STUDIES ON IMMUNITY TO TOXINS OF *CLOSTRIDIUM BOTULINUM*

III. Preparation, Purification, and Detoxification of Type E Toxin

MILTON GORDON, MARY A. FIOCK, ALLEN YARINSKY,[1] AND JAMES T. DUFF

Chemical Corps, Fort Detrick, Frederick, Maryland

Received for publication May 20, 1957

Although type E strains of *Clostridium botulinum* have been implicated in human botulism since 1936, no studies of the isolation or characterization of the toxin have been reported. Crude toxoids have been prepared by various workers for immunization of animals and preparation of antitoxin. Barron and Reed (1954) presented a systematic study of methods for preparation of crude alum precipitated type E toxoid, employing cultures grown in cellophane dialysis bags.

Publications from our laboratory have described procedures for production and purification of *C. botulinum* types A, B, and C toxins suitable for preparations of purified toxoids (Duff *et al.*, 1957*a*, 1957*b*; Cardella *et al.*, 1956). Duff *et al.* (1956) reported that the intraperitoneal toxicity for mice of crude and partially purified type E toxin was increased strikingly when the culture was incubated with trypsin. The present paper describes an extension of these studies to the preparation of partially purified type E toxoid. The purification and detoxification of nonactivated and trypsin activated type E toxins were compared, and the immunization of mice by aluminum phosphate toxoids was investigated.[2]

MATERIALS AND METHODS

The "VH" strain of *C. botulinum* type E, isolated by Dolman *et al.* (1950) from an outbreak of human botulism, was used. The methods for strain maintenance, production of toxin, and activation with trypsin have been described previously (Duff *et al.*, 1956). Toxicity titrations were carried out in 18 to 20 g white mice by intraperitoneal inoculation of 0.5 ml of dilutions of toxin in gelatin phosphate buffer. Procedures for total nitrogen determinations and flocculation titrations also have been described (Duff *et al.*, 1957*a*). Crude univalent type E antitoxin was supplied by the Microbiological Research Establishment of the Ministry of Supply at Porton, England. The aluminum phosphate suspension was prepared, with slight modifications, according to the procedure of Holt (1950). To 224 ml of $Na_3PO_4 \cdot 12H_2O$ (15.75 per cent wt/vol), 224 ml of $AlCl_3 \cdot 6H_2O$ (10.0 per cent wt/vol) was added slowly with constant stirring. To this mixture 1116 ml of distilled water was added and the pH adjusted to 5.5. After sterilization at 120 C for 15 min the precipitate was allowed to settle so that one-half the supernatant could be removed. The resulting suspension contained approximately 14 mg aluminum phosphate per ml.

RESULTS

Toxin production. Preliminary investigations of production of type E toxin led to use of a medium composed of proteose peptone, yeast extract, and dextrin. Several peptone preparations (Difco: Proteose Peptone #2, Proteose Peptone #3, Proteose Peptone #4, Tryptone, Neopeptone, Tryptose, Protone) were tried, but none produced as high a yield of toxin as proteose peptone (Difco). Hydrolyzed casein preparations (Baltimore Biological Laboratory: Trypticase, Lactalysate; Sheffield Farms: N-Z- Amine-A, N-Z-Amine-B, N-Z-Amine-E, Hycase, Edamin, N-Z-Case) were added to the proteose peptone medium. No increase in toxin production was observed. In the proteose peptone medium, dextrin was found to be slightly better than glucose, and considerably better than lactose, maltose, and sucrose.

Preliminary studies of the effect of temperature of incubation on toxin production in chopped meat medium revealed that after 5 days slightly higher toxicities were obtained at 30 C than at 26 C or 35 C. This observation is compatible with previous studies in which other media were used

[1] Present address: Department of Parasitology, School of Public Health, University of North Carolina, Chapel Hill, North Carolina.

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

(Dolman *et al.*, 1950; Hazen, 1937; Prévot and Huet, 1951).

The effect of various concentrations of constituents of the basal medium is summarized in table 1. Activation of the toxin was carried out for 1 hr at 37 C in the presence of 1.0 per cent trypsin (Difco 1:250). The pH values of the 5-day cultures ranged from 5.1 to 6.0; no adjustment was made before activation. When the proteose peptone and dextrin constituents were held constant, 2.0 per cent yeast extract was required for satisfactory toxin production. When the yeast extract and dextrin were held constant, a greater yield of toxin was obtained as the concentration of proteose peptone was increased from 0.1 per cent to 2.0 per cent. When the proteose peptone and yeast extract were held constant, satisfactory toxin production was obtained with 0.5, 1.0, or 2.0 per cent dextrin.

For routine production of the toxin used for purification, cultures were grown at 30 C in a medium composed of 2.0 per cent proteose peptone, 2.0 per cent yeast extract (BBL), and 1.0 per cent dextrin. On the basis of activation studies on type E toxin (Duff *et al.*, 1956), 7 days was selected as the growth time.

TABLE 1

*Effect of various concentrations of proteose peptone, yeast extract, and dextrin on type E production**

Basal Medium	Concentration of Variable	Primary LD_{50}/ml	Activated LD_{50}/ml
	%		
	Yeast extract		
Proteose peptone, 2.0%	0.1	200	20,000
Dextrin, 1.0%	0.5	200	20,000
	1.0	200	20,000
	2.0	4,000	250,000
	4.0	5,600	225,000
	Proteose peptone		
Yeast extract, 2.0%	0.1	200	20,000
Dextrin, 1.0%	0.5	250	58,000
	1.0	2,500	126,000
	2.0	4,000	250,000
	4.0	5,800	225,000
	Dextrin		
Proteose peptone, 2.0%	0.1	200	20,000
Yeast extract, 2.0%	0.5	5,600	225,000
	1.0	4,000	250,000
	2.0	5,000	225,000

* Cultures grown 5 days at 30 C.

Toxin purification. With types A and B botulinum toxins, acid precipitation was a satisfactory method for initial concentration and purification of toxin from whole culture (Snipe and Sommer, 1928; Lamanna and Glassman, 1947). However, type E toxin was not precipitated by 3 N H_2SO_4 in the range from pH 2 to pH 5. Accordingly, precipitation with alcohol was investigated. The temperature of the whole culture was lowered to 0 C, and 95 per cent ethanol was added slowly to a final concentration of 25 per cent at −7 C. Lower concentrations of ethanol precipitated less toxin from the culture. To aid settling of the precipitate, micron bentonite (Volclay Bentonite, BC grit free, American Colloid Company, Chicago), a highly refined diatomaceous earth, was added to a concentration of 1 g per L of culture. The mixture was allowed to stand 48 hr at −7 C. Approximately 80 per cent of the supernatant was siphoned off. The remaining precipitate was collected by centrifugation at −7 C, at 4000 rpm for 30 min. The precipitate was diluted with cold water to $\frac{1}{6}$ the culture volume and stirred for 1 hr at room temperature. This material is referred to as the first alcohol fraction.

The use of calcium chloride solutions for extraction of botulinum toxin from culture precipitates has been described for types A and B by Duff *et al.* (1957*a*, 1957*b*) and for type C by Cardella *et al.* (1956). The effect of calcium chloride concentrations from 0.015 M to 0.3 M at pH 5.2, 6.0, and 6.8 on extraction of type E toxin from nonactivated culture precipitate was investigated. The greatest recovery of toxin occurred with 0.3 M calcium chloride; however, as with the types A and B toxins, the type E toxin was not readily precipitable from the 0.3 M calcium chloride solution. The pH of extraction did not appear to be critical except at low concentrations of calcium chloride. Solubility of the toxin decreased with pH at 0.015 M calcium chloride. The following conditions and methods were selected for extraction of toxin. The alcohol precipitated toxin was diluted to $\frac{1}{4}$ the culture volume with water and 1.0 M $CaCl_2$ solution to a final concentration of 0.075 M $CaCl_2$. The pH was adjusted to 6.0 and the suspension was stirred for 2 hr at room temperature. The mixture was centrifuged at 20 C, at 4000 rpm for 30 min. The supernatant is called the $CaCl_2$ extract fraction.

The effect of ethanol on the precipitation of

toxin from the extract was studied. The greatest yield of toxin was obtained at −7 C with a final concentration of 25 per cent ethanol. The second alcohol precipitate was allowed to stand overnight at −7 C and collected by centrifugation at −7 C, at 4000 rpm for 30 min.

The effect of different concentrations of phosphate buffer in dissolving the second alcohol precipitate was investigated. The greatest recovery of toxin occurred when 0.08 M phosphate buffer was used for the resolution of the precipitate. The second alcohol precipitate was dissolved to ¼ the $CaCl_2$ extract volume in 0.08 M phosphate buffer, pH 6.0, and the solution was clarified by centrifugation at 20 C, at 4000 rpm for 30 min to remove a small amount of an inert insoluble material, probably calcium phosphate. The supernatant is called the second alcohol fraction.

The toxin was reprecipitated with ethanol at a final concentration of 25 per cent. Lower concentrations of ethanol resulted in lower yields of toxin. The precipitate was allowed to stand overnight at −7 C and collected by centrifugation at −7 C, at 4000 rpm for 30 min. The third alcohol precipitate was dissolved to ½ the volume of the second alcohol fraction in 0.2 M succinate buffer, pH 5.5. The product is called the third alcohol fraction.

The fractionation procedure was originally developed for nonactivated toxin and subsequently applied to the activated preparation. To compare the purification of activated and nonactivated toxins, whole cultures were divided into two equal portions. The first portion was activated before fractionation. The culture was adjusted to pH 6.0, brought to 37 C, and 0.1 per cent trypsin was added. Maximum activation was obtained in 75 min. The second portion, without trypsin, was incubated under the same conditions. Both batches of toxin were purified according to the procedure summarized in figure 1. Activation of the whole culture before fractionation led to easier manipulation throughout the purification. Settling of the precipitate at the first step was more compact, centrifugations were more effective, and the precipitates were more easily dissolved in buffer. With activated toxin gravity filtration using Eaton and Dikeman #193 filter paper could be substituted for centrifugation after extraction with calcium chloride.

Average purification and recovery data from three experiments are presented in table 2. Satisfactory purification and recovery were obtained with activated toxin at each step in the scheme. With nonactivated toxin, purification was satisfactory except at the final step, at which point no further purification occurred from the previous fraction. The specific activity of the final activated product (in terms of LD_{50}

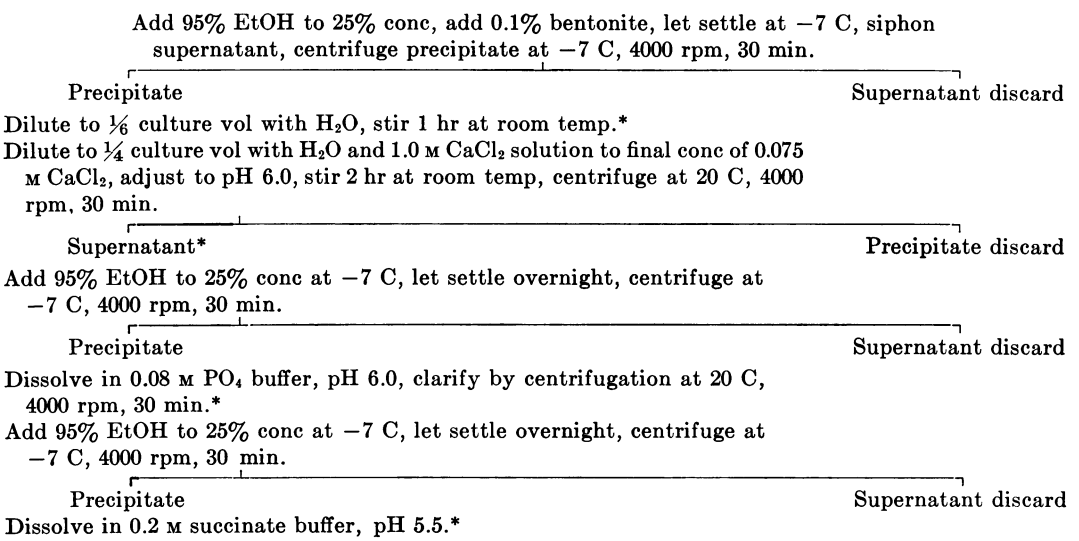

Figure 1. Fractionation scheme for *Clostridium botulinum* type E toxin. Asterisk (*) indicates points of assay (LD_{50} per ml).

TABLE 2

*Average purification and recovery during fractionation of activated and nonactivated toxins**

Fraction	Fold Concentration from Culture	Treatment of Culture†	LD_{50}/ml	LD_{50}/mg N	% Recovery from Culture	Fold Purification from Culture
Whole culture		A	200,000	47,000		
		NA	4,400	1,000		
First alcohol fraction	6	A	930,000	710,000	82	17
		NA	15,000	8,300	59	8
Calcium chloride extract	4	A	520,000	1,400,000	69	33
		NA	12,000	21,000	69	21
Second alcohol fraction	16	A	2,100,000	13,000,000	66	280
		NA	43,000	94,000	64	94
Third alcohol fraction	32	A	3,500,000	19,000,000	55	410
		NA	56,000	85,000	40	84

* The results are the average of 3 experiments.

† A refers to activated culture; NA refers to nonactivated culture.

per mg N) was about 200 times as great as the nonactivated preparation. When the nonactivated third alcohol fraction was activated with trypsin after purification, a specific activity of 4 million LD_{50} per mg N was obtained, excluding the nitrogen of the trypsin. The activity was about $\frac{1}{5}$ that of the products that were activated before purification. Preliminary ultracentrifugation studies indicated that the third alcohol fraction was not homogeneous.

Toxoids. The third alcohol fractions in 0.2 M succinate buffer were used for the preparation of toxoids. Detoxification of both activated and nonactivated toxins was carried out at pH 5.5 in the presence of 0.6 per cent formalin (Reagent Formaldehyde Merck Neutral) at 33 C. Samples were removed at various intervals during detoxification, dialyzed against succinate buffer to remove residual formalin, and tested for residual toxin by subcutaneous injection of 1.0 ml into each of 5 mice. The toxin was considered detoxified if all test animals survived for 10 days. In a comparison of four activated and nonactivated toxins, all nonactivated toxins were detoxified after 14 days and three of the four activated toxins were also nontoxic at this time. The fourth required between 18 and 22 days. Flocculation titrations were carried out before detoxification and after dialysis. No loss in flocculating activity (Lf) occurred during detoxification or dialysis of any of the toxoids.

Equal volumes of aluminum phosphate suspension and fluid toxoid were mixed so that the final concentration of $AlPO_4$ was 7 mg per ml and the final pH was 5.5. The $AlPO_4$ toxoids were titrated for antigenicity by immunization of mice with 1.0 ml doses of toxoid injected subcutaneously. The mice were challenged intraperitoneally 4 weeks later with varying doses of toxin. The animals were observed for 4 days and the protection was calculated by the method of Reed and Muench (1938). An activated toxin was selected as the challenge toxin because nonactivated toxin did not give a true picture of the antigenic value of the toxoid. In table 3 are compared the results of challenge of immunized mice with activated and nonactivated toxins (nonglycerinated). The two toxins were prepared from the same batch of culture. The toxoid was prepared from a nonactivated toxin. Similar results, which are not presented, were obtained with mice immunized with toxoid prepared from activated toxin. In subsequent studies, the challenge toxins were routinely stabilized by addition of 2 vol of glycerine and storage at −20 C. With these

TABLE 3

*Comparison of neutralization endpoint and protection in immune mice challenged with nonactivated and activated toxins**

Challenge Toxin		Dilution of Toxin Neutralized (50% End Point)	Protection in LD_{50}
Type	LD_{50}/ml		
Nonactivated	4×10^4	1–4	1×10^4
Activated	2×10^6	1–4	5×10^5

* Toxoid was prepared from a nonactivated toxin and was a 64-fold concentration of the culture.

TABLE 4

Immune response of mice to $AlPO_4$ integrated toxoids prepared from activated and nonactivated toxins

Exp. No.	Treatment of Culture*	LD_{50}/ml before Detoxification	Fluid Toxoid†			Lf in Immunizing Dose	Protection in LD_{50}
			MgN/ml	Lf/ml	Flocculating time (min)		
1	A	1×10^6	0.04	3	62	1.5	10,000
	NA	2.3×10^4	0.17	7	240	3.5	20
2	A	1.6×10^6	0.07	6	51	3.0	50,000
	NA	5.1×10^4	0.41	15	118	7.5	5,000
3	A	2.2×10^6	0.08	9	77	4.5	6,000
	NA	5.1×10^4	0.82	15		7.5	40,000
4	A	4.2×10^6	0.19	12	22	6.0	100,000
	NA	5.8×10^4	0.75	12	44	6.0	23,000

* A refers to activated culture; NA refers to nonactivated culture.

† All toxoids were a 32-fold concentration of the culture.

preparations, dilutions below 1 to 7 were not used for challenge to avoid deaths caused by glycerine.

The antigenic response of four $AlPO_4$ toxoids prepared from activated and nonactivated toxins purified according to the procedure described above is shown in table 4. Except in experiment 3, the toxoids prepared from activated toxins produced a greater antigenic response than did the corresponding toxoid prepared from nonactivated toxin. The highest antigenic response was obtained in experiment 4 with a toxoid prepared from an activated toxin. The toxoid protected against approx 100,000 LD_{50}. The purity, in terms of LD_{50} per mg N, was essentially constant in different batches of purified toxin, but the antigenicity of the toxoids varied. There was an inverse relationship between antigenicity and the time required for flocculation.

DISCUSSION

The medium described for the production of type E toxin consists of readily available and reasonably reproducible constituents. Toxicities of about 5000 mouse intraperitoneal LD_{50} per ml (nonactivated toxin) were routinely obtained from cultures grown at 30 C for 5 days. This compares favorably with published results. Dolman *et al.* (1950) obtained 4000 mouse MLD per ml in ground meat or peptone beef-infusion broth in 5 days at 37 C. Hazen (1937) reported a toxicity of about 3000 mouse LD_{50} per ml from a culture grown 5 days at 25 to 30 C in Van Ermengem's medium with 2.0 per cent peptone and 1.0 per cent calcium carbonate.

Most of the investigation on development of the medium was completed before the activation of toxin in trypsin was observed and studied. Accordingly, the composition of the medium was presumably adjusted not only for elaboration of protoxin, but also for partial spontaneous activation. Production and activation may be favored by different cultural conditions, however, and if trypsin activation is carried out after growth, additional investigation may suggest cultural conditions for increased production of toxin.

The fractionation of activated type E toxin as described above yielded products of considerable purity. In terms of LD_{50} per mg N, slightly greater than a 400-fold purification was obtained from whole culture. Preliminary analysis in the ultracentrifuge suggested that the products were not pure. The specific activity of the final activated product was essentially constant in different lots, and was about $\frac{1}{10}$ that obtained with purified types A and B toxins (Abrams *et al.*, 1946; Lamanna *et al.*, 1946; Lamanna and Glassman, 1947).

The protection afforded mice by type E toxoids prepared from activated toxins varied somewhat in the different lots, but approached that obtained with types A and B toxoids. The activated toxoids apparently afforded somewhat

greater protection than the preparations studied by Barron and Reed (1954). This difference is probably due, at least in part, to the use of nonactivated challenge toxins by the latter authors.

The results obtained in this study are similar to those reported by Batty and Glenny (1948) in their work with *C. welchii* epsilon toxins and toxoids. These workers found that activation of the epsilon toxin and toxoid by trypsin increased the antigenicity in rabbits and guinea pigs, despite a considerable loss in combining power. Three of four of our activated toxoids had a lower flocculating activity (Lf per ml) than their respective nonactivated controls. In three of the four experiments, however, the antigenicity of the activated toxoids was greater than that of the control. Trial in man will be required to determine their suitability for human immunization.

ACKNOWLEDGMENT

The authors are indebted to Dr. George G. Wright for his encouragement and guidance throughout these investigations and during the preparation of this manuscript.

SUMMARY

A simple and relatively reproducible medium has been developed for toxin production by *Clostridium botulinum* type E. The medium contained 2.0 per cent proteose peptone, 2.0 per cent yeast extract, and 1.0 per cent dextrin. Primary toxicities of approximately 5000 mouse intraperitoneal LD_{50} per ml were produced in 5 days at 30 C. This toxicity was increased to 200,000 LD_{50} per ml by activation with trypsin. The toxin was fractionated by a procedure involving precipitation with ethanol in the cold, extraction of the toxin with calcium chloride solution, and two additional ethanol precipitations. Fractionation of activated toxin produced a 400-fold increase in specific activity. Toxins were detoxified in the presence of formalin and adsorbed on aluminum phosphate. Toxoids prepared from both activated and nonactivated toxins were antigenic for mice.

REFERENCES

ABRAMS, A., KEGELES, G., AND HOTTLE, G. A. 1946 The purification of toxin from *Clostridium botulinum* type A. J. Biol. Chem., **164,** 63–79.

BARRON, A. L. AND REED, G. B. 1954 *Clostridium botulinum* type E toxin and toxoid. Can. J. Microbiol., **1,** 108–117.

BATTY, I. AND GLENNY, A. T. 1948 The antigenic efficiency of *Clostridium welchii* epsilon toxin and toxoid after treatment with trypsin. Brit. J. Exptl. Pathol., **29,** 141–148.

CARDELLA, M. A., DUFF, J. T., GOTTFRIED, C., AND BEGEL, J. S. 1956 Preparation of *Clostridium botulinum* type C from purified toxin. Bacteriol. Proc., **1956,** 90.

DOLMAN, C. E., CHANG, H., KERR, D. E., AND SHEARER, A. R. 1950 Fish-borne and type E botulism: Two cases due to home-pickled herring. Can. J. Public Health, **41,** 215–229.

DUFF, J. T., WRIGHT, G. G., KLERER, J., MOORE, D. E., AND BIBLER, R. H. 1957*a* Studies on immunity to toxins of *Clostridium botulinum*. I. A simplified procedure for isolation of type A toxin. J. Bacteriol., **73,** 42–47.

DUFF, J. T., KLERER, J., BIBLER, R. H., MOORE, D. E., GOTTFRIED, C., AND WRIGHT, G. G. 1957*b* Studies on immunity to toxins of *Clostridium botulinum*. II. Production and purification of type B toxin for toxoid. J. Bacteriol., **73,** 597–601.

DUFF, J. T., WRIGHT, G. G., AND YARINSKY, A. 1956 Activation of *Clostridium botulinum* type E toxin by trypsin. J. Bacteriol., **72,** 455–460.

GORDON, M., FIOCK, M. A., YARINSKY, A., AND DUFF, J. T. 1956 Purified *Clostridium botulinum* type E toxin and toxoid. Bacteriol. Proc., **1956,** 90.

HAZEN, E. L. 1937 A strain of *B. botulinus* not classified as type A, B, or C. J. Infectious Diseases, **60,** 260–264.

HOLT, L. B. 1950 In *Developments in diphtheria prophylaxis*. William Heinemann, Medical Books, Ltd., London, 173.

LAMANNA, C. AND GLASSMAN, H. N. 1947 The isolation of type B botulinum toxin. J. Bacteriol., **54,** 575–584.

LAMANNA, C., MCELROY, O. E., AND EKLUND, H. W. 1946 The purification and crystallization of *Clostridium botulinum* type A toxin. Science, **103,** 613–614.

PRÉVOT, A. R. AND HUET, M. 1951 Existence en France du botulisme humain d'origine pescaire et de *Clostridium botulinum* E. Bull. Acad. Natl. Med. (Paris), **135,** 432–435.

REED, L. J. AND MUENCH, H. 1938 A simple method of estimating fifty per cent endpoints. Am. J. Hyg., **27,** 493–497.

SNIPE, P. T. AND SOMMER, H. 1928 Studies on botulinus toxin. III. Acid precipitation of botulinus toxin. J. Infectious Diseases, **43,** 152–160.