STUDIES ON IMMUNITY TO TOXINS OF *CLOSTRIDIUM BOTULINUM*

I. A Simplified Procedure for Isolation of Type A Toxin

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Preparation of alum precipitated toxoids for immunization against type A and type B toxins of *Clostridium botulinum* has been described in previous reports from these laboratories (Nigg *et al.*, 1947; Hottle *et al.*, 1947; and Reames *et al.*, 1947). Although the bivalent toxoid produced satisfactory immunity in man as measured by the serum antitoxin concentration, undesirable local and systemic reactions to injection of the relatively crude antigen were encountered. Procedures for preparation of a purified polyvalent toxoid of improved clinical acceptability were therefore investigated.

Isolation of type A toxin has been reported by Lamanna *et al.* (1946*a, b*) and by Abrams *et al.* (1946). Although the fractionation methods permitted isolation of crystalline toxin of high activity, they appeared to be difficult to apply on a scale suitable for routine preparation of toxin for toxoid. Accordingly, the purification of type A toxin was reinvestigated (Duff *et al.*, 1952) and the simplified fractionation method described in the present report was developed.

MATERIALS AND METHODS

Medium. The medium for toxin production was composed of 2.0 per cent pancreatic digest of casein (N-Z-amine-type B, Sheffield Farms, Norwich, N. Y.), 0.5 per cent (in terms of dry solids) autolyzed yeast (Vico type #75, Vico Products Co., Chicago, Illinois), and 0.5 per cent glucose. The medium was adjusted to pH 7.2 before autoclaving. Glucose was autoclaved separately as a 20 per cent solution and added aseptically.

Production of toxin. A clone of the Hall strain of *Clostridium botulinum* type A, that had been selected for toxigenicity by Dr. Ralph E. Lincoln of these laboratories, was used. The stock strain was grown for 24–48 hr at 35 C in 200 ml vol of a medium composed of beef infusion, 1 per cent peptone, and chopped meat. After growth, the supernatants were distributed in small tubes, quick frozen at −60 C, and stored at −20 C. Since sporulation was not observed with this strain, subcultures have been prepared at yearly intervals. When inoculum was required for production of toxin, 2.0 ml of one tube of the stock strain was transferred to 10 ml of Brewer thioglycolate medium (Difco), and incubated at 37 C for 18–24 hr. Serial transfers were then made into larger volumes of the toxin production medium to obtain a volume of inoculum equal to 5 per cent of the final culture. These seed cultures were incubated at 35 C for 18–24 hr. Cultures for toxin production were grown at 35 C for 4 days in bottles containing 3 L of medium. After growth the pH was 5.6, almost complete lysis had occurred, and the toxicity averaged approximately 1.5×10^6 mouse intraperitoneal LD_{50} per ml.

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 6.8 with hydrochloric acid. White mice weighing 18–20 g were injected intraperitoneally with 0.5-ml aliquots of the dilutions of toxin and observed for four days. The number of mice per dilution ranged from 4 to 16, depending upon the accuracy desired. The LD_{50} was calculated by a graphic probit method (Weiss, 1948).

Neutralization titrations. Antitoxin diluted to contain either 0.1 or 0.01 units per 0.25 ml was mixed with an equal volume of varying dilutions of toxin. The mixtures were allowed to stand for 1 hr at room temperature and 0.5 ml was injected intraperitoneally into each of 4 mice. Antitoxins for types A, B, C, D, and E were supplied by the Microbiological Research Establishment of the Ministry of Supply Station at Porton, Wiltshire, England.

Flocculation titrations. Lf determinations were carried out in a water bath at 40 C with 1.0-ml

portions of diluted toxin and varying amounts of antitoxin. Crude, unprocessed antiserum containing 100 antitoxin units per ml was supplied by Lederle Laboratories Division, American Cyanamid Company.

Nitrogen determinations. Total nitrogen was determined by a colorimetric nesslerization procedure (Koch and McMeekin, 1924; Lanni *et al.*, 1945).

EXPERIMENTAL RESULTS

The acid precipitation procedure of Snipe and Sommer (1928) was used for concentration and initial purification of toxin in whole culture. Almost complete precipitation of toxin occurred between pH 3.5 and 4.0 when either 3 N hydrochloric acid or 3 N sulfuric acid were used. Accordingly, the cultures were adjusted to pH 3.5 with 3 N sulfuric acid and allowed to stand overnight at room temperature. The clear supernatants were discarded, and the precipitates were pooled and allowed to settle at 4 C to approximately $\frac{1}{40}$ the culture volume. A purification of approximately 25-fold from the culture was obtained. Specific activity was calculated in terms of total rather than protein nitrogen, and accordingly a large part of this purification represented removal of dialyzable nitrogen. These precipitates have been stored at 4 C for several months without significant loss of activity.

Further purification was accomplished by washing the culture acid precipitate by suspension in water. During early investigations, considerable difficulty gradually developed in obtaining satisfactory settling of this precipitate. This difficulty was traced to alterations in the stock culture which, during these initial experiments, had been subcultured periodically and stored at 4 C. Substitution of the original strain, which had been maintained at 4 C without subculture, produced satisfactory settling of the precipitate, which was desirable to facilitate handling large volumes of material. Subsequently this culture was stored at −20 C, and no alterations that influenced the purification of toxin were noted.

Conditions for extraction of inert material from the culture acid precipitate were studied. Comparison of extraction with water at pH 4.7, 5.0, 5.3, and 5.6 revealed that the removal of impurity increased with the pH. However, the optimum pH for settling of the toxic precipitate

was between pH 5.0 and 5.3, and pH 5.0 was selected as most satisfactory. A 4-fold dilution of the culture acid precipitate in water was used to permit easy handling of the desired volumes. After standing overnight at 4 C, the supernatant was decanted and the precipitate collected by centrifugation at 4 C at 3,500 rpm for 30 min. The precipitate was resuspended in distilled water to approximately $\frac{1}{40}$ the culture volume. This procedure resulted in an average purification of 2.5-fold and an 80 per cent recovery. In preliminary studies (Duff *et al.*, 1952) 0.02 M ethylenediamine tetraacetate was used for extraction of impurity at this step. Later it was determined that this solution had no consistent advantage over water.

Extraction of toxin from the washed acid precipitate was investigated. It had been observed in studies on extraction of impurity from the culture acid precipitate that addition of 0.01 M calcium chloride decreased the solubility of inert protein but did not alter significantly the solubility of toxin. This finding suggested that extraction of toxic activity from the culture acid precipitate with minimum extraction of impurity would be obtained in the presence of calcium chloride. Investigation of the effect of pH revealed that, in the presence of 0.15 M calcium chloride, the recovery of toxin from the culture acid precipitate increased between pH 4 and pH 6.5. At pH 6.5, alteration of the concentration of calcium chloride between 0.075 M and 0.3 M did not influence significantly the solubilities of the toxin or impurities.

Water suspensions of the acid precipitates at pH 6.5 were very turbid when calcium chloride was omitted and the insoluble material was not removed by ordinary centrifugation, whereas calcium chloride extracts were easily clarified by filtration or centrifugation. A 4-fold dilution was used at this step to permit satisfactory handling of the material during filtration. Extraction with 0.15 M calcium chloride solution at pH 6.5 resulted in 2-fold purification and recovery of approximately 66 per cent of the toxic activity. Repeated extraction with the calcium chloride solution did not dissolve appreciable additional toxin. Preliminary experiments indicated that substitution of lithium, barium, or magnesium chloride for calcium chloride led to similar recovery of toxin.

Toxin was not precipitated from the 0.15 M

calcium chloride extract by hydrochloric acid between pH 4.5 and pH 6.6, and only a slight precipitation of toxin occurred between pH 3.0 and 4.0. Addition of ethanol at −5 C to a final concentration of 15 per cent precipitated 80 per cent of the toxin and produced a purification of approximately 2.5-fold. The precipitated toxin dissolved readily in 0.06 M potassium phosphate buffer, pH 6.8.

Concurrent studies on fractionation of type B toxin (Duff *et al.*, 1954) indicated that toxin was extracted from the washed acid precipitate by 0.05 M calcium chloride at pH 6.0, and that the toxin in these extracts was precipitated on acidification; precipitation of toxin on acidification decreased as the molarity of calcium chloride was increased. Analogous studies with type A toxin revealed a similar relationship. Extraction of toxin in the presence of 0.075 M calcium chloride allowed almost complete precipitation of toxin when the extract was adjusted to pH 3.7. The precipitate dissolved readily in $\frac{1}{80}$ the culture volume of 0.03 M potassium phosphate buffer, pH 6.8. Recovery and purification of toxin were identical with the results obtained by ethanol precipitation. Because of the large volume of the
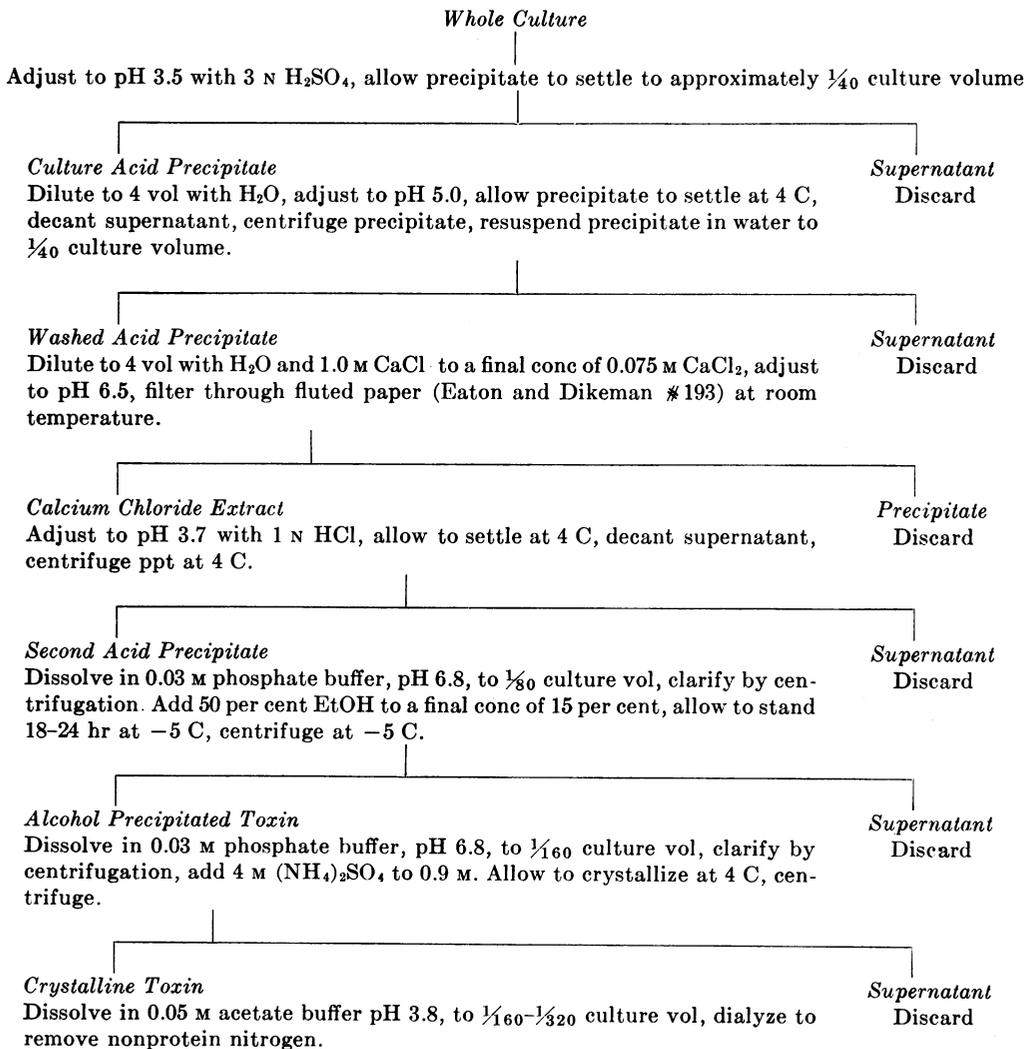
Whole Culture

Adjust to pH 3.5 with 3 N H₂SO₄, allow precipitate to settle to approximately $\frac{1}{40}$ culture volume

Culture Acid Precipitate   *Supernatant*
Dilute to 4 vol with H₂O, adjust to pH 5.0, allow precipitate to settle at 4 C,   Discard
decant supernatant, centrifuge precipitate, resuspend precipitate in water to
$\frac{1}{40}$ culture volume.

Washed Acid Precipitate   *Supernatant*
Dilute to 4 vol with H₂O and 1.0 M CaCl to a final conc of 0.075 M CaCl₂, adjust   Discard
to pH 6.5, filter through fluted paper (Eaton and Dikeman # 193) at room
temperature.

Calcium Chloride Extract   *Precipitate*
Adjust to pH 3.7 with 1 N HCl, allow to settle at 4 C, decant supernatant,   Discard
centrifuge ppt at 4 C.

Second Acid Precipitate   *Supernatant*
Dissolve in 0.03 M phosphate buffer, pH 6.8, to $\frac{1}{80}$ culture vol, clarify by cen-   Discard
trifugation. Add 50 per cent EtOH to a final conc of 15 per cent, allow to stand
18–24 hr at −5 C, centrifuge at −5 C.

Alcohol Precipitated Toxin   *Supernatant*
Dissolve in 0.03 M phosphate buffer, pH 6.8, to $\frac{1}{60}$ culture vol, clarify by   Discard
centrifugation, add 4 M (NH₄)₂SO₄ to 0.9 M. Allow to crystallize at 4 C, cen-
trifuge.

Crystalline Toxin   *Supernatant*
Dissolve in 0.05 M acetate buffer pH 3.8, to $\frac{1}{60}$–$\frac{1}{320}$ culture vol, dialyze to   Discard
remove nonprotein nitrogen.

Figure 1. Procedure for purification of *C. botulinum* type A toxin

calcium chloride extract, the acidification method was more convenient and was adopted in the final process.

An inert precipitate remained after the acid precipitated fraction was dissolved in phosphate buffer. This precipitate, probably calcium phosphate, was removed by centrifugation at 4 C at 3,000 rpm for 30 min. Further purification of toxin was accomplished by precipitation with ethanol. 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 overnight at −5 C, the preparation was centrifuged at −5 C at 3,500 rpm for 30 min. The precipitated toxin dissolved readily in 0.03 M phosphate buffer at pH 6.8 at $\frac{1}{160}$ the culture volume, and also in 0.2 M succinate buffer at pH 5.5 at $\frac{1}{40}$ the culture volume. The latter conditions were satisfactory when the purified toxin was used for toxoid preparations. Precipitation with ethanol resulted in an average purification of 1.5-fold and recovery of approximately 88 per cent of the toxic activity of the previous fraction. The over-all recovery from the culture was approximately 40 per cent.

The ethanol fraction was essentially pure, and the toxin could be crystallized from the phosphate buffer by addition of 4 M ammonium sulfate solution to a final concentration of 0.9 M. Crystals formed on standing at 4 C, and were similar in form and specific activity to those described previously (Abrams *et al.*, 1946; Lamanna *et al.*, 1946a). No effort was made to improve the recovery during crystallization of the toxin, because the purity of the preceding fraction was sufficient for preparation of toxoid.

Crystalline toxin was dissolved at a concentration of 0.3 per cent in 0.05 M sodium acetate buffer, pH 3.8, and examined in the ultracentrifuge. The toxin sedimented as a homogenous boundary with a sedimentation constant, s_{20}^{w}, of 14.5 Svedberg units, as in previous studies (Putman *et al.*, 1948; Wagman and Bateman, 1951; and Wagman, 1954). Both crude and purified toxins were neutralized by univalent type A antitoxin, but not by univalent antitoxin for types B, C, D, or E.

The fractionation procedure is summarized in figure 1. The average purification and recovery data of the fractions of toxin purified according to this method in eight experiments are presented in table 1. The per cent toxin in the various fractions was calculated from the specific activities, using the average specific activity of six crystalline samples as 100 per cent. It may be noted that satisfactory recovery and purification of toxin were obtained at each step.

DISCUSSION

The procedures described are adaptable to fractionation of toxin on a scale suitable for routine preparation of purified toxoid. Conditions were adjusted to allow settling of many of the precipitates, and difficult filtration or centrifugation procedures were avoided. With ordinary precautions the fractionation process could be carried out safely.

Purified toxoids for immunization of man usu-

TABLE 1
Average purification and recovery data of type A toxin

Fraction	LD₅₀/mg N	Lf/mg N	Per Cent Recovery Based on LD₅₀ from:		Purification Based on LD₅₀/mg N from Preceding Step	Per Cent Purity Based on:	
			Preceding step	Culture acid precipitate		LD₅₀/mg N*	Lf/mg N†
Culture.....................	0.5×10^6					2	
Culture acid precipitate.....	14×10^6		96		24	5	
Washed acid precipitate.....	35×10^6		80	80	2.5	13	
CaCl₂ extract...............	75×10^6	131	66	54	2.1	28	23
Second acid precipitate......	178×10^6	252	83	50	2.4	66	45
Alcohol precipitated toxin...	264×10^6	402	88	41	1.5	98	71
Crystalline toxin............	269×10^6	563		17	1.1	Taken as 100	Taken as 100

* 269×10^6 Mouse LD₅₀/mg N taken as pure toxin.

† 563 Lf/mg N taken as pure toxin.

ally have been prepared by fractionation of crude toxoid, rather than by detoxification of purified toxin. The stability of botulinum toxin under appropriate conditions made it practical to purify and concentrate the toxin, so that the volume during detoxification would be reduced greatly and the conditions for conversion to toxoid could be defined more precisely. Studies on the preparation and properties of toxoid will be presented in a subsequent paper.

The fractionation procedure differs from previous methods primarily in the use of calcium chloride solution for initial extraction of toxin from the crude precipitate, and in the use of precipitation with ethanol at -5 C for subsequent purification. Calcium chloride solution was employed by Burrell et al. (1948) for extraction of antigen from *Haemophilus pertussis* and subsequently has been found effective for fractionation of *C. botulinum* type B toxin (Duff et al., 1954). The greatest loss of toxic activity occurred at the calcium chloride extraction, and the procedure may deserve further investigation.

Precipitation of toxin with ethanol had been reported previously by Abrams et al. (1946), who carried out the process at 4 C. A lower temperature appeared to be desirable to avoid possible denaturation of toxin and subsequent reduction in antigenicity of toxoid prepared from the purified toxin. Although somewhat greater recovery of toxin was obtained at -5 C than at 4 C, the effect of temperature of precipitation on the antigenicity of the product has not been determined.

The flocculation reaction with antitoxin was not observed with the crude toxin but was obtained with the calcium chloride extract and subsequent fractions. It provided a rapid and convenient method for estimation of the recovery of toxin. Flocculation and toxicity titrations correlated rather well except with crystalline toxin. The Lf per mg N increased considerably as a result of crystallization, whereas the LD_{50} per mg N remained constant. No obvious explanation can be suggested for this observation, which suggests an inhomogeneity of the alcohol precipitated fraction.

The fractionation procedure was developed for isolation of toxin from cultures of one strain grown in a single medium, and the applicability of the procedure to other strains or production media has not been investigated. The early difficulties that were traced to spontaneous altera-

tions in the stock culture suggest that with other crude preparations of toxin, minor modification of the procedure may be necessary for optimum results. For example, to obtain satisfactory settling of precipitate during water washing of crude toxin it may be necessary to adjust the mixture to a pH below 5.0.

SUMMARY

A simplified procedure for purification of *Clostridium botulinum* type A toxin was described. The 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 precipitate with 0.075 M calcium chloride and precipitated from the extract at pH 3.7. The toxin was dissolved in phosphate buffer at pH 6.8 and precipitated with 15 per cent ethanol in the cold. This fraction was essentially pure toxin and could be crystallized by precipitation with ammonium sulfate. The crystalline toxin had a specific activity of 269×10^6 mouse intraperitoneal 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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