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Purification and Characterization of *Clostridium botulinum* Type B Toxin

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ABSTRACT

GERWING, JULIA (The University of British Columbia, Vancouver, B.C., Canada), CLAUDE E. DOLMAN, DAVID V. KASON, AND JACK H. TREMAINE. Purification and characterization of *Clostridium botulinum* type B toxin. J. Bacteriol. **91**:484–487. 1966.—A toxic component of low molecular weight has been isolated from a type B strain of *Clostridium botulinum* by methods involving ammonium sulfate precipitation and elution through diethylaminoethyl cellulose at *p*H 5.6. The material thus isolated was shown to be monophoretic and monodisperse in the ultracentrifuge. End-group analysis indicated the presence of a single N-terminal amino acid residue, which was identified as arginine. On the basis of biophysical studies and amino acid analyses, a molecular weight between 9,000 and 10,000 was calculated.

Relatively little has been reported on the purification of *Clostridium botulinum* type B. By performing a series of acid precipitations on type B toxic filtrates, Lammana and Glassman (9) produced a highly purified preparation, the molecular weight of which they estimated by diffusion methods to be around 60,000. In sedimentation studies on type B toxin, Wagman and Bateman (14) showed that an electrophoretically homogeneous preparation formed two boundaries in the ultracentrifuge. The major component had a molecular weight of about 500,000, whereas a more slowly sedimenting peak ($S_{20,w}$ = 10.7) represented about 30% of the material. This finding was later confirmed by Duff et al. (2), who obtained purified type B toxin with a potency of 2.6×10^8 LD_{50}/mg of N by precipitation with sulfuric acid, extraction with calcium chloride, and final precipitation in the cold with ethyl alcohol. These preparations contained a major component sedimenting at S = 14.9 and a smaller polydisperse boundary at S = 10.9.

Recent reports from this laboratory have indicated that types A and E toxins, in the unaggregated form, have molecular weights ranging between 12,000 and 16,000 (5, 7). A similar investigation of type B toxin points to its molecular weight being still lower than these figures.

MATERIALS AND METHODS

Toxin production. One strain ("Lamanna") of *C. botulinum* type B was used throughout the experiments. This strain consistently produced toxin of potencies between 300,000 and 1,000,000 mouse MLD per ml. The organisms were grown for toxin production by the method described previously for type E (4). Dextrose was added to the medium to a final concentration of 0.5%, and incubation was continued for 5 days. The toxin assay technique has been described previously (5).

Protein analysis. Total protein was estimated by the Lowry method as given by Kabat and Mayer (8). Sanger's technique for labeling and identifying N-terminal amino acid residues was employed with the use of method A described by Fraenkel-Conrat, Harris, and Levy (3). Procedures for protein hydrolysis and performic acid oxidation were as described previously (6). Quantitative amino acid analysis was carried out on a Beckman Spinco automatic amino acid analyzer. Tryptophan assays were performed by means of procedure K of Spies and Chambers (13) and the method of Patchornik et al. (11).

Physical measurements. The sedimentation coefficient was measured with a Spinco model E analytical ultracentrifuge, with the use of a synthetic boundary cell. Runs were made at 50,740 rev/min, and photographs were taken at 8-min intervals. Three runs were made under varying *p*H and ionic conditions with a protein concentration of 5.0 mg/ml in each case: one in 0.067 M citrate-phosphate buffer (*p*H 2.2), one in 0.5 M guanidine acetate buffer (*p*H 4.5), and one in 0.1 M guanidine acetate buffer (*p*H 5.5). The partial specific volume was calculated as 0.726 by the method described by Schachman (12). The electrophoretic mobility and the diffusion coefficient were determined by use of a Beckman Spinco model H electrophoresis-diffusion apparatus, with the 11-ml standard cell. In this instance, the toxin was in a concentration of 1.0 mg/ml and had been dialyzed exhaustively against

0.05 M guanidine acetate buffer (*p*H 4.5). The Raleigh integral fringe method was used in the evaluation of the diffusion coefficient (10). Seven pictures taken over a 24-hr period were used for the calculation of this value.

Purification procedures. Saturated ammonium sulfate solution was added to toxic filtrates to a final concentration of 50% (v/v). The precipitate was allowed to form overnight at 4 C and was collected by centrifugation at 7,000 × *g* at 4 C. The precipitate thus formed is slight and finely dispersed, as is the case with type A toxin, whereas similarly treated type E filtrates form heavy flocculent precipitates. The toxic sediment was resuspended in 0.067 M citrate-phosphate buffer (*p*H 5.6) in approximately 2.5% of the original volume. Under these conditions, the sediment went into solution without any difficulty of the kind encountered by Lamanna and Glassman (9) and Duff et al. (2). The material was then dialyzed against the same buffer at 4 C overnight, the dialysis sacs having been boiled in 0.1 M ethylenediaminetetraacetic acid (*p*H 7.0) and washed in distilled water. Diethylaminoethyl (DEAE) cellulose, which had been ionized previously for at least 24 hr in 2 M NaCl at 4 C and then acidified with 1 N HCl, was packed in dilute suspension into columns (diameter, 0.9 cm) to a final height of 30 cm. Equilibration was carried out with 0.067 M citrate-phosphate buffer (*p*H 5.6). Toxin concentrates, containing between 3 and 5 mg of protein per ml, were added in 2-ml quantities to such columns, and elution was carried out with the same buffer on a model V-10 fraction collector (Gilson Medical Electronics, Middleton, Wis.). Flow rate was regulated to approximately 45 ml/hr, and 3-ml fractions were collected.

This method permitted the frontal elution of a highly toxic component (Fig. 1). Several toxic samples thus isolated were pooled and concentrated with the use of polyethylene glycol as previously described (5). This method has likewise proved suitable for purifying type A toxin and, because of its greater simplicity, is for this purpose preferable to our earlier technique.

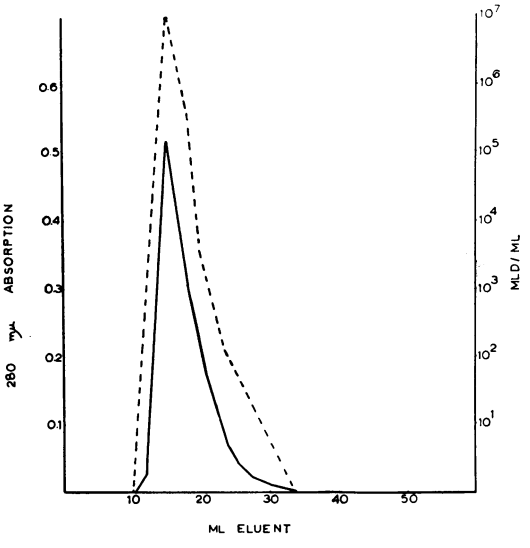

FIG. 1. *Elution profile of type B toxin through DEAE cellulose in 0.067 M citrate-phosphate buffer (pH 5.6). Solid line, absorbance at 280 mμ; broken line, MLD per milliliter.*

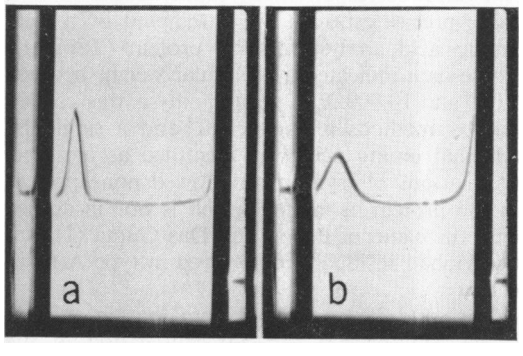

FIG. 2. *Representative photographs obtained in the sedimentation run on purified type B toxin in 0.1 M guanidine acetate buffer (pH 5.5). Speed, 50,740 rev/min; bar angle, 60°; protein concentration, 0.5%; times after obtaining full speed, (a) 0 min and (b) 48 min.*

RESULTS

The eluted toxic peak (Fig. 1) represents a recovery of 80 to 90% of the biological activity of the sample. This purified material had maximal ultraviolet absorption at 277 mμ, the extinction was $E_{277}^{1\,mg/ml} = 1.2$, and its potency was 2.0×10^7 MLD/mg of protein.

After further treatment by the concentration and dialysis procedures described above, three separate samples of the purified toxin, at *p*H 2.2, 4.5, and 5.5, respectively, were submitted to ultracentrifugal analysis. In each case, a single slow-moving boundary formed, with $S_{20,w}$ values falling between 0.65*S* and 0.69*S*. Representative photographs are shown in Fig. 2. Over a 96-min period, no shoulders or other boundaries appeared. Diffusion was not markedly evident, even in relatively high ionic strength buffers.

The homogeneity of this material was established with an electrophoresis run at *p*H 4.5, which demonstrated a single component migrating at 0.33×10^{-5} cm² per v-sec (Fig. 3). On the same solution, after sharpening the boundary, the diffusion coefficient was determined as $D_{20,w} = 8.89 \times 10^{-7}$ cm²/sec. With the formula

$$M = \frac{RTS}{D_{20}(1 - vp)}$$

we calculated a molecular weight of 6,900.

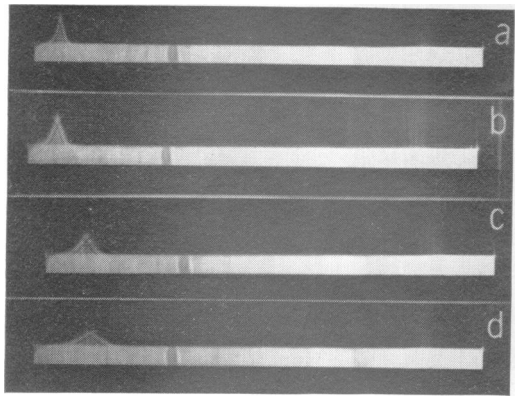

FIG. 3. *Representative photographs of the ascending electrophoretic boundary of type B toxin in 0.05 M guanidine acetate buffer (pH 4.5). Protein concentration, 0.10%; times after commencement of run, (a) 0 min, (b) 15 min, (c) 4.25 hr, and (d) 10.25 hr.*

In view of the inaccuracy of molecular-weight calculations based on biophysical measurements involving a material of very low sedimentation rate and correspondingly rapid diffusibility, a more precise estimate was attempted by a total amino acid analysis of this protein (Table 1). These data indicated a molecular weight between 9,000 and 10,000. End-group analysis was carried out by methods already cited, and a single N-terminal amino acid was identified as arginine. Estimations of tryptophan showed none present in the protein, a finding which is not in accord with the claim of Boroff and Das Gupta (1) that tryptophan residues are involved in type A toxin activity.

It should be mentioned here that in the *p*H range between 3.0 and 5.0 aggregation of the toxin occurred regardless of the ionic strength of buffers used, as evidenced by rapidly moving disperse boundaries in the untracentrifuge. At *p*H 5.5, under conditions of low ionic strength, the same observation was made. However, this occurrence was minimized if the toxin was maintained at either *p*H 2.2 or 5.6 in relatively strong buffer solutions. The use of guanidine acetate for buffering completely eliminated aggregation and yielded preparations which were homogeneous in terms of particle size and electrophoretic mobility.

DISCUSSION

The toxin of *C. botulinum* type B apparently falls into an even lower category of molecular weight than types A and E toxins. The discrepancy between the molecular-weight estimations based on physical measurements and total amino acid analysis is about 25%. The errors inherent in the use of physical measurements are magnified when substances of very low molecular weight are being examined. In this instance, the extremely low sedimentation coefficient and the correspondingly high rate of diffusion of the material inevitably render these methods somewhat inaccurate. Hence, we feel that the estimate of molecular weight based on total amino acid analysis, i.e., between 9,000 and 10,000, is closer to the actual value for the toxin.

Previous estimations of molecular weight for this toxin vary between 60,000 (9) and 500,000 (2, 14). The purification methods used by these workers involved precipitation with either acid or ethyl alcohol. It was shown earlier in this laboratory (5) that type A botulinum toxin showed a marked tendency to form aggregates, and during this study the same was found to be true for type B. Both Wagman and Bateman (14) and Duff et al. (2) found that their highly purified preparations of type B were heterogeneous in the ultracentrifuge, whereas they were homogeneous electrophoretically. Comparison of our values for biological activity per unit of weight with those obtained by previous workers shows that similar degrees of purity were obtained. However, the harsher methods previously used for purification probably lead to the formation of molecular aggregates, resulting in abnormally high molecular-weight values being attributed to this toxin.

TABLE 1. *Amino acid analysis of type B toxin*

Amino acid	Amt (μmoles)		Probable no. of residues
	Run 1	Run 2	
Cysteic acid*	0.0376		1
Aspartic acid	0.5881	0.5605	16
Threonine	0.1809	0.1746	5
Serine	0.2598	0.2477	7
Glutamic acid	0.2619	0.2480	7
Proline	0.0879	0.0858	3
Glycine	0.2020	0.1930	5
Alanine	0.1681	0.1586	4
Valine	0.1612	0.1531	4
Methionine*	0.0692		2
Isoleucine	0.2712	0.2601	7
Leucine	0.2595	0.2484	7
Tyrosine	0.1525	0.1498	4
Phenylalanine	0.1596	0.1498	4
Lysine	0.2346	0.2033	6
Histidine	0.0225	0.0204	1
Arginine	0.0773	0.0670	2

* Calculated from performic acid oxidation and hydrolysis of the protein.

ACKNOWLEDGMENT

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LITERATURE CITED

1. BOROFF, D. A., AND DAS GUPTA. 1964. Study of the toxin of *Clostridium botulinum*. VII. Relation of tryptophan to the biological activity of the toxin. J. Biol. Chem. **239**:3694–3698.
2. DUFF, J. T., J. KLERER, R. H. BIBLER, D. E. MOORE, C. GOTTFRIED, AND G. G. WRIGHT. 1957. Studies on immunity to toxins of *Clostridium botulinum*. II. Production and purification of type B toxin for toxoid. J. Bacteriol. **73**:597–601.
3. FRAENKEL-CONRAT, H., J. I. HARRIS, AND A. L. LEUNG. 1955. Recent developments in techniques for terminal and sequence studies on peptides and proteins. Methods Biochem. Analy. **2**:359–425.
4. GERWING, J., C. E. DOLMAN, AND D. A. ARNOTT. 1961. Purification and activation of *Clostridium botulinum* type E toxin. J. Bacteriol. **81**:819–822.
5. GERWING, J., C. E. DOLMAN, AND H. S. BAINS. 1965. Isolation and characterization of a toxic moiety of low molecular weight from *Clostridium botulinum* type A. J. Bacteriol. **89**:183–186.
6. GERWING, J., C. E. DOLMAN, and A. KO. 1965. Mechanism of tryptic activation of *Clostridium botulinum* type E toxin. J. Bacteriol. **89**:1176–1179.
7. GERWING, J., C. E. DOLMAN, M. E. REICHMANN, AND H. S. BAINS. 1964. Purification and molecular weight determination of *Clostridium botulinum* type E toxin. J. Bacteriol. **88**:216–219.
8. KABAT, E. A., AND M. M. MAYER. 1961. Experimental immunochemistry, p. 557–558. Charles C Thomas, Publisher, Springfield, Ill.
9. LAMANNA, C., AND H. N. GLASSMAN. 1947. The isolation of type B botulinum toxin. J. Bacteriol. **54**:575–584.
10. LONGSWORTH, L. G. 1952. Diffusion measurements at 1° aqueous solutions of amino acids, peptides and sugars. J. Am. Chem. Soc. **74**: 4155–4159.
11. PATCHORNIK, A., W. B. LAWSON, E. GROSS, AND B. WITKOP. 1960. J. Am. Chem. Soc. **82**:5923–5927.
12. SCHACHMAN, H. K. 1957. Ultracentrifugation, diffusion and viscometry, p. 32–103. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 4. Academic Press, Inc., New York.
13. SPIES, J. R., AND D. C. CHAMBERS. 1949. Chemical determination of tryptophan in proteins. Anal. Chem. 1249–1266.
14. WAGMAN, J., AND J. B. BATEMAN. 1951. The behaviour of botulinus toxins in the ultracentrifuge. Arch. Biochem. Biophys. **31**:424–430.