PURIFICATION AND MOLECULAR WEIGHT DETERMINATION OF CLOSTRIDIUM BOTULINUM TYPE E TOXIN

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Abstract

Gerwing, Julia (The University of British Columbia, Vancouver, British Columbia, Canada), Claude E. Dolman, M. E. Reichmann, and Hardial S. Bains. Purification and molecular weight determination of Clostridium botulinum type E toxin. J. Bacteriol. 88:216–219. 1964.—A method was developed whereby type E botulinus toxin can be obtained in a highly purified state by elution through acidified diethylaminoethyl-cellulose columns. The material thus isolated appears to be electrophoretically and ultracentrifugally homogeneous. A molecular weight of 18,000 was calculated for the toxin.

Increasing awareness of the hazards of type E botulism, and of the phenomenon of type E toxin activation by trypsin at pH levels around 6.0 (Duff, Wright, and Yarinsky, 1956), and by other proteolytic enzymes (Dolman, 1953; Dolman, 1957; Sakaguchi and Tohyama, 1955), have stimulated interest in the molecular size and character of this toxin. Previous attempts to purify type E toxin have had limited success. Products of quite high potency were obtained, but in no instance was it claimed that all criteria for homogeneity had been satisfied (Gerwing, Dolman, and Arnott, 1961, 1962; Gordon et al., 1957; Sakaguchi and Sakaguchi, 1959). When purification techniques previously reported from this laboratory were modified, by substituting ammonium sulfate for ethanol as initial precipitating agent, and by using columns packed with acidified diethylaminoethyl (DEAE) and equilibrated with sodium acetate buffer at pH 4.5, it proved possible to isolate from filtrates of a type E strain a highly toxic component of relatively low molecular weight. This material appears to be homogeneous by both ultracentrifugal and electrophoretic analyses.

Materials and Methods

The bacterial strain, culture media, and the potency and nitrogen determination methods, were described previously (Gerwing et al., 1961). The crude filtrates had an average potency of around 3,000 mouse MLD per ml.

Purification technique. A solution of saturated (NH₄)₂SO₄ was added to toxic filtrates to a final concentration of 60% (v/v). The precipitate was allowed to form overnight at 4 °C and was then collected by centrifugation at 5,000 × g at 4 °C. The sedimented material was dissolved in 0.01 M sodium acetate buffer at pH 5.5, to approximately 1/10 of the original volume, and was dialyzed against the same buffer for 24 hr at 4 °C. The small amount of insoluble material present after dialysis was removed by centrifugation. The soluble preparation contained 80% of the original toxin in a form concentrated enough to permit column chromatography to be carried out.

Columns packed with Selectacel (Brown Co., Berlin, N.H.) DEAE cellulose were used for further purification of the concentrated dialysate. In this technique, the column length is unimportant (within a range of 15 to 75 cm), but to obtain good resolution of the eluate the diameter must not exceed 1 cm. A finely suspended preparation of DEAE, pretreated with 2 N NaCl for 24 hr at 4 °C, was packed into columns, with slight positive pressure, to a height of about 25 cm. Columns thus prepared were washed with 1 N HCl until the eluate became strongly acidic (about pH 0.5), and were then equilibrated with 0.01 M sodium acetate buffer at pH 4.5.

To such columns, the concentrated toxin preparations, which contained approximately 2 mg of protein per ml, were added in 5.0-ml quantities. Elution was carried out with 0.01 M acetate buffer at pH 4.5 on a model V-10 fraction collector (Gilson Medical Electronics, Middleton, Wis.). The flow rate was regulated to 1.0 ml in 2 min. The operations were carried out at room temperature.

The eluate came through the acidified columns frontally, with a sharp, well-defined peak of high toxicity, followed by a small, easily separable
peak due to a nontoxic component (Fig. 1). The toxic peak samples thus isolated were pooled and treated again with (NH₄)₂SO₄ at 0.60 saturation. The precipitate was allowed to form for 2 hr at 4 C, and was then centrifuged and dissolved in 5 ml of 0.01 M acetate buffer at pH 5.5. This material was dialyzed for 24 hr at 4 C against the pH 5.5 buffer and passed through DEAE, as before at pH 4.5. A single sharp peak was now observed.

Treatment of dialysis paper. All dialysis paper used in this work was boiled for 5 min in a 0.01 M solution of ethylenediaminetetraacetic acid adjusted to pH 7.0, and washed in distilled water. This treatment prevented inactivation of toxin by surface-active elements on the dialysis paper. When untreated paper was used, up to 90% of toxic activity might be destroyed during dialysis.

Physical measurements. The sedimentation coefficient was measured with a Beckman-Spinco model E analytical centrifuge, with a synthetic boundary cell. Three runs were made, each using material from a different batch of toxin, for 96 min at 259,700 × g; photographs were taken at 8-min intervals. The solution was made up to contain 1% toxic protein in 0.05 M acetate buffer at pH 4.5. The electrophoretic mobility and the diffusion coefficient were determined with a Beckman-Spinco model H electrophoresis-diffusion apparatus by use of the 11-ml standard cell in the same buffer. In this instance, the toxin concentration was approximately 1 mg/ml. The Raleigh integral fringe method was used in the evaluation of the diffusion coefficient (Longsworth, 1952). Ten pictures taken over a 90-hr period were used for the calculation of this value.

RESULTS

The purified toxin had a maximal ultraviolet absorption at 277 mμ, and the extinction was 8.4 per mg of N₂. The total recovery of toxin was approximately 55%. The material yielded by the pooled toxic peak samples contained 7.5 × 10⁶ mouse MLD per mg of N₂.

In an analytical ultracentrifuge, a sharp peak was observed, with a sedimentation coefficient of S₂₀,ₐ = 1.70S. Virtually all the protein was contained within this boundary. However, an outline of a rapidly sedimenting component was also observed. The sharp symmetrical major peak suggested that the toxin was essentially homogeneous with respect to molecular size (Fig. 2).

![Fig. 1. Elution curve of type E toxin through acidified DEAE at pH 4.5. The large major peak contains the toxic activity.](http://jb.asm.org)

The electrophoretic pattern resulted in a single major peak migrating with an electrophoretic mobility of +5.66 × 10⁻⁵ cm² per v per sec (Fig. 3). A small, rapidly disappearing peak was observed both at the ascending and descending boundary.

The diffusion coefficient was determined on the same solution after sharpening the boundary, a value of D₂₀,ₐ = 8.87 × 10⁻⁷ cm²/sec being obtained. The molecular weight was calculated using the accepted formula

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M = \frac{RTS_{20, a}^2}{D_{20}(1 - Vp)}
\]

wherein M is the molecular weight, R the gas constant, T the absolute temperature, S₂₀,ₐ the sedimentation coefficient at 20 C in distilled water, V the partial specific volume which was taken as 0.75, p the solvent density, and D₂₀ the
FIG. 2. Representative photographs obtained in the sedimentation velocity run on purified type E toxin in 0.05 M acetate buffer at pH 4.5. Speed, 89,780 rev/min; bar angle, 40°; protein concentration, 1.0%; times after obtaining full speed, 0 min and 48 min.

diffusion coefficient at 20°C. A molecular weight of 18,600 was thus obtained.

DISCUSSION

The main principle involved in the column chromatography of the crude toxic concentrate is the frontal elution of the toxin under conditions leaving all other components strongly adsorbed on to the acidified DEAE, and only releasable therefrom by altering the pH or the molarity of the eluent. This technique is applicable only to toxins freed from undialyzable media constituents by growing the organisms in dialysis sacs. Attempts to purify toxins prepared in whole media by this method have been unsuccessful, because large amounts of impurities originating in the media are also liberated frontally from the columns with the toxin.

The electrophoretic and ultracentrifugal analyses of the material showed the presence of a single major homogeneous component giving a typical Gaussian distribution in the Schlieren optical systems used. However, in both cases a small percentage of apparent impurities was noted. In an ultracentrifuge, this took the form of a rapidly sedimenting small shoulder which appeared 16 min after full speed was attained. This may have been due to small amounts of either aggregated material or impurities. The small immobile peak seen in the electrophoretic analysis may have been due to protein-buffer interactions, or again to traces of impurities. Apart from these minor reservations, it may be claimed that the methods described permitted the isolation of a toxic component from filtrates of Clostridium botulinum type E which fulfilled the criteria for purity upon ultracentrifugal and electrophoretic analyses. Incidentally, studies on this material by use of high-voltage paper electrophoresis did not demonstrate these traces of apparent impurities, but showed a single migrating band at both pH 4.5 and 7.0.

It would appear that type E botulinus toxin is a relatively small protein molecule, with a molecular weight much lower than those reported previously for type A and B toxins (Kegeles, 1946; Lamanna and Glassman, 1947; Lamanna, McElroy, and Eklund, 1946; Putnam, Lamanna, and Sharp, 1946, 1948; Wagman, 1954). In an earlier report (Gerwing et al., 1962), a partially purified sample of type E toxin, having a potency of $6.0 \times 10^8$ MLD per mg of $N_2$, formed a boundary in an ultracentrifuge with a sedimentation constant of $S_{20,w} = 5.6S$. The discrepancy between that finding and the currently reported constant of 1.7 for a product of considerably higher potency ($7.5 \times 10^9$ MLD per mg of $N_2$) could be due to the former use of ethanol as precipitating agent, which possibly induced some degree of molecular degradation and aggregation. Moreover, lyophilization of the partially purified...
toxic materials in our earlier work introduced another factor which is well known to cause partial denaturation of certain proteins. Similar considerations may apply to some of the earlier reports on the molecular weight of type A and B toxins, especially to the very high values attributed to type A toxin. Indeed, Wagman and Bateman (1951) and Wagman (1954) showed that crystalline type A toxin was comprised of more than one component. The relatively gentle and simple methods reported here, which involve the use of (NH₄)₂SO₄ as precipitating agent and EDTA-treated dialysis sacs, and avoid exposure of the toxin at any stage to organic solvents or to destructive pH levels, should have minimized the chances of aggregation and denaturation.

Addendum

Since this paper was accepted for publication, Sakaguchi et al. (J. Bacteriol. 87:401, 1964) have reported that both precursor and trypsin-activated toxin may have molecular weights of 200,000 or larger. These claims are so much at variance with our findings that an immediate comment seems justified. Their conclusions were based on chromatographic results with CM-Sephadex G-200, which cannot be solely relied upon for molecular weight determination. Various factors may have contributed to the poor resolution evidently obtained by Sakaguchi et al. during elution. For example, their column height-to-diameter ratios seem quite inadequate; their use of sodium acetate eluate at pH 6.0 could have given negligible buffering action; and their columns may have been overloaded. In due time, a fuller explanation of these discrepancies may be forthcoming. Meanwhile, to avoid future confusion, we would urge that it is undesirable, on scientific and semantic grounds, to continue to designate as "toxin precursor" the lethal material present in either culture supernatants or bacterial cell extracts of toxigenic type E strains.

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Literature Cited


