DIAMPHOTOXIN

THE ARROW POISON OF THE !KUNG BUSHMEN

JONATHAN H. DE LA HARPE

Thesis submitted for the

degree of Doctor of Philosophy

University of Cape Town

August 1980

ABSTRACT

In this thesis I describe a toxic protein, diamphotoxin, that is present in the pupae of the beetle *Diamphidia nigro-ornata*. This insect is used as an arrow poison by the !Kung Bushmen inhabiting the savannah of eastern Namibia and western Botswana.

Preliminary investigations showed that the pupae contained a 3,7 S cationic protein which caused haemolysis and, after intramuscular injection, local paralysis followed by death. By intravenous lethality assay, one 200 mg pupa contained 20 000 mouse lethal doses.

Assays for the toxin were developed based upon haemolysis *in vitro* and lethality *in vivo*. These assays were used to monitor purification of the toxin.

Diamphotoxin was purified by acid extraction in 0,1M glycine-HCl pH 3,0 followed by ammonium sulphate fractionation, chromatography on hydroxyl apatite, phosphocellulose and, finally, on DEAE cellulose. A consistent increase in activity after the hydroxyl apatite chromatography pointed to the removal of an inhibitor during this step. A subsequent severe loss of activity after chromatography on phosphocellulose could neither be explained nor overcome. The phosphocellulose chromatography step yielded three peaks of toxic activity. Immunological studies revealed cross-reactivity but not identity between these three toxin species. The toxin in the first peak to elute from the phosphocellulose column was purified to electrophoretic homogeneity by chromatography on DEAE cellulose. Attempts to purify the toxin in the other two phosphocellulose peaks were not successful. The isolated molecule was confirmed to be the toxin by haemolysis in a blood-agarose underlay after SDS-gel electrophoresis.

The molecular weight estimate for the toxin by SDS-gel electrophoresis was 60 700 daltons and by analytical ultracentrifugation 62 100 daltons. The molecule appeared to exist as a single polypeptide chain. The amino acid composition showed a high proportion of hydrophobic amino acids. Isoelectric focussing showed an isoelectric point of pH 9,45.

Toxin mediated haemolysis was studied in detail. The haemolytic event could be broken down into two stages. In the first stage toxin bound irreversibly to the cell but, provided no divalent cations were present, no damage to the cell could be detected. The second stage required the presence of free calcium (or certain other divalent cations), with an optimum concentration at 1 mM. The interaction of calcium with the cell-bound toxin resulted in the cell membrane becoming highly permeable to Na^+ and K^+ ions.

Experiments designed to detect phospholipase or protease activity in toxin solutions gave negative results. Erythrocytes incubated with ^{125}I-labelled pure toxin in calcium-free medium retained a quantity of bound toxin which could not be removed by repeated washing. Incubation of erythrocytes with calcium-free toxin resulted in depletion of the activity of the toxin solution. The kinetics of the haemolytic action of the toxin were shown to be stoichiometric rather than catalytic. It was estimated that haemolysis by the toxin required a minimum of approximately 100 molecules per cell.

Studies using circular dichroism measurements and the fluorescent probe 8-anilino-1-naphthalene sulphonic acid (ANS) indicated that a conformational change occurred in the toxin upon exposure to calcium. The ANS studies indicated that upon the addition of calcium the toxin molecule became more hydrophobic.

It was concluded that the toxin functions as a calcium regulated Na^+ and K^+ ionophore in that it binds to the cell membrane and, in the presence of calcium or certain other divalent cations, assumes a conformation which mediates the free passage of Na^+ and K^+ ions. The resultant disruption of normal transmembranous ionic concentration gradients leads to cell lysis by loss of osmoregulation and, in the case of excitable membranes, disruption of electrophysiological activity.

ACKNOWLEDGEMENTS

I would like to thank Professor E.B. Dowdle for supervising this project, and for valuable guidance, support and encouragement during the experimental work and in preparing the manuscript.

I learned a great deal by working with Dr. E. Reich of Rockefeller University during his visit to our laboratory. It was on Dr. Reich's initiative that this project was started.

A number of people provided me with technical assistance during the course of the project. Clive Dutlow and Jill Small warrant special mention for outstanding ability.

The procurement of the pupae was made possible by the help of Ds. F. Weich and the assistance of a number of Bushmen in Namibia. I would like to express my thanks for this help and also to Dr. M-L. Penrith of the Windhoek Museum for helping to gather information on the biology of *Diamphidia nigro-ornata*.

My thanks to Angela Phillips for typing the drafts and final copy of this manuscript. She was always cheerful, accurate and quick.

I received assistance from neighbouring laboratories with some of the techniques used during the course of the experimental work. Professor M. Berman allowed me to use his equipment and as yet unpublished technique for measuring the uptake of calcium by sarcoplasmic reticulum vesicles. Professor L. Opie allowed me to use the flame photometer in his laboratory and his apparatus for setting up and monitoring the isolated perfused rat heart preparation. Dr. K. Ivanetich gave me access to the circular dichroism spectropolarimeter in the Department of Medical Biochemistry. I am most grateful for all this help.

Financial support was provided for the project by the U.C.T. Staff Research Fund and the Medical Research Council.

CONTENTS

CHAPTER ONE

INTRODUCTION

The !Kung Bushmen inhabit the savannah region of north eastern Namibia and western Botswana (Figure 1.1). Despite the inroads of civilisation and the many social and economic pressures to adopt a western way of life, groups of Bushmen may still be found living as hunter-gatherers. For hunting they use the bow and arrow.

The arrow comprises two parts - a shaft and a detachable head (Figure 1.2). The two are joined by a friction coupling that is sufficiently secure to maintain the junction during handling, transport and flight of the arrow. When, however, the arrow impales its target, the shaft becomes dislodged by the flight of the startled animals through the brush. The headless shaft serves to inform the hunter that a hit has been scored.

As shown in Figure 1.2, the hollow arrow shaft is made from a length of coarse grass bound at both ends with sinew. The one end is notched to receive the bowstring; the other end takes the pointed bone peg to which the wire arrow head is fixed by a short grass sleeve, gum and sinew binding. The assembled arrow, weighing approximately 10g, is a flimsy missile that would be ineffective were it not poisoned. The poison is applied to the sinew-bound length of wire immediately behind the pointed tip. The barb itself is left uncoated, so avoiding danger from an accidental scratch. If, however, an arrow penetrates the target by more than a centimetre, poison is introduced into the wound.

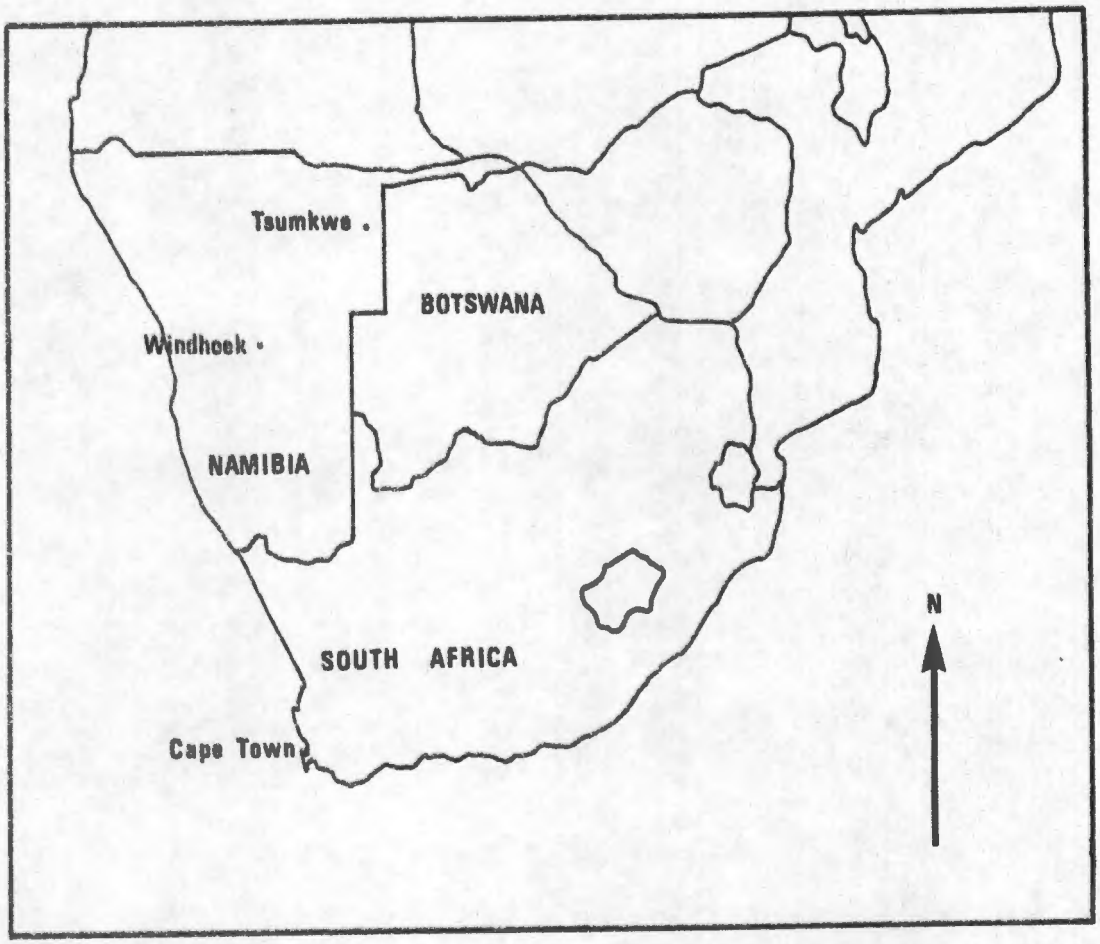


Figure 1.1 Map of Southern Africa showing localities relevant to this study.

The !Kung Bushmen inhabit the savannah region of north eastern Namibia and western Botswana. Pupae used in this project were obtained from cocoons excavated 20 km north of the mission station at Tsumkwe (20° 30' E, 19° 30' S).

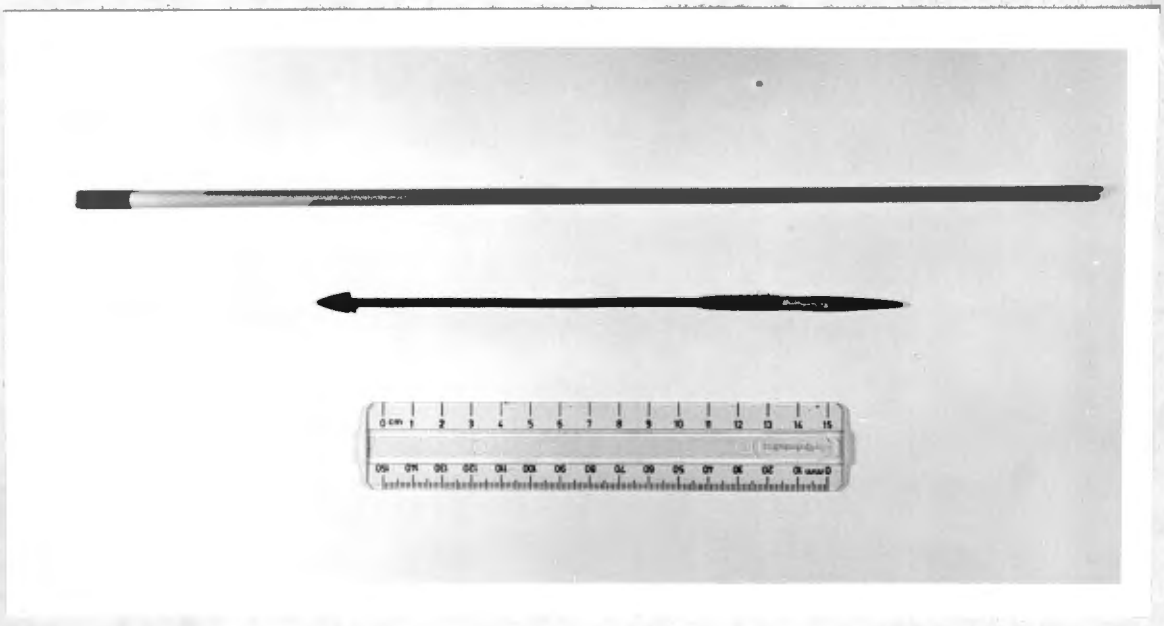


Figure 1.2 A Bushman arrow.

The arrow is constructed in two parts. The shaft (above) is fashioned from a coarse grass-stem, and the arrow-head (below) from a length of heavy gauge wire. The arrow-head is attached by sinew bindings to a bone peg which inserts into the front end of the shaft. The shaft is strengthened by sinew bindings at both ends, and has a notch at the back to take the bow-string. The arrow-head is bound with sinew along its length from behind the barb, and the poison is applied to this binding. The barb itself is not poisoned.

The poison applied to the arrow head is prepared by mixing about 2 ml of saliva produced after chewing the bark of the tree *Acacia mellifera* with about 0,1g of the roasted resinous pulp from the seed pod of the tree *Schwartzia madagascariensis*, and adding three or four *D. nigro-ornata* pupae. This concoction is thoroughly ground in a bone pestle and applied to the sinew-bound wire arrowhead with a flattened stick. Three or four applications are added with drying between each application.

Early descriptive reports of the poisoned arrows are to be found in the private and published writings of explorers, anthropologists, and naturalists. The earliest detailed description of the arrow poison that I have found is a report to the Royal Society by Lieut. William Paterson published in 1789:

> "Their method of making this pernicious mixture is by first taking the juice extracted from the Euphorbia, and a kind of caterpillar peculiar to another plant ..... They mix the animal and vegetable matter, and after drying it they paint their arrows with this composition".

In a report published in 1876 Lord and Baines describe how the Bushmen break open the sandy cocoon and:

> "taking out the grub, hold it between thumb and forefinger, and squeeze the entrails, or rather the internal juices, in small drops upon the arrow head".

From the reviews on Bushman arrow poison by Schapera (1924) and Shaw et al (1963) it is clear that a wide variety of materials were used as poisons. These included the juices from a number of different plant species; the venom from snakes, spiders, and scorpions; and the body juices of *Diamphidia* as well as at least two other species of beetle.

The Bushman has had a romantic appeal for many explorers and naturalists and some of the published information appears to be fanciful or anecdotal at best. For instance How (1970) quotes a statement that the venom of the mantis was used as an arrow poison!

In the work to be reported in this thesis I have restricted myself to a formal biochemical study of the toxin derived from *Diamphidia nigro-ornata*. This is a monophagous chrysomelid beetle which feeds exclusively on the shrub *Commiphera angolensis* found in Namibia, Botswana, and the northern Cape Province. The adult beetle, shown in figure 1.3, lays its eggs on the leaves of the *Commiphera*. When these hatch the caterpillar larvae feed on the leaves of the shrub. In autumn the fully grown caterpillars pupate about 20 cm below the surface of the sandy soil round the base of the shrub. In November, at the start of the rainy season, the adult beetles emerge from the cocoons to continue the life cycle.

Diamphidia nigro-ornata has, at various times, been mistakenly named *D. lesnei*, *D. locusta*, *D. simplex*, and *D. nigro-vitetta* (Koch 1958). Further entymological confusion has arisen from the fact that the *Diamphidia* pupa may be parasitised by the ectoparasytic wasp *Lebistina spp*. Thus, on opening a *Diamphidia* cocoon, one may sometimes find a *Lebistina* pupa and the dried out husk of the *Diamphidia*.

I have been unable to find reports of definitive studies of the Bushmen arrow poisons, although a number of pharmacological observations have been made. Shinz (1886) reported sending specimens of *Diamphidia* to Boehm who, in 1897 identified the toxic material as a "Toxalbumin" which caused haemoglobinuria. Breyer-Brandwijk (1937) noted that the toxin could be extracted with water; that it behaved as a protein; that it was inactivated by boiling; and that it caused haemolysis. She found that the pupae were a richer source of toxin

Figure 1.3 An adult beetle of *Diamphidia nigro-ornata*

The adult beetle weighs approximately 0,15g and measures approximately 1 cm. The eggs are laid on the leaves of the shrub *Commiphera angolensis*. The larvae which hatch from the eggs feed exclusively on the leaves of this shrub. In autumn they penetrate about 20 cm into the sandy soil below the plants where they pupate. At the start of the rainy season in November/December the adult beetles emerge from the cocoons to continue the life cycle. The Bushmen use the pupae (Figure 2.1) as a source of arrow poison.

than were the adults and she managed to raise an antibody to the toxin in rabbits. Steyn (1957) estimated the lethal dose for rabbits to be 0,25 to 0,50mg per kg body weight. Kundig and Charlton (1969) report that haemolysis by the toxin is not accelerated by lecithin (presumably intended as a test for phospholipase activity); that it has no action on the coagulation system; and that a slow contraction of intestinal and uterine smooth muscle could be demonstrated. There was no detectable action on vascular smooth muscle. They found the toxin to be heat labile; precipitable by 30% $(NH_4)_2SO_4$; denatured by alcohol or acetone; and excluded by Sephadex G50.

The present project was undertaken because the toxin appeared to be an interesting compound about which little was known. From the limited data available it seemed that it was a cytolysin and possibly a neurotoxin which might have interesting pharmacological properties, and which might prove to be a useful reagent for the study of cell membranes. This has been the case with a number of other toxins, both of biological and of synthetic origin.

CHAPTER TWO

PRELIMINARY INVESTIGATIONS ON THE EXTRACT FROM THE PUPAE

Pupae of *Diamphidia nigro-ornata* were obtained by arrangement with the Bushmen through the kind offices of Ds. F. Weich, a missionary in the area. Cocoons were excavated during the late winter and early spring months at a site approximately 20 km north of the mission station at Tsumkwe ($20^{\circ}30'E$, $19^{\circ}30'S$, Figure 1.1). The cocoons were transported by light aircraft to Windhoek and thence by commercial air-freight to Cape Town, where they arrived in excellent condition.

The pupae were removed from their cocoons and stored under liquid nitrogen. A small proportion of cocoons were found to contain adult beetles. The cocoons, formed from sand grains cemented together with a mucinous secretion from the pupae, were ellipsoid with axes measuring approximately 1,0 cm and 0,9 cm (Figure 2.1). Cocoons containing a pupa weighed approximately 475 mg. The orange pupae (Figure 2.2), measured 0,75 cm and weighed approximately 200 mg. The adult beetles (Figure 1.2) measured 1,0 cm and weighed 150 mg. They had a pair of hard outer wings and were yellow with eight dark markings on their back.

The following initial experimental observations provided useful preliminary information for subsequent studies.

Figure 2.1 Cocoons containing *Diamphidia nigro-ornata* pupae

A cocoon is egg-shaped with axes of 1,0 cm and 0,9 cm. Together with the encapsulated pupa it weighs approximately 0,5 g. The cocoon is formed from sand grains cemented together with a mucinous secretion by the pupa. Cocoons are found at a depth of approximately 20 cm below the surface of the sandy soil around the base of the shrub *Commiphora angolensis*.

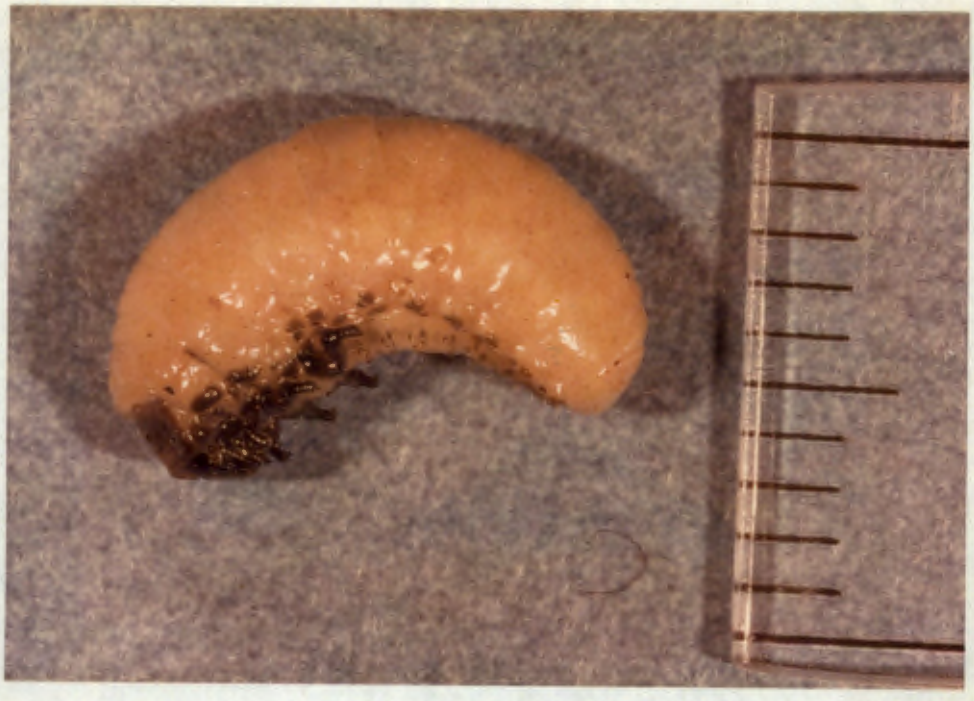


Figure 2.2 A *Diamphidia nigro-ornata* pupa

The pupa is orange in colour, measures about 0,75 cm and weighs about 0,2g. It has a small black head and six rudimentary black legs. It is this stage of the life cycle which the Bushmen use as a source of arrow poison. The scale is one centimetre ruled in millimetres.

Extracts of pupae injected intra-muscularly produced a local paralysis

When mice were injected into the spinal extensor muscles alongside the base of the tail with 100 µl of a 0,85% saline pupal extract containing 1 mg protein, they developed, as the first ill effect, a flaccid paralysis of the homolateral hind limb (Figure 2.3). Saline controls showed no ill effects.

Clarified crude aqueous extracts of pupae and beetles were toxic for mice when administered parenterally but not when given orally.

A homogenate of 1g pupae in 10 ml saline was centrifuged at 150xg for ten minutes at 4°C to separate a sediment, a cloudy aqueous phase and an orange fatty flotate. Mice were injected intramuscularly or intraperitoneally with 0,1 ml of the aqueous phase, either undiluted or in serial 10-fold dilution in 0,85% NaCl. Animals that received the undiluted material died within 30 min.; those injected with 1/10 or 1/100 dilutions died overnight. A homogenate of adult beetles prepared and tested similarly, was lethal at a dilution of 1/10 but no higher. Animals who received a concentrated extract of pupae by mouth suffered no adverse effects.

The toxin did not appear to be localised in the pupae in a manner that could usefully be exploited for preparative purposes.

A pupa was divided into 3 segments as indicated in Figure 2.4. Each segment was homogenized in 10 vols. of saline; the homogenates were centrifuged at 100xg for 10 min. and the aqueous phases tested at a dilution of 1/100 in each of five mice. The results given in Table 2.1, indicate that the toxin was not restricted to a particular segment of the pupa that could be isolated for subsequent purification.

Figure 2.3 Local paralysis induced by intramuscular injection of the toxin.

Mice were injected with 100 µl of pupal extract containing 1 mg protein (mark on head) or with 100 µl 0,9% saline (no mark). Injection was intramuscular into the right spinal extensor muscles alongside the base of the tail. Within 30 minutes the mice receiving the toxin extract showed a flaccid paralysis of the homo-lateral hind limb. Mice which received saline showed no ill effects.

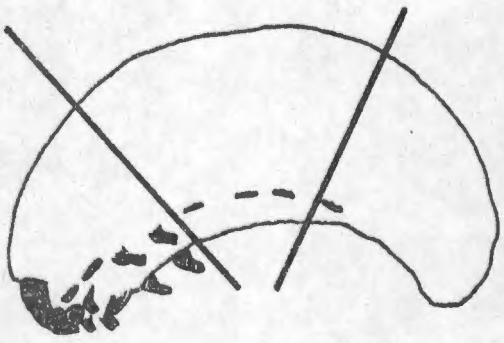


Figure 2.4 Localisation of the toxin in the pupae

A pupae was divided into 3 segments as indicated in the figure. Each segment was homogenised in 10 volumes of saline. The homogenates were centrifuged (1000xg, 10 min) and 0,1 ml of a 1/100 dilution of the aqueous phase in saline injected intramuscularly into each of 5 mice. The results, shown in Table 2.1, indicated that there was no restriction of the toxin to a particular region of the pupae.

TABLE 2.1

Segment	Mortality
head region	4/5
mid-region	5/5
tail region	2/5

Toxic activity in the saline pupal extracts resided in a component that was retained by a dialysis membrane and was heat labile

A saline extract of pupae that was lethal for mice (0,1 ml of a 1:100 dilution) was subjected to negative pressure ultrafiltration through a dialysis membrane. Toxic activity was concentrated in the retentate and was absent from the filtrate. Saline extracts of the pupae retained their toxic activity when stored overnight at 4°C and -20°C. As is evident from Table 2.2, the toxin was stable for 10 min at 56°C but was inactivated by a 2,5 minute exposure to 70°C.

Toxic activity precipitated from a saline extract of pupae between 30% and 70% saturated ammonium sulphate

A homogenate of 10g pupae in 100 ml of 0,85% NaCl was centrifuged (90 min.; 45 000 g; 4°C) to yield approximately 100 ml of aqueous phase. Three 10 ml aliquots of the solution were brought respectively, to 30%, 50% and 70% saturation with crystalline ammonium sulphate. The samples were stirred overnight at 4°C and then centrifuged (13 000xg; 20 min.; 4°C). The precipitates so obtained were dissolved in a minimal volume of 0,85% NaCl; these solutions and the supernatants were dialysed against 0,85% NaCl to remove ammonium sulphate and tested for lethality. The results, given in table 2.3, indicated that the toxic activity could be precipitated between 30% and 70% $(NH_4)_2SO_4$.

TABLE 2.2

HEAT LABILITY OF THE TOXIN

Extracts of pupae, prepared by homogenising pupae in 10 volumes of saline and centrifuging, were incubated at 56°C or 70°C for the indicated times. For each time-point 5 mice were injected intramuscularly with 0,1 ml of a 1/100 dilution of each solution in saline.

Time minutes	Mortality 56°C	70°C
0	3/5	
2,5	4/5	0/5
5	5/5	0/5
10	4/5	0/5

TABLE 2.3

PRECIPITATION WITH AMMONIUM SULPHATE

Three 10 ml 10% w/v saline extracts of pupae were brought to 30%, 50% and 70% saturation by adding 2,19 g, 3,65 g and 5,11 g crystalline $(NH_4)_2SO_4$ respectively. After stirring overnight at 4°C the suspensions were centrifuged (13 000 x g, 20 minutes, 4°C) and the precipitates so obtained were dissolved in a minimal volume of saline and dialysed against saline to remove the remaining $(NH_4)_2SO_4$. For each supernatant solution and each of the redissolved precipitates 4 mice were injected intramuscularly with 0,1 ml of a 1/100 dilution in saline.

$(NH_4)_2SO_4$	Mortality Supernatant	Precipitate
0%	4/4	
30%	1/4	0/4
50%	0/4	4/4
70%	0/4	3/4

Sucrose density gradient centrifugation of a pupae extract revealed toxic activity in a fraction with a sedimentation coefficient of 3,7

Approximately 100 µl of a 10% (w/v) saline extract of pupae was layered on a 4,5 ml, 5-20% sucrose gradient and centrifuged at 7°C for 15 hr at 115 000g. The gradient was then analysed for mouse lethality and for marker proteins to give the results depicted in Figure 2.5

Extracts of pupae contained a potent haemolysin

Samples (0,2 to 1,0 ml) of crude toxin solution containing 0,02 mg/ml protein were made to a final volume of 3,25 ml with veronal buffered saline (VBS) and 0,5 ml of a 4% suspension of washed sheep erythrocytes was added to each solution. After incubation at 37°C for 60 minutes the suspensions were centrifuged for 5 minutes at 1100xg and the optical densities of the supernatants were measured at 540 nm. The dose-dependent haemolysis which resulted is illustrated in Figure 2.6.

The toxin caused intravascular haemolysis in vivo

An anaesthetised dog was given an intravenous injection of toxin. The dog died approximately 15 minutes later. The haematocrit dropped from 40% to 5%, with intense haemoglobinaemia, ten minutes after administration of the toxin.

Chromatography of partially purified toxin on a column of Sephadex G100 revealed toxic activity in a peak with an elution volume close to that of haemoglobin.

Pupae (10g) were homogenized in 100ml 0,85% NaCl and the homogenate was clarified by centrifugation (45 000g; 90 min; 4°C).

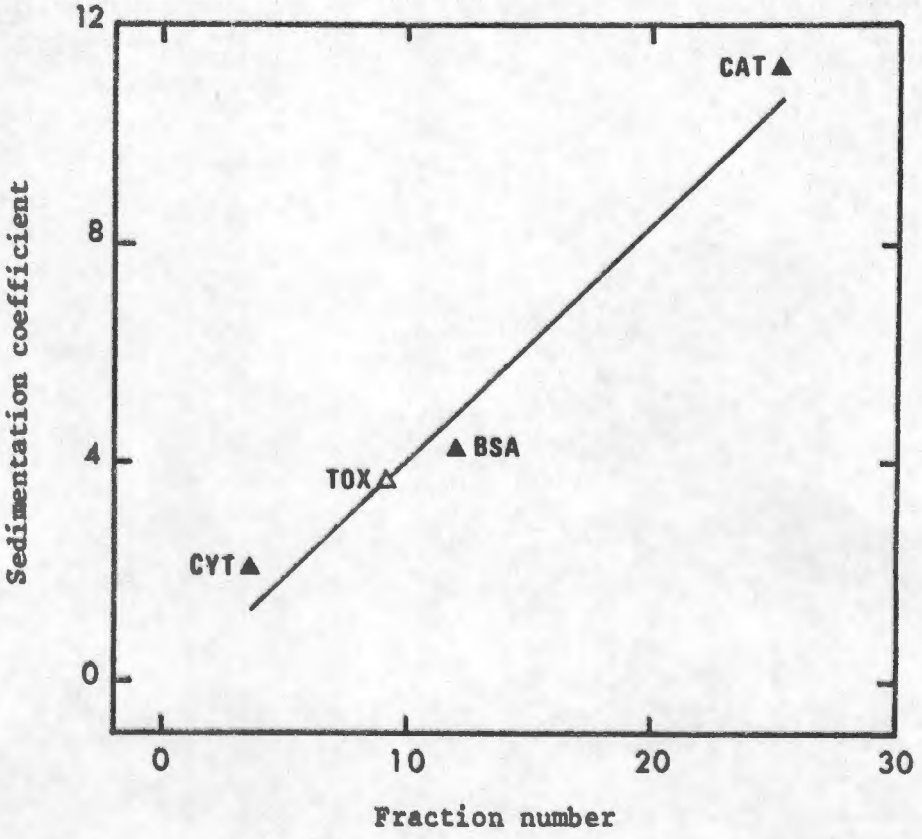


Figure 2.5 Sucrose density gradient centrifugation of a pupae extract.

Samples consisting of 25 µl ^{131}I-labelled BSA (65 000 cpm), 25 µl 100 mg/ml cytochrome C, 50 µl 0,6 mg/ml catalase, and 70 µl pupae extract were layered on 4,5 ml gradients of 5% to 20% sucrose in 5 ml centrifuge tubes. The gradients were centrifuged at 100 000 x g and 7°C. After 15 hours the tubes were punctured, and the gradients collected as 0,2 ml fractions. The position of the BSA peak was determined using a gamma counter; the position of the cytochrome peak was determined by measuring the O.D. of the fractions at 410 nm; and that of the catalase peak by the method of Beers and Sizer (1952). The toxin peak was localised by the mouse lethality assay.

The figure shows the relationship between S values of the marker proteins and their positions on the density gradient. An S value of 3,7 was calculated for the toxin by interpolation from the regression line.

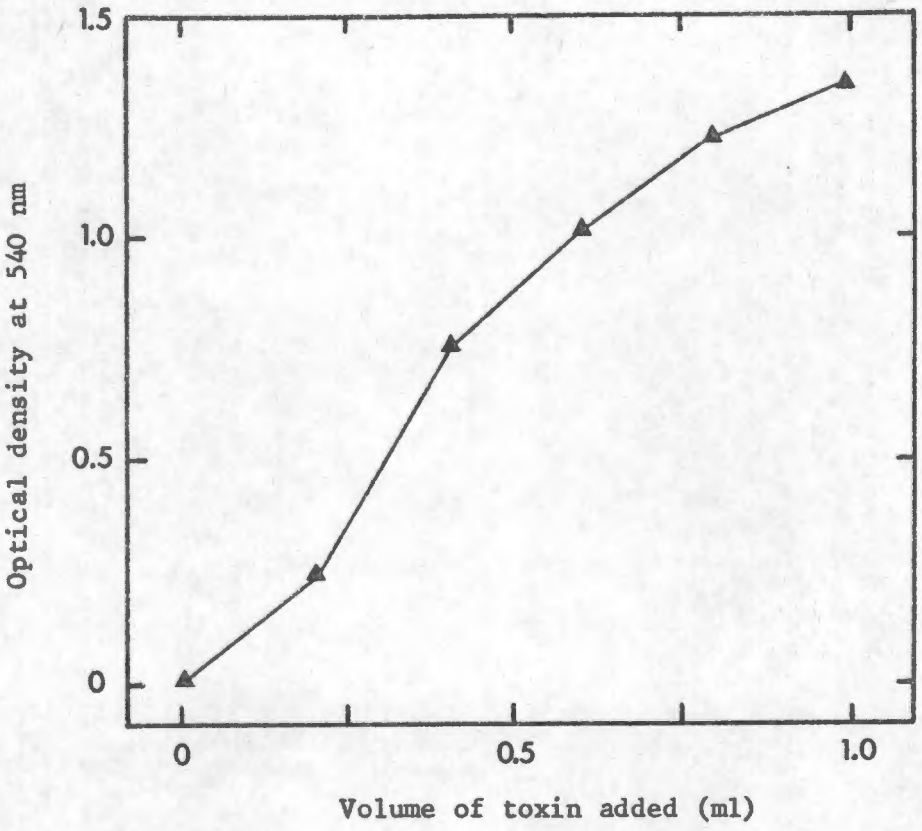


Figure 2.6 The haemolytic activity of the pupal extract

Aliquots of 0,2 to 1,0 ml of a toxin solution containing 0,02 mg/ml protein were made to 3,25 ml with VBS and 0,5 ml of a 4% suspension of SRBC in VBS was added to each solution. The tubes were incubated at 37°C for 60 minutes, centrifuged at 1100 x g for 5 minutes, and the optical density of the supernatant measured at 540 nm. The OD is plotted as a function of the volume of toxin solution added initially.

The aqueous phase (100ml) was treated with solid ammonium sulphate and the fraction precipitating between 30% and 70% saturation was collected. This was dissolved in 10ml of 0,01M potassium phosphate buffer pH 7,0 and 15, ml of the solution was applied to a 95 x 5 cm column of Sephadex G100. The column was equilibrated and developed with 0,01M potassium phosphate pH 7,0 buffer at 30 ml/hr. Fractions (10ml) were collected for protein determination and for haemolysis assays to give the results summarised in Figure 2.7.

Toxic activity did not bind to anion exchange cellulose whereas it was strongly absorbed by cation exchange cellulose

To 5 ml DEAE cellulose in 0,01M potassium phosphate buffer pH 7,0 was added 10 mg of the 70% $(NH_4)_2SO_4$ precipitate contained in 1 ml of the same buffer. After tumbling the mixture at 4°C for 90 minutes it was packed into a column and eluted with 10 ml aliquots of 0,01; 0,05; 0,10; 0,15 and 0,20M potassium phosphate pH 7,0. Figure 2,8 shows that the toxic activity was not retained by the resin.

To test for adsorption to cation exchange resins 20 mg protein in 1 ml 0,02M potassium phosphate 0,002M EDTA pH 6,0 was chromatographed on a 5 ml column of CM cellulose equilibrated in the same buffer. The column was developed with a NaCl concentration gradient. Figure 2.9 shows that the toxin activity was initially retained by the column and then eluted by the sodium chloride gradient. The conductivity of the peak fractions corresponded to a sodium chloride concentration of 0,1M.

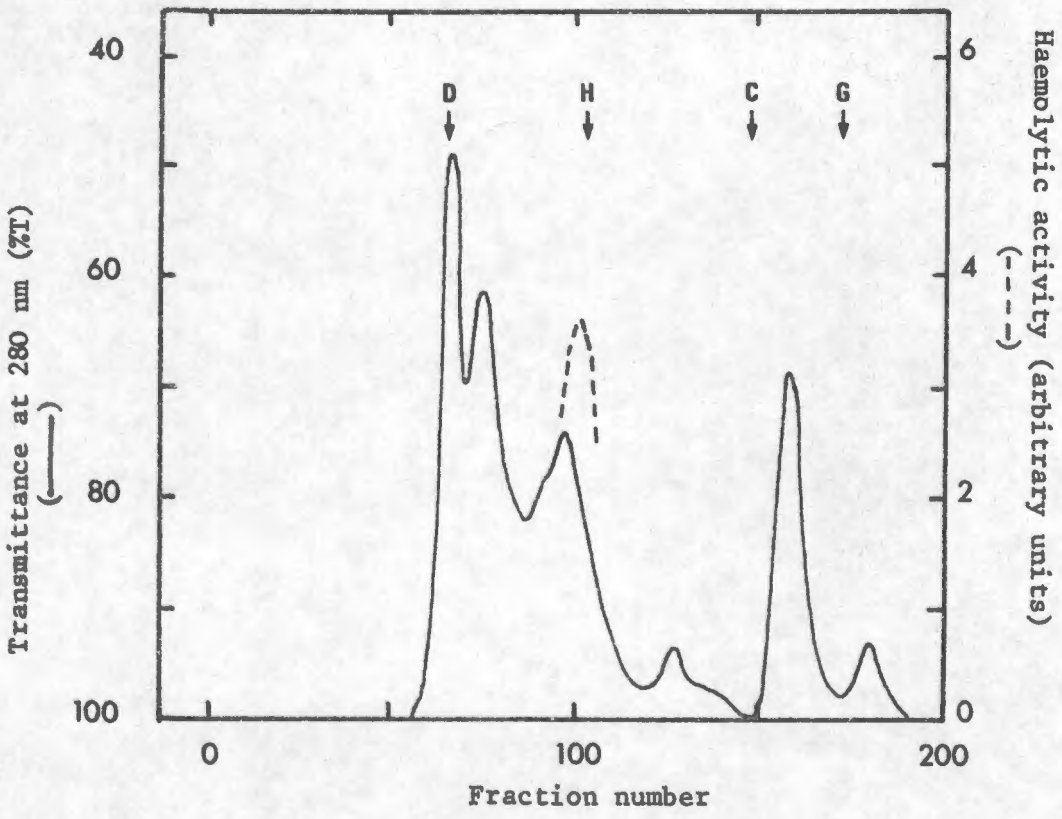


Figure 2.7 Chromatography of pupal extract on Sephadex G100

A sample of 30 ml (20mg/ml) of a 30% to 70% $(NH_4)_2SO_4$ fraction of the pupal extract, dissolved in 0,01M potassium phosphate buffer pH 7,0, was chromatographed on a 95 x 5 cm column of Sephadex G100. The column was equilibrated with 0,01M potassium phosphate pH 7,0 and developed with the same buffer at 30 ml/hr collecting 11 ml fractions. Protein (——) was determined by monitoring the percentage transmittance of the effluent at 280 nm. The position of the toxin peak (- - - -) was determined by assaying for haemolysis. In a second run on the same column a 30 ml sample containing blue dextran (B), haemoglobin (H), cytochrome C (C) and ^{14}C-glucose (G) was chromatographed. The elution volumes of these markers are shown by arrows.

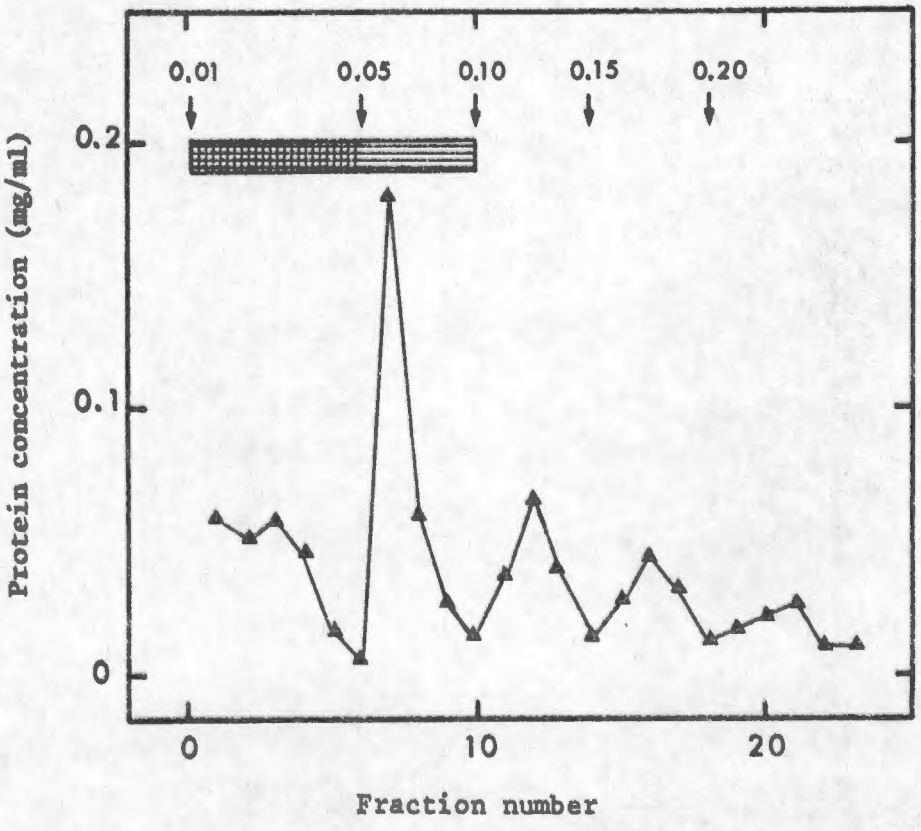


Figure 2.8 Chromatography on a trial DEAE cellulose column

A sample of 10 mg of the 70% $(NH_4)_2SO_4$ precipitate of the pupal extract dialysed into 0,01M potassium phosphate pH 7,0 was absorbed with 5 ml DEAE cellulose in the same buffer. After tumbling the mixture at 4°C for 90 minutes it was packed into a column and eluted with successive 10 ml steps of 0,01, 0,05, 0,10, 0,15, and 0,20 M potassium phosphate pH 7,0. The effluent was collected in 1 ml fractions which were tested for lethality by injecting 0,01 ml intramuscularly into a mouse.

Mice injected with material eluted with the 0,01 M buffer died in under 2,5 hours (▦). Injection with material eluted by 0,05 M buffer died in 4-18 hours (▤). There was no lethal activity in the remaining fractions.

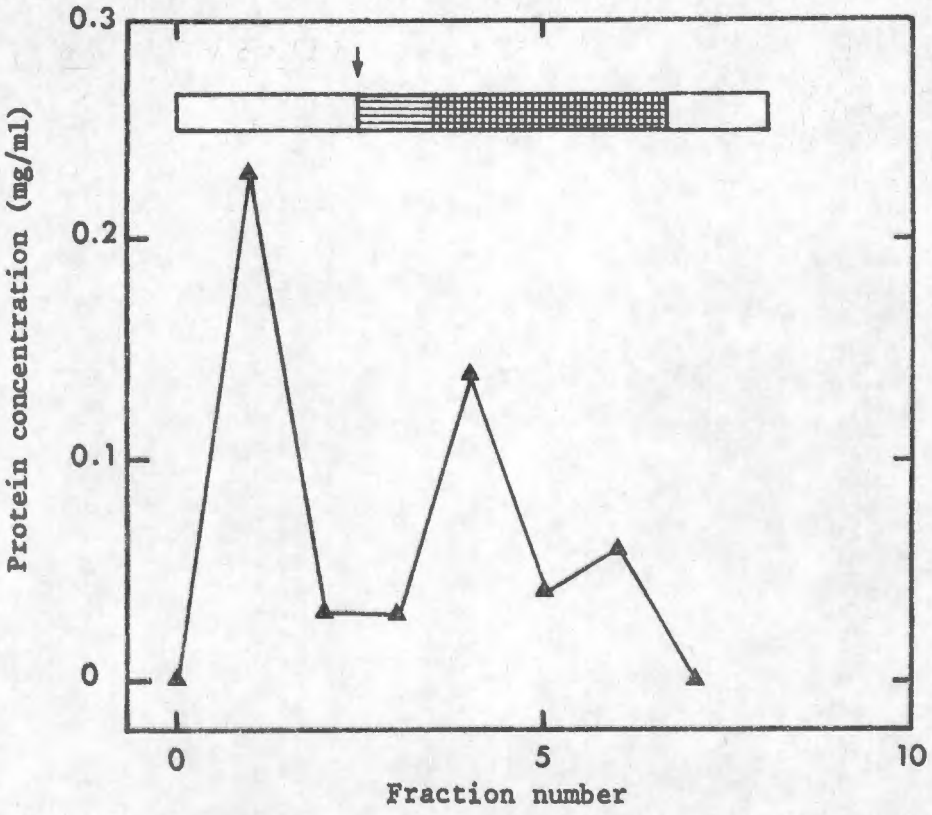


Figure 2.9 Chromatography on a trial CM cellulose column

A 1,0 ml toxin sample containing 2 mg protein was dialysed into 0,02M potassium phosphate, 0,002M EDTA pH 6,0 and chromatographed on a 5 ml column of CM cellulose equilibrated in the same buffer. The column was developed with an 80 ml concentration gradient of 0 to 1,0 M NaCl in the equilibration buffer. The arrow marks the start of the gradient. Fractions (5 ml) were collected and 1 µl aliquots assayed for haemolytic activity. Haemolysis was rated on an arbitrary scale: 0:□; 1:▤; 2:▦; 3:■. Protein was estimated by reading the optical density of the fractions at 260 and 280 nm, and the fractions were measured for conductivity.

The haemolytic activity was initially retained by the column and eluted at a conductivity of about 9 mmho.

Toxic activity could be destroyed by incubation with proteolytic enzymes

The haemolytic activity of a 1 mg/ml solution of toxin could be almost completely destroyed by incubation with 0,2 mg/ml pronase at 37°C for 90 minutes. Figure 2.10 shows the progress of this loss of activity and the less rapid loss of activity after incubation with 0,2 mg/ml trypsin.

SUMMARY AND CONCLUSIONS.

The preliminary studies reported in this chapter indicated that the concentration of toxin in the pupae exceeded that in the adult beetles.

The toxic component or components could be detected by their neuroparalytic or lethal effects on mice or by their ability to cause haemolysis *in vitro* and *in vivo*.

The apparent molecular size and heat lability of the toxin, its precipitation by 30% to 70% $(NH_4)_2SO_4$, its behaviour on ion exchange chromatography and its inactivation by proteolytic enzymes indicated that the toxin was a macromolecular compound and, in all probability a cationic protein. Further attempts to purify and characterise the toxin were based on these observations. For the sake of descriptive convenience I shall refer to the toxin from *Diamphidia nigro-ornata* as diamphotoxin.

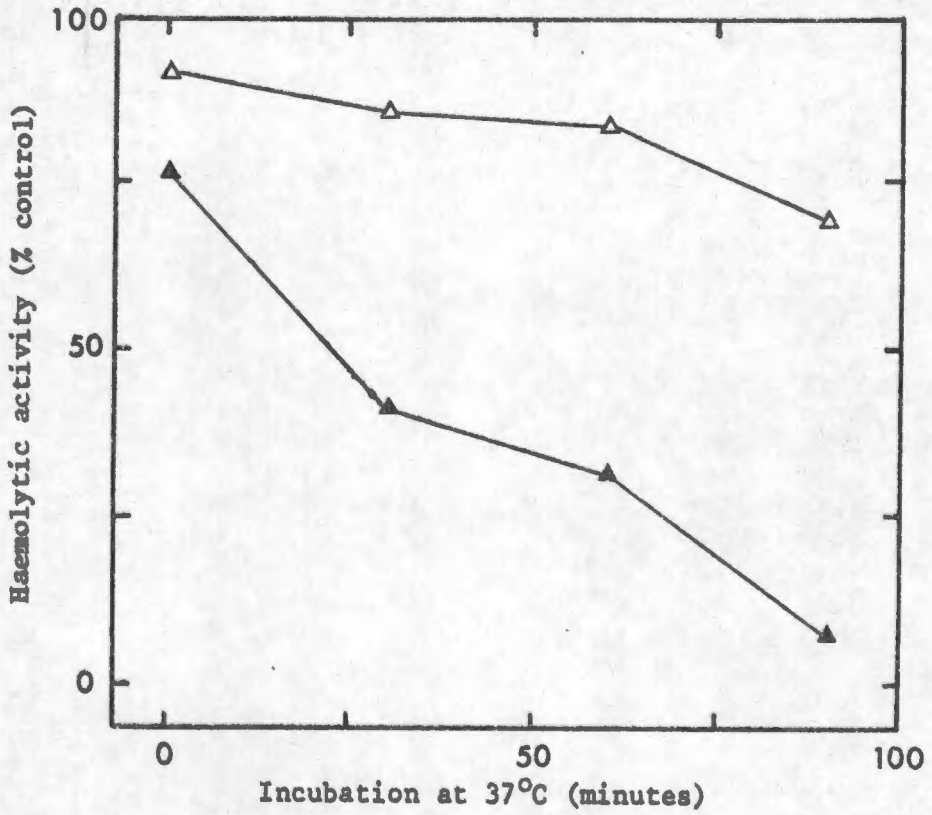


Figure 2.10 Inactivation of haemolytic activity after incubation with proteolytic enzymes.

To 4,5 ml 0,4 mg/ml crude toxin in VBS was added 0,5 ml of 2 mg/ml trypsin or pronase in VBS. In control tubes the toxin solution or the protease solution was replaced by VBS. The tubes were incubated at 37°C and at intervals of 30 minutes an aliquot of 10 µl was taken from each tube and diluted to 10 ml in VBS-G. An aliquot of 0,5 ml of this dilution was assayed for haemolytic activity. Haemolytic activity is expressed as a percentage of the activity in control tubes in which protease was omitted. Controls containing protease but no toxin did not exhibit haemolytic activity.

After 90 minutes exposure to the proteases the activity in the trypsin tube had dropped to 71% of the control, and that in the pronase tube to 8%.

CHAPTER THREE

ASSAYS FOR DIAMPHOTOXIN

Purification of the toxic components present in the crude pupal extract required the development of assay procedures for detection and quantification of toxic activity. Three such assays were devised; two of them were based on the ability of the toxin to cause haemolysis and the third on its lethality for mice.

The haemolytic assays, being more sensitive and less tedious, were used routinely to monitor purification procedures. The results were confirmed from time to time by means of the lethality assay. It transpired that lethality and haemolytic activity co-purified and could, eventually, be shown to be properties of the same compound.

HAEMOLYTIC ASSAYS

Solutions

Veronal buffered saline (VBS) was the basis of most solutions used for the assay procedures. It was made up as follows:

diethylbarbituric acid	4,0 mM
NaCl	143,0 mM
$NaHCO_3$	3,0 mM
$CaCl_2$	0,7 mM
$MgCl_2$	2,5 mM

This solution had a pH of 7,4 at room temperature and at 37°C. It was isotonic with sheep red blood cells.

Veronal buffered saline containing 0,5% w/v gelatin (VBS-G) was used for making dilutions of toxin solutions for assay. The addition of gelatin was found to protect against loss of toxin activity in dilute solutions, probably due to denaturation and absorption of the toxin molecules.

Alsever's solution was made up as follows:

glucose	114 mM
tri-sodium citrate	27 mM
NaCl	72 mM

titrated to pH 6,1 with 10% citric acid

Preparation of erythrocytes

Sheep erythrocytes (SRBC) were chosen as the haemolytic assay substrate. They were extremely sensitive to haemolysis by the toxin and were readily available from a standard source.

One sheep was maintained specifically to supply cells for this project. Using aseptic precautions the blood was taken from the jugular vein directly into an equal volume of sterile Alsever's solution. Cells were prepared for use by washing three times in ten volumes of VBS. The buffy layer was removed after the first wash. The cells could be stored, at $4^{o}C$, in Alsever's for one week and in VBS for 3 days without loss of sensitivity or reproducibility of the assay.

Haemolysis in test tubes (tube assay)

This assay, which proved to be the most reliable in terms of quantitative precision, was adapted from the standard assay for haemolytic complement (Kabat and Mayer, 1971). In principle this involved constructing a curve representing the relationship between extent of haemolysis and amount of toxin in the tube. One unit of toxin activity could then be defined as that amount giving 50% haemolysis under strictly standardized conditions. A curve was constructed for each sample by assaying five different dilutions of that sample.

A suspension of SRBC in VBS was prepared such that 0,5 ml of the cell suspension added to 3,25 ml water gave an optical density at 540 nm of 0,75. This corresponded to a 2,5% suspension of cells.

For the assay, dilutions of the toxin in VBS-G were brought to a volume of 3,25 ml with VBS-G in 5 ml plastic screw capped test tubes. To these were added 0,5 ml of the cell suspension after which the tubes were capped and inverted several times. After incubation in a 37°C waterbath for 60 minutes the tubes were centrifuged at 1000xg for 5 minutes and the optical densities of the supernatants measured at 540 nm.

For a toxin sample of unknown activity a preliminary assay was carried out on 0,1 ml of serial tenfold dilutions in VBS-G to find a dilution which would yield approximately 20% haemolysis in the assay system. The final assay would then be carried out using aliquots of this dilution.

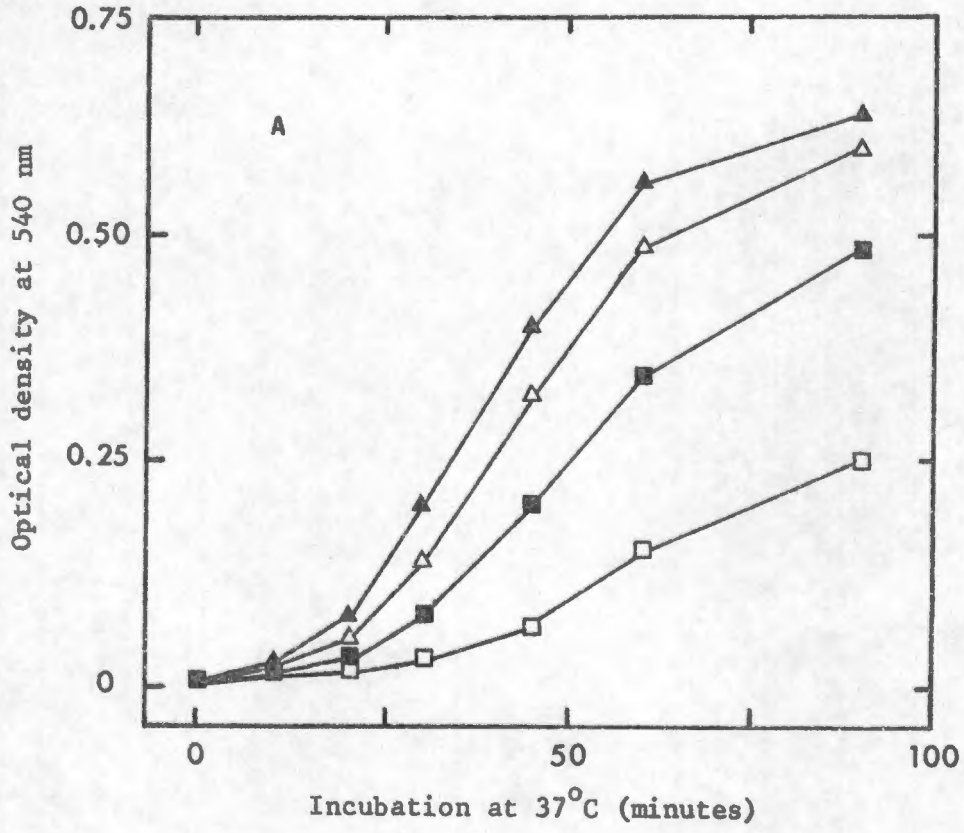
The extent of haemolysis showed a sigmoid relationship to toxin concentration and to time (Fig. 3.1). A straight line could be obtained by plotting the logit transform of the percentage haemolysis as a function of the log of the volume of toxin (Fig. 3.2.) From

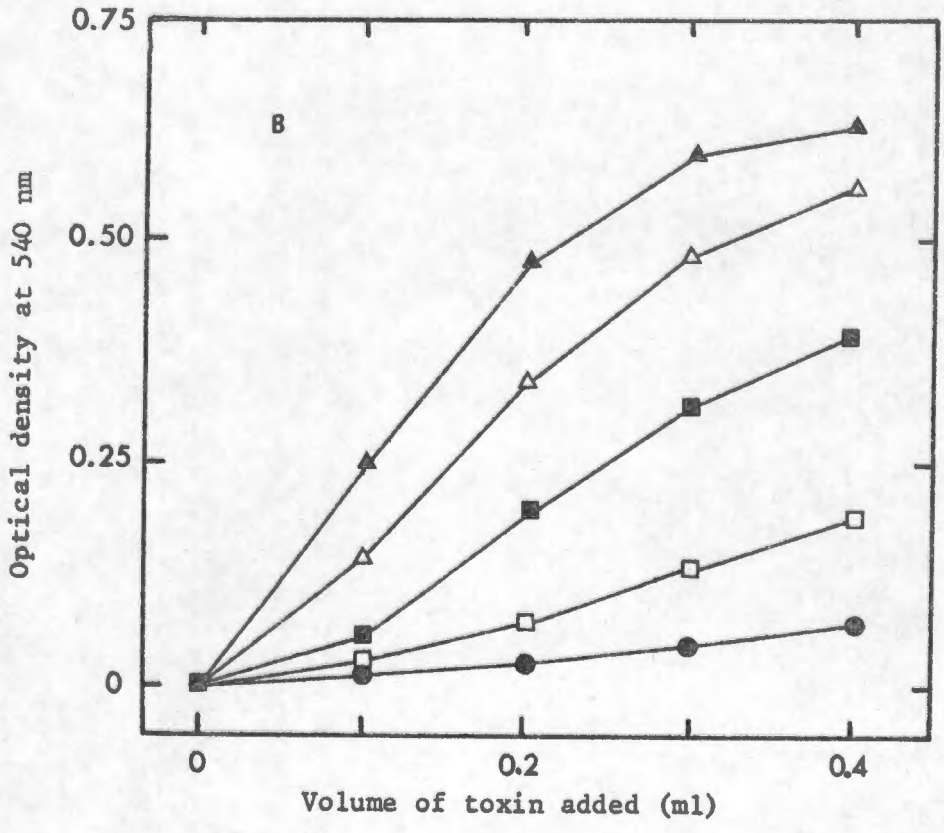
Figure 3.1

Figure 3.1 Haemolysis of SRBC by the toxin as a function of time and of toxin concentration

Aliquots of 0 - 0,4 ml of 0,2 µg/ml crude toxin in VBS-G were made to 3,25 ml using the same buffer. To these solutions were added 0,5 ml 2,5% SRBC in VBS. The tubes were incubated at 37°C for 0 to 90 minutes before being centrifuged at 1100 x g for 5 minutes. The optical density of the supernatant was read at 540 nm.

Haemolysis showed a sigmoid relationship to time (A), 0,4 ml: ▲ ; 0,3 ml: △ ; 0,2 ml: ■ ; and 0,1 ml: □ ; and, to a lesser extent, to toxin concentration (B), 90 min: ▲ ; 60 min: △ ; 45 min: ■ ; 30 min: □ ; 20 min: ● .


A
0.75
0.50
0.25
0
Optical density at 540 nm
0
50
100
Incubation at 37°C (minutes)


B
0.75
0.50
0.25
0
Optical density at 540 nm
0
0.2
0.4
Volume of toxin added (ml)

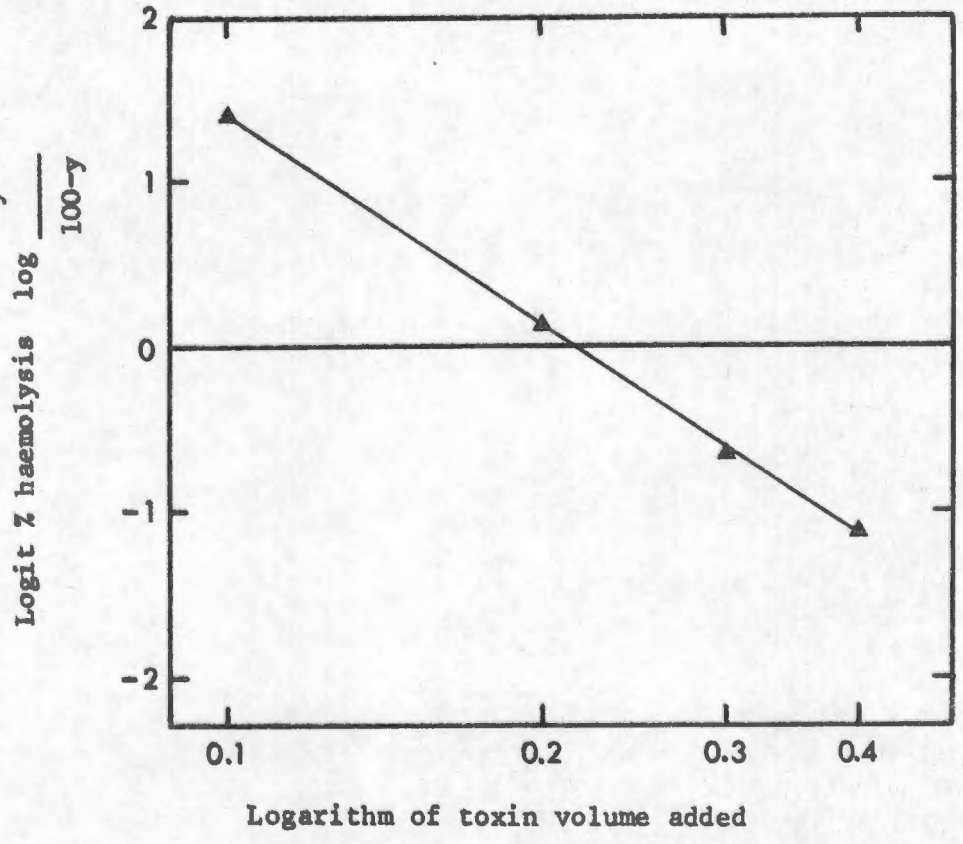


Figure 3.2 The linear relationship between the logarithm of the amount of toxin and the logit transform of the percent haemolysis

By plotting the logarithm of the toxin concentration against the logit transform of the percent haemolysis ($\log \frac{y}{100-y}$) a linear relationship was obtained. The volume of toxin yielding 50% haemolysis corresponded to the intercept on the ordinate ($\frac{50}{100-50} = 1$, $\log 1 = 0$). One haemolytic unit of toxin was defined as that amount yielding 50% haemolysis in this system.

this plot the volume of the toxin solution yielding 50% haemolysis could be obtained by interpolation. I defined one haemolytic unit (HU) of toxin as that amount of toxin giving 50% haemolysis with this assay procedure.

Comments

(i) The tube assay was used whenever accurate assay of toxin activity was required.

(ii) The sensitivity of the procedure was such that very low protein concentrations were required to remain in the assay range. To overcome the loss of activity due to denaturation or adsorption at these concentrations, 0,5% gelatin was incorporated in the buffer system. When the gelatin was omitted the results of assaying different dilutions of the same sample were inconsistent.

(iii) The use of plastic test-tubes instead of glass eliminated unacceptable variation in the results, possibly due to different degrees of adsorption to different types of glass.

(iv) The assay was more sensitive using VBS than in a number of other buffer systems tried. It transpired that this was due to the $CaCl_2$ and $MgCl_2$ in the VBS.

(v) It was not feasible to use the procedure described above for large numbers of samples, as in the case of fractions collected from a chromatography column. For this purpose a blood-agarose assay was developed.

Blood agarose gel assay

A suspension of 10 ml of 20% SRBC in VBS and 100 ml of a solution of 1% agarose (Indubiose A37) in VBS were brought to 40°C, rapidly mixed and poured into moulds fashioned from glass plates separated by perspex spacers, to give blood agarose gels measuring 100 x 90 x 1,5 mm . These were cooled on ice and the upper glass plate and perspex spacer removed to expose the gel slab. A pattern of wells 2,5 mm in diameter and 13 mm apart was punched in the gel. A 5 µl aliquot of toxin solution to be assayed was placed in a well. A standard curve was derived from a series of serial tenfold dilutions of a toxin solution whose activity had been measured with the tube assay. The gel was then incubated in a moisture chamber overnight at 4°C followed by 60 minutes at 37°C.

A clear zone of haemolysed cells developed around each well containing toxin. The area of this zone was linearly related to the log of the toxin concentration in the well. Figure 3.3 shows a standard curve for such an assay. The diameter of the haemolysed zone generated by a sample could be measured and the activity of the sample estimated by interpolation on the graph.

Comments

(i) Because of its simplicity the blood agarose assay could be used to assay a large number of samples in a single run. It was used routinely for assaying the fractions collected from chromatography columns.

(ii) The variance obtained with this assay was such that it was not suited to accurate quantitative work; the tube assay was used for this purpose.

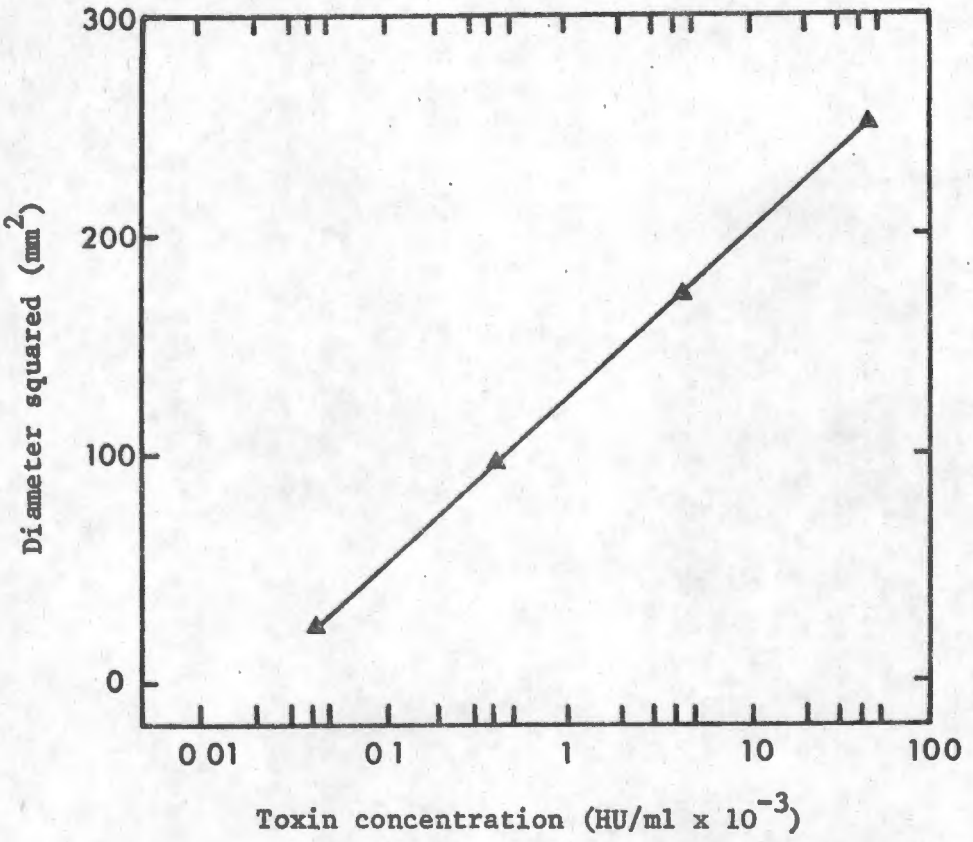


Figure 3.3 The standard curve for a blood agarose gel assay

A blood agarose gel consisting of 2% SRBC in 1% agarose was cast in a glass and perspex mould. A pattern of wells 2,5 mm in diameter and 13 mm apart was punched in the gel and aliquots of 5 µl of the toxin solutions to be assayed were injected into the wells. A standard curve was derived from serial tenfold dilutions of a toxin sample which had been standardised using the tube assay. The gel was incubated in a moisture chamber at 4°C overnight followed by 60 minutes at 37°C. The zone of haemolysis around each well was measured.

The square of the diameter of the zone of haemolysis was linearly related to the log of the toxin concentration in the sample.

(iii) The blood agarose gels could be stored in moist chambers at 4°C for up to five days before use.

(iv) If the gels were incubated at 37°C immediately after inoculation, diffusion and haemolysis took place simultaneously to give rings with ill-defined edges that were difficult to measure. By allowing diffusion to occur at 4°C - at which temperature haemolysis was inhibited - and subsequently incubating at 37°C for development of haemolytic zones, much sharper rings were obtained.

Mouse lethality assay

A volume of 0,05 ml of the toxin solution to be assayed was injected into the tail vein of a 20-30 g male ICR mouse. The time from injection to death was recorded. The time to death for the mice ranged from 0,5 minutes to 30 minutes. I defined one minimal lethal dose (MLD) as that amount of toxin which would kill 50% of injected mice within approximately 30 minutes. I found that mice injected with 1 MLD would either die after about 30 minutes or would show extreme discomfort and then recover. The time of death was linearly related to the log of the toxin dose, as illustrated in Figure 3.4. From this curve the activity of unknown samples could be estimated by interpolation.

Comments

(i) There was considerable variation in the activity of the same toxin sample determined by the mouse assay at different times. The source of this variation is not known.

(ii) Because of its unreliability the assay was used primarily to confirm the findings using the haemolytic assays and to watch for divergence of the haemolytic and lethality activities during the purification.

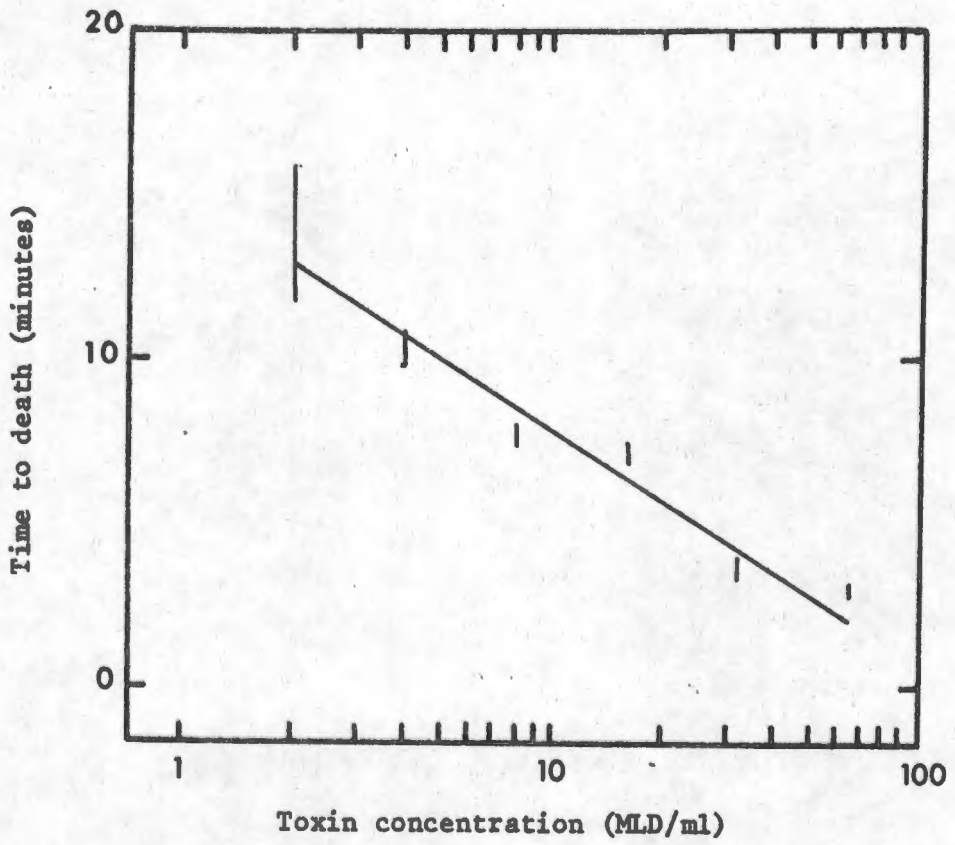


Figure 3.4 The dose-response curve for the injection of toxin into mice.

The solution to be assayed was taken up in a 1,0 ml plastic syringe which had been preincubated for 60 minutes with a solution of 10% foetal calf serum in 0,9% NaCl. A volume of 0,05 ml of the toxin solution was injected into the tail vein of the mouse and the time from injection to death was recorded. The vertical bars show the range of time to death for four mice for each toxin concentration.

The time of death was linearly related to the log of the toxin dosage.

(iii) Preincubating the syringes with 10% foetal calf serum eliminated anomalous underestimates of the activity of highly purified samples. It was assumed that the protein in the serum occupied binding sites on the syringe surface which would otherwise adsorb toxin molecules.

CHAPTER FOUR

PURIFICATION PROCEDURES

This chapter describes the procedures used for purifying the toxin. For the sake of brevity and clarity only the final protocol which was developed will be given here. Points of particular interest that emerged during the exploratory and initial phases of this work are mentioned in the comments on each section. The procedures are summarised in the flow diagram depicted in figure 4.1.

All steps were performed at 4°C unless otherwise stated.

The following techniques were used to monitor the purification procedure:

(i) Blood-agarose gels were used to monitor column effluents semi-quantitatively for haemolytic activity.

(ii) Tube assays were used for accurate quantitative work and to determine recovery of activity after each procedure.

(iii) Mouse lethality assays were used to establish coincidence between haemolytic activity and lethality.

(iv) Ultra-violet transmittance of column effluents was monitored at 280 nm by means of an LKB Uvicord.

(v) Rough determinations of protein concentration were carried out by measuring the optical density of the solution at 260 and at 280 nm. The method of Layne (1957) was used to derive protein concentrations from these readings.

(vi) Accurate determinations of protein concentration were done by the method of Lowry et al (1951) using BSA as a standard.

(vii) SDS-polyacrylamide gel electrophoresis was done by the

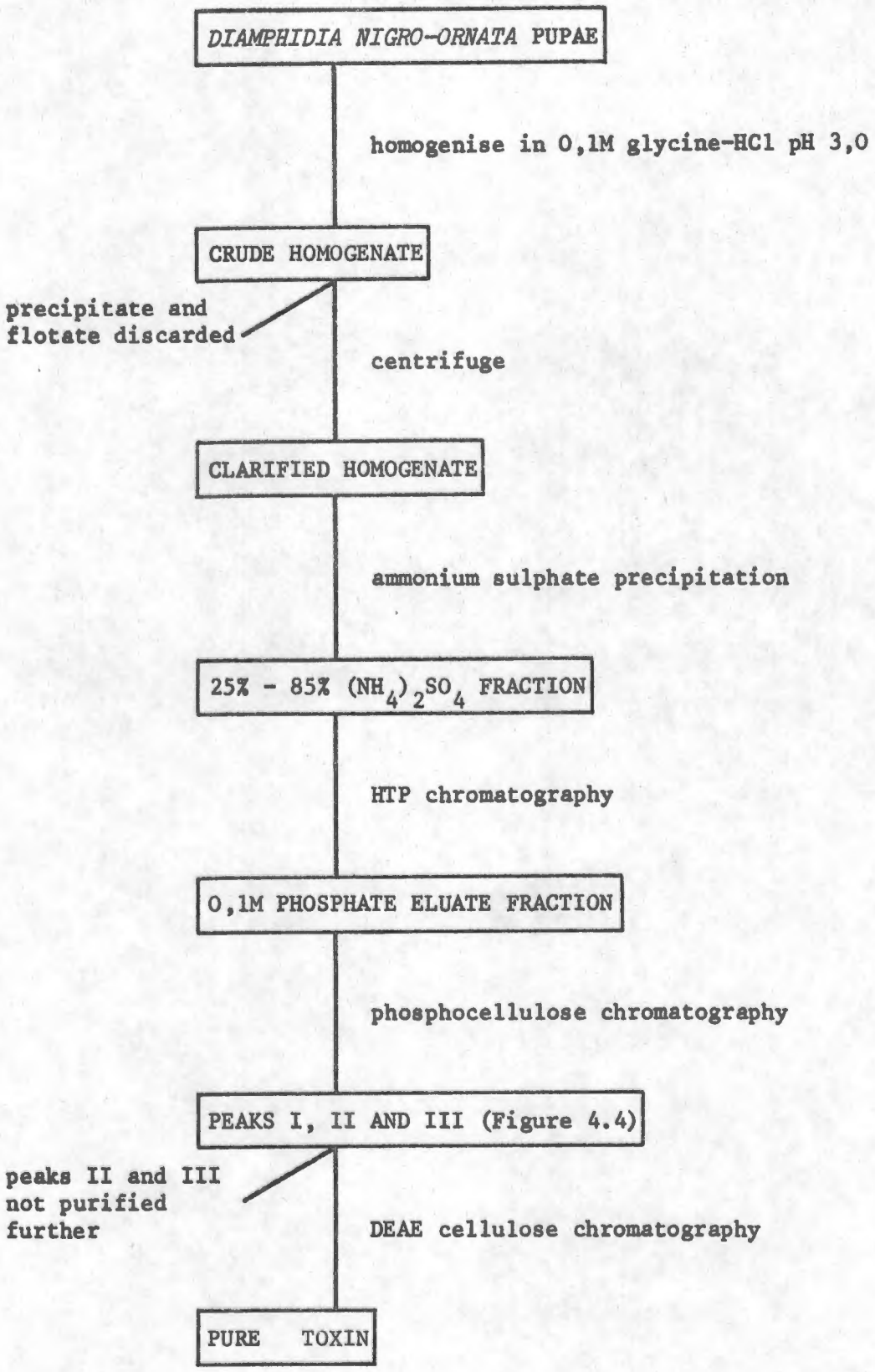


Figure 4.1 Schematic outline of the sequence of procedures for purifying the toxin.

method of Maizel (1971). Samples of 10 µg protein in 1% SDS were heated to 100°C for 1 minute then electrophoresed on 6 - 15% slab gels in 0,1% SDS. The gels were fixed with acetic acid:methanol:water 1:3:6 and stained with Coomassie brilliant blue R250. The sensitivity of the system was such that 0,05 µg of protein could be detected. Thus, if a protein was judged to be pure by the criterion of a single band on SDS-polyacrylamide gel electrophoresis, it was contaminated to the extent of less than 0,5% by any other protein.

(viii) A blood-agarose underlay technique was used to identify haemolytic activity in protein bands obtained after gel electrophoresis. Details of this procedure are given below.

(ix) The relationship between the different toxic components discovered was studied by immunoprecipitation and functional inhibition using a rabbit antibody to the purified toxin.

Extraction

Fifty grams of pupae were removed from the stock stored in liquid nitrogen. They were placed in 250 ml 0,1M glycine-HCl pH 3,0 at 4°C and left on ice for 30 minutes to thaw. The pupae were then homogenised in this buffer using a Virtis model 60K homogeniser and three 15 second bursts of 30 000 rpm. The crude homogenate was centrifuged (15 000 xg; 4°C; 20 min) to yield a pellet of grey-black solid material, an opalescent aqueous phase, and an orange fatty flotate. The aqueous phase was recovered by decantation and filtration through cotton wool to yield 260 ml containing 6630 mg protein and $1,34 \times 10^7$ HU of activity.

SDS-gel electrophoresis of this crude aqueous extract (Figure 4.2) showed a complex mixture of proteins with several prominent bands in the 60 000 - 70 000 M.W. region of the gel in which toxin was later to be localised.

Comments

(i) Initially the extraction was carried out in VBS at pH 7,4. It was found, however, that extraction at low pH gave the same yield of the toxic basic protein while removing less of the other proteins in the pupae. A two fold increase in specific activity was achieved by this manoeuvre.

(ii) A second extraction of the solid and fatty material yielded less than 5% of the amount of toxin in the first extract.

Ammonium sulphate fractionation

The clarified pH 3,0 extract (260 ml) was stirred at 4°C while 47,5 g crystalline $(NH_4)_2SO_4$ was added slowly over 30 minutes to give a final value of 25% saturation. This suspension was stirred for a further 30 minutes then centrifuged (15 000 x g; 4°C; 20 min). The supernatant (300 ml) was decanted and brought to 85% saturation by the addition of 113,9g $(NH_4)_2SO_4$. This suspension was stirred at 4°C overnight to ensure equilibrium then centrifuged (15 000xg; 4°C; 20 min). The precipitate was redissolved in a minimum volume of 0,01M sodium phosphate buffer pH 6,80 to yield approximately 100 ml solution containing 1820 mg protein and $1,15 \times 10^7$ HU of toxin activity.

SDS-gel electrophoresis revealed that, although the solution still contained a large number of protein species, the bands in the

Figure 4.2 SDS-Polyacrylamide gel electrophoresis of protein before and after ammonium sulphate fractionation

For each track 100 µg protein was electrophoresed on a 17 cm 5-15% polyacrylamide gradient gel with 0,1% SDS using the method of Maizel (1971), before (1), after (2).

60 000 to 70 000 M.W. region had been enriched (Figure 4.2). The specific activity of this material was 6320 HU/mg protein, representing a threefold increase over the specific activity of the crude aqueous extract (2020 HU/mg).

Ion exchange chromatography on hydroxyl apatite (Figure 4.3)

The toxin solution was dialysed exhaustively against 0,01M sodium phosphate pH 6,80. A sample of 60 ml containing 1500 mg protein was loaded on a 2,5 x 10 cm column of hydroxyl apatite (HTP) equilibrated in the same buffer. When the sample had been run into the column it was washed at 60 ml/hr with equilibration buffer until the transmittance of the effluent reached 95%. The toxin was then eluted with 0,1M sodium phosphate pH 6,80. When the transmittance again reached 95% the column was stripped with 1M K_2HPO_4.

The fractions eluted by the 0,1M buffer (200 ml) were pooled and concentrated using an Amicon ultrafiltration chamber with an XM50 membrane, to yield 60 ml of solution containing 660 mg protein and $1,83 \times 10^7$ HU toxin activity.

SDS-gel electrophoresis of the protein in the three peaks eluted from the HTP columns is illustrated in figure 4.3. The first peak was enriched with protein in the 30 000 M.W. region. The second peak with protein in the 10 000 to 20 000 and in the 60 000 to 70 000 M.W. region; and the third peak only in the 60 000 to 70 000 M.W. region.

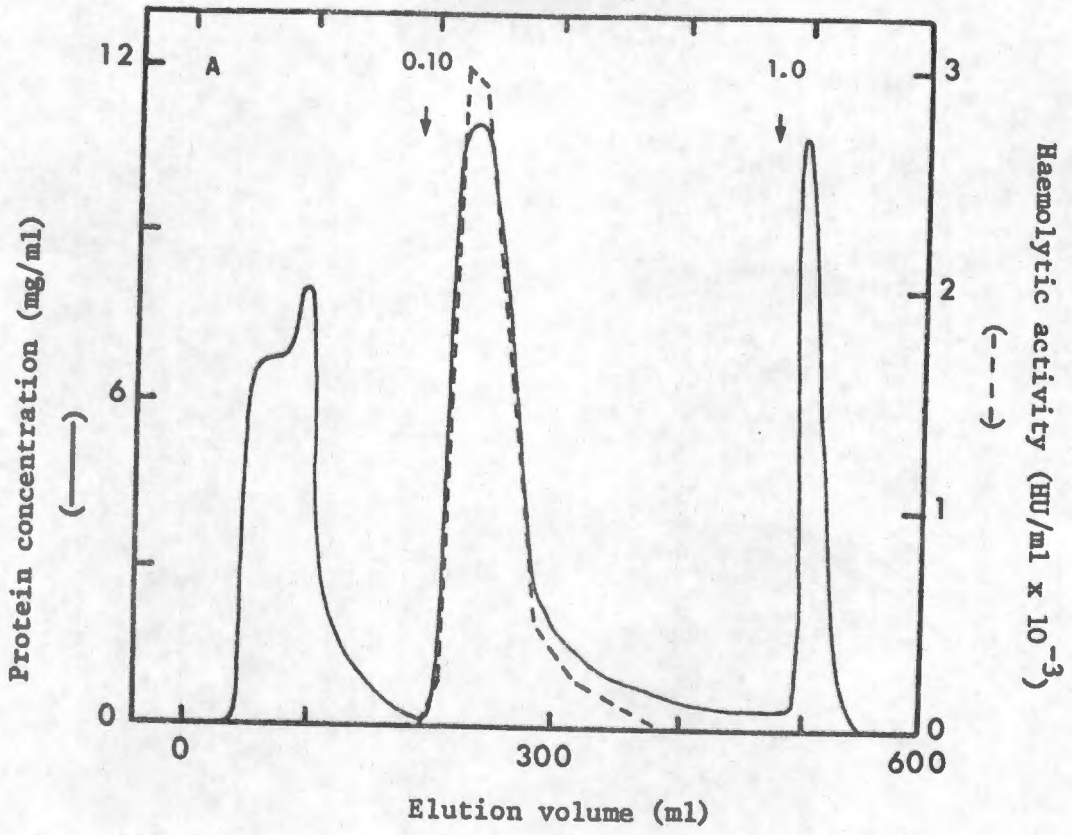
A consistent feature of this HTP chromatography and ultrafiltration step was an apparent nett gain in toxic activity. This I attribute to the removal of an inhibitor although I have no definitive data to substantiate this assumption. The specific activity of the material recovered was 27 800 HU/mg protein, representing a 4,4 fold purification for this step.

Figure 4.3

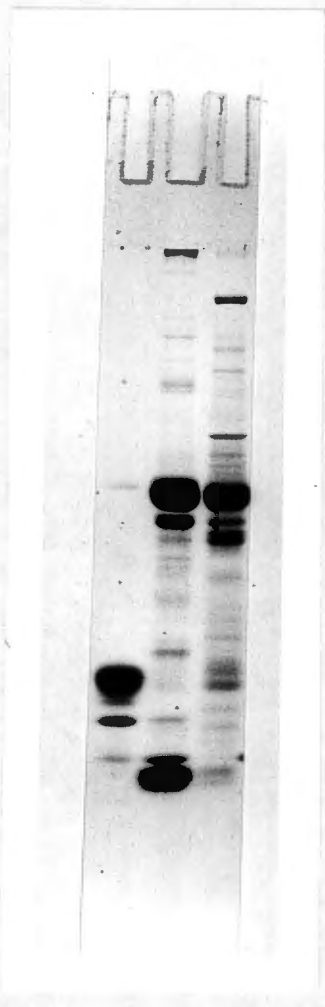
Figure 4.3 Ion exchange chromatography on hydroxylapatite

A. Toxin recovered from the ammonium sulphate fractionation was dialysed into 0,01M sodium phosphate pH 6,80. A sample containing 1500 mg protein was chromatographed at 60 ml/hr on a 2,5 x 10 cm column of hydroxylapatite equilibrated in the same buffer. The column was washed with equilibration buffer until the transmittance of the effluent returned to 95%. The toxin was then eluted with 0,10 M sodium phosphate pH 6,80. When the transmittance again reached 95% the remaining protein was eluted with 1,0M K_2HPO_4. The column effluent was monitored at 280 nm with an LKB Uvicord. Fractions of 10 ml each were collected and assayed for haemolytic activity using the blood-agarose assay. Protein (———); haemolytic activity (- - -).

B: Fractions corresponding to the three protein peaks were pooled and 10 µg protein from each pool was electrophoresed on 6-15% polyacrylamide gel, containing 0,1% SDS, by the method of Maizel (1971).


A
0.10
1.0
12
6
0
3
2
1
0
0
300
600
Protein concentration (mg/ml)
(——)
Haemolytic activity (HU/ml x 10^{-3})
(- - - -)
Elution volume (ml)

B



Comments

(i) One HTP column as described was not sufficient to chromatograph the protein obtained from 50 g of pupae. The excess protein was either chromatographed on a smaller column or pooled with equivalent protein from another extract.

(ii) The protein load of 30 mg protein per ml HTP was critical. Higher loading ratios resulted in some toxin not being bound while lower loading ratios resulted in excessive loss of activity.

(iii) Attempts to improve the effectiveness of these columns by eluting with a buffer concentration gradient were not successful.

Ion exchange chromatography on phosphocellulose (Figure 4.4)

The concentrated toxin solution from the HTP chromatography was dialysed into 0,01M sodium phosphate, 10% glycerol, pH 6,80. A 2,5 x 10 cm column of phosphocellulose equilibrated with the same buffer was loaded with 20 ml of the protein solution containing 200 mg protein. The sample was washed into the column with 10 ml of equilibration buffer after which the column was developed with a 500 ml linear gradient from 0,01M sodium phosphate, 10% glycerol pH 6,80 to 0,10 M sodium phosphate, 10% glycerol pH 6,80 followed by 50 ml 1,0 M K_2HPO_4.

The fractions collected were assayed by means of the blood-agarose assay and the mouse lethality assay. The fractions corresponding to the three toxin activity peaks (Figure 4.4) were pooled separately and each pool concentrated on an Amicon XM50 membrane to 4mg/ml. These pools were diluted 1:1 with 87% glycerol and stored

Figure 4.4

Figure 4.4 Ion exchange chromatography on phosphocellulose

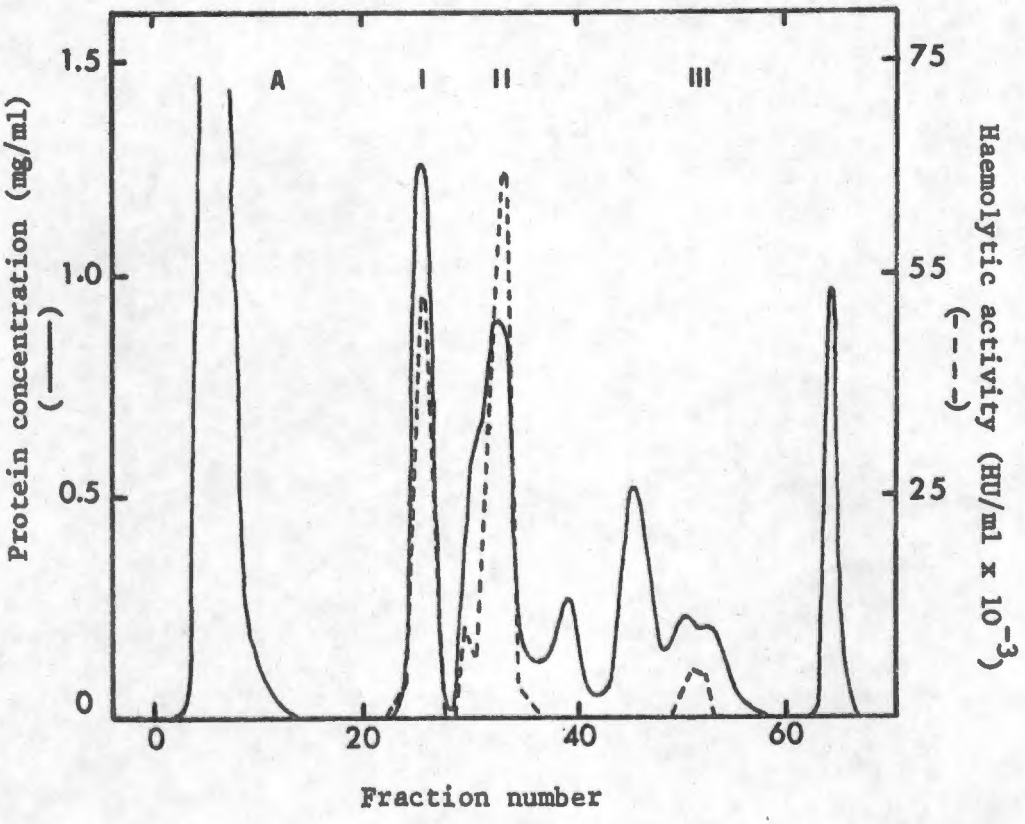
Toxin recovered from hydroxylapatite chromatography (Figure 4.3) was dialysed into 0,01M sodium phosphate, 10% glycerol, pH 6,80. A sample containing 200 mg of protein was chromatographed at 60 ml/hr on a 2,5 x 10 cm column of phosphocellulose equilibrated in the same buffer. The column was developed with a 500 ml gradient from 0,01M sodium phosphate, 10% glycerol, pH 6,80 to 0,10M sodium phosphate, 10% glycerol, pH 6,80, followed by 100 ml of 1,0M K_2HPO_4. The column effluent was monitored at 280 nm and 10 ml fractions were collected and assayed for haemolytic activity using the blood agarose assay and for mouse lethality.

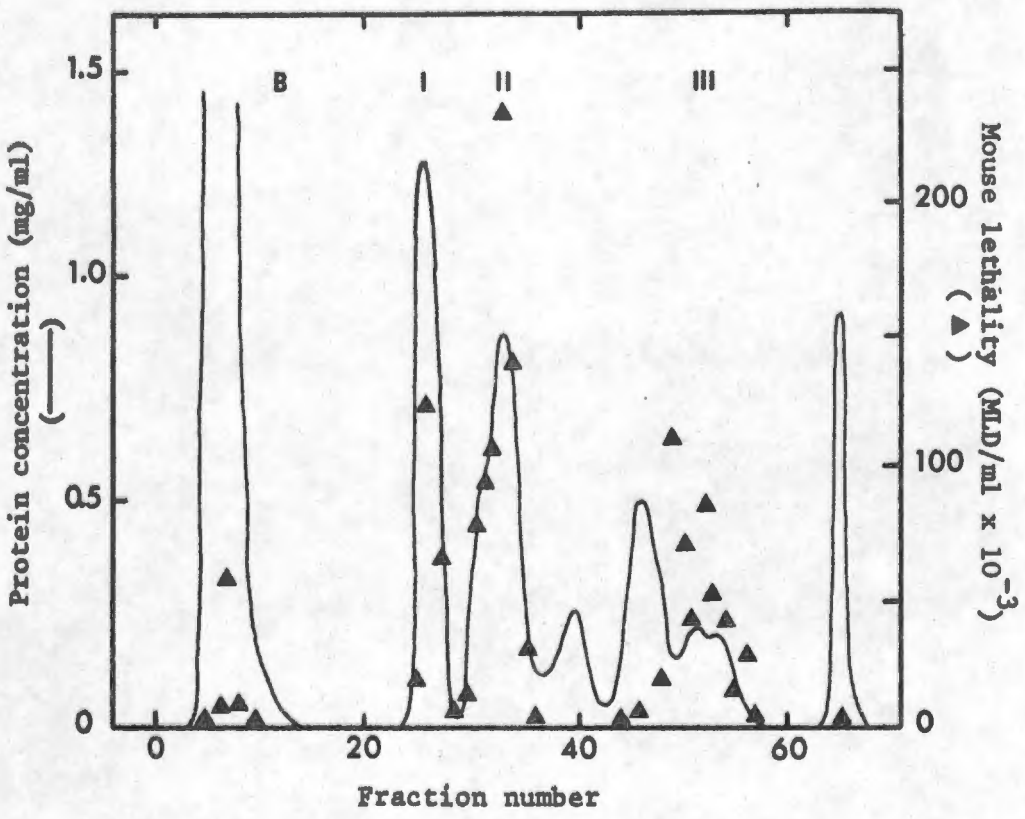
Fractions corresponding to the peaks marked I, II and III were pooled and concentrated using an Amicon XM50 membrane. Samples of 10 µg protein from each pool were electrophoresed on a 6% - 15% SDS polyacrylamide gel. The three activity peaks are referred to in the text by the numbering system shown here.

A: Protein concentration (———); haemolytic activity (- - - -)

B: Protein concentration (——); mouse lethality (▲)

C: SDS-polyacrylamide electrophoresis of protein from peaks I,II and III.


A
I
II
III
1.5
1.0
0.5
0
75
55
25
0
20
40
60
Protein concentration (mg/ml) (——)
Haemolytic activity (HU/ml x 10^{-3}) (- - - -)
Fraction number


B
I
II
III
1.5
1.0
0.5
0
200
100
0
0
20
40
60
Protein concentration (mg/ml) (——)
Mouse lethality (MLD/ml x 10^{-3}) (▲)
Fraction number

at -20°C. These three pools of toxin will be referred to as phosphocellulose peaks I, II, and III as indicated in figure 4.4.

Each of the three peaks contained the 62 000 M.W. protein band and material in the 20 000 M.W. region, although each peak had a different pattern of low molecular weight bands. Peak II contained an additional band at a position corresponding to a molecular weight of 66 000 and forming a doublet with the 62 000 M.W. band.

Peak I yielded 18,3 mg protein and 2,03 x 10^5 HU toxin activity; peak II 13,5 mg and 4,5 x 10^4 HU; and peak III 18,8 mg and 6,6 x 10^5 HU. This represented a considerable overall loss of toxin activity (table 4.1). Inactive fractions from a phosphocellulose column were pooled and added to aliquots of peaks I, II and III separately or in combination. These mixing experiments gave no reason to believe that the loss of activity was due to the separation of two synergistic components that were both required for activity.

In an attempt to attribute toxin activity to specific bands in the SDS gels, the blood underlay technique described in the next section was used. This indicated that the 62 000 M.W. bands in peaks I and III were active, but the zone of haemolysis in the blood agar gel was too diffuse to distinguish between the two bands in the doublet in peak II. To resolve this question an electrophoretic track containing separated components of peak II was cut from an SDS-acrylamide gel, washed in 0,25% Triton X100 for 45 minutes, in water for 20 minutes, and then cut into 2 mm sections. Each section was incubated overnight in 3,25 ml of VBS-G at 4°C. A 0,5 ml aliquot of 2,5% washed guinea pig red blood cells was added and the tubes incubated at 37°C for 60 minutes. Haemolysis of the cells, as measured by the release of haemoglobin, showed a clear peak corresponding to the position of the upper (66 000 M.W.) band of the doublet

as is illustrated in Figure 4.5. Similar procedures confirmed that the haemolytic activity in peaks I and III was associated with the 62 000 M.W. bands.

Some light was thrown on the relationship between the toxin molecules in the three phosphocellulose peaks by immunoprecipitation experiments. As described below the toxin molecule present in peak I was subsequently purified by chromatography on DEAE cellulose. A rabbit was injected with pure toxin which had been inactivated and insolubilised by cross-linking with glutaraldehyde. The IgG from the immune rabbit serum was isolated by a modification of the method of Weir (1973). This rabbit anti-toxin IgG was an effective inhibitor of the haemolytic activity of the toxin in peak I as illustrated in Figure 4.6. The partial inactivation of peaks II and III indicate cross-reactivity between the toxin molecules in these three peaks.

Immunochemical cross-reactivity between peaks I, II and III was further demonstrated by three techniques. Firstly the inhibitory effect of the IgG could be removed or reduced with immunoabsorbents prepared from phosphocellulose peaks I, II or III by the technique of Avrameas and Ternyck (1969). This is shown in Figure 4.7. In this experiment the amount of antigen added to the antibody was estimated in terms of haemolytic activity. Since the toxin was labile, denaturation may have occurred with the result that an abundance of antigenically intact, but functionally inactive, toxin competed with active toxin for antibody binding sites and so confused the result. Secondly, 5 µl aliquots of 1,6 mg/ml IgG antibody to peak I were placed in wells in a blood-agarose gel. Samples of peaks I, II and III were placed in adjacent wells and the gel was incubated at 4°C overnight and then at 37°C for 60 minutes. Figure 4.8 illustrates that the zones of haemolysis round all of the toxin wells were flattened on the side facing the antibody well, indicating that functional inhibition by the antibody had occurred with all three toxin samples. There was no inhibition by IgG

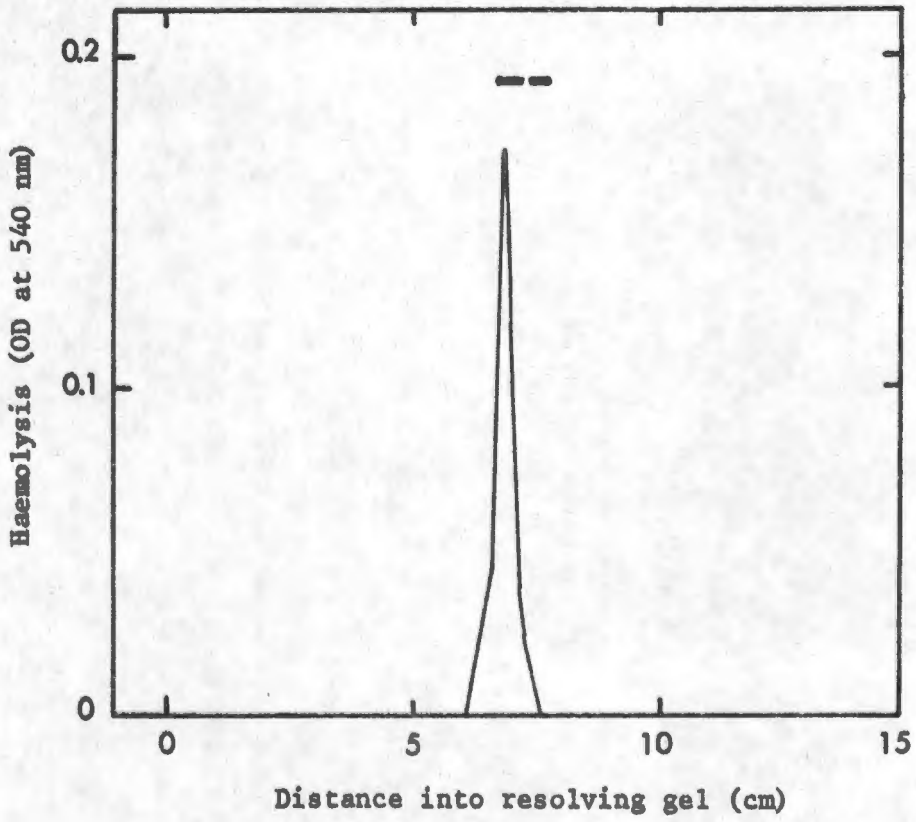


Figure 4.5 Haemolytic activity in slices cut from an SDS-polyacrylamide gel on which protein from phosphocellulose peak II had been electrophoresed.

Samples of 10 µg protein were electrophoresed in adjacent tracks on a 6-15% SDS-polyacrylamide gel. One track was fixed and stained while the other was cut into 2 mm sections. Each section was incubated in 3,25 ml VBS-G at 4°C overnight. A 0,5 ml aliquot of washed guinea pig red blood cells was added and the tubes incubated at 37°C for 60 minutes. The tubes were then centrifuged and the optical density of the supernatant measured at 540 nm.

There was a clear peak of haemolytic activity in the gel slices corresponding to the position of the upper band of the doublet in the 60 000 to 7000 M.W. region. The position of these two bands is indicated by bars at the top of the graph.

Figure 4.6 Inhibition of toxin haemolytic activity by IgG from the serum of a rabbit immunized with purified toxin.

Rabbits were immunized as described in the text with glutaraldehyde-treated toxin purified from phosphocellulose peak I. Immune IgG was isolated from the rabbit serum and added, in the amount shown, to tubes containing 10 HU of toxin obtained from each of the phosphocellulose peaks I, II and III. To each of a series of doubling dilutions of 100 µl IgG in VBS-G was added 100 µl of toxin containing 10 HU toxin activity. This was done for each of the three phosphocellulose peaks. The tubes were incubated at room temperature for 10 minutes after which 3,05 ml VBS-G and 0,5 ml 2,5% SRBC in VBS were added. The tubes were mixed, incubated at 37°C for 60 minutes and centrifuged (1100xg; 5 minutes). Percentage haemolysis estimated by measuring the optical density of the supernatant at 540 nm. Haemolysis was not inhibited in control tubes in which irrelevant rabbit IgG was substituted for the immune IgG. Haemolytic activity is expressed as a percentage of activity in tubes receiving irrelevant IgG.

All three toxin peaks exhibited inhibition when incubated with the antibody to peak I. The degree of inhibition was related to the antibody concentration but this relationship differed for each peak.

Peak I: ■ ; peak II: △ ; peak III: ▲ .

Figure 4.7 The effect of immunoabsorbents on the inhibitory activity of the rabbit IgG.

Immunoabsorbents were prepared by treating toxin protein (1 mg) from phosphocellulose peaks I, II or III and BSA (9 mg) with glutaraldehyde according to the method of Avrameas and Ternyck (1969). A control immunoabsorbent was prepared from 10 mg BSA treated in the same manner.

To each absorbent pellet was added 0,7 ml of a solution containing 1,6 mg/ml purified immune IgG. The suspensions were tumbled at room temperature for 3 hours and centrifuged to recover non-absorbed IgG. This IgG was then tested for inhibition of the activity of toxin from phosphocellulose peaks I, II and III by the method described in Figure 4.6. The figure shows the data for the haemolytic activity of 10 HU of toxin from phosphocellulose peak I after treatment with the absorbed IgG. Haemolytic activity is again described as a percentage of activity in tubes receiving irrelevant IgG.

The immunoabsorbent prepared from peak I removed all the specific antibody to peak I toxin, with the result that no inhibition of haemolysis was observed over the IgG concentration range tested (▲). The immunoabsorbent prepared from peak III removed most of the specific antibody to peak I toxin, and only slight inhibition of haemolysis was observed over the IgG concentration range tested (△). The immunoabsorbent prepared from peak III was less effective in removing specific IgG and a considerable inhibition of haemolysis by toxin from peak I was achieved with this IgG (■). There was some loss of inhibitory activity in antibody absorbed with the control immunoabsorbent (□).

Figure 4.6

Figure 4.7

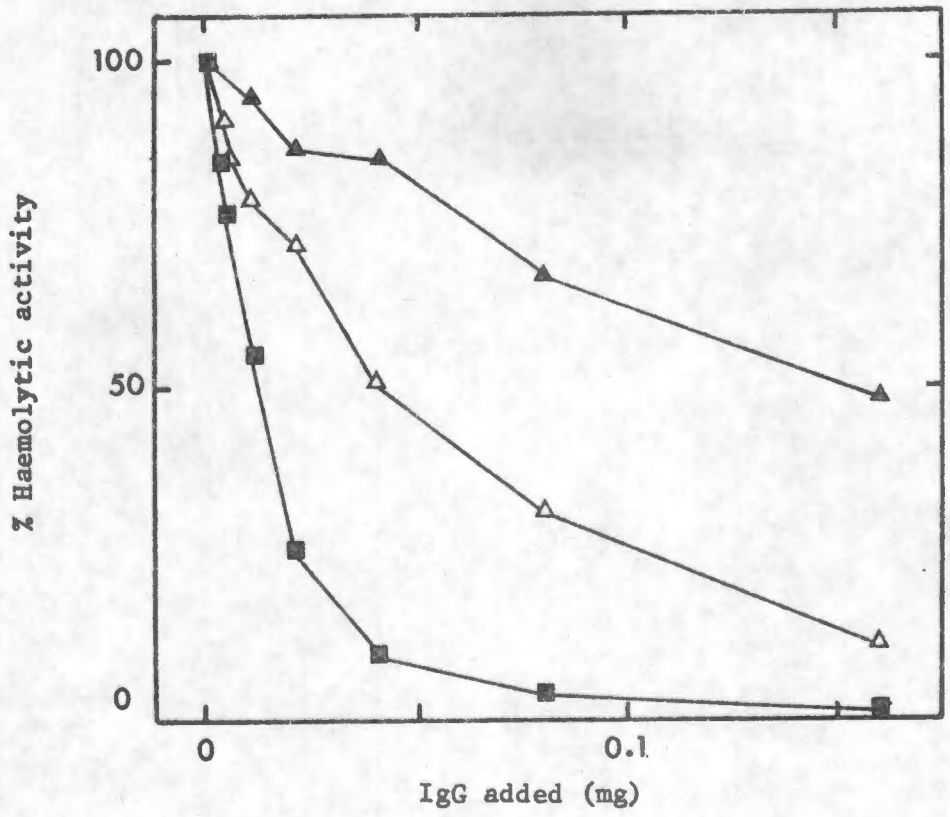


Figure 4.6

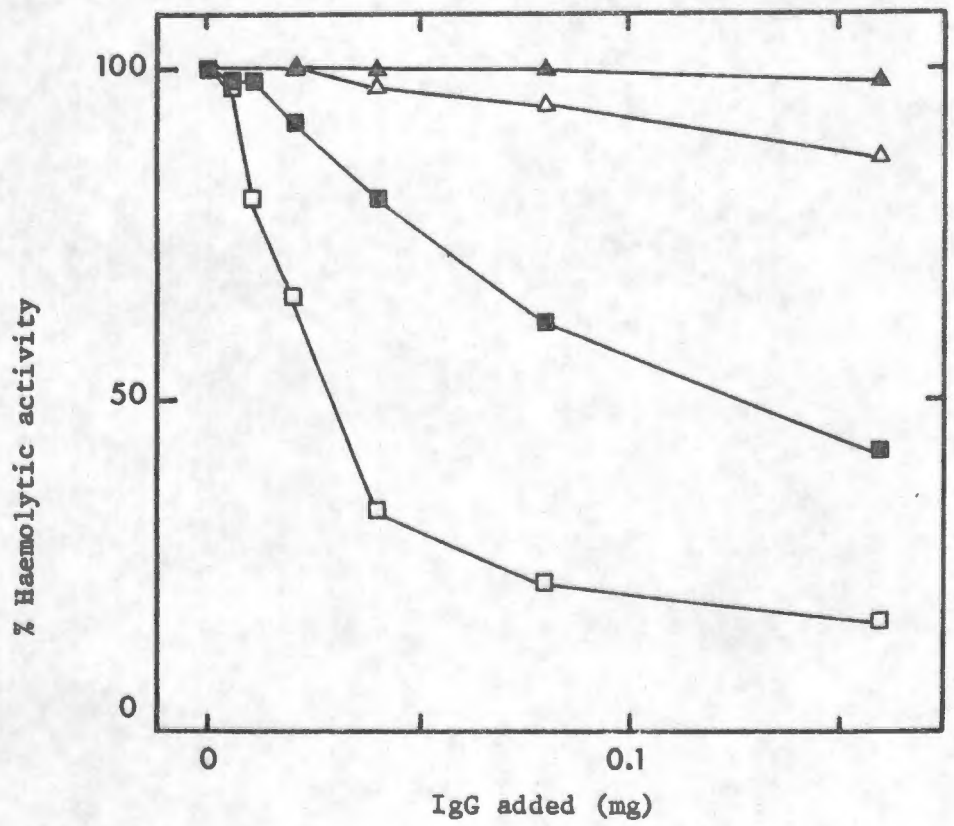


Figure 4.7

prepared from control rabbit serum.

Thirdly, the relationship between the three toxin fractions was also shown using the immunoprecipitation technique of Ouchterlony (1949). As may be seen in Figure 4.9 the three fractions all possessed common antigenic determinants. The spur at the junction of the precipitin line for peak III with both of the other peaks indicated that peak III lacked determinants present on the other two molecules while peaks I and II show a reaction of identity.

The first phosphocellulose peak was chosen as the primary medium for further attempts to purify the toxin. This decision was based on the fact that SDS-gel electrophoresis showed peak I to contain the least contaminating bands. It was subsequently discovered that haemolytic activity in peak III was underestimated by the blood-agarose gel assay. This assay proved to be tenfold less sensitive to activity in peak III than it was to activity in peak I. This difference in sensitivity was probably due to a restricted rate of diffusion of the peak III material in the blood-agarose. Measurements of the activity of the three peaks by the mouse lethality and the test-tube assays were consistent.

Comments:

(i) The performance of the phosphocellulose chromatography system depended very critically on the buffer pH and thorough equilibration of the resin.

(ii) The performance of the chromatography system and overall yield of toxin from this step were considerably improved by the incorporation of 10% glycerol in the buffers.

(iii) After the phosphocellulose step the toxin tended to precipitate irreversibly when frozen. This was overcome by adjusting the concentration of glycerol in solutions to be frozen to 50%.

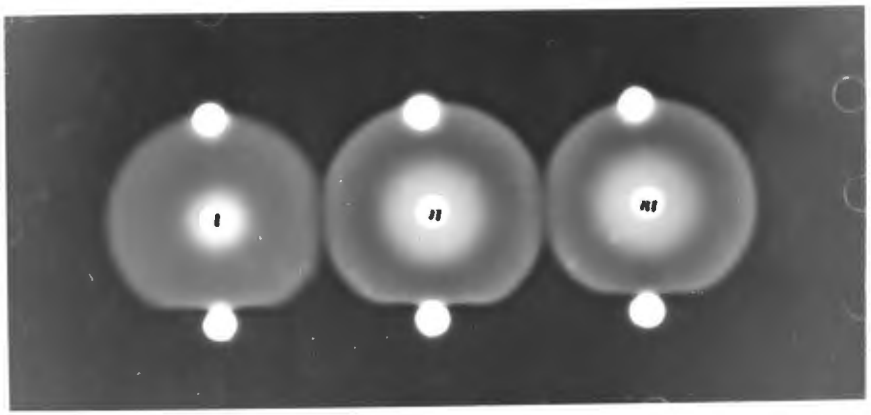


Figure 4.8 Inhibition of toxin haemolytic activity by antibody in blood-agarose gels.

Blood-agarose gels were prepared as for the blood-agarose gel assay. Aliquote of 5 µl of toxin solutions from phosphocellulose peaks I, II or III were injected into wells in the gel and adjacent wells received 5 µl of 1,6 mg/ml IgG recovered from antiserum to the toxin, or of IgG recovered from irrelevant serum. The gels were incubated overnight at 4°C in moisture chambers followed by 60 minutes at 37°C.

The zone of haemolysis round each toxin well was flattened on the side facing the immune IgG well but not on the side facing the irrelevant IgG. (irrelevant IgG above, immune IgG below.)

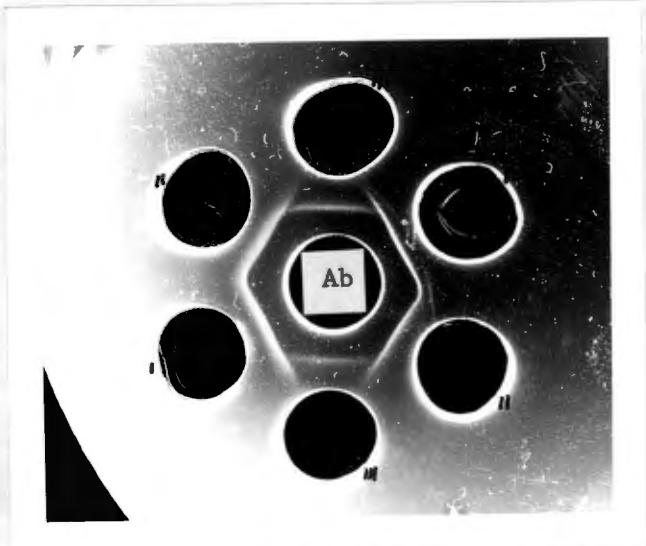


Figure 4.9 Double radial immunodiffusion of the antibody to toxin isolated from phosphocellulose peak I and toxin from the three phosphocellulose peaks.

(iv) When the peaks from the phosphocellulose columns were rechromatographed under the same condition they emerged from the column at the same ionic strength as in the first chromatography. There was no evidence of conversion of toxin from one form to another.

Chromatography on DEAE cellulose (Figure 4.10)

The peak I solution was dialysed into 0,01M Tris-HCl, 10% glycerol, pH 8,2 and concentrated to 2 mg/ml on an Amicon XM50 membrane. A 3ml sample containing 6 mg protein was chromatographed on a 1,25 x 20 cm column of DE 52 DEAE cellulose (Whatman) in 0,01M Tris-HCl, 10% glycerol pH 8,2 at 4°C. The column was pumped at 10ml per hour and 2 ml fractions collected. The effluent was monitored by an LKB Uvicord and the fractions containing the protein peak (Figure 4.10) were pooled and concentrated on an Amicon XM50 membrane to yield a total of 2,7mg protein and $3,4 \times 10^4$ HU of toxin activity.

The protein recovered in this way from peak I migrated as a single band on SDS-gel electrophoresis (Figure 4.10). Attempts to isolate the toxic components in peaks II and III using DEAE cellulose chromatography or rechromatography on phosphocellulose were not successful. Further studies were confined to the material isolated from peak I.

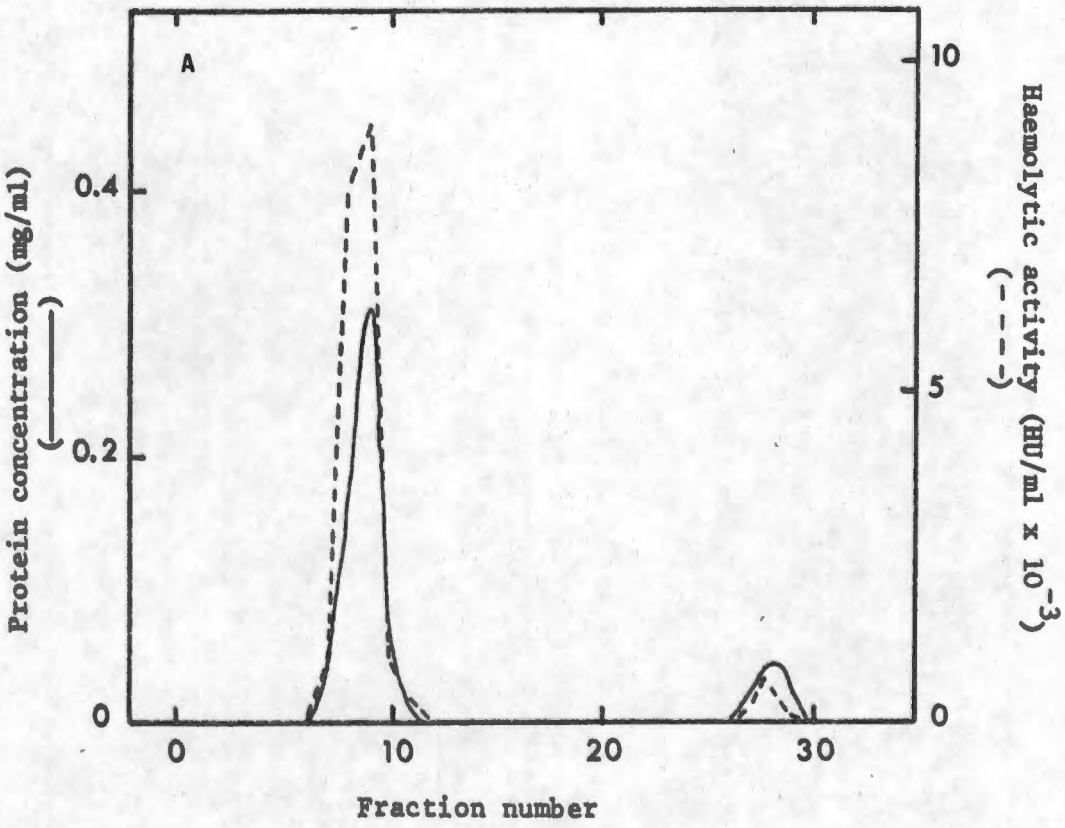
In an attempt to compare the CNBr-cleavage peptides from the 62 000 M.W. proteins from the three phosphocellulose peaks and the 66 000 M.W. protein from peak II these bands were cut from an SDS-gel and incubated with CNBr by an adaptation of the methods of Gross (1967) and Witkop (1968). The peptides were then electrophoresed on a 6-15% polyacrylamide gel in a buffer system adapted from Neville (1967) but modified to contain 2M urea and 0,1% SDS. The fixed and stained gels showed 25 or more bands in each track. The toxin in peak I was found to have only 6 methionine residues (Table 5.3) and I assumed that the excessive number of peptide bands was due to incomplete cleavage

Figure 4.10 Ion exchange chromatography on DEAE cellulose

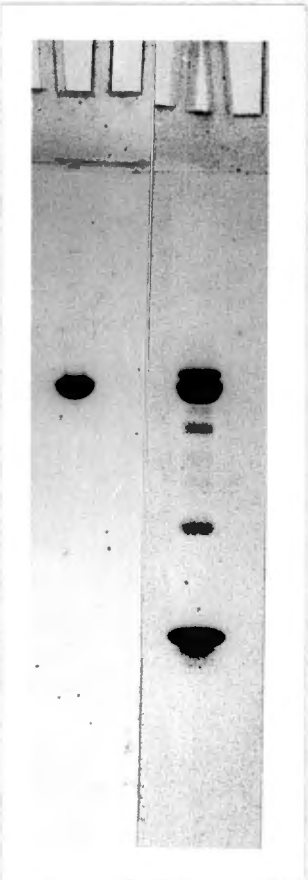
A. Toxin recovered from the first phosphocellulose peak was dialysed into 0,01M Tris-HCl, 10% glycerol, pH 8,2. A sample containing 6 mg protein was chromatographed at 10 ml/hr on a 1,25 x 20 cm column of DEAE cellulose equilibrated in the same buffer. The column was developed with the equilibration buffer and 2 ml fractions collected. When the effluent protein concentration, monitored at 280 nm, returned to baseline the retained protein was eluted from the column with 1M NaCl in 0,01M Tris-HCl, 10% glycerol, pH 8,20. Fractions were assayed for haemolytic activity using the blood-agarose assay. Protein (———); haemolytic activity (– – –).

B. Protein samples (10 µg) from each of the two protein peaks were electrophoresed on a 6-15% SDS-polyacrylamide gel by the method of Maizel (1971). The protein in the first protein peak was shown to be electrophoretically homogeneous.

Figure 4.10


A
Protein concentration (mg/ml)
(——)
Haemolytic activity (HU/ml x 10^{-3})
(- - - -)
0.4
0.2
0
10
5
0
0
10
20
30
Fraction number

B



resulting from oxidation of some of the methionine residues. My attempts to overcome this oxidation problem by pre-electrophoresis with reducing agents were not successful. Similar problems have been reported by other workers (Alfageme et al, 1974; Nikodem, 1979).

To confirm the identity of the material isolated from peak I as the toxin molecule a blood agarose underlay electrophoresis technique was used. This was based on the observation by Granelli-Piperno and Reich (1978) that activity could be restored to biological molecules after SDS gel electrophoresis by washing with a non-ionic detergent.

Samples of pure toxin or albumin were made to 1% SDS and aliquots containing 30 µg protein were electrophoresed on a 7,5% SDS-polyacrylamide gel until the phenol-red tracking dye reached the bottom of the gel. An 0,5 cm strip was removed from the bottom of the gel, then it was agitated in 0,01M Tris-HCl, 0,1% Triton X-100, 10% glycerol, pH 8,0 for 20 minutes, water for 20 minutes, and finally VBS for 10 minutes. The washed gel was laid on a blood-agarose gel containing 2% washed guinea pig erythrocytes in VBS and 1% agarose. The gel assembly was incubated overnight at $4^{o}C$ and then for 60 minutes at $37^{o}C$. A duplicate acrylamide gel was fixed and stained in 30% methanol, 10% acetic acid, 0,05% Coomassie brilliant blue R250. The photographs of the blood-agarose underlay and the stained gel in figure 4.11 show that the cells were lysed in a zone corresponding to the position of the toxin band while there was no haemolysis in the region of the albumin band.

Comments:

(i) Guinea pig erythrocytes were used in place of SRBC in the blood-agarose underlay because of their greater sensitivity to haemolysis by the toxin.

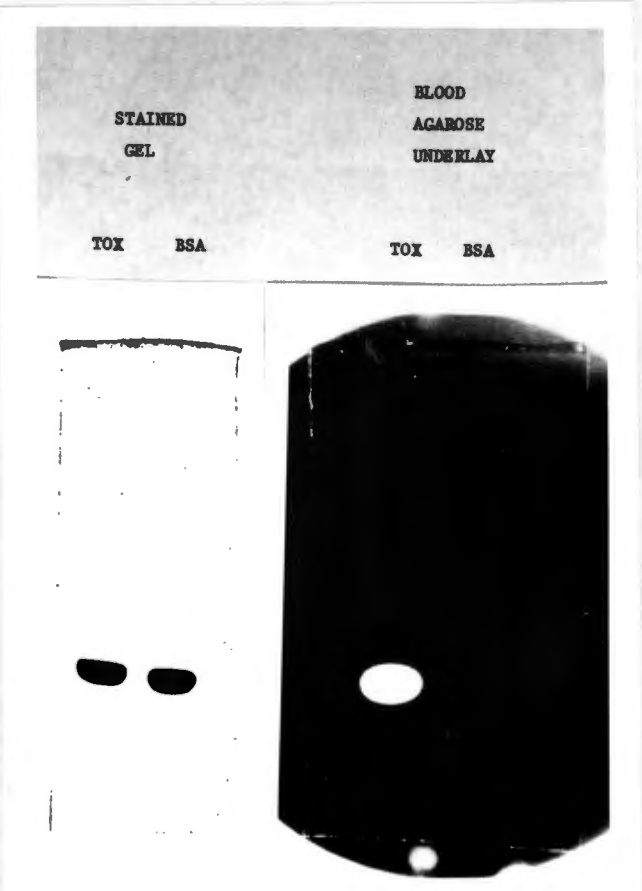


Figure 4.11 Haemolysis of a blood-agarose underlay by purified toxin after SDS-gel electrophoresis

Samples of pure toxin or albumin were made to 1% SDS and 30 µg electrophoresed on a 7,5% SDS-polyacrylamide gel until the phenol red tracking dye reached the bottom of the gel. An 0,5 cm strip was cut from the bottom of the gel which was then agitated in 0,01M Tris-HCl, 0,1% Triton X-100, 10% glycerol, pH 8,0 for 20 minutes, water for 20 minutes, and finally VBS for 10 minutes. The washed gel was laid on a blood-agarose gel containing 2% washed guinea pig erythrocytes in VBS and 1% agarose and incubated in a moisture chamber overnight at 4°C and then for 60 minutes at 37°C. A duplicate acrylamide gel was fixed and stained in 30% methanol 10% acetic acid, 0,05% Coomassie brilliant blue. The washed gel on the blood-agarose underlay was photographed with dark-field illumination.

The erythrocytes in the blood-agarose gel were lysed in the region corresponding to the toxin band, but not in the region corresponding to the albumin band. Toxin: T; albumin: A.

(ii) In the course of developing the purification protocol described above a wide range of techniques were tried without success. These included: molecular sieve chromatography on several mesh sizes of Sephadex and Biogel; ion exchange chromatography at various pH values on SP Sephadex, CM cellulose, and QAE Sephadex; affinity chromatography on Con A linked sepharose; preparative iso-electric focussing; and preparative scale SDS polyacrylamide gel electrophoresis. Only the preparative SDS electrophoresis was effective in isolating the toxin molecule but the technique was abandoned because the SDS could not be satisfactorily removed from the toxin after isolation.

Conclusion

The recovery of protein and toxin activity throughout the purification procedure is summarised in table 4.1. The poor recovery of activity attests to the instability of the isolated toxin and the tendency of non-specific losses to accumulate with the removal of protecting proteins.

The toxin appeared to exist in at least three molecular forms as judged by consistent behaviour on phosphocellulose chromatography. The immunochemical cross-reactivity of the material in the three peaks suggests that they are derived from a common precursor molecule or that the 66 000 M.W. material in peak II is a precursor for the 62 000 M.W. material in peaks I and III resulting from proteolytic cleavage of the parent molecule.

As described in the next chapter the 62 000 and 15 000 M.W. bands in peak I migrated as a monodisperse system in sedimentation

TABLE 4.1

RECOVERY OF PROTEIN, HAEMOLYTIC ACTIVITY, AND LETHALITY AT EACH STAGE OF PURIFICATION

The figures are those for a typical purification run starting with 50g pupae. Protein concentration was determined by the method of Lowry et al (1951); haemolytic activity by the tube assay; and lethality by the mouse assay.

	Protein mg	H.U ($\times 10^{-6}$)	% Recovery (HU)	Specific activity (HU/mg $\times 10^{-3}$)	Purification factor (HU)	MLD ($\times 10^{-6}$)	% Recovery (MLD)	Specific activity (MLD/mg $\times 10^{-3}$)	Purification factor (MLD)
Homogenate	6630	13,4	100	2,02	1	48,0	100	7,24	1
25%-85% $(NH_4)_2SO_4$	1820	11,5	86	6,32	3,1	27,2	57	14,9	2,1
HTP	800	22,2	166	27,8	13,8	50,22	105	62,8	8,7
Phospho-cellulose 1	73	0,81	6	11,1	5,5	1,92	4	26,3	3,6
2	54	0,18	1,3	3,4	1,7	1,44	3	26,7	3,7
3	75	2,62	19,5	34,9	17,3	2,40	5	32,0	4,42
DEAE cellulose	33	0,41	3	12,4	6,1	1,44	3	43,6	6

velocity ultracentrifugation. Equilibrium ultracentrifugation of this material yielded a molecular weight estimate of 76 000, corresponding to a 1:1 complex formed by the two molecules. This coacervate form of the toxin probably explains the extreme difficulty I experienced in the final stages of isolating the toxin using non-dissociating systems. Incorporation of dissociating factors such as urea or SDS into the chromatography systems resulted in irreversible loss of activity by the toxin.

CHAPTER FIVE

THE BIOCHEMICAL CHARACTERISATION OF THE TOXIN MOLECULE

In this chapter I report on some of the physical and chemical characteristics of the toxin molecule isolated from peak I of the phosphocellulose columns (Figure 4.4).

SDS polyacrylamide gel electrophoresis

The toxin, together with standard globular proteins of known molecular weight, was electrophoresed on a 6-15% polyacrylamide gel in 0,1% SDS using the method of Maizel (1971). Figure 5.1 shows the rf values for the protein bands plotted as a function of the logarithm of their molecular weights. By interpolation the toxin was estimated to have a molecular weight of 60 700 $\pm$ 3700 daltons based on 9 determinations.

Comment

The electrophoretic mobility of the toxin was not affected by incubation with 5% β-mercaptoethanol nor by reduction and acetylation by the method of Waxdal et al (1968).

Analytical ultracentrifugation.

Centrifugation was carried out in a Beckman model E analytic ultracentrifuge fitted with schlieren and interference optics and using an AN-D rotor. Temperature was controlled at 20,0°C throughout all spins. Images were photographed on Kodak metallographic plates (catalogue 156 7427) for schlieren spins and on Kodak spectroscopic type IIG plates (catalogue 166 2196) for equilibrium spins. The

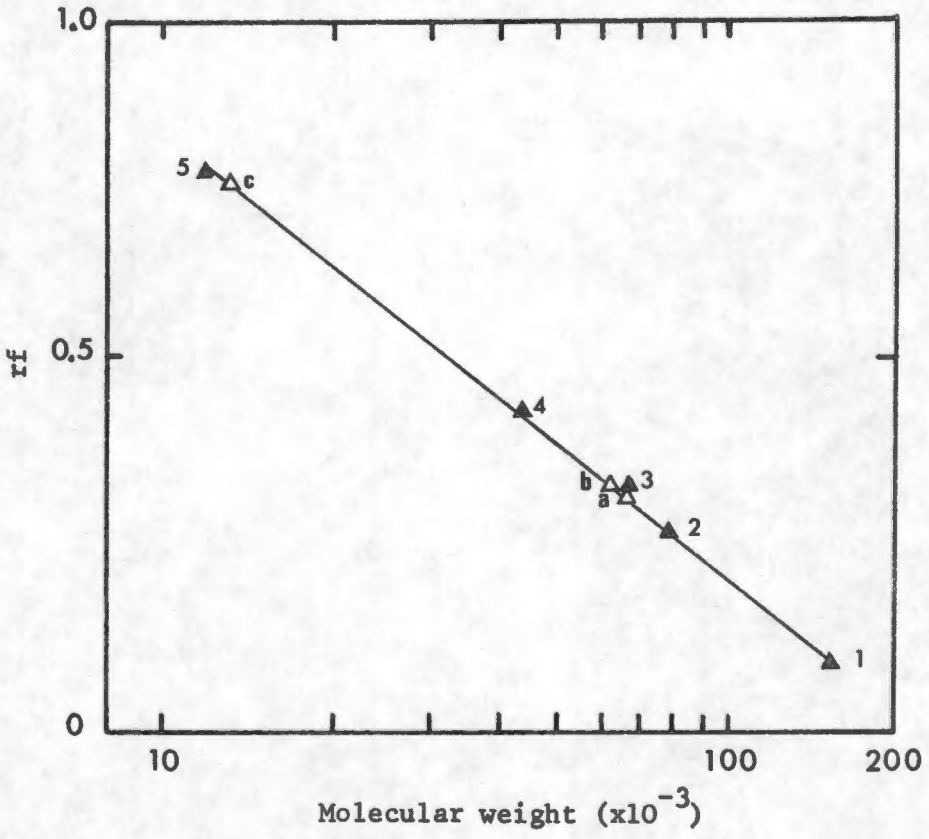


Figure 5.1 Estimation of the molecular weight of the toxin by electrophoresis on SDS-polyacrylamide gels.

The purified toxin was electrophoresed on a 6-15% polyacrylamide gel containing 0,1% SDS together with a series of marker proteins in adjacent tracks. The graph is a plot of the rf values (relative to the tracking dye) of proteins as a function of the logarithms of their molecular weights. The following marker proteins were used: (1) IgG, 150 000; (2) lactoperoxidase, 78 000; (3) BSA, 67 000; (4) ovalbumin, 43 000; (5) cytochrome C, 11 700.

By interpolation the toxin was estimated to have a molecular weight of 62 000 (b); the upper band in peak II a molecular weight of 66 000 (a); and the low molecular weight contaminant in peak I a molecular weight of 15 000 (c).

images were measured on a Nikon profile projector at a magnification of 20x using the micrometer facilities of the instrument.

Sedimentation velocity measurements were carried out by the method of Chervenka (1969). Pure toxin was centrifuged at 2 mg/ml in 0,10M sodium acetate pH 4,8 or in 0,10M ethanolamine-HCl pH 9,5. Figure 5.2 shows the schlieren peak generated by the toxin after 32 minutes at 60 000 rpm and in table 5.1 and figure 5.3 the measurements derived from a schlieren run and the calculation of the sedimentation coefficient are shown.

Sedimentation velocity experiments were also carried out at pH 4,8 on semi-purified toxin from phosphocellulose peak I. The sedimentation coefficient for the pure toxin was 3,9 S both at pH 4,8 and at pH 9,5. The semi-purified material from phosphocellulose peak I migrated as a single peak with a sedimentation coefficient of 5,0 S.

For equilibrium measurements the meniscus depletion technique of Yphantis (1964) was used. A solution of 1 mg/ml pure toxin in 0,10M sodium acetate 0,10M NaCl pH 4,8 was centrifuged at 30 000 rpm for 24 hours. The image of the interference fringes was photographed at 18 hours and at 24 hours. Table 5.2 shows the measurements of the fringe displacement and in figure 5.4 the value of $\ln \overline{\Delta y}$ is plotted as a function of r^2. The calculation of the molecular weight of the toxin is based on the gradient of this line. Analysis of 7 similar spins at pH 4,8 or in 0,10M ethanolamine-HCl pH 9,5 gave a calculated mean molecular weight of 62 100 $\pm$ 2800 daltons.

Equilibrium centrifugation of the semi-purified material from phosphocellulose peak I at pH 4,8 yielded a molecular weight estimate of 77 600 daltons.

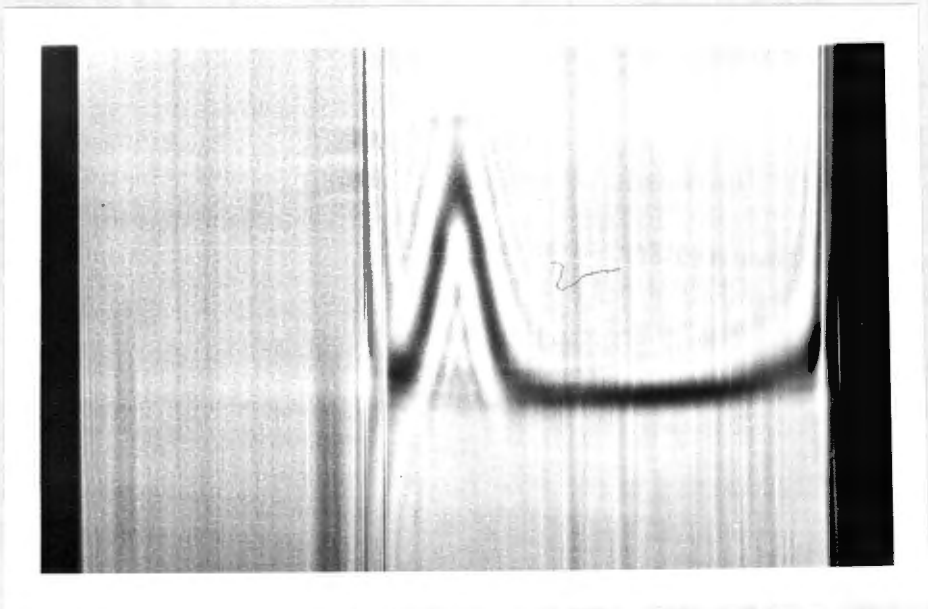


Figure 5.2 Analytical ultracentrifugation of pure toxin (2 mg/ml) dissolved in 0,1 M sodium acetate pH 4,8. The photograph shows the schlieren peak observed after 32 mins at 60 000 rpm and 20°C.

TABLE 5.1

THE SEDIMENTATION VELOCITY OF THE TOXIN

Pure toxin (2mg/ml) isolated from phosphocellulose peak I was dialysed against 0,10 M sodium acetate pH 4,8 and centrifuged at 60 000 rpm and 20°C in a Beckman Model E analytical ultracentrifuge using schlieren optics. The table shows the logarithm of the distance in centimetres from the centre of rotation to the boundary position of the toxin in the cell calculated from photographs taken at 8 minute intervals (Figure 5.2).

Figure 5.3 shows a plot of log x against elapsed time. The gradient of the straight line so obtained was $3,946 \times 10^{-4}$. After correction for buffer density and viscosity (Chervenka, 1969) a sedimentation coefficient ($S_{20,w}$) of 3,9 was obtained.

Time (minutes)	$\log_{10} x$
0	1,1321
7,78	1,1351
15,78	1,1382
23,78	1,1412
31,80	1,1445
39,82	1,1477
47,82	1,1506
55,83	1,1539
63,87	1,1571
71,87	1,1605
79,88	1,1636

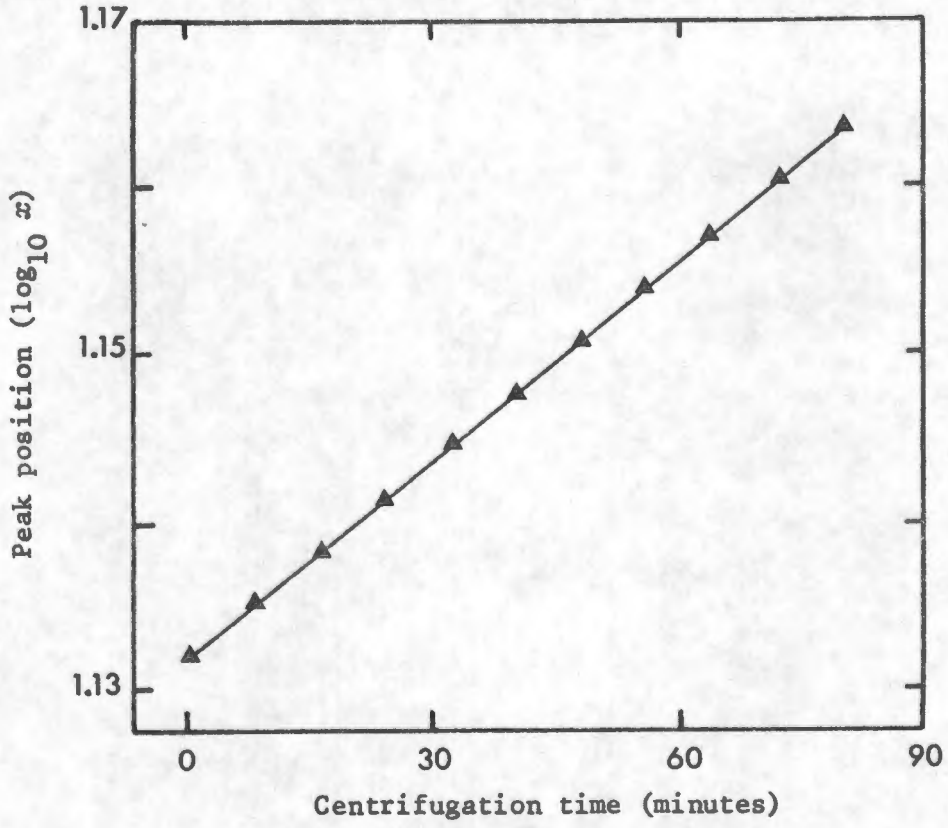


Figure 5.3 Estimation of the sedimentation coefficient of the toxin

A solution of 2 mg/ml pure toxin was centrifuged at 60 000 rpm in a Beckman Model E analytical ultracentrifuge using schlieren optics. The schlieren peak was photographed at 8 minute intervals and the radial position of the peak calculated (designated x). The graph shows a plot of $\log_{10} x$ as a function of time. Zero time was taken as the rotor speed passed through 40 000 rpm.

From the gradient of the line ($3{,}9460 \times 10^{-4}$) a value of $S_{20,w} = 3{,}9$ was calculated for the toxin.

TABLE 5.2

SEDIMENTATION EQUILIBRIUM CENTRIFUGATION OF THE TOXIN

A solution of 1 mg/ml pure toxin in 0,10M sodium acetate 0,10M NaCl pH 4,8 was centrifuged by the method of Yphantis (1964) in a Beckman Model E analytical ultracentrifuge using a double sector cell. The reference soltuion was 0,10M sodium acetate 0,10M NaCl pH 4,8. After 18 hours at 30 000 rpm the cell was photographed using interference optics and the protein concentration gradient in the cell estimated by measuring the lateral displacement of the interference fringes at increasing distances from the centre of rotation.

The table shows the displacement in microns (Δy) for five fringes at increasing distances (x) from the meniscus in cm. In figure 5.4 the logarithm of the mean displacement for 5 fringes ($\ln\Delta y$) is plotted against r^2 calculated from x. From the gradient of the straight line so obtained (3,1753) a molecular weight of 60 000 daltons was calculated for the toxin.

x	Δy	Δy_2	Δy_3	Δy_4	Δy_5	r^2	$\ln\Delta y$
,3000	10379	10524	10659	10813	10952		
,3500	10270	10408	10544	10698	10837	37,0355	5,1930
,3600	10226	10382	10516	10671	10798	37,0926	5,3604
,3700	10200	10337	10463	10623	10757	37,1498	5,5389
,3800	10141	10279	10430	10572	10715	37,2070	5,7170
,3900	10078	10227	10368	10522	10653	37,2643	5,8911
,4000	10014	10152	10292	10441	10582	37,3216	6,0758
,4100	9917	10058	10200	10358	10499	37,3789	6,2634
,4200	9801	9954	10091	10246	10376	37,4363	6,4580
,4300	9684	9832	9977	10116	10259	37,4937	6,6304
,4400	9545	9695	9823	9982	10111	37,5512	6,8026
,4500	9309	9479	9611	9794	9922	37,6087	7,0107
,4600	9185	9282	9382	9528	9677	37,6663	7,1858

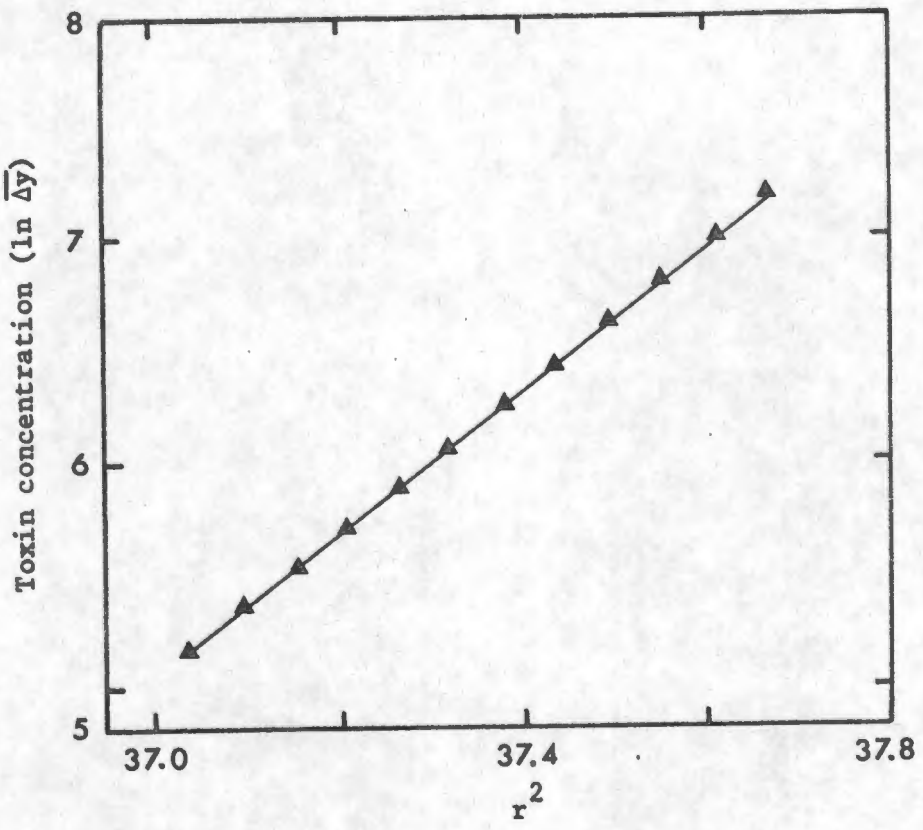


Figure 5.4 Estimation of the molecular weight of the toxin by equilibrium centrifugation

A solution of 1 mg/ml pure toxin in 0,10M sodium acetate, 0,10M NaCl, pH 4,8 was centrifuged at 30 000 rpm in a Beckman Model E analytical ultracentrifuge using interference optics. The interference pattern was photographed after 18 hours and the fringe displacement (Δy) measured at 100 μ increments in radial position (r) for 5 fringes (Table 5.1). The graph shows a plot of ln $\overline{\Delta y}$ as a function of r^2.

From the gradient of the line (3,175) an estimated molecular weight of 60 000 was obtained for the toxin.

Comment

(i) A protein concentration of 5 mg/ml in the sedimentation velocity spins would have yielded clearer schlieren peaks. However the pure toxin was inclined to come out of solution at concentrations much above 2 mg/ml unless the buffer contained 10% glycerol.

(ii) Buffer densities and viscosities were calculated from tables (Weast 1974).

(iii) The camera was focussed on a plane 2/3 from the base of the cell (Svensson, 1954).

(iv) The partial specific volume of the toxin was calculated from its amino acid composition (Table 5.3) using the method of van Holde (1975) and Cohn and Edsall (1943).

(v) From photographs taken at intervals during the course of an equilibrium spin as described above it was established that concentration equilibrium was established after 7 hours. Molecular weights calculated from photographs taken at 18 hours and 24 hours did not differ.

(vi) The semi-purified protein from phosphocellulose peak I showed two major bands on SDS gel electrophoresis corresponding to molecular weights of 60 000 and 15 000 daltons (Figure 5.1). This material migrated as a single peak in the sedimentation velocity spins indicating a homogeneous dispersion of a single species of macromolecules. The equilibrium spins on this material yielded a molecular weight estimate of 77 600, which corresponds closely to the expected mass of a 1:1 complex of the 60 000 and the 15 000 dalton components found by SDS electrophoresis.

Amino acid composition

A Beckman 120C amino acid analyser was used to analyse acid hydrolysates of toxin samples that had been treated with performic acid to oxidise methionine and cysteine residues. In general procedures followed were those of Spackman et al (1958), Moore (1963), and Toepfer (1965).

For each analysis pure toxin (usually approximately 50 nanomoles) was freeze-dried in a small round-bottomed flask. Protein was determined gravimetrically and by the method of Lowry et al (1951). The protein was taken up in 2 ml of cold performic acid and stored on ice. After 4 hours 0,3 ml 48% hydrobromic acid was added and the solution taken to dryness in a rotary evaporator. The residue was taken up in 3,10 ml constant boiling HCl and 1,00 ml aliquots dispensed into 3 glass hydrolysis vials. The vials were flushed with N_2 and the solution degassed then frozen in a dry ice/acetone bath. The vials were evacuated to 50 μ Hg, sealed, and then incubated for 24, 48 or 72 hrs at 110($\pm$ 1)°C.

After a brief low-speed centrifugation the vials were opened and accurately measured aliquots of the internal standards α-amino β-guanidinoproprionic acid hydrochloride (AGPA) and nor-leucine were added. The hydrolysates were evaporated to dryness, taken up in 0,750 ml of amino acid analyser pH 3,28 buffer, and 0,250 ml loaded on each of the two columns (AA15 acidics and PA35 basics) of a Beckman 120C Amino Acid Analyser. The development of the columns and analysis of the results were as described by Toepfer (1965). The analyser was calibrated with Beckman or BDH standard amino acid solutions to which were added accurately measured quantities of the internal standards AGPA and nor-leucine.

To measure phenyl-alanine and tyrosine, which are partially degraded by the performic acid oxidation procedure described above, duplicate hydrolysates were analysed without the oxidation step.

To measure tryptophan, which is degraded by acid hydrolysis, the method of Barman and Koshland (1967) was used. The protein was reacted with 2-hydroxy-5-nitrobenzyl bromide (HNB), precipitated with TCA and washed thoroughly to remove unreacted HNB. The tryptophan-HNB product was estimated by measuring the optical density at 410 nm (Σ=18 000). To relate the tryptophan estimate to the other amino acids a measured volume of the HNB reacted protein solution was hydrolysed and analysed on the PA35 basics column of the analyser.

The calculation of the amino acid composition of the toxin, based on the 24 hour hydrolysate, is shown in table 5.3. Similar calculations were done for the 48 and 72 hour hydrolysates. Table 5.4 shows the results of those calculations and the values finally assigned for each amino acid.

Comment

(i) There is partial conversion of methionine to methionine sulphone or sulphoxide and of cystein to cysteic acid during the acid hydrolysis procedure. To overcome the inaccuracy this introduced the performic acid oxidation technique of Moore (1963) was used. This ensures that all methionine is converted to methionine sulphoxide and all cystein to cysteic acid. The two amino acids are then measured as their oxidation products.

(ii) AGPA elutes between ammonia and arginine on the PA35 basics column and nor-leucine elutes between leucine and tyrosine on the AA15 acidics column.

(iii) A number of attempts were made to detect a carbohydrate

TABLE 5.3

AMINO ACID ANALYSIS OF THE 24 HOUR TOXIN HYDROLYSATE

A sample of 0,688 mg of pure toxin (0,01148 µM) was hydrolysed for 24 hours as described in the text. The hydrolysed amino acids were dissolved in the pH 3,28 buffer for the Beckman Model 120C amino acid analyser (Toepfer, 1965) and aliquots corresponding to 0,00358 µM protein were chromatographed on the AA15 acidics column and on the PA35 basics column. The peak areas were integrated and the amino acid concentrations were calculated as described by Toepfer (1965). The estimate of the number of residues of each amino acid present in the parent molecule was based on these calculated concentrations.

Residue	Peak Height x width	Colour constant	µM	Number residues
TRP	,881	73,42	,012	3,4
LYS	13,928	112,32	,1240	34,7
HIS	2,064	95,56	,0216	6,0
NH_3	26,434	92,49	,2858	
AGPA	10,896	87,38	,1247	
ARG	5,698	96,41	,0591	16,5
ASP	21,699	105,90	,2049	57,3
THR	6,555	105,73	,620	17,3
SER	12,702	109,50	,1160	32,4
GLU	16,319	107,15	,1523	42,6
PRO	2,017	30,47	,0662	18,5
GLY	13,976	105,16	,1329	37,1
ALA	12,903	108,89	,1185	33,1
CYS				
VAL	13,426	107,32	,1251	35,0
MET	2,608	113,89	,0229	6,4
ILE	12,800	119,29	,1073	30,0
LEU	14,564	115,68	,1259	35,2
u-LEU	14,571	116,66	,1249	
TYR	13,450	113,79	,1182	33,0
PHE	10,228	112,64	,0908	25,4

TABLE 5.4

THE AMINO ACID COMPOSITION OF THE TOXIN

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

Residue	hydrolysis time			assigned value
	24	48	72	
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 sulphoxide

component in the purified toxin molecule. These included periodic acid Schiff staining of toxin after gel electrophoresis, estimation of total hexose using the anthrone procedure described by Kabat and Mayer (1971), and incubating gel electrophoresed toxin with an ^{125}I-labelled lectin from *Canavalia virosa* which has an affinity for mannose and glucose. I was unable to detect a carbohydrate component in the toxin molecule by any of these methods.

(iv) Examination of table 5.4 reveals that the hydrophobic amino acids (TRP, VAL, ILE, LEU, TYR, PHE) constitute 36% of the total number of residues. The albumin molecule, which exhibits extensive hydrophobic interaction with many different compounds, has only 27% of its amino acid residues in this group. This confirms observations made while working with the toxin that it had a strong tendency to absorb to surfaces of test tubes, syringes, and pipettes. Hydrophobic interaction with other components of the pupal extract would also explain the difficulty experienced in isolating the toxin molecule. The improved solubility and stability of the toxin in buffers containing a proportion of glycerol is presumably due to the interaction between hydrophobic regions in the toxin molecule and the relatively non-polar glycerol molecule.

(v) No unusual amino acids such as those encountered by Rosenberg and Ennor (1959) in their study on *D. lombricine* were found in the toxin molecule.

Identification of the N-terminal amino acid

The "dansyl chloride" procedure of Gray (1967) was used with modifications introduced by Gross et al (1969) and Weiner et al (1972). To 0,5 mg toxin was added 500 µl 8M urea, 150 µl 0,4M sodium phosphate pH 8,2, 250 µl dimethyl formamide, and 100 µl 20 mg/ml 1-dimethylamino-naphthalene-5-sulphonyl chloride (dansyl chloride) in acetone. The solution was vortexed immediately upon the addition of the dansyl chloride. After 60 minutes at room temperature the protein was precipitated by the addition of 10 ml 10% trichloracetic acid, centrifuged, and washed twice with 1N HCl. The pellet was taken up in 5,7 N HCl and hydrolysed under vacuum at 110°C for 16 hours in a glass vial. The vial was then opened, the HCl taken off under vacuum and the hydrolysate taken up in 100 µl pyridine.

The hydrolysate was spotted on a 5 cm x 5 cm polyamide thin layer chromatography plate (Schleiger and Schüll, F 1700 TLC plates, catalogue 356 000) and a cocktail of standard dansylated amino acids spotted on the reverse side of the plate. The plate was developed, in the first instance, with 1,5% formic acid in water; then at right angles to the first dimension, with benzene:acetic acid 7:1; ethyl-acetate:acetic acid:methanol 20:1:1 and 0,05M Na_3PO_4 in 25% aqueous ethanol.

The hydrolysate generated two fluorescent spots whose chromatographic behaviour corresponded to ε-dansylated lysine and dansyl glutamic acid. It was concluded that the N-terminal amino acid was glutamic acid or glutamine.

Isoelectric point

An aliquot of purified toxin solution containing 0,5 mg protein was analysed by electrofocussing in an LKB 110 column using

1% pH 9-11 ampholines at an energy dissipation of approximately 2 watts. After 36 hours the column was bled collecting 2,5 ml fractions. The pH of each fraction was read immediately upon collection and toxin activity was assayed by means of a blood agarose gel.

The elution profile showed a single clearly defined peak of activity corresponding to a pH of 9,45 (figure 5.5).

Comment

After 36 hours the resistance of the column was still increasing, indicating that the system had not yet stabilised at equilibrium. Focussing was stopped at this point because a band of flocculent protein could be seen at a point in the column corresponding to the subsequently determined activity peak.

SUMMARY

The data presented in this chapter indicate that the major toxic component present in the pupae of *D. nigro-ornata* is a hydrophobic protein with a molecular mass of 62 000 daltons and an isoelectric point of 9,5.

Only one N-terminal amino acid (glx) could be detected and the apparent molecular weight of the toxin was unaffected by reduction and alkylation, indicating that it consists of a single polypeptide chain. Analysis of the amino acid composition showed a high proportion of hydrophobic residues which accounted for the tendency of the toxin to absorb onto the surfaces of containers.

The phospho-cellulose chromatography described in chapter 4 indicated that, in the pupae, at least 3 molecular forms of the toxin might exist, each of which contains a component immunochemically identical to the 60 000 molecular weight protein isolated from phosphocellulose

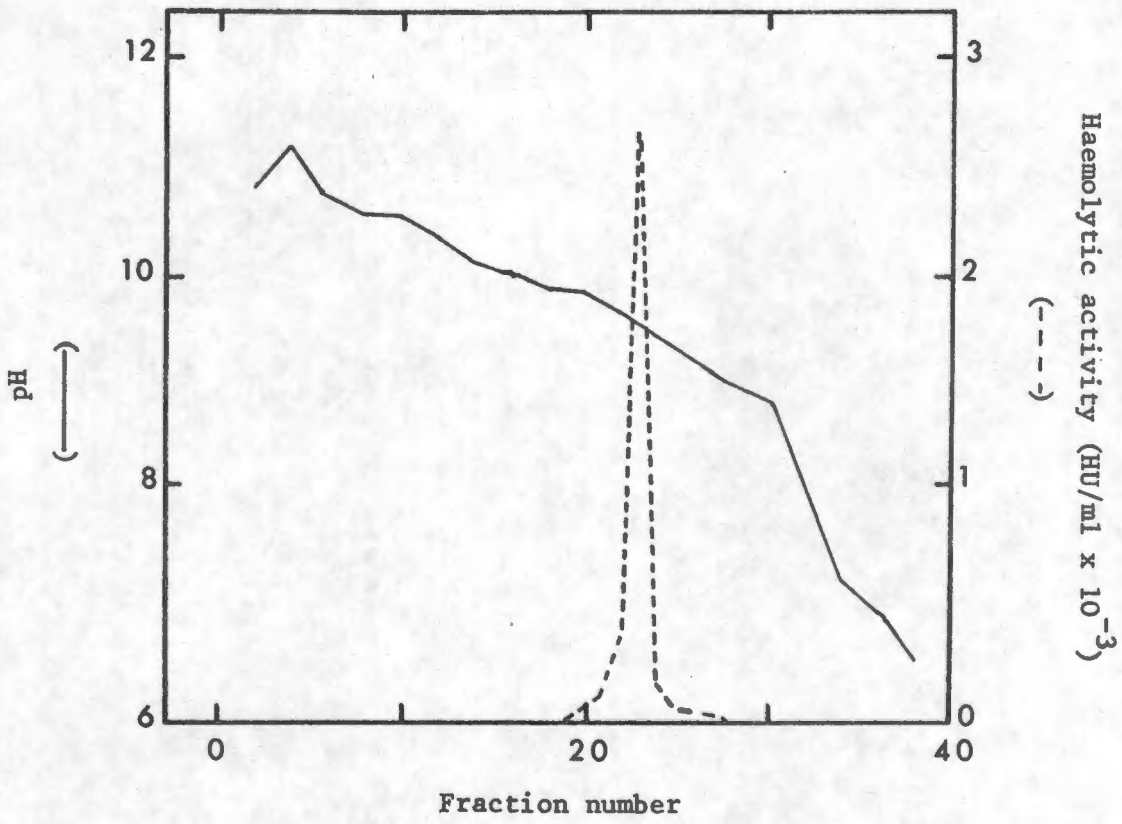


Figure 5.5 Determination of the isoelectric point of the pure toxin

A sample of 0,5 mg pure toxin was focussed in an LKB 110 electrofocussing column using 1% pH 9-11 ampholines and an energy dissipation of approximately 2 watts. After 36 hours the column was bled and 2,5 ml fractions were collected. For each fraction the pH was measured and haemolytic activity was determined using the blood-agarose gel assay.

The figure shows a clear peak of activity corresponding to a pI of 9,45.

peak I. Minor post-translational modification of the parent toxin molecule may have been responsible for these findings. Such modification could alter the molecular mass, charge, or tendency to form coacervates with other proteins and hence its behaviour on cation exchange chromatography. This speculative assumption could not be verified by formal sequence analysis owing to lack of resources and adequate material.

CHAPTER SIX

THE TOXICOLOGY OF DIAMPHOTOXIN

I observed at an early stage, that *Diamphidia nigro-ornata* pupae contained material that was intensely haemolytic *in vivo* and *in vitro*. The haemolytic effect formed the basis for the assay that was used to monitor purification of the toxin, and co-purified with the lethal effect of the toxin for mice. There was, therefore, reason to believe that haemolysis and lethality resided in the same molecule and that elucidation of the manner in which the purified toxin caused lysis of the red cell would contribute to a more general understanding of its toxic action on other cells. For these reasons and for the obvious reasons of ease and convenience, I used sheep erythrocytes as targets for most of the experiments performed to define the cytolytic action of diamphotoxin in quantitative and mechanistic terms.

In the initial stages of the work, I studied the haemolytic reaction only to the extent that was necessary for the development of a reliable assay to monitor the purification procedure. The tube assay (p. 27), in which haemoglobin released by damaged erythrocytes was determined by spectrophotometry of the supernatant fluid after centrifugation, gave a useful measure of overall haemolysis but was limited in the extent to which it could be used to study the kinetics of cell lysis or the events that preceded the release of haemoglobin. For these purposes additional investigative procedures were developed that are described in detail in the Methods section that follows.

The results that I have obtained are consistent with the notion that diamphotoxin, by virtue of its amphipathic character, is readily incorporated into cellular membranous structures. In the

presence of calcium, the toxin molecules embedded in the lipid bilayer undergo a conformational change, so producing a lesion in the membrane through which Na^+ and K^+ ions may pass. Ionic gradients involved in osmoregulation, cellular haemostasis, or the maintenance of transmembrane potentials are lost. Cellular death ensues by osmotic effects or disruption of essential metabolic processes; and the physiological functions of conducting, contractile or other excitatory tissues are disrupted.

In the sections which follow the toxin used corresponded to the material eluted from the HTP columns by 0,1M phosphate, except where it is indicated that pure toxin was used.

METHODS

Haemolytic assays

Two assay procedures were used for studying haemolysis: the first was the tube assay, described in Chapter 3, in which sheep erythrocytes were exposed to toxin in isotonic veronal buffered saline containing 0,5% gelatin. After incubation unlysed cells were sedimented by centrifugation and haemolysis was quantitated by reading the optical density of the supernatant at 540 nm.

The second assay was based on the change of light scattering properties of erythrocytes with lysis. A suspension of 10^7 SRBC/ml in isotonic buffer had an apparent optical density of approximately 1 at 700 nm. The same suspension of cells, when lysed, showed a negligible absorbance at 700 nm relative to a buffer control.

The assay was performed as follows: to 1 ml VBS-G at 37°C in a 1,3 ml quartz cuvette (light path 1,0 cm) was added 10 µl of the toxin solution to be assayed and 10 µl of a suspension of 10^9 SRBC/ml in VBS. The cuvette was placed in a Beckman DBG-T recording spectrophotometer operated in single beam mode, with the cuvette chamber thermostatted at 37°C. The turbidity of the cell suspension, as OD

units at 700 nm, was recorded as a function of time. Samples to which toxin was added showed an initial lag phase of 2-5 minutes - during which the turbidity sometimes increased marginally - followed by a steady drop in turbidity (Figure 6.1). The gradient of the steepest portion of the curve, measured as ΔOD/min, was linearly related to the final concentration of the toxin over the range 10 to 1000 HU/ml (Figure 6.2).

Although tedious and unsuitable for large numbers of samples, this assay proved extremely useful for studying the kinetics of the toxin-mediated haemolysis. The linear relationship between cell concentration and turbidity was lost above OD readings of 1,0. The presence of 0,5% gelatin in the buffer system effectively prevented settling of the cells over a period of fifteen minutes. In assays extending over more than 15 minutes the cells were resuspended at intervals.

Versilube procedure

In order to measure the passage of solute between medium and cells, a procedure was devised for achieving the rapid separation of cells from the medium in which they were suspended. In this method, based upon that described by Werkheiser and Bartley (1957) for the separation of mitochondria from their incubation medium, the cells were centrifuged through a layer of silicone oil, Versilube F50 (General Electric Corporation) that was immiscible with water and had a density (1,050 g/l) intermediate between that of the cells and the medium. Experiments in which the incubation medium contained ^{125}I-IgG as an extracellular marker showed that less than 2% of the medium passed through the Versilube with the cells.

In practice 0,2 ml aliquots of experimental or control cell

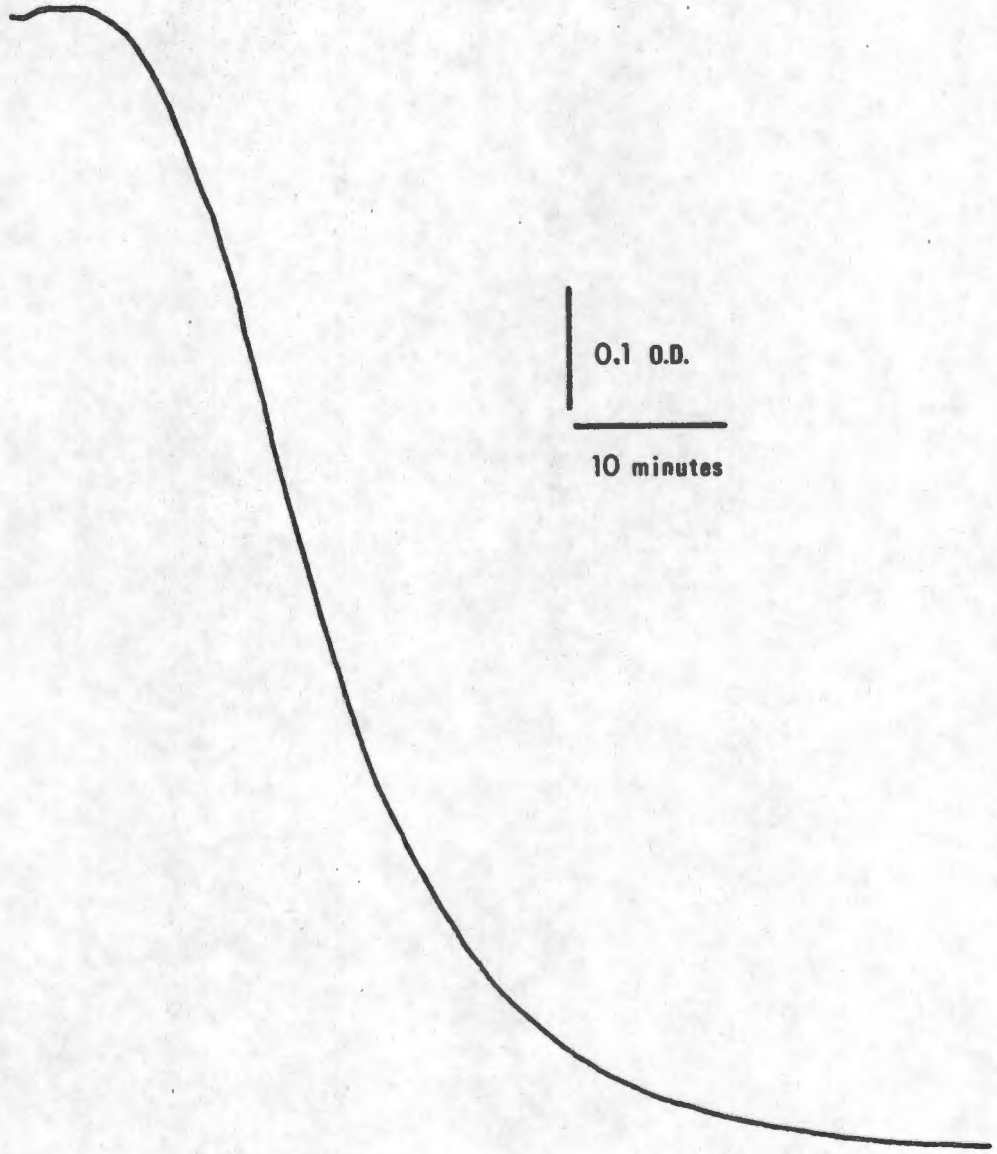


Figure 6.1 The turbidity assay for haemolysis

The assay relied on the decrease in turbidity of a suspension of erythrocytes with haemolysis. The apparent optical density at 700 nm of a suspension of 10^7 SRBC/ml in VBS-G was plotted as a function of time using a Beckman DBG-T recording spectrophotometer in single beam mode. The cuvette chamber was maintained at $37^{\circ}C$.

Upon the addition of toxin the turbidity showed an initial lag phase of 2 to 5 minutes (during which the turbidity sometimes increased marginally) followed by a steady fall in turbidity.

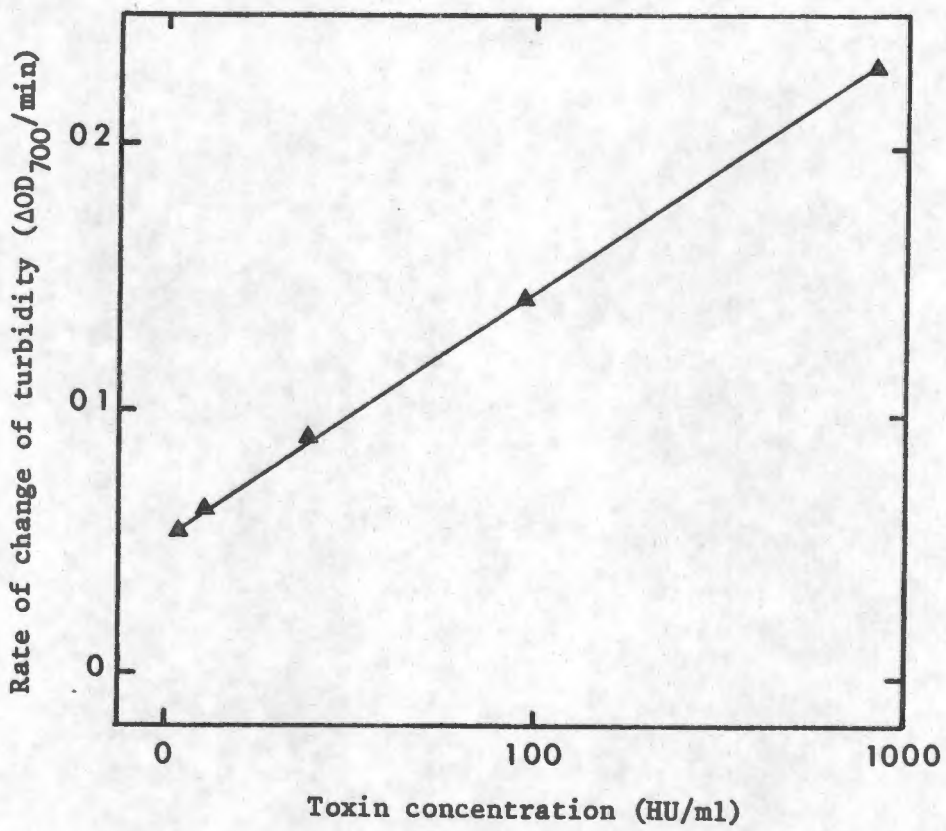


Figure 6.2 The rate of change of turbidity as a function of toxin concentration

The gradient of the steepest portion of the curve relating turbidity to time (Figure 6.1) was linearly related to the concentration of toxin in the cuvette over the range 10 to 1000 HU/ml. A toxin concentration of 100 HU/ml gave a convenient rate of haemolysis in this system and in most experiments using this assay, this was the concentration used.

suspensions were layered over 0,1 ml Versilube which was, in turn, layered over 0,1 ml of 0,3 M sucrose in a plastic 400 µl Microfuge tube. The tube was centrifuged in a Beckman model 152 Microfuge at 5000 x g for 60 seconds. After centrifugation the tube was immersed in a dry ice-acetone freezing mixture to freeze the aqueous layers, and then cut through the unfrozen Versilube layer. The separated aqueous phases were thawed for measurement of sodium, haemoglobin, potassium or ^{45}Ca as was called for by the particular experiment.

Binding studies

For the purpose of investigating the binding of the toxin to the cell membrane approximately 1 mg of purified toxin was labelled with ^{125}I using the chloramine-T method of Greenwood et al (1963).

Proteolytic assays

Two techniques were used to examine toxin preparations for proteolytic potential.

The first was the electrophoretic procedure described by Heussen and Dowdle (1980) in which protein substrates are incorporated into an acrylamide gel during polymerisation. The sample to be tested for proteolytic activity was electrophoresed in the gel in a Tris buffer system containing 0,1% SDS. After electrophoresis the gel was washed in 2,5% Triton X100, incubated at 37°C in 0,1M glycine-NaOH pH 8,3 for 3-5 hours, fixed, and stained with amido black. Gelatin in the region of a protease band was digested during the incubation period, leaving a clear zone after the amido black staining.

In a further attempt to detect proteolytic activity associated with the toxin the method of Unkeless et al (1974) using ^{125}I-labelled fibrin was used. In this procedure the sample to be assayed was

incubated in the well of a Linbro plate (Falcon) which had been coated with insoluble ^{125}I-labelled fibrin. Proteolysis was detected by testing for the release of soluble ^{125}I-labelled peptides into the medium. The toxin was also tested for activity as a plasminogen activator by including the zymogen, plasminogen, in the incubation medium. Aliquots of 50 µl of semi-purified toxin corresponding to 3000 HU were tested.

Phospholipase assays

Two approaches were used to test the toxin for phospholipase activity.

In the first, toxin samples were examined directly for phospholipases A, C and D by the method of Grossman et al (1974) using ^{14}C-labelled lecithin as a substrate. Thin layer chromatography was used to detect ^{14}C-lysolecithin released by hydrolysis by phospholipase A. Choline or choline phosphate produced by hydrolysis by phospholipases C or D were detected by their preferential aqueous solubility.

For the assay, 0,6 µCi of phosphatidyl [N-methyl-^{14}C] - choline (^{14}C-lecithin; 82 µCi/mg) was added to 50 mg non-radioactive lecithin. The chloroform:methanol (2:1) solvent was evaporated under a stream of nitrogen and the phospholipid resuspended in 1 ml 5% Triton X100 + 0,25 ml 1M $CaCl_2$ + 10 ml 0,1M Tris.HCl pH 7,5. For the assay 25 µl toxin solution (2500 HU) was added to 1,125 ml of this stock diluted to 2,5 ml with water. Controls received 25 µl buffer in place of the toxin. After 10 minutes incubation at 30°C the reaction was stopped by adding 1 ml 6% v/v perchloric acid and the aqueous phase extracted three times with 5 ml ether. The ether extracts were pooled and evaporated under N_2 at 50°C before being taken up in 500 µl

chloroform:methanol (1:1). Five microlitres of this solution were spotted on a 10 cm by 10 cm silica TLC plate (Merck silica gel 60, 0,25 mm) which had been pre-chromatographed with 0,2% ammonium sulphate. The second solvent system of Bowyer and King (1977) (Chloroform:acetone:methanol:acetic acid:water 45:46:45:11:6) was used to develop the plate. The positions of the lecithin and lysolecithin spots were determined by running standards and the corresponding areas of silica for the extract scraped into counting vials, 10 ml Instagel added, and counted for ^{14}C. A 1 ml aliquot of the aqueous phase after ether extraction was dissolved in 10 ml Instagel and counted for ^{14}C.

In a further attempt to detect phospholipase activity, a suspension of 1 ml 20% SRBC/ml in VBS was lysed by incubation with 100 HU of toxin at 37°C for 60 minutes. A control suspension was incubated in the absence of toxin. The suspensions were then extracted with n-butanol by the method of Bjerve et al (1974) or with chloroform: methanol by the method of Bligh and Dyer (1959). The extracts were evaporated to dryness under nitrogen and the residue taken up in 100 µl 1:1 chloroform:methanol. Approximately 1 mg phospholipid was chromatographed on silica gel TLC plates by the method of Bowyer and King (1977).

Guinea pig ileum longitudinal muscle-myenteric plexus preparation

The guinea pig ileum longitudinal muscle-myenteric plexus preparation was set up as described by Paton and Vizi (1969).

The preparation was stimulated supramaximally with biphasic, 2 msec, 45V square wave pulses delivered at a frequency of 0,1 Hz. The isometric response was measured with a force displacement transducer with a resting tension of 0,3g, and recorded on a chart recorder.

The preparation was bathed in a 7,5 ml solution of Krebs Ringers solution (118mM NaCl, 4,7mM KCl, 12 mM NaH_2PO_4, 25 mM NaH_2CO_3, 30 µM choline chloride, 11mM glucose, 1,2 mM $MgCl_2$, 2,5mM $CaCl_2$) through which was bubbled 90% O_2 10% CO_2 to maintain a pH of 7,4.

After establishing a stable baseline response 75 µl aliquots of toxin were added to the bathing medium.

In another series of experiments the preparation was stimulated in Krebs Ringers to establish a stable baseline and then washed and bathed in modified Krebs Ringers solution containing no Ca^{++} or Mg^{++}. After 5 minutes 75 µl 100 HU/ml toxin was added to the bathing solution. After 10 minutes exposure to the toxin the preparation was washed and bathed in Ca^{++}- and Mg^{++}-free medium for 5 minutes, then returned to the standard Krebs Ringers solution.

Perfused rat heart preparation

A 250 - 300 g hooded rat was anaesthetised with ether and injected intravenously with 200 units of heparin. The heart was excised and suspended by ligaturing the aorta over a glass tube. The heart was perfused with Krebs Ringers solution pH 7,4 (bubbled with 90% O_2 10% CO_2) at a hydrostatic pressure of 100 cm. After a 15 minute stabilisation period the heart was perfused with Krebs Ringers containing 1 HU/ml toxin. At time points 0 minutes (immediately prior to toxin exposure), 2, 5 and 10 minutes after the start of the toxin perfusion the heart rate and coronary flow were measured. The perfusate was also assayed for the release of LDH. The electrocardiogram was continuously monitored on an oscilloscope and photographic records of the trace taken at the sampling times.

Sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles (SR vesicles) were prepared from rabbit skeletal muscle as described by Boland and Martonosi (1974), and McIntosh and Berman (1978).

Calcium uptake by the vesicles was measured by recording the rate of addition of a 0,1M solution of $CaCl_2$ required to maintain an extra-vesicular concentration of 10 µM Ca^{++}.

In practice, a reaction vessel containing a final volume of 2 ml 20 mM histidine, 50 mM KCl, 5 mM $MgCl_2$, 5 mM potassium oxalate pH 4,0 and 20 mg/ml SR vesicle protein was maintained at 25°C and the contents stirred with a magnetic stirrer. The calcium concentration in the vessel was monitored with a Ca^{++} electrode and maintained at 10 µM by an electrically driven injection pump supplying 10 mM $CaCl_2$ and controlled by an electronic feedback loop from the Ca^{++} electrode.

The experiment was started by adding to the reaction vessel 100 µl of 100 mM ATP. After a lag period of approximately 10 seconds there was a steady uptake of Ca^{++} leveling out after 90 seconds.

Conformational studies

Changes in the conformation of the toxin induced by the addition of calcium or lanthanum were studied by means of fluorescent probes and by circular dichroism.

The circular dichroism studies were carried out using a JASCO J-40A automatic recording spectropolarimeter. Pure toxin at 0,4 mg/ml in 0,01M Tris-HCl, 10% glycerol pH 8,2 was placed in a fused quartz cell of 2,0 cm path length. The toxin solutions contained either 1 mM EDTA or 10 mM $CaCl_2$. The circular dichroism spectra of these solutions were recorded in the region from 190 to 300 nm. Reference spectra

were run on solutions of buffer containing no toxin.

Studies using the fluorescent dye 8-anilino-1-naphthalene sulphonic acid (ANS) were based upon the findings of Stryer, L. (1965) who showed that the specific fluorescence of the dye increases with increasing hydrophobicity of its environment. From this they inferred that an increase in the specific fluorescence of a protein-dye mixture reflects the binding of ANS molecules to hydrophobic residues newly exposed on the surface of the protein molecule.

For the experiments 0,5 ml solution of 0,4 mg/ml pure toxin in 0,01M Tris-HCl, 0,1M NaCl, pH 8,3 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 using a Perkin Elmer MPF-4-A fluorescence spectrophotometer. The fluorescence, measured in arbitrary units, was recorded for toxin solutions containing 1 mM EDTA or 1 to 10 mM $CaCl_2$. Similar recordings were made with toxin solutions containing 0,01 to 1 mM $LaCl_3$.

RESULTS

The kinetics of haemolysis and the effect of sucrose

When haemoglobin release was studied as a function of time after the addition of toxin, a lag period of approximately 15 min was observed during which the rate of haemolysis was slow. The rate of cell lysis then rose rapidly to reach a maximum after approximately 35 minutes and then fell as 100% lysis was approached. A sigmoid plot was therefore obtained (Figure 6.3).

A sigmoid relationship was also observed when % haemolysis after exposure to toxin for 1 hour was plotted as a function of toxin concentration (Figure 6.4).

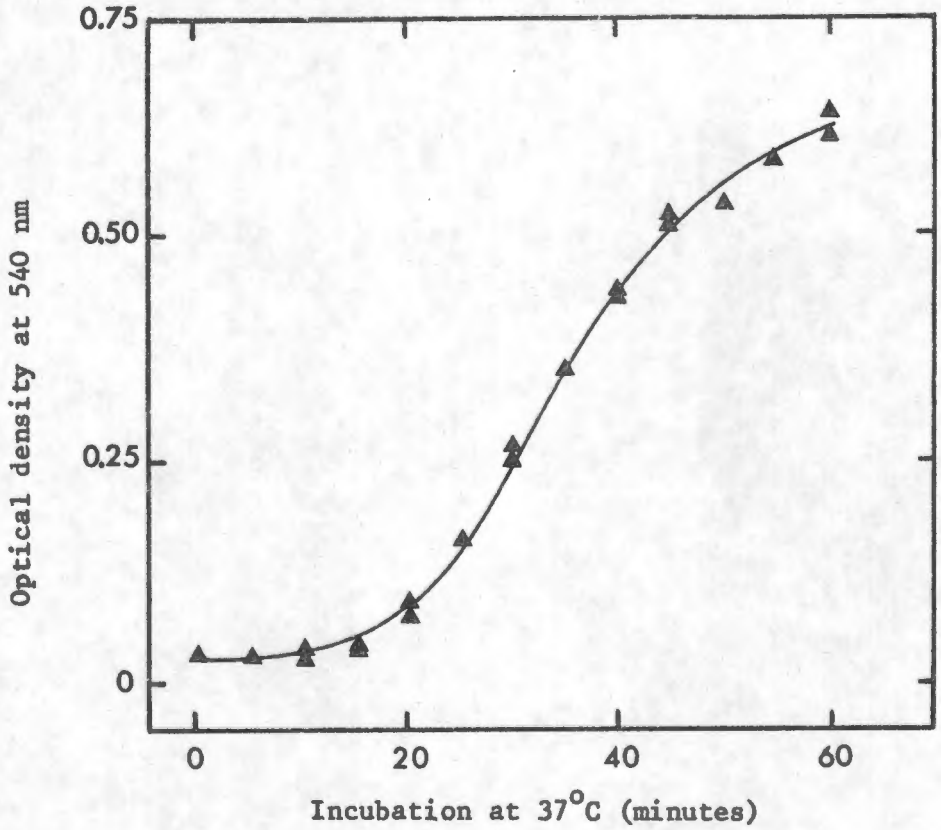


Figure 6.3 The release of haemoglobin from erythrocytes exposed to toxin as a function of time.

To each of a series of tubes containing 3 HU of toxin in 3,25 ml VBS-G was added 0,5 ml 2,5% SRBC in VBS. The tubes were incubated in a 37°C water bath, and at 5 minute intervals a pair of tubes was centrifuged for 2 minutes at 2000 x g and the OD of the supernatant read at 540 nm.

The release of haemoglobin plotted against time produced a sigmoid curve.

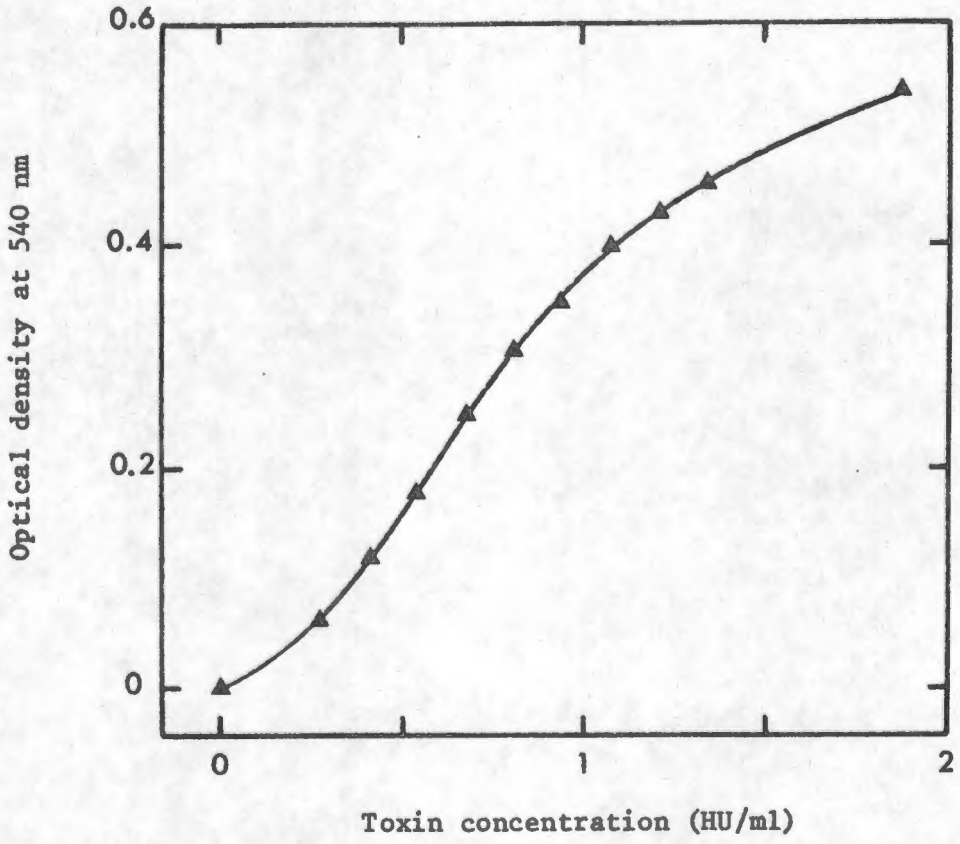


Figure 6.4 The release of haemoglobin from erythrocytes exposed to toxin as a function of toxin concentration

A series of tubes was prepared containing 0 to 2 HU of toxin in 3,25 ml VBS-G and to each tube was added 0,5 ml 2,5% SRBC in VBS. The tubes were incubated at 37°C for 60 minutes and then centrifuged at 1100 x g for 5 minutes. The optical density of the supernatant was read at 540 nm.

The release of haemoglobin plotted against the amount of toxin added produced a sigmoid curve.

When the versilube procedure was used to study sodium, potassium and calcium fluxes across the cell membrane as a function of time after addition of toxin, it was found that toxin induced a rapid release of K^+ and uptake of Na^+ that was virtually complete by 20 minutes. Release of haemoglobin started at approximately 15 minutes and approached completion at approximately 45 minutes (Figure 6.5). There was no apparent nett flux of Ca across the cell membrane as a result of exposure to toxin.

The results of the experiments described above suggested that the toxin acted initially to produce a lesion in the erythrocyte membrane that allowed the free passage of sodium and potassium ions. Since cellular osmoregulation requires the maintenance of physiological ionic gradients across the cell membrane, one might suspect that cellular swelling would have resulted with subsequent release of haemoglobin by osmotic rupture. To examine this suggestion, the effects of the osmotically active disaccharide, sucrose, were studied.

As can be seen from the experimental results depicted in Figure 6.6 addition of sucrose to toxin-treated erythrocytes resulted in inhibition of haemoglobin release. The extent of this inhibition was concentration dependent over the range 0 - 0,3M sucrose. Sucrose had no effect upon the release of K^+.

Requirement for divalent cations

In view of the fact that many haemolytic agents show a functional requirement for calcium ions (Schanne, 1979) it was of interest to examine diamphotoxin in this context.

An experiment was accordingly performed in which 100 HU of toxin were added to an erythrocyte suspension in the presence of 1mM EDTA and the turbidity of the mixture was monitored continuously

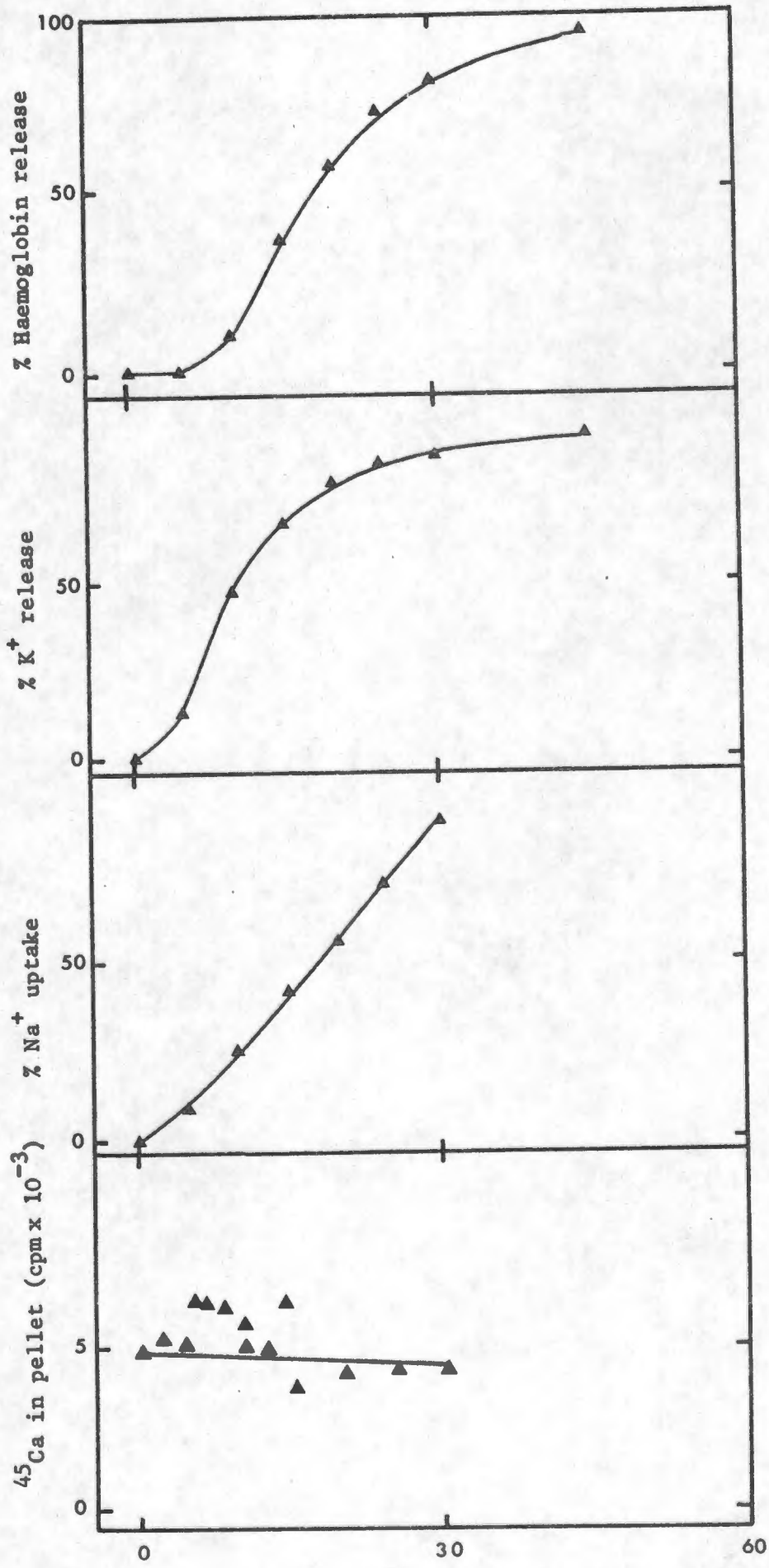
Figure 6.5 The movement of Na^+, K^+, Ca^{++} and haemoglobin across the erythrocyte membrane after exposure to toxin.

An 0,2 ml sample containing cells (10^9 SRBC/ml) and toxin (1000 HU/ml) in VBS was layered over 0,1 ml Versilube which was, in turn, layered over 0,1 ml 0,3M sucrose in a 400 µl microfuge tube. After incubation at 37°C for the appropriate period of time the tube was centrifuged at 5000xg for 60 seconds, resulting in intact cells passing through the Versilube layer into the 0,3M sucrose. The tubes were immersed in a dry ice-acetone freezing mixture to freeze the aqueous layers and cut through the Versilube layer.

Loss of K^+ by the cells was monitored by determining the K^+ concentration in the upper aqueous layer by flame photometry. Loss of haemoglobin was determined spectrophotometrically from the upper aqueous layer or, by inference, from the lower aqueous layer after the cells had been lysed by the addition of 15 µl 10% saponin and transferred to a separate tube. Uptake of Na^+ by the cells was determined by flame photometry of this same lysate and the application of a correction factor, based on the release of haemoglobin in the upper aqueous phase, to compensate for ruptured cells not passing through the Versilube layer. To determine the uptake of Ca^{++} by the cells ^{45}Ca (0,2 mCi/µM) was added to the cells prior to the incubation with toxin. The lower aqueous phase was then incubated overnight with 5 ml Instagel to dissolve the cell pellet and counted for ^{45}Ca after adding 5 ml Soluene. Counts were corrected for quenching.

The Na^+ and K^+ fluxes could be detected immediately upon the exposure of the cells to toxin. The loss of haemoglobin by the cells began between 5 and 10 minutes after exposure to the toxin. No nett flux of ^{45}Ca could be detected.

Figure 6.5


100
50
0
% Haemoglobin release
50
0
% K+ release
50
0
% Na+ uptake
5
0
45Ca in pellet (cpm x 10-3)
0
30
60
Incubation with toxin (minutes)

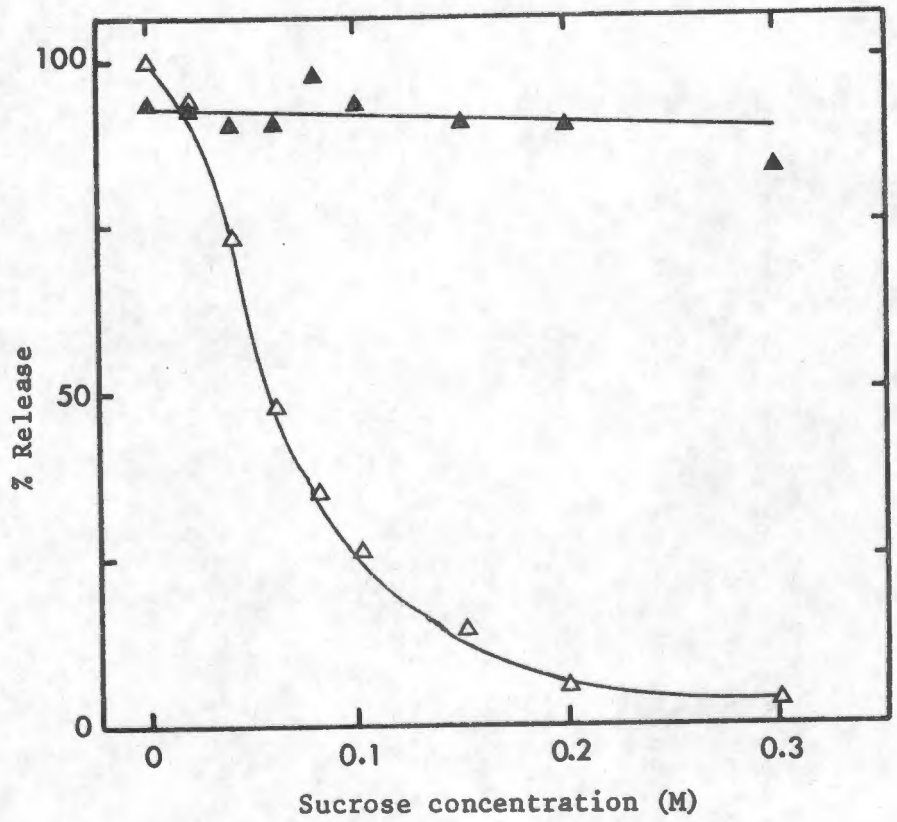


Figure 6.6 Effect of extracellular sucrose on the release of haemoglobin and K^+ from toxin-treated cells

A series of tubes were prepared containing 0,45% SRBC, 100 HU of toxin, and 0 to 0,3M sucrose in a total volume of 5 ml VBS-G. The tubes were incubated at 37°C for 60 minutes, centrifuged at 1100 x g for 5 minutes, and the haemoglobin and potassium concentration determined in the supernatant by spectrophotometry and flame photometry respectively.

The release of K^+ was unaffected by the sucrose while the release of haemoglobin decreased with increasing sucrose concentration.

Release of K^+: ▲ ; and haemoglobin: △ .

at 700 nm.

As can be seen from the data presented in Figure 6.7, EDTA inhibited haemolysis and the turbidity of the mixture remained constant for 20 minutes. Addition of 1 mM EDTA + 10 mM $CaCl_2$ resulted in haemolysis at the rate of 0,06 OD U/min when measured using the turbidity assay. Cells in the control cuvette (i.e. toxin in VBS-G) lysed at the rate of 0,07 OD U/min.

The calcium dependence of the toxic haemolytic reaction was confirmed by showing, in the tube assay, that no haemolysis occurred in VBS prepared without Ca^{++} or Mg^{++} (CMF-VBS) or in VBS to which 1 mM EDTA was added. Activity could be restored by adding calcium.

It could similarly be shown (Figure 6.8) that toxin-induced release of K^+ ions required the presence of Ca^{++} in the medium. In this experiment, red cells were suspended at a concentration of $2,5 \times 10^8$/ml in CMF-VBS-gelatin and were treated with either toxin alone followed 30 minutes later with $CaCl_2$ or with toxin and $CaCl_2$ added simultaneously. Samples taken at intervals after the addition of toxin were centrifuged and the supernatant fluid was assayed for K^+ content by flame photometry. Release of intracellular potassium required the combined action of both toxin and calcium.

Although these experiments showed unequivocally that Ca^{++} was required for the haemolytic action of diamphotoxin, they gave no indication of whether the haemolytic reaction required the simultaneous presence of toxin and calcium throughout or whether toxin and calcium acted sequentially.

To resolve this issue, erythrocytes were exposed to toxin in the absence of calcium and then washed extensively (5x). Exposure of these cells to VBS containing 1 mM Ca^{++} resulted in lysis.

It was possible, therefore, to distinguish two sequential

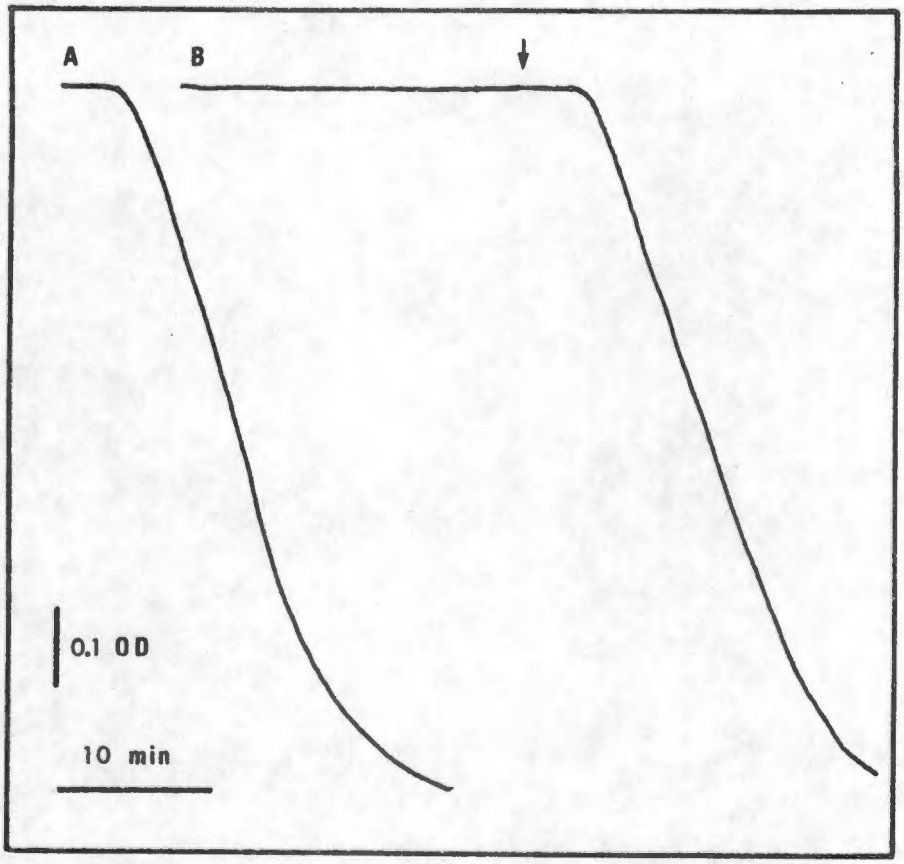


Figure 6.7 The haemolytic activity of diamphotoxin was inhibited by EDTA and restored by the addition of an excess of calcium

The presence of 100 HU/ml in the turbidity assay resulted in a fall in turbidity such as that shown in curve A. If the assay solution was made 1 mM in EDTA before addition of the toxin, no change in turbidity was seen (curve B). However, if calcium was added to the latter system to bring it to 10 mM with respect to calcium, haemolysis proceeded in the same manner as shown in curve A. The time of addition of calcium is marked on curve B with an arrow.

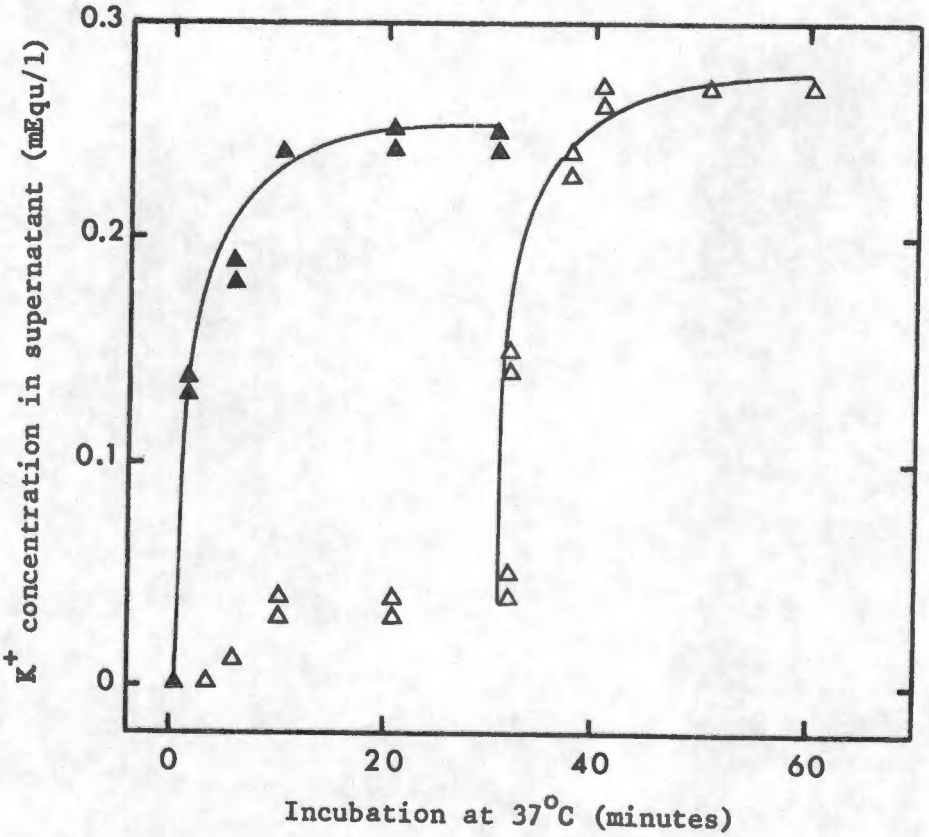


Figure 6.8 The release of K^+ from diamphotoxin-exposed erythrocytes requires the presence of Ca^{++}.

Two series of tubes were prepared containing 2,5 x 10^8 SRBC and 25 HU toxin in 1 ml calcium- and magnesium-free VBS-G (CMF-VBS-G). The tubes were incubated at 37°C and at intervals tubes from each series were centrifuged at 2000 x g for 1 minute, the supernatant removed, and the release of K^+ by the cells determined by flame photometry. For one series of tubes (▲) 20 µl 0,1M $CaCl_2$ was added to each tube at the start of the incubation; for the other series (△) the calcium was added after 30 minutes of incubation at 37°C.

The release of K^+ by the cells only proceeded in the presence of Ca^{++}.

phases to the cytolytic action of the toxin. The first, a calcium-independent step, led to the production of an irreversible but incomplete lesion of the cell membrane. In the second step Ca^{++} ions interacted with the sensitized cells to complete the lesion and allow flux of Na^{+} and K^{+} ions to take place.

Having defined two sequential phases in the haemolytic reaction that could be distinguished on the basis of their calcium-dependence, further experiments could be performed to study these two phases in more detail. In these experiments the following general protocol was used:

- washed SRBC were incubated with toxin in CMF-VBS-G containing 1 mM EDTA,
- the cells were then washed extensively with CMF-VBS, by repeated centrifugation and resuspension to remove all free toxin;
- the cells were finally resuspended in CMF-VBS, calcium was added and haemolysis was measured - either by quantitating haemoglobin release or by monitoring turbidity at 700 nm.

For the sake of convenience I shall refer to the two critical experimental stages, namely the calcium-free preincubation with toxin, and the subsequent haemolytic phase requiring Ca^{++}, as the toxin phase and the calcium phase respectively.

Using this protocol the following observations were made:

(a) The rate of haemolysis during the calcium phase was directly related to the concentration of toxin and to the duration of exposure to toxin during the toxin phase (Figure 6.9 and Figure 6.10).

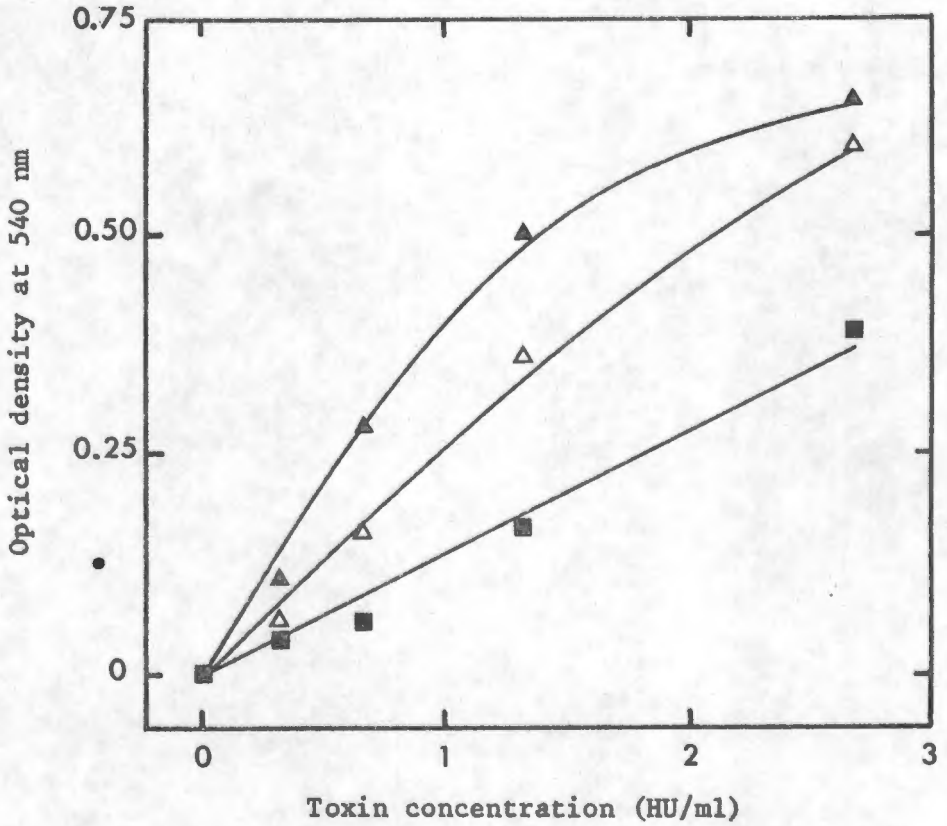


Figure 6.9 Effect of duration of exposure to toxin and toxin concentration, in the absence of calcium, upon subsequent haemolysis following addition of calcium.

Tubes were prepared containing 0,33% SRBC in a final volume of 3,75 ml of calcium and magnesium-free VBS-G containing 1 mM EDTA. Toxin was added to give the concentrations shown and the tubes were placed at 37°C. After 10 min (■), 20 min (△) and 30 min (▲) a representative tube from each toxin concentration series was removed from the waterbath and centrifuged. The pelleted cells were washed once with CMF-VBS-G, resuspended in VBS containing calcium and magnesium and re-incubated at 37°C for a further 60 min for haemolysis to proceed.

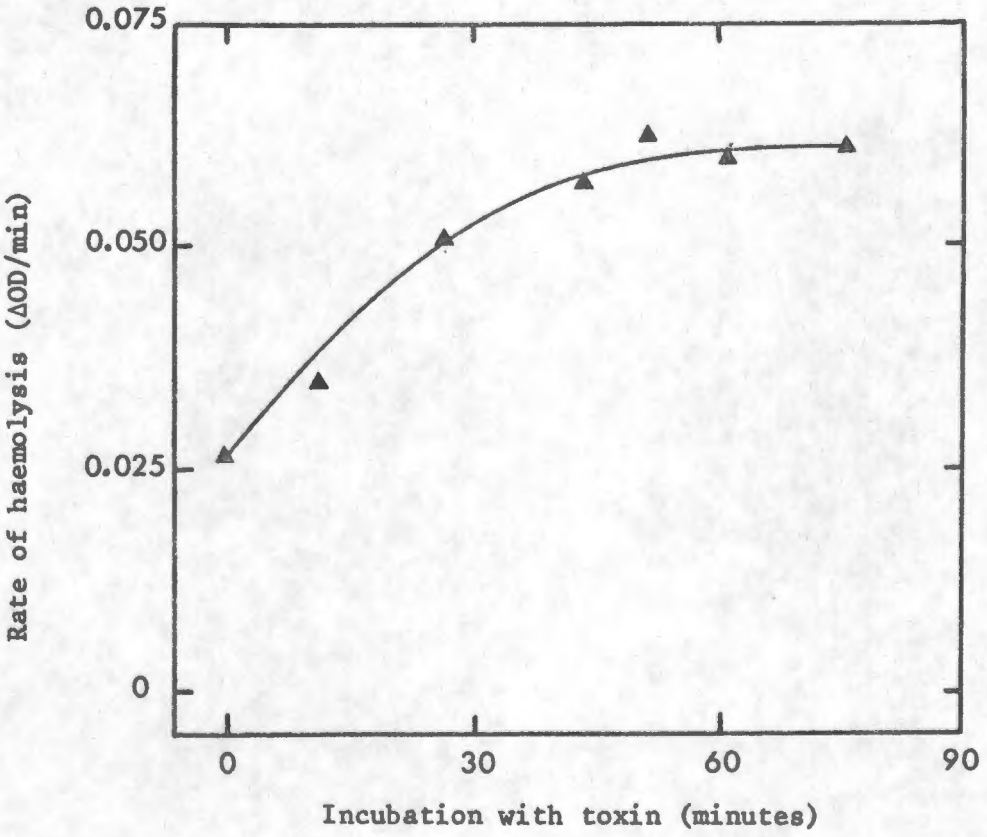


Figure 6.10 The rate of haemolysis during the calcium phase was related to the duration of exposure to toxin during the toxin phase

A 10 ml solution containing 10^7 SRBC/ml and 25 HU/ml toxin in CMF-VBS was incubated at 37°C. At times of 0 to 75 minutes after the start of the incubation aliquots of 1 ml were transferred to a cuvette containing 10 µl 0,1M $CaCl_2$ and the turbidity monitored as described for the turbidity assay.

The rate of haemolysis was a function of the duration of incubation with toxin in the range 0-60 minutes.

(b) After exposure to 25 HU/ml of toxin for 30 min at 37°C during the toxin phase, optimal lysis was observed during the calcium phase with a Ca^{++} concentration of 1 mM (Figure 6.11).

(c) Other divalent cations substituted for Ca^{++} during the calcium phase. Their efficacy decreased in the order $Sr^{++} > Ca^{++} > Ba^{++} > Mg^{++} > Mn^{++}$ (Figure 6.12).

(d) Lanthanum (as $LaCl_3$) inhibited the haemolysis caused by 100 HU/ml of toxin in the presence of 1 mM $CaCl_2$. This inhibition was evident at 10^{-7}M $LaCl_3$ and complete at concentrations higher than 10^{-5}M (Figure 6.13).

(e) Maximal haemolysis was observed when the pH during the toxin phase was 6,7 and the pH during the calcium phase was 8,6 (Figure 6.14).

(f) As can be seen from the data summarised in Figure 6.15, both phases were temperature dependent. An increase of temperature from 25°C to 45°C at the toxin stage increased haemolysis 4,2 fold. A similar increase at the calcium stage increased haemolysis 5,9 fold.

Incubation at 0°C during the toxin phase resulted in no detectable haemolysis when the washed cells were subsequently incubated at 37°C during the calcium phase. Incubation at 0°C during the calcium phase following exposure to toxin at 37°C during the preincubation, resulted in a considerably depressed rate of cytolysis.

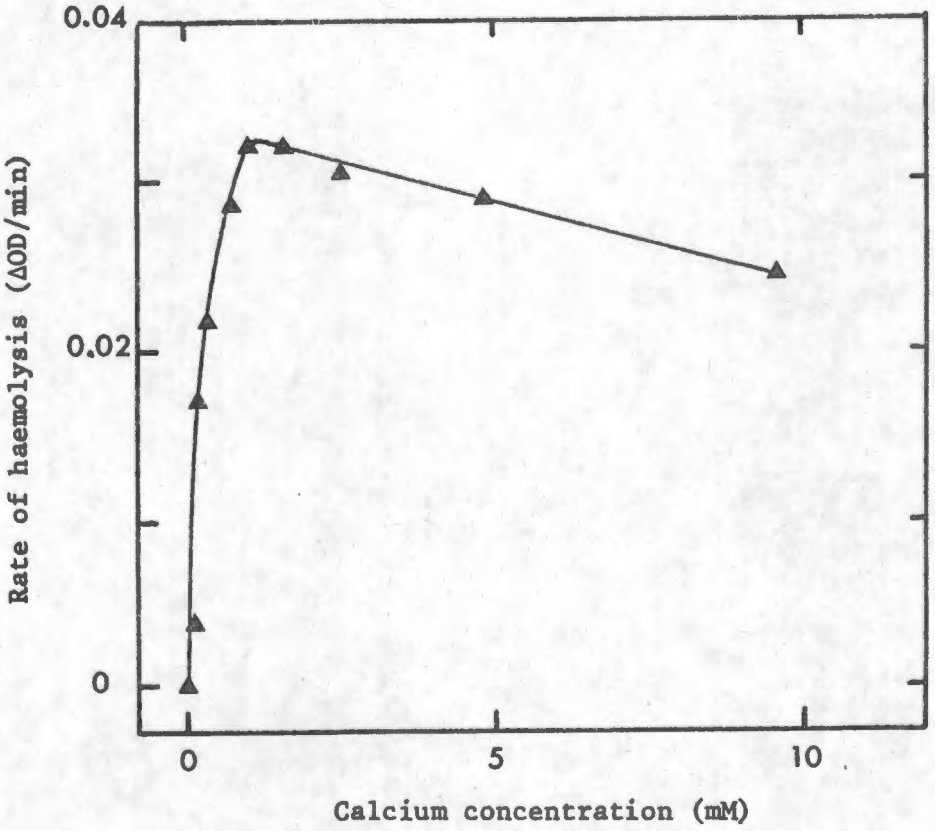


Figure 6.11 Calcium requirement for haemolytic activity

Tubes containing 10^7 SRBC and 25 HU toxin in 0,9 ml CMF-VBS-G were incubated at 37°C. After 30 minutes the calcium concentrations were adjusted to those shown by the addition, to the tubes, of 0,1 ml of $CaCl_2$ solution of the required concentration. The solution was then transferred to a cuvette for the turbidity assay.

The maximum rate of haemolysis was observed with 1 mM Ca^{++}.

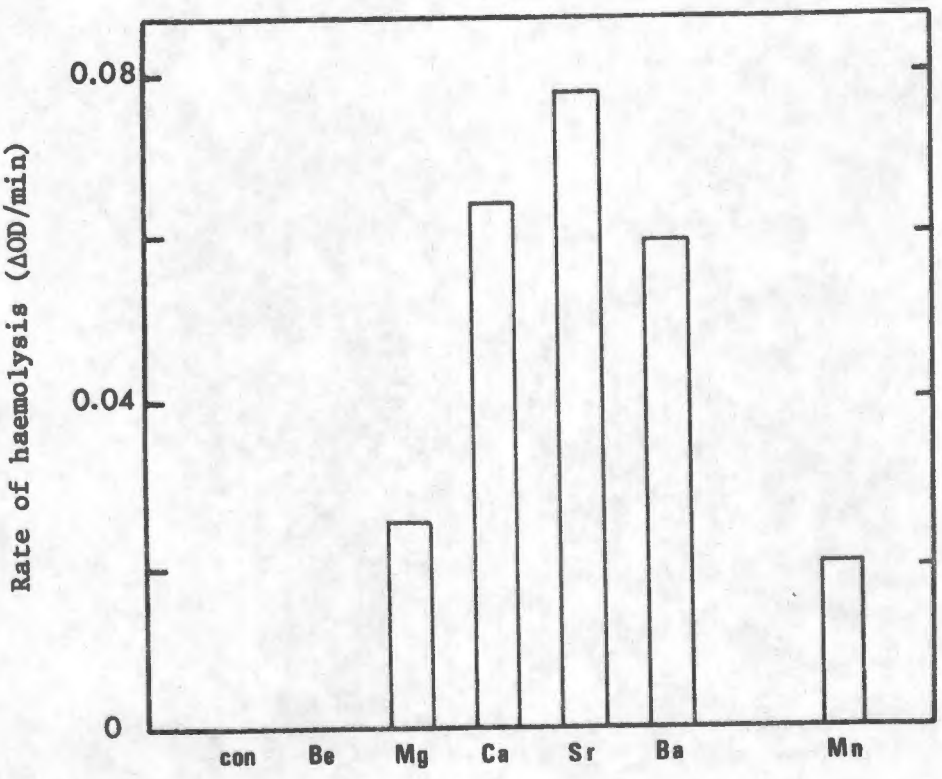


Figure 6.12 Effect of divalent cations other than calcium during the calcium phase

Tubes containing 10^7 SRBC/ml and 25 HU/ml toxin in 1,0 ml of CMF-VBS-G were incubated at 37°C for 60 minutes. The contents of each tube were then transferred singly to a cuvette and 10 µl of a 1M solution of the chloride salt of the divalent cation to be tested was added. The change in turbidity of the suspension was monitored.

When $BeCl_2$ was added to the cells the suspension became flocculent and settled very rapidly to the bottom of the cuvette. The other cations tested all facilitated haemolysis, a maximal rate being obtained with strontium.

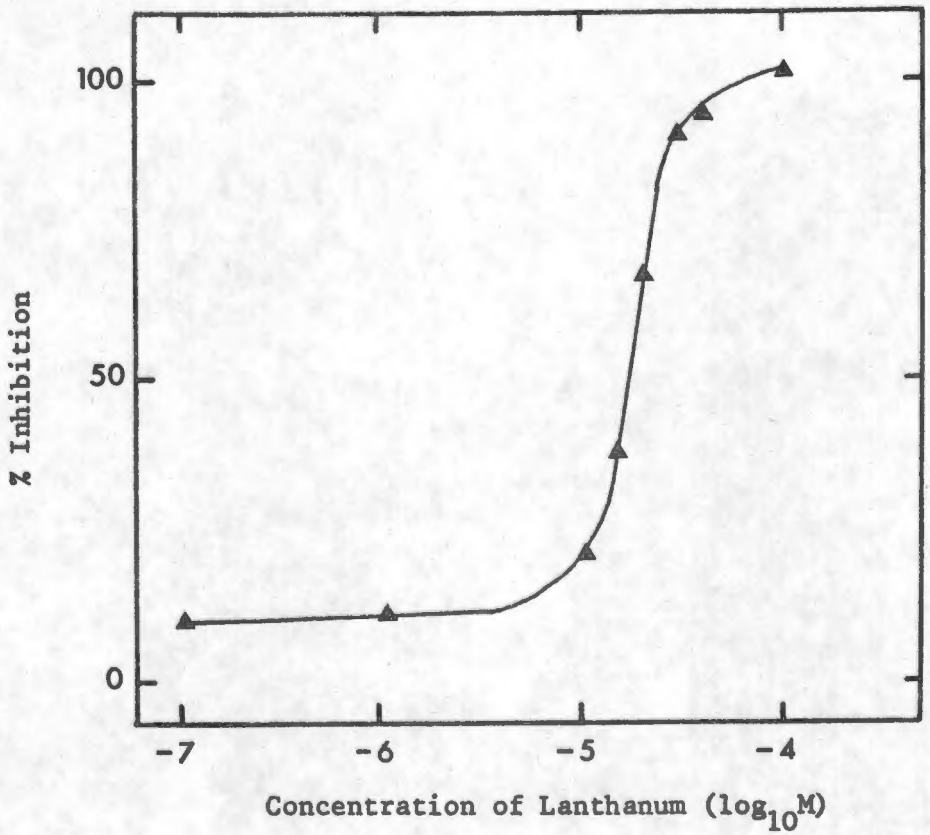


Figure 6.13 Haemolysis was inhibited by 10^{-4}M lanthanum chloride

Using the turbidity assay the haemolytic activity of 100 HU/ml of toxin was determined in the presence of 0 to 10^{-4}M $LaCl_3$. The inhibitory effect of La^{+++} rose sharply from concentrations above 10^{-5}M, becoming absolute at 10^{-4}M.

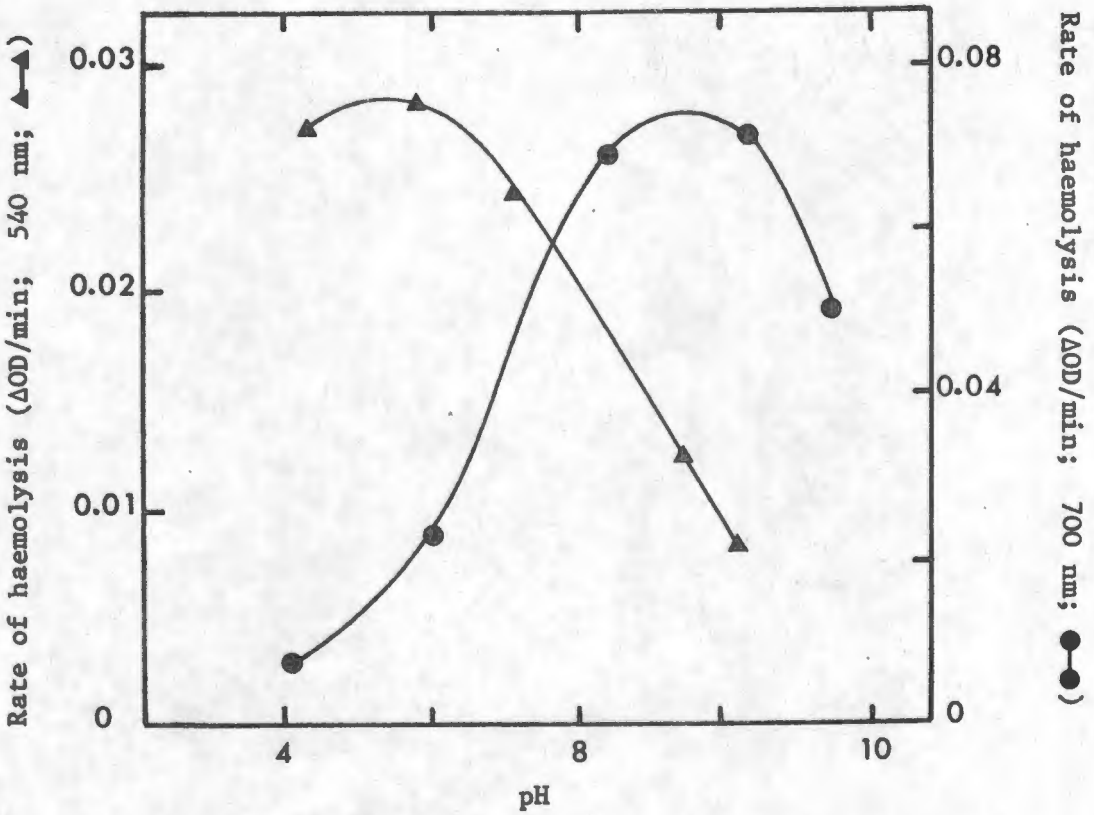


Figure 6.14 The pH optima for the toxin phase and the calcium phase.

To study the effect of pH on the toxin phase, erythrocytes (10^7/ml) were incubated with toxin (8 HU/ml) at 37°C for 0 to 30 minutes in isotonic NaCl, 1 mM EDTA buffered at pH 6 or 7 by 0,01 M citrate or at 8, 9 or 10 by 0,01M borate. The erythrocytes were then pelleted and resuspended in VBS. After a further incubation of 60 minutes at 37°C the cells were again pelleted and the OD of the supernatant measured at 540 nm. The rate of increase of OD with increasing durating of the first incubation was calculated for each pH. This was taken as a measure of the rate of the toxin phase at that pH.

To study the effect of pH on the calcium phase a suspension of 10^7 SRBC was incubated with 10 HU of toxin in 1 ml CMF-VBS-G, 1 mM EDTA at 37°C for 30 minutes. This incubation was terminated by adding 4 ml isotonic saline buffered at pH 6 or 7 by 0,01M citrate or at pH 8, 9 or 10 by 0,01M borate, centrifuging for 1 minute at 2000 x g, and resuspending the cells in 1 ml of saline solution of the appropriate pH. After the addition of 20 µl 0,1M $CaCl_2$ the cell suspension was transferred to a cuvette and the rate of haemolysis measured as described for the turbidity assay.

The rate for the toxin phase (▲—▲) showed an optimum at pH 6,7 and that for the calcium phase (●—●) at pH 8,6.

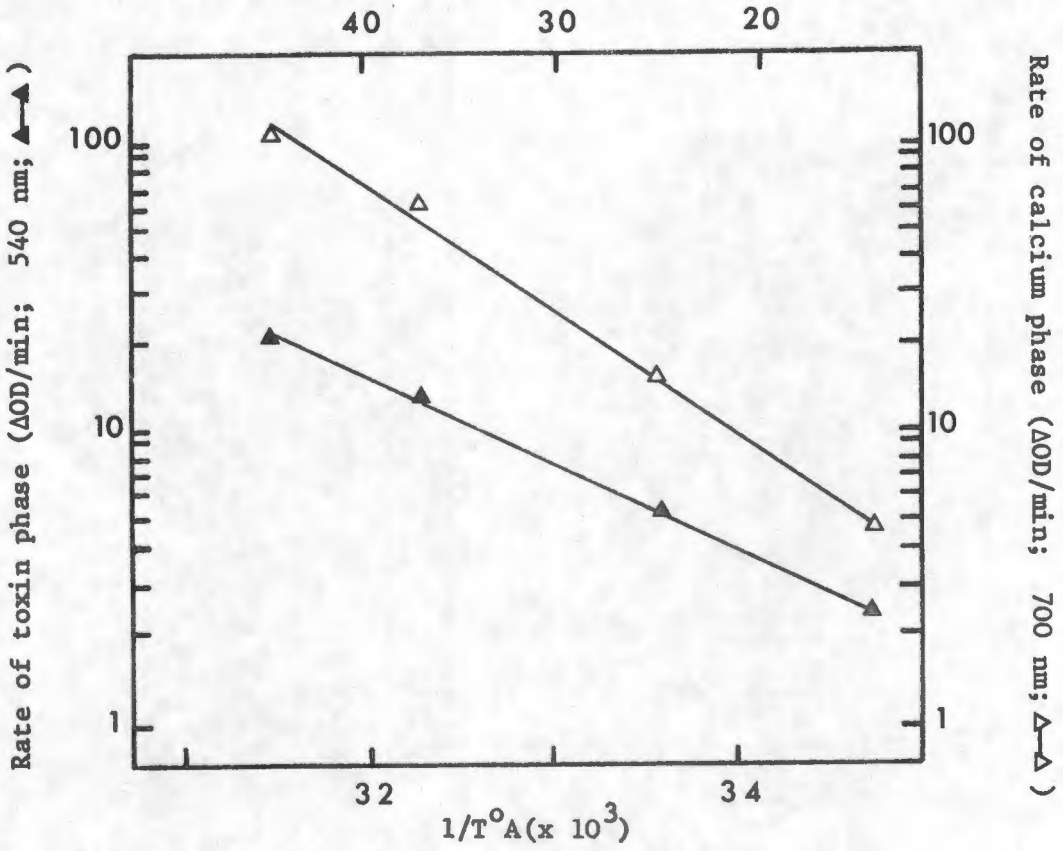


Figure 6.15 The effect of temperature on the rates of the toxin phase and the calcium phase of haemolysis

To study the effect of temperature on the toxin phase 1 ml suspensions of 10^8 SRBC/ml in CMF-VBS-G, 1 mM EDTA and 10 HU/ml toxin were incubated at 45°C, 37°C, 25°C or 15°C for times ranging from 1 to 60 minutes. The incubation was terminated by pelleting the cells, washing them in 4 ml CMF-VBS, and resuspending them in 1 ml VBS. A second incubation of 60 minutes at 37°C was then carried out, after which the cells were again pelleted and the OD of the supernatant at 540 nm was determined. The rate of increase of OD with increasing duration of the first incubation was calculated for each temperature. This was taken as a measure of the rate of the toxin phase at that temperature.

To study the effect of temperature on the calcium phase 1 ml suspensions of 10^7 SRBC/ml in CMF-VBS-G, 1 mM EDTA, and 100 HU/ml toxin were incubated at 37°C. After 30 minutes 25 µl 0,1 M $CaCl_2$ was added and the suspension was transferred to a cuvette. The rate of haemolysis was monitored as described for the turbidity assay, except that the temperature of the cuvette chamber was maintained at 45°C, 37°C, 25°C or 15°C.

The graph shows a plot of the logarithm of the rate of the toxin phase (▲—▲) and the calcium phase (△—△) as a function of the reciprocal of the temperature in °K.

Phospholipase activity

A number of cytolytic toxins have been shown to have phospholipase activity (Wells and Hanehan, 1969; Hessinger and Lenhof, 1974). It was accordingly important to examine diamphotoxin for a similar mode of action.

Attempts to demonstrate the ability of the toxin to hydrolyse ^{14}C-lecithin to lysolecithin or to liberate choline or choline phosphate from this substrate were unsuccessful (Table 6.1). Similarly, erythrocyte membrane, following exposure to haemolytic concentration of toxin, showed no alteration in phospholipid composition as detected by organic solvent extraction and thin layer chromatography (Figure 6.16).

When phospholipase inhibitors were examined for their effects on the action of diamphotoxin, the results, summarised in Figures 6.17 and 6.18 were obtained.

Rosenthal's inhibitor, an analogue of lecithin, showed an interesting inhibition effect that increased gradually and linearly from 0% inhibition at 10^{-8}M to approximately 10% inhibition at 6×10^{-6}M. Between 6×10^{-6}M and 7×10^{-6}M, however, a sharp increase in inhibition occurred to approximately 100%. This "step-function" relationship between concentrations and % inhibition was observed in three separate experiments. On each occasion the inflection occurred between 6×10^{-6}M and 7×10^{-6}M. A typical experiment is depicted in Figure 6.17. Rosenthal's inhibitor only acted in this way when dissolved and added in chloroform:methanol (1:19). No effect of the compound was observed if it was dissolved in methanol alone or in chloroform:methanol (1:1).

Neither lecithin nor sphingomyelin had any effect on haemolysis by diamphotoxin when used at a concentration of 10^{-4}M in exactly the same manner as Rosenthal's inhibitor.

TABLE 6.1

ASSAY FOR PHOSPHOLIPASE ACTIVITY IN A TOXIN SOLUTION

A solution containing 2500 HU toxin and 5 mg ^{14}C-labelled lecithin (N-methyl-^{14}C, 48 000 cpm) in 0,2% Triton X100, 0,04M $CaCl_2$ and 4 mM Tris-HCl pH 7,5 was incubated at 30°C for 10 minutes. In control solutions the toxin was omitted. The reaction was stopped by adding 1 ml 6% v/v perchloric acid and the aqueous phase extracted three times with ether. As described in the text the phospholipid in the ether extract was chromatographed on a silica-gel TLC system and the lecithin and lysolecithin recovered and quantitated by determining ^{14}C. Aliquots of the aqueous phase were counted for ^{14}C to determine the release of choline from the lecithin. The table shows the results for an experiment carried out in duplicate.

	Toxin			Control		
	cpm		%	cpm		%
lecithin	41905	40941	86	39979	38055	81
lysolecithin	1445	2400	4	1440	1925	3,5
Aqueous (choline)	344	234	0,6	225	248	0,5

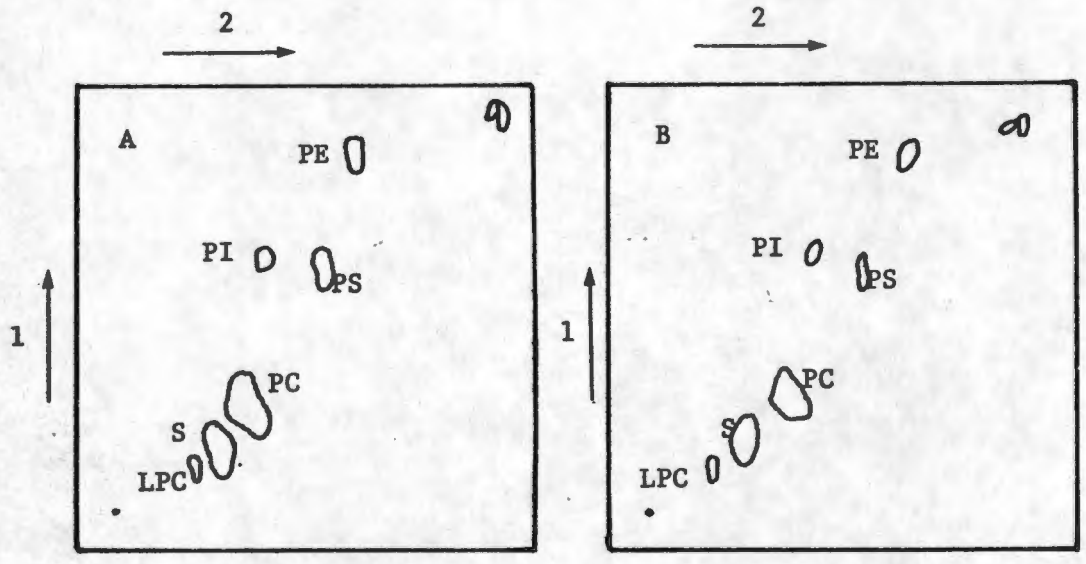


Figure 6.16 The composition of erythrocyte membrane phospholipid after exposure to toxin.

A 1 ml suspension of 20% SRBC in VBS was lysed by incubation with 1000 HU of toxin at 37°C for 60 minutes. A control suspension was incubated without toxin. These suspensions were then extracted with n-butanol by the method of Bjerve et al (1974) and the extracts evaporated to dryness under nitrogen. The residue was taken up in chloroform:methanol 1:1 and chromatographed on silica gel TLC plates by the method of Bowyer and King (1977). Phospholipid spots were visualised by spraying first with 2,7 dichlorofluorescein followed by ninhydrin and incubation at 110°C for 10 minutes. A: control cells; B: toxin lysed cells; PC: phosphatidylcholine; LPC: lysophosphatidylcholine; PE: phosphatidylethanolamine; S: sphingomyelin; PI: phosphatidylinositol; PS: phosphatidylserine.

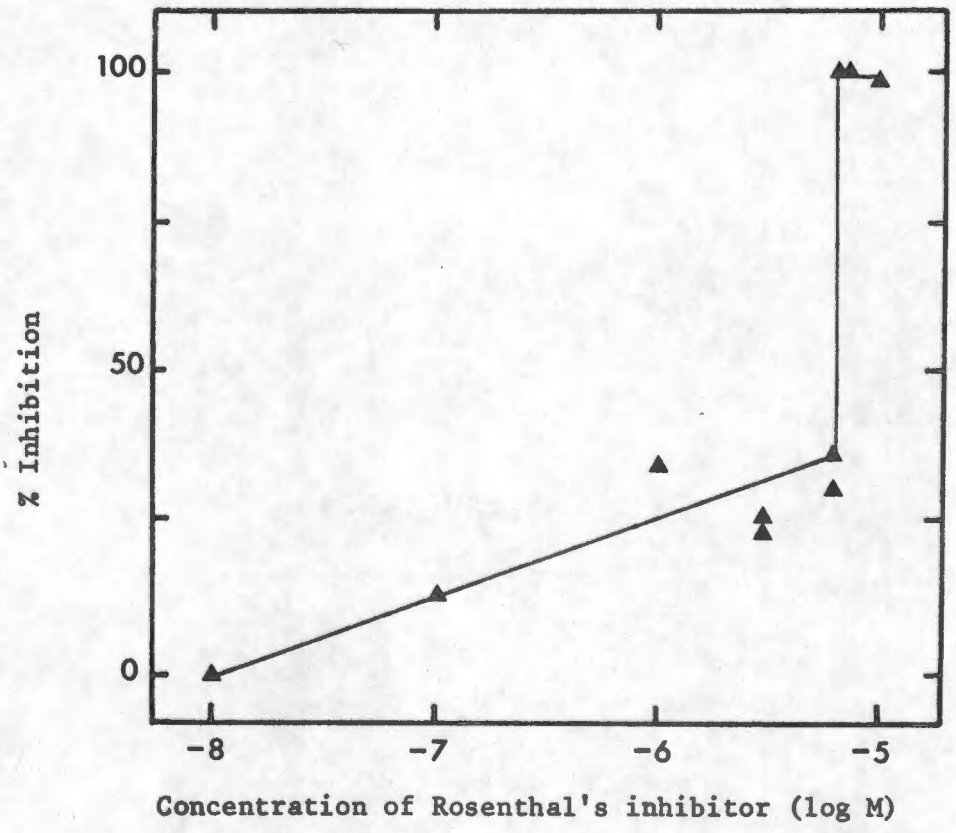


Figure 6.17 The effect of Rosenthal's inhibitor on haemolysis by the toxin

A 10 µl aliquot of Rosenthal's inhibitor dissolved in chloroform: methanol 1:19 was added to 1 ml VBS-G at 37°C in a glass 5 ml test tube and vortexed immediately. The solution was then transferred to a quartz cuvette, 10 µl 10^4 HU/ml toxin added, the contents of the cuvette mixed, and finally 10 µl 10^9 SRBC/ml added. The suspension was again mixed and then placed in the spectrophotometer. The rate of haemolysis was monitored as described for the turbidity assay. The concentration of Rosenthal's inhibitor in the $CHCl_3$:CH_3OH solutions ranged from 10^{-6}M to 10^{-2}M.

The figure shows a plot of the rate of haemolysis as a function of the final concentration of Rosenthal's inhibitor.

The reagent p-bromophenacyl bromide has been used, as an inhibitor that modifies histidine, to indicate that a number of phospholipases possess histidine residues at the active site (Volwerk et al, 1974; Halpert et al, 1976; Viljoen et al, 1977). When toxin was incubated in the presence of $3,3 \times 10^{-7}$M p-bromophenacyl bromide, a progressive loss of haemolytic activity with time was observed (Figure 6.18).

Proteolytic activity

Attempts to demonstrate proteolytic activity associated with the toxin were negative. Electrophoresis of a toxin sample containing 600 HU of activity failed to generate any proteolytic bands in a gelatin SDS polyacrylamide gel. In the ^{125}I-fibrin procedure 50 µl samples of toxin solution containing 3000 HU of activity were added to Linbro wells in the presence or absence of plasminogen. In neither case did the release of ^{125}I-labelled fibrin degradation peptides differ from the background level in buffer controls. Furthermore, the SDS-gel protein banding pattern for erythrocyte ghosts incubated in 300 HU/ml toxin (37°C, 60 minutes) did not differ from that for a control suspension incubated with heat-inactivated toxin.

Stoichiometry of the toxin-erythrocyte interaction

In order to decide whether the cytolytic action of the toxin was stoichiometric or catalytic in nature, it was necessary to search for experimental conditions where it could be shown that a given amount of toxin would lyse a certain number of cells and no more, despite prolonged exposure to the toxin. These conditions could be met by incubating 10^9 SRBC/ml for 5 hours in the presence of 0,01 HU of toxin/ml. As will be seen from Figure 6.19 under these conditions

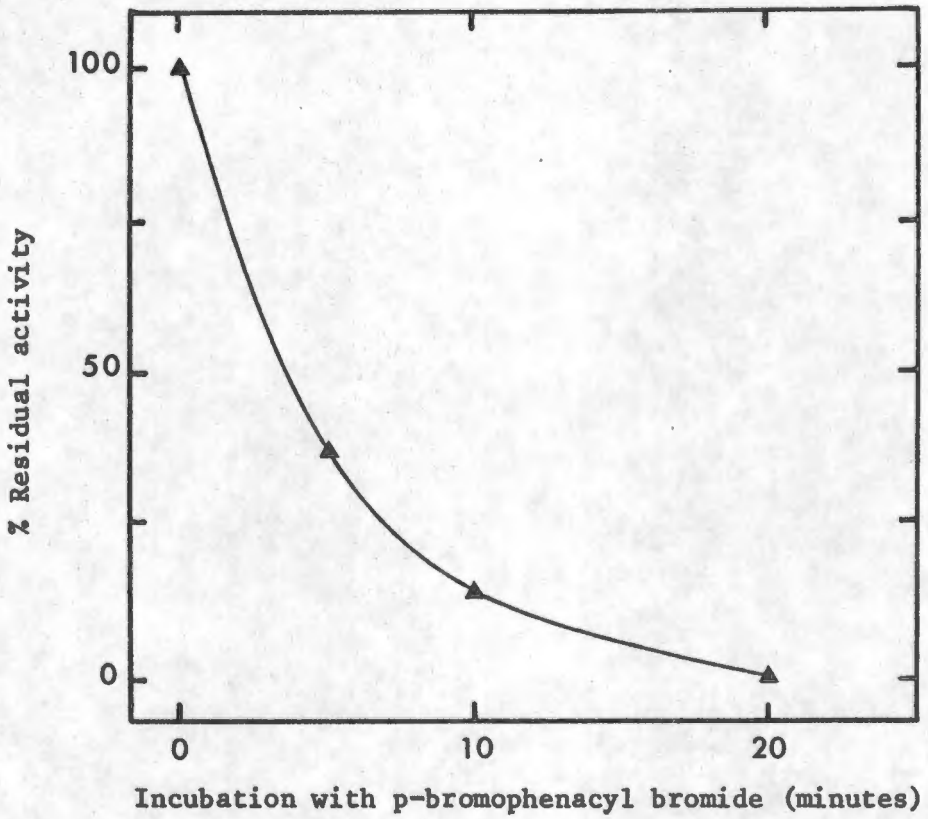


Figure 6.18 The effect of incubating the toxin with p-bromophenacyl bromide

Toxin (1000 HU/ml) was incubated with 3,3 x 10^{-7}M p-bromophenacyl bromide in 0,1M cacodylate, 0,1M NaCl, pH 6,0 at 30°C. At intervals aliquots of 0,8 ml were removed and the reaction in these aliquots stopped by adding 25 µl glacial acetic acid and cooling on ice. A control sample did not have p-bromophenacyl bromide present during the incubation. The aliquots were assayed for haemolytic activity by the tube assay and the residual activity of the treated toxin expressed as a percentage of the activity in the corresponding control sample.

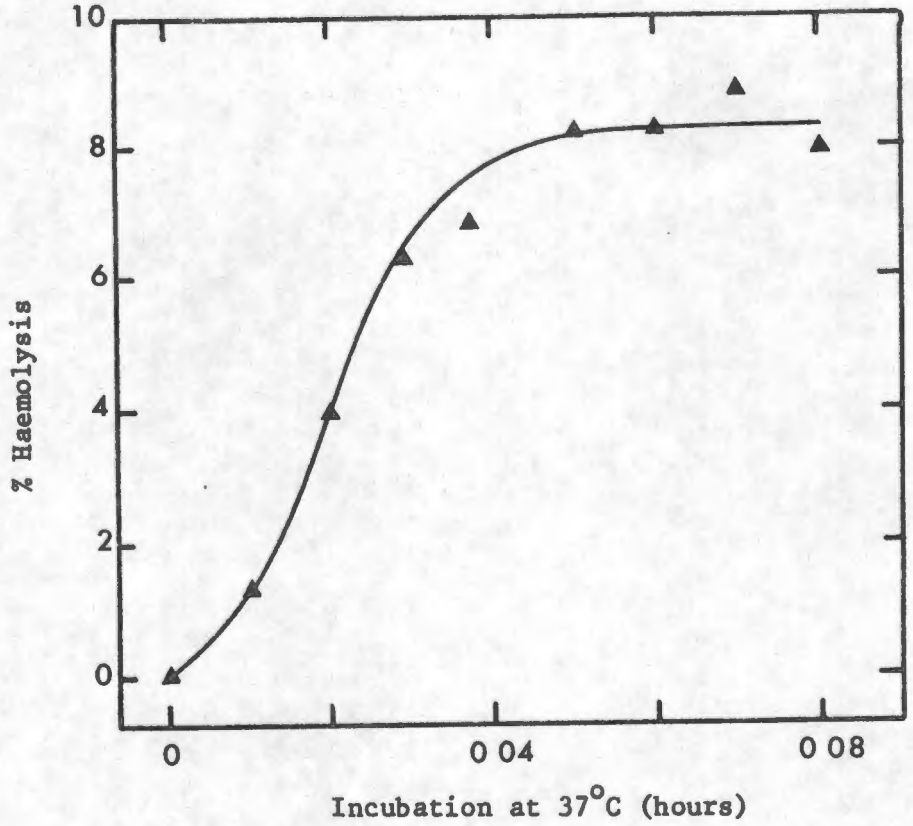


Figure 6.19 Haemolysis as a function of time of incubation of erythrocytes with toxin

A series of tubes containing 0,5 ml 0,02 HU/ml toxin in VBS-G was incubated at 37°C for 8 hours. At intervals of 1 hour 0,5 ml of 2×10^9 SRBC/ml in VBS was added to successive tubes. At the end of the 8 hour 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 haemolysis dropped to approach zero after approximately 5 hours.

haemolysis reached a plateau at 5 hrs. Figure 6.20 shows that the level of this plateau was a direct function of the initial toxin concentration on the range 0 to 0,1 HU/ml.

Further evidence for stoichiometry was provided by absorbing a solution of 1 HU/ml of toxin in CMF-VBS-G containing 1 mM EDTA with pellets of 10^5 to 10^{10} SRBC. The results, depicted in Figure 6.21 show that the activity of the toxin solution was depleted after incubation with SRBC, and that this depletion was a function of the number of cells in the preliminary incubation.

The ^{125}I-labelled toxin was used for direct demonstration of toxin binding to cells. Pellets of 0,2 x 10^9 to 1,0 x 10^9 SRBC were incubated in 1 ml CMF-VBS-G containing 1 mM EDTA and 18 000 cpm of labelled toxin. After 30 minutes at 37°C the cells were pelleted, washed four times in CMF-VBS containing 1 mM EDTA, and counted. Figure 6.22 shows that the radioactivity recovered was proportional to the number of cells in the pellet.

Conformational changes induced by calcium

The circular dichroism (CD) spectra of the toxin in 1 mM EDTA, or 10 mM Ca^{++}, or 1 mM La^{+++} showed a strong negative peak of optical activity in the 200 to 250 nm region. The amplitude of the peak in the region of 210 to 230 nm was considerably increased by the presence of Ca^{++} (Figure 6.23) or La^{+++} (Figure 6.24). This is indicative of a conformational change in the protein, probably reflecting an increase in the α-helical component of the secondary structure (Freifelder, 1976). The signal in the 250 - 300 nm region was too weak to allow any conclusions to be drawn about the tyrosine and tryptophan residues, which are said to be active at these wavelengths.

Fluorescence of the dye 8-anilino-1-naphthalene sulphonate

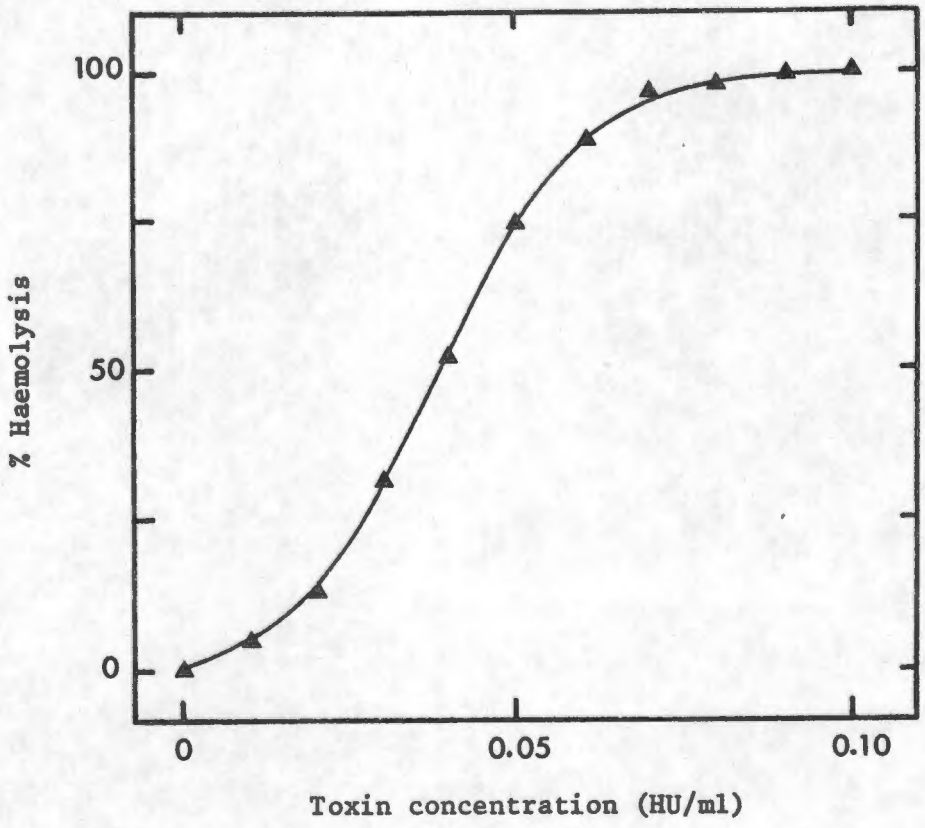


Figure 6.20 Haemolysis by different concentrations of toxin after prolonged incubation

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

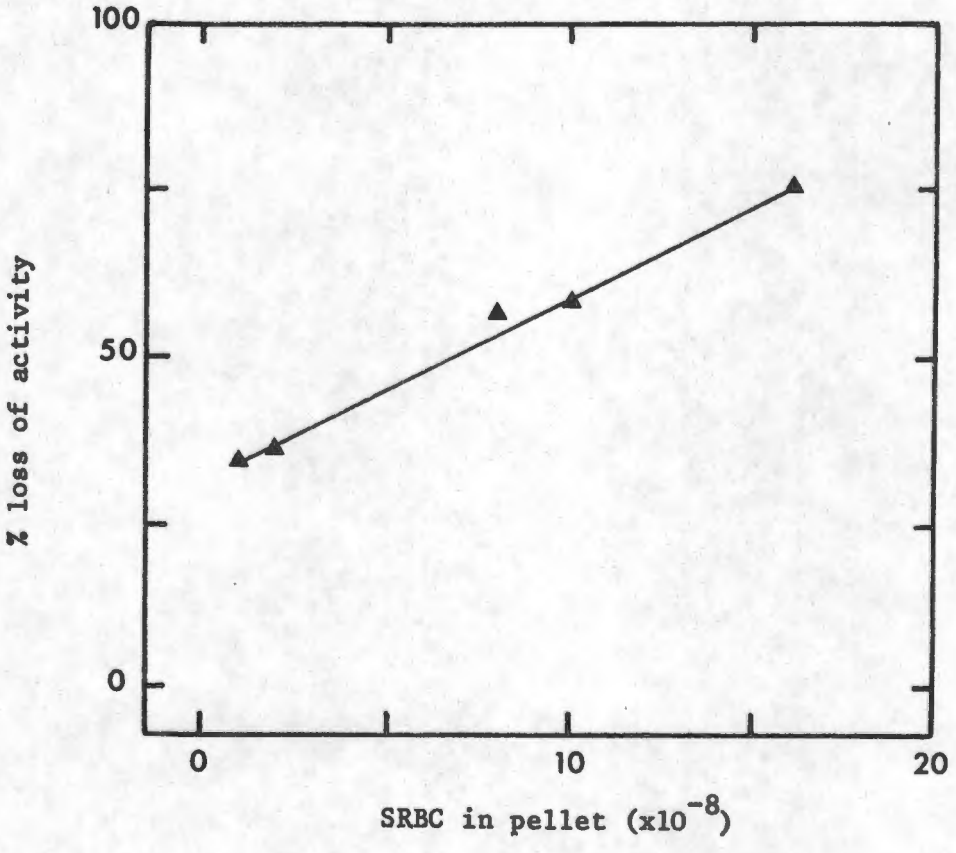


Figure 6.21 The loss of activity of a toxin solution after exposure to cells in the absence of calcium

Aliquots of 1 ml 10 HU/ml toxin in CMF-VBS-G were incubated with 10^8 to 10^9 SRBC at 37°C for 30 minutes. The cells were then pelleted and the supernatant assayed for activity using the turbidity assay. The figure shows the loss of activity of the toxin solution, expressed as a percentage of the activity of a control solution incubated without cells, plotted against the number of cells in the preincubation.

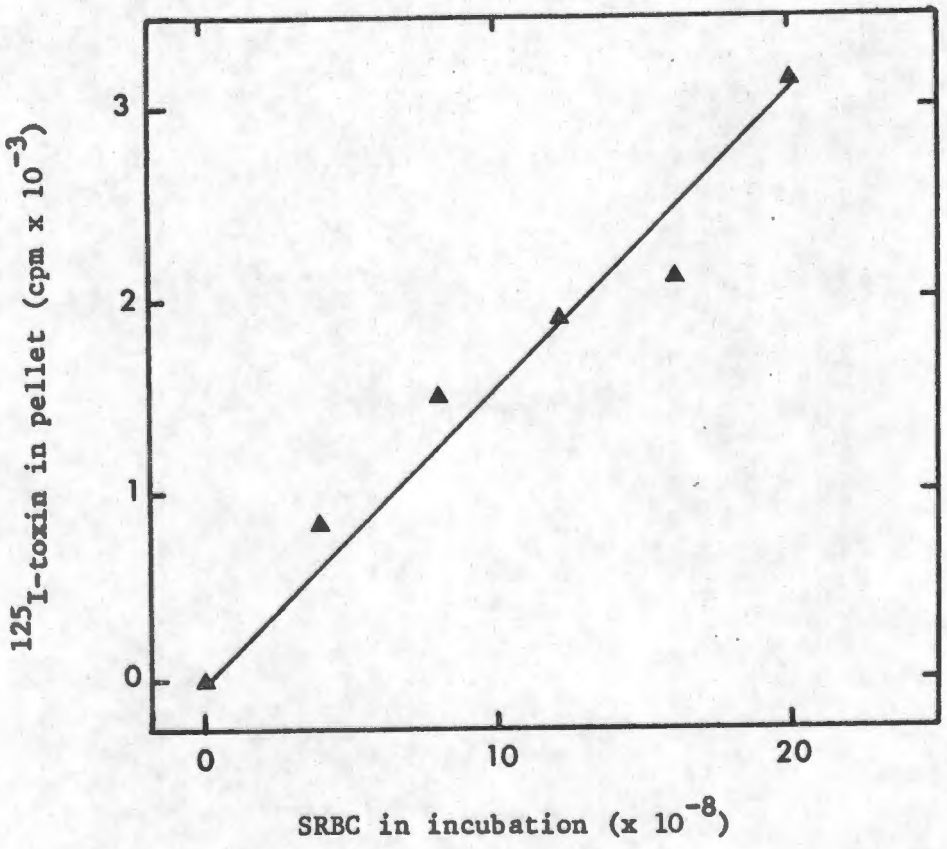


Figure 6.22 The binding of ^{125}I-labelled toxin to erythrocytes.

Pure toxin (1 mg) was labelled with ^{125}I by the chloramine T method of Greenwood et al (1963). The recovery of haemolytic activity after labelling was approximately 10%.

Pellets of 0,4 x 10^9 to 2 x 10^9 SRBC were suspended in 1 ml CMF-VBS-G containing 1 mM EDTA and 18 000 cpm of labelled toxin. After incubation at 37°C for 30 minutes the cells were pelleted, washed 4 times in CMF-VBS containing 1 mM EDTA, and counted for ^{125}I.

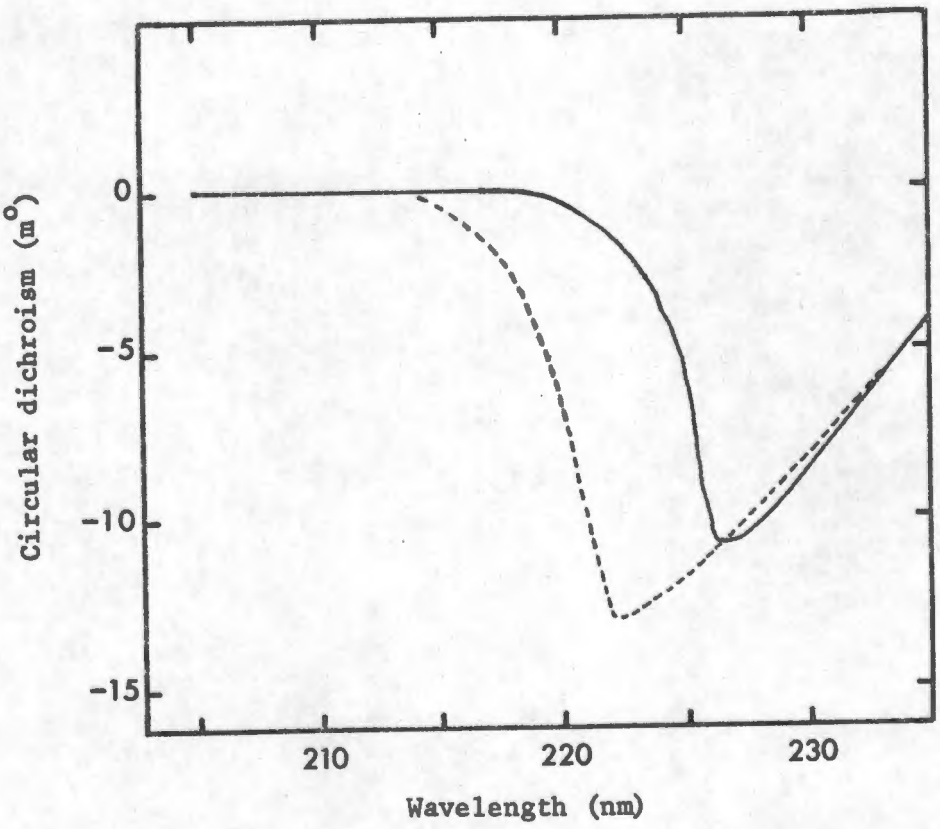


Figure 6.23 The effect of calcium on the circular dichroism spectrum of the toxin

A solution of 0,4 mg/ml pure toxin in 0,01M Tris-HCl, 10% glycerol, 1 mM EDTA, pH 8,2 was placed in a fused quartz cuvette of 2,0 cm light path. The optical activity of the solution was measured in a JASCO J40A spectropolarimeter in the range 300 to 190 nm. The toxin solution was then made to 10mM $CaCl_2$ and the procedure repeated.

There was a marked change in the CD spectrum in the range 200 to 230 nm, as is illustrated in the figure. Buffer controls showed no activity in this region of the spectrum (EDTA: —— ; CaCl : - - -).

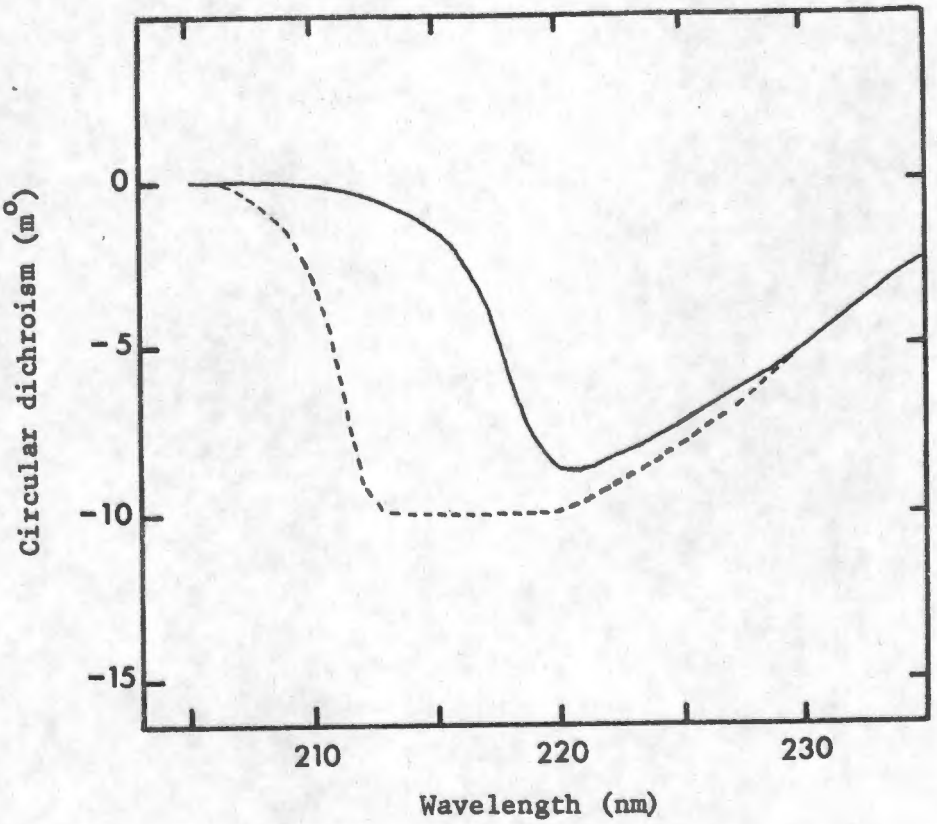


Figure 6.24 The effect of lanthanum on the circular dichroism spectrum of the toxin.

A solution of 0,4 mg/ml pure toxin in 0,01M Tris-HCl, 0,1M NaCl, 1 mM EDTA, pH 8,2 was placed in a fused quartz cuvette of 1,0 cm light path. The optical activity of the solution was measured as described for Figure 6.23. The solution was then made 0,1mM with respect to $LaCl_3$ and the procedure repeated.

As in the case of calcium, the lanthanum caused a marked change in the CD spectrum in the range 200 to 230 nm. Buffer controls again showed no activity in this region (EDTA:——; $LaCl_3$:---).

in the presence of toxin solutions was markedly increased by the addition of Ca^{++} or La^{++} . Figures 6.25 and 6.26 show that, taking the fluorescence of a toxin-ANS solution containing 1 mM EDTA as 100%, the presence of 10 mM Ca^{++} resulted in a 900% increase in fluorescence and 1 mM La^{+++} in a 1600% increase.

Species specificity

Erythrocytes from a variety of species were substituted for SRBC in the turbidity assay and used to assay a standard solution of 100 HU/ml of toxin. Figure 6.27 illustrates that there was a wide range in the rate of haemolysis for erythrocytes from the species studied. No correlation could be found between published data on the membrane phospholipid composition (Rowser et al, 1968; Wessels and Veerkamp, 1973) and the rate of haemolysis for the different species (Figure 6.28).

The effect of toxin on other biological systems

A marked change in the response of the guinea pig ileum myenteric plexus-longitudinal muscle preparation to electrical stimulation was seen after the addition of toxin at a concentration of 1 HU/ml in the bathing solution.

As illustrated in Figure 6.29 there was a transient increase in tonicity followed by a rapid fall in response until the preparation ceased to respond to the stimulus. The preparation did not recover after several washes with toxin-free medium.

The muscle response to electrical stimulation was severely inhibited by the absence of Ca^{++} and Mg^{++} in the medium but recovered fully when these ions were restored (Figure 6.30). Preparations exposed to 1 HU/ml toxin in the absence of Ca^{++} and Mg^{++} recovered

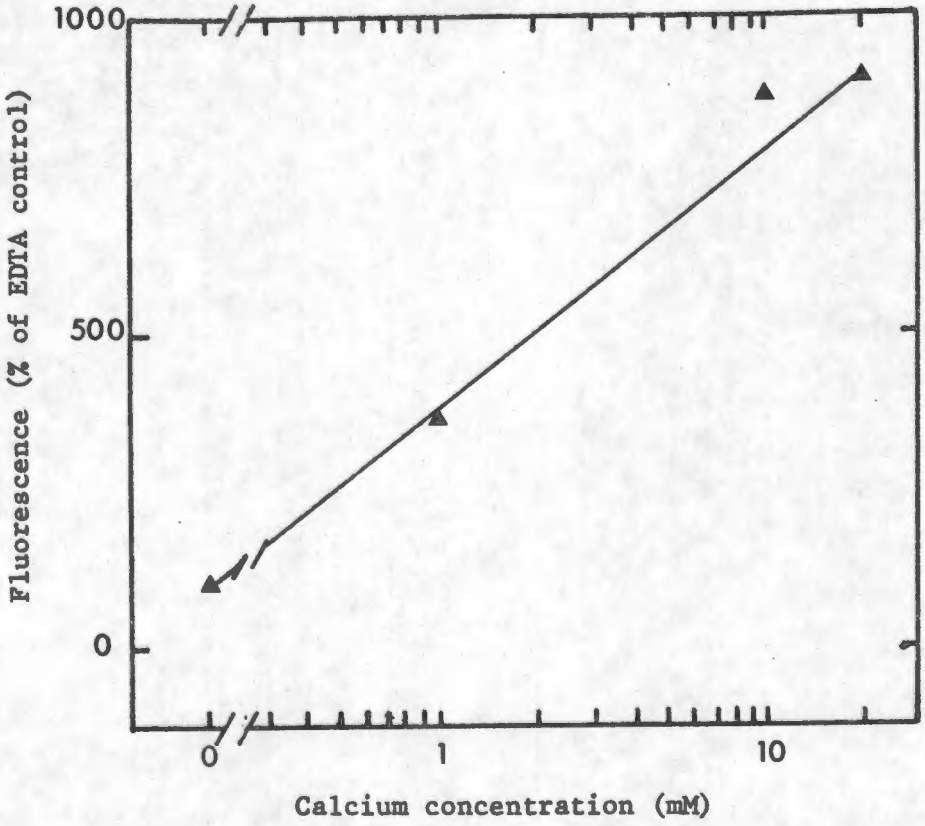


Figure 6.25 The effect of calcium on the specific fluorescence of 8-anilino-1-naphthalene sulphonate in the presence of toxin.

Solutions of 0,4 mg/ml pure toxin in 0,01M Tris-HCl, 0,1M NaCl, pH 8,2 and containing 40 µM ANS were made to 1 mM EDTA, or 1 to 10 mM $CaCl_2$. The fluorescence of the solutions was measured using a Perkin Elmer MPF-4-A fluorescence spectrophotometer with excitation at 396 nm and monitoring emission at 468 nm. The fluorescence of the preparation containing 1 mM EDTA was taken as 100%. The graph shows the percentage fluorescence of the calcium preparations plotted against the concentration of calcium.

Control solutions of ANS omitting toxin showed no change in fluorescence on the addition of $CaCl_2$.

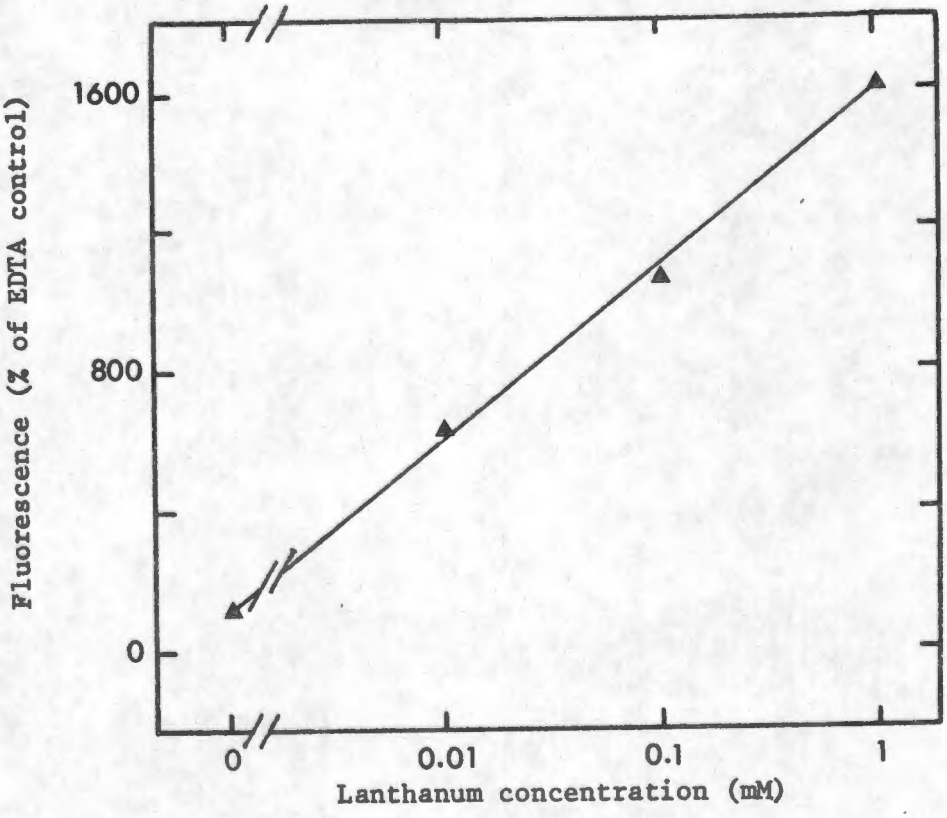


Figure 6.26 The effect of lanthanum on the specific fluorescence of 8-anilino-1-natpthalene sulphonate in the presence of toxin.

Solutions of 0,4 mg/ml pure toxin in 0,01M Tris-HCl, 0,1 M NaCl, pH 8,2 and containing 40 µM ANS were made to 1 mM EDTA, or 0,01 to 1 mM $LaCl_3$. The fluorescence of the solutions was measured at an excitation wavelength of 396 nm and an emission wavelength of 468 nm. The fluorescence of the preparation containing 1 mM EDTA was taken as 100%. The graph shows the percentage fluorescence of the lanthanum preparations plotted against the concentration of lanthanum.

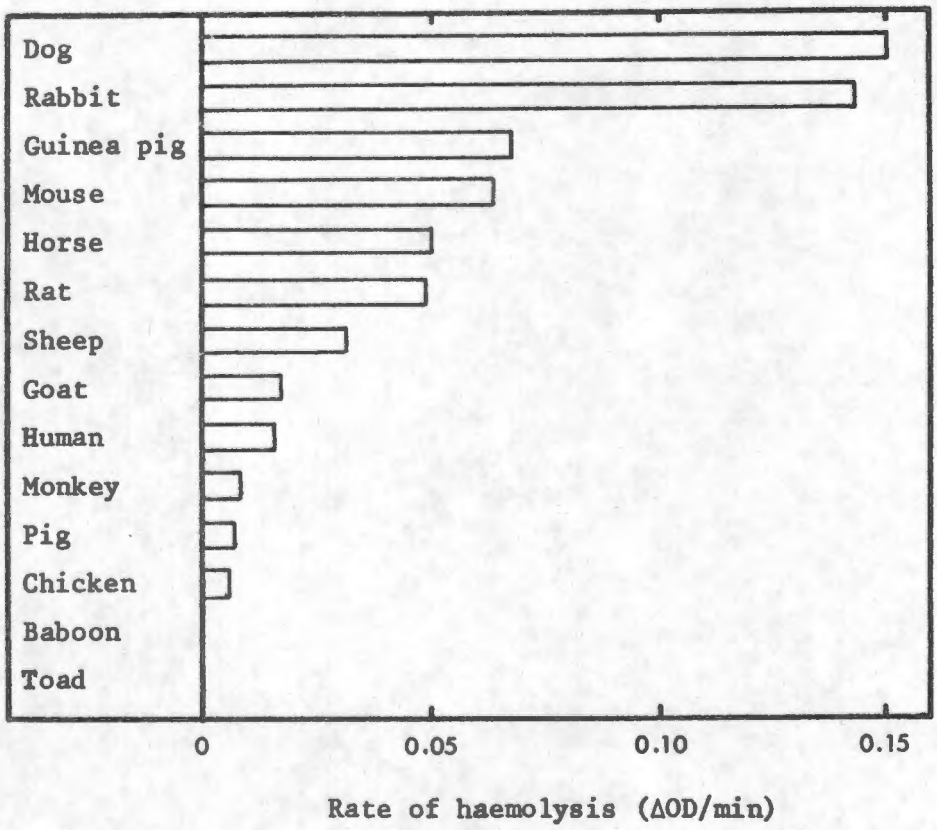


Figure 6.27 The rate of haemolysis of erythrocytes from various different species

Erythrocytes from a variety of species were substituted for SRBC in the turbidity assay and used to assay a standard solution of 100 HU/ml of toxin.

Figure 6.28

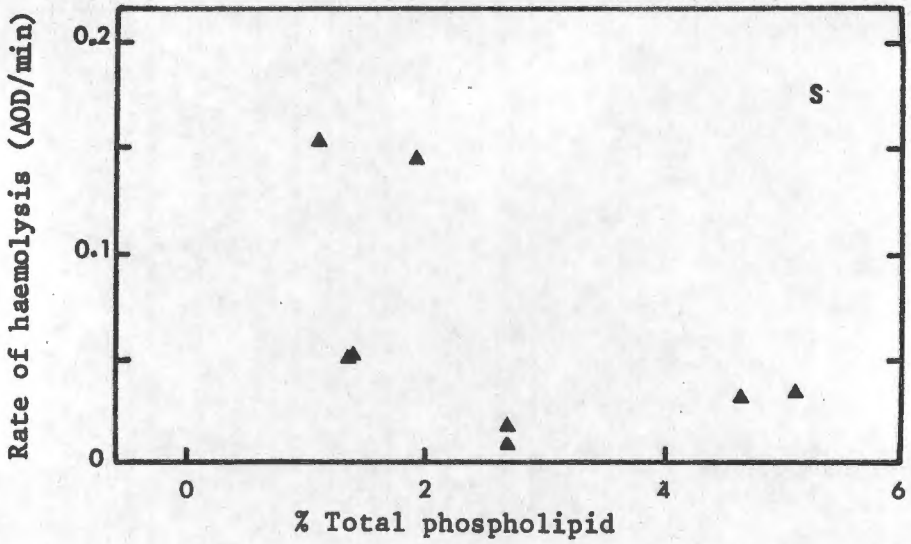
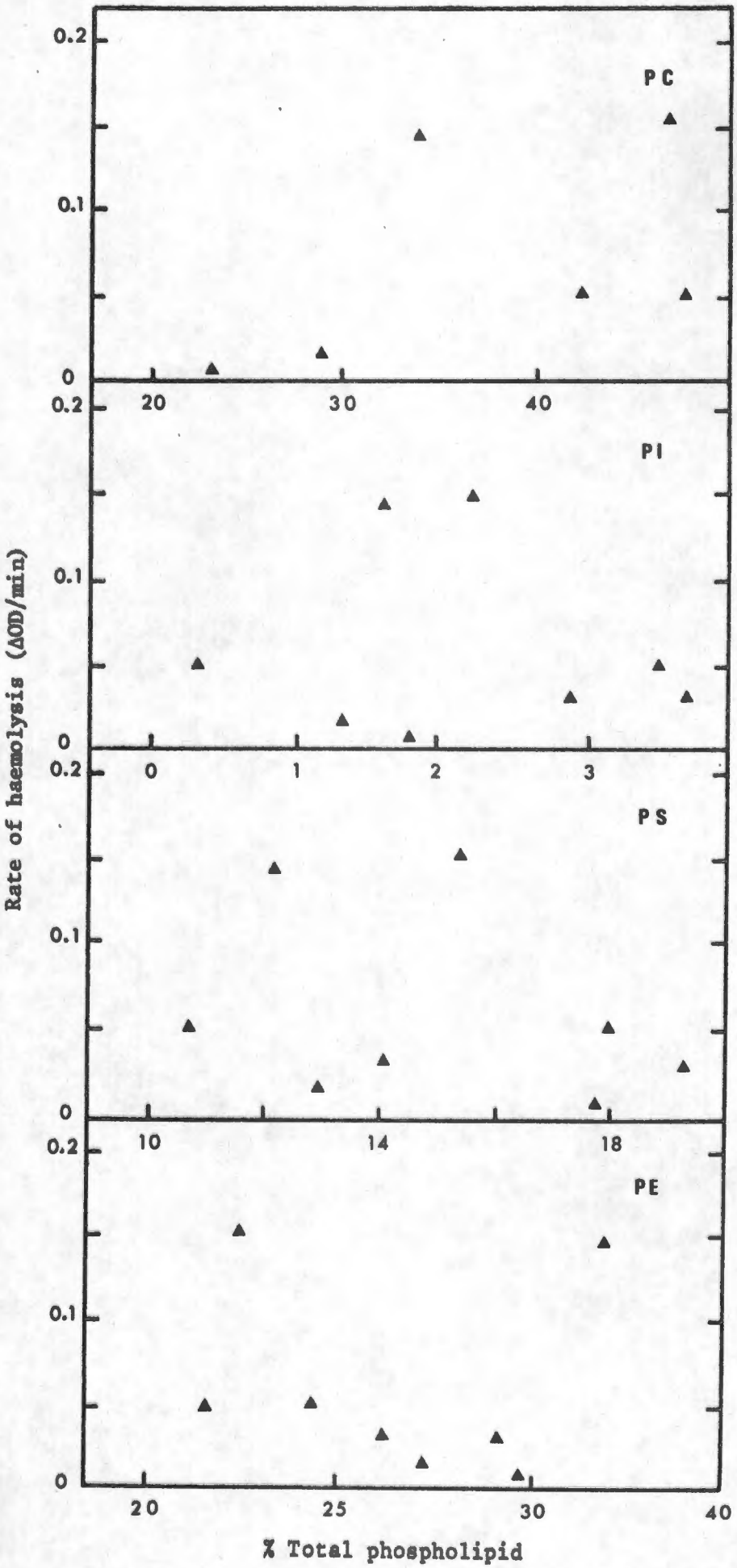


Figure 6.28 Relationship between erythrocyte membrane phospholipid composition and the rate of haemolysis by the toxin for various species

The figures are scattergrams using published data on membrane phospholipid composition (Rowser et al, 1968; Wessels and Veerkamp, 1973), and the data depicted in Figure 6.27.
S: sphingomyelin; PC: phosphatidylcholine; PI: phosphatidylinositol; PS: phosphatidylserine; PE: phosphatidylethanolamine. The phospholipid composition is expressed as a percentage of the total membrane phospholipid.


PC
PI
PS
PE
Rate of haemolysis (ΔOD/min)
% Total phospholipid
0.2
0.1
0
20
30
40
0
1
2
3
10
14
18
25

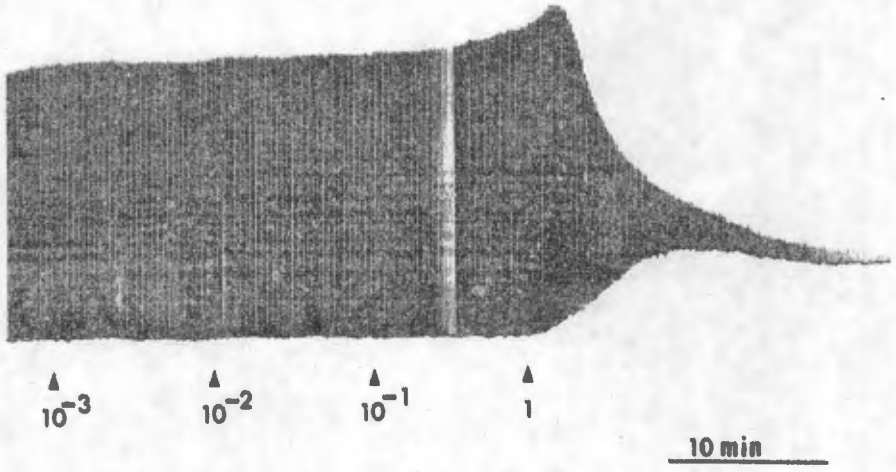


Figure 6.29 The effect of diamphotoxin on the response of the guinea pig ileum longitudinal muscle-myenteric plexus to electrical stimulus.

The preparation was set up as described by Paton and Vizi (1969) and stimulated with biphasic, 2 msec, 45V square wave pulses at a frequency of 0,1 Hz. The isometric response was measured with a force displacement transducer with a resting tension of 0,3g and recorded on a chart recorder. The preparation was bathed in 7,5 ml Krebs Ringers solution bubbled with 90% O_2, 10% CO_2 to maintain a pH of 7,4.

After establishing a stable baseline response, 75 µl aliquots of toxin solution were added to the bath at 10 minute intervals to give toxin concentrations in the bath of 10^{-3}, 10^{-2}, 10^{-1}, and 1 HU/ml. The figure shows the recording obtained from the chart recorder. The arrows mark the additions of toxin. The response of the preparation could not be restored in spite of repeated washing.

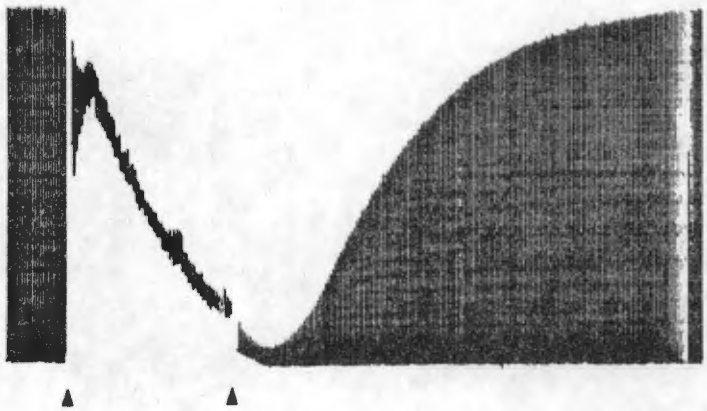


Figure 6.30 The effect of Ca^{++}- and Mg^{++}-free Krebs Ringers on the guinea pig ileum longitudinal muscle-myenteric plexus preparation.

The preparation was set up as described for figure 6.29 and stimulated until a stable baseline was obtained. The bathing-medium was then substituted by a modified Krebs Ringers containing no Ca^{++} or Mg^{++}. After 10 minutes the preparation was returned to standard Krebs Ringers containing 1,2 mM $MgCl_2$, and 2,5 mM $CaCl_2$. The Ca^{++}- and Mg^{++}-free period is marked by arrows.

to about 50% of their pre-treatment response when the toxin was washed out and the calcium and magnesium restored (Figure 6.31). In the case of one preparation this recovery was virtually 100%. This was in contrast to the complete absence of recovery for preparations exposed to toxin in the presence of Ca^{++} and Mg^{++}.

The response of the preparation to chemical stimulation by nicotine or acetyl choline was also reduced by about 50% after exposure of the preparation to toxin in the absence of Ca^{++} and Mg^{++}. In preparations exposed to toxin in the presence of these ions the response to chemical stimulation was totally abolished.

When the perfused rat heart was exposed to 1 HU/ml of toxin an increase in the P-R interval in the ECG (first order heart block) was observed in the first 5 minutes after the start of the toxin perfusion. This developed into a 2:1 block progressing rapidly through a 3:1 block to independent atrial and ventricular activity (Figure 6.32). After about 15 minutes ventricular activity ceased.

The heart rate (Figure 6.33) and coronary flow (Figure 6.34) decreased steadily from the start of the toxin exposure. An increase in LDH release could only be detected after 10 minutes (Figure 6.35).

The addition of 250 HU/ml toxin to the sarcoplasmic reticulum vesicle preparation, either during the course of the Ca^{++} uptake, or during a 10 minute pre-incubation at $25^{o}C$, had no detectable effect on the uptake of Ca^{++} by the vesicles (Figure 6.36).

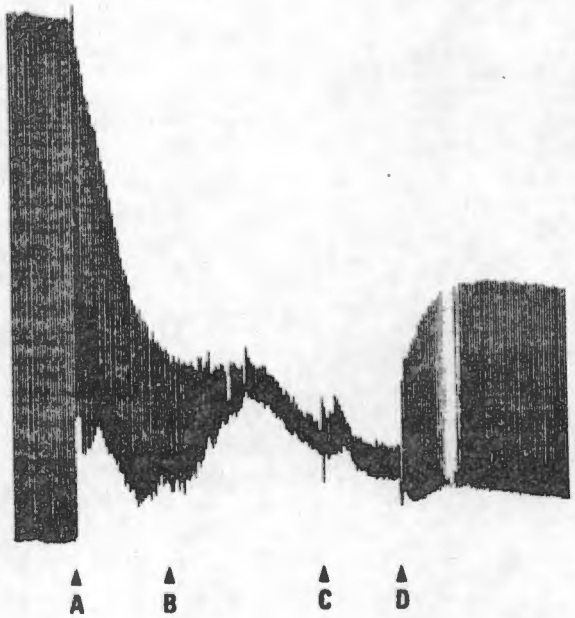


Figure 6.31 The effect of exposing the guinea pig ileum longitudinal muscle-myenteric plexus preparation to toxin in the absence of Ca^{++} and Mg^{++}

The preparation was set up and stimulated as described for Figure 6.29. When a stable baseline had been established the preparation was washed and bathed in modified, Ca^{++}- and Mg^{++}-free Krebs Ringers for 5 minutes. Toxin was then added to the bathing medium to give a final concentration of 1 HU/ml and the preparation stimulated for a further 10 minutes. The preparation was again washed and bathed in Ca^{++}- and Mg^{++}-free Krebs Ringers for 5 minutes, then returned to the standard Krebs Ringers solution. The arrows mark the changes of solution and the addition of toxin; A: change to Ca^{++}- and Mg^{++}-free Krebs Ringers; B: add toxin; C: wash out toxin with Ca^{++}- and Mg^{++}-free Krebs Ringers; D: restore to standard Krebs Ringers.

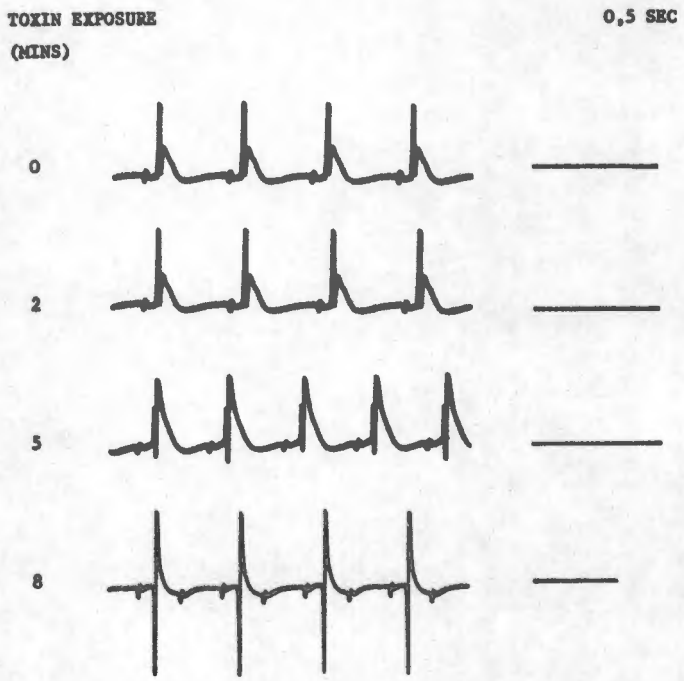


Figure 6.32 The effect of diamphotoxin on the electrocardiogram of the isolated perfused rat heart.

A 300g rat was anaesthetised with ether and injected intravenously with 200 units of heparin. The heart was excised and suspended by ligaturing the aorta over a glass tube through which it was perfused with Krebs Ringers pH 7,4 at a hydrostatic pressure of 100 cm. After a 15 minute stabilisation period the heart was perfused with Krebs Ringers containing 1 HU/ml toxin. The figure shows photographic recordings of the ECG immediately prior to the toxin perfusion (0), and after 2, 5 and 8 minutes of toxin perfusion. Note, among other changes, the development of 2:1 atrio-ventricular block after 8 mins.

Figure 6.33

Figure 6.34

Figure 6.35

Figure 6.33 The effect of diamphotoxin on the heart rate of the isolated perfused rat heart.

In the preparation described in Figure 6.32 the heart rate was estimated from the average interval between ventricular beats recorded by the ECG equipment. The figure shows the heart rate at 0, 2, 5 and 10 minutes after exposure to the toxin.

Figure 6.34 The effect of diamphotoxin on the coronary flow in the isolated perfused rat heart.

In the preparation described in Figure 6.32 a one minute collection of the perfusate was made starting at 0, 2, 5 and 10 minutes after the initiation of the toxin exposure. The volume of perfusate collected in one minute was taken as a measure of coronary flow and in the figure is plotted against duration of toxin exposure.

Figure 6.35 The release of LDH by heart tissue after exposure to diamphotoxin

The samples of perfusate collected as described in figure 6.34 were assayed for LDH activity. The figure shows LDH activity plotted against the duration of exposure to toxin.

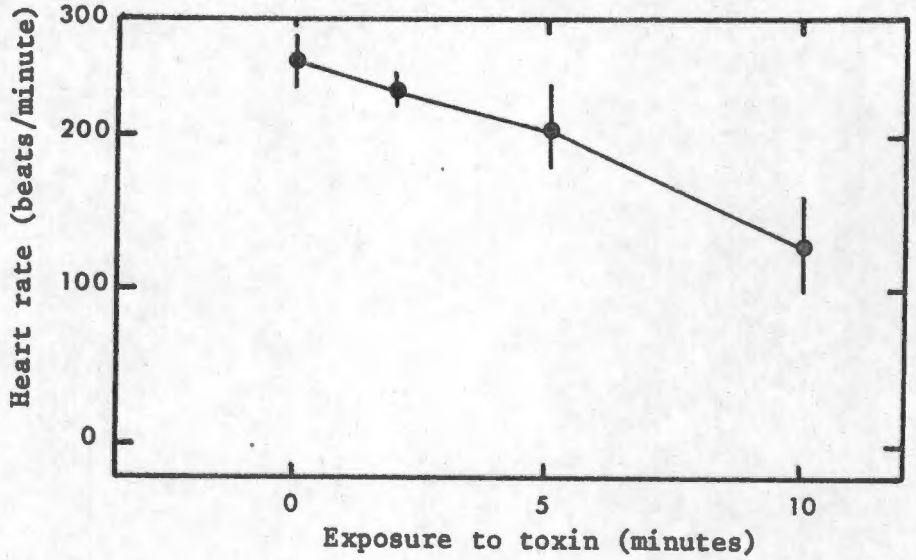


Figure 6.33

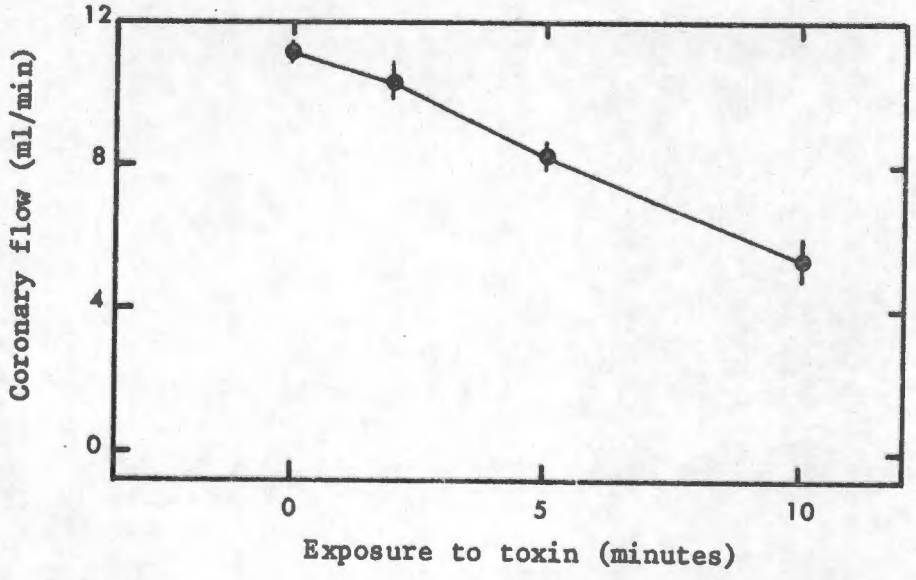


Figure 6.34

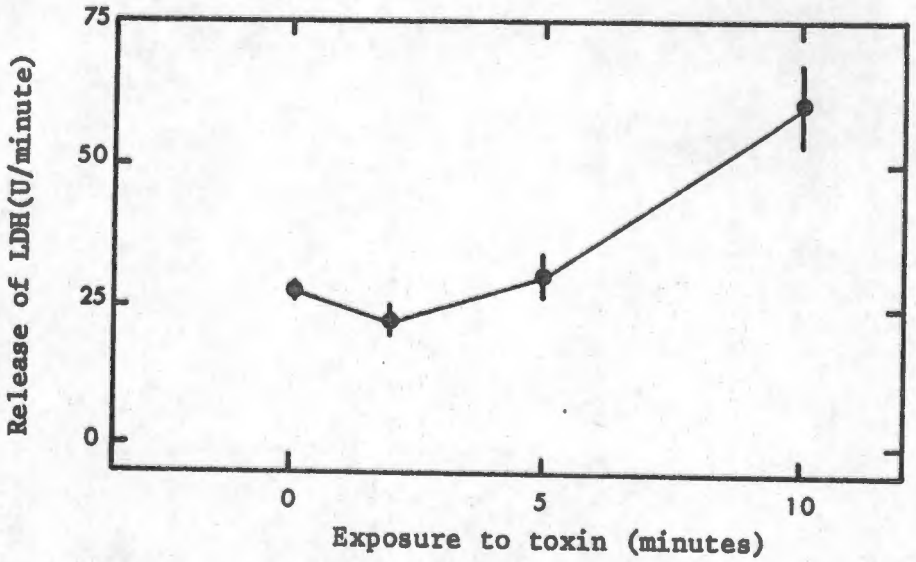


Figure 6.35

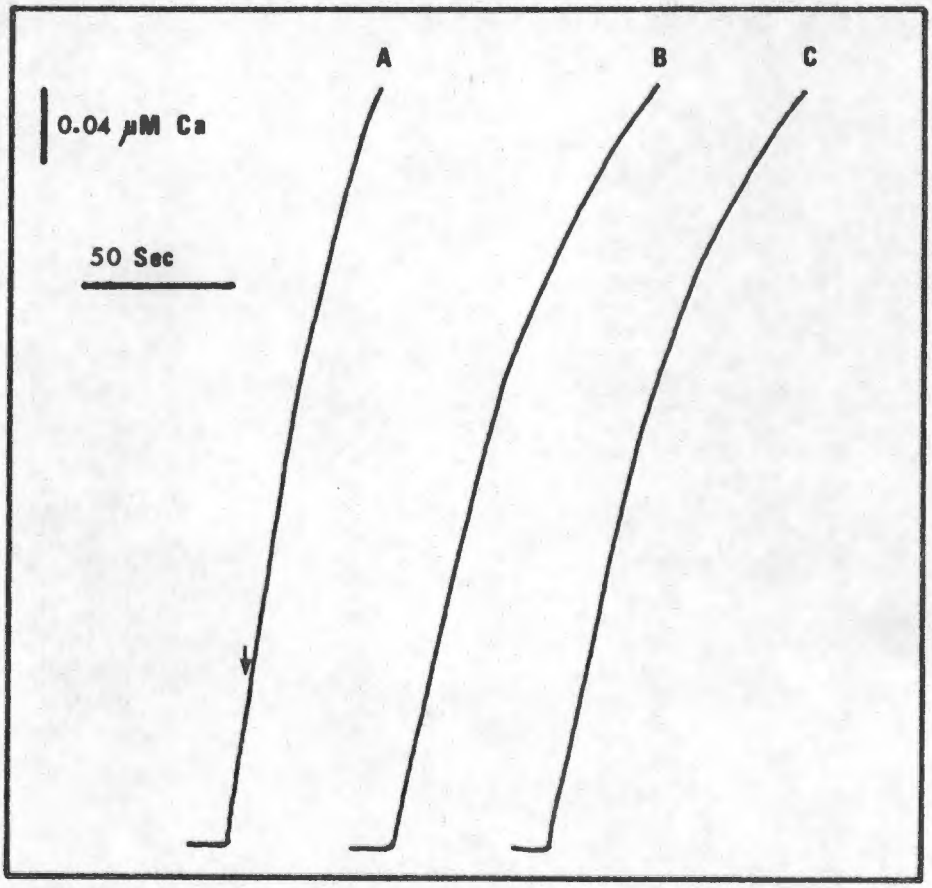


Figure 6.36 The effect of diamphotoxin on the uptake of Ca^{++} by sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle as described by Boland and Martonosi (1974) and by McIntosh and Berman (1978). Calcium uptake by the vesicles was measured by recording the rate of addition of a 0,1M solution of $CaCl_2$ required to maintain an extra-vesicular concentration of 10 μM Ca^{++}. The experimental conditions and apparatus are described in the text. The figure shows the uptake of Ca^{++} by: vesicles to which toxin was added during the Ca^{++} uptake, to give a final toxin concentration of 250 HU/ml (A): vesicles incubated for 10 minutes at 25°C prior to the Ca^{++} uptake experiment (B); and vesicles incubated with 250 HU/ml toxin for 10 minutes prior to the Ca^{++} uptake experiment (C).

DISCUSSION

The scientific literature is rich in reports on biologically derived toxins. For many of these the mechanism of action has now been elucidated and the specificity of the cellular targets has been defined. Based upon these characteristics, toxins may be divided into two broad categories. The first contains the cytolysins which usually act on the cell membrane and are lethal to almost all cell types. They include the phospholipases in snake and bee venom (Condrea and de Vries, 1964; Shipolini et al, 1971); the surfactants such as cardiotoxin and melittin, also found in snake and bee venom (Louw and Visser, 1978; Habermann and Jentsch, 1967); the ionophores from anemones and *Streptomyces conglobatus* (Michaels, 1979; Lin and Hermann, 1978); and the toxins found in *Crotalus* venom (Bjarnason and Tu, 1978).

The second comprises toxins having highly specific cellular targets. In this category one finds many of the neurotoxins such as α-bungarotoxin which binds to the post-synaptic acetylcholine receptor (Mebs et al, 1972); botulinus toxin which blocks acetylcholine release at the neuromuscular junction (Rášková and Mašek, 1970); black widow spider toxin which causes massive and uncontrolled release of neurotransmitter (Grasso, 1976); or the polypeptide in scorpion venom that blocks the closure of the action potential Na^+-ionophore in the axon (Caterall, 1977). Other toxins in this group, with specific targets outside the nervous system, include diphtheria toxin, which blocks protein synthesis by the inactivation of elongation factor 2 (Pietrowski and Stephen, 1978) and cholera toxin which stimulates adenylate cyclase activity and so disrupts intracellular control mechanisms (Sharpe and Hynie, 1971).

The striking and immediate haemolytic activity of diamphotoxin

provided a useful initial pointer to its cytolytic mode of action, and it seemed unlikely, for a number of reasons, that the toxin affected some cellular metabolic process that was essential for structural integrity. Although the red cell does possess enzymatic and other active metabolic functions that contribute to its survival *in vivo*, there is no precedent to suggest that disruption of these functions may lead to acute haemolysis *in vitro*. Ouabain, for example, inhibits erythrocyte Na^+-K^+-ATPase at 10^{-3} - 10^{-7}M but does not lead to haemolysis after 6 hours (Hülser et al, 1974).

If one considers the many haemolytic agents of biological origin three groups may be distinguished on the basis of their mechanism of action; namely the phospholipases; the ionophores; and the surfactants. The characteristics of each of these groups are pertinent to the study of the mechanism of diamphotoxin-mediated haemolysis.

The phospholipases are all proteins. Where molecular weights have been reported they fall within the range of 10 000 - 20 000 daltons (Shipolini et al, 1971; Tu et al, 1970; Botes et al, 1974). Studies of these enzymes have shown that in many cases their haemolytic activity is potentiated by synergistic interaction with the surfactant polypeptide direct lytic factor (DLF)(Condrea et al, 1964; Louw and Visser, 1977; Hessinger and Lenhof, 1976) or with detergents (Taguchi and Ikezawa, 1975). The specificity of the phospholipases appears to be dependent on the environment of the substrate molecule, since Taguchi and Ikezawa (1976) have shown that phospholipase C from *Clostridium perfringens* selectively hydrolyses phosphatidylcholine in horse erythrocyte membranes but sphingomyelin in sheep erythrocyte membranes.

The ionophores are a more heterogeneous group than the

phospholipases, and vary both in size and in mechanism of action. The relatively small polyether, ionomycin, for example is believed to act by forming a 1:1 complex with calcium ions so permitting them to cross hydrophobic barriers (Lin and Hermann, 1978). The somewhat larger polyene antibiotic amphotericin B, on the other hand complexes with cholesterol to form transmembranous hydrophilic channels (van Hoogevest, 1978). The short polypeptide gramicidin A appears to enter the membrane to form similar channels (Hladky and Haydon, 1972). The macromolecular ionophores appear to be permanently resident in the membrane where they provide hydrophilic channels whose activity may be modulated by other molecules or factors (Catterall, 1977; Michaels, 1979).

Surfactant molecules are found in a number of animal venoms. Although these can be shown to be cytolytic in their own right this usually requires very high concentration of the surfactant (Alouf and Raynaud, 1968; Mayer, 1972; Louw and Visser, 1977). Lower concentrations may potentiate the action of phospholipases. This is well illustrated by the synergistic haemolytic action achieved by the surfactant, DLF, and a phospholipase that are found together in animal venoms (Condrea et al, 1964; Hessinger et al, 1973; Hessinger and Lenhoff, 1976; Louw and Visser, 1978). Melittin and DLF, both relatively short polypeptide surfactants, have been sequenced. Habermann and Jentsch (1967) showed that melittin comprises 20 hydrophobic residues, which they propose would enter the lipid bilayer, followed by 6 basic hydrophilic residues which would remain on the surface of the membrane. Lauterwein and Wüthrich (1978) have pointed out the extensive homology in the sequences of DLF from different sources. They propose a tertiary structure for these molecules which would result in three hydrophobic loops suitable for insertion into the

membrane leaving hydrophilic basic groups at the surface.

The relationship between Diamphotoxin and other known cytolysins may be deduced from my experimental results.

After exposure of the erythrocytes to toxin there was an initial lag period during which there was a negligible release of haemoglobin. This lag period lasted 1 - 15 minutes and its duration was an inverse function of toxin concentration. The sigmoid curve depicting the subsequent release of haemoglobin as a function of time is shown in Figure 6.3.

Studying the Na^+ and K^+ fluxes after exposure of the cells to toxin provided a clearer picture than did the release of haemoglobin. After exposure to toxin the cells showed an immediate loss of K^+ and uptake of Na^+ from the medium (Figure 6.5). These fluxes clearly preceded the loss of haemoglobin. It seemed likely therefore, that the release of haemoglobin was a secondary phenomenon and resulted from osmotic swelling and rupture of the cells secondary to the disruption of osmoregulatory ionic gradients. This notion was supported by the experiment shown in Figure 6.6 in which sucrose, when present at a concentration of 0,3M in the medium, prevented the release of haemoglobin, but did not prevent the Na^+ and K^+ fluxes. The inference drawn from these results is that the toxin rendered the cell membrane permeable to the small cations Na^+ and K^+ (Stokes radii about 1) but not to sucrose and haemoglobin (Stokes radii 4,4 and 31 respectively) (Pappenheimer, 1953; Boyle et al, 1979). The extracellular, non-permeant sucrose exerted an osmotically protective effect that prevented complete erythrocytolysis. Similar results have been obtained in studies of haemolysis by complement (Green et al, 1959; Frank et al, 1965; and Boyle et al, 1979); by staphylococcal α-toxin (Madoff et al, 1964; Cooper et al, 1964); and by the haemolysin form *Listeria monocytogenes* (Kingdon, 1970).

The discovery that the toxin required divalent cations for haemolytic activity proved valuable in designing experiments to study the mechanisms involved in cytolysis. By incubating erythrocytes with toxin in calcium- and magnesium-free medium, washing free of unbound toxin, and then adding calcium, two clearly defined, sequential stages in the haemolytic process could be identified. In the first, toxin interacted with the cells to leave them intact but sensitized to the subsequent haemolytic action of calcium, or other divalent cations during the second stage.

These and other experiments have provided data to suggest that the toxin acts by becoming inserted into the cell membrane in such a manner that subsequent interaction with divalent cations brings about a conformational change in the molecule that creates an ionophoric channel for Na^+ and K^+ ions to pass.

Studies with ^{125}I-labelled toxin (Figure 6.22) showed that binding to erythrocytes did, indeed, take place in the absence of calcium. Furthermore, the action of the toxin was stoichiometric and not catalytic (Figures 6.20 and 6.21). This is as one would have expected on the basis of the model I propose above.

Calcium-independent binding of the toxin to the cells failed to take place at 0^oC, presumably because, at this temperature, the membrane phospholipid was in a largely crystalline configuration that would not allow penetration of the toxin molecules.

I was able to show, by two independent techniques, that calcium ions induce a conformational change in the toxin molecule which might render it active as an ionophore. The circular dichroism measurements indicate that calcium ions caused a change in secondary structure (Figure 6.23) while the studies using the fluorescent probe ANS, showed that calcium ions increased the number of hydrophobic

residues exposed at the surface of the toxin molecule (Figure 6.25). The latter results suggest that the molecule would penetrate deeper into the hydrophobic environment of the membrane in the presence of calcium, and provide a plausible explanation of the activating effects of this ion.

The observed inhibition of the toxin by La^{+++} was also consistent with the conformational change hypothesis. In at least two other systems La^{+++} is thought to occupy preferentially a divalent cation binding site and, because of its high charge density, distort the molecule into an inactive form. The molecules referred to are the $Na^{+}K^{+}$ ATPase (Naylor and Harris, 1976) and brain adenylate cyclase (Nathanson et al, 1976).

In a recent paper Schanne et al (1979) reported that ten different toxic compounds, including the calcium ionophore A 23187, lysolecithin, amphotericin B, melittin and silica, caused death of hepatocytes in a strictly calcium-dependent manner. They concluded that disruption of the integrity of the plasma membrane by widely differing mechanisms was followed by a common final lytic process initiated by influx of Ca^{++} into the cell across the damaged cell membrane. In their view this resulted in intracellular free calcium concentration, normally strongly buffered in the range 10^{-5}M or less (Hodgkin and Keynes, 1957; Dipolo et al, 1976; Valverde et al, 1979), rising to reach lethal levels. Unfortunately they did not do the sequential experiments in which calcium was added after the toxin compound had been withdrawn nor did they attempt to measure Ca^{++} fluxes directly.

The observations of these workers, therefore, contributed little to my studies with diamphotoxin. In experiments designed to quantitate directly the flux of ^{45}Ca across erythrocyte membranes I

could detect no measurable effect of diamphotoxin (Figure 6.5). Similarly, the results of the experiments with sarcoplasmic reticulum vesicles showed no effect of the toxin on membrane permeability for calcium (Figure 6.36).

Although there was good reason to believe that the toxin exerted its haemolytic effect by acting as a calcium regulated ionophore, other haemolytic mechanisms had to be explored. Since a number of cytolytic toxins have been shown to possess phospholipases (Condrea and de Vries, 1964; Shippolini et al, 1971; Hessinger and Lenhoff, 1974) and many phospholipases have shown a requirement for millimolar Ca^{++} (Tu et al, 1970; Hessinger et al, 1973; Martin et al, 1975; Taguchi and Ikezawa, 1975) it was important to study diamphotoxin for similar enzyme activity. Using a sensitive direct assay based on the hydrolysis of radioactively labelled lecithin, I was unable to detect any phospholipase activity in toxin preparations (Table 6.1). Since phospholipases may exhibit considerable substrate specificity, and since this specificity may be influenced by the environment of the substrate phospholipid (Taguchi and Ikezawa, 1976), it was possible that the toxin was a phospholipase with specificity for a substrate other than lecithin. To explore this possibility, erythrocyte membrane phospholipids were used as substrate.

As shown in Figure 6.16, thin layer chromatographic analysis of toxin-treated erythrocyte membranes showed no change in membrane phospholipid composition as a result of exposure to diamphotoxin. Quantitative studies using haemolytic phospholipases have shown that extensive hydrolysis of membrane phospholipids takes place before haemolysis occurs. Martin et al (1975) found up to 70% hydrolysis of membrane phosphatidylcholine by phospholipase A_2 without haemolysis. Colley et al (1973) found 75% to 80% hydrolysis of membrane sphingo-

myelin by sphingomyelinase without haemolysis. I would expect, therefore, to have been able to detect an alteration in phospholipid composition of the cell membrane had toxic activity been mediated by phospholipase.

In a number of reports haemolysis by phospholipase action has been inferred from the results of studies in which membrane components have been used as inhibitors. Streptolysin O, for example, is inhibited by lecithin (Hewitt and Todd, 1939) and cholesterol (Howard et al, 1953; Halbert et al, 1970). The cytolytic protein extracted from the sea anemone *Metridium senile* is inhibited by cholesterol (Bernheimer and Avigad, 1978), while that from *Stoichactus helianthus* is inhibited by sphingomyelin (Bernheimer and Avigad, 1976; Linder et al, 1977). The cytolytic bacterial exotoxin from *Aeromonas hydrophila* is inhibited by incubation with osmotically lysed erythrocyte membranes (Bernheimer and Avigad, 1974).

Haemolysis by diamphotoxin was unaffected by the addition of lecithin, sphingomyelin, cholesterol, or erythrocytes that had been lysed by freezing and thawing.

I concluded, therefore, that diamphotoxin does not act as a cytolysin by virtue of phospholipase activity.

This conclusion was supported by the finding that the kinetics of haemolysis were stoichiometric and were not characteristic of enzymatic catalysis (Figure 6.19 and Figure 6.20).

The conclusion that the toxin was not a phospholipase may justifiably be questioned in view of the results I obtained using p-bromophenacyl bromide and Rosenthal's inhibitor. Both of those compounds are generally regarded as phospholipase inhibitors, and both inhibited the haemolytic action of the toxin.

The inhibitor p-bromophenacyl bromide inhibits the phospholipase A_2 present in porcine pancreas (Volwerk et al, 1974); the venom of *Notechis scutatis scutatis* Halpert et al, 1976); and the venom of *Bitis gabonica* (Viljoen et al, 1977) and reacts covalently with histidine residues in the enzyme (Volwerk et al, 1974). The inactivation of diamphotoxin by this reagent cannot be interpreted to mean that the toxin is a phospholipase, since p-bromophenacyl bromide is by no means a specific inhibitor of phospholipases. It does, however, indicate an essential role for at least one histidine residue in the action of the toxin.

Rosenthal's inhibitor is an analogue of lecithin in which the positions of the choline and phosphate groups have been interchanged. It has been shown to be a competitive inhibitor of phospholipase (Rosenthal 1960). The results I obtained with this inhibitor (Figure 6.17) were interesting and unlike those one would have expected from a competitive inhibitor of the toxin inasmuch as there was a sharp step in the effect of the inhibitor between a concentration of 6×10^{-6} and 7×10^{-6}M. The most plausible explanation for this abrupt change in inhibitory capacity over this very narrow range in concentration is that, under the experimental conditions used, this concentration range spanned the critical micelle concentration of the inhibitor, and that the toxin was efficiently taken up by the micelles. The lack of the effect of the inhibitor when added in solvents other than 1:19 $CHCl_3$:CH_3OH could be due to critical requirements for a phase transition as the inhibitor is transferred from one solvent to another.

It is not clear why lecithin was not active as an inhibitor while its analogue did show activity. Once again the explanation possibly lies in the extent to which the experimental conditions

used satisfied different requirements for micelle formation by these compounds.

In their studies on the haemorrhagic venom from *Crotalus atrox* Bjarnason and Tu (1978) found significant proteolytic activity in the venom which they felt was sufficient to account for its necrotic effects on tissues. In view of these observations it seemed possible that haemolysis by diamphotoxin might be mediated by a proteolytic action. I was, however, unable to detect proteolytic activity in toxin preparations using two sensitive direct assays nor could I detect a change in the banding pattern of membrane proteins on SDS electrophoresis polyacrylamide gels after toxin induced haemolysis.

The final haemolytic mechanism that needs to be discussed is that of the toxin acting as a detergent or surfactant, in a manner similar to that in which saponin causes disruption of the plasma membrane and release of cellular contents. A number of considerations allow me to exclude the possibility with reasonable confidence.

In the first instance, a number of studies on surfactant mediated haemolysis have shown a requirement for between 10^7 and 10^{10} surfactant molecules per cell for 50% lysis (Alouf and Raynaud, 1968; Mayer, 1972; and Louw and Visser, 1977). Taking the data presented in Figure 6.20 I have calculated that approximately 100 toxin molecules per cell are sufficient to cause 50% haemolysis. These calculations are based on the observation that 0,04 HU of toxin ($3,24 \times 10^{-9}$g; $5,41 \times 10^{-14}$ moles; $3,26 \times 10^{10}$ molecules) will lyse $0,5 \times 10^9$ cells, i.e. 65 molecules/cell. Quantitatively, therefore, toxin and surfactant-induced haemolysis are different.

Secondly, toxin-induced haemolysis was relatively species specific (Figure 6.27). This is not generally the case with surfactant-

induced haemolysis.

Finally, surfactants do not show the temperature-dependence that I demonstrated with the toxin (Figure 6.15) nor do they require Ca^{++} for this haemolytic effect.

Although most of the experimental investigations of the toxin's action were carried out on sheep erythrocytes, a number of studies were performed on other biological preparations to examine the question of whether or not the toxin acted consistently on cells from other tissues.

Using the rat heart preparation it was found that exposure to the toxin caused a block in atrio-ventricular conduction followed by cessation of ventricular activity. These findings could be explained by assuming that the toxin acted as an ionophore for Na^{+} and K^{+} ions and so disrupted the trans-membrane gradients essential for coordinating electrical activity in the atrio-ventricular conduction system. Release of the macromolecular LDH molecule could only be detected after 10 minutes, parelleling the events during haemolysis. The calcium dependence of the cardiac effect of the toxin could not be tested in this preparation, since Ca^{++} deprivation on its own leads to irreversible malfunction of the heart.

The toxin disrupted the functioning of the guinea pig ileum longitudinal muscle-myenteric plexus preparation but the experiments described did not permit the identification of the site of action of the toxin. They did, however, demonstrate that the action of the toxin is calcium dependent in this system as well.

The effect of the toxin on the axonal transmembrane Na^{+} and K^{+} gradients may have accounted for the local paralysis observed in mice receiving intramuscular injections of toxin (Figure 2.3).

The studies reported in this project have brought our know-

ledge about the toxin to an advanced stage. We are able to conclude that diamphotoxin is a basic, hydrophobic protein of molecular weight 62 000 daltons. Its toxicity is the result of its insertion into the cell membrane where, in the presence of Ca^{++} or other divalent cations, it assumes a configuration which renders the membrane permeable to Na^{+} and K^{+} but not to larger molecules such as sucrose. This permeability disrupts the ionic gradients essential to physiological functions such as electrical excitability as well as leading to osmotic swelling of the cell and eventually to rupture of the membrane and release of macromolecular material from the cell.

Further studies on the toxin should examine the detailed events occurring in the membrane. These could be carried out using erythrocyte ghosts, liposomes, and artificial bilayer lipid membranes. Such techniques would allow a detailed study of the size and charge characteristics of the membrane lesion generated by the toxin and the kinetics of the movement of ions or molecules able to pass through the lesion.

A question which has not been addressed in this project concerns the role of the toxin in the organism which manufactures it - *Diamphidia nigro-ornata*. The toxin constitutes a significant proportion of the protein content of the pupae from which it is extracted, indicating that it probably plays an important functional role in these pupae. Whether this role is related to the properties of the molecule studied in this project is not clear. Clearly if the molecule does act as a $Na^{+}K^{+}$ ionophore in its parent organism it must do so under closely controlled regulation by some other factor - otherwise the toxin would be as lethal to its parent organism as it is to so many others. Some indication of a regulatory factor was gained during the purification of the toxin, where a consistent increase in

activity was observed after chromatography on HTP. This is presumably the result of the separation of the toxin from some inhibitory molecule present in the pupa.

A study of the functional role played by the toxin in its parent organism would prove a fascinating field of study.

Alouf, J.E. and Raynaud, M. (1968). Action de la streptolysine o sur les membranes cellulaires. 1. Fixation sur la membrane erythrocytaire. Annales de L'Institut Pasteur 114, 812-827.

Alfageme, C.R., Swiedler, A., Mahowald, A., and Cohen, L.H. (1974). Histones of *Drosophila* embryos. Electrophoretic isolation and structural studies. J. Biol. Chem. 249(12), 3729-3736.

Avrameas, S. and Ternyck, T. (1969). The cross-linking of protein with glutaraldehyde and its use for the preparation of immunoadsorbents. Immunochemistry 6, 53-56.

Barman, T.E. and Koshland, D.E. (1967). A colorimetric procedure for the quantitative determination of tryptophan residues in protein. J. Biol. Chem. 242(24), 5771-5776.

Beers, R.F. Jr., and Sizer, I.W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195, 133-140.

Bernheimer, A.W. (1974). Interactions between membranes and cytolytic bacterial toxins. Biochim. Biophys. Acta. 344, 27-50.

Bernheimer, A.W. and Avigad, L.S. (1976). Properties of a toxin from the sea anemone *Stoichactus helianthus*, including specific binding to sphingomyelin. Proc. Nat. Acad. Sci. U.S.A. 73(2), 467-471.

Bernheimer, A.W. and Avigad, L.S. (1978). A cholesterol-inhibitable cytolytic protein from the sea anemone *Metridium senile*. Biochim. Biophys. Acta 541, 96-106.

Bjarnason, J.B. and Tu, A.T. (1978). Hemorrhagic toxins from Western Diamond black rattlesnake (*Crotalus atrox*) venom: isolation and characterization of five toxins and the role of zinc in hemorrhagia toxin e. Biochemistry 17(16), 3395-3404.

Bjerve, K.S., Daad, L.N.W. and Bremer, J. (1974). The selective loss of lysophospholipids in some commonly used extraction procedures. Anal. Biochem. 58, 238-245.

Bligh, E.G. and Dyer, W.J. (1959). A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37(8), 911-917.

Boehm, R. (1897). Ueber das Gift der Larven von *Diamphidia locusta*. Archiv. Exp. Pathol. a. Pharm. 38, 424-427.

Boland, R. and Martonosi, A. (1974). Developmental changes in the composition and function of sarcoplasmic reticulum. J. Biol. Chem. 249(2), 612-623.

Botes, D.P. and Viljoen, C.C. (1974). *Bitis gabonica* venom. The amino acid sequence of phospholipase A. J. Biol. Chem. 249(12), 3827-3835.

Bowyer, D.E. and King, J.P. (1977). Methods for the rapid separation and estimation of the major lipids of arteries and other tissues by thin layer chromatography on small plates followed by micro-chemical analysis. J. Chromatog. 143, 473-490.

Boyle, M.D.P. and Borsos, T. (1979). Studies on the terminal stages of immune hemolysis. V. Evidence that not all complement-produced transmembrane channels are equal. J. Immunol. 123(1), 71-76.

Boyle, M.D.P., Gee, A.P. and Borsos, T. (1979). Studies on the terminal stages of immune hemolysis. VI. Osmotic blockers of differing stokes radii detect complement-induced transmembrane channels of differing size. J. Immunol. 123(1), 77-82.

Breyer-Brandwijk, M.G. (1937). A note on the Bushmen arrow poison, *Diamphidia simplex* Péringuey. Bantu Studies 11, 279-284.

Catterall, W.A. (1977a). Membrane potential-dependent binding of scorpion toxin to the action potential Na^+ ionophore. J. Biol. Chem. 252(23), 8660-8668.

Catterall, W.A. (1977b). Activation of the action potential Na^+ ionophore by neurotoxins. An allosteric model. J. Biol. Chem. 252(23), 8669-8676.

Chervenka, C.H. (1969). A manual of methods for the analytical ultracentrifuge. Spinco Division of Beckman Instruments, Palo Alto.

Cohn, E.J. and Edsall, J.T. (1943). Proteins, amino acids and peptides as ions and dipolar ions. N.Y. Reinhold Publishing Corporation.

Colley, C.H., Zwaal, R.F.A., Roelofsen, B. and van Deenen, L.L.M. (1973). Lytic and non-lytic degradation of phospholipids in mammalian erythrocytes by pure phospholipase.

Condrea, E. and de Vries, A. (1964). Hemolysis and splitting of human erythrocyte phospholipids by snake venom. Biochim. Biophys. Acta 84, 60-73.

Cooper, L.Z., Madoff, M.A. and Weinstein, L. (1964). Haemolysis of rabbit erythrocytes by purified staphylococcal alpha-toxin. I. Kinetics of the lytic reaction. J. Bacteriol. 87(1) 127-135.

Cooper, L.Z., Madoff, M.A. and Weinstein, L. (1964) Hemolysis of rabbit erythrocytes by purified staphylococcal alpha-toxin. II. Effects of inhibitors on the hemolytic sequence. J. Bacteriol. 87(1), 136-144.

Dipolo, R., Requena, J., Brinley, F.J. Jr., Mullins, L.J., Scarpa, A. and Tiffet, T. (1976). Ionised calcium concentrations in squid axons. J. Gen. Physiol. 67, 433-467.

Fahey, J.L. and Terry, E.W. (1973). Ion exchange chromatography and gel filtration. In: Handbook of experimental immunology, second edition. Ed. D.M. Weir, Blackwell, Oxford.

Frank, M.M., Rapp, H.J. and Borsos, T. (1965). Studies on the terminal stages of immune hemolysis II. Resolution of the E transformation reaction into multiple steps. J. Immunol. 94(2), 295-300.

Freifelder, D. (1976). Physical biochemistry. W.H. Freeman and Co., San Fransisco.

Granelli-Piperno, A. and Reich, E. (1978). A study of proteases and protease inhibitor complexes in biological fluids. J. Exp. Med. 148, 223-234.

Grasso, A. (1976). Preparation and properties of a neurotoxin purified from the venom of black widow spider (*Latrodectus mactans tredecimguttatus*). Biochim. Biophys. Acta 439, 406-412.

Gray, W.R. (1967). Dansyl chloride procedure. In: Methods in Enzymology (Ed. C.H.W. Hirs) Vol. II, 139-151. N.Y., A.P.

Green, H., Barrow, P. and Goldberg, B. (1959). Effect of antibody and complement on permeability control in ascites tumor cells and erythrocytes. J. Exp. Med. 110, 699-713.

Greenwood, F.C., Hunter, W.M. and Glover, J.S. (1963). The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem. J. 89, 114-123

Gros, C. and Labouesse, B. (1969). Study of the dansylation reaction of amino acids, peptides and proteins. Europ. J. Biochem. 7, 463-470.

Gross, E. (1967). The cyanogen bromide reaction. In: Methods in Enzymology (Ed. C.H.W. Hirs), Vol XI, 238-255, N.Y., A.P.

Grossman, S., Oestreicher, G., Hogue, P.K., Cobley, J.G. and Singer, T.P. (1974). Microanalytical determination of the activities of phospholipases A, C, and D and of their mixtures. Anal. Biochem. 58, 301-309.

Habermann, V.E. and Jentsch, J. (1967). Sequenzanalyse des Melittins aus den tryptischen und peptischen Spaltstücken. Hoppe Seyler's Z. Physiol. Chem. 348, 37-50.

Halbert, S.P. (1970). Streptolysin O. In: Microbial toxins (Eds. T.C. Montie, S. Kadis, and S.J. Ajl) Vol. III, 69-98, A.P., N.Y.

Halpert, J., Eaker, D. and Karlsson, E. (1976). The role of phospholipase activity in the action of a presynaptic neurotoxin from the venom of *Notechis scutatus scutatus* (Australian tiger snake). FEBS LETT 61(1), 72-76.

Hessinger, D.A. and Lenhoff, H.M. (1973). Assay and properties of the haemolysis activity of pure venom from the nematocysts of the acontia of the sea anemone *Aiptasia pallida*. Arch. Biochem. Biophys. 159, 629-638

Hessinger, D.A. and Lenhoff, H.M. (1974). Degradation of red cell membrane phospholipids by sea anemone nematocyst venom. Toxicon, 12, 379-383.

Hessinger, D.A., Lenhoff, H.M. (1976). Membrane structure and function. Mechanism of hemolysis induced by nematocyst venom: roles of phospholipase A and direct lytic factor. Arch. Biochem. Biophys. 173, 603-613

Heussen, C. and Dowdle, E.B. (1980). Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulphate and copolymerised substrates. Analytical Biochemistry 102, 196-202.

Hewitt, L.R. and Todd, E.W. (1939). The effect of cholesterol and sera contaminated with bacteria on the haemolysis produced by haemolytic streptococci. J. Pathol. Bacteriol. 49, 45-51.

Hladkey, S.B. and Haydon, D.A. (1972). Ion transfer across lipid membranes in the presence of gramicidin A. 1. Studies on the unit conductance channel. Biochim. Biophys Acta 274, 294-312.

Hodgkin, A.L. and Keynes, R.D. (1957). Movements of labelled calcium in squid giant axons. J. Physiol. (London) 138, 253-281.

How, M.W. (1970). The mountain Bushmen of Basutoland. Pretoria: J.L. van Schaik Ltd.

Howard, J.G., Wallace, K.R. and Payling Wright, G. (1953). The inhibitory effects of cholesterol and related sterols on hemolysis by streptolysin D. Br. J. Exp. Pathol. 34, 174-180.

Hülser, D.F., Ristow, H-J., Webb, D.J., Pachowsky, H., and Frank, W. (1974). Fibroblastoid and epitheliod cells in tissue culture; differences in sensitivity to ouabain and to phospholipid composition. Biochim. Biophys. Acta, 372, 85-89.

Kabat, E.A. and Mayer, M.M. (1971). Experimental Immunochemistry (second edition). Charles C. Thomas, Springfield.

Kingdon,J.R. (1970). Effects of *Lysteria monocytogenes* hemolysin on phagocytic cells and lysosomes. Infection and Immunity I(4), 356-362.

Koch, C. (1958). Preliminary notes on the coleopterological aspect of the arrow poison of the Bushmen. S. Afr. Biol. Soc. pamphlet No. 20 49-54.

Kundig, H. and Charlton, R.W. (1969). The pharmacology of the Bushman arrow poison obtained from *Diamphidia* beetle larvae. Fourth International Congress on Pharmacology, Basel.

Lauterwein, J. and Wüthrich, K. (1978). A possible structural basis for the different modes of action of neurotoxins and cardiotoxins from snake venoms. FEBS LETT. 93(2), 181-184.

Layne, E. (1957). Spectrophotometric and turbidometric methods for measuring proteins. In: Methods in Enzymology (Eds. S.P. Colowick and N.O. Kaplan) Vol. III, 447-454 A.P. N.Y.

Linder, R., Bernheimer, A.W. and Kim, K-S (1977). Interaction between sphingomyelin and a cytolysin from the sea anemone *Stoichactus helianthus*. Biochim. Biophys. Acta. 467, 290-300.

Lin, C-M. and Hermann, T.E. (1978). Characterisation of ionomycin as a calcium ionophore. J. Biol. Chem. 253(17), 5892-5894.

Lord, W.B. and Baines, T. (1876). Shifts and expedients of camp life, travel and exploration. Reprinted 1975. Africana Book Society, Johannesburg.

Louw, A.I. and Visser, L. (1977). Kinetics of erythrocyte lysis by snake venom cardiotoxins. Biochim. Biophys. Acta 498, 143-153.

Louw, A.I. and Visser, L. (1978). The synergism of cardiotoxin and phospholipase A_2 in haemolysis. Biochim. Biophys. Acta 512, 163-171.

Lowry, D.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Madoff, M.A., Cooper, L.A. and Weinstein, L. (1964). Hemolysis of rabbit erythrocytes by purified staphylococcal alpha-toxin. III Potassium release. J. Bacteriol. 87 (1), 145-149.

Maizel, J.V. (1971). Polyacrylamide gel electrophoresis of viral proteins. In: Methods in Virology 5, 179-246 Ed. K. Maramorosch and H. Koprowskie. Acad. Press. N.Y.

Martin, J.K., Luthra, M.G., Wells, M.A., Watts, R.P. and Hanahan, D.J. (1975). Phospholipase A_2 as a probe of phospholipid distribution in erythrocyte membranes. Factors influencing the apparent specificity of the reaction. Biochemistry 14(25), 5400-5408.

Mayer, M.M. (1972). Mechanism of cytolysis by complement. Proc. Nat. Acad. Sci. U.S.A. 69(10), 2954-2958.

McIntosh, D.B. and Berman, M.C. (1978). Calcium ion stabilisation of the calcium transport system of sarcoplasmic reticulum. J. Biol. Chem. 253(14) 5140-5146.

Mebs, D., Narita, K., Iwanoga, S., Samejuma, Y. and Lee C-Y (1972). Purification, properties and amino acid sequence of α-bungarotoxin from the venom of *Bungarus multicinctus*. Hoppe Seyler's Z. Physiol. Chem. 353, 243-262.

Michaels, D.W. (1979). Membrane damage by a toxin from the sea anemone *Stoichactus helianthus*. I. Formation of transmembrane channels in lipid bilayers. Biochim. Biophys. Acta 555, 67-78.

Moore, S. (1963). On the determination of cystine as cysteic acid. J. Biol. Chem. 238(1), 235-237.

Nathanson, J.A., Freedman, R. and Hoffer, B.J. (1976). Lanthanum inhibits brain adenylate cyclase and blocks noradrenergic depression of Purkinje cell discharge independent of calcium. Nature 261, 330-332.

Nayler, W.G. and Harris, J.P. (1976). Inhibition by lanthanum of the Na^+ and K^+ activated, ouabain-sensitive adenosine triphosphatase enzyme. J. Mol. Cell. Cardiol. 8, 811-822.

Neville, D.M. Jr. (1967). Fractionation of cell membrane protein by disc electrophoresis. Biochim. Biophys. Acta 133, 168-170.

Nikodem, V. and Frisco, J.R. (1979). Protein fingerprinting by SDS-gel electrophoresis after partial fragmentation with CNBr. Anal. Biochem. 97, 382-386.

Ouchterlony, Ö, and Nilsson, L.Å. (1967). Immunodiffusion and immunoelectrophoresis. In: Handbook of Experimental Immunology, second edition. Ed. Weir, D.M., Blackwell Scientific Publications, Oxford.

Pappenheimer, J.R. (1953). Passage of molecules through capillary walls. Physiol. Rev. 33, 387-423.

Paterson, W. (1789). A narrative of four journeys into the country of the Hottentots and Caffraria. London: J. Johnson pp. 62,162,169.

Paton, W.D.M. and Vizi, E.S. (1969). The inhibitory action of noradrenalin and adrenalin on acetylcholine output by guinea-pig ileum longitudinal muscle strip. Br. J. Pharmacol. 35, 10-28.

Pietrowski, R.A. and Stephen, J. (1978). Reconstitution of fully active diphtheria toxin from purified fragments A and B. FEBS LETT 87(2), 311-314.

Rásková, H. and Mašek, K. (1970). Pharmacology of bacterial protein toxins. In: Microbial toxins. Ajl, S.J., Kadis, S. and Montie, T.D. (eds) Academic Press, N.Y.

Rosenthal, A.F. and Geyer, R.P. (1960). A synthetic inhibitor of venom lecithinase A. J. Biol. Chem. 235(8), 2202-2206.

Rowser, G., Nelson, G.J. and Eleischer, S. (1968). Lipid composition of animal cell membranes, organelles and organs. In: Biological Membranes. Physical fact and function. Ed. D. Chapman, Academic Press, N.Y.

Schanne, F.A.X., Kane, A.B., Young, E.E. and Farber, J.L. (1979). Calcium dependence of toxic cell deaths on final common pathway. Science 206, 700-702.

Schapera I. (1924). Bushman Arrow Poisons. London: Benn Brothers Ltd. reprint from Discovery 6(65).

Schinz, H. (c 1886). On the arrow poison of the Kalahari-San. Unpublished typed manuscript in S.A. Museum Library, Cape Town. ref. E. p. 280.

Sharpe, G.W.G. and Hynie, S. (1971). Stimulation of intestinal adenyl cyclase by cholera toxin. Nature 229, 266-269.

Shaw, E.M., Wooley, P.Q.L. and Rae, F.A. (1963). Bushmen arrow poisons. Cimbebasia 7, 2-41.

Shipolini, R.A. (1971). Phospholipase A from bee venom. Eur. J. Biochem. 20, 459-468.

Spackman, D.H., Stein, W.H., and Moore, S. (1958). Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30(7), 1190-1206.

Steyn, D.G. (1957). A Bushmen arrow poison. S. Afr. Med. J. 31, 119-120.

Stryer, L. (1965). The interaction of a naphthalene dye with apomyoglobin and apohemoglobin. A fluorescent probe of non-polar binding sites. J. Mol. Biol. 13, 482-495

Svensson, H. (1954). The second-order aberrations in the interferometric measurement of concentration gradients. Optica Acta 1(1), 25-32.

Taguchi, R. and Ikezawa, H. (1975). Phospholipase C from *Clostridium novyi* type AI. Biochim. Biophys. Acta 409, 75-85.

Taguchi, R. and Ikezawa, H. (1976). Studies on the hemolytic and hydrolytic actions of phospholipases against mammalian erythrocyte membranes. Arch. Biochem. Biophys. 173, 538-545.

Toepfer, H. (1965). Model 120C amino acid analyser instruction manual. Spinco Division, Beckman Instruments Inc., Palo Alto, California.

Tu, A.T., Passey, R.B., and Toom, P.M. (1970). Isolation and characterisation of phospholipase A from sea snake *Laticauda semifasciata* venom. Arch. Biochem. Biophys. 140, 96-106.

Unkeless, J.C., Danø, K., Kellerman, G.M. and Reich, E. (1974). Fibrinolysis associated with oncogenic transformation, partial purification and characterisation of the cell factor, a plasminogen activator. J. Biol. Chem. 249, 4295-4305.

Valverde, I., Van der Meers, A., Anjaneyulu, R. and Malaisse, W.J. (1979). Calmodulin activation of adenylate cyclase in pancreatic islets. Science 206, 225-227.

van Holde, K.E. (1975). Sedimentation analysis of proteins. In: The Proteins. 3rd edition Vol. 1 (eds) H. Neurath, R.L. Hill and C-L Boeder. New York, Academic Press.

van Hoogevest, P. and de Kruijff, B. (1978). Effect of amphotericin B on cholesterol-containing liposomes of egg phosphatidylcholine and didecosenoyl phosphatidylcholine. A refinement of the model for the formation of pores by amphotericin B in membranes. Biochim. Biophys. Acta 511, 397-407.

Viljoen, C.C., Visser, L. and Botes, D.P. (1977). Histidine and lysine residues and the activity of phospholipase A_2 from the venom of *Bitis gabonica*. Biochim. Biophys. Acta 483, 107-120.

Volwerk, J.J., Pieterson, W.A. and de Haas, G.H. (1974). Histidine at the active site of phospholipase A_2. Biochem. 13(7), 1446-1454.

Waxdal, M.J., Konigsberg, W.H., Henley, W.L. and Edelman, G.M. (1968). The covalent structure of a human γG-immunoglobulin. II Isolation and characterisation of the cyanogen bromide fragments. Biochemistry 7(5), 1959-1966.

Weast, R.C. (1974). Handbook of Chemistry and Physics. 55th edition. CRC Press, Cleveland.

Weiner, A.M., Platt, T. and Weber, K. (1972). Amino terminal sequence analysis of proteins purified on a nanomole scale by gel electrophoresis. J. Biol. Chem. 247(10), 3242-3251.

Wells, M.A. and Hanahan, D.J. (1969). Studies on phospholipase A. I. Isolation and characterisation of two enzymes from *Crotalus adamanteus* venom. Biochemistry 8(1), 414-424.

Werkheiser, W.C. and Bartley, W. (1957). The study of steady-state concentrations of internal solutes of mitochondria by rapid centrifugal transformation to a fixation medium. Biochem. J. 66, 79-91.

Wessels, J.M.C. and Veerkamp, J.H. (1973). Some aspects of the osmotic lysis of erythrocytes. III. Comparison of glycerol permeability and lipid composition of red blood cell membranes from eight mammalian species. Biochim. Biophys. Acta. 291(1), 190-196.

Witkop, B. (1968). Chemical cleavage of proteins. Science, 162, 318-326.

Yphantis, D.A. (1964). Equilibirum ultracentrifugation of dilute solutions. Biochemistry 3 (3), 297-317.