

## THE PARTIAL PURIFICATION OF *CLOSTRIDIUM PERFRINGENS* BETA TOXIN

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### ABSTRACT

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An attempt was made to purify *Clostridium perfringens* Beta toxin. Crude toxin prepared by ammonium sulphate precipitation of culture supernatants was purified by chromatography on Sephadex G50, Sephadex G100 and DEAE cellulose. This material, although highly purified, was not homogeneous on polyacrylamide gel electrophoresis. It had a toxicity of 800 000 mouse MLDs/mg N, a typical protein absorption spectrum in the UV region, an iso-electric point of 5.6 and the main component had a molecular mass of  $42\ 000 \pm 2\ 000$  (estimated by electrophoresis in sodium dodecyl sulphate containing polyacrylamide gels).

### INTRODUCTION

*Clostridium perfringens* organisms produce a variety of toxic factors which cause animal diseases. The Beta toxin is produced by *C. perfringens* types B and C. (Brooks, Sterne & Warrack, 1957). According to Hauschild (1971) the combined effects of Beta and Epsilon toxins are responsible for the diseases caused by *C. perfringens* type B, which include lamb dysentery and enterotoxaemia of foals, goats, sheep and calves. Beta toxin is responsible for the diseases caused by *C. perfringens* type C which include Struck of sheep and enterotoxaemia of lambs, calves and piglets and necrotic enteritis of man and fowls. (For review see Hauschild, 1971.) Most of the conditions in which Beta toxin plays the major role are characterized by ulceration of the intestines or acute haemorrhagic enteritis. Epsilon toxin is primarily responsible for classical enterotoxaemia (pulpy kidney) of sheep characterized by nervous symptoms and sudden deaths.

The significance of Beta toxin has been understood for many years, and the toxin can readily be identified by toxin neutralization tests in mice (Glenny, Barr, Llewellyn-Jones Dalling & Ross, 1933; Brooks Sterne & Warrack, 1957). Jansen (1961) gave a very detailed account of the production of Beta toxin and lamb dysentery vaccine. Beta toxin has, however, never been successfully purified and the object of this investigation was to attempt to purify Beta toxin.

### MATERIALS AND METHODS

#### *Production of crude toxin*

*Clostridium perfringens* type B, strain 1930 (Mason 1935), a strain which produces Beta toxin but virtually no epsilon toxin, was used for toxin production. The organism was cultivated for a period of 5½ h in 10 l of Wright's broth according to the standard methods used for the production of lamb dysentery vaccine at the Veterinary Research Institute, Onderstepoort. At this stage the culture supernatant contained 250 flocculation units (LF)/ml of toxin. The culture broth was centrifuged at  $4\ 000 \times g$  for 30 min and ammonium sulphate added to the supernatant at a rate of 400g/l, which was then stored overnight at 4 °C to allow the precipitate to sediment. All subsequent steps were carried out at 4 °C. The bulk of the supernatant was siphoned off and the precipitate further concentrated by centrifugation. The precipitate was redissolved in distilled water and dialyzed against distilled water (4 changes of 20 l) and freeze-dried. An

8,0 g aliquot of this dried material was redissolved in 0,01 M phosphate buffer of pH 6,0 and centrifuged at  $20\ 000 \times g$  to remove insoluble material. The clear supernatant was freeze-dried giving a total yield of 4,2 g of dried material. Solutions of this material were tested with Nessler's reagent and found to be free of ammonia. It will be referred to as crude toxin.

#### *Gel filtration chromatography*

In all chromatography experiments on Sephadex G50 and G100 0,01M phosphate buffer of pH 6,0 was used for swelling the Sephadex, dissolving the toxin and for column elution. Details of column dimensions, flow rates, hydrostatic pressure and fraction sizes are given in the subscripts to Fig. 1 and 2. All fractionation procedures were carried out at 4 °C.

#### *DEAE cellulose chromatography*

DEAE cellulose was activated according to the manufacturer's\* instructions and equilibrated with 0,01 M acetate buffer, pH 4,5. The same buffer was used for elution. Details of flow rates and fraction sizes are given in Fig. 3. All fractionation procedures were carried out at 4 °C.

#### *Iso-electric focussing*

Iso-electric focussing experiments were done in an LKB 8101 Ampholine column. The anode solution (bottom) consisted of 0,2 ml phosphoric acid, 12 g sucrose and 14 ml water. The cathode solution consisted of 0,2 ml ethanolamine in 10 ml water. Between anode and cathode solution the column was filled with a sucrose gradient containing 1,2% of ampholytes\*\*. Pilot experiments were done with ampholytes with a pH range 3,5-10 and 4-6. In the experiment described in this paper 1,5 ml each of 40% ampholyte solutions of range 5-8 and 4-6 and 0,35 ml of ampholytes with a pH range of 3,5-10 were included in the sucrose gradient. The toxin sample was introduced into the middle of the sucrose gradient during the gradient formation. The voltage applied across the column was 400 V at the start of the experiment which was gradually increased to 600 V in about 4 h. This voltage was maintained for a further 16 h after which it was increased to approximately 900 V for a further 2 hours. The power was never allowed to rise above 2 W and the column was cooled by pumping water at 4 °C through the cooling jacket. Fractions of about 2,5 ml were collected at the end of the run and the pH of each fraction measured immediately it came off the column. The absorbance of each fraction was monitored at 280 nm and the toxicity of fractions

\* Whatmans

\*\* LKB—Produkter AB

tested by mouse toxicity tests. For these toxicity tests fractions were diluted in 0,5% peptone in 0,1 M phosphate buffer pH 7,2.

#### Nitrogen determinations

These were performed by a standard micro Kjeldahl method with determination of ammonia being done by Nesslerisation (C. v Holt Dept. of Biochemistry, University of Cape Town, personal communication, 1969).

#### Mouse toxicity tests

Toxin was suitably diluted in a peptone saline solution [1% peptone (m/v) in 0,25% (m/v) NaCl] and 0,5 ml amounts injected intravenously into mice. In most titrations doubling dilutions were used and a MLD was taken as the least amount of toxin which killed two out of three mice within 24 hours of injection.

#### Flocculation tests

Flocculation tests were done by the method described by Jansen (1961) using standardized anti-beta toxin gamma globulin kindly supplied by the Director of the Veterinary Research Institute, Onderstepoort.

#### Carbohydrate analysis

The carbohydrate content was estimated by the phenol sulphuric acid method described by Williams & Chase (1968). A glucose solution was used as a carbohydrate standard.

#### Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis was done as described previously for Epsilon toxin (Worthington, Mülders & Van Rensburg 1973).

#### Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

Estimation of molecular mass by electrophoresis on SDS gels was done according to the method of Weber, Pringle & Osborn (1972). Toxin and marker proteins were dissolved so as to contain 10 mg/ml of protein in buffer containing 3% SDS and 1% mercaptoethanol (ME). The solutions were placed in a boiling waterbath for 5 min before applying to the gels. Cytochrome C\*, Chymotrypsin\*, ovalbumin\*, and aldolase\* were used as markers.

#### RESULTS

Crude Beta toxin was first fractionated on Sephadex G50. The result of a typical run is shown in Fig. 1. The technical details of the fractionation is given in the relevant subscript. Mouse toxicity tests on the eluted material showed that the toxin was concentrated in the first peak. The fractions indicated by the cross-hatched area in Fig. 1 were therefore pooled and freeze-dried. Virtually 100% of the toxicity applied to the column was recovered in the dried material which represented only 26% of the 280 nm absorbing material and 37% of the nitrogen was recovered.

\* Boehringer Mannheim

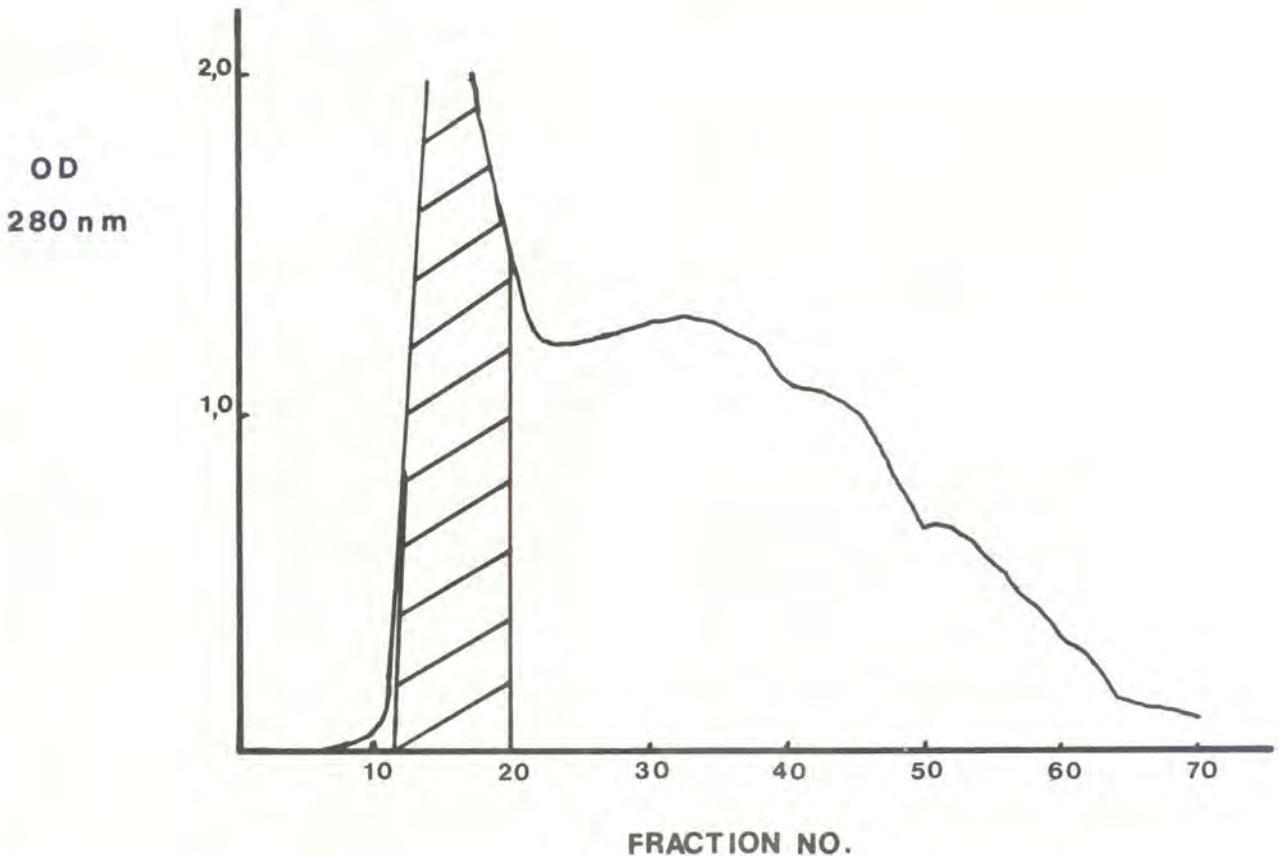


FIG. 1 Fractionation of 400 mg of crude toxin on a 40×2,5 cm column of Sephadex G50. Hydrostatic pressure 20 cm, flow rate 40 ml/h. Fractions 3 ml

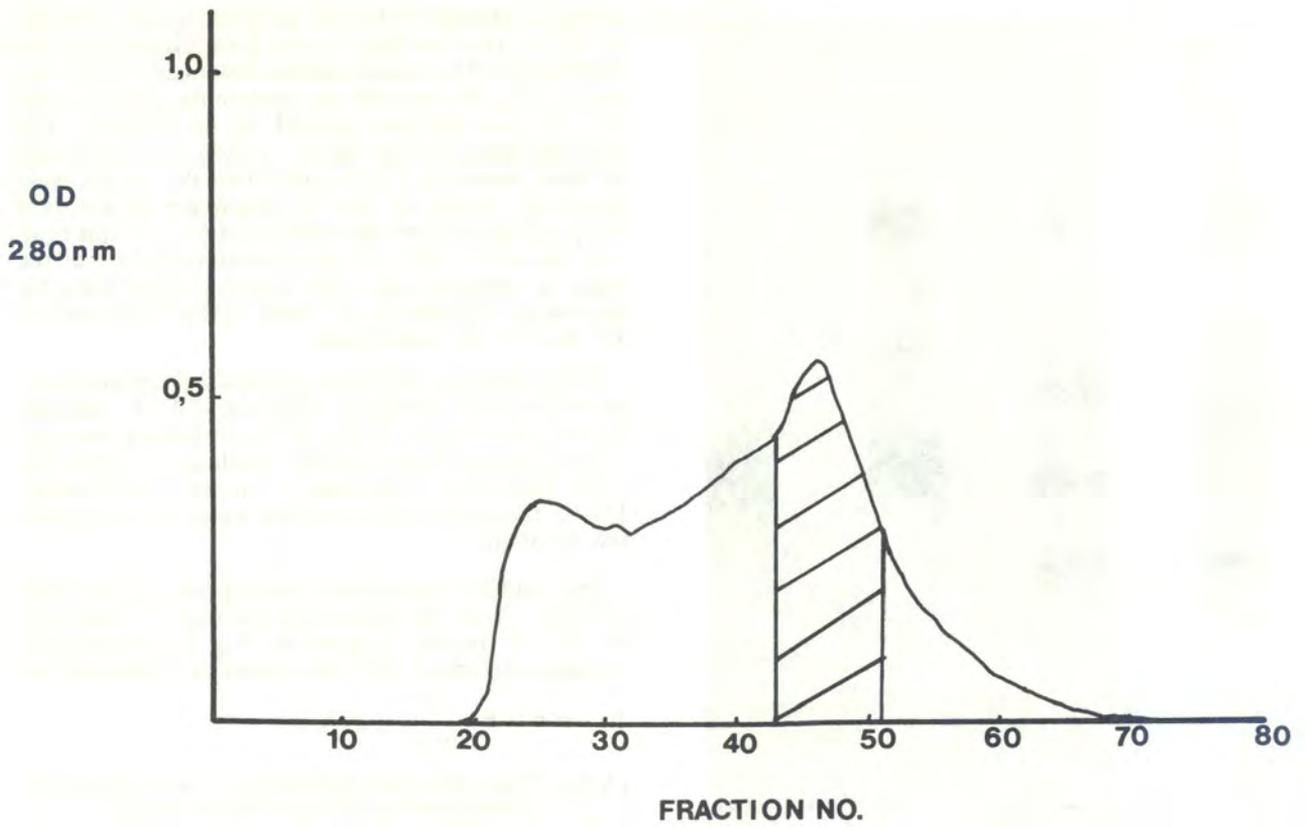


FIG. 2 Fractionation of 300 mg of toxin from a Sephadex G50 column on a  $95 \times 2,5$  cm column of Sephadex G100. Hydrostatic pressure 20 cm. Flow rate 14 ml/h. Fractions 5 ml

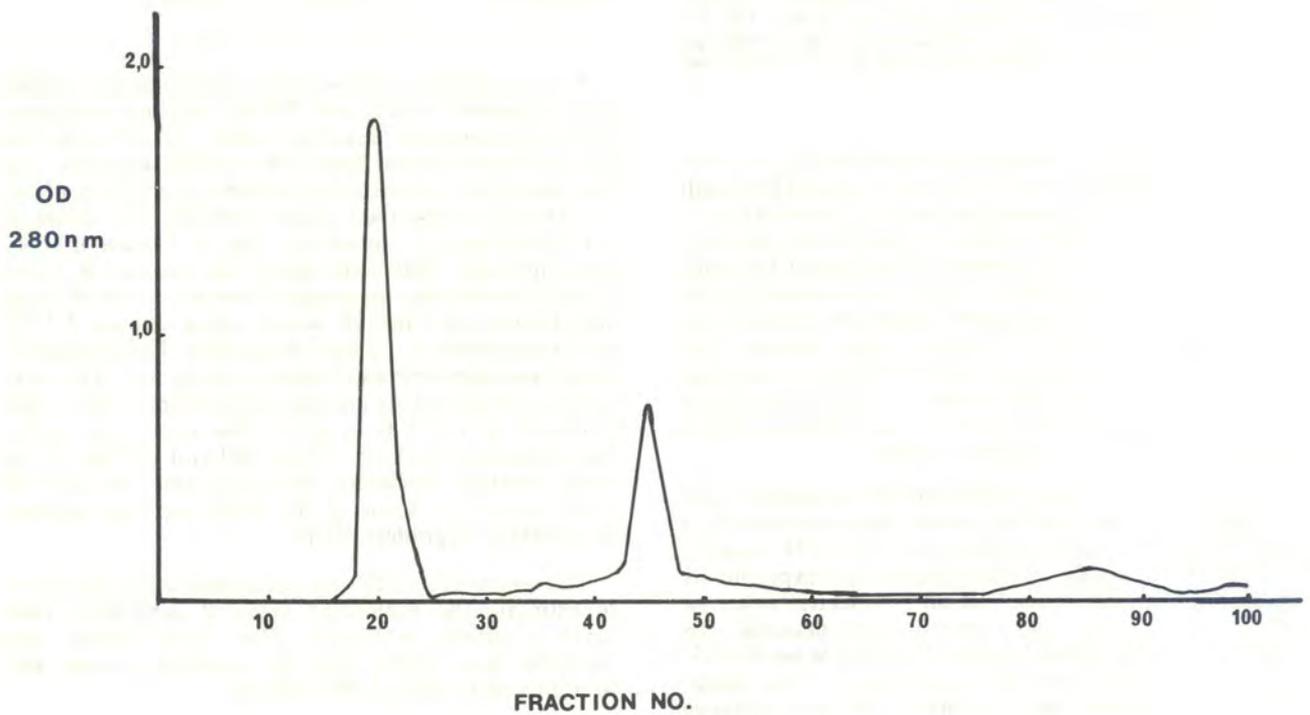


FIG. 3 Fractionation of 110 mg of toxin from a Sephadex G100 column on a  $45 \times 1,5$  cm column of DEAE cellulose equilibrated and eluted with 0,01 M acetate buffer pH 4,5. Flow rate 15 ml/h. Fractions 2,5 ml



FIG. 4 Polyacrylamide gel electrophoresis in 7.5% polyacrylamide, reservoir buffer tris/glycine pH 8.9; current 2mA per gel; time of electrophoresis 30 min. Gel 1=crude Beta toxin; gel 2=Sephadex G50 Beta toxin; gel 3=Sephadex G100 Beta toxin; gel 4=DEAE cellulose Beta toxin

Toxin recovered from Sephadex G50 columns was further fractionated on Sephadex G100. The result of a typical fractionation experiment is shown in Fig. 2. Technical details of the run are given in the subscript to Fig. 2. Mouse toxicity tests on the eluted fractions showed that the majority of the toxin material was concentrated in the fractions which are indicated by cross-hatching. These fractions were pooled and freeze-dried. Approximately 55% of the toxin applied to the column was recovered in the freeze-dried material which contained 27% of the 280 nm absorbing material and 23% of the nitrogen.

The dried toxin recovered from the Sephadex G100 column was then submitted to iso-electrophoresis in a sucrose gradient using ampholytes with a pH range of 3.5–10.0. The results of these preliminary experiments indicated that the toxin had an iso-electric point of about 5.5 and that the contaminating material was more acidic. The details of more definitive iso-electric focussing experiments are given below. This result, however, indicated that conditions for ion exchange chromatography on DEAE cellulose could be set up in which the toxin would pass straight through the column while the acidic contaminating material would be retained. Dried toxin from Sephadex G100 columns was fractionated on DEAE cellulose as described under

methods. Details of the run are given in the subscript to Fig. 3. The first peak eluted from the column was freeze-dried. The dried material contained 63% of the toxin, 32% of the 280 nm absorbing material and 51% of the nitrogen applied to the column. The fractionation described above was the most successful of three attempts. In the other two the results were essentially similar in that the toxin was eluted as a sharp initial peak but the recovery of toxin in this peak was only about 10%. It does therefore seem that the toxin is unstable and that optimal conditions for preventing degradation of toxin during fractionation will have to be investigated.

Polyacrylamide gel electrophoresis of crude toxin, Sephadex G50, Sephadex G100 and DEAE cellulose fractions are shown in Fig. 4. It can be seen that the toxin obtained from DEAE cellulose contains one main band with a number of minor components. This is the most highly purified toxin we have been able to obtain.

The results of the purification steps are summarized in Table 1 and the absorption spectrum of the toxin in the UV region is given in Fig. 5. Assuming a nitrogen content of 16% the extinction coefficient for

$$\text{the toxin is } E_{278 \text{ nm}}^{1\%} = 2,7.$$

TABLE 1 Purification data; purification of Beta toxin by gel filtration and ion exchange chromatography

	MLD/OD	MLD/mg N	% carbohydrate
Crude toxin.....	8 000	25 000	0,6
G50 toxin.....	32 000	70 000	1,1
G100 toxin.....	64 000	160 000	0,4
DEAE toxin.....	128 000	208 000	1,9

Iso-electric focussing was done on Beta toxin from both Sephadex G100 and DEAE cellulose columns. In the experiments described below DEAE cellulose purified material was used. The iso-electric point was determined by iso-electric focussing in a pH gradient of pH 4–8 as described under methods. The result of this experiment is shown in Fig. 6. Mouse toxicity tests indicated that some toxin was present in tubes 21 to 32 but by far the greatest concentration of toxin was located in tube 28 which when diluted 1/1250 killed mice within  $\pm 5$  min of injection. The iso-electric point was therefore estimated as being 5.6. This was further confirmed in another experiment using a pH gradient of 4.0–6.0 in which the iso-electric point was found to be 5.65. Toxin did not appear to be stable during iso-electric focussing and recovery of toxin was poor. Some of the faster moving material is probably degraded toxin.

The results of an SDS gel electrophoresis experiment to estimate the molecular mass of denatured Beta toxin is shown in Fig. 7. This determination was repeated three times and the molecular mass was found to be  $42\,000 \pm 2\,000$  Daltons.

The carbohydrate content of all our toxin preparations was less than 2%. The most highly purified toxin from DEAE cellulose contained 1.9% carbohydrate while other preparations contained from 0.4 to 1.1% carbohydrate.

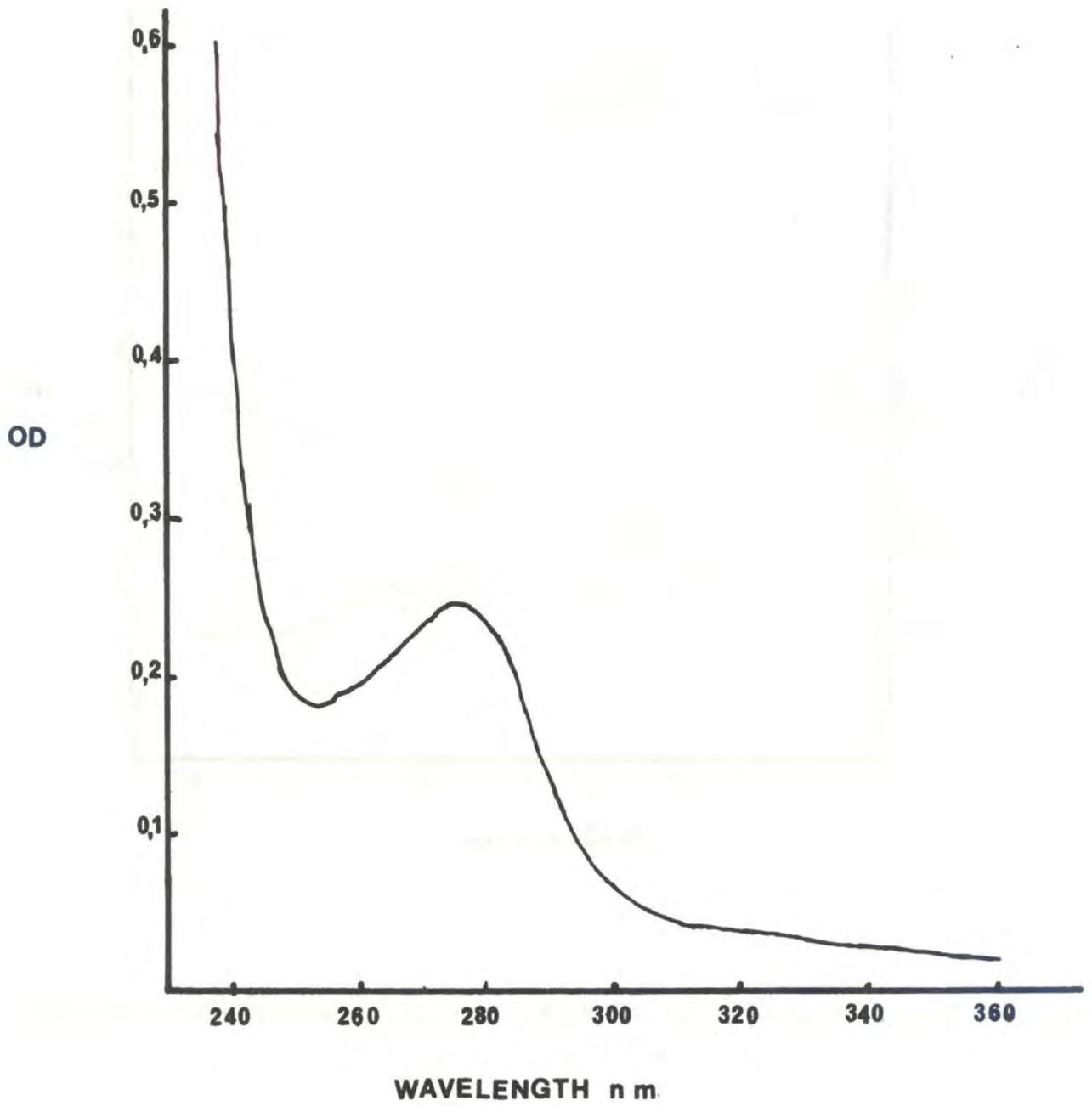


FIG. 5 U.V. absorbance spectrum of DEAE cellulose Beta toxin

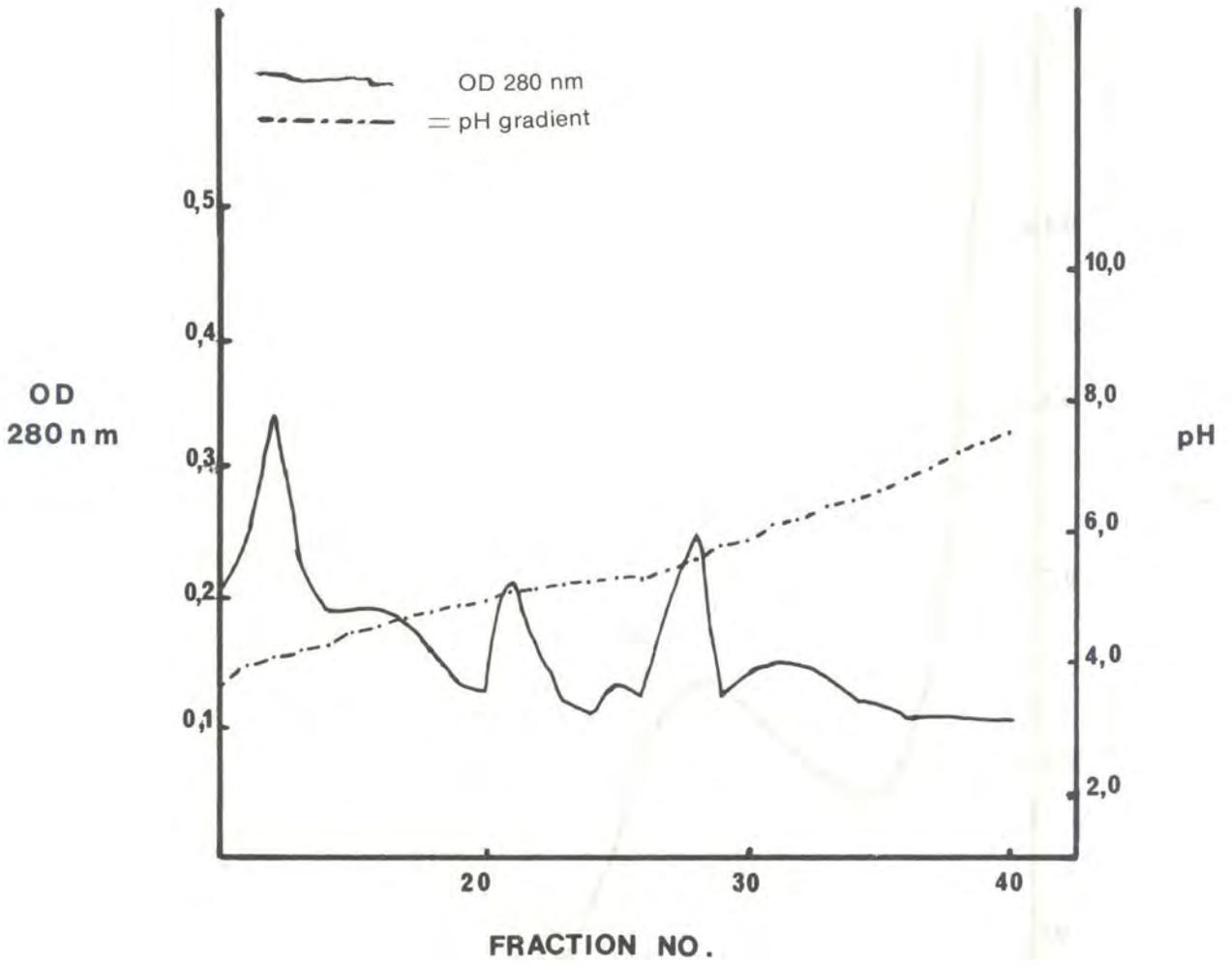


FIG. 6 Iso-electric focussing of Beta toxin on a pH gradient of pH 4-8. Fractions  $\pm 2$  ml. The highly toxic material was concentrated in fraction 28

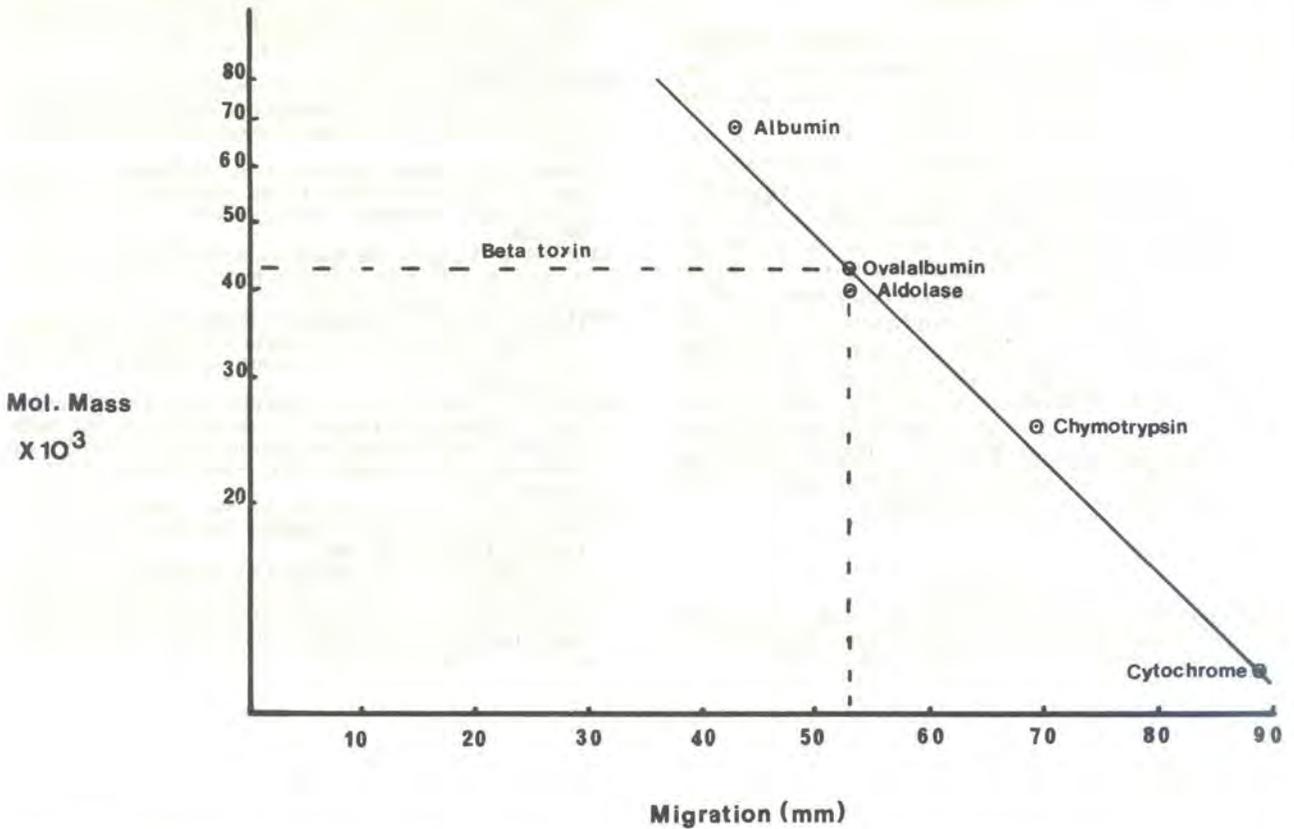


FIG. 7 Plot of distance of migration against log mol. mass of marker proteins and Beta toxin in SDS gel electrophoresis

#### DISCUSSION

Chromatography of crude toxin in Sephadex G50 eliminated a considerable amount of 280 nm absorbing material of small molecular mass. Further chromatography on Sephadex G100 eliminated large molecular mass substances and some material of a lower molecular mass than the toxin. In the latter step it was clear that the most highly toxic material was eluted in a relatively narrow band but that other fractions which are eluted over a much wider range contain material of low toxicity. In view of the fact that Beta toxin is known to be labile (Roberts 1959; Jansen 1961) we believe that this low toxicity material may be partially degraded Beta toxin. Jansen 1961 found that Beta toxin was unstable when stored at 37 °C after addition of toluol as preservative. He found the optimal pH for the storage of Beta toxin to be 6,0. At this pH as much as 53% of the toxin present in liquid Beta toxin cultures was still present after one week's storage at 37 °C while at other pH values the stability was considerably lower. In Jansen's studies the flocculation test was used to measure toxin and as it is possible that partially degraded toxin molecules may retain some antigenic activity these estimates may be somewhat high. In view of the obviously greater stability at pH 6,0 all our gel filtration experiments were carried out at this pH.

The purification on DEAE cellulose was designed to allow the rapid passage of Beta toxin through a short DEAE cellulose column at a pH value which is lower than its iso-electric point, but somewhat higher than most of the contaminating material. In this way the toxin is maintained at a low pH for a minimum length of time. Although this resulted in the elution of a sharp peak of highly toxic material which is well

separated from other material eluted from the column it was still not homogeneous on gel electrophoresis and contained a number of minor bands (Fig. 4). The possibility exists that some of the contaminating protein may also represent degraded toxin. DEAE cellulose chromatography was preferred as a preparative method to iso-electric focussing, because it is faster and less loss of protein occurred. The entire separation of the first peak was completed within four hours. The iso-electric focussing procedure takes 24 hours and must then be followed by dialysis or gel chromatography to separate the toxin from the ampholytes. In addition larger amounts of protein could be purified by DEAE cellulose chromatography.

The toxin has a typical protein absorption spectrum in the UV region. It is, however, unusual in that the 280 nm absorbance is lower than that seen with many other proteins  $E_{278 \text{ nm}}^{1\%} = 2,7$ ). The ratio of the absorbance at 278 nm to the absorbance at 253 nm is also low ( $=1,4$  see Fig. 5). It therefore seems likely that the protein has a rather low content of aromatic amino acids. The carbohydrate content of the toxin is very low and can be regarded as insignificant. The toxin therefore appears to be a protein with a molecular mass of about 42,000 and an iso-electric point of 5,6.

#### ACKNOWLEDGEMENTS

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## Résumé

WORTHINGTON, R. W. & MÜLDERS, MARIA S. G., 1975. La purification partielle de la toxine beta de *Clostridium perfringens*. *Onderstepoort J. vet. Res.* 42 (3), 91-98

Une tentative de purification de la toxine beta de *Clostridium perfringens* a été faite. De la toxine brute, précipitée par le sulfate d'ammonium à partir de surnageants de cultures, a été purifiée par chromatographie sur Sephadex G50, Sephadex G100 et cellulose DEAE. Cette substance, quoique hautement purifiée, n'était pas uniforme en électrophorèse sur gélose de polyacrylamide. Sa toxicité se trouve à 800 000 DML/mgN. Son spectre d'absorption dans la zone UV était de nature typiquement protéinique, son point iso-électrique était de 5,5 et son composant principal avait un poids moléculaire de  $42\,000 \pm 2\,000$  (apprécié par électrophorèse sur sulfate de dodecyl sodium additionné de géloses de polyacrylamide).

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