STUDIES ON IMMUNITY TO TOXINS OF *CLOSTRIDIUM BOTULINUM*

IV. Production and Purification of Type C Toxin for Conversion to Toxoid

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Received for publication October 11, 1957

Clostridium botulinum type C has been implicated in outbreaks of botulism in fowl, aquatic wild birds, and domestic and fur-bearing animals (Meyer, 1956). Although type C intoxication is usually assumed to occur only in animals, two human outbreaks with one fatality have been described (Meyer, 1953; Prévot *et al.*, 1955). Purification of type C toxin by extraction from washed cells has been reported by Boroff *et al.* (1952) and by Katitch (1952). Sterne and Wentzel (1950) have described a method for the production of high potency type C toxin by growth of type C strains in cellophane sacs immersed in nutrient medium. These and other authors (Bennetts and Hall, 1938; Oxer, 1940; Moberg, 1953) have described the preparation of effective type C toxoids for large-scale immunization of animals. Prévot and Brygoo (1950) produced type C antitoxin in man by immunization with toxoid prepared from toxin extracted from bacterial cells.

Previous reports from our laboratory have described procedures for the production and purification of the toxins of *C. botulinum* types A, B, and E suitable for preparation of purified toxoids (Duff *et al.*, 1957*a*, *b*; Gordon *et al.*, 1957). The present paper describes similar investigations on the type C toxin, and the immunization of mice and guinea pigs with fluid and aluminum phosphate adsorbed toxoids.[1]

MATERIALS AND METHODS

The type C strain of *C. botulinum* isolated by Theiler and Robinson (1928) was used. This strain was obtained from Dr. R. C. Jansen of the Onderstepoort Laboratories, Union of South Africa. The method for maintenance of cultures was essentially that described by Duff *et al.* (1957*a*) for the type A strains. The type C strains were grown at 33 C and stored at −20 C. Cultures stored in this manner have maintained their toxigenicity and viability for over two years. Procedures for nitrogen determinations and toxicity, flocculation, and neutralization titrations also have been described (Duff *et al.*, 1957*a*). Aluminum phosphate suspension was prepared as described by Gordon *et al.* (1957). Crude univalent type C antitoxin, supplied by the Microbiological Research Establishment of the Ministry of Supply Station at Porton, England, was used as the standard in all neutralization titrations. The toxin used in the assay of guinea pig sera was a partially purified preparation diluted with two parts of glycerol and stored at −20 C. Under these conditions the toxin retained its toxicity and combining power over a period of several years. The working dilution of toxin was equivalent to 0.4 unit per ml.

EXPERIMENTAL RESULTS

Toxin production. In preliminary investigations large and consistent yields of toxin were obtained when the South African strain of *C. botulinum* type C was grown in a veal infusion medium (Difco) containing proteose peptone and 0.1 per cent agar and supplemented with 2.0 per cent yeast extract (B.B.L.) and 1.0 per cent glucose. The presence of agar in the commercially prepared medium prevented concentration of precipitates of whole culture by settling or centrifugation, as had previously been observed with the type B toxin (Duff *et al.*, 1957*b*). Laboratory preparation of veal infusion was cumbersome, and successive lots gave inconsistent results. Various commercial peptones and hydrolyzed casein preparations were investigated as replacements for the veal infusion; N-Z-Amine-type B (Sheffield Farms) and an increased concentration of proteose peptone (Difco) were satisfactory. The medium developed for produc-

[1] A preliminary report of these studies has been published (Cardella *et al.*, 1956).

tion of toxin was composed of 4.0 per cent proteose peptone (Difco), 2.0 per cent pancreatic digest of casein (N-Z-Amine-type B, Sheffield Farms, Norwich, New York), 2.0 per cent yeast extract (Baltimore Biological Laboratories, Baltimore, Maryland), and 1.0 per cent glucose. Addition of the glucose to the medium prior to autoclaving led to more prompt and consistent initiation of growth. The pH of the medium before autoclaving was 7.6. When inoculum was required for production of toxin, the stock culture was inoculated into a beef infusion-peptone medium and thence through serial transfers in increasing volumes of production medium. A 10 per cent volume of inoculum was found most satisfactory in all transfers. Seed cultures were incubated at 33 C for 18 to 24 hr. Cultures for toxin production were grown at 33 C for 5 days in 4-L bottles containing 3 L of medium. After growth the pH was 5.5 to 6.0, and the toxicity averaged 0.8×10^6 mouse intraperitoneal LD_{50} per ml.

Toxin purification. (1) Alcohol procedure:— Fractionation procedures described for the types A and B botulinum toxins (Snipe and Sommer, 1928; Lamanna and Glassman, 1947; Duff *et al.*, 1957*a*, *b*) have included the use of an acid precipitation technique for initial concentration and purification of toxin from culture. Application of this procedure to type C cultures resulted in low recovery of toxin and only 2-fold purification. With type E botulinum toxin (Gordon *et al.*, 1957), precipitation with ethanol was used for initial concentration and purification of toxin from culture. Type C toxin also could be precipitated from culture by addition of ethanol to a final concentration of 25 per cent at −5 C. Precipitation at 4 C or with lower concentrations of ethanol at −5 C resulted in lower yields of toxin. Precipitation was most complete in the pH range 5.5 to 6.0, which was the pH of the cultures at the time of harvesting.

Accordingly, the whole culture was cooled to −5 C, and 95 per cent ethanol was run in through a cooling coil at −5 C to a final concentration of 25 per cent. Ethanol-precipitable material settled out on overnight standing at −5 C. The supernatant was siphoned off and the precipitate collected by centrifugation for 30 min at 4000 rpm and −5 C. The precipitate was resuspended in cold water to ⅛ the culture volume. This represented the first alcohol fraction.

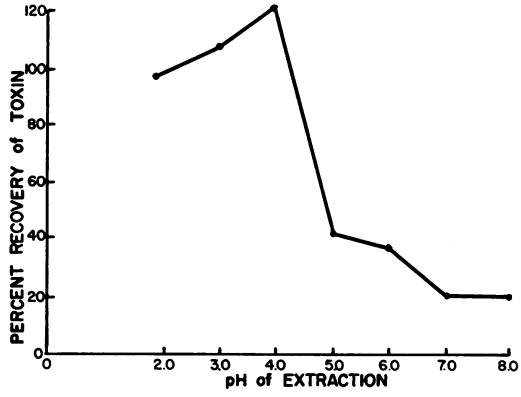

Figure 1. Effect of pH on extraction of toxin in 0.075 M CaCl solution.

Previous reports from our laboratories (Duff *et al.*, 1957*a*, *b*); Gordon *et al.*, 1957) described use of calcium chloride solution at pH 6.0 to 6.5 for the extraction of types A, B, and E toxins from culture precipitates. This method was applied to extraction of type C toxin from the culture alcohol precipitates. Extraction of toxin with 0.075 M $CaCl_2$ solution was investigated in the pH range 2.0 to 8.0. The data from several experiments are summarized in figure 1. Complete recovery of toxin occurred in the pH range of 2.0 to 4.0. The reason for recovery of toxin in excess of 100 per cent is not known. It is possible that an activation phenomenon occurs with the type C toxin, but attempts to substantiate this hypothesis have been unsuccessful.

Investigation of the lower pH range indicated that pH 3.0, 0.075 M calcium chloride, and room temperature were optimum conditions for recovery of toxin at this step. After further purification these fractions were relatively insoluble in various buffers at pH 5.0 or above. When the toxin was extracted at pH 5.0 to 8.0, a somewhat lower yield of toxin was obtained, but fractions purified further were found to be soluble at pH 5.0. Further investigations at pH 5.0 indicated that 0.05 M calcium chloride and room temperature were optimum conditions for extraction. Although extraction at pH 5.0 resulted in a lower recovery of toxin, this pH was selected to provide toxin more suitable for preparation of toxoid. Accordingly, the ethanol precipitated toxin was diluted to ¼ culture volume with distilled water and 1.0 M $CaCl_2$ solution to 0.05 M $CaCl_2$; the pH was adjusted to 5.0. The suspension was stirred intermittently for 1

WHOLE CULTURE

Add 95 per cent EtOH to a final conc of 25 per cent at −5 C, allow to stand 18 to 24 hr at −5 C, siphon supernatant, centrifuge precipitate at −5 C.

Precipitate	Supernatant
Dilute to ⅛ culture vol with H_2O	Discard
FIRST ALCOHOL FRACTION	
Add H_2O and 1.0 M $CaCl_2$ to a final conc of 0.05 M $CaCl_2$ to ¼ culture vol, adjust to pH 5.0, stir 1 hr at room temperature, centrifuge at 4 C.	
Supernatant ($CaCl_2$ EXTRACT FRACTION)	Precipitate Discard
Adjust to pH 6.0 with 1 N NaOH, add 50 per cent EtOH to a final conc of 15 per cent at −5 C, allow to stand 18 to 24 hr at −5 C, centrifuge at −5 C.	
Precipitate	Supernatant
Dissolve in 0.4 M succinate buffer pH 5.0, clarify by centrifugation at 4 C.	Discard
SECOND ALCOHOL FRACTION	

Figure 2. Procedure for purification of *Clostridium botulinum* type C toxin.

hr at room temperature, then centrifuged at 4 C at 4000 rpm for 30 min. The supernatant represented the $CaCl_2$ extract fraction.

Further concentration and purification was accomplished by precipitation of toxin from the $CaCl_2$ extracts and resuspension of precipitates in buffer. Toxin could be precipitated from the $CaCl_2$ extract by addition of alcohol, by addition of ammonium sulfate, or by adjustment of the pH to 6.0. The greatest recovery and purification of toxin were obtained on addition of 50 per cent alcohol to a final concentration of 15 per cent at pH 6.0 and −5 C. The supernatant was drawn off and the precipitate was collected by centrifugation for 30 min at 4000 rpm and −5 C.

The alcohol precipitated toxin was only slightly soluble in 0.1 M succinate buffer at pH 5.0; the solubility increased with an increase in succinate concentration. Dissolving the alcohol precipitate in 1/16 the culture volume of 0.4 M succinate buffer at pH 5.0 resulted in complete recovery of toxin and a purification of 10-fold from the $CaCl_2$ extract step. The solution was clarified by centrifugation for 30 min at 4000 rpm and 4 C to remove an insoluble precipitate, probably calcium succinate. The supernatant is referred to as the second alcohol fraction.

TABLE 1

Average purification and recovery data of type C toxin

Fraction	LD_{50}/mg N	Per Cent Recovery Based on LD_{50} from:		Purification Based on LD_{50}/mg N from:	
		Preceding step	Culture	Preceding step	Culture
Culture	0.14×10^6	—	—	—	—
First alcohol	3.9×10^6	104	104	56	56
$CaCl_2$ extract	2.5×10^6	41	45	0.7	36
Second alcohol	22×10^6	99	43	10	308

The fractionation procedure is summarized in figure 2. Purification and recovery of toxin at the various steps in the purification procedure are summarized in table 1; the results are the average of five experiments. It may be observed that initial concentration of toxin by precipitation with alcohol in the cold resulted in complete recovery of toxin and a purification of 56-fold. Although a considerable loss of toxin and no purification occurred at the pH 5.0, $CaCl_2$ extract step, toxin purified further was soluble at pH 5.0 and therefore suitable for the preparation of toxoid. Extraction of toxin at lower pH values resulted in greater recovery and purification, but toxin purified further was much less soluble at pH 5.0. The second alcohol fraction represented a 43 per cent recovery of toxin and a purification of 308-fold from culture.

(2) $(NH_4)_2SO_4$ procedure:—Substitution of $(NH_4)_2SO_4$ for alcohol in the initial concentration of toxin from culture was more convenient and permitted satisfactory handling of larger volumes of culture. Saturated $(NH_4)_2SO_4$ solution was added to whole culture at room temperature with intermittent swirling to a final concentration of 40 per cent saturation. Ammonium sulfate-precipitable material settled out on overnight standing at room temperature. The supernatant was siphoned off and the precipitate collected by

centrifugation at 4000 rpm and 4 C. The precipitate was resuspended in water to ¼ the culture volume. Toxin was further fractionated by the procedures developed for fractionation of toxin from culture alcohol precipitates.

Recovery of toxin at all the steps in the ammonium sulfate procedure was essentially identical with results obtained by the alcohol procedure. The over-all purification of toxin from culture and the specific activity of the final product were approximately three times that of the final products prepared by the alcohol procedure.

Toxoids. Second alcohol fractions in 0.4 M succinate buffer at pH 5.0, prepared by the alcohol procedure, were used for preparation of toxoids. Toxin was converted to toxoid in the presence of 0.6 per cent formalin (Reagent Formaldehyde Merck Neutral) at 33 C. Detoxification was considered complete when 1.0 ml of a 1 to 2 dilution injected subcutaneously did not kill mice in 10 days or guinea pigs in 21 days. About 20 days was required for detoxification under these conditions. After detoxification, the toxoids were dialyzed against 0.4 M succinate buffer pH 5.0 to remove residual formalin. No loss in total nitrogen or Lf occurred during detoxification and dialysis. Fluid toxoids were integrated with an equal volume of aluminum phosphate suspension so that the product contained 7 mg $AlPO_4$ per ml.

The integrated toxoids were tested for antigenicity in mice and guinea pigs. Mice were immunized subcutaneously with 1.0 ml of toxoid and challenged intraperitoneally after five weeks. The mice were divided into two groups. The first group was challenged with crude toxin, a first alcohol fraction; and the second group challenged with purified toxin, a second alcohol fraction. The mice were observed for four days and the protection in LD_{50} was calculated by the method of Reed and Muench (1938). Guinea pigs were immunized with 1.0 ml of integrated toxoid and bled 9, 12, and 15 weeks after immunization. The antitoxin titers of the sera were measured by neutralization titrations in mice.

Table 2 summarizes the antigenic response in mice to a single injection of integrated toxoid. It may be observed that protection was afforded against approximately 1×10^6 mouse intraperitoneal LD_{50} of crude or purified toxins. Even the product that contained 2 Lf produced considerable immunity. Table 3 shows the antigenic response elicited in mice and guinea pigs to a single injection of integrated and fluid toxoid diluted to contain an equal immunizing dose. The response in mice to integrated toxoid was 25 times that induced by fluid toxoid. The responses in guinea pigs to fluid and integrated toxoids were essentially the same, and the maximum titers were reached after approximately 12 weeks.

TABLE 2

Immunological response of mice to one injection of $AlPO_4$ integrated type C toxoid

LD_{50}/ml Before Detoxification	Lf/ml in Integrated Toxoid	Protection in LD_{50}	
		Challenge toxins	
		Crude	Purified
0.3×10^6	2	0.4×10^6	0.6×10^6
0.6×10^6	5	0.9×10^6	1.0×10^6
1.3×10^6	9	0.9×10^6	1.6×10^6
2.6×10^6	19	1.3×10^6	1.1×10^6

TABLE 3

Immunological response of mice and guinea pigs to one injection of fluid and integrated toxoids

Toxoid	Mice	Guinea Pigs		
	Protection in LD_{50} at 5 weeks	Titer in units/ml of serum		
		9 weeks	12 weeks	15 weeks
Fluid	4×10^4	8	23	22
Integrated	1×10^6	10	21	18

Protection in mice and immune response in guinea pigs obtained with toxoids prepared by the $(NH_4)_2SO_4$ procedure were essentially the same as those obtained from toxoids purified by the alcohol procedure.

DISCUSSION

The medium developed for the production of *C. botulinum* type C toxin consisted of commercially available constituents, and the production method could be applied readily on a scale suitable for routine production of a biological. Toxicities of approximately 1×10^6 mouse intraperitoneal LD_{50} per ml were regularly obtained in this medium when cultures were grown at 33 C for five days. These values were greater than those obtained by Boroff *et al.* (1952) and Prévot *et al.* (1950, 1953), and approximated

those obtained in dialysis cultures by Sterne and Wentzel (1950).

Fractionation of type C toxin by the alcohol method increased specific activity approximately 300-fold. The specific activity, which was essentially constant in separate experiments, was about one-tenth that of types A and B toxins (Abrams *et al.*, 1946; Lamanna *et al.*, 1946; Lamanna and Glassman, 1947) and essentially the same as the toxicity of activated type E toxin (Gordon *et al.*, 1957). Substitution of ammonium sulfate in the initial precipitation yielded a product with approximately three times the specific activity of the alcohol precipitated toxin. The type C toxin was similar to type B toxin in that exposure to low pH during fractionation yielded a product that was relatively insoluble in the region of neutrality (Lamanna and Glassman, 1947; Duff *et al.*, 1957*b*).

Type C toxin was converted to toxoid under conditions similar to those that were satisfactory for the types A, B, and E toxins. The fluid toxoids provided greater protection in mice than the type C toxoids prepared by Prévot and Brygoo (1950) from toxin obtained by extraction of bacterial cells. The protection approached that obtained by Rice *et al.* (1947*a*) with alum precipitated type A toxoids and by Gordon *et al.* (1957) with purified type E toxoids. The increase in antigenicity for mice when type C toxoid was adsorbed on aluminum phosphate was similar to the effects noted by Sterne and Wentzel (1950) in the immunization of cattle with types C and D toxoids. These workers observed that the antitoxin response to adsorbed toxoids was 30 times that to fluid toxoids. Alum precipitation was also found to increase the antigenicity of types A and B toxoids in mice (Hottle *et al.*, 1947; Rice *et al.*, 1947*a*, *b*). Guinea pigs evidently respond similarly to unadsorbed and adsorbed type C toxoids, in agreement with the results of Rice *et al.* (1947*a*, *b*).

The observation that the protection conferred on mice by the purified toxoid is equally effective against crude and purified toxins indicates that the fractionation procedure does not eliminate any significant toxic product of the type C organisms.

ACKNOWLEDGMENT

Acknowledgment is made to Dr. George G. Wright for his advice throughout the course of the work and his assistance with the preparation of the manuscript.

SUMMARY

A medium was developed for the production of *Clostridium botulinum* type C toxin. The medium contained 4.0 per cent proteose peptone, 2.0 per cent N-Z-Amine-type B, 2.0 per cent yeast extract, and 1.0 per cent glucose. Cultures grown five days at 33 C contained 800,000 mouse intraperitoneal LD_{50} per ml. Toxin produced in this medium could be purified by procedures that included either alcohol or ammonium sulfate precipitation, followed by a calcium chloride extraction and an additional alcohol precipitation. Specific activity of the purified toxin was 20 to 60 $\times$ 10^6 LD_{50} per mg N. Toxin was converted to toxoid in the presence of formalin and after adsorption on aluminum phosphate was highly antigenic for mice and guinea pigs.

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