

## Purification and Some Properties of Progenitor Toxins of *Clostridium botulinum* Type B

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Received for publication 15 May 1974

Purification of progenitor toxin of *Clostridium botulinum* type B strain Okra was undertaken by sequential steps of acid precipitation, extraction, ammonium sulfate precipitation, ribonuclease digestion, acid precipitation, protamine treatment, sulphopropyl-Sephadex chromatography, and Sephadex G-200 gel filtration. Two different molecular-sized toxins, named large (L) and medium (M) toxins, were obtained. L toxin was centrifugally homogeneous but electrophoretically heterogeneous. It contained  $2.5 \times 10^8$  to  $3.0 \times 10^8$  mean lethal doses per mg of nitrogen, and its sedimentation constant was 16S. M toxin was centrifugally and electrophoretically homogeneous. It contained  $5.5 \times 10^8$  to  $6.0 \times 10^8$  mean lethal doses per mg of nitrogen, and its sedimentation constant was 12S. The presence of both L and M toxins in spent culture was demonstrated. It seems justified, therefore, to call both progenitor toxins. Both consisted of toxic and nontoxic components. The toxic components of L and M toxins appeared to be identical with each other. The nontoxic component of L toxin was 12S and possessed a hemagglutinin activity of about 0.5% that of type A crystalline toxin; that of M toxin was 7S and possessed no hemagglutinin activity. They were antigenically related but not identical.

*Clostridium botulinum* type A toxin was crystallized and its molecular weight was estimated to be 900,000 (12, 15). It consists of two components, a neurotoxin and a hemagglutinin (11). The neurotoxic component was obtained as a single entity with a molecular weight of 150,000, whereas the hemagglutinin component was obtained in three different forms with molecular weights of 290,000, 500,000, or 900,000 (4).

Type E progenitor toxin, molecular weight of 350,000, consists of neurotoxic and nontoxic components of the same molecular weight, 150,000. The nontoxic component contains no hemagglutinin activity (8). Although the dissociated toxic component plays the crucial role in parenteral toxicity, only progenitor toxin, defined as the complex of the toxic and nontoxic components, is significant as an oral toxin (9).

The molecular weights reported for type B toxin vary from 9,000 to 500,000 (6, 7, 10, 19, 20). Homogeneous type B toxin with a molecular weight of 165,000 or 167,000 was obtained by diethylaminoethyl (DEAE)-cellulose chromatography at pH 8.0 (1, 5). This toxin must have been the dissociated toxic component analogous to those of types A and E.

No previous publication has identified which purified toxin is type B progenitor toxin, nor

clarified its molecular structure. We attempted, therefore, to purify type B progenitor toxin without inducing possible molecular aggregation or disaggregation during the processes of purification. Different molecular-sized progenitor toxins were obtained. We attempted to characterize the molecular structure of each progenitor toxin in comparison with those of other types.

### MATERIALS AND METHODS

**Bacterial strain.** *C. botulinum* type B strain Okra was used. A suspension of about  $10^7$  viable spores per ml was kept frozen and used as the inoculum for toxin production.

**Culture for toxin production.** The medium for toxin production was composed of 2% peptone (for toxin production; Mikuni Kagaku Sangyo Co., Tokyo), 0.5% yeast extract (Oriental Yeast Kogyo Co., Osaka), 0.5% glucose, and 0.025% sodium thioglycolate. It was adjusted before autoclaving to pH 7.0 with a 10% NaOH solution. For toxin production, 1 ml of the spore suspension was inoculated into 5 liters of medium, which was incubated at 30 C for 4 days.

**Chemicals and reagents.** SP-Sephadex, C-50 (medium), DEAE-Sephadex, A-50 (medium), and Sephadex G-200 (particle size, 40 to 120  $\mu$ m) were purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Crystalline pancreatic ribonuclease was obtained from Worthington Biochemical Corp., Freehold, N.J.; protamine sulfate (salmon sperm) from Wako Pure

Chemical Industries, Osaka); and twice crystallized trypsin from Sigma Chemical Co., St. Louis, Mo. Trypsin was dissolved in 0.001 M HCl at 2 mg/ml; the solution was kept in a refrigerator for not longer than 10 days. Crystalline type A toxin was a gift from E. J. Schantz, Food Research Institute, University of Wisconsin, Madison. Type E progenitor toxin was purified in this laboratory by the method described by Kitamura et al. (8). Type B horse antitoxin (anti-large [L] and -medium [M] toxins) of 800 IU/ml was a gift from H. Kondo, Chiba Serum Institute, Ichikawa, Chiba.

**Determination of toxin potency.** Toxin potency was determined mostly by the time-to-death method by intravenous injection into mice (2, 17). Tryptic activation was performed in the same way as for type E progenitor toxin (8). From a standard dose response curve prepared with trypsinized type B progenitor toxin containing both L and M toxins, the intraperitoneal mean lethal dose ( $LD_{50}$ ) per milliliter was estimated.

Since the quantity of toxin in each fraction of sucrose density gradient centrifugation was too small to be determined by the bioassay, the reversed passive hemagglutination test was performed with sheep erythrocytes sensitized with anti-M toxin immunoglobulin purified from crude type B antitoxin on a column of CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals) conjugated with purified M toxin. The details of affinity chromatography, sensitization of sheep erythrocytes, and hemagglutination tests are described elsewhere (18).

**Determination of protein.** Protein was determined by the method of Lowry et al. (14), with bovine serum albumin as the standard.

**Polyacrylamide gel electrophoresis.** Polyacrylamide gel of 4.5% and the tray buffer were prepared according to Reisfeld et al. (16). Electrophoresis was carried out by applying a current of 2 to 3 mA per column for 3 h at room temperature. After electrophoresis, the gel columns were stained in a 1% amido black 10B solution in 7% acetic acid.

**Agar gel double diffusion.** About 4 ml of 1% agar (Special agar A; Wako Pure Chemical Industries) gel in 0.05 M acetate buffer (pH 6.0) was spread over a glass plate (5 by 5 cm) with seven stainless-steel cylinders (2 by 10 mm) placed upright at the center, with each angle of a 7-mm hexagon in one side. The cylinders were removed after the agar solidified. After each well was filled, the agar plates were incubated in a moist chamber at 20 C for 2 days.

**Sucrose density gradient centrifugation.** Linear density gradients of 5 to 20% sucrose were prepared in Beckman cellulose nitrate tubes (0.5 by 1 inch) [about 1.25 by 2.5 cm] at 0 C by mixing 2.5 ml each of 5 and 20% sucrose solutions in 0.05 M phosphate buffer, pH 6.0. A 0.2-ml portion of a sample was loaded on top of the sucrose gradient. Three tubes were centrifuged at a time in an RPS 40 rotor in a Hitachi 55P ultracentrifuge at  $120,000 \times g$  for 6 h at 4 C. After centrifugation, 11-drop fractions were collected manually.

**Purification of type B progenitor toxin.** The flow sheet for purification of type B progenitor toxin is given in Fig. 1.

For step 1 (acid precipitation [6]), a whole culture was adjusted to pH 4.0 with 3 N  $H_2SO_4$  and kept standing overnight at room temperature to allow the precipitate to settle. The supernatant fluid was removed by siphoning; to the remaining precipitate about 4 volumes of distilled water was added to dissolve acid-soluble materials, and the mixture was allowed to stand overnight. The supernatant fluid was removed by siphoning; the precipitate was packed by centrifugation at  $3,800 \times g$  for 10 min at 4 C.

For step 2 (extraction), the washed acid precipitate obtained from a 10-liter culture was extracted two or three times with 150 ml of 0.2 M phosphate buffer, pH 6.0, by centrifugation at  $8,600 \times g$  for 20 min at 4 C.

For step 3 (ammonium sulfate precipitation), 0.25 volumes of a saturated ammonium sulfate solution kept at room temperature was added to the cold extract. The mixture was allowed to stand for 1 h at room temperature and centrifuged at  $3,800 \times g$  for 10 min at 4 C. To the supernatant fluid, taken by decantation, solid ammonium sulfate was added to 60% saturation. The mixture was allowed to stand overnight at 4 C. The toxin precipitate was collected by centrifugation at  $3,800 \times g$  for 10 min at 4 C.

For step 4 (ribonuclease digestion), the precipitated toxin was dissolved in 50 ml of 0.2 M phosphate buffer, pH 6.0; it was dialyzed against 2 liters of the same buffer for 24 h at 4 C. The toxic solution in the dialysis casing was digested with ribonuclease (50  $\mu g/ml$ ) for 5 h at 30 C.

For step 5 (second acid precipitation and extraction), the precipitate formed during the ribonuclease digestion was removed by centrifugation. The supernatant fluid containing the toxin was adjusted to pH 4.2 with 1 M acetic acid and dialyzed against 0.05 M acetate buffer, pH 4.2, for 48 h at 4 C. A white precipitate appeared during dialysis. It contained the toxin and was collected by centrifugation at  $8,600 \times g$  for 10 min at 4 C. The toxic precipitate was washed twice, first with 50 ml of 0.05 M acetate buffer, pH 4.2, and then with 50 ml of the same buffer containing 0.1 M NaCl. The washed precipitate was extracted with 0.05 M acetate buffer (pH 4.2) containing 0.5 M NaCl for 10 min at 37 C followed by centrifugation at  $8,600 \times g$  for 10 min at 10 C. The extraction was repeated two more times, and the three extracts were combined.

For step 6 (protamine treatment), a 2% protamine sulfate solution was added dropwise to the extract, which was kept stirred on a mechanical stirrer to a final protamine concentration of 0.06% (wt/vol). The mixture was allowed to stand for 20 min at room temperature and centrifuged at  $8,600 \times g$  for 10 min at 4 C. The toxic supernatant was percolated through a sulphopropyl (SP)-Sephadex column (2 by 3 cm) equilibrated with 0.05 M acetate buffer, pH 4.2, containing 0.5 M NaCl, to remove the excess protamine that was adsorbed onto the column.

For step 7 (SP-Sephadex chromatography), the column effluent was diluted 2.5-fold in 0.05 M acetate buffer, pH 4.2, to lower the sodium chloride concentration to 0.2 M. The diluted effluent was applied to an SP-Sephadex C-50 column (2 by 15 cm) equilibrated with the same buffer containing 0.2 M NaCl.

Whole culture  
 |  
 Precipitation at pH 4.0 adjusted with 3 N H<sub>2</sub>SO<sub>4</sub>  
 |  
 Extraction with 0.2 M phosphate buffer, pH 6.0  
 |  
 Precipitation at 20% saturation of ammonium sulfate  
 |  
 Precipitation at 60% saturation of ammonium sulfate  
 |  
 Ribonuclease treatment for 5 h at 30 C  
 |  
 Precipitation at pH 4.2 adjusted with 1 N acetic acid  
 |  
 Extraction with 0.05 M acetate buffer, pH 4.2, containing 0.5 M NaCl  
 |  
 Protamine treatment  
 |  
 SP-Sephadex (C-50) chromatography at pH 4.2  
 |  
 Gel filtration on Sephadex G-200 at pH 4.2

FIG. 1. Purification procedure for *Clostridium botulinum* type B toxin.

The toxin, adsorbed onto the column, was eluted with a linear gradient of NaCl from 0.2 to 0.4 M in 1,000 ml of the same buffer (Fig. 2).

For step 8 (molecular sieving on Sephadex G-200), the toxic fractions eluted from SP-Sephadex were pooled and concentrated by ultrafiltration through an Amicon UM-10 membrane (Amicon Corp., Lexington, Mass.). The concentrate was applied to a Sephadex G-200 column (2.5 by 96 cm) and eluted with 0.05 M acetate buffer, pH 4.2, containing 0.5 M NaCl. Two toxin peaks emerged; the early eluted toxin was named L toxin and the late eluted toxin was named M toxin (Fig. 3).

**Separation of toxic from nontoxic component.** L and M toxin pooled fractions were chromatographed on DEAE-Sephadex A-50 (0.9 by 10 cm) equilibrated with 0.01 M phosphate buffer, pH 8.0, with a linear gradient of NaCl from 0 to 0.5 M in 200 ml of the same buffer (9).

## RESULTS

**Purification of type B progenitor toxin and examinations for purity.** The recovery and specific toxicity at each step of purification are shown in Table 1. After separation by molecular sieving on Sephadex G-200, M toxin contained  $6.04 (5.5 \text{ to } 6.0) \times 10^8$  LD<sub>50</sub> per mg of nitrogen, and L toxin contained  $2.63 (2.5 \text{ to } 3.0) \times 10^8$  LD<sub>50</sub> per mg of nitrogen (the bottom two lines of Table 1). Through all the steps of purification, the activation ratio (the ratio of the toxicity after trypsinization to that before) did not change appreciably. Toxicity of L and M toxins increased by 4- to 30-fold by tryptic activation. M toxin at 192 μg/ml showed no hemagglutinin activity, whereas L toxin at 123 μg/ml or higher concentrations showed positive agglutination of chicken erythrocytes. Under the same conditions, crystalline type A toxin at 0.6 μg/ml or

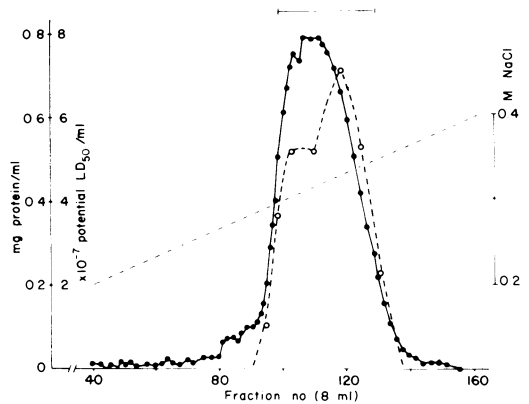


FIG. 2. Chromatography of protamine-treated toxin from an SP-Sephadex column (2 by 15 cm) equilibrated with 0.05 M acetate buffer, pH 4.2, containing 0.2 M NaCl. Elution with a linear gradient of sodium chloride in the buffer. The sample contained 328 mg of protein and a potential toxicity of  $1.34 \times 10^{10}$  LD<sub>50</sub>. Symbols: ●, protein contents; ○, toxicity; ----, NaCl concentration.

higher concentrations gave positive hemagglutination.

In polyacrylamide gel electrophoresis at pH 4.0, purified L and M toxins formed a major and one or more minor bands, one of which possessed a relative mobility corresponding to that of the other toxin. After another run of gel filtration of each, M toxin showed a single band, but L toxin still showed two to four bands (Fig. 4).

In ultracentrifugal analysis at pH 4.2, each toxin formed a single symmetrical boundary. The sedimentation constants of M and L toxins were calculated to be 11.5 and 16.1S, respec-

tively. In gel filtration at pH 4.2, M toxin was eluted at the same position as type E progenitor toxin. The molecular weight of M toxin, therefore, was estimated to be about 350,000.

**Demonstration of L and M toxins in culture.** The two different molecular-sized toxins were demonstrated by gel filtration of supernatant fluid of 2-, 4-, and 7-day cultures on Sephadex G-200 with, as eluant, 0.05 M acetate buffer (pH 6.0) containing 0.1 M NaCl. We observed that the longer the incubation period, the larger the relative amount of L toxin appearing in the supernatant.

In sucrose density gradient centrifugation of the supernatant of a 4-day culture, two toxin peaks appeared; the heavy one corresponded to L and the light one corresponded to M toxin in sedimentation (Fig. 5).

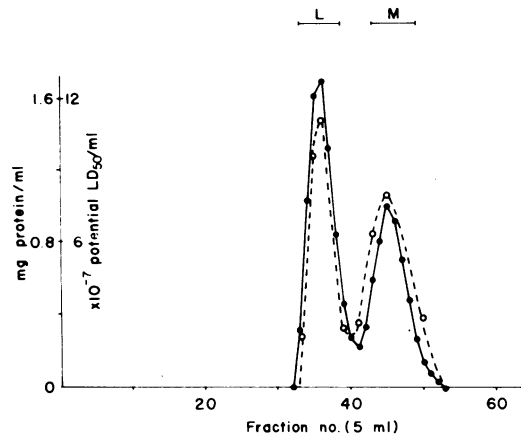


FIG. 3. Gel filtration of SP effluent on a Sephadex G-200 column (2.5 by 96 cm) with 0.05 M acetate buffer, pH 4.2, containing 0.5 M NaCl as eluant. The sample contained 79.5 mg of protein and a potential toxicity of  $8.25 \times 10^6$  LD<sub>50</sub>. Symbols: ●, protein contents; ○, toxicity.

**Molecular dissociation of L and M toxins.** In DEAE-Sephadex chromatography at pH 8.0, M toxin was eluted in two protein peaks of the same area. The first peak was toxic and the second was nontoxic. L toxin was eluted also in toxic and nontoxic peaks under the same conditions, but the area of the second peak was about

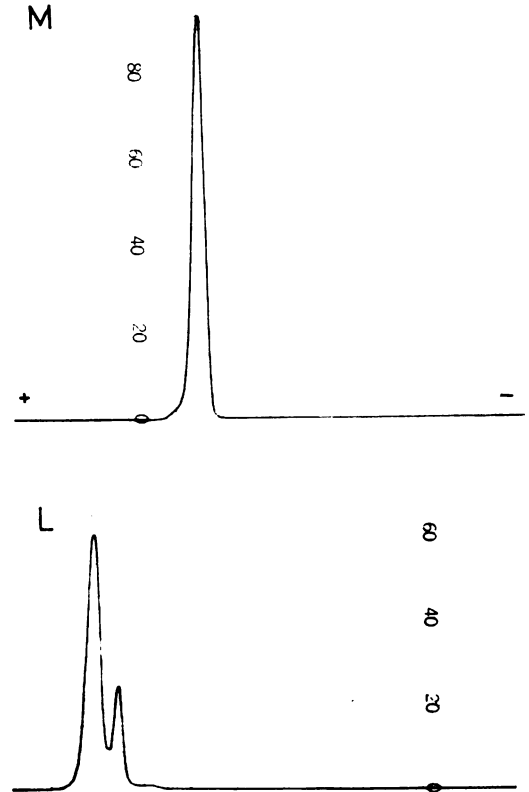


FIG. 4. Polyacrylamide gel electrophoresis of the purified L and M toxins at pH 4.0. Samples: L toxin, 70 μg; M toxin, 110 μg.

TABLE 1. Purification of *C. botulinum* type B toxin from a 10-liter culture

Step	Protein		Potential ( $\times 10^{-6}$ )		Potential ( $\times 10^{-6}$ ) (LD <sub>50</sub> /mg of nitrogen)
	Amt mg	%	LD <sub>50</sub>	%	
Whole culture	— <sup>a</sup>	—	70.0	100	—
1st acid precipitate	7,300	100	62.4	89	0.53
Extract	3,200	44	62.0	89	1.21
Ammonium sulfate precipitate	930	13	36.0	51	2.42
Ribonuclease digest	572	8	34.0	48	3.72
2nd acid precipitate	429	6	28.3	40	4.12
Extract	334	5	19.0	27	3.56
Protamine-treated	286	4	16.1	23	3.52
SP effluent	125	2	11.0	15	5.50
G-200 effluent					
M toxin	30	0.4	2.9	4	6.04
L toxin	38	0.5	1.6	2	2.63

<sup>a</sup> —, Not determined.

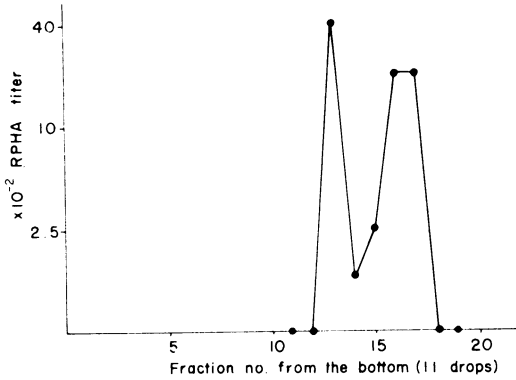


FIG. 5. Ultracentrifugation of whole-culture supernatant in a sucrose density gradient (5 to 20%) at pH 6.0. A 0.2-ml sample contained  $1.6 \times 10^6$  LD<sub>50</sub>. RPHA titer denotes the reciprocal of the highest dilution of the fraction giving positive hemagglutination.

four times as large as that of the first one (Fig. 6).

In Sephadex G-200 gel filtration at pH 8.0, dissociated M toxin was eluted in a single peak at a position close to that for alcohol dehydrogenase, its molecular weight being 150,000. The elution position of the toxic peak was identical to that of the single peak of M toxin (Fig. 7).

In ultracentrifugal analysis at pH 6.0 of each M toxin component separated chromatographically, a sedimentation constant of 7S was obtained for each.

In agar gel double-diffusion tests at pH 6.0, M toxin formed two precipitin lines. The single lines formed with the toxic components of each toxin coalesced, whereas the lines of the non-toxic components of M and L toxins only partially coalesced (Fig. 8).

## DISCUSSION

We intended to purify and characterize the progenitor toxin of *C. botulinum* type B, which had not been obtained in an ultimately pure state. To prevent molecular dissociation, any process that had to be performed at pH 7 or above was avoided. For the first step of purification, acid precipitation (6, 10) seemed to be simplest and most economical for handling a large batch of culture. Extraction of the toxin from the acid precipitate was accomplished most efficiently with 0.2 M phosphate buffer, pH 6.0, which had been used to extract progenitor toxin from type E organisms (8). The extract became insoluble, however, when equilibrated with a buffer below pH 5, probably due to the presence of a large amount of ribonucleic acid in the acid precipitate. To make the toxic extract soluble below pH 5, which is suitable for SP-

Sephadex chromatography, treatments with ribonuclease and protamine were applied. The ribonuclease treatment removed a large amount of RNA but prevented precipitation only partially. The protamine treatment, on the other

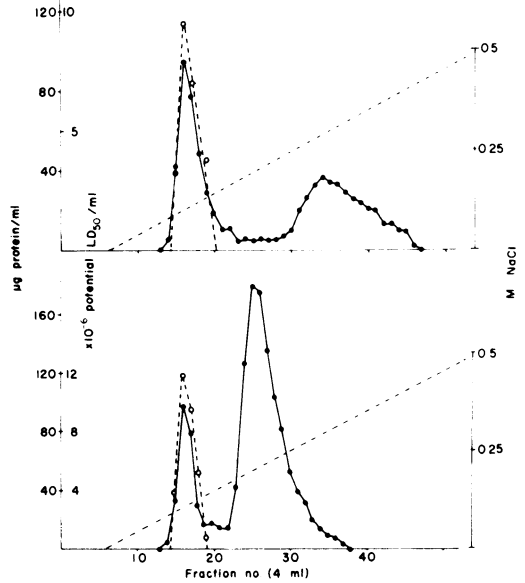


FIG. 6. Chromatography of M (top) and L (bottom) toxins on DEAE-Sephadex (A-50) columns (0.9 by 10 cm) equilibrated with 0.01 M phosphate buffer, pH 8.0. Elution with a linear gradient of sodium chloride in the same buffer. M toxin (3.3 ml) contained 2.2 mg of protein and a potential toxicity of  $1.98 \times 10^6$  LD<sub>50</sub>; L toxin (4.0 ml) contained 5.4 mg of protein and a potential toxicity of  $2.38 \times 10^6$  LD<sub>50</sub>. Symbols: ●, protein contents; ○, toxicity; ----, NaCl concentration.

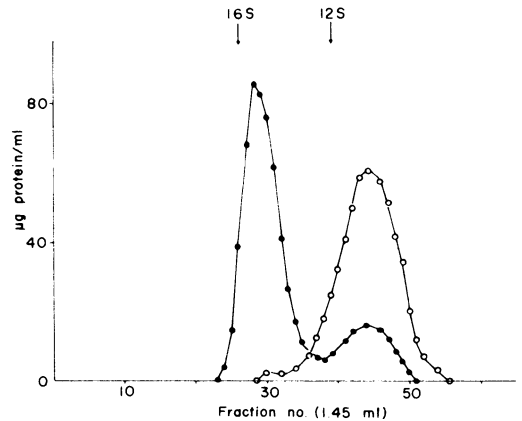


FIG. 7. Gel filtration of L (●) and M (○) toxins on a Sephadex G-200 column (1.5 by 80 cm) with 0.01 M phosphate buffer (pH 8.0) containing 0.2 M sodium chloride as eluant. Each L and M toxin sample contained 1.2 mg of protein.

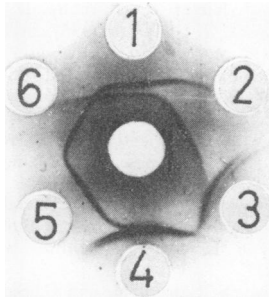


FIG. 8. Agar gel double-diffusion tests with purified L and M toxins and their toxic and nontoxic components. Center well received horse antitoxin type B with a potency of 355 IU/ml. Lateral wells: (1) M toxin, 439  $\mu\text{g/ml}$ ; (2) nontoxic component of M toxin, 387  $\mu\text{g/ml}$ ; (3) nontoxic component of L toxin, 200  $\mu\text{g/ml}$ ; (4) L toxin, 436  $\mu\text{g/ml}$ ; (5) toxic component of L toxin, 235  $\mu\text{g/ml}$ ; and (6) toxic component of M toxin, 250  $\mu\text{g/ml}$ .

hand, completely prevented precipitation of the material when brought to pH 5 or below, but an excess amount caused partial precipitation of the toxin. The use of both ribonuclease and protamine prevented precipitation and loss of the toxin. Such a weakly cationic exchanger as carboxymethyl-Sephadex, used for purification of type E progenitor toxin (8), did not adsorb type B toxin; hence a more strongly cationic exchanger, SP-Sephadex, was adopted. The use of an anionic exchanger such as DEAE- or diethyl-(2-hydroxypropyl)aminoethyl-Sephadex was avoided, because it is used generally above pH 7. Gel filtration on Sephadex G-200 resolved the toxin into two peaks L and M toxins. Each toxin gave a single symmetrical boundary in ultracentrifugation at pH 4. In polyacrylamide gel electrophoresis, however, L toxin gave multiple bands, whereas M toxin gave a single band. It is not now known whether all of the multiple bands of L toxin represent toxin molecules with slightly different electric charges or whether some represent contaminating substances.

The overall recovery of the toxin including L and M toxins through the present purification procedures was 5 to 10%. The specific potential toxicity of L toxin was  $2.5 \times 10^8$  to  $3.0 \times 10^8$  LD<sub>50</sub> per mg of nitrogen, and that of M toxin was  $5.5 \times 10^8$  to  $6.0 \times 10^8$  LD<sub>50</sub> per mg of nitrogen. The former is on the same level as those reported previously by other workers (6, 10), but the latter is at least 2 to 2.5 times higher than the highest value ever reported for type B toxin (6). It should be mentioned, however, that in the past, tryptic activation was not performed to titrate type B toxin.

There is no reason to believe that either one or both of L and M toxins were artifacts, because both were demonstrated in supernatants of spent cultures by Sephadex G-200 gel filtration and sucrose density gradient centrifugation performed at a pH value and ionic strength simulating the spent culture. The physiological significance of L and M toxins for the organisms is not known. The sedimentation constants of L and M toxins were found to be 16S and 12S, respectively. The figure for L toxin agrees with the one reported by Duff et al. (6), Wagman and Bateman (20), and Schantz and Spero (19). The molecular weight of L toxin, therefore, was estimated to be about 500,000. From the same elution volume in gel filtration and the same sedimentation constant as for type E progenitor toxin (8), the molecular weight of M toxin was estimated to be about 350,000.

Schantz and Spero (19) estimated the sedimentation constant of type B toxin in culture as 16S. Duff et al. (6) reported that their purified type B toxin contained a minor component of 10.9S in addition to the major one of 14.9S, and that the main toxin purified by extracting the acid precipitate with 0.2 M succinate buffer, pH 5.5, instead of 0.05 M calcium chloride solution at pH 6.0 was 12.7S. The distribution of different molecular-sized toxins in culture, however, was not examined by these authors. In the present investigation, the presence of both L and M toxins in the starting material was demonstrated; therefore, it does not seem justified to assume that L or M toxin, or both, is an artifact resulting from aggregation or disaggregation of some other toxin molecules. Another strain of *C. botulinum* type B, strain QC, which is a nonproteolytic strain, was also found to produce two different molecular-sized toxins with the same sedimentation constants as those of L and M toxins of strain Okra (unpublished data).

In gel filtration at pH 4.2 and 8.0, in DEAE-Sephadex chromatography at pH 8.0, and in agar gel double diffusion, M toxin behaved identically with type E progenitor toxin (8). We therefore conclude that the M toxin molecule is a complex of one molecule of the toxic component of 7S with a molecular weight of about 150,000 and one molecule of the nontoxic component with an equivalent molecular weight and sedimentation constant. On the other hand, L toxin was separated into two peaks in Sephadex G-200 gel filtration and DEAE-Sephadex chromatography at pH 8.0. The elution position of the toxic component of L toxin was identical to the counterpart component of M toxin in gel filtration. The molecular weight of the toxic

component of L toxin, therefore, was estimated to also be about 150,000, and that of the nontoxic component was estimated to be 350,000, assuming that the molecular weight of L toxin is 500,000 (6, 20).

Agar gel double-diffusion tests indicate that the nontoxic component of L toxin may be composed of two subcomponents, one antigenically related to the nontoxic component of M toxin and the other not contained by M toxin and associated with the hemagglutinin activity. Under conditions that allow M toxin to dissociate into the two components, L toxin also dissociates into the two, but the nontoxic component of L toxin does not dissociate further. This suggests that the bond connecting the two subcomponents is a stronger one.

At the present moment, both L and M toxins seem to be entitled to be called progenitor toxins constituting oral toxins to man and animals according to the definition described by Lamanna and Sakaguchi (13).

#### ACKNOWLEDGMENT

This investigation was supported by a grant for scientific research from the Ministry of Education.

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