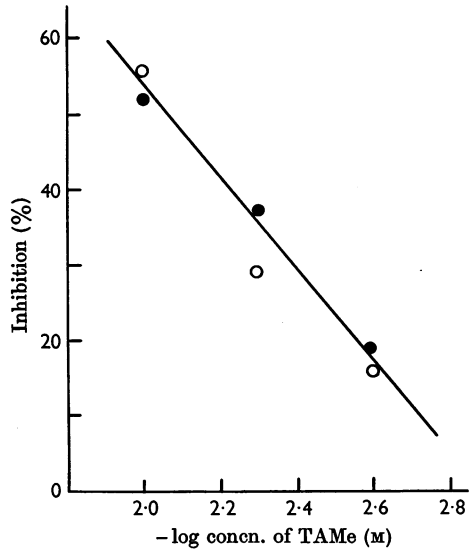

Fig. 11. Effect of TAMe on the activation of the bovine-serum fraction by the venom protein. In Expt. *A* (●) the reaction mixture consisted of 0·1 ml. of bovine-serum fraction containing: 4·0 mg. of protein/ml. in 0·15 M-sodium chloride; 0·1 ml. of purified venom protein containing 2·0 μg./ml. in tris–saline buffer, pH 8·0; 0·1 ml. of 0·05 M-tris–saline buffer, pH 8·0, containing TAMe in sufficient quantity to give the final concentrations noted in the Figure; 0·1 ml. of buffered 33 mM-calcium chloride, pH 8·0. After 1 min. at 37°, 0·1 ml. of the reaction mixture was mixed with 0·9 ml. of 0·01 M-tris–saline buffer, pH 7·3, and 0·1 ml. of this mixture was assayed for coagulant activity. In Expt. *B* (○), the reaction mixture was the same as in Expt. *A* except that the final volume was 2·0 ml. After 1 min., the reaction mixtures were chilled in ice, and then dialysed for 10 hr. at 4° against 0·01 M-tris–saline buffer, pH 7·3. After dialysis 0·1 ml. of the reaction mixture was assayed for coagulant activity.

7 mM-calcium chloride. The effect of pH on the coagulant activity is given in Fig. 10. Maximum activity was attained in reaction mixtures incubated at between pH 7·0 and 9·0. Below pH 7·0 there was a rapid loss in activity.

*Effect of toluene-*p*-sulphonylarginine methyl ester on the activation of the serum fraction.* Since both TAMe and bovine-serum fraction appeared to be substrates for the purified venom protein, the effect of TAMe on the activation of the serum fraction was investigated. The incubations were carried out at pH 8·0 because at this pH the venom protein hydrolysed TAMe and activated the bovine-serum fraction at the optimum rate. Two different types of experiments were performed.

In Expt. *A* (Fig. 11), TAMe was added to reaction mixtures containing purified venom protein, bovine-serum fraction and calcium chloride, and the coagulant activity formed was compared with that found in reaction mixtures not containing TAMe. The effect of TAMe on the assay system was controlled by adding TAMe of the appropriate concentration to the tris–saline buffer used before the assay for dilution of the reaction mixtures incubated without TAMe. No inhibition of the assay system by the concentrations of TAMe used could be detected. Fig. 11 shows that TAMe inhibited the formation of coagulant activity and that this inhibition was dependent on the concentration of TAMe. In similar experiments with lysine ethyl ester no inhibition could be detected.

In Expt. *B*, reaction mixtures consisting of purified venom protein, bovine-serum fraction and calcium chloride with or without different concentrations of TAMe were incubated at 37° for 1 min. and then chilled in melting ice to stop the reaction. The reaction mixtures were kept cold and were immediately dialysed at 4° for 10 hr. against tris–saline buffer to remove TAMe and Ca^{2+} ions. At the end of the dialysis the reaction mixtures were assayed (Fig. 12) in a system identical with that used in the previous experiment.

DISCUSSION

A coagulant protein has been obtained from crude Russell's-viper venom after chromatography on DEAE-cellulose. The protein fraction amounted

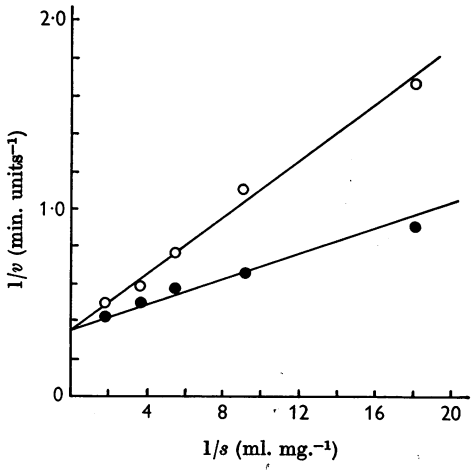

Fig. 12. Effect of TAMe on the rate of formation of coagulant activity from different concentrations of bovine-serum fraction. The reciprocal of the bovine-serum-fraction concentration in mg./ml., s, is plotted against the reciprocal of the initial rate in units/min., v. The reaction mixtures were prepared as in Expt. *A* except that the purified venom-protein solution used contained 0·2 μg./ml. and the bovine-serum fraction was added to give the final concentrations shown in the Figure. After 1 min., 0·1 ml. of the reaction mixture was assayed for coagulant activity as outlined in the text. TAMe was added to the reaction mixture (○) at a final concentration of 1 mM; in Expt. *A* (●) the reaction mixture contained no TAMe. To control possible effects of TAMe on the assay system in Expt. *A* (●) the phospholipid emulsion contained TAMe at a final concentration of 0·2 mM. In these experiments TAMe had no detectable effect on the assay system.

to 10 % of the protein applied to the column, but had an apparent specific activity 12 times that of the crude venom. This increase in specific activity over the theoretical value may be due to the presence of an anticoagulant fraction in the crude venom (Grasset & Schwartz, 1955). No anticoagulant activity was detected in any of the protein fractions obtained from the column, but it could have been masked by the small amount of coagulant activity present in some of the fractions.

The performance of the columns was completely reproducible, and the coagulant protein was always contaminated with the second protein, even with different solvent systems. This, however, might be expected, since both the proteins behaved similarly on electrophoresis. The centrifuging of the protein into the sucrose density gradient proved a useful adjunct to column chromatography, and with the technique described was reproducible. The only limitation to this technique was the relatively small amount of protein (15–20 mg.) that could be centrifuged in each tube.

The protein preparations fitted the normally accepted criteria for physical homogeneity and in addition the protein fraction was devoid of other enzyme activities which were present in the crude venom. This is in contrast with the finding that the other protein fractions contained various proportions of different enzyme activities. The reason for the distribution of one enzymic activity in different fractions has not been investigated, but it may arise from the choice of unsuitable solvents for the elution of these particular enzymes.

The purified venom protein behaves as an enzyme in the activation of a coagulation factor present in bovine serum, which supports the suggestion made by Macfarlane (1961). The purified coagulant protein hydrolysed the synthetic proteinase substrates, TAMe and lysine ethyl ester. The esterase activity of this protein was greater with TAMe than with lysine ethyl ester, which was hydrolysed at only 7 % of the rate found with TAMe. This suggests some structural requirement for the substrate. The esterase activity of the coagulant protein resembles that found for thrombin (Sherry & Troll, 1954). Both these enzymes hydrolyse TAMe, have little or no activity with lysine ethyl ester and are unaffected by soya-bean trypsin inhibitor. The Michaelis constant for the reaction between the venom protein and TAMe was 0·59 mM, which is lower than that found for thrombin, 4·3 mM (Sherry & Troll, 1954). The major difference between thrombin and the venom protein is that venom protein has no coagulant action on fibrinogen.

The esterase activity and coagulant activity of the purified venom protein appear to be related, because TAMe competitively inhibited the activation of the bovine-serum coagulation factor by the venom protein. Lysine ethyl ester had no inhibitory action.

SUMMARY

1. The coagulant protein has been obtained from Russell's-viper venom as a physically homogeneous component with a specific activity eight times that of the crude venom.

2. The coagulant protein functions as an enzyme in activating a coagulation factor present in bovine serum, and catalyses the hydrolysis of a synthetic proteinase substrate, toluene-*p*-sulphonylarginine methyl ester.

3. Toluene-*p*-sulphonylarginine methyl ester functions as a competitive inhibitor of the activation of a coagulation factor in bovine serum by the purified coagulant protein.

We are indebted to Dr H. Blaschko for his help in the assay of α-amino acid-oxidase activity and to Dr S. G. Waley for advice on the determination of proteinase activity.

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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,