THE ISOLATION OF TYPE B BOTULINUM TOXIN

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High yields of toxin have not been obtained with the nonproteolytic, and most of the proteolytic, strains of *Clostridium botulinum*, type B, in our culture collection. An exceptionally toxic type B culture designated as strain "okra" was received from the National Institute of Health. It is a proteolytic strain capable of producing, under certain conditions, one million minimum lethal doses of toxin for the mouse per ml of medium. From cultures of the "okra" strain a highly toxic and immunologically distinct protein has been isolated. Though this protein has not been crystallized it appears to be essentially a highly purified, single substance. The method of isolation for crystalline type A botulinum toxin of Lamanna, McElroy, and Eklund (1946) has not been applicable. This is probably a reflection of the physical and chemical differences that exist between the two serological types of toxin. Our methods and observations with the type B toxin are recorded in the following sections.

Maintenance of the stock culture. The stock culture is kept in chopped-beef infusion medium consisting of meat fragments submerged in a double-strength beef infusion including 0.5 per cent sodium chloride and 1 per cent Difco proteose peptone. Once a month the culture is transferred using a 1-ml inoculum per test tube. Incubation is at 34 C for 48 hours, and storage is at room temperature.

Medium and growth for toxin production. The organism is grown in 16-liter lots in 5-gallon pyrex glass carboys. The medium is composed of 1 per cent technical grade casein, 1 per cent alkaline-treated cornsteep liquor (47 to 52 per cent solids), and 0.5 per cent technical grade glucose. The cornsteep liquor is a filtrate of raw cornsteep treated by the addition of 1 part of water to 11 parts of cornsteep, alkalinizing with 40 per cent sodium hydroxide to pH 8.5, and heating at 65 C for a half hour. The casein is brought into solution by agitation at pH 9.5 or higher. The cornsteep liquor is added, and the pH adjusted to 7.2. The mixture is autoclaved at 120 C for 1 hour. Upon cooling, the proper amount of glucose solution, which has been autoclaved separately, is added.

A flask with 500 ml of the same medium is preheated to remove dissolved air, cooled, and inoculated with the contents of a test tube of a 1-month-old stock culture. After overnight incubation at 34 C, 10 ml are transferred to 500 ml of medium in flasks corresponding in number to the carboys to be used for toxin production. These flasks in turn are incubated at 34 C overnight and then are used as inoculum, one per carboy. The carboys are incubated for 2 weeks at 34 C.

As a consequence of vigorous fermentation during the first 24 hours of growth the pH drops rapidly to values of 5.3 to 5.5. The casein comes out of solution both as a sediment and as a thick, firm pellicle floating at the surface as a result

of the entrapment of gas bubbles. At the beginning of the third day a decrease in the evolution of gas and a rise in pH are noticeable. The pH continues to rise slowly to 6.3 to 6.5 and may reach a value as high as 6.7 in two weeks' time. The decrease in acidity is accompanied by digestion of the casein. The bulk of the casein becomes soft in consistency, friable, and smaller in quantity. By the time the pH has risen to 6.0, toxin production in quantity becomes evident. A maximum titer (1 million MLD per ml or greater) is generally obtained within 10 days of incubation. No exact time for maximum accumulation of toxin can be stated as it has varied for each batch studied. As long as the pH does not rise above 6.5, there seems to be little loss of toxicity. Some batches have been incubated for 22 days without reduction in titer. The digestion of casein proceeds beyond the period of maximum toxin production and is presumably responsible for the continual decrease in acidity. It is of some advantage in the purification procedure to get rid of as much of the casein as possible by means of the natural proteolysis.

Determination of toxicity. The toxicity of all preparations is determined by intraperitoneal injection of 0.5-ml quantities of solution into 20-gram white mice (± 2 g). The mice are observed for a period of 4 days. The term MLD is used in this report to signify the least amount of material killing all mice injected (usually 4 mice per dilution). LD_{50} is calculated by the method of Reed and Muench (1938) and is based on the use of 6 to 10 mice per dilution.

In making up dilutions of toxic solutions for titration purposes, 0.2 per cent gelatin buffered at pH 6.5 by the use of 1 per cent phosphate salts has been employed as diluent. It is a peculiarity of the toxin that it is relatively insoluble at pH values above 4.5. Therefore, to keep the toxin in solution during dilution, if a solution contains more than 5×10^6 MLD per ml, it is more satisfactory to dilute with acidified water in the lower dilutions and with gelatin diluent in the higher dilutions.

Flocculation tests with commercially available horse antitoxins were studied briefly and discarded as an impractical means of following toxin concentration. The chief reason for this lies in the relative insolubility of the toxin at pH values greater than 4.5. Inasmuch as flocculation tests are generally performed near the neutrality point, the appearance of a flocculus with the toxin may be an index of loss of solubility rather than of a specific serological reaction. It is highly probable that the nature and quantity of other proteins present affect the solubility of this toxin. Depending on the stage of purity and the character of the serum in use, flocculation could, therefore, at times be the result of loss in solubility and at other times the result of the formation of specific toxin-antitoxin aggregates. Combined with our meager knowledge of the toxin-antitoxin reaction for the particular system, this complex state of affairs made the flocculation test a less practical means of following toxin concentration than titration in the mouse.

ISOLATION PROCEDURE

The method developed for the isolation of the toxin from the culture medium is fundamentally a series of acid precipitations. Salting out was tried at various

stages, but it was found to offer no advantages over acid precipitation of the toxin from solution. Slight additions of salt, such as 1 per cent saturation with $(\text{NH}_4)_2\text{SO}_4$ or NaCl to 0.1 M concentration, result in large decreases in solubility. This is different from the experience with type A toxin of Lamanna, Eklund, and McElroy (1946). The following is a description of the procedure. Unless noted otherwise, work was conducted at room temperature.

Step 1: Acid precipitation of toxin from culture medium. The 14-day-old cultures are acidified with a strong acid (2 N HCl) to pH 4.0. As a result the bacterial cells, undigested casein, toxin, and other acid-precipitable material slowly settle out. The carboys are permitted to stand overnight for the purpose of collecting as much of the insoluble matter as possible. The supernatant is siphoned off, and the acid precipitates are pooled and freed of liquid by centrifugation (1,800 rpm in 250-ml cups). The recovery of toxin in the acid mud may be 90 per cent or more effective. With some batches of culture pH 4.0 has not given quantitative recovery. It must be emphasized that the solubility of the toxin is probably greatly influenced by other proteins and products of bacterial growth. Inasmuch as the bacterial culture is a dynamic system, and all the variables affecting growth and proteolysis are not rigidly controlled, differences in composition between batches may exist at the time of acid precipitation of the cultures. Be this as it may, the empirically established fact is that an acid pH value exists at which the solubility of the toxin in the culture medium is at a minimum. This value is pH 4.0, or a neighboring one. Snipe and Sommer (1928) and Sommer (1937) made the original observation that botulinus toxins are precipitated from medium by acid.

Step 2: Extraction of the toxin from the acid mud. The precipitate from step 1 is resuspended in distilled water to one-fortieth the volume of the mother culture. This reduced volume will be referred to as the original volume. A strong acid (2 N HCl) is added to bring the pH to 2.0. As much as possible of the undissolved material is centrifuged off. The remainder is removed by filtration through a filter paper of fine porosity, which was previously wet with water acidified to pH 2. The clear filtrate contains about 80 per cent of the toxin.

At this stage, before filtration but after centrifugation, if the suspension is rotated an anisotropic appearance is noted. Under the microscope the suspended material is observed to be a mixture of amorphous and extremely small needle-shaped material. It gives typical chemical tests for a protein. The specific gravity of this material must be nearly that of the suspending solvent, for it can be removed only with great difficulty by repeated high-speed centrifugation. A Sorval angle centrifuge rotating at 13,000 rpm or the multispeed attachment of the International Equipment Company's refrigerated centrifuge (model PR-1) has been used. The anisotropic material is insoluble at all pH values studied (2.0 to 7.0). It remains finely divided at pH 2.0 to 4.5, aggregates above 4.5, and stays so up to pH 7.0, the highest pH value studied. This phenomenon is curious because the purified toxin itself in fairly concentrated solution remains soluble up to pH 4.5 and comes out of solution in clumps at higher pH values. The anisotropic material is not considered to be toxin since its potency per milligram of nitrogen is considerably lower than that for the

purified toxin, but it is definitely toxic. Repeated washing with acidified water does not entirely remove its toxicity. Thus, if the material itself is not toxic it strongly adsorbs toxin. The color of this material is nearly white in contrast to the purified toxin which has a grayish-yellow cast. The material must be associated with the growth of the organism, inasmuch as it is not obtainable upon similar treatment of unautoclaved and autoclaved medium in which the organism has not been grown.

The choice of distilled water acidified to pH 2 for redissolving the toxin from the acid precipitate of culture rests on a series of experiments with the toxic acid-precipitated mud which showed: (a) The toxin was rapidly detoxified at

TABLE 1

Influence of pH, glycine, and salt on the resolution of toxin from an acid precipitate of medium

pH	SALT ADDED (FINAL CONC.)	DILUTIONS (IN MILLIONS) OF SOLUTION KILLING 20-GRAM MICE			
		10	20	30	40
2.0	0	4/4 (26)*	2/4 (26) 2/4 (41)	4/4 (41)	2/4 (48) 1/4 (68)
	glycine 0.1 M	3/4 (26) 1/4 (41)	2/4 (26) 2/4 (41)	1/4 (41) 2/4 (48)	1/4 (48)
	NaCl 0.1 M	4/4 (26)	2/4 (26) 2/4 (41)	3/4 (41) 1/4 (48)	1/4 (48)
3.0	0	4/4 (26)	2/4 (26) 2/4 (41)	4/4 (41)	0/4
	glycine 0.1 M	3/4 (26) 1/4 (41)	3/4 (49) 1/4 (69)	0/4	0/4
	NaCl 0.1 M	0/4	0/4	0/4	0/4

$$\text{* Ratio} = \frac{\text{no. of mice dying}}{\text{no. of mice injected}}$$

Figure in parentheses is time in hours of observed death of mice.

pH values above 6.5. Thus, a suspension of 20×10^6 MLD per ml lost none of its potency in 3 days at room temperature at pH values of 2, 3, 4, and 5. At pH 6.5 less than 10 per cent was lost; at pH 7.5 there was a loss of about 80 per cent; and at pH 8.5 there was more than a 90 per cent loss. (b) Resolution was poor in the pH range 4.5 to 6.5 and was quantitative only below pH 4. A significant difference existed even at pH 3 and 2 (table 1). (c) The addition of sodium chloride or glycine to the extracting solvent decreased the amount of toxin going back into solution, the effect being more notable at the higher pH values (table 1).

Step 3: Precipitation of toxin from the extract of acid mud. The clear filtrate

of step 2 is brought to pH 4.0 by slow addition of 2 N NaOH and cooled to refrigerator temperature. A flocculent precipitate, which is collected by centrifugation, forms.

Recovery of toxin in this step has varied considerably. As the toxin is purified, it seems to become progressively more soluble at pH values on the acid side of the isoelectric range. This would definitely point to impurities as influencing the solubility of the toxin. If, because of variations between cultures, the nature and quantities of impurities vary, then the differences in recovery of toxin from separate batches at this stage are explicable. More recently it has been found that precipitation at pH 5.0 will result in higher and more consistent yields.

Step 4: Washing of the precipitate of step 3. The precipitate of step 3 is washed by resuspension for 5 to 10 minutes in one-fourth original volume of a solution of 1.5 M NaCl at pH 2.0. The washed toxin is recovered by centrifugation. The solubility of the toxin in this solvent is low, being less than 100,000 MLD per ml.

Step 5: Reprecipitation of the toxin. The centrifuged material of step 4 is redissolved in one-fourth original volume of acidified distilled water at pH 2.0. Difficulty with resolution of the toxin will be experienced if much sodium chloride is carried over from the preceding step. The toxin is then reprecipitated by bringing the solution to pH 5.0 to 5.5 and collected by centrifugation.

The precipitate of step 5 represents purified toxin. It should be noted that except for the original toxic culture medium the solutions of the toxin are worked with at pH values on the acid side of the isoelectric zone. The method permits recovery of 50 per cent or more of the toxin.

PROPERTIES OF PURIFIED TOXIN

The purified toxin is an odorless, slightly grayish-yellow-colored solid. Solutions appear yellow brown in color. During electrophoresis the color travels with the toxin boundary and shows no tendency to separate from it. No characteristic absorption spectrum is shown in the range from 800 to 340 $m\mu$. It reacts positively in qualitative tests for protein such as the biuret, ninhydrin, Millon's, xanthoproteic, and Hopkins-Cole. The Molisch test for carbohydrate is negative. The phloroglucinol test for nucleic acid is negative, and the orcinol test (Bial's reagent) slightly positive or negative. The absorption spectrum with ultraviolet light shows no evidence of the presence of nucleic acid. Absorption is maximum at 277 $m\mu$. Extinction plotted against wave length gives a curve typical for a simple protein (figure 1). Chemical analyses for iron and metals precipitable by H_2S were negative. Thus no evidence for the presence of a prosthetic group has been obtained. The microkjeldahl nitrogen of toxin dried at 120 C in a vacuum oven is 15.5 per cent. Amide nitrogen has not been detected. Nitrogen of free amino groups was found to be 5.9 per cent of total nitrogen.

An estimate of molecular size was made using the Northrop and Anson (1929) sintered glass membrane diffusion apparatus and their method of calculation. The cell constant was determined by the use of NaCl. The diffusion constant

was calculated from analyses for nitrogen diffusing from the cell at 20 C in 12-hour intervals. The toxin was dissolved in acidified water at pH 2. The diffusion constant was 0.0624 cm² per day or 7.22×10^{-7} cm² sec⁻¹. Assuming a spherical shape this indicates a radius of 2.9×10^{-7} cm and a molecular weight of about 60,000. This is in contrast to 900,000, the figure obtained by Putnam *et al.* (1946), and 1,200,000, obtained by Kegeles (1946) by different methods, for the molecular weight of crystalline type A toxin.

FIG. 1. ULTRAVIOLET LIGHT ABSORPTION SPECTRUM OF PURIFIED TYPE B TOXIN

It is difficult to place the toxin in the American system of classification of the proteins. On heating, it is denatured and will coagulate above pH 4.5, but it does not have easily classified solubility properties. Electrophoretic mobility shows that pH 4.5 and lower values represent the acid side of the isoelectric range. The toxin is quite soluble in water on the acid side of its isoelectric range and only slightly soluble on the alkaline side. At pH 5.0 and 5.5 at 7 C, 0.043 mg of toxin nitrogen per ml was found in solution. At 20 C at pH 6.0, only 0.056 mg of nitrogen per ml was present. To date no electrophoretic studies between pH 4.5 and 7.0 have been possible because of inability to get sufficient material into solution.

Unlike the globulinlike type A toxin, purified B toxin in water suspensions does not significantly increase in solubility upon the addition of salts. On the other

hand, addition of NaCl to the mother culture can increase the solubility of impure toxin. Table 2 shows that increasing additions of NaCl to the medium after toxin production has occurred results in an initial rise in solubility, followed by a decrease. At the same time, the pH of minimum solubility shifts to more acidic values. Thus without addition of salt the minimum solubility is at pH 4.0, but with 2 M NaCl pH 3.0 is the point of minimum solubility. The changes in solubility that are being measured in the case of table 2 represent toxicities of a few hundred thousand LD₅₀ per ml, that is, extremely small concentration

TABLE 2

Influence of NaCl on solubility of toxin in mother culture at three pH values

SALT ADDED (FINAL CONC.)	pH	TOXICITY OF DILUTIONS (IN THOUSANDS) OF SUPERNATANTS AFTER CENTRIFUGING OUT INSOLUBLE MATTER IN CULTURE				
		50	100	200	400	800
0	4.5	1/2*	3/4	2/4	0/4	0/4
	4.0	0/2	0/4	0/4	0/4	0/4
	3.0	1/2	4/4	4/4	2/4	0/4
0.1 M	4.5	2/2	4/4	3/4	4/4	0/4
	4.0	2/2	3/4	1/4	0/4	0/4
	3.0	2/2	3/4	4/4	4/4	0/4
0.5 M	4.5	1/2	4/4	4/4	3/4	0/4
	4.0	1/2	4/4	4/4	1/4	0/4
	3.0	2/2	4/4	4/4	2/4	4/4
1.0 M	4.5	2/2	4/4	3/4	4/4	2/4
	4.0	2 2	4/4	2/4	0/4	0/4
	3.0	2/2	4/4	4/4	4/4	0/4
2.0 M	4.5	2/2	4/4	4/4	3/4	0/4
	4.0	2/2	2/4	0/4	0/4	0/4
	3.0	0/2	0/4	0/4	0/4	0/4

* Ratio = $\frac{\text{no. of mice dying}}{\text{no. of mice injected}}$.

differences. A milliliter of solution of 100,000 LD₅₀ contains 5×10^{-4} mg of toxin nitrogen or a concentration of toxin of approximately 0.0003 per cent.

Electrophoretic studies in glycine buffer at pH 1.8 reveal a single boundary on the ascending side. In addition to the major boundary a small boundary, which rapidly moves off the field of view, occurs on the descending side and does not reappear on reversal of the current (figure 2). Electrophoresis at pH 3.8 in glycine buffer showed a single moving boundary; the fast-moving boundary noted on the descending side at pH 1.8 was not seen. Whether the latter is an anomaly or has some special significance remains to be determined.

Identity and potency of toxin. Toxin in culture medium and purified toxin were typed by using commercially available horse antitoxins in mouse protection

FIG. 2. ELECTROPHORETIC PATTERN OF PURIFIED TYPE B BOTULINUM TOXIN (CONC.

0.64%) DISSOLVED IN 0.1 M GLYCINE-HCl BUFFER AT pH 1.8 AT 3.5 C

The moving boundary marked T is the toxin. At 1,920 seconds the measurement of mobility was 10.1×10^{-5} cm²/sec/volt on the descending side and 11.6×10^{-5} cm²/sec/volt on the ascending side. Note a second moving boundary on the descending side labeled 1 in the 1,920 seconds' photo. This boundary rapidly moved off the field of view so that it is no longer visible in the 3,720 seconds' photo. Its mobility was 25.3×10^{-5} cm²/sec/volt. For this run the field strength was 4.31 volts per cm and the current 25 milliamperes. The stationary delta and epsilon boundaries are marked accordingly. The illustration is a composite of drawings made from tracings of the original photographs.

tests. Monovalent types A and C antisera did not protect, but type B monovalent antisera did. Rabbit antisera available as a result of immunization with

crystalline type A toxin, or toxoid prepared from crystalline toxin, showed no protective activity. Immunologically, there can be no doubt as to the identity of the toxin produced by the strain "okra."

On titration of one batch of the toxin in the mouse and the guinea pig, the following values were obtained:

- 6.2×10^{-9} mg nitrogen = LD₅₀ per 20-g mouse;
- 31.2×10^{-9} mg nitrogen = LD₅₀ per 300-g guinea pig;
- 310×10^{-9} mg nitrogen = LD₅₀ per kilo mouse;
- 103×10^{-9} mg nitrogen = LD₅₀ per kilo guinea pig.

These figures show that on a body weight basis the guinea pig is only three times as sensitive to the toxin as the mouse. This conclusion is in sharp contrast to the recent report of Stevenson, Helson, and Reed (1947) that the guinea pig is 6,000 to 8,000 times as sensitive as the mouse to the type B toxin. These workers have used strains other than "okra" and relatively impure toxin. The identity of similar serological types of toxin from different bacterial strains has been assumed, but final proof will rest with studies on pure materials isolated from different strains.

Separately prepared batches of purified toxin have given values of toxicity from 5 to 9×10^{-9} mg of nitrogen per mouse LD₅₀. This is of the same order of magnitude as 4.5×10^{-9} mg nitrogen obtained with crystalline type A toxin. On a weight basis the two toxins appear equipotent; but if future research confirms the finding that the B toxin is 10 or more times smaller in molecular size, then on a molar basis the B toxin is considerably less toxic.

The stability of the purified B toxin has presented itself as a serious problem. It appears more labile than the impure material. As a result we have not succeeded in storing solutions in the refrigerator for periods greater than two weeks without serious losses in potency. Interestingly enough, one batch of reduced potency studied appeared to have unaltered electrophoretic properties at pH 2.

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SUMMARY

A method for the purification of the type B toxin from the proteolytic "okra" strain of *Clostridium botulinum* is described. Essentially the purification depends upon working with the toxin on the acid side of its isoelectric zone and upon a series of acid precipitations. The purified toxin appears to be a slightly colored, simple protein, soluble in water on the acid side of the isoelectric range and relatively insoluble on the alkaline side and within the isoelectric range. Slight additions of salt do not favor increased solubility of the purified toxin.

Serologically, chemically, and physically the purified B toxin differs from type A crystalline toxin. Its toxicity per milligram of nitrogen is only slightly less than that of the type A, but on a molar basis, it would appear to be 10 times less potent. By the intraperitoneal route the guinea pig is about three times more susceptible to the toxin than is the white mouse.

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