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# Isolation and Characterization of a Toxic Moiety of Low Molecular Weight from *Clostridium botulinum* Type A

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ABSTRACT

GERWING, JULIA (The University of British Columbia, Vancouver, B.C., Canada), CLAUDE E. DOLMAN, AND HARDIAL S. BAINS. Isolation and characterization of a toxic moiety of low molecular weight from *Clostridium botulinum* type A. J. Bacteriol. **89:** 1383–1386. 1965.—A toxic moiety of low molecular weight has been isolated from a type A strain of *Clostridium botulinum*, by a method involving ammonium sulfate precipitation and elution through diethylaminoethyl cellulose at *p*H 5.6. By means of electrophoresis and ultracentrifugation, the toxic substance was shown to be homogeneous; a molecular weight of 12,200 was calculated.

Early attempts to purify *Clostridium botulinum* type A toxin involved the adsorption of the toxic material onto colloidal aluminum hydroxide and subsequent elution with ammonium phosphate (Sommer, Sommer, and Meyer, 1926; Sommer and Snipe, 1928). Snipe and Sommer (1923) demonstrated that the toxin precipitated under acidic conditions, thus establishing a basis from which subsequent attempts at purification have proceeded. Stockinger and Ackerman (1941) used acid precipitation and enzyme digestion to remove carbohydrate- and phosphorus-containing material from the crude toxin. In none of the foregoing instances was any claim to purity made.

In 1946, the crystallization of type A botulinus toxin was reported from two separate laboratories (Abrams, Kegeles, and Hottle, 1946; Lamanna, Eklund, and McElroy, 1946*a;* Lamanna, McElroy, and Eklund, 1946*b*). The methods used were slightly different, but each entailed preliminary acid precipitation followed by repeated solution and reprecipitation. The crystalline material obtained in both cases appeared to be electrophoretically and ultracentrifugally homogeneous, and a molecular weight of approximately 1 million was calculated.

Duff et al. (1957) purified type A toxin by repeated acid precipitation, extraction by calcium chloride, and reprecipitation with ethyl alcohol. They obtained a product with a sedimentation coefficient of $S_{20,w}$ = 14.5; the biological activity of this product was slightly higher than that shown by the preparations of previous workers.

Having found that type E toxin had a molecular weight of about 18,000 (Gerwing et al., 1964), we were led to investigate by similar methods the characteristics of type A toxin. Initial precipitation of the toxin with ammonium sulfate was followed by ion-exchange chromatography on diethylaminoethyl (DEAE) cellulose, and a homogeneous toxic entity was thus isolated with a molecular weight of 12,200. The discrepancies between this finding and previous claims are discussed.

## MATERIALS AND METHODS

*Toxin production.* One strain of *C. botulinum* type A ["corn T" isolated by Dolman (1954)] was used throughout. This strain reliably and consistently produced toxin of potencies between 100,000 and 300,000 mouse minimal lethal doses (MLD) per ml. The organisms were grown for toxin production by the method described previously for type E toxin (Gerwing, Dolman, and Arnott, 1961). Modifications in the method included the addition of 0.5%, instead of 2%, dextrose to the medium and the continuance of incubation for 7 days.

*Assay technique.* White mice weighing approximately 20 g were injected intraperitoneally with 0.5-, 0.3-, 0.2-, or 0.1-ml amounts of the given toxic preparation diluted decimally in physiological saline. Groups of five mice were used per dose, and the MLD was calculated as the highest dilution by which all mice were killed within 72 hr.

*Nitrogen determination.* Total nitrogen determination was carried out by direct nesslerization of Kjeldahl digests, with the use of the modification described by Minar and Zilversmit (1963).

*Purification procedures.* Saturated ammonium sulfate solution was added to toxic filtrates to a final concentration of 50% (v/v). The precipitate was allowed to form overnight at 4 C and was then collected by centrifugation at 5,000 × *g* at 4 C. The sedimented material was resuspended in 0.01 M citric acid in approximately 5% of the original volume. DEAE cellulose, which had previously been ionized for 24 hr in 2 M NaCl at 4 C and acidified with 1 N HCl, was packed in dilute suspension, under slight positive pressure, into columns 1 cm in diameter to a height of 100 cm. Equilibration was carried out with 0.067 M phosphate-citrate buffer (*p*H 5.6). Toxic concentrates, containing between 4 and 6 mg of protein per ml, were added in 1-ml quantities to such columns, and elution was carried out with the same buffer on a model V-10 fraction collector (Gilson Medical Electronics, Middleton, Wis.). Flow rate was regulated to 30 ml/hr, and 3-ml fractions were collected.

A highly toxic moiety was isolated by this technique (Fig. 1). Toxic samples thus isolated were pooled and dialyzed at room temperature against a concentrated solution of polyethylene glycol (molecular weight, 8,000; approximately 25%, w/v), until the volume had decreased to 3 ml. The concentrated material was then dialyzed overnight in 0.01 M citric acid at 4 C.

All dialysis paper was pretreated with ethylenediaminetetraacetic acid (EDTA) as previously described (Gerwing et al., 1964).

*Physical measurements.* The sedimentation coefficient was measured with a Beckman Spinco model E analytical ultracentrifuge, with the use of a synthetic boundary cell. Runs were made at 50,740 × *g*, and photographs were taken at 8-min intervals. The electrophoretic mobility and the diffusion coefficient were determined with a Beckman Spinco model H electrophoresis-diffusion apparatus with the use of the 11-ml standard cell. In this instance, the toxin was in a concentration of 1.5 mg/ml and had been dialyzed exhaustively against 0.05 M guanidine acetate buffer (*p*H 4.5). The Raleigh integral fringe method was used in the evaluation of the diffusion coefficient (Longsworth, 1952). Eight pictures taken over a 96-hr period were used for the calculation of this value.

## RESULTS

Under the chromatographic conditions described, type A toxin was eluted frontally to give a well-defined peak; the biological activity of the toxin indicated a high degree of purity (Fig. 1.) The purified material had a maximal ultraviolet absorption at 278 mμ, the extinction was 4.0 per mg of nitrogen, and its potency was 2.7 × $10^8$ mouse MLD per mg of nitrogen. Total recovery of toxin was 40 to 50%.

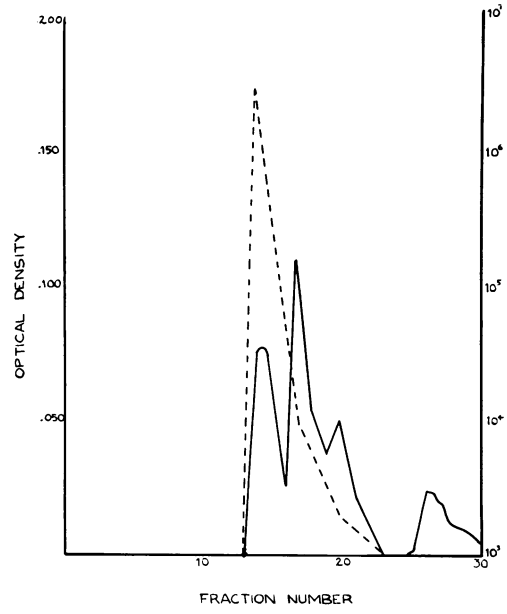


FIG. 1. *Elution profile of type A toxin through DEAE cellulose in 0.067 M phosphate-citrate buffer (pH 5.6). Solid line shows absorbance at 280 mμ; broken line shows MLD/ml.*

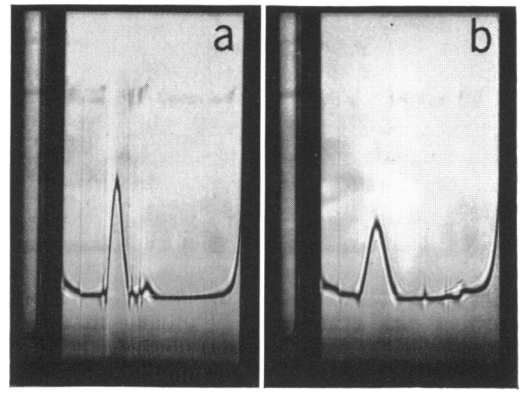


FIG. 2. *Representative photographs obtained in the sedimentation velocity run on purified type A toxin in 0.01 M citric acid. Speed, 50,740 × g; bar angle 60°; protein concentration 1.5%; times after obtaining full speed, (a) 0 min and (b) 40 min.*

Pooled samples from numerous runs were concentrated by dialysis against polyethylene glycol and equilibrated by further dialysis with 0.01 M citric acid (*p*H 2.2). When this material was analyzed in an ultracentrifuge with the use of the synthetic boundary cell, a major slow-moving component was observed with a sedimentation coefficient of $S_{20,w}$ = 0.93. However,

several other boundaries of lesser concentration were apparent (Fig. 2). As our toxic preparations also showed a troublesome tendency to be partially insoluble, which suggested the possible presence of aggregates of the toxin, the material was dialyzed further, with 0.5 M guanidine acetate buffer (*p*H 4.5) as a deaggregating agent. Under these conditions, the "contaminating" boundaries disappeared (Fig. 3). The major component maintained a sedimentation coefficient of $S_{20,w}$ = 0.93 and showed no excessive diffusion after 96 min, thus indicating that the material was homogeneous in molecular size.

The electrophoretic pattern resulted in a single sharp peak migrating at $+2.05 \times 10^{-5}$ cm² per v per sec (Fig. 4). On the same solution, after sharpening the boundary, the diffusion coefficient was determined as $D_{20,w} = 6.128 \times 10^{-7}$ cm² per sec. Employing the formula

$$M = \frac{RTS_{20,W}}{D_{20}(1-V\rho)}$$

we obtained a molecular weight of 12,200.

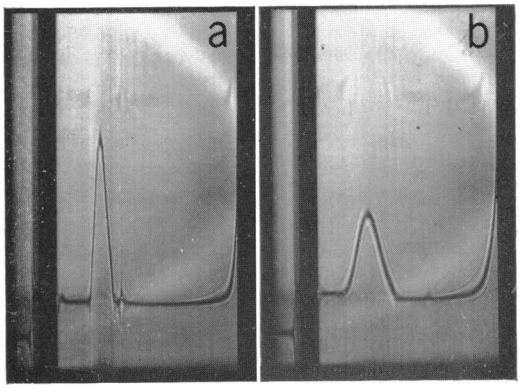


FIG. 3. *Representative photographs obtained in the sedimentation velocity run on purified type A toxin in 0.5 M guanidine acetate buffer (pH 4.5). Speed, 50,740 × g; bar angle, 60°; protein concentration, 1.5%; times after obtaining full speed, (a) 0 min and (b) 40 min.*

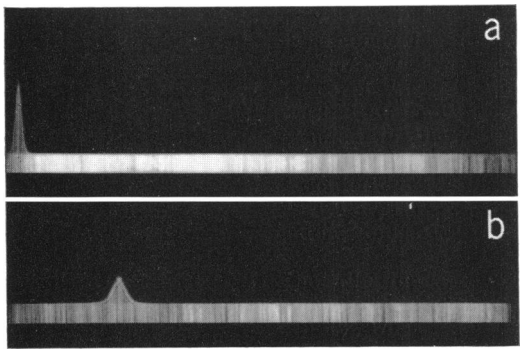


FIG. 4. *Representative photographs of the ascending electrophoretic boundary of type A toxin in 0.05 M guanidine acetate buffer (pH 4.5). Protein concentration, 0.15%; times after commencement of run, (a) 0 min and (b) 127 min.*

## DISCUSSION

Previous findings on the molecular weight and electrophoretic mobility of crystalline type A toxin were based on materials prepared by acid precipitation, repeated salting out, and treatment with organic solvents (Abrams et al., 1946; Lamanna et al., 1946*a*, *b*). The material thus isolated had a sedimentation coefficient of $S_{20,w}$ = 17.3 (Kegeles, 1946; Putnam, Lamanna, and Sharp, 1946), and the molecular weight was estimated to be about $10^6$. However, Putnam et al. (1946) noted that in the ultracentrifuge the boundary spread of the crystalline toxin was too great to be attributed to diffusion alone. The same workers later confirmed the high sedimentation coefficient of the material (Putnam et al., 1948), but again the polydispersed character of their preparation was shown by some degree of boundary spread.

The validity of these high molecular weight estimates came into question when Buehler, Schantz, and Lamanna (1947) conducted a chemical analysis of the crystalline toxin and, on the basis of the amino acid content, showed that the possible minimal molecular weight was 45,000. Subsequently, Wagman and Bateman (1951) and Wagman (1954), in reports with an importance not fully appreciated, showed that the sedimentation rates of the crystalline toxin varied with its concentration, and that dissociation of the material occurred when the *p*H of the buffer was raised to 5.6. Dialysis of the toxin in buffers on the alkaline side of its isoelectric point (between *p*H 6.5 and 8.0) yielded a product with a sedimentation coefficient of $S_{20,w}$ = 6.5, which contained two to three times the specific activity of the crystalline material. In addition, Wagman (1954) showed that this light component would polymerize at *p*H 3.8 to give a product whose dimensions were similar to those of the crystalline material.

These earlier indications of a tendency of the type A toxin molecule to polymerize were strongly reinforced by our own findings. For instance, insoluble aggregates continually formed during the process of repeated precipitation, which considerably reduced the yields of active material. This complication occurred especially when we were working under acidic conditions, which we were

obliged to do because of the instability of the toxin at *p*H values above 7.0. The emergence of a series of small boundaries when toxic preparations were submitted to ultracentrifugation (Fig. 2) suggested that the apparent impurities might be accounted for by molecular aggregates. Confirmation of this concept was afforded by the low sedimentation coefficient, $S_{20,w}$ = 0.93, and the virtual absence of contaminating boundaries, shown by the product obtained after dialysis against guanidine acetate, an agent which owes its deaggregating effect to a capacity to break hydrogen bonds (Fig. 3).

We contend, therefore, that determination of the true molecular weight of type A botulinus toxin entails taking steps to avoid or inhibit the marked polymerizing potentialities of its molecule. These steps include the avoidance, throughout the process of purification, of techniques which are conducive to aggregation, e.g., frequent and harsh methods of precipitation; and also the employment of an active deaggregating agent, such as guanidine acetate. The disparity between a molecular weight of 1 million, as formerly estimated, and the figure of 12,200 now indicated is wide, but not inconsistent with the molecular aggregation hypothesis.

Finally, we emphasize that we do not claim to have achieved significantly higher degrees of biological activity than those reported by previous workers. Thus, Lamanna et al. (1946*a*, *b*) obtained preparations with a potency of $2.2 \times 10^8$ mouse $LD_{50}$ per mg of nitrogen, whereas the corresponding figures cited by Abrams et al. (1946), Halliwell (1954), and Duff et al. (1957) were $2.2 \times 10^8$, $2.2 \times 10^8$, and $2.7 \times 10^8$, respectively. The maximal potency of our purified product was $2.7 \times 10^8$ mouse MLD per mg of nitrogen. This remarkable concordance does not run counter to our main contention that the lowest common divisor of the type A botulinus toxin molecule has a molecular weight of approximately 12,200.

## ACKNOWLEDGMENTS

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