STUDIES ON IMMUNITY TO TOXINS OF *CLOSTRIDIUM BOTULINUM*

II. Production and Purification of Type B Toxin for Toxoid

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Isolation of *Clostridium botulinum* type B toxin was reported by Lamanna and Glassman (1947). The purified product differed from crystalline type A toxin not only in serological specificity, but also in chemical and physical properties. The type B toxin was soluble in water and dilute salt solutions only on the acid side of the isoelectric zone, and membrane diffusion measurements suggested a molecular weight of the order of 60,000, in contrast with the well established value of about a million for type A toxin. Wagman and Bateman (1951), however, determined by sedimentation and diffusion measurements that the molecular weight of the major component of similar preparations of type B toxin was about 500,000.

From the practical standpoint, the insolubility of the purified type B toxin at neutral pH made it unsuitable for preparation of a purified toxoid. It appeared possible that the chemical differences between the type A and type B toxins were a result of the different procedures employed in their isolation, and that the rather acid conditions used in fractionation of type B toxin had altered the toxin. Accordingly, isolation of type B toxin was reinvestigated and a new procedure was devised. The purified product appears to be like type A toxin in chemical and physical properties (Duff *et al.*, 1954).

MATERIALS AND METHODS

Strain. The strain of *C. botulinum* type B designated "beans" was used for these investigations. The strain was grown for 24 to 48 hr at 35 C in 200-ml volumes of a medium composed of beef infusion, 1 per cent peptone, and chopped meat. The culture supernatant was distributed in small tubes, frozen rapidly, and stored at −20 C. The viability and toxigenicity of the stock strains have been maintained for more than 2 years under these conditions.

Toxicity titrations. The buffer used for dilution of toxin contained 0.2 per cent gelatin and 0.4 per cent dibasic sodium phosphate, and was adjusted to pH 6.2 with hydrochloric acid. White mice weighing 18 to 20 g were injected intraperitoneally with 0.5-ml aliquots of the dilutions of toxin and observed for 4 days. Usually 8 mice per dilution were used and the LD_{50} was calculated by a graphic probit method (Weiss, 1948).

The procedures for neutralization titrations and nitrogen determinations have been described previously (Duff *et al.*, 1957).

EXPERIMENTAL RESULTS

Toxin production. In preliminary investigations, it was observed that high yields of toxin were obtained by growth of the "beans" strain of *C. botulinum* type B in fluid thioglycolate medium (Baltimore Biological Laboratory). The presence of agar in the above medium interfered with subsequent purification of the toxin by preventing the precipitate obtained on acidification from concentrating to the desired volume. When agar was omitted from the medium, satisfactory yields of toxin were obtained, and upon acidification of the culture greater concentration of precipitate occurred. The effects of alteration in the concentration or omission of the other constituents were studied. The medium devised for routine toxin production was composed of 1.5 per cent "trypticase," 0.5 per cent yeast extract (both procured from the Baltimore Biological Laboratory), 0.075 per cent cysteine hydrochloride, and 0.5 per cent glucose. The pH of the medium was adjusted to 7.1 before autoclaving. Glucose was autoclaved separately in the form of a 20 per cent solution and added aseptically. When inoculum was required for production of toxin, 2.0 ml of a stock strain was transferred to 15 ml of fluid thioglycolate medium and incubated at 37 C for 18 to 24 hr. After incubation, 7.5 ml was transferred to 150 ml of the toxin production

medium and incubated at 25 C for 18 hr. Serial transfers were then made into larger volumes of toxin production medium to obtain a volume of inoculum equal to 5 per cent of the final culture. Routine toxin production was carried out using 3 L of medium in 4-L bottles, which were incubated at 35 C for 5 days. The cultures contained 2.0×10^6 mouse intraperitoneal (IP) LD_{50} per ml or greater.

Toxin purification. The method was essentially that described for the purification of type A toxin (Duff *et al.*, 1957). The type A toxin was precipitated from the culture by acidification at pH 3.5 and the precipitate was washed with water at pH 5. Toxin was extracted from the washed acid precipitate with 0.075 M calcium chloride at pH 6.5 and precipitated from the extract at pH 3.7. The toxic precipitate was dissolved in 0.03 M phosphate buffer at pH 6.8 and precipitated with 15 per cent ethanol in the cold. The precipitate was dissolved in either 0.03 M phosphate buffer at pH 6.8 or 0.2 M succinate buffer at pH 5.5.

With the type B toxin, investigation indicated that 85 to 100 per cent of the culture toxin could be precipitated with 3 N H_2SO_4 in the pH range 2.5 to 5.0. The best settling of the precipitate was observed at pH 4.2 and this pH was selected. All acid precipitable material settled out on overnight standing at room temperature. The supernatant was siphoned off, and the precipitates were pooled and allowed to settle at room temperature to a 30-fold concentration. Acid soluble constituents of the whole culture were removed by suspending the acid precipitate in 4 vol of water and allowing the precipitable material to settle out at 4 C. The supernatant was drawn off and the precipitate packed by centrifugation and resuspended in water to a volume equal to that of the culture acid precipitate.

It was observed that the toxin could be extracted from the water washed acid precipitate with calcium chloride solutions ranging from 0.05 M to 0.3 M in the pH range 1.5 to 6.5. With each molarity, the greatest recovery of toxin was at pH 2.0, but after further purification this fraction was insoluble in various buffers at pH 5.5. When the toxin was extracted at pH 5.0 to 6.5, a somewhat lower recovery of toxic activity was obtained, but fractions purified further were found to be soluble in the range of pH 5.0 to pH 6.8. Further investigations in the higher pH range indicated that 0.05 M calcium chloride, pH 6.0, and a temperature of 30 to 35 C were optimum conditions for extraction of the toxin at this step. The washed acid precipitate was diluted to 4 vol with distilled water and 1.0 M calcium chloride solution to a final concentration of 0.05 M calcium chloride and adjusted to pH 6.0. The solution was stirred intermittently for 1 hr at 30 to 35 C, then filtered by gravity through fluted paper (Eaton and Dikeman No. 193) at room temperature. Approximately 50 per cent of the toxic activity was recovered from the washed acid precipitate.

The toxin could be precipitated from the calcium chloride extract either by acidification or by addition of alcohol. Approximately equal purification and recovery of toxin were obtained, and it appeared that precipitation with acid rather than alcohol was more practical. The acid precipitation of the toxin became less effective as the calcium chloride concentration was increased. In the presence of 0.15 M calcium chloride, acid precipitation gave poor recovery of toxin. In the presence of 0.05 M or 0.075 M calcium chloride, acid precipitation was satisfactory. The calcium chloride extract was adjusted to pH 3.7 with 1 N HCl and allowed to settle overnight at 4 C. The supernatant was decanted and the precipitate collected by centrifugation at 4 C at 4,000 rpm for 30 min.

The second acid precipitate was dissolved in 0.1 M phosphate buffer, pH 6.8. An inert precipitate, presumably calcium phosphate, remained after the acid precipitated fraction was dissolved in phosphate buffer, and was removed by centrifugation at 4 C at 4,000 rpm for 30 minutes. Further purification and concentration of the toxin was accomplished by three methods: (1) a third precipitation with acid, (2) salting out with ammonium sulfate, or (3) precipitation with ethanol in the cold. The latter method was selected since it provided the most effective means of purification. The temperature of the solution was lowered to -2 C, and 50 per cent ethanol was run in through a cooling coil at -5 C to a final concentration of 15 per cent. After standing 18 to 24 hr at -5 C, the preparation was centrifuged at -5 C at 4,000 rpm for 30 min.

The alcohol precipitated fraction was readily soluble either in 0.1 M phosphate buffer, pH 6.8, or 0.2 M succinate buffer, pH 5.5. For toxoid studies, the alcohol precipitate was dissolved in

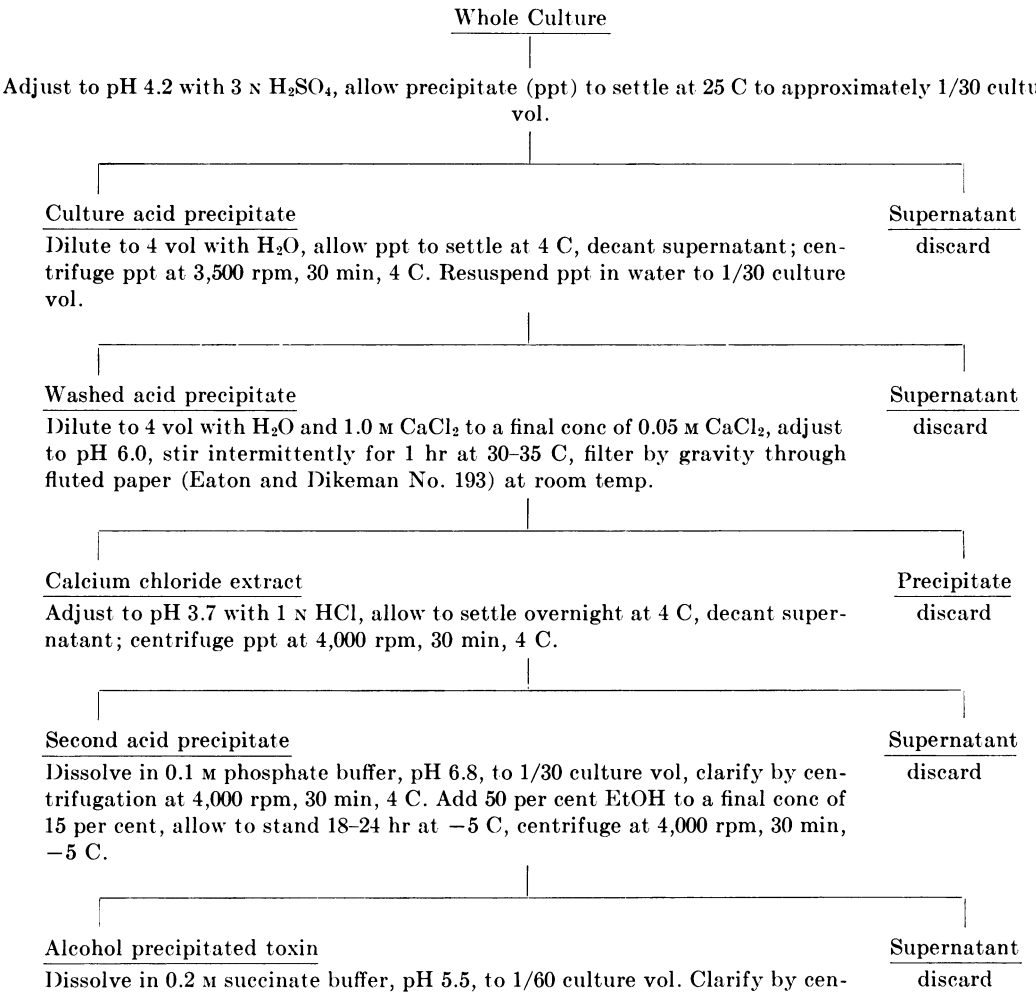

Figure 1. Procedure for purification of *Clostridium botulinum* type B toxin.

the succinate buffer at 1/60 the culture vol. Further fractionation of the toxin provided no additional increase in specific activity and attempts to crystallize the protein by slow precipitation with ammonium sulfate were unsuccessful.

The fractionation procedure is summarized in figure 1. The average purification and recovery data of toxin purified according to this fractionation procedure are presented in table 1. It may be observed that almost complete recovery of toxin and appreciable purification resulted from precipitation with acid and washing the acid precipitate with water. Since specific activity is given in terms of total rather than protein nitrogen, a large part of this purification in the initial step represents removal of the dialyzable constituents of the whole culture. Although the effectiveness of the calcium chloride extraction step is not shown by the data in this table, other experiments have indicated that less effective purification was obtained in the following step when the toxin was extracted in the absence of calcium chloride. The alcohol precipitated fraction represented approximately a 20 per cent recovery of toxic activity, and a 200-fold purification from the culture.

Properties of the purified toxin. The specific activity of 262×10^6 mouse LD_{50} per mg N represents an average for 20 samples. The toxin

was neutralized by type B antitoxin but not by univalent antitoxins of types A, C, D, and E. The toxin was readily soluble in 0.1 M phosphate buffer, pH 6.8, or 0.2 M succinate buffer, pH 5.5.

The purified toxin was examined in the analytical ultracentrifuge. The sample represented by the diagrams in figure 2 can be seen to consist of a main component for which a sedimentation constant (s^w_{20}) of 14.9 Svedberg units was calculated, and a diffusely sedimenting minor component with an s^w_{20} value of 10.9. The results are similar to those reported by Wagman and Bateman (1951) for a type B toxin preparation dissolved in 0.1 N citrate buffer, pH 3.0.

Figure 3 shows the sedimentation of a sample type B toxin prepared by a slight modification of the fractionation scheme described above. For this preparation the crude toxin was precipitated from the whole culture by acidification at pH 3.5, and the precipitate was washed with water at pH 5.0. The remainder of the purification was carried out according to the regular procedure, and the product was dissolved in 0.2 M succinate buffer, pH 5.5. It will be noted that the toxin sedimented as a single homogeneous boundary with a sedimentation constant of 12.7 Svedberg units. The specific activity of this fraction was 313 × 10^6 mouse IP LD_{50} per mg N.

The absorption spectrum of the toxin in the ultraviolet showed a maximum at 277 mμ and a minimum at 250 mμ, and was similar to the spectrum of type A toxin and to the spectrum reported for previous preparations of type B toxin (Lamanna and Glassman, 1947).

TABLE 1

Average purification and recovery data of type B toxin

Fraction	LD_{50}/mg N	Per Cent Recovery Based on LD_{50} from:		Purification Based on LD_{50}/mg N from Culture
		Preceding step	Culture acid precipitate	
Culture	1.2×10^6	—	—	—
Culture acid precipitate	17×10^6	97	—	14
Washed acid precipitate	32×10^6	97	97	28
$CaCl_2$ extract	34×10^6	43	40	28
Second acid precipitate	105×10^6	69	27	88
Alcohol precipitated toxin	262×10^6	70	19	218

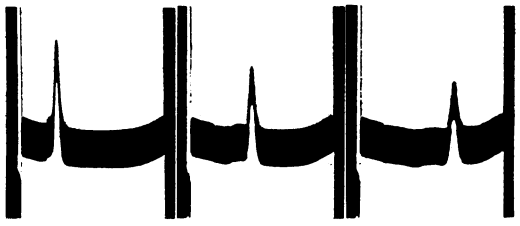

Figure 2. Sedimentation of purified type B toxin. Protein conc, 0.5 per cent; solvent, 0.1 M phosphate buffer, pH 6.0; centrifugal field, 260,000 × G; average temp, 24 C. Time after reaching full speed: left, 12 min; center, 22 min; right, 34 min.

Figure 3. Sedimentation of purified type B toxin. Protein conc, 0.25 per cent; solvent, 0.2 M succinate buffer, pH 5.5; centrifugal field, 110,000 × G; average temp, 24 C. Time after reaching full speed: left, 11 min; center, 22 min; right, 65 min.

DISCUSSION

The results of the present study indicate that in essentially all properties except serological specificity, type B toxin is similar to type A toxin. The value 262 × 10^6 LD_{50} per mg N for type B toxin is in close agreement with the 269 × 10^6 LD_{50} per mg N recently reported for type A toxin (Duff *et al.*, 1957). It is somewhat greater than the value of 161 × 10^6 obtained by Lamanna and Glassman (1947). The solubility properties of the toxins were not observed to be different; 0.5 per cent solutions of type B toxin were readily obtained in dilute salt solutions at pH 6. Similarity of the ultraviolet absorption spectra suggests that the proteins had similar proportions of aromatic amino acids and were both essentially free of nucleic acid. The similarity of the sedimentation constant to the value of 14.5 that was obtained for crystalline type A toxin (Duff *et al.*, 1957) suggests that the molecu-

lar weights of the two toxins are not greatly different, although diffusion measurements will be required before a definite molecular weight can be calculated. Efforts to crystallize the type B toxin have failed thus far, and in this respect it appears to differ from the readily crystallizable type A toxin.

The type B toxin was converted to toxoid by incubation in the presence of formalin, and closely resembled type A toxin with respect to this reaction. Further details of the detoxification of the purified toxins will be presented in a subsequent paper.

Our results confirm the observation of Lamanna and Glassman (1947) that type B toxin fractionated by procedures involving exposure to pH 2.0 is relatively insoluble in the region of neutrality. Evidently the previously reported chemical differences between the type A and type B toxins resulted at least in part from the fractionation methods employed in their isolation. It is possible that differences between present and previous preparations of type B toxin resulted from use of different stains and culture media; this possibility appears unlikely but has not been excluded.

Differences in serological specificity doubtless reflect differences in chemical structure, and accordingly it is to be expected that refined observations will eventually detect chemical differences between the type A and type B toxins. It seems evident, however, that the differences are less profound than was originally suggested.

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SUMMARY

A procedure was developed for the production and purification of *Clostridium botulinum* type B toxin. The toxin was produced in a medium composed of "trypticase," yeast extract, cysteine hydrochloride, and glucose. The cultures contained 2.0×10^6 mouse intraperitoneal (IP) LD_{50} per ml or greater. The toxin was precipitated from the culture by acidification at pH 4.5, and the precipitate was washed with water. Toxin was extracted from the washed precipitate with 0.05 M calcium chloride solution at pH 6.0 and 30 to 35 C. The toxin was precipitated from the extract by acidification at pH 3.7. The toxin was dissolved in 0.1 M phosphate buffer at pH 6.8 and precipitated with ethanol to 15 per cent concentration at −5 C. The purified toxin was soluble in 0.2 M succinate buffer, pH 5.5, or 0.1 M phosphate buffer, pH 6.8, had a specific activity of 262×10^6 mouse LD_{50} per mg N, and was essentially homogeneous when examined in the analytical ultracentrifuge. The fractionation procedures were reproducible and could be applied on a scale suitable for routine isolation of toxin for conversion to toxoid.

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