

CLOSTRIDIUM PERFRINGENS TYPE D EPSILON PROTOTOXIN. SOME CHEMICAL, IMMUNOLOGICAL AND BIOLOGICAL PROPERTIES OF A HIGHLY PURIFIED PROTOTOXIN

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ABSTRACT

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Highly purified *Cl. perfringens* type D epsilon prototoxin was prepared by ammonium sulphate precipitation and DEAE cellulose chromatography of culture filtrate of cultures of *Cl. perfringens* type D (Strain ET 468). Preparations of prototoxin were electrophoretically heterogeneous. The protein bands demonstrable in polyacrylamide gel electrophoresis were, however, all immunologically identical and toxic. The faster moving bands were shown to be degradation products of the main prototoxin band which was the slowest moving of the major bands. There was an inverse relationship between electrophoretic mobility and the activation ratio of these degradation products. The undegraded prototoxin could be separated from its degradation products by CM cellulose chromatography but degradation appears to be a continual process and isolation of an absolutely pure product was not achieved.

INTRODUCTION

Clostridium perfringens type D produces two major lethal toxins and a number of minor lethal or non-lethal toxins (Brooks, Sterne & Warrack, 1957). The principal toxin is epsilon toxin which causes enterotoxaemia, a disease of great economic importance in all sheep breeding countries. Epsilon toxin is produced in the form of a non-toxic prototoxin which is activated by proteolytic enzymes.

Epsilon prototoxin has been purified by methanol precipitation (Verwoerd, 1958, 1960) and by ion exchange chromatography (Thomson, 1963; Orleans, Richards & Jones, 1960; Hauschild, 1965; Habeeb, 1969). Prototoxin prepared by Habeeb was of higher toxicity than prototoxin prepared by other workers but was not electrophoretically homogeneous. The electrophoretically distinguishable fractions were, however, immunologically identical. Habeeb (1969) estimated the molecular mass of his prototoxin to be 23 200 to 25 000 which is considerably less than the estimates of Orleans *et al.* (1960) and Thomson (1963) which were 38 000 and 40 000 respectively.

The results reported in this paper largely confirm and extend the results reported by Habeeb (1969).

METHODS

Production of epsilon prototoxin

Cl. perfringens type D (Strain ET 468) was grown at 37°C in Wright's broth medium from which meat particles had been removed by filtration through gauze and to which 3% insoluble dextrin had been added (Jansen, 1965). The culture was grown in a 20 l fermentation tank with the pH automatically controlled at 7.0 by the addition of concentrated NaOH by an automatic titrator. Alternatively toxic

culture was kindly supplied by the Onderstepoort vaccine production section where epsilon toxin is produced from the same strain in a large 800 l fermentation tank. Growth in this type of system (as measured by NaOH consumption) is usually complete in 8 to 12 hours and the cultures are harvested at 24 hours. The toxin production was measured by a flocculation test (Jansen, 1961, 1965) and yields of 250 to 400 Lf/ml were generally obtained. Cultures producing lower yields of toxin were found to be less suitable for prototoxin production and were not used.

"Crude prototoxin" was obtained by centrifuging the culture fluid to remove cells and debris, followed by ammonium sulphate precipitation (350 g/l), dialysis of the redissolved precipitate against distilled water and freeze-drying (Habeeb, 1969). All the above steps were carried out at 4°C.

"Purified prototoxin" was prepared on a DEAE cellulose column which was equilibrated with 0.005 M phosphate buffer, pH 7.2. The total yield of crude freeze-dried prototoxin from about 5 l of culture was dissolved in 100 ml of 0.005 M phosphate buffer, pH 7.2 and applied to a 2.5 × 40 cm column of DEAE cellulose. The column was eluted with the same buffer and the eluate monitored at 280 nm. The first peak eluted was immediately freeze-dried. In this manner about 400 mg of "purified" prototoxin could be prepared in a single run.

Nitrogen determinations

Kjeldahl nitrogen was determined by a standard micro Kjeldahl method (C. von Holt, Dept. Biochemistry, University of Cape Town, personal communication 1969).

Preparation of a monospecific anti-epsilon toxin serum

A monospecific horse anti-epsilon toxin serum was prepared by re-immunization of a horse which already had a basic immunity (Jansen, 1965). The horse was

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immunized with formalinized, trypsinized ammonium sulphate precipitated epsilon toxoid containing 700 Lf/ml. It was given 2, 4, 8 and 16 ml of the toxoid at weekly intervals by subcutaneous injection and bled 3 days after the last injection.

The serum obtained gave only a single precipitin line in agar double diffusion precipitin tests against crude prototoxin and contained about 650 Lf/ml of antibody.

Flocculation tests

Flocculation tests were done according to the method of Jansen (1961, 1965). Standardised flocculation serum (625 Lf/ml) was kindly supplied by Prof. B. C. Jansen*.

Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was carried out according to the method of Habeeb (1969), in 7.5% polyacrylamide gels with Tris-glycine buffer, pH 8.9 (5.16 g Tris and 3.48 g glycine per litre) in the electrode wells. Gels 65 mm long and 5 mm in diameter were used. After pre-electrophoresis for 60 min at a current of 2 mA per gel the sample containing 60 to 120 µg of prototoxin dissolved in 30% (m/v) sucrose or 50% (v/v) glycerol was layered on top of the gel and electrophoresis was continued for about 75 min at a current of 2 mA per gel. Gels were stained in 0.3% (m/v) amido black in 25% (v/v) ethanol and 7% (v/v) acetic acid, and destained electrophoretically in 25% (v/v) ethanol and 7% (v/v) acetic acid.

Polyacrylamide gels to be used for toxicity or radial diffusion experiments were placed in test tubes which were dipped into an ethanol dry ice mixture until the gels were thoroughly frozen. Frozen gels were stored at -20°C. When required they were sliced into 2 mm sections with a gel slicer or laid next to stained gels, which had been run in parallel, and the required sections excised with a scalpel blade.

Immuno-electrophoresis

Immuno-electrophoresis was conducted on microscope slides in 1.5% agarose dissolved in Tris-glycine buffer, pH 8.9 (5.16 g Tris and 3.48 g glycine per litre). Two ml of molten agarose solution was pipetted onto each slide and a central trough and two antigen wells cut in the agar with a suitable pattern cutter. Samples of 1.5 µl (± 20 mg/ml of prototoxin) were placed in the antigen wells and a current of 2 mA per slide applied for 40 min. The central trough was filled with monospecific anti-epsilon toxin serum and the slides incubated at 37°C in a humidity chamber for 20 hours. Slides were then repeatedly washed in isotonic saline, stained in 0.3% (m/v) amido black dissolved in acetic acid and methanol (one volume to nine volumes) and destained in acetic acid methanol solution (1:9).

Radial diffusion tests

1.0 Gramme of agarose was dissolved in 100 ml of phosphate buffer, pH 7.2 at 105°C and cooled to 60°C in a waterbath, 1 ml of suitably diluted monospecific

anti-epsilon toxin serum and 1 ml of 1% (m/v) merthiolate solution were added and well mixed. The entire mixture was spread on a 20 × 20 cm glass chromatography plate and allowed to gel. Frozen polyacrylamide gels were cut into 2 mm slices and the slices laid out in rows on the agarose plate. The plate was incubated at 37°C in a humidity chamber for 72 hours and the precipitin rings which developed measured with a vernier calliper. If a permanent record of the plate was required it was stained in the same way as described for immuno-electrophoresis and photographed.

Mouse toxicity tests

Mouse toxicity tests were done according to the method of Habeeb (1969) using a rather low grade commercial preparation of Trypsin for toxin activation. The activation ratio is defined as:

Maximal dilution of activated toxin which kills 2 out of 3 mice

Maximal dilution of non-activated prototoxin which kills 2 out of 3 mice

Sections excised from frozen polyacrylamide gels were crushed with a glass rod and 9.5 ml of 0.1 M phosphate buffer, pH 8.0, added. The suspensions were allowed to stand for 30 min at room temperature and two aliquots taken from the supernatant. To one aliquot a tenth volume of 5% (m/v) trypsin and to the other a tenth volume of buffer was added. Both aliquots were incubated at 37°C for 30 min, doubling dilutions were made in peptone saline solution 1% (m/v) Oxoid peptone in 0.25% (m/v) NaCl and 0.5 ml amounts were injected intravenously into mice. Three mice were injected with each dilution and the MLD was taken as the amount which killed two out of three mice.

Degradation of toxin

Purified prototoxin was dissolved at the rate of 20 mg/ml water and dialyzed against 0.01 M Tris-HCl buffer of pH 7.5; 8.0; 8.5 and 9.0 in the absence or presence of mercaptoethanol, glycerol, EDTA and sodium bisulphite (see Results). The solutions were incubated at 37°C for up to 14 days. Aliquots were removed from the samples at varying intervals and submitted to polyacrylamide gel electrophoresis.

Carbohydrate analysis

The carbohydrate content was estimated by the phenol, sulphuric acid method described by Williams & Chase (1968).

Cation exchange chromatography of purified prototoxin

A 1.5 × 30 ml column of CM cellulose was equilibrated with 0.02 M phosphate buffer pH 6.3. Forty mg of purified prototoxin was dissolved in 0.01 M phosphate buffer, pH 6.3, and dialyzed against the same buffer before applying it to the column. The column was eluted with this buffer.

RESULTS

Purification of prototoxin

Purification of culture supernatant by ammonium sulphate precipitation followed by DEAE cellulose chromatography resulted in an approximately ninety-fold purification of epsilon toxin as judged by the flocculation titre per mg N in the preparations (Table 1).

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TABLE 1 Purification of epsilon prototoxin in terms of flocculation units of epsilon toxin per mg N (2 batches of prototoxin)

Purification step	Lf/mg N	
	Batch 1	Batch 2
Culture supernatant.....	153	155
Ammonium sulphate precipitate (crude prototoxin).....	3 500	3 900
DEAE cellulose chromatography (purified prototoxin).....	11 700	13 700

Properties of purified prototoxin

The purified prototoxin had the properties of a typical protein. Ultraviolet spectrophotometry showed that it had a typical protein absorption spectrum with a peak at 278 nm. For the purposes of this investigation the nitrogen content multiplied by 6,25 was regarded as the protein mass. It was found that a solution containing 1 mg/ml of protein ($N \times 6,25$) had an optical density (OD) of 1,01 OD units (standard deviation: 0,05) at 278 nm in a 1 cm cuvette. The

extinction coefficient $E_{278 \text{ nm}}^{1\%} = 10$ was therefore generally used for estimating protein content. Purified prototoxin contained about 5% carbohydrate as estimated by the phenol, sulphuric acid method.

The toxicity of purified prototoxin batches varied from $1,6 \times 10^6$ to $3,2 \times 10^6$ MLD/mg protein. The activation ratio of various batches was invariably $\geq 1 000$ indicating that most of the toxin was in the form of prototoxin. Flocculation titres varied from 1 875 to 2 187 Lf/mg protein.

Electrophoretic and immunological properties

Polyacrylamide gel electrophoresis showed that different batches of purified prototoxin contained three to five main protein bands. The first band (slowest moving component) was always the major component and the available evidence indicates that this is the undegraded prototoxin. The other bands could be identified by measuring their mobility relative to this major band. Typical electrophoretograms of a number of batches of purified toxin are shown in Fig. 1. The relative mobilities of the five bands in Fig. 1(3) are given in Table 2.

TABLE 2 Electrophoretic mobility of the five major bands of protein in polyacrylamide gel electrophoresis of purified prototoxin

Band No.	Relative mobility*	Standard deviation
1.....	1,0	—
2.....	1,74	0,05
3.....	2,43	0,06
4.....	3,23	0,10
5.....	4,02	0,11

* Relative mobility = $\frac{\text{distance migrated by Band}}{\text{distance migrated by Band 1}}$

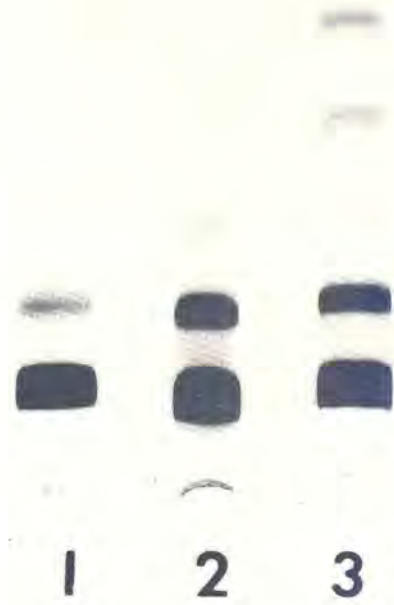


Fig. 1 Polyacrylamide gel electrophoresis of three batches of purified prototoxin. Electrophoresis at pH 8,9

Sometimes one or two small bands of protein that moved somewhat more slowly than the main prototoxin band were seen. These bands vanished if the purified prototoxin was prepared for electrophoresis by dissolving it in 8 M urea. It is therefore assumed that they were aggregation products of one or more of the other bands. In some preparations a small band was found with a relative mobility between that of Bands 1 and 2.

Gels were cut into 2 mm slices and each slice examined by radial diffusion against a fixed concentration of monospecific anti-epsilon toxin serum (see Methods). Fig. 2 depicts a typical example of the rings that formed from a gel containing two protein bands (Fig. 1, No. 1). The results of an experiment in which a gel containing five protein bands (Fig. 1, No. 3) was used, is shown in Fig. 3 where the square of the radius of the precipitin ring is plotted against the gel slice number. The five peaks indicate that there were five bands of antigenic material. These bands had relative mobilities (Fig. 3) which were essentially the same as those of the five protein bands demonstrated in the standard electrophoresis procedure (Table 2). This experiment was repeated three times with different batches of purified toxin with similar results.

The method used for immunoelectrophoresis separated the purified prototoxin into a number of protein components, which varied according to the batch of prototoxin used. These components were immunologically identical as indicated by the fact that the precipitin arcs joined each other with no sign of crossover. The multiple spur formation at the anodal end of the immunoelectrophoretogram seems to indicate that there were some small rapidly migrating peptides of decomposition present which showed partial identity to the main bands. This spur formation was not visible in unstained preparations.

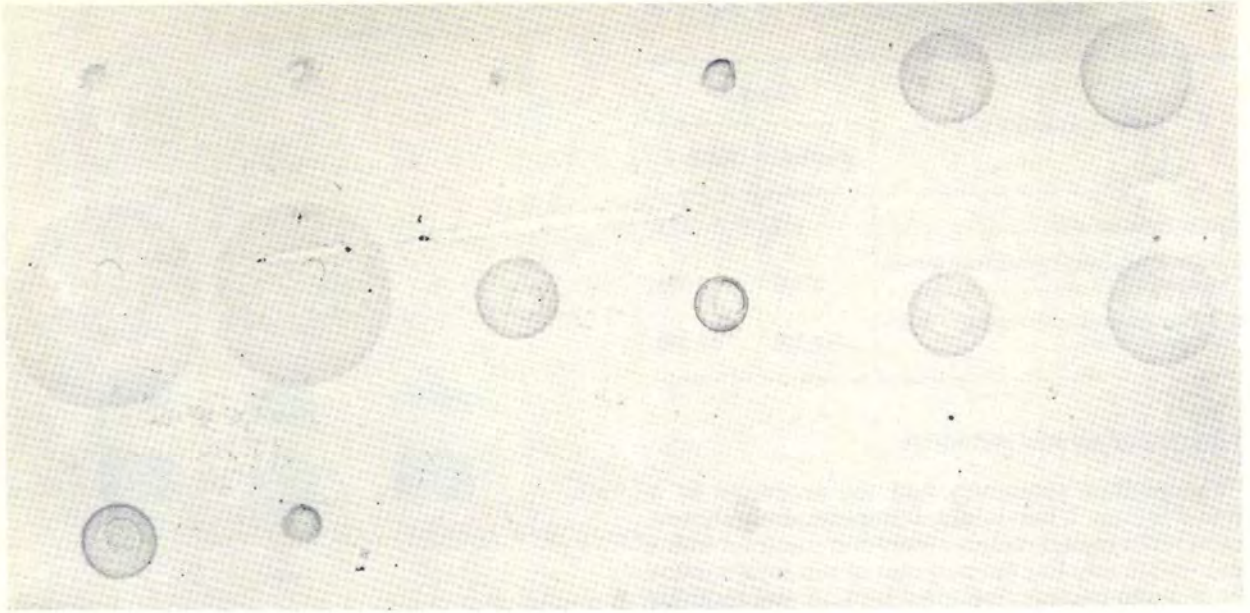


FIG. 2 Precipitin rings formed by gel slices laid on a slab of agarose containing monospecific anti-epsilon toxin serum. A stained gel showing the electrophoretic pattern of the same batch of purified prototoxin can be seen in Fig. 1.1

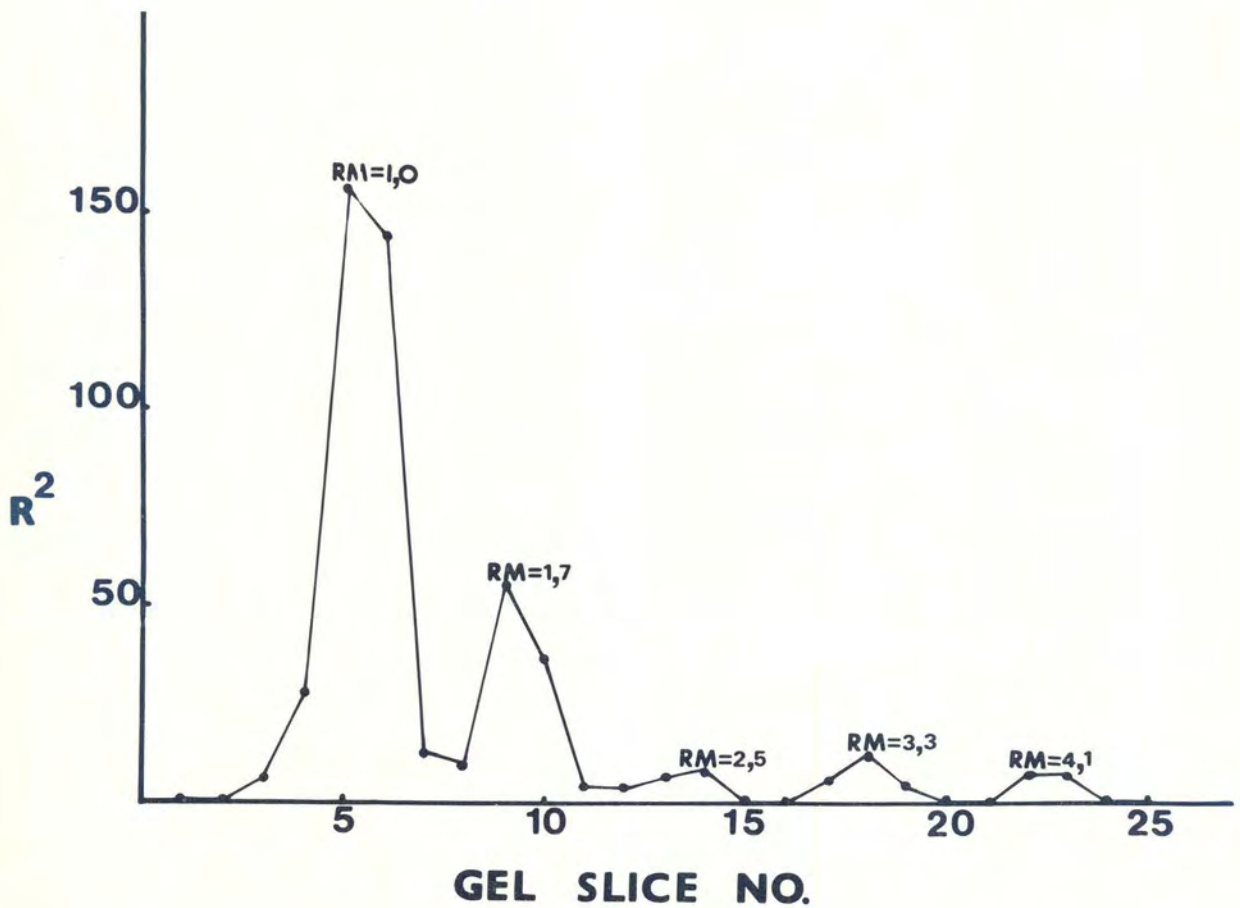


FIG. 3 Plot of the square of the radius of the precipitin ring against the gel slice number. The relative mobility (RM) of each peak is given on the figure

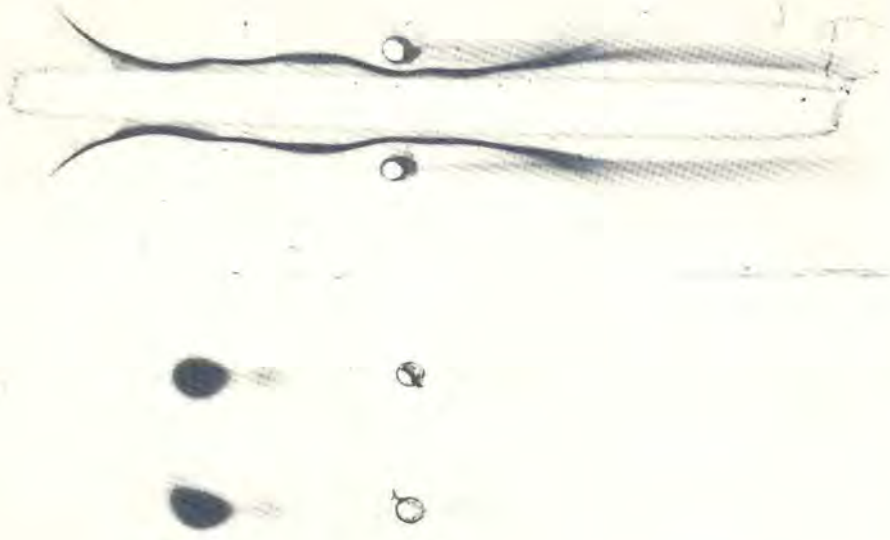


FIG. 4 Immunoelectrophoresis and electrophoresis of purified prototoxin in agarose. Electrophoretic separation is shown in the stained gel (below) and immunoelectrophoresis against monospecific anti-epsilon toxin serum above

Toxicity of electrophoretically separated components

The results of mouse toxicity tests done on protein components 1 to 5 which were excised from the gels are summarised in Table 3. In this experiment doubling dilutions of activated and non-activated toxin were tested. Activation ratios could not always be determined because some bands were non-toxic in the non-activated state. It was therefore only possible to state that the activation ratios for Bands 2 and 3 were >200 and >12 respectively (Table 3).

TABLE 3 Toxicity and activation ratios of the protein bands of purified prototoxin, separated by polyacrylamide gel electrophoresis

Band No.	Relative mobility	Total toxicity of the Band (mouse MLD)		Activation ratio
		Trypsin activated	Non-activated	
1.....	1,0	32 000	40	800
2.....	$\pm 1,74$	2 000	< 10	> 200
3.....	$\pm 2,43$	120	< 10	> 12
4.....	$\pm 3,23$	320	80	4
5.....	$\pm 4,02$	320	80	4

This experiment was repeated three times with essentially similar results. It was clear that all bands were toxic and that the activation ratios decrease as the mobility of the bands increases. The toxicity of all the bands could be increased by trypsin activation.

Degradation of toxin

A preparation of purified prototoxin which contained mainly electrophoretic Bands 1 and 2 with only traces of Band 3 was used in this experiment. Purified prototoxin was dissolved in water and dialyzed against 0,01 M Tris buffers of pH 7,5; 8,0; 8,5 and 9,0 and allowed to stand at 37°C (see Methods). Degradation of the prototoxin occurred in all the preparations.

The original preparation contained mainly Band 1, but on standing this band decreased in intensity and there was a marked increase in the concentration of the faster moving bands (Fig. 5). The pH of the buffer had no apparent influence on the extent of degradation that occurred. Similar degradation occurred when purified prototoxin was dissolved in water and filtered through a Millipore filter to sterilise it. In yet another experiment purified prototoxin was dissolved in 0,1 M Tris buffer pH 8,0. To 0,25 ml aliquots of this solution the following additions were made: 0,25 ml glycerol, one drop 0,5 M mercaptoethanol, one drop 0,015 M EDTA, one drop of 0,05 M sodium bisulphite solution. Degradation of prototoxin was not noticeably inhibited by any of these substances.

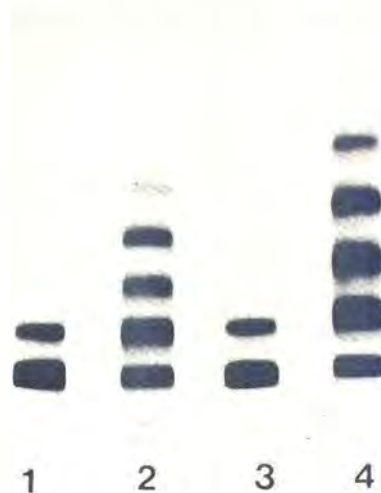


FIG. 5 Degradation of purified prototoxin in solution. Gels 1 and 3 are undegraded controls. Gels 2 and 4 show the degradation after standing for 10 days and 14 days respectively at 37°C

Cation exchange chromatography of purified prototoxin

The results of a typical experiment to fractionate purified prototoxin on CM cellulose are shown in Fig. 6. Polyacrylamide gel electrophoresis showed that the major peak (B) contained mainly undegraded prototoxin (electrophoretic Band 1) but minor contamination with Band 2 still occurred (Fig. 7).

Peak A contained mainly the faster moving bands. It is clear that undegraded prototoxin can be well separated from its degradation products but we are still unable to prepare an absolutely pure preparation of undegraded prototoxin. Degradation of prototoxin may continue to occur at a slow rate thus making the preparation of an absolutely pure preparation of prototoxin difficult.

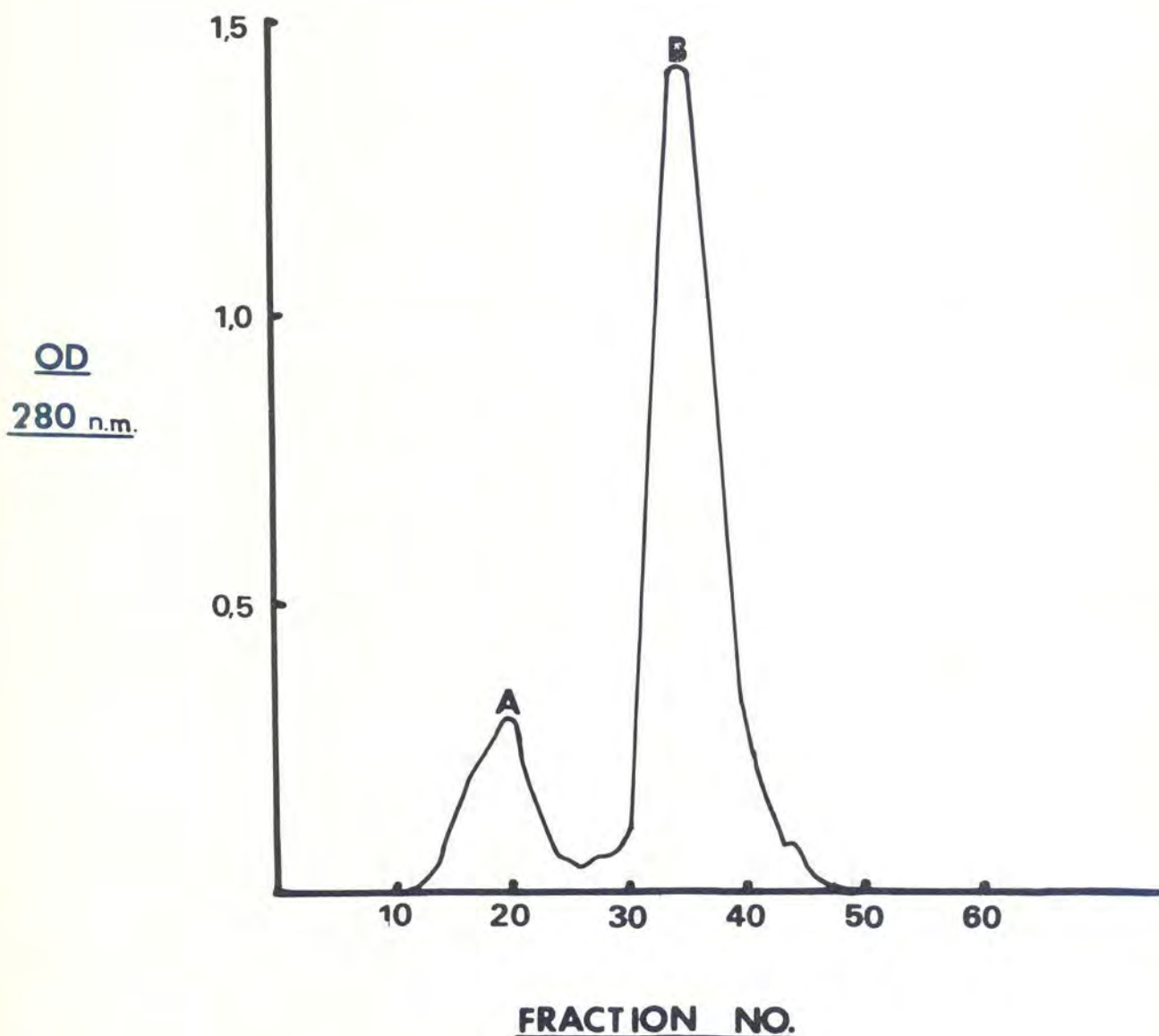


FIG. 6 Separation of purified prototoxin on a $1,5 \times 25$ cm column of CM cellulose. The column was equilibrated with 0,01 M phosphate buffer pH 6,3 and eluted with the same buffer

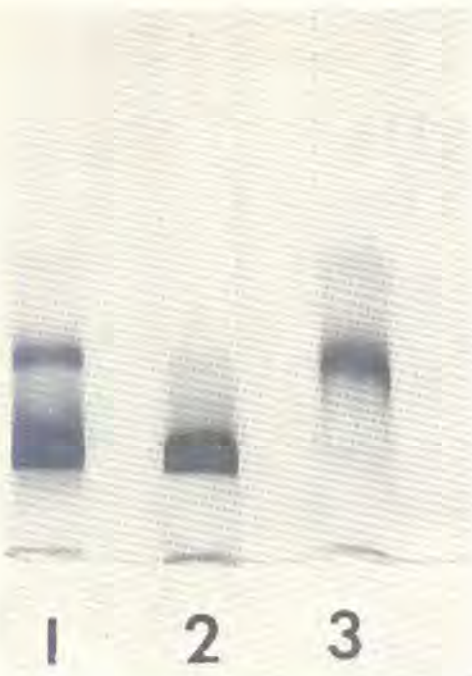


FIG. 7. Polyacrylamide gel electrophoresis of the fractions obtained from the CM cellulose fractionation of purified prototoxin shown in Fig. 6. Gel 1 shows the purified prototoxin applied to the column, Gel 2 shows Fraction B and Gel 3 shows Fraction A.

Purification of prototoxin by cation exchange chromatography did not significantly alter the biological properties of this preparation from those of purified prototoxin; toxicity, flocculation titres and nitrogen content of the products were essentially similar.

DISCUSSION

Purification of *Cl. perfringens* type D culture supernatant by ammonium sulphate precipitation followed by DEAE cellulose chromatography results in the preparation of a highly purified epsilon prototoxin. Although the preparation is not electrophoretically homogeneous it was shown that all the protein bands were immunologically identical.

It was also demonstrated that the electrophoretically faster moving bands were degradation products of the main band which is generally the slowest moving band and is regarded as the undegraded prototoxin. The slowest-moving band has an activation ratio of ± 800 indicating a minimal content of activated toxin (Table 3). The faster-moving degradation products of the prototoxin are all toxic but the activation ratio of these proteins are inversely related to their electrophoretic mobilities (Table 3). The marked increase in electrophoretic mobility of the degradation products could be due to a decrease in the iso-electric point of the molecules resulting from a loss of basic residues, or to a decrease in molecular size. Iso-electric focussing experiments (Worthington, unpublished data, 1973)

have shown that the effect is mainly due to a decrease in iso-electric point. The reason for the degradation of the protein is not known. The possibility that it is caused by minute contamination with proteolytic enzymes must be considered although we do not have any evidence to support this idea. It is, however, also possible that the molecule is inherently unstable due to an unusual primary structure and conformation.

Undegraded prototoxin could be easily separated from its degradation products by cation exchange chromatography but degradation appears to continue at a slow rate and absolute purification has not been achieved (Fig. 6 and 7). Similar results have been obtained using preparative gel electrophoresis and iso-electric focussing techniques (Worthington, unpublished data, 1973). Purified prototoxin prepared by DEAE chromatography was extremely toxic (1.6 to 3.2×10^6 MLDs/mg protein). Chromatography on CM cellulose did not alter the properties or toxicity of the preparation to any significant extent. This result is in contrast to the results reported by Habeeb (1969) who found a distinct increase in the toxicity after CM cellulose chromatography and postulated the removal by CM cellulose chromatography of a toxin inhibitor. The toxicity of our products is, however, similar to that of Habeeb (1969) and considerably higher than that reported by other workers (Verwoerd, 1958, 1960; Orlans, Richards & Jones 1960; Thomson, 1963; Hauschild, 1965).

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