

## Purification and Characterization of Two Components of Botulinum C<sub>2</sub> Toxin

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Two dissimilar proteins, designated as components I and II, of botulinum C<sub>2</sub> toxin elaborated by strain 92-13 were purified to a homogeneous state. The molecular weights determined by sodium dodecyl sulfate gel electrophoresis were 55,000 for component I and 105,000 for component II. Whereas each component showed no or feeble toxicity even after being treated with trypsin, the toxicity was elicited when these two components were mixed and trypsinized. The toxicity of the mixture of components I and II at a ratio of 1:2.5 on a protein basis was  $2.2 \times 10^4$  mouse intraperitoneal 50% lethal doses per mg of protein and increased by 2,000 times or more when treated with trypsin. These results indicate that the molecular characteristics of botulinum C<sub>2</sub> toxin differ from those of the toxin of *Clostridium botulinum* types A through F in that C<sub>2</sub> toxin is constructed with two separate protein components, which are not covalently held together, and that its toxicity is elicited by cooperation of the two components.

*Clostridium botulinum* type C and D strains produce three antigenically different toxins, C<sub>1</sub>, C<sub>2</sub>, and D. The C<sub>2</sub> toxin is produced as a protoxin by certain strains of *C. botulinum* types C and D and those cured of their prophages (3, 5, 12), so that toxicity can be demonstrated only after treatment with trypsin (3, 6). It has been reported that the trypsinized or endogenously nicked toxin molecules of *C. botulinum* types A through F are composed of two polypeptide chains (1, 10, 13, 18) which can be dissociated by treatment with a reducing agent in the presence of a detergent (8, 19). However, the association of these two chains is essential for eliciting the toxicity (19). In a previous paper, we reported that C<sub>2</sub> toxin consists of two cooperative components which are resolved by ion-exchange chromatography or gel filtration (4). These two components individually show very feeble toxicity even after treatment with trypsin, but full toxicity can be attained simply by mixing these two components. This suggests that the molecular construction of botulinum C<sub>2</sub> toxin differs from that of the toxin of *C. botulinum* types A through F. In the present paper, we describe the purification and characterization of these two components of C<sub>2</sub> toxin and compare the molecular construction of C<sub>2</sub> toxin with those of the other botulinum toxins.

### MATERIALS AND METHODS

**Bacterial strain and toxin production.** Strain 92-13, resembling *C. botulinum* type C but producing only C<sub>2</sub> toxin (11), was provided by S. Nakamura, Kanazawa University, Kanazawa, Japan. Medium for

toxin production was the chopped-meat medium described previously (4). A 1-ml inoculum containing  $10^6$  refractile spores was inoculated into 5,000 ml of the medium in a flat-bottomed spherical flask, and the culture was incubated for 2 days at 37°C.

**Activation and toxicity assay of C<sub>2</sub> toxin.** Tryptic activation was carried out in 0.05 M phosphate buffer, pH 8.0, for 30 min at 35°C unless otherwise indicated. Purified components I and II and C<sub>2</sub> toxin were activated at a toxin-to-trypsin ratio of 2:1 on a protein basis. Crude materials were treated with trypsin at a final concentration of 200 µg/ml. Trypsinization was terminated by adding an amount of soybean trypsin inhibitor that was twice the weight of trypsin. The toxicity in intraperitoneal mean lethal doses was determined in mice by the time-to-death method (4). When necessary, serial twofold dilutions of each sample were made in 0.05 M phosphate buffer, pH 8.0, containing 0.1% gelatin, and 0.1 ml was injected intraperitoneally or intravenously into separate groups of four mice. The 50% lethal dose was calculated from deaths within 4 days by the method of Reed and Muench (15).

**Protein determination.** Protein was determined by the method of Lowry et al. (9) with bovine serum albumin as the standard.

**Polyacrylamide gel electrophoresis.** Electrophoresis in a 7.5% polyacrylamide gel at pH 4.0 was carried out by the method of Reisfeld et al. (16). Neutral red was used as a marker dye to measure relative mobility. Sodium dodecyl sulfate gel electrophoresis and molecular weight determinations were performed in a 6.0% polyacrylamide gel by the method of Dunker and Rueckert (2). The molecular weight was determined in sodium dodecyl sulfate gel electrophoresis from the mobility rate relative to that of  $\alpha$ -chymotrypsinogen A (molecular weight = 25,000) by using the following protein standards: gamma globulin (molecular weight = 150,000), phosphorylase a (molec-

ular weight = 94,000), bovine serum albumin (molecular weight = 66,000), and ovalbumin (molecular weight = 45,000).

**Anti-component I and II sera.** Antiserum specific for each component was prepared. Purified component at 200  $\mu$ g/ml was detoxified by dialysis against 0.05 M phosphate buffer, pH 8.0, containing 0.4% Formalin for 48 h at 30°C. A 0.5-ml (100  $\mu$ g) portion of a toxoid was emulsified in an equal volume of Freund complete adjuvant (Difco Laboratories, Detroit, Mich.) and was injected subcutaneously into a rabbit. After 4 weeks, a 0.5-ml (100- $\mu$ g) portion each of the untreated and homologous components was injected subcutaneously. The animals were bled after 2 weeks.

**Neutralization test and immunodiffusion.** A mixture of antiserum and toxin was held at room temperature (20 to 25°C) for 30 min and injected intravenously into mice to determine toxicity not neutralized. The agar gel double-diffusion test was performed by the method reported previously (7).

**Purification of botulinum type C and D toxins.** Botulinum type C and D progenitor toxins were purified from the culture of *C. botulinum* type C strain CB19 and type D strain CB16, respectively (10, 14).

**Chemicals.** Phosphorylase a and ovalbumin (Pentex Inc., Kankakee, Ill.), bovine serum albumin (Fraction V, Armour Pharmaceutical Co., Chicago, Ill.), gamma globulin (Schwarz/Mann, Orangeburg, N.Y.), and  $\alpha$ -chymotrypsinogen A (Sigma Chemical Co., St. Louis, Mo.) were purchased from Wako Pure Chemical Industries, Osaka, Japan. Soybean trypsin inhibitor was the product of Worthington Biochemicals Corp., Freehold, N.J.; trypsin (type III, twice crystallized) was from Sigma Chemical Co.

## RESULTS

**Purification of C<sub>2</sub> toxin.** All procedures were performed at 4°C unless otherwise stated.

**Step 1: precipitation with ammonium sulfate.** Solid (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was added to the whole culture to 58% saturation (380 g/liter), and the mixture was adjusted to pH 7.5 with 4 N NaOH. The precipitate formed by standing the mixture overnight was collected by centrifugation for 20 min at 4,650  $\times$  g. The precipitate from a 5-liter culture was suspended in 80 ml of 0.1 M tris-(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.5, and the suspension was centrifuged for 20 min at 4,650  $\times$  g. This extraction procedure was repeated, and the supernatants were combined.

**Step 2: acid precipitation.** The supernatant was dialyzed against 3 liters of 0.05 M acetate buffer, pH 4.5, for 24 h. The precipitate formed during dialysis was collected by centrifugation for 10 min at 8,600  $\times$  g and extracted twice with 50 ml of acetate buffer containing 0.5 M NaCl. The toxic extract was dialyzed against 0.05 M Tris-hydrochloride, pH 7.5.

**Step 3: diethylaminoethyl Sephadex chromatography.** The precipitate formed dur-

ing dialysis was removed by centrifugation for 10 min at 8,600  $\times$  g. The supernatant was applied to a column of diethylaminoethyl Sephadex A-50 (4 by 24 cm) equilibrated with the Tris-hydrochloride buffer. After sample application, the column was washed with 700 ml of the same buffer and then eluted with 1,000 ml of 0.3 M NaCl in 0.05 M Tris-hydrochloride buffer, pH 7.5. The eluted fractions were pooled, concentrated to 60 ml by ultrafiltration with a PM-30 membrane, and dialyzed against 0.01 M acetate buffer, pH 6.0.

**Step 4: carboxymethyl Sephadex chromatography.** The dialyzed fraction was applied to a column of carboxymethyl Sephadex C-50 (2 by 10 cm) equilibrated with 0.01 M acetate buffer, pH 6.0. The column was washed with 60 ml of the buffer and eluted with 150 ml of the same buffer containing 0.3 M NaCl (Fig. 1). Each of the nonadsorbed and the eluate fractions had a toxicity of less than 1% of that applied. However, most toxicity was recovered when the two fractions were combined. Therefore, the nonadsorbed and the eluate fractions were designated as components I and II, respectively. These two fractions were concentrated separately by ultrafiltration through a PM-30 membrane.

**Step 5: gel filtration on Sephadex G-100.** The concentrated components were applied to separate Sephadex G-100 columns (2.5  $\times$  95 cm) equilibrated with 0.05 M Tris-hydrochloride buffer, pH 7.5, and eluted with the same buffer (Fig. 2); the component I was rechromatographed on the Sephadex G-100 column. High toxicity was demonstrated when components I and II were mixed and trypsinized, whereas each component showed a very low toxicity.

The overall purification is summarized in Table 1. The recovery of toxicity was 20% of that in the culture as determined by the toxicity obtained by trypsinizing a mixture that was made by combining components I and II in the ratio of their concentrate volumes. The degree of the purification on a toxicity basis was 95-fold relative to the toxic preparation obtained by precipitation with (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>.

**Polyacrylamide gel electrophoresis.** Purified components I and II each showed a single band in disc electrophoresis at pH 4.0. Electrophoretic mobilities relative to the marker dye were 0.51 and 0.42, respectively (Fig. 3). In sodium dodecyl sulfate gel electrophoresis, the purified components before and after treatment with 2-mercaptoethanol each showed a single band (Fig. 4). The molecular weights estimated by sodium dodecyl sulfate gel electrophoresis were 55,000 for component I and 105,000 for component II.

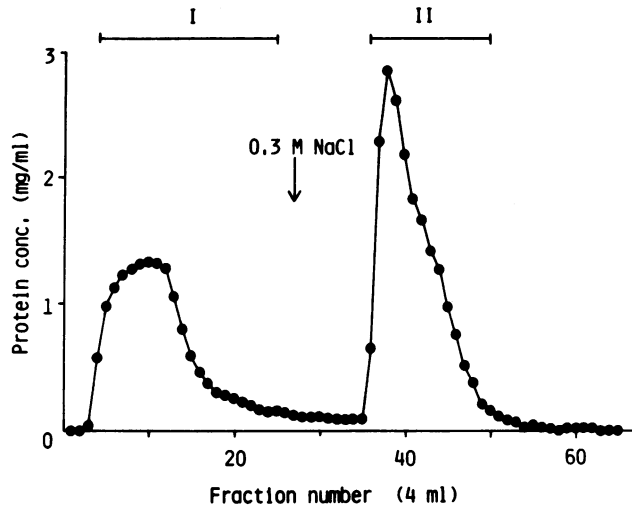


FIG. 1. Carboxymethyl Sephadex chromatography of  $C_2$  toxin. The toxin fractions obtained by diethylaminoethyl Sephadex chromatography were applied to a carboxymethyl Sephadex C-50 column, and 4-ml fractions were collected. The unabsorbed fractions were pooled as component I, and the eluate fractions were pooled as component II (horizontal bars).

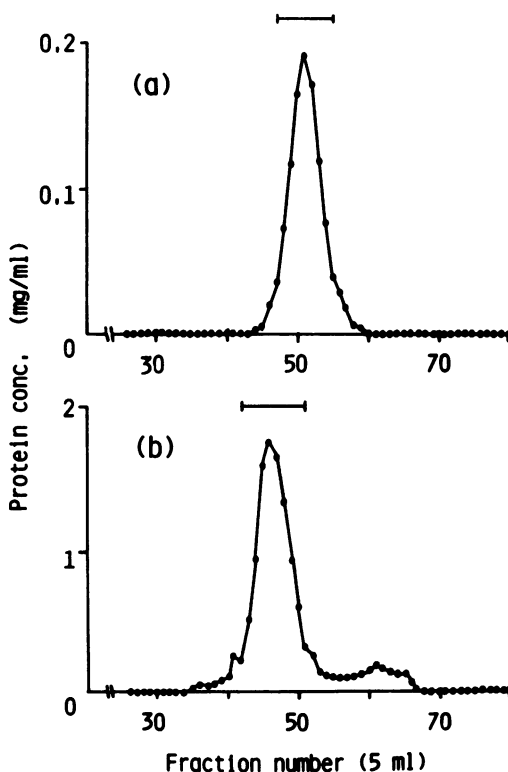


FIG. 2. Gel filtration of components I and II on Sephadex G-100. Components I and II from carboxymethyl Sephadex chromatography were applied separately to a Sephadex G-100 column. The fractions indicated by the horizontal bars were pooled. (a)

**Toxicities.** The toxicities of  $C_2$  toxin were studied in a mixture made by adding various amounts of component I to a fixed amount of component II and by treating with trypsin (Fig. 5). The maximum toxicity was obtained when the ratio of component I to component II was from 1:2.0 to 1:2.5. Mixtures of these ratios increased 2,000-fold or more in toxicity when treated with trypsin. From these results,  $C_2$  toxin was defined in the present experiments as a mixture of components I and II at a ratio of 1:2.5 on a protein basis. Component II showed a very low toxicity after treatment with trypsin, whereas component I before or after trypsin treatment was not lethal to mice when 100  $\mu$ g was injected intravenously (Table 2).

The toxicities of different combinations of trypsinized and untrypsinized component I or II were examined. A mixture of trypsinized component I and untrypsinized component II was not lethal to mice, whereas that of untrypsinized component I and trypsinized component II showed 82% of the toxicity of trypsinized  $C_2$  toxin (Table 3).

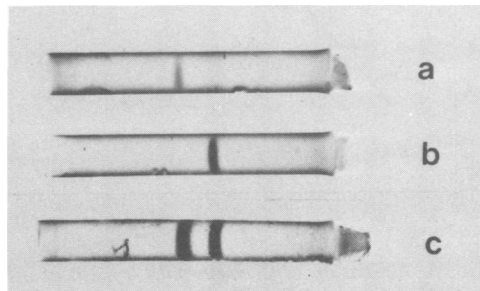
The toxicity of purified  $C_2$  toxin to mice by the intravenous route was higher than that by the intraperitoneal route; one 50% lethal dose required 5.4 ng of protein by the intravenous route and 49 ng by the intraperitoneal route.

**Activation conditions.** The purified  $C_2$  toxin

*Elution pattern of the second gel filtration of component I; (b) elution pattern of gel filtration of component II.*

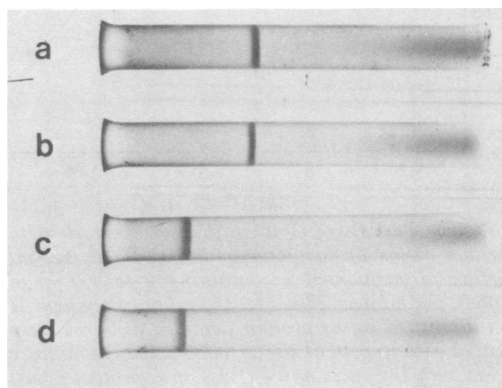
TABLE 1. *Purification of components I and II of botulinum C<sub>2</sub> toxin*

Step	Volume (ml)	Protein (mg)	Potential toxicity ( $\times 10^{-4}$ i.p. LD <sub>50</sub> ) <sup>a</sup>	Recovery (%) <sup>b</sup>	Specific toxicity ( $\times 10^{-2}$ i.p. LD <sub>50</sub> /mg of protein) <sup>a</sup>
Whole culture	5,000		472	100	
Ammonium sulfate precipitation	100	24,600	460	98	1.9
Extract of ammonium sulfate precipitate	160	17,500	451	96	2.6
Extract of acid precipitate	100	4,570	394	84	8.6
Diethylaminoethyl effluent	60	686	400	85	58.3
Carboxymethyl Sephadex					
Nonabsorbed (component I)	120	82	— <sup>c</sup>	NS <sup>d</sup>	
Eluate (component II)	150	166	3.3	0.7	2.0
Mixture of I and II <sup>e</sup>			320	68	129.0
Sephadex G-100					
Component I	45	4.8	* <sup>f</sup>	NS <sup>d</sup>	
Component II	50	48.5	3.1	0.7	6.4
Mixture of I and II <sup>e</sup>			96	20	180.1

<sup>a</sup> i.p. LD<sub>50</sub>, Intraperitoneal 50% lethal dose.<sup>b</sup> Toxicity of whole culture was taken as 100%.<sup>c</sup> —, Mice survived for 100 min but not 6 h after intravenous injection of 4  $\mu$ g of trypsinized protein.<sup>d</sup> NS, Not significant.<sup>e</sup> Toxicity after trypsinizing a mixture made of components I and II in a ratio corresponding to volumes of the two fractions.<sup>f</sup> \* Mice survived after intravenous injection of 4  $\mu$ g of trypsinized protein.FIG. 3. *Polyacrylamide gel electrophoresis of purified components I and II. A 10- $\mu$ g portion of purified component I or II was applied to a gel column and electrophoresed for 210 min at 3 mA per column. (a) Component I, (b) component II, and (c) a mixture of I and II.*

was treated with trypsin at different pH's for 30 min at 35°C, and the toxicities were determined. The optimum condition of activation was pH 8.0. Maximum activation of C<sub>2</sub> toxin was attained by incubation for 30 min at a trypsin-toxin ratio of 1:2 (Fig. 6). Toxicity persisted on the same level for 180 min.

**Immunodiffusion and neutralization tests.** In agar gel double-diffusion tests, purified components I and II each gave a single precipitin line against a mixture of rabbit anti-component I and II sera that crossed each other. The C<sub>2</sub>

FIG. 4. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of components I and II. A 5- $\mu$ g portion of purified component I or II was applied on a gel column (4 by 70 mm) and electrophoresed for 240 min at 6 mA per gel. (a) Component I before and (b) after reduction; (c) component II before and (d) after reduction.*

toxin, a mixture of components I and II, formed two precipitin lines, of which one fused in line of identity with the line of component I, and the other fused with that of component II (Fig. 7). Neither anti-component I nor II serum formed a precipitin line with 100  $\mu$ g of the progenitor toxins of *C. botulinum* types C or D. A 10- $\mu$ l

portion of anti-component I or II serum completely neutralized 20 mouse intraperitoneal 50% lethal doses of activated C<sub>2</sub> toxin but did not neutralize the same amount of type C or D toxin.

### DISCUSSION

The evidence of the present study shows that the two dissimilar protein components, designated as components I and II, are requisite for the lethality of C<sub>2</sub> toxin in mice; a markedly higher toxicity was obtained when components I and II were mixed and treated with trypsin, whereas the components individually show a very low toxicity even after trypsinization. In a previous paper, we reported that these two components in culture supernatant can be separated by ion-exchange chromatography or gel filtration without treatment with detergent, reducing agent, or protease (4), indicating that components I and II exist as separate forms in culture fluid. This was confirmed in the present study

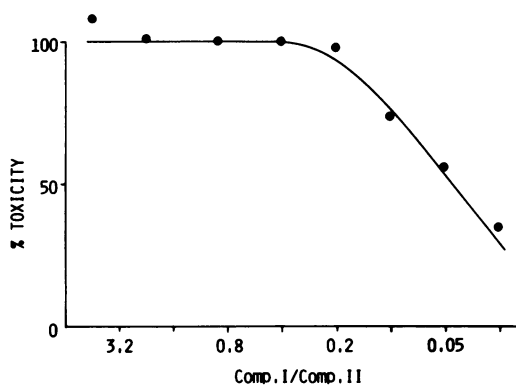


FIG. 5. Toxicity of C<sub>2</sub> toxin in mixture made with various ratios of components I and II. A 0.25-ml portion of component I containing 2 to 400  $\mu$ g of protein per ml was added to 0.25 ml of component II containing 80  $\mu$ g of protein per ml. Mixtures were treated with trypsin at 200  $\mu$ g/ml. Toxicity in mixture made with the ratio of 0.4 (1:2.5 on a protein basis) was taken as 100%.

TABLE 2. Toxicities of components I and II and C<sub>2</sub> toxin before and after trypsinization

Toxin	Toxicity (i.p. LD <sub>50</sub> /100 $\mu$ g of protein) <sup>a</sup>	
	Trypsinized	Untrypsinized
Component I	* <sup>b</sup>	*
Component II	50	*
C <sub>2</sub> toxin <sup>c</sup>	2,200	— <sup>d</sup>

<sup>a</sup> i.p. LD<sub>50</sub>, Intraperitoneal 50% lethal dose.

<sup>b</sup> \*, Mice survived after intravenous injection.

<sup>c</sup> Components I and II were mixed at the ratio of 1:2.5 on a protein basis.

<sup>d</sup> —, Mice survived for 100 min but not 6 h.

TABLE 3. Toxicity in mixtures made with different combinations of trypsinized and untrypsinized components I and II<sup>a</sup>

Mixture	Toxicity (i.p. LD <sub>50</sub> /ml) <sup>b</sup>	Ratio <sup>c</sup>
T-I + T-II <sup>d</sup>	17.9	1.0
T-I + UT-II <sup>e</sup>	—	
UT-I + T-II <sup>e</sup>	14.6	0.82
UT-I + UT-II <sup>f</sup>	—	

<sup>a</sup> Equal volumes of trypsinized (T) and untrypsinized (UT) component I (2.3  $\mu$ g/ml) and component II (5.8  $\mu$ g/ml) were mixed, and the toxicity was determined. Trypsinization of each component was carried out as described in the text.

<sup>b</sup> i.p. LD<sub>50</sub>, Intraperitoneal 50% lethal dose.

<sup>c</sup> Toxicity of T-I + T-II taken as 1.0.

<sup>d</sup> Both components trypsinized.

<sup>e</sup> Trypsinized component I plus untrypsinized component II.

<sup>f</sup> —, Mice survived for 100 min but not 6 h.

<sup>g</sup> Untrypsinized component I plus trypsinized component II.

<sup>h</sup> Neither component trypsinized.

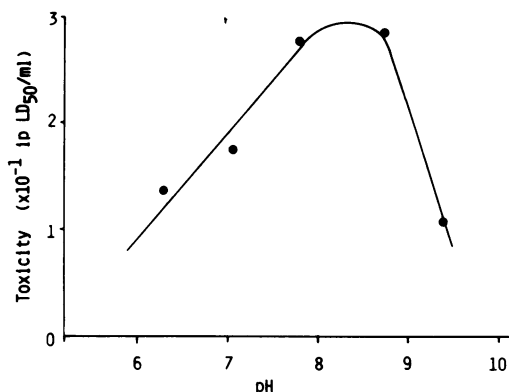


FIG. 6. Activation of C<sub>2</sub> toxin with trypsin at different pH values. A 0.25-ml portion of C<sub>2</sub> toxin containing 5  $\mu$ g of component I and 12.5  $\mu$ g of component II was mixed with an equal volume of each of the buffer solutions at different pH values containing 400  $\mu$ g of trypsin. The buffers used were 0.05 M phosphate buffer, pH 6.3 and 7.1, and 0.1 M Tris-hydrochloride, pH 7.9, 8.8, and 9.4.

by the successful purification of components I and II without such treatments. It has been reported that the trypsinized or endogenously nicked toxic components of types A through F progenitor toxins consist of two fragments with approximate molecular weights of 100,000 and 50,000 linked together with a disulfide bridge(s) (1, 10, 13, 17, 18). The fragments of type B and C toxins can be resolved by chromatography only when treated with both a detergent and a reducing agent (8, 19). The toxicity is lost concomitant to dissociation; it is restored on reassociation of these two fragments by removing

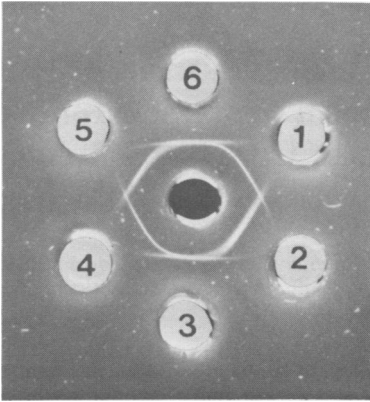


Fig. 7. Agar gel double-diffusion tests with purified components I and II. Center well, a mixture of equal volumes of anti-component I and anti-component II sera; lateral wells (1) and (4), component I, 55 µg/ml; (2) and (5), component II, 100 µg/ml; (3) and (6), C<sub>2</sub> toxin, 155 µg/ml.

the detergent and the reducing agent (19). The toxicity of botulinum C<sub>2</sub> toxin, however, was regenerated simply by mixing components I and II, indicating that the in vitro binding of component I and II molecules is not required for toxicity. The molecular construction of C<sub>2</sub> toxin is entirely different from that of the toxic component of progenitor toxins of *C. botulinum* types A through F in that its components are not covalently held together, so the components exist naturally as separate molecules.

Gel filtration, polyacrylamide gel electrophoresis, and sucrose density ultracentrifugation were tried in attempts to see whether purified components I and II of C<sub>2</sub> toxin form a complex in vitro. But all attempts to demonstrate the complex failed. As we reported previously, the separation of components I and II of botulinum C<sub>2</sub> toxin was observed with 1- or 2-day cultures of *C. botulinum* types C and D and strains producing only C<sub>2</sub> toxin (4). It was also demonstrated in sodium dodecyl sulfate gel electrophoresis that components I and II each gave a single band even after reduction. These results indicate that C<sub>2</sub> toxin intrinsically constitutes two separate polypeptides, although this does not rule out the possibility that these two components form a complex at the site where biological activity occurs.

In agar gel diffusion, purified components I and II each formed a single precipitin line; they crossed each other. This shows that components I and II are immunologically distinct protein molecules. Anti-component I or II serum gave no precipitin line with the progenitor toxin of *C. botulinum* types C and D and did not neutralize

both of these toxins. This indicates that the components of C<sub>2</sub> toxin have no antigenic relation with these botulinum toxin types.

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