The Isolation of a Component of the Venom of *Trimeresurus okinavensis* that Causes the Aggregation of Blood Platelets

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1. A substance has been isolated from the venom of the Okinawan pit viper (*Trimeresurus okinavensis*) that causes the aggregation of human blood platelets. The reaction resembles that between platelets and thrombin or connective tissue, in that the platelets release ADP. 2. The purified venom fraction is a protein-polysaccharide, and after Pronase digestion had mol.wt. 4×10^6 , containing 10% peptide, 85% neutral sugars and 5% hexosamines. It is distinct from the proteolytic, esterolytic and coagulant materials also present in the venom, and has not been shown to possess any enzymic activity. How it reacts with platelets is not yet known.

The venom of the Okinawan pit viper, or himedabu, *Trimeresurus okinavensis*, is one of several venoms of snakes belonging to the family Crotalidae that can cause the aggregation of human platelets and the release of some of their constituents (Davey & Lüscher, 1965b, 1967). These venoms can also clot purified fibrinogen. It was at first thought that an enzyme resembling thrombin (EC 3.4.4.13) was responsible both for the clotting and for the effects on platelets (Davey & Lüscher, 1965a). However, treatment of the venoms with di-isopropyl phosphorofluoridate inhibited their coagulant and esterolytic activities without altering their actions on platelets; also, the coagulant and platelet-aggregating activities could be separated by electrophoresis in agarose or starch gels (Davey & Lüscher, 1968). The present paper presents an account of the purification of the platelet-aggregating material from *T. okinavensis* venom, and describes some of its properties.

MATERIALS AND METHODS

T. okinavensis venom. Dried venom was purchased from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Different batches, nos. 8897 and 33217, were used for preparations A and B respectively. These were similar in activity and in behaviour on electrophoresis in polyacrylamide gels and immunoelectrophoresis.

Buffers. Reagent-grade tris (British Drug Houses Ltd., Poole, Dorset) was recrystallized from ethanol. Recrystallized tris was used for all buffers. Solutions were adjusted

to the required pH with either N-HCl (molarity refers to final concentration of tris) or N-H₃PO₄ (molarity refers to concentration of phosphate).

Plasma. Platelet-rich plasma was usually obtained from blood treated with the citric acid-sodium citrate-glucose solution (ACD) of Aster & Jandl (1964): 1 vol. to 4 vol. of blood. The whole blood concentration of citrate was 30 mM, and the final plasma pH about 6.5. The blood was centrifuged at 145g for 20 min. at 0°, and the separated plasma centrifuged again to remove residual cells. Sometimes the plasma pH was brought back to about 7 by adding 5 N-NaOH dropwise with brisk stirring (neutralized ACD-plasma). Other anticoagulants occasionally employed were EDTA (disodium salt), 5 or 10 mM in whole blood, and heparin (Pularin; Evans Medical Ltd., Liverpool), 20 units/ml. of whole blood.

Measurements of platelet aggregation. A modification of the method of Born (1962) was used. The plasma, in a flat-bottomed cylindrical glass tube, 7.2 cm. $\times$ 0.9 cm., was placed in the path of a narrow (0.7 cm. diam.) beam of white light, and scattered light was measured at right angles to the incident beam by a cadmium sulphide photoconductor. The changes in resistance of the photoconductor were recorded on a potentiometer recorder. The plasma was stirred by a small piece of plastic-covered steel wire, and rotated by a magnet placed just below the sample. The tests were always done at room temperature.

The recorder deflexion obtained with platelet-rich plasma was adjusted to a convenient value. The solution to be tested was added and platelet aggregation was accompanied by a decrease in turbidity of the solution, which resulted in a decrease in the recorder deflexion. The recording obtained was interpreted empirically in terms of a 'rate' of platelet aggregation, which was related to the concentration of active material (see the Results section).

Tests were usually performed with 0.8 ml. of platelet-rich plasma to which 0.1 ml. of 0.1 M-CaCl₂ and 0.1 ml. of test solution were added.

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TEAE-cellulose* chromatography. Columns of TEAE-cellulose were prepared by making a slurry of the cellulose in 2M-NaCl buffered with 8mM-tris phosphate, pH 6. The mixture was then poured into a glass column, packed and washed with 25–50 column vol. of 2M-NaCl buffered as above, then equilibrated with the required starting buffer. All chromatography was performed at room temperature.

Three columns were used for the procedures reported. For chromatography of crude venom columns (15 cm. $\times$ 1.8 cm. or 24 cm. $\times$ 2.6 cm.) of cellulose were used, for 60–80 mg. or 300–400 mg. samples of venom, and were eluted at 60 or 150 ml./hr. respectively. The starting buffer was 1.5 mM-tris phosphate, pH 8.5, and the columns were eluted initially with stepwise decreases in pH, then with stepwise increases in phosphate concentration at pH 6.0. Details are given in the text.

For purification and concentration of digested and gel-filtered venom material, a column 9 cm. $\times$ 4.5 cm. was equilibrated with 0.1 M-NaCl buffered with 0.01 M-tris-HCl, pH 8.0, and eluted at 105 ml./hr. with stepwise increases of NaCl concentration at pH 8.0, as described in the text. Elution of protein from all columns was followed by measuring the extinction of fractions at 280 m μ in a 1 cm. cell.

The columns after use were washed as above with 2M-NaCl at pH 6.0, then re-equilibrated with starting buffer.

Gel filtration. Bio-Gel P-200 (Calbiochem, Los Angeles, Calif., U.S.A.) was suspended in 0.1 M-NaCl buffered with 0.01 M-tris-HCl, pH 8.0, and stirred intermittently for 24 hr., with several changes of the supernatant buffer. It was then poured into a column 110 cm. $\times$ 1.5 cm., and allowed to settle slowly to a height of 97 cm.; the outlet of the column was 20 cm. below the inlet. The column was washed with the same buffer overnight, at a flow rate of 15–20 ml./hr., under the same pressure gradient. Samples from the TEAE-cellulose column were concentrated to 3–10 ml. by ultrafiltration and dialysed against the starting buffer for at least 24 hr. before application to the column and were eluted at 15–20 ml./hr. as above.

An 8% agarose gel, in bead form, was obtained as Sagarose SAG-8 from Seravac Laboratories Ltd., Maidenhead, Berks. The suspension as supplied was diluted, to a suitable consistency, with 0.1 M-NaCl buffered with 0.01 M-tris-HCl, pH 8.0, poured into a column 100 cm. $\times$ 1.7 cm., and allowed to settle to 90 cm. It was washed with the same buffer at 8–10 ml./hr., with a total head of 1.3 m. of water. Samples (2–4 ml.), again pre-equilibrated by dialysis against starting buffer, were eluted at the same flow rate. In some procedures, 1 mM-EDTA was added to the eluting buffer.

The void volumes of the Bio-Gel and Sagarose columns were determined with Blue Dextran 2000 (Pharmacia Ltd., Uppsala, Sweden): 5 mg. in 2–4 ml. of the eluting buffer was applied to the columns and eluted as just described. The extinction of these fractions was determined at 620 m μ .

Pronase digestion. Crude venom and Pronase (*Streptomyces griseus* proteinase; obtained from Seravac Laboratories Ltd.) were dissolved together in 0.1 M-NaCl, buffered with 0.01 M-tris-HCl, pH 8.0, to final concentrations of 50 and 5 mg./ml. respectively. The solution was placed in 8/32 in. dialysis tubing, and incubated in 200 vol. of the same buffer,

with stirring, at 37° for 60–72 hr. The buffer was changed about every 12 hr.

Esterase activity. This was determined by the method of Schwert & Takenaka (1955), with 1 mM-benzoyl-L-arginine ethyl ester (Sigma Chemical Co., St Louis, Mo., U.S.A.) as substrate, in 0.05 mM-tris-HCl buffer, pH 8.0, or 0.08 M-tris phosphate buffer, pH 6.0, at 37°, the rate of change of extinction at 253 m μ being measured in a Gilford model 220 Absorbance Indicator (Gilford Instrument Laboratories, Oberlin, Ohio, U.S.A.).

The molar extinction difference coefficient, ΔE , for benzoyl-L-arginine ethyl ester and benzoylarginine was found to be 1000 at either pH.

TEAE-cellulose fractions were screened at pH 6, as this eliminated the need to adjust the pH of the fractions: the pH optimum for the venom esterases is, however, in the vicinity of 8.

Esterase activity was also measured according to Martin, Golubow & Axelrod (1959), with 0.02 mM-benzoyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester as substrate, in 0.05 M-tris-HCl buffer, pH 8.0, the rate of change of extinction being measured at 400 m μ . For the nitrophenol produced, $E = 15000$ at pH 8. The results obtained with the two techniques were parallel; that using benzoyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester is the more sensitive, especially for the detection of Pronase (specific activity at pH 8: benzoyl-L-arginine ethyl ester, 19.5 moles/min./mg.; benzoyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester, 64.3 moles/min./mg.).

Caseinolytic activity. Hammarstein casein (British Drug Houses Ltd.) was suspended in 0.2 M-tris-HCl, pH 7.4, at a concentration of 20 mg./ml., and left in a water bath at 37° until it dissolved. Test solutions (0.2 ml.) were added to 1.8 ml. of casein solution, and incubated for 60 min. at 37°; 1 ml. of 1.8 M-perchloric acid was added, samples were centrifuged at 600 *g* for 10 min., the supernatants neutralized with 2N-KOH, and their extinctions at 280 m μ determined after letting the potassium perchlorate settle out overnight at 0°. Results were expressed as moles of tyrosine released/hr., assuming E 1200.

Coagulant activity. Bovine fibrinogen (grade B; Kabi Pharmaceuticals Ltd., London, W. 5) was dissolved in 0.02 M-tris-HCl, pH 7.4, at a concentration of 20 mg./ml. Clotting mixture contained 0.1 ml. of fibrinogen, 0.1 ml. of 25 mM-CaCl₂, 0.7 ml. of buffer and 0.1 ml. of test solution, and clotting times were determined at 37°. In the screening of TEAE-cellulose fractions the presence of phosphate, even in diluted fractions, so interfered with fibrin formation that results could not be accurately evaluated.

Ultrafiltration. Solutions were concentrated by ultrafiltration in 8/32 in. dialysis tubing immersed in buffer; pressure was maintained in the container at 40–60 mm. Hg.

High-speed centrifugation. Preparative high-speed centrifugation of concentrated preparations was performed in an MSE Superspeed 50 refrigerated centrifuge, with either the 3 $\times$ 5 ml. swing-out rotor at 40000 rev./min. (176000 *g*) or a 10 $\times$ 10 ml. angle rotor at 50000 rev./min. (200000 *g*).

Electrophoresis. Polyacrylamide (disc) electrophoresis was carried out as described by Davis (1964) except that concentrating (large-pore) gels were not used. Acrylamide concentrations in the separating (small-pore) gels were 4.75 or 7%. The buffer used was 0.01 M-tris-glycine, pH 9.2.

Immunoelectrophoresis. Antiserum against crude *T. okenavensis* venom was produced in rabbits by injecting

* Abbreviation: TEAE-cellulose, triethylaminoethyl-cellulose.

25 mg. of venom, adsorbed on 1 ml. of a polystyrene latex particle suspension (Pontyclun Chemical Co., London, W. 4), with 1 ml. of incomplete Freund's adjuvant, intraperitoneally at weekly intervals for 3 weeks. The serum was stored in portions at -20° .

Immunoelectrophoresis was performed by the micro-method of Scheidegger (1955), in layers of 1% agarose gel in 0.05 M-sodium barbital-HCl buffer, pH 8.6. The chamber buffer was 0.1 M-sodium barbital-HCl, pH 8.6, and a potential of 6–7 v/cm. (current 8–10 mA/gel) was applied for 45 min. Precipitin reactions with dilute antigens were sometimes reinforced by placing the antigen solutions in troughs out along the sides of the slide so that it diffused through the region of electrophoretic separation towards the antiserum trough. After diffusion, washing and drying, the electrophoresis diagrams were stained with Amido Black 10B as described by Grant & Everall (1965).

Concentrations. The concentration of purified materials was determined in a differential refractometer (Brice-Phoenix, Phoenix Precision Instrument Co., Philadelphia, Pa., U.S.A.), assuming a specific refractive increment of 0.145 ml./g. This figure was obtained by assuming that the carbohydrate had a specific refractive increment of 0.140 ml./g. and the corresponding figure for the polypeptide portion was 0.180 ml./g.

Physical measurements. The measurements were made on solutions of the purified venom fraction in 0.1 M-NaCl buffered with 0.01 M-tris-HCl, pH 8.0. Sedimentation-velocity studies were carried out in a Beckman model E analytical ultracentrifuge at a rotor speed of 50 740 rev./min. at 20° . Photographs were taken at 3 min. intervals. The molecular weight (M_w) was calculated from sedimentation-equilibrium experiments (Lansing & Kraemer, 1935) employing a 3 mm. column of solution (1.4 mg./ml.) at a rotor speed of 61 666 rev./min.

Amino acid analysis. This was done in a Technicon amino acid analyser (Technicon Instruments Ltd., Chertsey, Surrey) with the elution procedure of Thomson & Miles

(1964). Samples for analysis were hydrolysed with 6 N-HCl at 108° for different periods of time.

Carbohydrate analyses. Uronic acid was detected by a modified carbazole reaction (Bitter & Muir, 1962); sialic acid was detected by the thiobarbituric acid reaction (Warren, 1959).

Trimethylsilyl derivatives of the carbohydrates present were prepared according to Bolton, Clamp & Hough (1964) and analysed by gas-liquid chromatography (Sweeley, Bentley, Makita & Wells, 1964) on 5% Apiezon L (Edwards High Vacuum, Crawley, Sussex) at 168 – 170° at a nitrogen flow of 80 ml./min. The components were identified by co-chromatography with known standards.

Other materials. Crystalline α -chymotrypsin (EC 3.4.4.5), trypsin (EC 3.4.4.4) and ficin (EC 3.4.4.12) were purchased from the Sigma Chemical Co., and neuraminidase (EC 3.2.1.18, 100 units/ml.) and hyaluronidase (ovine, 300–500 units/mg.) from British Drug Houses Ltd. Di-isopropyl phosphorofluoridate (Koch-Light Laboratories Ltd.) and phenylmethylsulphonyl fluoride (British Drug Houses Ltd.) were dissolved in propan-2-ol to concentrations of 0.2 M. *N*-Ethylmaleimide, 2,3-dimercaptopropan-1-ol and mercaptoethanol were from British Drug Houses Ltd.; *p*-chloromercuribenzenesulphonic acid was from Calbiochem; adenosine was from Sigma Chemical Co.; pyruvate kinase (EC 2.7.1.40; 100 units/mg. of protein) and phosphoenolpyruvate were from Koch-Light Laboratories Ltd.

Periodate oxidation. A sample of preparation A was incubated with 0.002 M-sodium periodate at pH 7.0 for 24 hr. at room temperature in the dark, and then dialysed against several changes of distilled water. The non-diffusible material was assayed for platelet-aggregating activity.

RESULTS

Assay of platelet-aggregating activity. With crude venom, a linear relationship was found between the reciprocal of the initial rate of change of recorder deflexion and the reciprocal of the venom concentration. Such plots were used to relate the activity of purified fractions to that of crude venom. It was also observed that for small amounts of added venom (less than $10 \mu\text{g./ml.}$ of platelet-rich plasma) the square of the rate of change of recorder deflexion was proportional to the venom concentration. This relationship was used to estimate the activity in venom fractions after chromatography or gel filtration. In either case, results obtained with crude venom in the same platelet-rich plasma were used for the estimation. In some studies the delay between the addition of venom and the onset of aggregation and the total recorder deflexion were measured; neither parameter was consistently related to the concentration of venom used.

TEAE-cellulose chromatography of crude venom. The fractionation of crude venom on a column of TEAE-cellulose, with stepwise elution with tris phosphate buffers, is shown in Fig. 1. Eight major protein fractions were obtained. About 80% of platelet-aggregating activity was eluted with 0.32 M-phosphate (fraction F). The distribution of

Fig. 1. Fractionation of *T. okinavensis* venom (320 mg.) on a column (24 cm. $\times$ 2.6 cm.) of TEAE-cellulose. The chromatogram was developed at room temperature by stepwise elution with tris phosphate buffers at the molarities and pH values indicated on the Figure. The rate of elution was 150 ml./hr. and 25 ml. fractions were collected.

Table 1. *Activities of venom fractions obtained by TEAE-cellulose chromatography*

Chromatography of 75 mg. of crude venom was carried out on a column, of size and flow rate shown in Fig. 2.

Fraction	Eluted at		Benzoyl-L-arginine ethyl ester esterase (μ moles/min.)	Caseinolysis (μ moles of tyrosine/hr.)	Clotting of fibrinogen	Platelet aggregation (relative rates)
	pH	Phos- phate (M)				
A I	8.5	0.0015	0.25	62.8	—	0
II			0.06	0	—	0
B	7.0	0.006	2.08	11.4	—	0
C	6.0	0.015	5.60	105.2	+	0
D I	6.0	0.08	29.31	56.9	++	0
II			10.30	13.0	+++	0
E	6.0	0.12	0.73	3.5	+	0.01
F	6.0	0.32	4.15	5.0	±	0.80
G	6.0	0.48	0.09	2.5	—	0
Starting material			377.1	957.2		1.0

Table 2. *Purification of platelet-aggregating factor from venom*

	Aggregating activity (% of crude venom)	Yield (% w/w) of crude venom	Purification relative to crude venom
Preparation A			
1. TEAE-cellulose chromatography	78	2.80*	28
2. Bio-Gel P-200 filtration	75	1.34†	55
Preparation B			
1. Pronase digestion and Bio-Gel P-200 filtration	100	2.23*	45
2. TEAE-cellulose chromatography	100	1.17*	85
3. Agarose (SAG-8) filtration	90	0.92†	100

* Concentrations estimated from E_{280} (see the text).

† Concentrations determined by differential refractometry.

esterase, caseinolytic and coagulant activities in the fractions from a similar separation is set out in Table 1. Fraction F usually contained less than 2% of the esterase and about 0.1% of the caseinolytic activity of the starting material, and only a trace of coagulant activity. Purification was nearly 30-fold, as judged by ultraviolet-absorbing material (Table 2). The accuracy of this estimate is doubtful, as the isolated fractions, particularly D and F, were appreciably turbid, although the crude venom solution was relatively free of insoluble material ($E_{1\text{cm}}^{1\%}$ at $280\text{m}\mu = 11.12 \pm 0.03$).

The second error in estimates based on extinction values is due to the lower specific extinction of the finally purified fraction ($E_{1\text{cm}}^{1\%}$ at $280\text{m}\mu = 2.90$). No allowance has been made for this error in calculating intermediate yields; final yields were determined from measurements made in the differential refractometer.

Attempts were made to redissolve the material responsible for the turbidity in 5M-urea, or 30% glycerol; although there was some increase in extinction, 109% and 51% respectively, the solutions showed no increase in their platelet-aggregating

activity. After high-speed centrifugation (176 000g, 30 min.) all activity was present in the supernatant, none in the sediment. Fractions were therefore centrifuged before further purification.

Gel filtration on Bio-Gel P-200. The elution of concentrated peak F material from TEAE-cellulose off a column (97 cm. $\times$ 1.5 cm.) of Bio-Gel P-200 is shown in Fig. 2(a). Although the peak of platelet-aggregating activity coincided with the first protein peak, some activity was eluted before the protein. The second protein peak contained less than 4% of the activity applied to the column. After pooling of the active fractions, concentration and high-speed centrifugation (200 000g, 30 min.), 96% of the activity applied was recovered, and the purity was further increased (Table 2).

After dialysis and concentration, the preparation contained 75% of the platelet activity and 1.4% by weight of the starting material. This preparation (preparation A) showed two precipitin lines on immunoelectrophoresis (Fig. 3) and a single diffuse band on electrophoresis in 4.75% polyacrylamide gel (Fig. 4). Preparation A was used for carbohydrate analyses and for studies of the effects of proteolytic

Fig. 2. Gel filtration, on a column (97 cm. $\times$ 1.5 cm.) of Bio-Gel P-200, of: (a) fraction F (see Table 1) from TEAE-cellulose chromatography of 400 mg. of venom; (b) a Pronase digest of 100 mg. of venom. Elution was carried out as described in the text. $\bullet$, E_{280} ; $\circ$, rate of platelet aggregation; Δ , esterase activity (benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester)

Fig. 3. Immunoelectrophoreses of preparations A and B, with antiserum to crude *T. okinavensis* venom. (a) Prep. A; (b) and (d) crude venom; (c) prep. B. The pattern of (c) and (d) has been reinforced by the diffusion of prep. B from two lateral troughs: a reaction of identity is also evident with a venom component having the same mobility as the purified fraction.

and other enzymes, and of various inhibitors, on its platelet-aggregating activity.

Preparation B. It was found that 98% of the venom protein could be digested with Pronase (Table 2) without affecting the platelet-aggregating activity. The esterase activity of the Pronase digest was only 0.2% that of the original venom. Crude venom (500 mg.) was digested with 50 mg. of Pronase for 70 hr. at 37°; after dialysis the digest was filtered on a column of Bio-Gel P-200 by the procedure used in the previous preparation. The aggregating activity appeared in the same volume of eluent as the TEAE-cellulose fraction F and contained 25% of the applied esterase activity (Fig. 3b). The first 35 ml. of the eluent was discarded. The next 80 ml. was further purified and concentrated on a column (9 cm. $\times$ 4.5 cm.) of TEAE-cellulose equilibrated with 0.1 M-sodium chloride buffered to pH 8.0 with 0.01 M-tris-hydrochloric acid, and eluted as shown in Fig. 5. All the platelet-aggregating activity was eluted by 0.5 M-sodium chloride, with less than 0.1% of the esterase activity of the digest.

The 0.5 M-sodium chloride peak material was dialysed and concentrated to 5 ml. in 0.1 M-sodium chloride buffered to pH 8.0 with 0.01 M-tris-hydrochloric acid. It was applied to a column (90 cm. $\times$ 1.7 cm.) of 8% agarose gel (Sagarose SAG-8) equilibrated with the same solvent, and eluted as shown in Fig. 6. Two protein peaks were

obtained, the first of which had platelet-aggregating activity. This was concentrated in the same buffer, and centrifuged (200 000g, 30 min.). The yield and purification achieved are shown in Table 2. Preparation B was used for physical studies and for amino acid analyses.

Preparation A was found to have a contaminant since two lines were found on immunoelectrophoresis (Fig. 3) and a single diffuse band in 4.75% polyacrylamide gels (Fig. 4). Preparation B gave a single precipitin line on immunoelectrophoresis, corresponding to the component of higher mobility in preparation A, and one band in polyacrylamide. When the concentration of acrylamide in the gels was increased to 7%, both preparations showed rather sharper single bands at 2–3 cm. from the origin.

Physical studies. Sedimentation-velocity experiments on preparation A (1.5 mg./ml.) revealed two components, which had S_{20} values of about 8s and 16s. Preparation B (1.4 mg./ml.) on the other hand showed only one component with S_{20} 10.3s. From

Fig. 4. Polyacrylamide-gel electrophoresis (4.75% acrylamide) of (a) prep. A, (b) prep. B, (c) crude *T. okinavensis* venom. The heavily stained line towards the bottom of each gel is the buffer front.

Fig. 5. Fractionation on a column of TEAE-cellulose (9cm. $\times$ 4.5cm.) of the first protein peak obtained by Bio-Gel filtration of a Pronase digest of 400mg. of venom (see Fig. 2b). Elution was carried out with a single increase in NaCl concentration, as shown, in 0.01M-tris-HCl, pH 8.0, at 105ml./hr., collected in 17.5ml. fractions. $\bullet$, E_{280} ; $\circ$, rate of platelet aggregation; Δ , esterase activity (benzyloxycarbonyl-L-phenylalanine *p*-nitrophenyl ester).

Fig. 6. Gel filtration, on a column (90cm. $\times$ 1.7cm.) of Sagarose SAG 8, of the platelet-aggregating material obtained by TEAE-cellulose chromatography as in Fig. 5. Elution was as described in the text. $\bullet$, E_{280} ; $\circ$, rate of platelet aggregation.

Table 3. Amino acid composition of purified venom fraction after pronase digestion

Amino acid	Amount found (μ mole)	Moles/ 10^5 g. of sample
Asp	0.123	10.2
Thr*	0.044	3.7
Ser*	0.109	9.1
Glu	0.154	12.8
Pro	0.046	3.8
Gly	0.071	5.9
Ala	0.078	6.5
Cys	0.047	3.9
Val	0.078	6.5
Met	0.019	1.6
Ile	0.044	3.7
Leu	0.068	5.7
Tyr	0.035	2.9
Phe	0.070	5.8
His	0.035	2.9
Lys	0.080	6.7
Arg	0.046	3.8

Total wt. of amino acid residues recovered (μ g.) ... 128.3 (9.5%)

Total wt. of material applied to the column (μ g.) ... 1201

* Values corrected for destruction during hydrolysis by extrapolation; all other figures are means of values after hydrolysis for 21 and 69hr.

sedimentation-equilibrium studies on preparation B (1.4mg./ml.) a value for the M_w of 4×10^6 was obtained.

Amino acid content. The amino acid analyses of preparation B after 21 and 69hr. hydrolysis are

Table 4. Carbohydrate composition of purified venom fraction

	Composition (%)
Arabinose	39.7
Fucose	8.2
Mannose	45.7
N-Acetylglucosamine	4.1
N-Acetylgalactosamine	2.4

given in Table 3. Values for serine and threonine were obtained by extrapolation to zero time. All other amino acids seemed to have been fully liberated after only 21 hr. hydrolysis, so means of the two values were taken. Tryptophan was not estimated. On the basis of these figures, peptide accounts for only 9.5% by weight of the preparation.

Carbohydrate content. Carbohydrate analyses were carried out on preparation A, because B was slightly contaminated with galactose eluted from the agarose gel. The carbohydrate composition as determined by gas-liquid chromatography is given in Table 4; it is seen that there is a predominance of neutral sugars, mainly mannose and arabinose. Colorimetric methods failed to detect uronic acid and sialic acid, indicating that these could constitute not more than 2% of the preparation.

Action of purified venom fractions on platelets. In heparinized plasma (20 units of heparin/ml. of whole blood) crude venom caused some platelet aggregation at a concentration of 2.5×10^{-8} g./ml. Similarly, purified preparations A and B caused some aggregation at 10^{-9} g./ml. of neutralized citrated plasma.

At higher concentrations of the fraction, the rate of aggregation could be related to its concentration empirically as for crude venom. Aggregates, once formed, did not break up spontaneously even when observed for more than 24 hr. at room temperature or at 4°.

Aggregation by the venom fraction was prevented in plasma containing EDTA, but occurred when Ca^{2+} ions were added in excess. Aggregation, once it had begun, could be stopped by the addition of an excess of EDTA, but the aggregates already formed did not disperse.

If platelet-rich plasma was incubated with the venom fraction and Ca^{2+} ions for several minutes, without stirring, no aggregation occurred. When the plasma was stirred aggregation began at once, and the rate of aggregation was the same as in plasma that had been stirred from the addition of the venom. The plasma after the removal of the platelets that had been aggregated by venom fraction contained all the aggregating activity added; no decrease was evident up to 2 hr.

Aggregation was inhibited by the addition of adenosine, or of pyruvate kinase with phosphoenol-

Fig. 7. Effect of platelet aggregation by venom of systems that inhibit ADP aggregation. Platelet-rich plasma used was prepared from blood treated with the disodium salt of EDTA (5 mm in whole blood) to prevent clotting. The venom preparation (0.5 μ g./test) was fraction F from TEAE-cellulose chromatography (Table 1). Additions (added 5 min. before the venom fraction unless otherwise specified) were: I, pyruvate kinase, 5 units, phosphoenolpyruvate to 50 μ M; II, adenosine to 0.4 mM; III, adenosine to 0.2 mM; IV, adenosine to 0.4 mM, at arrow (200 sec.); V, none.

pyruvate, to the plasma before the venom factor. Under these conditions pyruvate kinase and phosphoenolpyruvate had no effect on platelet aggregation when added singly. Adenosine added during aggregation reduced its rate but did not stop it (Fig. 7).

The thiol reagents *N*-ethylmaleimide and *p*-chloromercuribenzenesulphonic acid both inhibited aggregation if added before the venom fraction, and stopped and reversed it if added while it was going on, or even when it was complete. Reversal took place after a delay inversely proportional to the concentration of the inhibitor, and continued over 10–15 min. Minimum effective concentrations were: for inhibition, 0.1 mM-*N*-ethylmaleimide or 0.5 mM-*p*-chloromercuribenzenesulphonic acid; for reversal from maximum aggregation, 0.5 mM-*N*-ethylmaleimide or 5 mM-*p*-chloromercuribenzenesulphonic acid (in experiments with 0.6 μ g. of preparation B/ml. of neutralized citrated plasma). The dependence on plasma pH of platelet-aggregating activities of crude and purified venom is shown in Fig. 8. The curves are similar, with an optimum activity between pH 7.2 and 7.5.

Aggregating activity of the purified venom fractions was stable in solution at pH 7–8 for months at 0°, or for days at 25°. It was also stable

Fig. 8. Effect of plasma pH on platelet aggregation by *T. okinavensis* venom: samples of ACD-plasma were adjusted to different pH values by adding 1N-HCl or 5N-NaOH dropwise with vigorous stirring. Rates of aggregation were determined with 20 μ g. of crude venom (●) and 1 μ g. of a TEAE-cellulose fraction F (see Table 1) (Δ).

at 37°, at pH 6, for 30 min., but at that temperature activity was lost rapidly in solutions of lower pH, slowly at pH 7 or 8, more rapidly at pH 10, and very rapidly above that value (Table 5). Activity was lost after 30 min. at 100°, irrespective of the pH of the solution. Inactivation of the fraction was also obtained on incubation of the fraction with periodate at neutral pH. Several procedures were undertaken that had no effect on the platelet-aggregating activity of the venom, crude or purified, at pH 7.5: incubation with di-isopropyl phosphorofluoridate (5 mM), phenylmethylsulphonyl fluoride, sodium iodoacetate, *N*-ethylmaleimide, *p*-chloromercuribenzenesulphonic acid, mercaptoethanol or dimercaptopropanol (all 10 mM). Solutions were diluted 1:100 for assay to prevent effects of the inhibitors on platelets. Also, as already noted, 5 M-urea or 30% glycerol in venom solutions did not alter their activity. Activity was also unchanged by incubation of portions of TEAE-cellulose fraction F (40 μ g./ml.) at pH 8.0 with α -chymotrypsin (0.5 mg./ml.), trypsin, Pronase and ficin (all at 0.8 mg./ml.), neuraminidase (20 units/ml.), or at pH 6.0 with hyaluronidase (2 mg./ml.). The proteolytic enzymes used were inhibited before the assay of platelet-aggregating activity by adding 5 mM-*p*-chloromercuribenzenesulphonic acid to the ficin, and 12.5 mM-di-isopropyl phosphorofluoridate to the other enzymes, and inhibition was verified by determining esterase activities, with benzoyl-L-

Table 5. *Stability of platelet-aggregation activity*

Portions of prep. A, 30 μ g./ml., were incubated in the solvent shown for 30 min. at 37°, then diluted to 6 μ g./ml. at pH 7, and activity was assayed in ACD-plasma.

Solvent	pH	Activity remaining (%)
0.1 M-Acetic acid	4.0	0
0.05 M-Tris phosphate	6.0	100
0.05 M-Tris phosphate	7.0	73
0.05 M-Tris-HCl	8.0	43
0.1 M-Tris	10.0	27
0.01 M-NaOH	12.0	0

Table 6. *Release of adenine nucleotides from washed platelets by T. okinavensis venom and a part-purified platelet-aggregating fraction*

Nucleotide release was measured spectrophotometrically as described by Davey & Lüscher (1965a). The platelet suspensions used contained 11.3 mg. of platelet protein in 0.05 ml., with 5 mM-CaCl₂, and were incubated with the substances tested for 15 min. at 37°. The partly purified fraction used was prepared by TEAE-cellulose chromatography of whole venom (Fig. 1) and corresponds to fraction F in Table 1; it was dissolved in 0.1 M-NaCl, pH 7.5; E_{280} (obs.) = 0.01.

Substance tested	Amount	Nucleotides in supernatant (%)
Buffer		29.3
Venom	5 μ g.	57.2*
Venom	10 μ g.	62.1*
Fraction F	0.05 ml.	50.5*
Fraction F	0.1 ml.	54.1*

* Platelet aggregation had occurred in these mixtures.

arginine ethyl ester. The other inhibitors were diluted out, as above, to assay the action on platelets. Neuraminidase and hyaluronidase alone were shown in control tests not to cause platelet aggregation themselves, nor to modify platelet reactions with the intact venom fraction.

The release of adenine nucleotides from washed platelets after incubation with the venom fraction was also measured (Table 6), by a method described by Davey & Lüscher (1965a).

Studies of the purified fraction have also been kindly undertaken by workers in other Laboratories. Their results are presented here in summary. Platelet aggregation, measured in another optical system, resembled that caused by connective-tissue suspensions, and was inhibited by the same substances (Dr J. R. A. Mitchell and Dr M. J. G. Harrison, Department of the Regius Professor of Medicine, Radcliffe Infirmary, Oxford). The purified fraction caused the same pattern of change in the electrophoretic mobility of platelets as do thrombin, connective tissue and noradrenaline

(Dr J. Hampton, Department of the Regius Professor of Medicine, Radcliffe Infirmary, Oxford). Systemic application of solutions *in vivo* does not affect platelet thrombus formation in injured blood vessels of the rabbit brain (Dr A. J. Honour, Department of the Regius Professor of Medicine, Radcliffe Infirmary, Oxford) or of the hamster cheek pouch (Dr A. G. Sanders, Sir William Dunn School of Pathology, Oxford). Its action is inhibited by amitriptyline (Mr D. C. G. Mills, Department of Pharmacology, Royal College of Surgeons, London), which prevents release of ADP from platelets (Mills, 1968). In certain patients the platelets do not aggregate when they are incubated with connective tissue; these platelets appear to be unaffected by incubation with the venom fraction (Dr R. M. Hardisty, Department of Haematology, Hospital for Sick Children, London). In all these investigations our findings that very small amounts of the venom fractions cause changes in platelets have been confirmed.

DISCUSSION

The platelet-aggregating material from *T. okinavensis* venom is distinct from the fractions responsible for the proteolytic, esterolytic and coagulant activities of the venom and so far no enzymic activity has been found associated with it.

Previous studies (Davey & Lüscher, 1965b, 1967) have indicated that similar components are present in the venoms of *Crotalus terrificus* (tropical rattlesnake) and of *Trimeresurus purpureomaculata* (purple-spotted pit viper). Active material from the former has been partly purified (Makwardt, Barthel, Glusa, Hoffmann & Walsman, 1966); it is also of high molecular weight, and has been separated from the coagulant, proteolytic and phospholipase activities of the whole venom.

The *T. okinavensis* fraction resembles thrombin, trypsin, connective-tissue suspensions and a number of other agents in causing aggregation of platelets by releasing ADP from them (Haslam, 1964; Hovig, 1963). Aggregation by venom, as well as by ADP, requires Ca^{2+} ions (Born & Cross, 1964). It is, however, irreversible for any concentration of venom used, whereas aggregation by added ADP is spontaneously reversible except when large amounts of ADP are used, and is always reversed by addition of excess of EDTA to the plasma (Sharp, 1965).

Inhibition and reversal of aggregation by thiol reagents such as *N*-ethylmaleimide and *p*-chloromercuribenzenesulphonic acid have also been described for ADP, thrombin, connective tissue, noradrenaline and 5-hydroxytryptamine (Robinson, Mason & Wagner, 1963; Harrison, Emmons & Mitchell, 1966). *N*-Ethylmaleimide also inhibits the release of platelet nucleotides by thrombin

or by connective tissue (Kowalski, Kopec, Węgrzynowicz, Hurwic & Budzynski, 1966). The concentrations required for inhibition, in platelet-rich plasma, were similar to those we found effective against the venom fraction.

Although aggregation of platelets by venom was prevented by the absence of Ca^{2+} ions, its reaction with platelets was not: the delay in onset of aggregation could be abolished by preincubating the venom fraction with stirred platelet-rich plasma containing EDTA, or with unstirred platelet-rich plasma. The venom fraction (Davey & Lüscher, 1965b), like thrombin and collagen (Ireland, 1967), causes the release of nucleotides from platelets in the absence of Ca^{2+} ions.

The results presented suggest a reaction of platelets with the venom fraction that resembles reactions with thrombin or collagen. In other studies it has been suggested that the venom is more like thrombin, in releasing both nucleotides and specific proteins; connective tissue releases much less protein (Davey & Lüscher, 1968).

We found no evidence that the venom fraction was appreciably inactivated by plasma or by platelets, nor that it was irreversibly bound to platelets.

Although the activity of the venom fraction was unaffected by storage for several days at neutral pH at 25°, outside the range pH 6–10 at 37° activity was rapidly lost. The activity was also unimpaired by digestion with several proteolytic enzymes, which suggests either that the peptide moiety is not related to the aggregating activity of the fraction, or that it is protected from hydrolysis because it is shielded by a polysaccharide envelope.

The observation that periodate oxidation rapidly destroys the activity of the fraction suggests that it is the carbohydrate moiety that is responsible for the platelet reaction.

There appear to be certain similarities between the venom fraction and a number of protein-polysaccharide compounds that have been isolated from various tissues. These compounds contain 83–97% of carbohydrate and the principal components are: chondroitin sulphates, or kerato-sulphate (Castellani, 1965; Partridge, Whiting & Davis, 1965), or sialomucoids (Barnes, 1965). In all instances the composition of the peptide fragments has been similar. The most plentiful amino acids are glutamic acid, aspartic acid, glycine and serine, which, with the exception of glycine, also predominate in the Pronase-digested venom fraction. These residues have been implicated in covalent carbohydrate-protein bonds, in connective-tissue mucopolysaccharides (Meyer, Anderson, Seno & Hoffman, 1965; Roden, 1965), in collagen (Seifter, Franzblau, Harper & Gallop, 1965) and in blood-group substances (Morgan, 1956). The

composition of the venom polysaccharide is, however, quite dissimilar to that found in tissue protein-polysaccharides. It lacks uronic acid and sialic acid and contains a large proportion of arabinose, a pentose that has not been previously found as a major component of vertebrate polysaccharides.

It is already well known that platelets adhere to connective tissue or to purified collagen fibres (Zucker & Borrelli, 1962; Hughes & Lapiere, 1964) even in the absence of Ca^{2+} ions (Spaet & Zucker, 1964).

Purified collagen has been shown to contain as much as 1% of its weight as carbohydrate (Banga, 1966), which may be present as mucoid compounds. These are protein-polysaccharides containing 15–20% of polypeptide and the polysaccharide component resembles chondroitin sulphate.

It may be this component that causes platelets to adhere to collagen and subsequently to release nucleotides and aggregate (Hovig, 1963; Davey & Lüscher, 1967).

The venom protein, although not resembling collagen in its carbohydrate composition, may well bear some similarity to collagen in its physical dimensions. Citrate-extracted collagen is a rod-shaped molecule with a value for the frictional coefficient f 6.5, which corresponds to a rod 3000 Å long and 13.6 Å in diameter (Boedtker & Doty, 1956). A calculation of the apparent frictional coefficient for the venom fraction with $M_w = 4 \times 10^6$, $S_{20} = 10.3$ s and assuming $\bar{v} = 0.65$, gives $f/F_0 = 12$. This value is probably too large to be accounted for solely in terms of a large axial ratio; it may indicate that there is a considerable degree of hydration. Nevertheless this large value of the frictional coefficient would be compatible with a rod-shaped molecule. It should be emphasized that biological assays for the aggregating activity of the fraction were carried out at concentrations as low as 10^{-9} g./ml., and since the venom fraction appeared to associate at concentrations greater than about 1.5 mg./ml., no precise estimate can be made of the molecular dimensions at such high dilution.

There appears to be a similar biological reaction between platelets and the venom fraction or collagen, and it will be of interest to study the reaction of platelets taken from patients whose platelets do not normally react with collagen (Hardisty & Hutton, 1967).

We have estimated that a concentration of less than five molecules of the venom fraction per platelet will produce detectable aggregation in platelet-rich plasma. The corresponding figures for the activity of other aggregation agents are: thrombin about 1500 (Davey & Lüscher, 1968), ADP about 10^5 molecules per platelet (O'Brien,

1962; Mitchell & Sharp, 1964). It is likely that the venom fraction is acting at a very early stage of the aggregation reaction, and it may be that a long rod-like polysaccharide molecule with the associated peptide causes a distortion of the platelet membrane, which leads to the release of ADP and subsequent aggregation.

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