THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 258, No. 19, Issue of October 10, pp. 11924-11931, 1983
Printed in U.S.A.

Diamphotoxin

THE ARROW POISON OF THE !KUNG BUSHMEN*

(Received for publication, March 31, 1983)

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We have purified the arrow poison extracted from *Diamphidia nigro-ornata* pupae by the !Kung Bushmen of Southern Africa, and named it diamphotoxin. The toxin is a single chain polypeptide of M_r = 60,000 with an isoelectric point of pH 9.5. It blocks neuromuscular function and is cardiotoxic and hemolytic, and the minimum lethal dose for mice is 25 pg (<0.5 fmol).

The toxin binds tightly to cells, permitting the resolution of two distinct phases. Erythrocytes exposed to toxin in the absence of divalent cations show no apparent lesion (phase I). After washing and addition of 1 mM Ca^{2+}, there occurs a rapid efflux of K^+ followed by the loss of hemoglobin (phase II). The pH optimum for phase I is pH 6.7 and for phase II pH 8.6. The action of the toxin is noncatalytic, requiring a solution concentration of approximately 65 toxin molecules/cell for hemolysis of sheep erythrocytes under standard conditions. Ca^{2+} ions induce a conformational change in the free, purified toxin molecule. We propose that this change also occurs in membrane-bound toxin. Hemolysis would result from the formation of channels permitting the diffusion of small cations.

The !Kung Bushmen who inhabit the Kalahari Desert and the adjacent savannah of northwestern Botswana use poisoned arrows to hunt large mammalian prey. In this region, the poison comes primarily from the pupae of the chrysomelid beetles *Diamphidia nigro-ornata* and *Polyclada flexuosa*. The cocoons of these insects (Fig. 1*a*)[1] are excavated by the Bushmen who extract the enclosed pupae (Fig. 1*b*) to obtain the arrow poison. In a typical preparation, four or five fresh pupae (each weighing approximately 0.2 g) are macerated together with a knife point of baked and powdered seed pods of the tree *Schwarzia madagascariensis* and a salivary extract of acacia bark. This concoction is then applied sparingly to the sinew binding immediately behind the barbs of each of a dozen arrows. When air-dried, such arrows retain their lethal potency for at least 1 year.

Reports on the use of beetle pupae in Bushman arrow poisons date back to the early observations of Paterson (1), and reviews of the relevant literature have been published by Schapera (2) and by Shaw *et al.* (3). Boehm (4) identified the toxic material in the pupae as a "toxalbumin" which caused hemoglobinuria. Breyer-Brandwijk (5) raised an antibody to the toxin in rabbits. She reported that the toxin behaved as a protein and was inactivated by boiling and that the pupal extracts caused hemolysis.

We undertook to purify the toxin and examine its pharmacological and biochemical characteristics because its great potency (a single arrow suffices to kill an adult giraffe weighing over one-half ton) suggested that it might have novel properties. The results of this work are presented below.

EXPERIMENTAL PROCEDURES

Materials

Cocoons containing *D. nigro-ornata* pupae were collected by the Bushmen and promptly despatched by air to Cape Town. All arrangements for collection and shipment were kindly completed by Ds. F. Welch. On receipt in the laboratory the the pupae were removed from the cocoons and stored in liquid nitrogen until processed.

Reagents used in this study were the best commercially available. Indubiose A37 was obtained from L'Industrie Biologique Francaise, 35 Quai de Moulin de Cage 92231, Gennevilliers, France; Versilube F50 was obtained from General Electric Corporation, Cranford, N.J., USA. Veronal buffered saline (VBS) was 4 mM diethylbarbituric acid, 143 mM NaCl, 3 mM $NaHCO_3$, 0.7 mM $CaCl_2$, 2.5 mM $MgCl_2$.

Methods

Assays

1) Spectrophotometric assay. A suspension of washed sheep red blood cells (SRBC) in VBS was prepared such that the addition of 0.5 ml of the suspension to 3.25 ml water gave an optical density of 0.75 at 540 nm after lysis of the cells. This sock corresponded to a 2.5% cell suspension. Aliquots of the toxin solution to be assayed were brought to 3.25 ml in plastic tubes using VBS containing 0.5% gelatin (VBS-G). After adding 0.5 ml of the SRBC suspension the tubes were capped, inverted several times, incubated at 37°C for 60 minutes, then centrifuged and the extent of hemolysis estimated by measuring the absorbance of the supernatant at 540 nm.

The dose-response curve for toxin-induced hemolysis was sigmoid and could be linearized by applying the von Krogh equation (6); the use of a log-logit plot (Fig. 2) facilitated the estimation of the toxin concentration needed to produce 50% hemolysis under the standardized conditions of erythrocyte concentration and incubation. This quantity was defined as one hemolytic unit (HU) of toxin and used as a reference standard throughout. Erythrocytes from different sheep varied up to 20% in their sensitivity to lysis by the toxin. These variations showed no correlation with the intracellular K^+ concentration (7) as measured by flame photometry of cell lysates.

2) Blood agarose assay. The spectrophotometric assay was sensitive, precise and reproducible, but time consuming to perform. It was limited to the range 1 - 20 HU/ml and required a preliminary estimate of toxin activity to make an appropriate dilution for accurate assay. For assaying large numbers of samples such as the fractions of column eluates, we developed an assay which relied on the radial diffusion of a toxin sample into a suspension of SRBC in an agarose gel.

Aliquots of 5 µl of toxin solution were placed in wells within a gel containing 2% SRBC and 1% agarose (Indubiose A37) in VBS. The gels were incubated overnight at 4°C and then for 60 minutes at 37°C. The area of the resultant zone of hemolysis around each well was proportional to the logarithm of the concentration of the toxin solution. Aliquots of tenfold dilutions of a toxin solution standardised by the spectrophotometric assay provided a calibration curve in each gel. This assay provided linear results in the range 20 to 100,000 HU/ml. (Fig. 3).

3) Turbidometric assay. For studying the kinetics of toxin action it was convenient to monitor continuously the extent of hemolysis. We utilised the difference in light-scattering properties of intact and of lysed erythrocytes. The change in turbidity of a suspension of 10^7 SRBC/ml upon exposure to toxin in VBS-G at 37°C was monitored by measuring the apparent OD at 700 nm with a recording spectrophotometer. The suspension showed an apparent OD of approximately 1 when intact, and 0 when lysed. Incorporation of

* This work was supported by grants from the John Simon Guggenheim Foundation, the South African Medical Research Council, and the Staff Research Fund of the University of Cape Town. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] Portions of this paper (including "Experimental Procedures," Figs. 1-18, and Tables I-IV) are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 83M-865, cite the authors, and include a check or money order for $14.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

0.5% gelatin in the buffer system minimized the rate of settling of the cells. After introduction of the toxin sample the system showed an initial lag phase of 2 to 5 minutes followed by a steady drop in apparent OD (Fig. 12a). The gradient of the steepest portion of the curve was linearly related to the final toxin concentration in the range 10 to 1000 HU/ml. Toxin activity was expressed in terms of change in OD per minute.

4) Mouse lethality. An independent standardized assay was developed based on the lethality of the toxin for mice. Injection of 0.05 ml of toxin solution into the tail vein of a 20 - 30 g mouse resulted in death within a period ranging from 30 sec to 30 min. The time to death was linearly related to the log of the toxin dose over the range 2 to 80 MLD (Fig. 4). Mice which received a dosage insufficient to kill them within 30 min would show signs of distress over a period of about one hour and then usually recover with no apparent permanent ill-effects.

Extraction and purification

All procedures were carried out at 4°C unless otherwise indicated. Loss of activity was minimized by incubating all plasticware in 1% Tween 80 followed by rinsing with water, and by incorporating 10% glycerol in all buffers when working with dilute protein solutions.

Fifty grams of pupae were homogenized in 250 ml of cold 0.1 M glycine-HCl pH 3.0. The homogenate was centrifuged for 20 minutes at 15,000 x g and the aqueous phase decanted and filtered through cotton wool. To this clarified extract (260 ml) was added 47.5 g crystalline $(NH_4)_2SO_4$. The resultant suspension was stirred for 30 minutes and then centrifuged for 20 minutes at 15,000 x g. The supernatant was stirred overnight with 113.9 g $(NH_4)_2SO_4$ and again centrifuged for 20 minutes at 15,000 x g. The precipitate was dissolved in a minimum volume of 0.01 M sodium phosphate pH 6.80, and dialysed exhaustively against this buffer.

The dialysed material was applied in batches containing 1500 mg protein to 2.5 x 10 cm columns of HTP equilibrated with the same buffer. After loading, the columns were washed until the absorbancy of the effluent at 280 nm had returned to baseline. The adsorbed toxin was then eluted in a single step with 0.10 M sodium phosphate pH 6.80, and concentrated to 10 mg protein per ml using an Amicon XM50 ultra-filtration membrane.

The toxin recovered from HTP chromatography was dialysed into 0.01 M sodium phosphate, 10% glycerol, pH 6.80 and chromatographed in batches of 200 mg protein on 2.5 x 10 cm columns of phosphocellulose previously equilibrated in the same buffer. These columns were first washed and then developed with a 500 ml gradient of 0.01M to 0.10 M sodium phosphate pH 6.80 containing 10% glycerol. Fractions corresponding to the toxin activity peaks (Fig. 5) were pooled and again concentrated to 2 mg/ml of protein using an Amicon XM50 ultra-filtration membrane.

Protein from the first activity peak eluted from the phosphocellulose columns was dialysed into 0.01 M Tris-HCl, 10% glycerol, pH 8.2. Samples containing 6 mg of this material were chromatographed on 1.25 x 20 cm columns of DEAE cellulose equilibrated with the same buffer. Fractions corresponding to the toxin activity, which was not retained by these columns, were pooled and concentrated to 4 mg/ml by XM50 ultra-filtration, then added to an equal volume of 87% glycerol and stored at -20°C.

Throughout the purification we tested for separation of the hemolytic and lethal activities in the extract, but at no point did we observe such separation. The results of a typical purification run, starting with 50 g of pupae taken from the liquid nitrogen store are summarized in Table I. Extraction and centrifugation yielded a clear solution of 260 ml containing 6630 mg of protein with an activity of 51,500 HU/ml and 184,600 MLD/ml. Ammonium sulfate fractionation yielded a two-fold enrichment of activity while reducing the volume to 100 ml.

Fractionation on columns of HTP provided a further two-fold enrichment of activity and an increase of 160% in the total activity. This consistent increase in activity was presumably due to the removal of an inhibitory factor. Developing the HTP columns with a buffer concentration gradient did not improve the purification.

Chromatography on phosphocellulose was carried out in 10% glycerol, which enhanced the resolution and the recovery of activity. This step resolved three peaks of activity (Fig. 5). The combined activity recovered was only 27% of the starting activity, with only 6% being recovered in peak I. Separate rechromatography of active material from each of the three phosphocellulose peaks yielded single peaks of activity consistent with the original behavior of the material. The blood agarose assay consistently under-estimated the activity in phosphocellulose peak III when compared with results obtained using the spectrophotometric assay.

Further chromatography on DEAE cellulose of the protein in phosphocellulose peak I produced active material which migrated as a single band (Fig. 6) on polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate (SDS) (8). This step resulted in a further 50 % loss of activity. The overall recovery of activity in the purified material was 3%. When corrected for the activity fractionated in phosphocellulose peaks II and III, and not used in the final stage of purification, the recovery was 13%. Attempts to use similar strategies to purify the active components of phosphocellulose peaks II and III were not successful. The purified protein was inactivated by freezing and thawing, but could be stored in 50% glycerol at -20°C without loss of activity for at least a month.

The relatively low purification factor achieved for the toxin is consistent with the observation that electrophoresis of the crude extract showed dominant protein bands in the region of the gel corresponding to the molecular weight of the toxin.

In the studies described below purified toxin refers to material recovered from DEAE chromatography (Fraction V). Where this is not specified the material used was from phosphocellulose peak I (Fraction IV).

Characterisation of the purified molecule.

The molecular weight of the purified toxin was estimated in SDS polyacrylamide gels (8) using globular proteins of known molecular weight as references. The molecular weight was also determined by equilibrium centrifugation using the meniscus depletion method (9).

The isoelectric point was determined by electrofocussing in an LKB 110 column using 1% pH 9-11 ampholines.

The amino acid composition was determined by hydrolysis in constant boiling HCl at 110°C for 24, 48, or 72 hours and analysis on a Beckman model 120C amino acid analyser. Tryptophan was determined colorimetrically (10).

The N-terminal amino acid was identified by dansylation and chromatography on polyamide plates (11).

Immunological studies. Polyclonal antibodies to the toxin were raised in a rabbit by subcutaneous injection of purified diamphotoxin which had been inactivated and insolubilised by cross-linking with glutaraldehyde. Immunoglobulin from the immune rabbit serum was isolated by $(NH_4)_2SO_4$ fractionation and ion exchange chromatography (12, 13) and tested for neutralisation of hemolytic activity of peaks I, II and III from the phosphocellulose chromatography (Fig. 5). Aliquots of 100 µl of immune or irrelevant immunoglobulin solution and of toxin solution containing 10 hemolytic units (HU) were mixed and incubated at room temperature for 10 minutes. To each tube were then added 3.05 ml VBS-G and 0.5 ml 2.5% SRBC in VBS. These suspensions were incubated at 37°C for 60 minutes, centrifuged, and the percentage hemolysis estimated.

The immunological cross-reactivity of the material in the three peaks was compared using radial immunodiffusion (14).

Association of hemolytic activity with the isolated molecule was achieved by electrophoresing samples of pure toxin or albumin in 0.1% SDS on a 7.5% polyacrylamide gel (8). After electrophoresis the gel was washed in 0.1% Triton X100 for 20 minutes, water for 10 minutes and finally in VBS for 10 minutes (15). The washed gel was laid on a 2 mm thick blood agarose gel containing 2% washed guinea-pig erythrocytes and 1% agarose (Indubiose A37) in VBS. The gel assembly was incubated overnight at 4°C and then for 60 minutes at 37°C. A polyacrylamide gel with duplicate protein tracks was fixed and stained with Coomassie brilliant blue.

Phospholipase assays. Two methods were used to test the toxin for phospholipase activity. In the first, toxin samples were examined directly for phospholipases A, C and D using [^{14}C]lecithin as a substrate (16). In the second, a suspension of 1 ml 20% SRBC in VBS was lysed by incubation with 100 HU toxin at 37°C for 30 minutes. A control suspension was incubated in the absence of toxin. The suspensions were then extracted with n-butanol (17), or with chloroform:methanol (18). The extracts were evaporated to dryness under nitrogen and the residues taken up in 100 µl 1:1 chloroform:methanol. Approximately 1 mg phospholipid was chromatographed on silica gel thin layer chromatography (TLC) plates (19).

Protease assays. Purified toxin was tested for proteolytic activity by an electrophoretic procedure (20) and by using [^{125}I]fibrin as a substrate (21) in the presence and in the absence of plasminogen.

Conformational changes in the toxin on exposure to calcium were monitored by spectropolarimetry and by measuring the specific fluorescence of solutions of toxin containing 8-anilino-1-naphthalene sulfonic acid (ANS). The circular dichroic spectrum of purified toxin (0.4 mg in 1 ml of 0.01 M Tris-HCl, 10% glycerol, 1mM EDTA, pH 8.2) was measured in the range of 300 to 190 nm using a JASCO J-40A automatic recording spectropolarimeter at 20°C. The toxin was then brought to 10 mM Ca^{++} by the addition of 10 µl of a 1 M solution of $CaCl_2$ and the spectrum recorded again.

For the ANS experiments 0.5 ml solutions of 0.4 mg/ml purified toxin in 0.01 M Tris-HCl, 0.1 M NaCl, pH 8.2 were made to 40 µM ANS and the fluorescence was measured at an excitation wavelength of 396 nm and an emission wavelength of 468 nm. The fluorescence, measured in arbitrary units, was recorded for toxin solutions containing 1 mM EDTA or 1 to 10 mM $CaCl_2$.

The influx of Na^+ ions into SRBC. A suspension of 10^9 SRBC/ml was incubated with 1000 HU/ml of toxin at 37°C. At intervals aliquots of 0.2 ml were layered over silicone oil (Versilube F50) in Beckman Microfuge tubes and centrifuged for 60 seconds at 5000 x g (22). The tubes were then frozen in dry ice-acetone and cut in the region of the silicone oil layer. Intracellular Na^+ was determined by flame photometry on the lysate of the cell pellet found under the silicone oil after centrifugation. Values were corrected for lysed cells, which did not traverse the silicone layer, on the basis of hemoglobin released in the upper aqueous phase.

Cell cultures. Chick embryo myotube cultures were prepared from muscle tissue of 12 day old chick embryos (23).

The perfused rat heart preparation was set up as described (24). The heart of an anaesthetised 250-300 g hooded rat was excised and suspended by ligaturing the aorta over a glass tube. The heart was perfused at 20°C with Ringer's solution pH 7.4 at a hydrostatic pressure of 100 cm H_2O. The heart rate and coronary flow were measured and the electrocardiogram monitored on an oscilloscope. The perfusate was assayed for the release of lactate dehydrogenase (24).

The guinea pig ileum longitudinal muscle myenteric plexus preparation was set up as described (25). The preparation was bathed in a 7.5 ml solution of Krebs-Ringer's pH 7.4 and stimulated supramaximally with biphasic, 2 msec, 45 V square wave pulses at a frequency of 0.1 Hz. The isometric response was measured with a force displacement transducer and recorded on a chart recorder.

Sarcoplasmic reticulum vesicles (SR vesicles) were prepared from rabbit skeletal muscle as described (26, 27). Ca^{++} uptake by the vesicles in the presence or absence of 250 HU/ml of toxin was measured by recording the rate of addition of a 0.1 M solution of $CaCl_2$ required to maintain an extra-vesicular concentration of 10 µM Ca^{++} (28).

RESULTS

Preliminary studies on extracts of *D. nigro-ornata* pupae confirmed the older reports on their lethality and hemolytic action. We found the pupae to be a 10-fold richer source of toxin activity than the adults on a weight basis, and we were unable to detect any activity in extracts of the leaves of *Commiphera angolensis* which could account for the presence of the toxin in the pupae by direct ingestion. Intramuscular injection of a lethal quantity of pupal extract in mice caused a local paralysis in the region of the injection. Death followed after 12–18 h, and we found local necrosis of the tissue in the immediate region of the site of injection. Addition of pupal extract at a final dilution of 10^4 to cultures of chick embryo myotubes resulted in an immediate loss of the cell's contractile response to acetylcholine stimulation and in visible disintegration of the cells in the monolayer within 20 min.

Physical Characterization

The purified toxin recovered from DEAE-chromatography was electrophoresed on a 6–15% polyacrylamide gel in 0.1% SDS[2] (8). Staining the gel with Coomassie blue revealed a single protein band (Fig. 6) whose migration corresponded to M_r = 60,000. Equilibrium centrifugation of the purified toxin yielded a molecular weight estimate of 62,000. The electrophoretic behavior of the toxin in SDS-polyacrylamide gels was not affected by reduction and alkylation.

Isoelectric focussing of the purified molecule yielded a sharp peak of activity at a pH of 9.5, in accord with the observation that the toxin behaved as a basic protein. The results of amino acid analysis are shown in Table II. Only one NH_2-terminal amino acid (glutamine or glutamic acid) was detected.

The isolated protein was identified as the toxic factor by two procedures. Rabbit antibodies raised to this protein after inactivation with glutaraldehyde completely blocked the hemolytic activity of the material from phosphocellulose peak I. These antibodies also inhibited the hemolytic activity in peaks II and III, although less effectively than that of peak I (Fig. 7). Addition of irrelevant antibodies did not detectably inhibit hemolysis. Note that reducing the extent of hemolysis by 10

[2] The abbreviations used are: SDS, sodium dodecyl sulfate; FCS, fetal calf serum; MLD, minimal lethal dose; SRBC, sheep red blood cells; HU, hemolytic unit; HTP, hydroxylapatite; dansyl, 5-dimethylaminonaphthalene-1-sulfonyl.

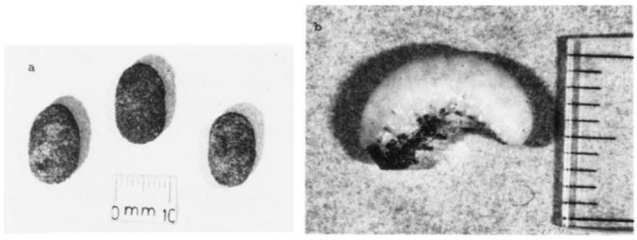

Figure 1. a) Cocoons containing D. nigro-ornata pupae.

The cocoon is egg-shaped with axes of 1.5 and 0.9 cm. Together with the encapsulated pupa it weighs approximately 0.5 g. The cocoons are formed from sand grains cemented together with a mucinous secretion by the pupa and may be found at a depth of approximately 20 cm below the surface of the sandy soil around the base of the shrub Commiphera angolensis.

b) A D. nigro-ornata pupa.

The pupa is orange in colour, measures about 1 cm and weighs about 0.2 g. It has a small black head and six rudimentary black legs. Scale: 1 cm, ruled in mm.

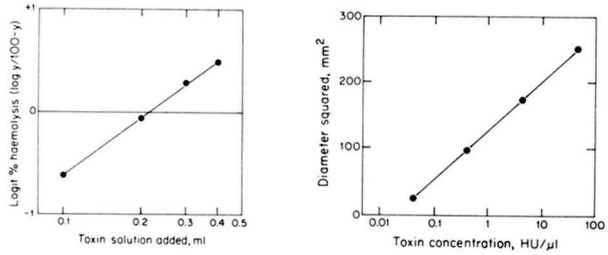

Figure 2. The spectrophotometric assay for diamphotoxin.

Suspensions of SRBC were incubated with dilutions of toxin, centrifuged, and hemolysis estimated from the absorbance of the supernatant at 540 nm (Methods). A log-logit transformation of the data yielded a linear plot which intercepted the abscissa at 50% hemolysis, in this case effected by the addition of 0.215 ml of the toxin solution being assayed. The abscissa is a logarithmic scale.

Figure 3. The standard curve for a blood agarose assay.

Aliquots of 5 µl of toxin solution (Fraction IV) were placed in wells in a gel of 2% SRBC, 1% agarose in VBS. After incubation at 4°C overnight followed by 60 min at 37°C the area of the clear zone of lysed erythrocytes around each well was proportional to the logarithm of the toxin concentration placed in that well. The standard curve was constructed using a toxin solution previously assayed in the spectrophotometric assay. The abscissa is a logarithmic scale.

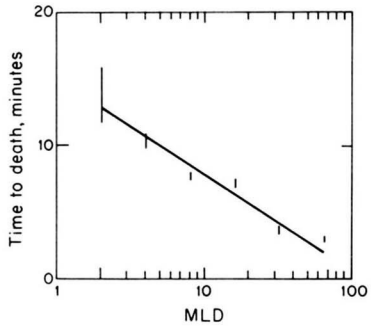

Figure 4. The dose-response curve for the intravascular injection of diamphotoxin in mice.

The toxin solution to be assayed was diluted in 0.9% NaCl, 10% FCS and 0.05 ml was injected into the tail vein of a mouse. The vertical bars represent the range of the time to death for four mice for each concentration. The abscissa is a logarithmic scale.

TABLE I

RECOVERY OF PROTEIN, HAEMOLYTIC ACTIVITY AND LETHALITY.

The figures are those for a typical purification run starting with 50g pupae.

	Fraction	Protein mg	HU**	MLD**	specific activity HU*/mg	specific activity MLD*/mg	percent recovery HU	percent recovery MLD
I	Homogenate	6630	13.4	48.0	2.02	7.24	100	100
II	25%-85% $(NH_4)_2SO_4$	1820	11.5	27.2	6.32	14.9	86	57
III	HTP	800	22.2	50.2	27.8	62.8	166	105
IV	P-cellulose 1♦	73	0.81	1.92	11.1	26.3	6	4
	2	54	0.18	1.44	3.4	26.7	1	3
	3	75	2.62	2.40	34.9	32.0	20	5
V	DEAE-cellulose♦♦	33	0.41	1.44	12.4	43.6	3	3

* $(x10^{-3})$
** $(x10^{-6})$
♦ P-cellulose 1, 2 and 3 refer to activity peaks 1, 2 and 3 in Fig. 2.
♦♦ Only phosphocellulose peak I was used as starting material for DEAE cellulose chromatography

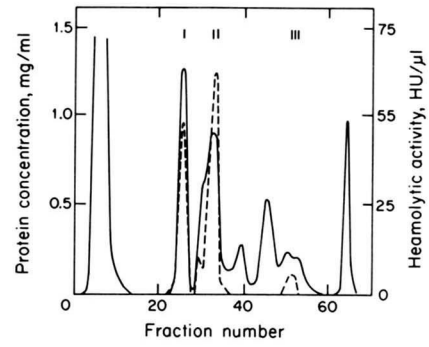

Figure 5. Phosphocellulose chromatography of diamphotoxin.

Details of the procedure are described in the text. The absorbance was monitored at 280 nm (——) and 10 ml fractions were assayed for hemolytic activity (– – –) using the blood agarose assay. Reference is made in the text to peak I (fractions 22 - 24), peak II (fractions 25 - 27) and peak III (fractions 44 - 46).

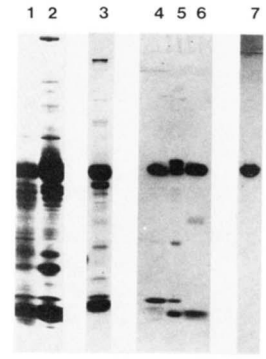

Figure 6. SDS polyacrylamide gel electrophoresis.

1: Clarified extract (Fraction I); 2: after $(NH_4)_2SO_4$ fractionation (Fraction II); 3: after HTP chromatography (Fraction III); 4 - 6: phosphocellulose peaks I, II and III (Fraction IV); 7: after DEAE cellulose chromatography (Fraction V). For tracks 1 and 2 100 ug of protein was loaded on a gel 15 cm long and 1.5 mm thick. For the remaining tracks 10 ug of protein was loaded on gels 7.5 cm long and 0.75 mm thick. The gels were stained with Coomassie blue.

TABLE II

THE AMINO ACID COMPOSITION OF DIAMPHOTOXIN

On the basis of analyses of toxin samples hydrolysed for 24, 48 and 72 hrs an integer value was assigned for the number of residues of each amino acid in the toxin molecule.

Residue	Hydrolysis time (hours) 24	48	72	Assigned value
TRP	3.3			10(1)
LYS	34.3	34.1	33.2	34
HIS	6.0	6.2	6.1	6
ARG	16.3	17.1	15.4	16
ASP	56.9	55.7	61.0	58
THR	17.2	17.1	17.2	17
SER	32.2	27.4	25.9	35(2)
GLU	42.3	39.8	42.7	42
PRO	18.4	17.6	19.2	18
GLY	36.9	33.2	37.9	36
ALA	32.9	30.8	32.9	32
CYS	2.1	4.2	3.6	4(3)
VAL	34.7	36.3	39.2	37
MET	6.4	7.0	5.4	6(3)
ILE	29.8	28.5	33.1	30
LEU	35.0	31.8	35.5	34
TYR	32.8	30.8	33.7	32
PHE	25.2	24.7	24.8	25

(1) Determined colorimetrically.
(2) Extrapolated to zero hydrolysis time.
(3) Determined as cysteic acid and methionine sulfone.

HU to 50% in this system, as is the case for the lowest value for peak III, represents a 90% inhibition of activity. The relationship between the active factors in the three phosphocellulose peaks was further elucidated by double radial immunodiffusion. The precipitin lines shown in Fig. 8 indicate immunological identity between the antigens in peaks I and II, while the spurs at both junctions with peak III indicate that this material lacks antigenic determinants found on the molecules in the other two peaks. The active factors in all three peaks are obviously very closely related, and their structures probably differ only in minor respects.

Further confirmation of the identity of the isolated molecule was provided by the hemolytic underlay procedure (Fig. 9). The underlay showed a clear zone of hemolysis limited to the region of the band of isolated protein. There was no lysis under a band of an equivalent quantity of albumin, showing that the lytic zone was probably not attributable to a local concentration of SDS. The underlay gel technique was used to examine the material from the three phosphocellulose activity peaks (Fig. 5). These gels showed lytic activity in the M_r = 60,000–70,000 range for each peak, providing further evidence that the active factors in the three peaks are closely related.

Functional Characterization

Hemolytic Activity—To identify the mechanism of action of diamphotoxin, we considered principally two functional activities: phospholipase action and selective modification of membrane permeability. The rapid lethality and hemolytic effect (exposure of erythrocytes to relatively high toxin concentrations produced lysis within a few minutes) suggested that the toxin was probably not acting as a metabolic poison.

The toxin was inactivated by 3.3×10^{-7} M *p*-bromophenacylbromide under conditions known to inactivate phospholipases (29–31). A number of phospholipases require mM Ca^{2+} (32–35), and toxin-mediated hemolysis had an absolute requirement for divalent cations. This finding led us carefully to examine toxin preparations for phospholipase activity.

When assayed under conditions (see "Methods") which detected trace amounts of phospholipase A, C, or D, the toxin preparations showed no phospholipase activity using either [^{14}C]lecithin or erythrocyte membrane phospholipid as a substrate (data not shown). Proteolytic activity was not detected in purified toxin preparations either by the zymographic gel procedure or with ^{125}I-fibrin as substrate. The kinetics of hemolysis also indicated that the hemolytic action was probably not enzymatic. When the spectrophotometric assay was modified to increase the ratio of cells to toxin and the period of incubation was extended beyond 6 h, conditions were found where an apparently stoichiometric relationship could be shown between the quantity of toxin in the medium and the number of cells lysed (Fig. 10*a*). Under these conditions, 0.04 HU of toxin (3.24×10^{-9} g or 3.26×10^{10} molecules) lysed 0.5×10^9 cells (Fig. 10*b*). Thus, a ratio of 65 toxin molecules/erythrocyte gave 50% lysis of the sample in 6 h. We conclude that the toxin is not a phospholipase.

Another modification of the spectrophotometric assay was used to examine the extent to which hemolysis could be related to disruption of the cellular osmoregulatory mechanisms. Samples of a 0.45% SRBC suspension were incubated with 20 HU/ml of toxin and 0–0.3 M sucrose in Veronal-buffered saline containing 0.5% gelatin at 37 °C for 60 min. The release of hemoglobin and K^+ from the cells was estimated by spectrophotometry and flame photometry, respectively. The efflux of K^+ from cells exposed to toxin was unaffected by the sucrose concentration in the medium, while the release of hemoglobin decreased with increasing sucrose concentration, falling to zero at 0.3 M sucrose (Fig. 11). This suggested that the primary action of the toxin was to produce a channel that permitted the transmembrane diffusion of small ions and water. In analogy to the effect of complement, this would lead to hemolysis as a secondary consequence of osmotic deregulation.

The omission of divalent cations from the routine Veronal-buffered saline completely blocked the hemolytic activity of the toxin; a comparable effect was also obtained by addition of adequate concentrations of EDTA (Fig. 12*b*). Optimal rates of hemolysis were obtained at 1 mM Ca^{2+}, a concentration very close to the level of free Ca^{2+} in vertebrate body fluids. The rate of hemolysis decreased sharply below 1 mM Ca^{2+} and decreased less rapidly at higher Ca^{2+} concentrations (Fig. 13). The effects of other divalent cations on the rate of hemolysis are shown in Table III. Hemolysis in the presence of 1 mM Ca^{2+} was reduced progressively by La^{3+} concentrations above 10^{-6} M and completely blocked at 10^{-4} M La^{3+}.

Once the requirement for divalent cations in the action of diamphotoxin had been identified, we were able to define two phases in the hemolytic event. Erythrocytes exposed to an excess of toxin (25 HU/ml) in the absence of divalent cations and then extensively washed to remove all free toxin remained intact for several hours (phase I), but would lyse within minutes after the medium was supplemented with 1 mM Ca^{2+} (phase II).

Flame photometry was used to monitor the release of K^+ by the erythrocytes after exposure to toxin. No increase in K^+ release could be detected during phase I, but a rapid and massive release of K^+ coincided with the start of phase II (Fig. 14*a*) and preceded the escape of hemoglobin from the cells (Fig. 14*b*). Using the silicone oil procedure (see "Methods"), we observed a Na^+ influx apparently coincident with the K^+ efflux.

The percentage hemolysis during phase II depended both on the duration of the preceding incubation and on the toxin concentration present during phase I. Phase I had a pH optimum at pH 6.7, while the optimum for phase II was at pH 8.6 (Fig. 15). Toxin solutions preincubated with SRBC in medium free of divalent cations were depleted of hemolytic activity. The extent of this depletion was proportional to the number of cells present during the preincubation period (Fig. 16).

The toxin did not affect Ca^{2+} distribution across the erythrocyte membrane. Using the silicone oil procedure, we were unable to detect any change in permeability to $^{45}Ca^{2+}$ in toxin-exposed SRBC. Ca^{2+} uptake by the sarcoplasmic reticulum vesicle preparation was not affected by the addition of 250 HU/ml of toxin, either during the course of Ca^{2+} uptake or during a 10-min preincubation period (data not shown). Factors which render the sarcoplasmic reticulum vesicle membrane permeable to Ca^{2+}, such as A23187, allow accumulated Ca^{2+} to leak out of the vesicles and appear to block Ca^{2+} uptake in this system. Our data thus indicate that diamphotoxin renders the SRBC membrane permeable to Na^+ and K^+ (ionic radii 2.76 and 2.32 Å, respectively) but not to sucrose and hemoglobin (Stokes radii 4.4 and 31 Å respectively) and that it does not affect Ca^{2+} (ionic radius 3.21 Å) permeability of SRBC or sarcoplasmic reticulum vesicle membranes (36, 37).

Conformational Studies—These studies on the toxin showed an increase in amplitude of the negative CD peak in the region of 210–230 nm and a shift of the peak toward shorter wavelength when the toxin was exposed to Ca^{2+} ions (Fig. 17). The CD spectrum of a buffer blank was not affected by the addition

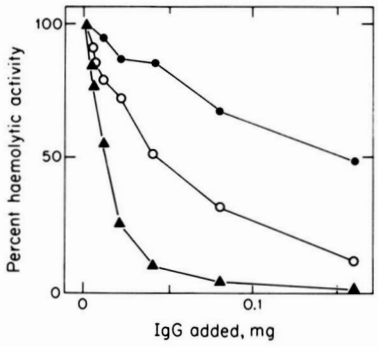

Figure 7. Inhibition of hemolytic activity by IgG from a rabbit immunised with highly purified diamphotoxin.

Aliquots containing 10 HU from phosphocellulose peaks I (▲), II (○) or III (●) were incubated with immune rabbit IgG and then assayed for hemolytic activity. The ordinate represents the percentage of cells lysed in the spectrophotometric assay. No inhibition of hemolytic activity was seen after incubation with irrelevant rabbit IgG.

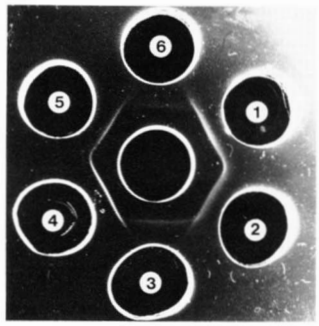

Figure 8. Analysis of phosphocellulose peaks I, II and III by double radial immunodiffusion.

The centre well contained immune IgG from a rabbit injected with highly purified diamphotoxin isolated from phosphocellulose peak I. The outer wells contained semi-purified material from phosphocellulose peaks I (wells 1 and 4), II (wells 2 and 5) and III (wells 3 and 6).

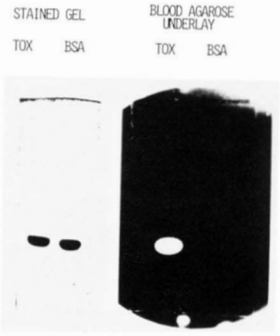

Figure 9. Hemolysis in a blood agarose underlay by highly purified toxin after SDS polyacrylamide gel electrophoresis.

Samples of 30 ug of toxin or albumin were electrophoresed on a 7.5% polyacrylamide gel in 0.1% SDS. The gel was washed, placed on a blood agarose underlay and incubated as described in the text. A gel with duplicate tracks was fixed and stained with Coomassie blue. The gel on the blood agarose underlay was photographed with dark field illumination.

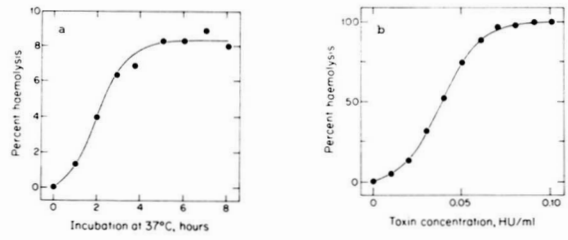

Figure 10. a) Hemolysis as a function of time of incubation of erythrocytes with toxin.

A series of tubes containing 0.5 ml 0.02 HU/ml toxin in VBS-G was incubated at 37°C for 8 hrs. At intervals of 1 hr 0.5 ml of 2 x 10^9 SRBC/ml in VBS was added to successive tubes. At the end of the 8 hr incubation the tubes were all centrifuged and the optical density of the supernatants measured at 540 nm. The percentage of cells lysed is plotted against the duration of exposure of cells to toxin. The rate of hemolysis dropped to approach zero after approximately 5 hrs incubation. The period of incubation at 37°C was the same for all toxin samples. The lysis for the 2 hour time point took place during the last two hours of the incubation period, since the cells were added to these tubes after 6 hrs. The data indicate that there was no hemolysis in the tubes for the 8 hour time point during these last two hours. This experimental design precludes the inactivation of the toxin by incubation at 37°C as an explanation for the limited hemolysis. Separate experiments established that the toxin was not inactivated by the products of SRBC hemolysis (data not shown).

b) Hemolysis by different concentrations of toxin after prolonged incubation.

Aliquots of 1 ml 10^9 SRBC/ml in VBS-G containing 0 to 0.1 HU of toxin were incubated at 37°C for 6 hrs. The tubes were then centrifuged and the release of hemoglobin measured by spectrophotometry. The percentage of cells lysed is plotted against the toxin concentration.

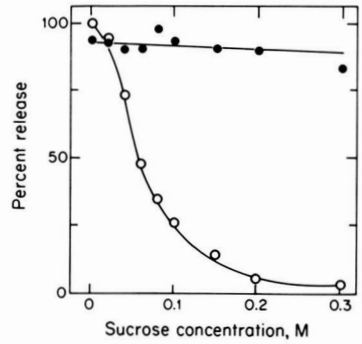

Figure 11. The effect of extracellular sucrose on the release of hemoglobin and K^+ by toxin-treated erythrocytes.

Suspensions of 0.45% SRBC, 20 HU/ml toxin and 0 - 0.3 M sucrose in VBS-G were incubated at 37°C for 60 min, centrifuged, and the escape of hemoglobin (○) and K^+ (●) from the cells estimated by spectrophotometry and flame photometry respectively. Results are expressed as a percentage of the values for hypotonically lysed cells.

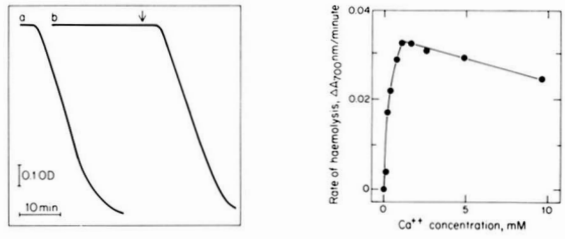

Figure 12. Inhibition of hemolysis by EDTA in the turbidity assay.

A suspension of 10^7 SRBC/ml had an apparent OD at 700 nm of 1 when intact and 0 when lysed. Suspensions of cells exposed to toxin showed a steady decrease in OD after an initial lag period (a). If the assay solution was made 1 mM EDTA before the addition of toxin no change in turbidity was seen (b). If the latter system was brought to 10 mM Ca^{++} hemolysis proceeded in the same manner as in curve A. The time of addition of Ca^{++} is marked on curve B with an arrow.

Figure 13. The effect of Ca^{++} on the rate of hemolysis.

SRBC were incubated with 25 HU/ml toxin at 37°C in modified VBS-G containing no divalent cations. After 30 minutes Ca^{++} was added to a final concentration of 0 - 10 mM and the rate of hemolysis measured using the turbidity assay.

TABLE III

THE RATE OF HEMOLYSIS DURING PHASE II USING DIFFERENT DIVALENT CATIONS

ion	OD/min.
control	0
Be	0
Mg	0.0249
Ca	0.0640
Sr	0.0771
Ba	0.0592
Mn	0.0197

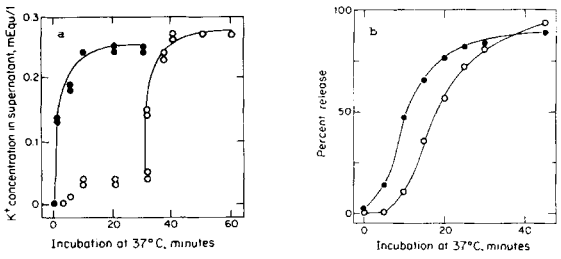

Figure 14. a) The release of K^+ from toxin-treated cells during phase I and phase II of hemolysis.

SRBC were exposed to toxin in medium free of divalent cations and incubated at 37°C. The medium was brought to 2 mM Ca^{++} at the start of the incubation (●) or after 30 min at 37°C (○). At intervals tubes were removed, centrifuged, and the escape of K^+ from the cells estimated by flame photometry.

b) The release of hemoglobin and K^+ from cells treated with 1000 HU/ml toxin.

A suspension of 10^9 SRBC/ml in VBS was treated with 1000 HU/ml of toxin and incubated at 37°C. At intervals aliquots were centrifuged and the escape of K^+ (●) and hemoglobin (○) estimated by flame photometry and spectrophotometry respectively.

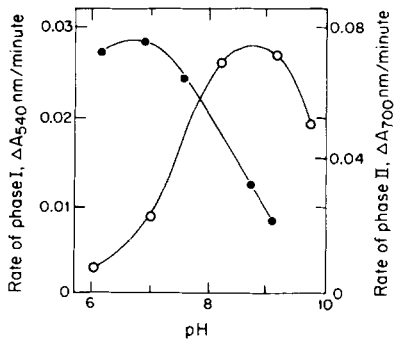

Figure 15. The pH optima for phase I and for phase II of toxin-mediated lysis of sheep erythrocytes.

To study the effect of pH on phase I SRBC were incubated with toxin in citrate or borate buffered saline, 1 mM EDTA pH 6 - 10 for 0 - 30 min. At the end of this period the cells were pelleted, resuspended in VBS, incubated at 37°C for 60 min and again centrifuged. The release of hemoglobin (measured by spectrophotometry) was a linear function of te duration of the first incubation, and the change in final OD per min of the first incubation was taken as a measure of the rate of phase I at that pH (●).

To study the effect of pH on phase II SRBC were incubated in 10 HU/ml toxin in modified VBS-G, 1 mM EDTA containing no divalent cations for 30 min at 37°C. The cells were then pelleted, washed, and resuspended at 10^7/ml in saline buffered with citrate or borate at pH 6 - 10. The suspensions were brought to 2 mM Ca^{++} and the rate of hemolysis measured at 37°C using the turbidity assay (○).

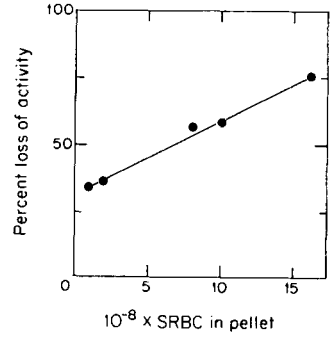

Figure 16. The depletion of the hemolytic activity of a toxin solution by exposure to SRBC in the absence of divalent cations.

Aliquots of 1 ml of 10 HU/ml toxin in modified VBS-G containing no divalent cations were incubated with 10^8 to 10^9 SRBC for 30 min at 37°C. The cells were then pelleted and the supernatant assayed for residual activity using the turbidity assay. There was no lysis of the cells in the preliminary incubation.

of Ca^{2+}. The specific fluorescence of the toxin/8-anilino-1-naphthalenesulfonate solutions increased with increasing Ca^{2+} concentration. At 10 mM $CaCl_2$, this increase was 9-fold. Control solutions from which toxin was omitted showed no change in specific fluorescence with changing Ca^{2+} concentration. Addition of La^{3+} to the toxin/8-anilino-1-naphthalenesulfonate solutions also resulted in an increase of specific fluorescence with 0.1 mM La^{3+} yielding an increase of the same magnitude as was generated by 10 mM Ca^{2+}. Exposure of pure toxin to La^{3+} at 0.1 mM likewise increased the amplitude of the negative CD peak.

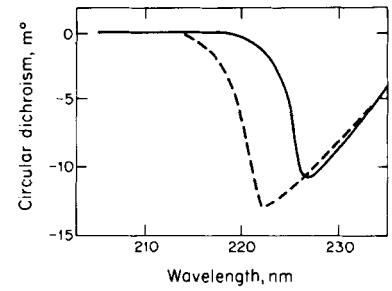

Figure 17. The effect of Ca^{++} on the CD spectrum of highly purified toxin.

A solution of 0.4 mg/ml toxin from Fraction V in 0.01 M Tris-HCl, 10% glycerol, 1 mM EDTA pH 8.2 was placed in a quartz cuvette of 2 cm lightpath. The optical activity was measured in the range 300 to 190 nm (—) and again after bringing the solution to 10 mM Ca^{++} (--). The plot shows only the range 210 to 235 nm in which a change in the spectrum was observed.

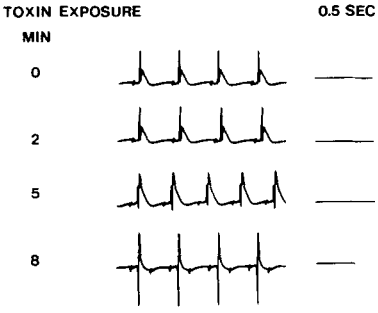

Figure 18. The effect of diamphotoxin on the electrocardiogram of the isolated perfused rat heart.

The heart was excised and suspended by ligaturing the aorta over a glass tube through which it was perfused with Krebs Ringer solution. After a 15 min stabilisation period the perfusate was made 1 HU/ml with toxin. The figure shows photographic recordings of the electrocardiogram immediately prior to the toxin perfusion (0), and after 2, 5 and 8 min of perfusion with toxin.

TABLE IV

THE RATE OF HEMOLYSIS OF ERYTHROCYTES FROM DIFFERENT SPECIES

Erythrocytes from various species were substituted for SRBC in the turbidity assay and exposed to 100 HU/ml toxin

species	OD/min.
dog	0.1508
rabbit	0.1433
guinea pig	0.0676
mouse	0.0635
horse	0.0506
rat	0.0489
sheep	0.0312
goat	0.0172
human	0.0156
vervet monkey	0.0077
pig	0.0062
chicken	0.0053
baboon	0
toad (X. laevis)	0

The Isolated Perfused Rat Heart—This developed a 2:1 atrioventricular block after 8 min of exposure to 1 HU/ml of toxin in the Krebs solution perfusate (Fig. 18) and progressed through a 3:1 block to independent atrial and ventricular activity. Ventricular activity ceased after about 15 min. An increase in lactate dehydrogenase levels in the perfusate, detectable after 10 min, signaled cellular disruption by the toxin in this system as well. In the control condition where toxin was omitted from the perfusate, the heart activity remained stable for at least 30 min. Toxin concentrations exceeding 1 HU/ml provoked a very rapid deterioration of cardiac activity.

The Guinea Pig Ileum Longitudinal Muscle Myenteric Plexus Preparation—When exposed to 1 HU/ml toxin in the bathing medium, this preparation showed a transient increase in tonicity followed by a rapid fall in the response to the stimulus over a period of about 10 min. Repeated washing of the preparation with toxin-free medium did not restore its responsiveness. If, however, the preparation was exposed to 1 HU/ml of toxin in medium containing no Ca^{2+} or Mg^{2+}, preceded and followed by 5-min washes in this divalent ion-

free medium, and then returned to the standard Krebs-Ringer solution, it regained 50–100% of its initial responsiveness to electrical stimuli.

Species Susceptibility to Hemolysis—Erythrocytes from a number of species were substituted for SRBC in the turbidity assay and used to assay a standard solution of 100 HU/ml of toxin. Table IV shows that there was a wide range in susceptibility, with dog erythrocytes being most rapidly lysed, while those of the baboon and the toad showed extremely slow hemolysis that was only detectable after prolonged incubation. No correlation could be found between published data on membrane phospholipid composition (38, 39) and the rate of hemolysis for the different species.

P. flexuosa—Exploratory studies on extracts of *P. flexuosa* pupae showed them to be lethal to mice and powerfully hemolytic. However, the profile of protein bands in SDS-polyacrylamide gels for *P. flexuosa* extracts did not show the dominant bands at M_r = 60,000 found in *D. nigro-ornata* extracts. The extracts also differed in that the toxic activity from *P. flexuosa* could be adsorbed on DEAE-cellulose.

DISCUSSION

The protein that we have isolated is a potent lethal and hemolytic toxin; the minimal lethal dose for adult mice is around 25 pg, and it produces red cell lysis at a solution concentration of approximately 5×10^{-11} M, corresponding to 65 molecules/cell in the assay system used. When the losses associated with purification are allowed for, the highly purified molecule, together with the closely related species in phosphocellulose peaks II and III, easily account for all of the lethal and hemolytic activity in the pupal extract. Additional considerations that identify diamphotoxin as the major biologically active factor in pupal extracts include the following 1) Protective rabbit antisera raised against highly purified toxin cross-react strongly with the closely related and as yet incompletely purified species separated on phosphocellulose. 2) Throughout all of the fractionation procedures tested, it proved impossible to separate the lethal and hemolytic factors and there was no evidence indicating that either activity was associated with unrelated molecules. 3) Hemolytic activity was clearly associated with the single band of highly purified protein seen in SDS-polyacrylamide gels. For all of these reasons, there appears to be little doubt that the molecule we have isolated is responsible for the activity of Bushman arrow poison.

Our best preparations of diamphotoxin (Fraction V) were highly pure as indicated by the following criteria. 1) Considering the high potency of the material, it would be surprising if the lethal and/or hemolytic activities were entirely due to trace contaminants with the bulk of the protein being biologically inert. 2) The protein behaved as a homogeneous single species of molecular weight approximately 60,000 both in the ultracentrifuge and on SDS-polyacrylamide gel electrophoresis, and it gave a single band on immunodiffusion. 3) Only a single NH_2-terminal amino acid, glutamic acid or glutamine, was detected. Taken together, these considerations support the conclusion that diamphotoxin is both highly purified and represented by the bulk of the isolated protein species.

On the assumption that diamphotoxin is indeed highly purified and correctly identified as the M_r = 60,000 protein, our results may be considered with two further questions in mind. The first is, how does the toxin bring about red cell lysis? We could not obtain any evidence for the involvement of phospholipase activity, either intrinsic to the toxin itself or being activated as a product of the toxin-erythrocyte interaction. The toxin appeared also to be devoid of protease activity. Furthermore, the rapidity of its lethal and hemolytic actions suggests that it is not acting as a specific enzyme inhibitor nor interfering with some essential metabolic function.

Any useful working hypothesis concerning the nature of toxin action should be expected to accommodate the following facts. 1) Direct assay of residual toxin (Fig. 16) and the kinetics of red cell lysis (Fig. 10) show that the toxin acts in a stoichiometric fashion and seems to be consumed during the hemolytic reaction. A small number of toxin molecules, fewer than 100, suffice to cause the lysis of a sheep red cell. 2) The hemolytic reaction is biphasic, with a divalent cation requirement limited to the second phase. 3) Toxin-induced hemolysis is prevented when erythrocytes are osmotically stabilized by addition of sucrose, but enhanced red cell permeability to K^+ persists.

With these facts in mind, we offer the following tentative working hypothesis, recognizing that the limited evidence available does not yet permit all other possibilities to be rigorously excluded. This hypothesis is attractive because it is consistent with all of the salient facts and suggests a number of experimental tests. 1) We propose that diamphotoxin interacts with erythrocytes, and presumably with other cell types, to form channels that permit the rapid diffusion of small monovalent cations across the plasma membrane. 2) Channel formation would occur in two steps. In the first stages, the toxin attaches to the cell surface without perturbing the normal permeability barriers. The firmness of this association, which survives repeated washing and prolonged incubation at 37 °C in Ca^{2+}-free media, would be compatible with the incorporation of toxin molecules into the lipid bilayer. 3) In the second phase, exposure of toxin-bearing erythrocytes to suitable concentrations of divalent cations would promote the formation of ion-permeable channels by inducing a conformational change in the attached (or incorporated) toxin molecules.

This hypothesis is compatible with (*a*) the noncatalytic nature of toxin action and with the apparent consumption of toxin that occurs during incubation with erythrocytes, (*b*) the observation that different pH optima apply to each of the two phases of toxin-provoked hemolysis, and (*c*) the large changes in 8-anilino-1-naphthalenesulfonate fluorescence and toxin CD spectra that accompany exposure to Ca^{2+}.

The second question concerns the lethal action of the arrow poison, and our hypothesis also provides a reasonable explanation for this. Intravascular hemolysis leading to hemoglobinuria could promote death by renal failure or, more acutely, by disturbances of electrolyte balance. Furthermore, toxin-mediated perturbations of ion distribution are not limited to red cells. The rapid disruption of cardiac rhythm already referred to, the destructive effect on myotubes in culture, and the local paralysis that regularly follows intramuscular injection all indicate that several vital functions which depend on excitable cells can be blocked by the toxin. It seems likely that the predominant cause(s) of death in an individual case will be determined by the site of injection, rate of absorption, and total dose of toxin administered.

Finally, the toxin raises a series of questions about its obscure role in the life cycle of *D. nigro-ornata*. Because it is one of the major pupal proteins and its concentration is at least 10-fold lower in the adult, diamphotoxin seems likely to perform some function important during the pupal phase. Related questions of interest concern the localization of the toxin within the pupa and the way in which the pupa copes with its potentially lytic action. All of these points are intriguing subjects for future work.

Acknowledgments—We thank Prof. L. Opie for help with the perfused rat heart preparation, Prof. M. C. Berman and Dr. A. Aderem for the use of their Ca^{2+} uptake apparatus, Dr. R. Maske for help with the guinea pig ileum longitudinal muscle-myenteric plexus apparatus, Drs. Jay Unkeless and Jay Valinsky for critical reading of the manuscript, and Clive Dutlow and Jill Bleakley for excellent technical assistance.

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J. Biol. Chem. 1983, 258:11924-11931.
