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Molecular Construction of *Clostridium botulinum* Type A Toxins

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Two *Clostridium botulinum* type A toxic fractions, named large (L) and medium (M) toxins, were eluted from Sephadex G-200. Sucrose density gradient centrifugation resolved L toxin (2.5×10^8 to 3.0×10^8 mean lethal doses per mg of N) into two fractions, 19S and 16S. The same procedure performed at pH 8 resolved it into three fractions; the heavier two were both nontoxic and hemagglutinin positive, and the lightest one (7S) was toxic. M toxin (12S) (4.5×10^8 to 5.0×10^8 mean lethal doses per mg of N) was homogeneous in electrophoresis and centrifugation at pH 6. The latter procedure performed at pH 8 demonstrated that it dissociated into uniform 7S components. The nontoxic component of M toxin was free from hemagglutinin. M toxin alone was demonstrated in culture by sucrose density gradient centrifugation at pH 6. Dialysis of the culture supernatant resulted in partial formation of 16S toxin. Centrifugation of the crystalline toxin in 1 M NaCl demonstrated 16S toxin only. The toxic components of L, M, and crystalline toxins were antigenically identical. The nontoxic components of the crystalline and L toxins, consisting of two distinct antigens, were antigenically identical; that of M toxin was identical with one of these two antigens.

Clostridium botulinum type A toxin, the first crystallized bacterial toxin (1, 15), has a molecular weight of 900,000 and an *S* value of 17.3 (22, 23). Duff et al. (8) reported an *S* value of 14.5 for their preparation of type A toxin. The crystalline toxin behaves as a homogeneous protein in electrophoresis and ultracentrifugation below pH 5.5, but its *S* value varies from 13 to 20 depending upon the solvent used (28).

It is, at present, generally accepted that type A toxin has a molecular weight of 900,000 and an *S* value of 19 (16) and consists of two components, neurotoxin and hemagglutinin (13, 16, 19). Molecular dissociation occurs when the crystalline toxin is exposed to pH higher than 7.5 and ionic strength higher than 0.13 (29). The two components are separable from each other by diethylaminoethyl (DEAE)-Sephadex chromatography at pH 8.0. The neurotoxic component was obtained as a homogeneous protein with a molecular weight of 150,000; the hemagglutinin component occurred in three different forms with molecular weights of 290,000, 500,000, and 900,000 (4, 6).

Schantz and Spero (26) analyzed the molecular size of type A toxin in spent culture and found the 19S toxin only. Hauschild and Hilsheimer (9) demonstrated two different type A toxins with molecular weights of about 200,000 and 500,000 in culture and cell extracts. Lamanna and Sakaguchi (16) proposed the term "progenitor toxin" to designate the toxin first appearing in culture of foodstuffs and regarded the 19S toxin as type A progenitor toxin.

Type E progenitor toxin of uniform molecular size has a molecular weight of 350,000 and an *S* value of 11.6 whether it is activated with trypsin or not. It is composed of one molecule each of the neurotoxic and nontoxic components of the same molecular weight, 150,000 (11). Two different sized type B progenitor toxins, 16S and 12S, have been reported (12). Type F progenitor toxin of uniform molecular size has an *S* value of 10.3 and a molecular weight of 235,000 (21).

The accepted molecular size of type A progenitor toxin seems to be exceptionally large compared with those of other types (25). To authenticate the molecular size of type A progenitor toxin and elucidate its molecular construction, attempts were made to purify it by processes similar to those used to purify progenitor toxins of other types. It was indicated that the toxin in culture had exclusively a molecular size of 12S, which is common in type B, E, and F progenitor toxins, but the purification processes produced

two toxins of different molecular size, 16S and 19S, in addition to the 12S toxin. Characterization of each was attempted.

MATERIALS AND METHODS

Toxin production. *C. botulinum* type A strain Hall was provided by E. J. Schantz, Food Research Institute, University of Wisconsin, Madison. The medium had the same ingredients as that used for *C. botulinum* type B (12). For toxin production, 10 ml of an overnight culture in liver infusion broth was inoculated into 5 liters of medium, which was incubated at 30 C for 5 days.

Chemicals and reagents. Sulfopropyl (SP)-Sephadex C-50 (medium), DEAE-Sephadex A-50 (medium), and Sephadex G-200 (particle size, 40 to 120 μm) were the products of Pharmacia Fine Chemicals, Uppsala, Sweden. Protamine sulfate (salmon sperm), ribonucleic acid (RNA), and deoxyribonucleic acid (DNA) (both sodium salts) were purchased from Wako Pure Chemical Industries, Osaka. Type A crystalline toxin was given by E. J. Schantz. Type B and E progenitor toxins were purified by the methods reported by Kozaki et al. (12) and Kitamura et al. (11), respectively. Type A antitoxic horse plasma containing 1,280 IU/ml was given by H. Kondo, Chiba Serum Institute, Ichikawa-shi, Chiba.

Determination of toxicity. Toxicity was determined mostly by the time-to-death method by intravenous injection into mice (2). From the standard curve prepared with a partially purified preparation (a mixture of L and M toxins) of type A toxin, intraperitoneal mean lethal dose (LD_{50}) per milliliter was estimated. The same standard curve was found to be applicable to 19S, 16S, 12S, and 7S toxins despite the report of dependence of time to death on the molecular size of botulinum toxin (17).

Determination of protein, RNA, and DNA. Protein was determined by the method of Lowry et al. (20) with crystalline bovine serum albumin (fraction V, Armour Pharmaceutical Co., Chicago, Ill.) as standard. RNA was determined by the method of Kerr and Seraidarian (10), and DNA was determined by the method of Burton (3).

Determination of direct hemagglutinin. Samples were diluted twofold serially in 0.075 M NaCl-0.075 M phosphate buffer, pH 7.2. The reaction mixture contained 0.05 ml of dilution and 0.05 ml of a 0.5% suspension of chicken erythrocytes. Hemagglutination was read after incubation for 2 h at 4 C (18). Hemagglutinin titer denotes the reciprocal of the highest dilution of a sample giving positive hemagglutination.

Polyacrylamide gel electrophoresis. Polyacrylamide gel (4.5%) and the tray buffer (pH 4.5) were prepared by the method of Reisfeld et al. (24). Electrophoresis was carried out by applying a current of 2 to 3 mA per column for 3 to 3.5 h at room temperature. After electrophoresis, the gel column was stained with 1% amido black 10B in 7% acetic acid.

Agar gel double diffusion. The method reported previously (12) was used.

Sucrose density gradient centrifugation. A linear density gradient of 5 to 20% sucrose was prepared at 0 C in a Beckman cellulose tube (1.25 by 2.5 cm) by mixing 2.5 ml each of 5 and 20% sucrose solutions in 0.05 M phosphate buffer (KH_2PO_4-Na_2HPO_4), pH 6.0 or pH 8.0, or in 0.05 M acetate buffer, pH 6.0, containing 1 M NaCl. A 0.2-ml portion was loaded on top of a sucrose gradient. Six tubes were centrifuged at a time in an RPS 40 T-2 rotor in a Hitachi 65P ultracentrifuge at 123,000 × *g* and 15 C for 6 h for pH 6.0 gradient or 145,000 × *g* and 4 C for 8 h for pH 8.0 gradient. After centrifugation, 13-drop fractions were collected manually from the bottom of the tube.

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

Step 1: Acid precipitation. The whole culture was adjusted to pH 3.5 with 3 N H_2SO_4 and kept standing overnight at room temperature. The supernatant fluid was siphoned off; the bottom fluid was added with 4 volumes of distilled water. The mixture was allowed to stand overnight at room temperature. After siphoning off the supernatant fluid, the bottom fluid was centrifuged at 4,650 × *g* for 20 min at 4 C. The packed precipitate from a 10-liter culture was resuspended in 500 ml of distilled water. The suspension, adjusted to pH 4.7 with 0.1 N NaOH, was centrifuged at 4,650 × *g* for 20 min at 4 C.

Step 2: Extraction. The washed acid precipitate was suspended in 800 ml of 0.05 M acetate buffer, pH 6.5, containing 1 M NaCl, and the suspension was adjusted to pH 6.5 with 0.1 N NaOH. It was centrifuged at 4,650 × *g* for 20 min at 4 C.

Step 3: Protamine treatment. A 2% protamine sulfate solution was added to the extract while the extract was stirred on a magnetic stirrer until an RNA-protamine ratio of 1:4 was attained. The mixture was allowed to stand for 20 min at room temperature, and the precipitate formed was removed by centrifugation at 4,650 × *g* for 20 min 4 C.

Step 4: Ammonium sulfate precipitation. The protamine-treated extract was added with solid ammonium sulfate to 50% saturation (38 g/100 ml). The mixture was allowed to stand overnight at 4 C. The precipitate formed was collected by centrifugation for 20 min at 4,650 × *g* and 4 C, dissolved in 50 ml of 0.05 M acetate buffer, pH 3.8, and dialyzed against 3 liters of the same buffer for 24 h at 4 C. The nontoxic precipitate formed during dialysis was removed by centrifugation for 10 min at 7,800 × *g* and 4 C. The toxic supernatant fluid was added with NaCl to 0.55 M and percolated through an SP-Sephadex C-50 column (2 by 10 cm) equilibrated with 0.05 M acetate buffer, pH 3.8, containing 0.55 M NaCl. The excess protamine was adsorbed onto the column and the toxin was recovered in the percolate.

Step 5: SP-Sephadex chromatography. The percolate was diluted 1.6-fold with 0.05 M acetate buffer, pH 3.8, to bring the NaCl concentration to 0.35 M. The diluted percolate was applied to another SP-Sephadex C-50 column (2 by 15 cm) equilibrated with the same buffer containing 0.35 M NaCl. The toxin was adsorbed onto the column and eluted in a

linear gradient with NaCl from 0.35 to 0.55 M in the same buffer.

Step 6: Gel filtration on Sephadex G-200. The toxic fractions eluted from SP-Sephadex were pooled and precipitated with ammonium sulfate at 50% saturation. The toxic precipitate, dissolved in 10 to 15 ml of 0.05 M acetate buffer, pH 3.8, containing 0.5 M NaCl, was subjected to gel filtration on a Sephadex G-200 column (2.5 by 97 cm) with the same buffer as eluant.

RESULTS

Purification of type A toxins. Recovery in protein, toxicity, and hemagglutinin activity at each step of purification are shown in Table 1. The extract of the washed acid precipitate contained a large amount of RNA, which was removed by the protamine treatment without appreciable loss of toxicity. The protamine treatment made the toxin soluble at pH 3.8. Thus, the toxin was successfully adsorbed onto SP-Sephadex equilibrated with 0.05 M acetate buffer, pH 3.8, containing 0.35 M NaCl. The adsorbed toxin was eluted in two peaks at NaCl concentrations of approximately 0.43 and 0.45 M NaCl (Fig. 2). In gel filtration of the mixture of the two toxic fractions, two toxin peaks were eluted, one at a position corresponding to the void volume and the other at a retarded position. The first peak was named large (L) and the second one medium (M) toxin (Fig. 3). The L toxin fraction contained 2.7 (2.5 to 3.0) $\times 10^8$ LD_{50}/mg of N and gave positive hemagglutination at 3.5 μg/ml or higher concentrations. The M toxin fraction contained 4.6 (4.5 to 5.0) $\times 10^8$ LD_{50}/mg of N and gave negative hemagglutination at a concentration of 184 μg/ml. Under the same conditions, the crystalline toxin gave positive hemagglutination at 3.1 μg/ml or higher concentrations.

Purities and molecular sizes of the purified toxins. L toxin was further resolved into two toxic peaks by centrifugation in sucrose density gradient in 0.05 M phosphate buffer, pH 6.0. The heavier peak sedimented to the same position as did the main component of crystalline toxin (19S), and the lighter one sedimented to the same position as did type B L

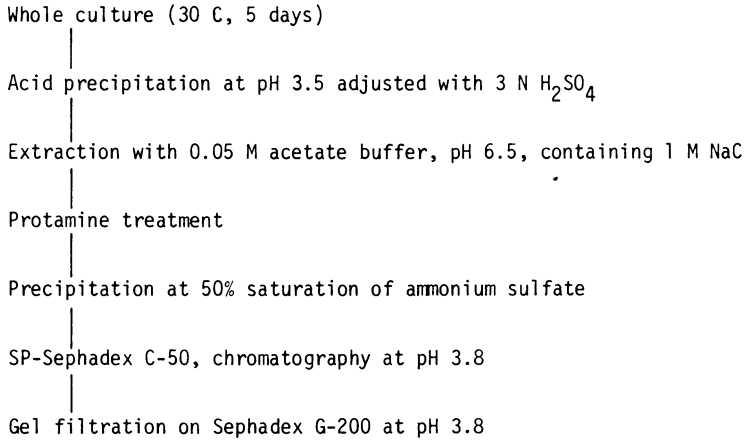

FIG. 1. *Purification procedures for Clostridium botulinum type A toxin.*

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

Culture fraction	Protein (mg)	LD_{50}/ml ($\times 10^{-9}$)	HA titer/ml[a] ($\times 10^{-4}$)	LD_{50}/mg of N ($\times 10^{-8}$)
Whole culture	—[b]	25.5 (100)[c]	320 (100)	—
Acid precipitate	5,010 (100)	23.6 (92.5)	270 (84)	0.3
Extract	554 (11)	13.2 (52)	20 (6.5)	1.4
Protamine treated	392 (7.8)	10.2 (40.6)	17 (5.2)	1.6
$(NH_4)_2SO_4$ precipitate	208 (4.1)	7.1 (28)	12 (3.7)	2.1
SP-Sephadex effluent	124 (2.5)	5.6 (22)	1.6 (0.5)	2.8
G-200 effluent				
L toxin	33 (0.6)	1.4 (5.4)	0.5 (0.1)	2.7
M toxin	26 (0.5)	1.9 (7.4)	0.04 (0.01)	4.6

[a] HA, Hemagglutination.
[b] —, Not determined.
[c] Numbers in parntheses are percentages.

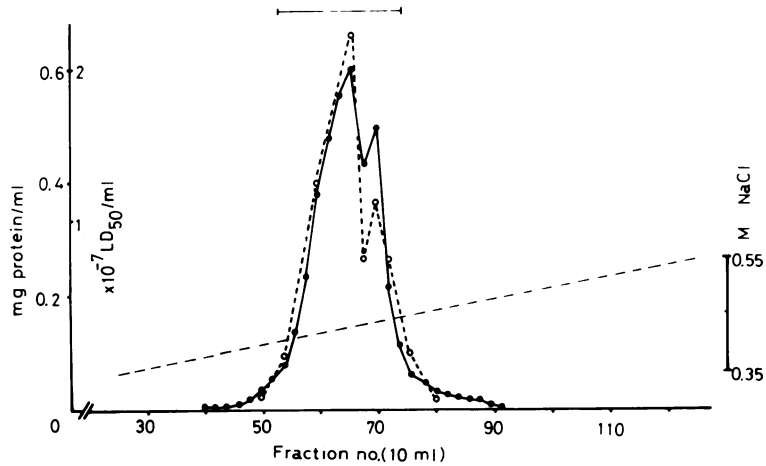

FIG. 2. *Chromatography of protamine-treated toxin on an SP-Sephadex column (2 by 15 cm) equilibrated with 0.05 M acetate buffer, pH 3.8, containing 0.35 M NaCl in linear gradient increase in NaCl concentration. The sample contained 170 mg of protein and 2.8 × 10⁹ LD_{50}. Symbols: ●, Protein contents; ○, toxicity; -----, NaCl concentration.*

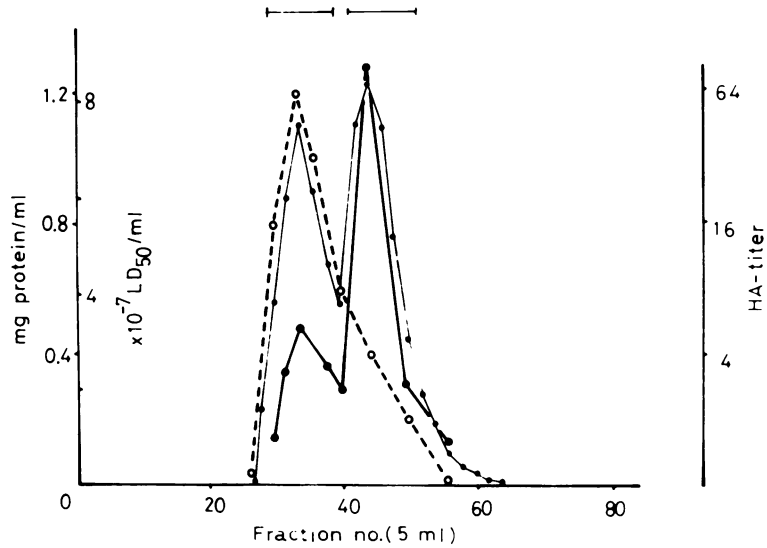

FIG. 3. *Gel filtration of SP-Sephadex effluent on a Sephadex G-200 column (2.5 by 97 cm) with 0.05 M acetate buffer, pH 3.8, containing 0.5 M NaCl, as eluant. The sample contained 78 mg of protein and 1.4 × 10⁹ LD_{50}. Symbols: ●, Protein contents; ●, toxicity; ○, hemagglutination titer.*

toxin (16S). In sucrose density gradient in the same buffer supplemented with 1 M NaCl, both crystalline and L toxins sedimented in a single peak to the same position as did type B L toxin (16S). M toxin sedimented in a single peak to the same position as did type B M (12S) and type E progenitor toxins (12S) (Fig. 4). These results showed the presence of at least three different sized type A toxins, 19S, 16S, and 12S, and showed that 19S toxin was transformed into 16S at pH 6.0 in the presence of 1 M NaCl.

In polyacrylamide gel electrophoresis at pH 4.0, purified M toxin showed a single band; L and crystalline toxins showed three to four bands. The least mobile peaks of L and crystalline toxins seemed to possess identical relative mobilities (Fig. 5).

Molecular size of the toxin in culture. In gel filtration of the crude culture supernatant on a Sephadex G-200 column (1.5 by 85 cm) with 0.05 M acetate buffer, pH 6.0, containing 1 M NaCl, as eluant, the toxic activity was eluted in

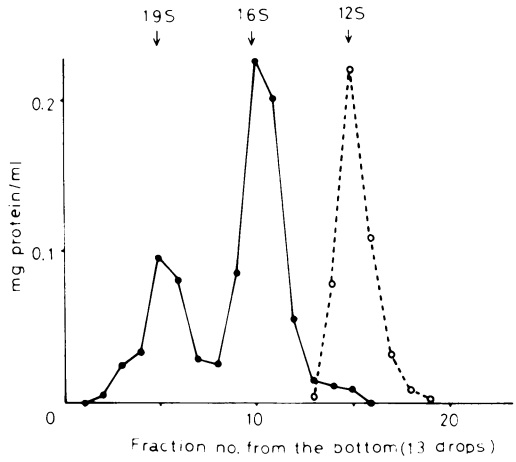

FIG. 4. *Ultracentrifugation of purified toxins in sucrose density gradients (5 to 20%) in 0.05 M phosphate buffer, pH 6.0; a 0.2-ml sample contained 290 μg of L toxin or 200 μg of M toxin. Symbols: ●, L toxin; ○, M toxin.*

two peaks (Fig. 6). The front peak eluted in void volume contained hemagglutinin activity. In sucrose density gradient centrifugation at pH 6.0 of the crude culture supernatant or its concentrate, only 12S toxin was detected. Upon dialysis of the crude culture supernatant against 0.05 M acetate buffer, pH 6.0, containing 1 or 0.2 M NaCl, however, both 16S and 12S toxin peaks appeared (Fig. 7). No 19S toxin was found by sucrose density gradient centrifugation. The ratio of 16S to 12S toxin was larger when the material was dialyzed against 0.05 M acetate buffer, pH 6.0, containing 0.2 M NaCl than against buffer containing 1 M NaCl.

Molecular dissociation of L and M toxins. Upon chromatography of L toxin on DEAE-Sephadex at pH 8.0, two peaks emerged (Fig. 8, bottom). The first peak was toxic and hemagglutinin negative, and the second one was nontoxic but hemagglutinin positive; the area of the second peak was about seven times larger than that of the first. M toxin was also eluted in two peaks under the same conditions (Fig. 8, top); the first one was toxic and at the same position as the corresponding toxic peak of L toxin, and the second one was nontoxic and at a position slightly different from that of the corresponding nontoxic peak of L toxin. Neither peak contained hemagglutinin activity. The areas of the two peaks were nearly the same.

In sucrose density gradient centrifugation at pH 8.0, L toxin sedimented in three peaks (Fig. 9). Both the heaviest and the intermediate peaks were nontoxic and hemagglutinin positive, whereas the lightest one was toxic and hemagglutinin negative. The sedimentation position of the toxic peak was the same as those of type B M and type E progenitor toxins centrifuged simultaneously under the same conditions. M toxin sedimented in a single peak under the same conditions, and the sedimentation position was identical to that of the toxic peak of L toxin (Fig. 9).

Antigenicities of the components of L and M toxins. In agar gel double diffusion, type A crystalline toxin formed two precipitin lines. The toxic components separated from either L or M toxin formed a single precipitin line and formed lines of identity with each other. They coalesced also with the precipitin line of the corresponding toxic component of crystalline toxin (Fig. 10A). Apparently two precipitin lines were formed with the nontoxic components separated from L and the crystalline toxins, and each line coalesced; that separated from M toxin appeared to form a single line, and it coalesced with one of the two lines of the nontoxic components of L and crystalline toxins (Fig. 10B).

DISCUSSION

Attempts were made to purify type A progenitor toxin. The use of 0.05 M acetate buffer, pH 6.5, containing 1 M NaCl (14, 15) to extract the toxin from the acid precipitate gave a higher recovery than 0.075 M calcium chloride (8). The crystalline toxin is adsorbed onto sulfoethyl (SE)-Sephadex at pH 3.8 (5), but the toxin in the extract of the acid precipitate becomes insoluble when dialyzed against a buffer of pH 3.8. Nucleoproteins play a role in acid precipitation of botulinum toxin (27). The protamine treatment at pH 6.5 removed RNA almost completely without causing appreciable loss of the toxin. Two toxic peaks emerged in gel filtration on Sephadex G-200, one in the void volume and the other at a slightly retarded position. The front peak (the L toxin fraction) apparently contained two different-sized toxins. In sucrose density gradient centrifugation at pH 6.0, the heavier toxin sedimented to the same position as did the main component of the crystalline toxin; therefore its molecular size was estimated to be 19S (molecular weight, 900,000) (22, 23, 28, 29). The lighter one sedimented to the same position as did type B L toxin; therefore the molecular size should be 16S (molecular weight, 500,000 (7, 12, 28). M toxin sedimented in a single peak to the same position as did type E progenitor and B M toxins; therefore its molecular size should be 12S (molecular weight, 350,000) (11, 12). Thus, type A, B, E, and F cultures all produce 12S toxin solely or partly.

In polyacrylamide gel electrophoresis at pH

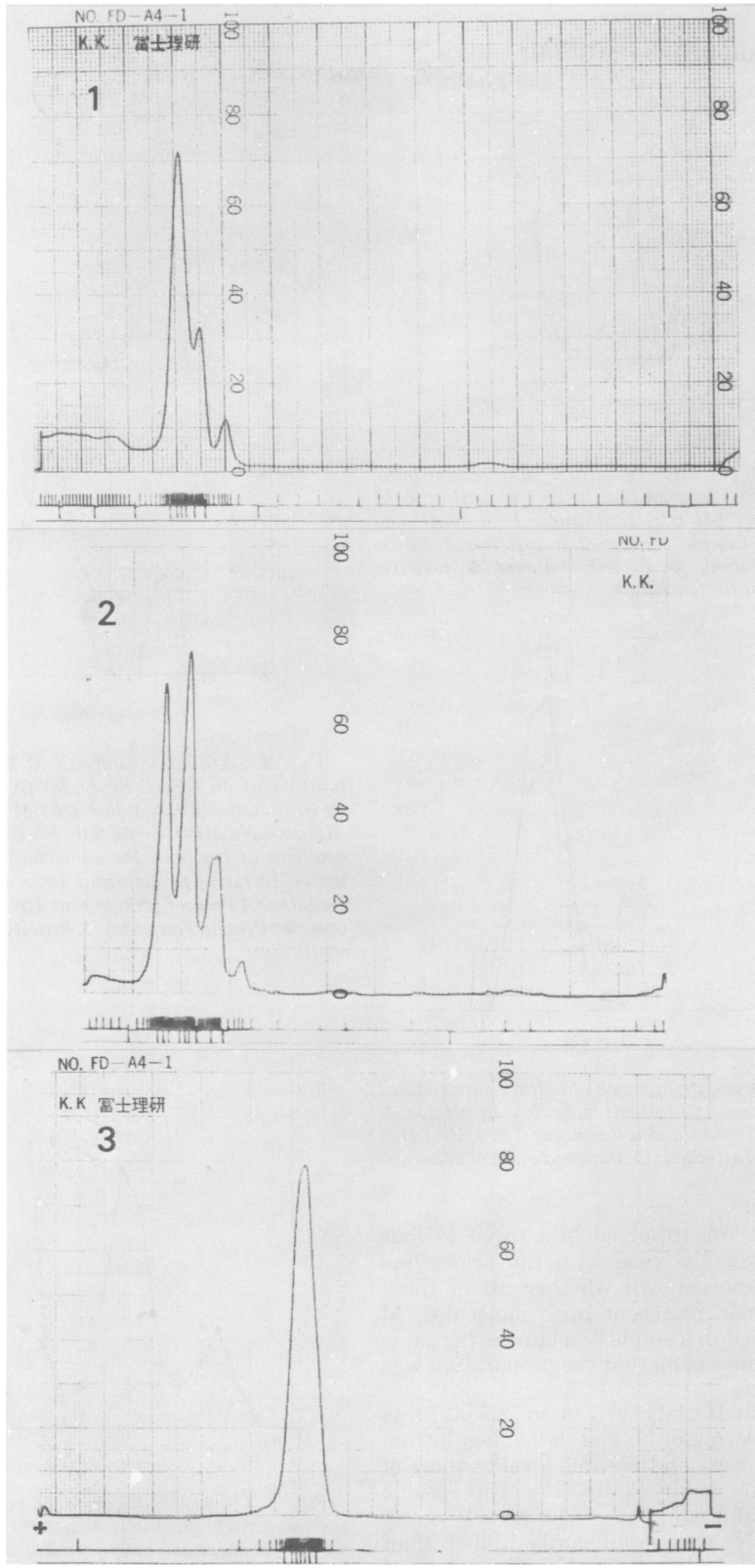

FIG. 5. *Polyacrylamide gel electrophoreses of crystalline, L, and M toxins at pH 4.0. Samples: (1) crystalline toxin, 100 μg; (2) L toxin, 105 μg; (3) M toxin, 98 μg.*

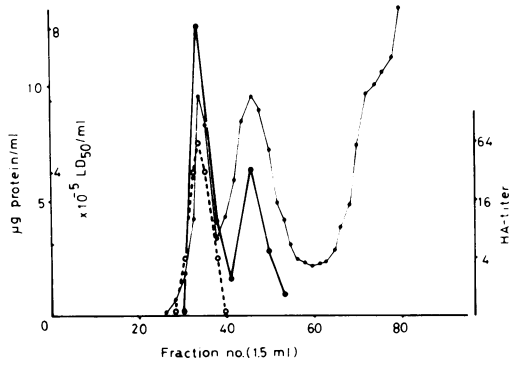

FIG. 6. *Gel filtration of culture supernatant on a Sephadex G-200 column (1.5 by 85 cm) with 0.05 M acetate buffer, pH 6.0, containing 1 M NaCl, as eluant. The sample contained a toxicity of 4.6 × 10^6 LD_{50}. Symbols: ●, Protein contents; ●, toxicity; ○, hemagglutination titer.*

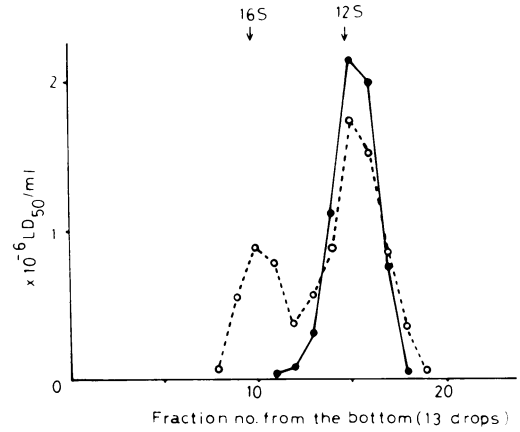

FIG. 7. *Ultracentrifugation of culture supernatant in a sucrose density gradient (5 to 20%) at pH 6.0. A 0.2-ml sample contained a toxicity of 3.6 × 10^6 LD_{50}. Symbols: ●, Untreated; ○, dialyzed culture supernatant.*

4.0, L toxin was resolved into three to four bands, as was the case with the crystalline toxin. It is not known whether all of these multiple bands represent toxic molecules. M toxin migrated in a single band under the same conditions, indicating that the preparation was homogeneous.

The specific toxicity of L toxin, 2.5 × 10^8 to 3.0 × 10^8 LD_{50}/mg of N, and its hemagglutination activity were on the same level as those of the crystalline toxin (8, 14, 15). The specific activity of M toxin, 4.5 × 10^8 to 5.0 × 10^8 LD_{50}/mg of N, was significantly higher than that of the crystalline toxin. M toxin contained little or no hemagglutination activity.

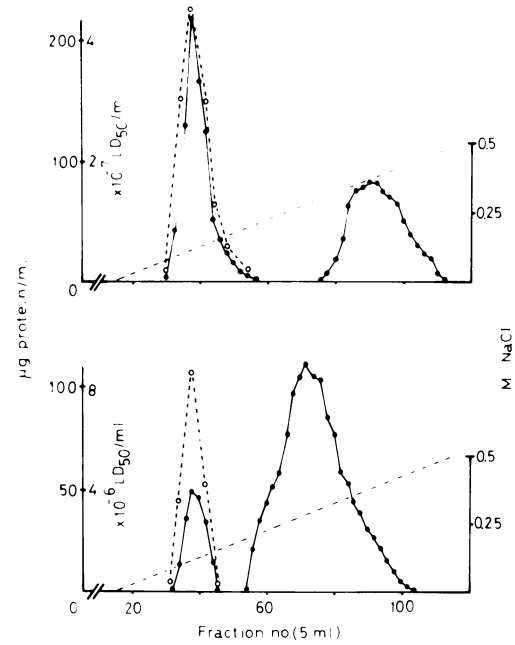

FIG. 8. *Chromatography of M (top) and L (bottom) toxins on DEAE-Sephadex (A-50) columns (1.5 by 10 cm) equilibrated with 0.01 M phosphate buffer (KH_2PO_4-Na_2HPO_4), pH 8.0. Elution with a linear gradient of NaCl in the same buffer. M toxin contained 10 mg of protein and 4.8 × 10^8 LD_{50}; L toxin contained 14 mg of protein and 4.1 × 10^8 LD_{50}. Symbols: ●, Protein contents; ○, toxicity; -----, NaCl concentration.*

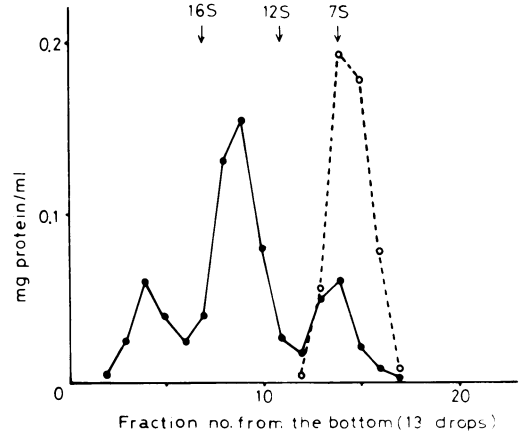

FIG. 9. *Ultracentrifugation of purified toxins in sucrose density gradients (5 to 20%) at pH 8.0. A 0.2-ml sample of L toxin contained 338 μg of protein, and that of M toxin contained 236 μg of protein. Symbols: ●, L toxin; ○, M toxin.*

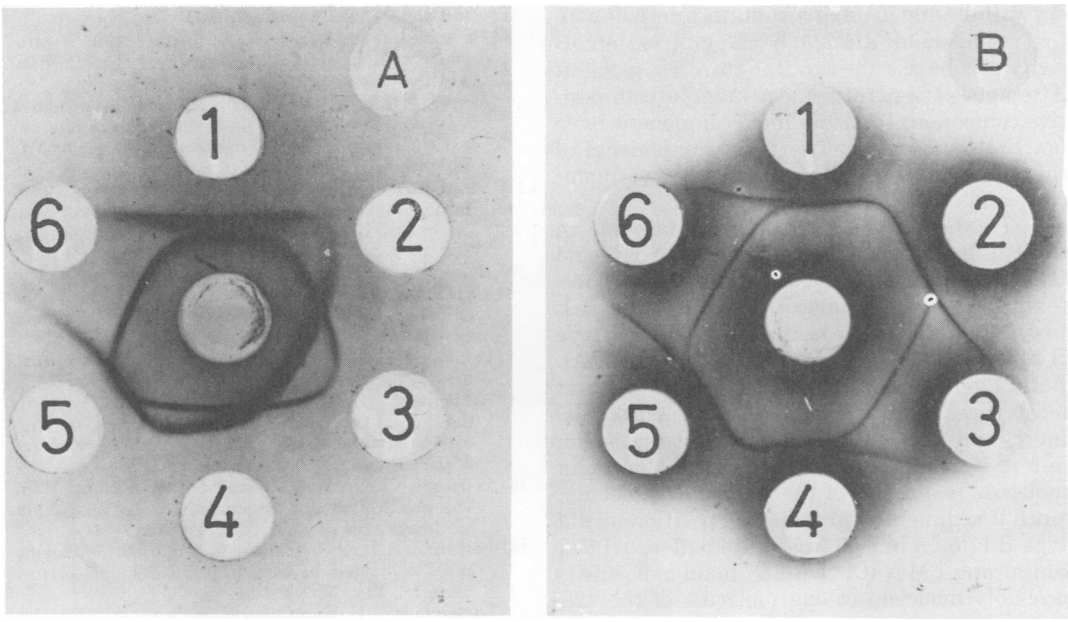

FIG. 10. *Agar gel double-diffusion tests with crystalline toxin and toxic and nontoxic components of crystalline and purified L and M toxins. Center well received type A horse antitoxin with a potency of 208 IU/ml. (A): wells 1 and 4, crystalline toxin, 400 μg/ml; well 2, toxic component of M toxin, 201 μg/ml; well 3, nontoxic component of M toxin, 109 μg/ml; well 5, nontoxic component of L toxin, 205 μg/ml; well 6, toxic component of L toxin, 205 μg/ml. (B): wells 1 and 4, nontoxic component of crystalline toxin, 540 μg/ml; wells 2 and 5, nontoxic component of L toxin, 409 μg/ml; wells 3 and 6, nontoxic component of M toxin, 109 μg/ml.*

When the type A culture supernatant was subjected to gel filtration on Sephadex G-200 at pH 6.0, two toxic fractions were eluted at positions corresponding to those of L and M toxins. In sucrose density gradient centrifugation of the culture supernatant at pH 6.0, however, only 12S toxin was found. Hauschild and Hilsheimer (9) demonstrated two different-sized toxins with molecular weights of 200,000 and 500,000 by gel filtration of type A culture supernatant and cell extract. Their smaller toxin may correspond to our M toxin and their larger toxin to our 16S toxin. The present results indicate that type A toxin in culture is solely in the form of 12S, which is transformed into the 16S form when some small-molecular-sized substance(s) is removed by dialysis or gel filtration on Sephadex G-200. Schantz and Spero (26) reported the presence of 19S toxin alone in type A spent culture. The method for determining the molecular size used by them may have caused the same effect as gel filtration or dialysis. It is also likely that, in earlier work for purification of type A toxin, M toxin was overlooked because it may not have been precipitated at 20% saturation of ammonium sulfate (15), 30% saturation of sodium sulfate (1), or 15% ethanol (8).

The results of DEAE-Sephadex chromatography at pH 8.0 showed that M toxin consists of one molecule each of the toxic and nontoxic components of the same molecular weights, as does type E progenitor toxin (11). Under the same conditions, the toxic component of L toxin was eluted at the same position as the corresponding toxic component of M toxin; the nontoxic component was eluted earlier than the corresponding nontoxic component of M toxin. The ratio of toxic to nontoxic peaks of L toxin was approximately 1:7.

Agar gel double-diffusion tests demonstrated that the nontoxic components of the crystalline and L toxins are antigenically identical. The nontoxic component of M toxin does not contain hemagglutinin and is only partially identical with those of the crystalline and L toxins. The generally accepted notion that "type A crystalline toxin consists of two components, neurotoxin and hemagglutinin" will have to be changed. A 7S nontoxic component containing no hemagglutination activity is involved commonly in the crystalline, L, and M toxins. The

crystalline and L toxins contain another nontoxic component, and the hemagglutination activity can be ascribed to this third component. Attempts at separating a hemagglutinin-positive component from the inert component have not been successful. The toxic components of the three toxins are identical physicochemically and antigenically.

C. botulinum type A toxin is obtainable in three different molecular forms, 19*S*, 16*S*, and 12*S*. Only 19*S* is characteristic of type A; 16*S* toxin is the same molecular size as type B L toxin (12); 12*S* toxin is the same size as type B M (12), E (11), and F progenitor toxins (21). Both 19*S* and 16*S* toxins are composed of at least three different components, neurotoxic, inert, and hemagglutinin. It may be possible, but it is not proven, that the the 19*S* toxin molecule is a dimer of the 16*S* toxin molecule, since it sedimented to the same position as did type B L toxin in 0.05 M acetate buffer, pH 6.0, containing 1 M NaCl. The 16*S* toxin molecule is probably made up of one molecule of the 12*S* toxin plus another component with the hemagglutination activity. It seems justifiable to call M toxin the progenitor toxin, since it seems to be the form first appearing in culture and since later the hemagglutinin component is bound to it.

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