Technical Papers

The Purification and Crystallization of *Clostridium botulinum* Type A Toxin [1]

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A preliminary report is presented of a method which has permitted the isolation at will of highly toxic needle-shaped crystalline protein material from a Type A culture of *Clostridium botulinum*. Electrophoretic analysis shows a single component.[3] The capacity of this material to react in a flocculation test with commercial antitoxin and to have its pharmacological activity neutralized by such antitoxin suggests the isolation of the Type A toxin concerned in food poisoning. The method builds upon the basic observations of Snipe and Sommer (*3*) and Sommer (*4*) that the toxin is acid precipitable from culture medium and can be eluted from the acid precipitate by buffer solutions at appropriate pH values.

Five-gallon carboy lots of a 0.3-per cent casein, 0.5-per cent glucose, and 1.0-per cent alkali-treated corn steep liquor medium (*1*) at pH 7.2 are inoculated and held at 34° C. until a maximum titer of toxin appears. This usually takes 72 to 80 hours. The method of purification and crystallization is as follows:

Step 1: The cultures are precipitated at pH 3.5 by addition of 2 N HCl. All acid-precipitable material, which includes toxin, nucleic acid, undigested casein, material from corn steep, and the organisms, is allowed to settle out overnight. The supernatant is siphoned off, and the precipitate collected in centrifuge tubes (250 ml.) at 1,800 r.p.m. The mud is washed once by resuspension in distilled water and recentrifugation.

Step 2: The washed acid mud is resuspended with the help of a Waring blender in distilled water to a volume of 300–400 ml. for each carboy of culture originally precipitated. This volume is referred to as original volume. Sodium chloride is added to one molar and sodium acetate to 0.075 molar concentration, and the pH adjusted to 6.5.

Step 3: The suspension is centrifuged at 1,800 r.p.m. to remove the coarse particles. To the supernatant one-sixth to one-tenth of its volume of c.p., chloroform is added and the mixture shaken vigorously for five minutes under CO_2. The pH drops to 5.8–6.1. The gel and excess chloroform are centrifuged off. If the original culture has 800,000 or more intraperitoneal 20-gram mouse MLD/ml., the aqueous supernatant will contain 15–30,000,000 MLD/ml. After the chloroform shaking, the pH of acid precipitation of the toxin in the presence of 1-per cent salt solutions changes from 3.5 to 5.0. The new pH value of acid precipitation is used in Step 5. In a future paper we hope to present data that permit some understanding of the function of shaking with chloroform in the purification procedure and the reason for the new pH value of acid precipitation.

Step 4: To the supernatant of Step 3, $(NH_4)_2SO_4$ is added to 20 per cent saturation (14 grams $(NH_4)_2SO_4$/100 ml. of solution). After refrigeration overnight the toxic precipitate which forms is centrifuged off. This step and the following one remove the bulk of nucleic acid present.

Step 5: The toxic precipitate of Step 4 is resuspended to three-fourths original volume in 0.075 M sodium acetate at pH 6.5–6.7. The pH is dropped to 5 with HCl, and the resulting precipitate allowed to settle out in the refrigerator. The pH 5 precipitate is centrifuged out and resuspended at pH 6.5 in molar sodium chloride and 0.075 M sodium acetate to one-third the original volume. In this mixture of salts the toxin is no longer precipitable at pH 5.

Step 6: The pH is then dropped to 5. Solid $(NH_4)_2SO_4$ is added in increments of 5 per cent saturation (3.5 grams/100 ml. of solution) up to 20 per cent saturation. The precipitates appearing on each addition of 5 per cent saturation are collected separately by centrifugation. The 20-per cent saturation precipitate is usually pure white and upon resuspension in 0.075 M sodium acetate gives a perfectly clear solution. The color of the 15-per cent saturation precipitate will vary from batch to batch. The 10 per cent precipitate is usually highly colored. The 5 per cent precipitate is usually not very abundant, is deep brown in color, and is discarded.

Step 7: The 20 per cent and 15 per cent precipitates are redissolved in 1/300 of the original volume (at least 240,000,000 MLD/ml.) of 0.075 M sodium acetate at pH 6.5. $(NH_4)_2SO_4$ is added, 0.1 gram/10 ml. at a time, to a total of 0.9 gram/10 ml. of solution. Any brown material remaining usually precipitates and can be centrifuged off. The resulting clear liquid has additional $(NH_4)_2SO_4$ added to give tur-

[1] Studies conducted at Camp Detrick, Frederick, Maryland, from June 1944 to August 1945.

[2] With the technical assistance of PhM1c J. F. Valentine, PhM3c N. Harmand, and HA1c S. Biscardi.

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bidity. After standing overnight in the refrigerator, the material coming out of solution is crystalline.

Step 8: The color of the precipitates of Step 6 has varied from batch to batch. In those cases where the precipitates remained fairly highly colored they were reworked in the following fashion. The precipitates are resuspended in molar NaCl and 0.075 M sodium acetate at pH 6.5. Insoluble material is centrifuged off and discarded. The pH is dropped to 4.0, and precipitated material is centrifuged off. This precipitate can be reworked as many times as is necessary for complete recovery of toxin. To the supernatant at pH 4.0 after centrifugation enough solid $(NH_4)_2SO_4$ is added to give 15 per cent saturation. After standing in the refrigerator the white toxic precipitate is centrifuged off and can be crystallized (Step 7). In the salt solution down to pH 3.5 the toxin apparently remains more soluble than the brown contaminating material. This fact makes the separation possible. The pH of acid precipitation of the toxin is dependent on the nature and quantities of salts present.

Crystallization from the acetate buffer has been obtained in the pH range of 5.5–7.1. It appears as though supersaturation on the alkaline side of the isoelectric point by a variety of means will result in crystallization. Adding sufficient $(NH_4)_2SO_4$ to reduce the solubility of solutions of amorphous material given the most rapid and consistent results. But $MgSO_4 \cdot 7H_2O$ and Na_2SO_4 may be used in place of $(NH_4)_2SO_4$. In some cases slow evaporation at room temperature of concentrated solutions of the toxin in acetate buffer at pH 6.5 results in crystallization.

Crystal size has been found to be dependent on the speed with which the toxin is salted out from the supersaturated solution. Apparently the fewer nuclei present, the larger the crystals obtained. Two rapid methods of obtaining large crystals are used: (a) In the case of a solution in which crystallization is going on, centrifuge off the crystals coming out first. Place the clear supernatant in the refrigerator. The crystals then coming out of solution are of a larger size than the first material. (b) Place a drop of slightly turbid crystalline suspension on a clean glass slide and cover with a cover slip. At room temperature let some of the solution evaporate from the sides of the cover slip. Then seal the preparation with paraffin and place in the refrigerator. After the large crystals have formed (few hours to overnight) by growth from smaller ones, the preparation can be kept at room temperature without disruption of the crystals. Crystals 125 μ in length and 7 μ in width have been obtained. Others average about 85 μ by 5 μ. A tendency for the crystals to line up in parallel rows with no tendency to collect in rosettes has been observed.

Recrystallization has been accomplished by supersaturation of distilled water solutions. A minimum amount of distilled water at room temperature at pH 7–8 is used to dissolve the material to be recrystallized. The pH tends to drop as the toxin goes into solution. When placed in a refrigerator, the material coming out of solution appears as the same-shaped needle-like crystals noted for the buffer solutions.

The degree of purity attained by the method is suggested by the data in Table 1.

TABLE 1

SOME BASIC DATA FOR PURIFIED *Clostridium botulinum* TYPE A TOXIN

Observation		Batch of Toxin	
		C42E	Crystalline
Nature of materials		Amorphous	C38
Electrophoresis data* pH 4.44, acetate buffer	No. of components	One	One
	Mobility, $V_D(10)^5$ Cm.²/volt/sec.	+3.2	+3.4
MLD/mg. nitrogen†		120.7×10^6	.....
LD_{50}/mg. nitrogen‡		198.5×10^6	239.9×10^6
Mg. nitrogen/MLD		8.3×10^{-9}	.....
Mg. nitrogen/LD_{50}§		5×10^{-9}	4.2×10^{-9}
Adamkiewicz test (tryptophane)		Positive	Positive
Molisch test		Negative	Negative

* Data obtained by Lt. G. Kegeles.

† MLD is defined as the smallest amount of material injected intraperitoneally which will kill six out of six 18- to 20-gram white mice within four days time.

‡ LD_{50}—calculated by R. A. Tiede by the method of Bliss (*Ann. appl. Biol.*, 1935, **22**, 134, 307).

§ Pickett, Hoeprich, and Germain (*J. Bact.*, 1945, **49**, 515–516) have reported the isolation of tetanus toxin giving $2.3–5.7 \times 10^{-8}$ mg. N_2/mouse MLD. This is the most potent toxic material known to us that has been previously reported.

The similar electrophoretic mobility for both amorphous and crystalline material is a strong argument for the isolation of a single toxic substance. The rapidity of crystallization and the agreement of data on the LD_{50}/mg. of N_2 for both types of materials are equally suggestive of a high degree of purity. The ordinary chemical tests for protein such as the Biuret and Millon's are positive. The nitrogen content is 14.3 per cent. The molecular size as indicated by the membrane diffusion method of Northrop and Anson (*2*) is rather large. Assuming a spherical shape, the radius of the molecule lies between 7.7 and 9.1×10^{-7} cm. These data suggest a molecular weight between 1,000,000 and 2,000,000. The immoderate spread in these data is attributable to a large variation in the animal titrations used for measuring the toxic diffusate. Both impure and crystalline toxin were used in making the determinations.

References

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