

# Ultracentrifugal Analysis of the Crystalline Toxin and Isolated Fractions of *Clostridium botulinum* Type A\*

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## SUMMARY

Following the chromatographic separation of the crystalline toxin of *Clostridium botulinum* type A into one fraction ( $\alpha$ ) containing 5 times the specific activity of the unfractionated preparation and a second fraction ( $\beta$ ), only feebly toxic, the native toxin and the two components were analyzed in the ultracentrifuge for homogeneity and Fraction  $\alpha$  was analyzed for molecular weight. Under all experimental conditions, crystalline toxin resolved into two peaks with  $s_{20,w}$  values of about 7 and 13, respectively. Fraction  $\beta$  aggregated to a heavier component, with  $s_{20,w}$  of 16 or 23 S, depending upon the pH and ionic strength of the solvent and the concentration of the protein. Fraction  $\alpha$ , however, remained homogeneous. The molecular weight of Fraction  $\alpha$ , as determined by Yphantis' method, was  $128,000 \pm 10\%$ . This value is in reasonable agreement with the molecular weight of 150,000 established for this component on Sephadex G-200 columns by Andrews' method. The possibility that Fraction  $\alpha$  is a polymer of lower molecular weight units cannot yet be ruled out, nor do we know whether  $\beta$  is the inactive "subunit" proposed by Wagman, an inactive precursor as postulated by Bonventre and Kempe, or a nonspecific contaminant which crystallizes with the toxic  $\alpha$  fraction.

One of the most powerful of biologically active substances is the toxin of *Clostridium botulinum*, of which six serological types, designated A to F, are known. Of these, only type A has been crystallized and characterized (1). The crystalline material has the potency of  $2.8 \times 10^8$  LD<sub>50</sub> per mg of protein nitrogen for a 20-g mouse. It has been reported to be a simple homogeneous protein of molecular weight 900,000 (1, 2). The 900,000-molecular weight moiety was, however, reported to be an aggregate of smaller units which, on the basis of cysteine and cystine content

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of the molecules, were calculated to have a minimum molecular weight of 45,000 (3). Crystalline toxin, according to Wagman (4), sediments in the ultracentrifuge at pH 9.2 as two components with sedimentation coefficients,  $s_{20,w}$ , of 7.2 and 13.7, respectively. Wagman considered these two components to be products of dissociation of the large aggregate. In our hands, however, serological reactions by the Ouchterlony method between crystalline toxin and its homologous antiserum invariably resulted in two precipitation lines, indicating the presence of at least two distinct components. Electrophoresis of this toxin on agar in 0.05 M tris(hydroxymethyl)aminomethane buffer at pH 8.6 also showed two bands having different mobilities. Eluted, one band proved to be toxic while the other had only feeble activity. Treatment of crystalline toxin with 2-hydroxy-5-nitrobenzyl bromide or photo-oxidation by visible light in the presence of methylene blue resulted in loss of toxicity and disappearance of one serologically reactive component and one electrophoretically demonstrable band, with concurrent loss of the ability to stimulate formation of protective antibody when injected into rabbits (5). The above observations cast doubt on the homogeneity as well as on the previously reported molecular weights of the crystalline toxin preparations.

Attempts to separate the two components of the toxin succeeded when crystalline preparations were chromatographed on diethylaminoethyl Sephadex with 0.07 M Tris-HCl buffer of pH 7.2, and eluted with a gradient of NaCl. A component ( $\alpha$ ) emerged which contained 5 times the specific activity of the original but only 20% of its protein content. A second component ( $\beta$ ), which was eluted in higher concentrations of NaCl, contained 80% of the protein but was only feebly toxic. These components appeared to be identical with the two components observed when crystalline toxin preparations were allowed to react against the anticrystalline rabbit serum in gel double diffusion tests (6).

Successful separation of the crystalline preparation into two components prompted reinvestigation of the molecular weight and the homogeneity of the native toxin, as well as investigation of the separated fractions in the analytical ultracentrifuge.

## MATERIALS AND METHODS

Three times crystallized toxin of *C. botulinum* type A was kindly supplied by Dr. E. J. Schantz, Fort Detrick, Frederick, Maryland. This toxin was used to obtain the toxic Fraction  $\alpha$  and the nontoxic Fraction  $\beta$  by means of chromatography on

TABLE I  
Sedimentation coefficients of crystalline toxin and isolated fractions of *C. botulinum* type A

Material	Concentration mg/ml	Solvent <sup>a</sup>	r/2	pH	Peaks			
					s <sub>20,w</sub> value (% of total)			
Crystalline toxin	2.6	Acetate	0.05	3.8	6.1 (5)	13.1 (90)	16.0 (5)	
Crystalline toxin	2.8	Phosphate	0.23	7.0	8.2 (17)	11.5 (6)	16.0 (11)	23
Crystalline toxin	2.0	Guanidine-SCN	5 M	9.5		14.0 (60)	15.7 (25)	23.7 (15)
Crystalline toxin	2.6	Carbonate	0.12	9.35	6.6 (20)	13.1 (80)		
Crystalline toxin	2.7	Tris-ClO <sub>4</sub>	0.02	9.5	6.1 (20)	13.5 (54)	16.0 (26)	
Crystalline toxin	2.7	Tris-ClO <sub>4</sub>	0.02	9.5	6.5 (15)	13.1 (65)	16.0 (20)	
Crystalline toxin	1.9	Tris-ClO <sub>4</sub>	0.05	9.5	7.4 (23)	11.1 (66)		
Crystalline toxin	0.27	Tris-ClO <sub>4</sub>	0.05	9.5	7.0	12.8		
Fraction α	0.27	Acetate	0.0011	3.4	7.2			
Fraction α	0.27	Tris-ClO <sub>4</sub>	0.05	9.5	7.25			
Fraction α	0.3	Tris-ClO <sub>4</sub>	0.05	9.5	7.3			
Fraction β	1.0	Acetate	0.05	3.8		14.3		
Fraction β	0.27	Acetate	0.05	3.8		14.0		
Fraction β	1.0	Tris-ClO <sub>4</sub>	0.05	9.5		13.1		
Fraction β	0.27	Tris-ClO <sub>4</sub>	0.05	9.5		13.3		

<sup>a</sup> Compositions given in the text.

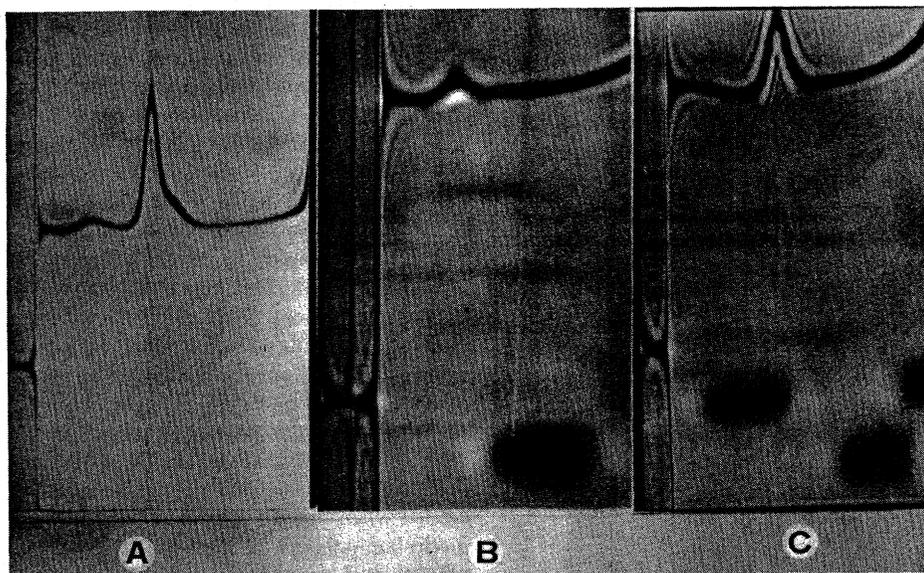


FIG. 1. Ultracentrifuge photographs of crystalline toxin and isolated fraction of *C. botulinum* type A at 50,700 rpm, 32 min after reaching full speed, in 0.05 M Tris-ClO<sub>4</sub>, pH 9.5, at 25°. A, crystalline toxin; B, toxic fraction (α); C, nontoxic fraction (β).

DEAE-Sephadex as previously described (6). Gel electrophoresis and Ouchterlony double diffusion tests were performed by standard methods in 1.0% Noble agar (Difco). Toxicities of all preparations were established by the intravenous inoculation of mice with 0.1 ml of solution of the substance.

Ultracentrifugal velocity analyses were done at 50,700 rpm in a Spinco model E instrument<sup>1</sup> equipped with temperature control. Aluminum-filled Epon cells with sapphire windows were used for equilibrium sedimentation runs, which were done at 17,250 rpm with 3-mm liquid columns.

Interference plates were analyzed and equilibrium molecular weight was calculated by the application of the Yphantis technique (7). Relative areas of multiple peaks seen in the schlieren runs were established by the projection of the photographs on

heavy filter paper by means of a photographic enlarger. These projection patterns were then traced, cut out, and weighed. The buffers used were acetate, sodium acetate, molarity specified, titrated to desired pH with glacial acetic acid; phosphate, 63.6 ml of 0.05 M Na<sub>2</sub>HPO<sub>4</sub> and 36.4 ml of 0.05 M NaH<sub>2</sub>PO<sub>4</sub> with resultant pH 7.0 and ionic strength 0.227; carbonate, 92 ml of 0.05 M NaHCO<sub>3</sub> and 8 ml of 0.05 M NaHCO<sub>3</sub>, pH 9.3, ionic strength 0.116; and Tris-perchlorate, molarity specified, tris-(hydroxymethyl)aminomethane titrated to desired pH with 70% HClO<sub>4</sub>. Protein concentrations were established by the method of Lowry *et al.* (8).

#### RESULTS AND DISCUSSION

Ultracentrifugal analysis was done on the crystalline toxin and the chromatographically separated fractions α and β. Results obtained in the experiments are presented in Table I and Fig. 1. Examination of the data for the crystalline toxin shows distinct

<sup>1</sup> Mention of specific products does not imply endorsement by the United States Department of Agriculture to the detriment of other manufacturers in the same business.

inhomogeneity, with at least two peaks under every experimental condition used. The experiments at the lower pH values (3.8 to 7) were less reproducible than those at the higher pH values (9 and above), because of large convective disturbances which could be seen moving ahead of the fast peaks. These disturbances, seen as transient spikes throughout the plateau region of the cell, indicated extensive interaction with solvent or buffer components (9). These disturbances were minimized or completely eliminated at alkaline pH values.

A common factor in all the experiments with the whole toxin was the presence of two major peaks with  $s_{20,w}$  values near 7 and 13.5 S. This is in agreement with Wagman's findings. The presence of a third component with  $s_{20,w}$  values near 16 indicates that these components (7 and 13.5) can undergo further aggregation. The aggregation to the 16 S complex is probably reversible, since an increase in ionic strength from 0.02 to 0.05, with a concomitant decrease in protein concentration from 2.7 to 1.9 mg per ml at the same pH (pH 9.5, Tris-perchlorate), eliminated this peak entirely. That this is the case was also indicated by the observed reappearance of a 16 S peak at lower pH values, where even greater aggregation to material of 23 S was seen.

A single ultracentrifugal experiment was carried out at pH 9.5 in 5 M guanidine thiocyanate, which would be expected to act as a dissociating agent (10). The 6 S peak, however, completely disappeared, and a peak with sedimentation coefficient of 23.7 S appeared, in addition to 13 and 16 S peaks as seen in other experiments. The isolated  $\alpha$  and  $\beta$  fractions, when analyzed in the ultracentrifuge at pH 9, appeared to be homogeneous (Fig. 1). At pH 3.8, however, the heavier  $\beta$  component showed an indication of containing still heavier material. This is in agreement with results obtained with the whole toxin. There was, however no evidence in all our experiments of any heterogeneity of the  $\alpha$  fraction, although the possibility that this fraction is a polymer and consists of units of lower molecular weight cannot yet be,

ruled out. The  $s_{20,w}$  values of Fractions  $\alpha$  and  $\beta$  were identical with  $s_{20,w}$  values of the two peaks observed by Wagman, who, however, regarded them as dissociation products of the homogeneous crystalline toxin. Furthermore, the similarity of the  $s_{20,w}$  values obtained on chromatographically separated fractions to the values of the two major components seen in the crystalline toxin strongly indicates that these components were isolated without denaturation. This is corroborated by the observation that the  $\alpha$  fraction, having only 20% of the protein concentration of the crystalline preparation, retained nearly 100% of the original toxicity.

Under no conditions did our experiments yield peaks with values less than 6 S. This is in disagreement with the results reported by Gerwing, Dolman, and Bains (11).

The molecular weight of the toxic  $\alpha$  fraction was established in the ultracentrifuge, with the use of the meniscus-depletion technique of Yphantis, in 0.05 M Tris-perchlorate solvent at pH 9.5. Equilibrium was attained in less than 24 hours. Data obtained showed the fraction to have a molecular weight of  $128,000 \pm 10\%$ . This is in reasonable agreement with the value for molecular weight of 150,000 obtained in our laboratory (6) by Andrews' method (12) on Sephadex G-200, and with the value of 158,000 assigned to this component by Wagman. The two components,  $\alpha$  and  $\beta$ , along with the unfractionated toxin, were allowed to react by Ouchterlony technique against the anticycrystalline toxin rabbit serum. The precipitation lines formed by the antiserum and the two components show identity with lines formed by crystalline toxin against the same antiserum (Fig. 2).

The relation of the nontoxic  $\beta$  component in the crystalline toxin to the  $\alpha$  component has not yet been established. Whether  $\beta$  is the inactive "subunit" proposed by Wagman or the inactive precursor postulated by Bonventre and Kempe (13) is not yet clear. This component, however, reacts differently from  $\alpha$  in the Ouchterlony test, has a different electrophoretic mobility, and fails to induce the formation of protective antibody when administered to rabbits. The nature of the fraction is presently under investigation.

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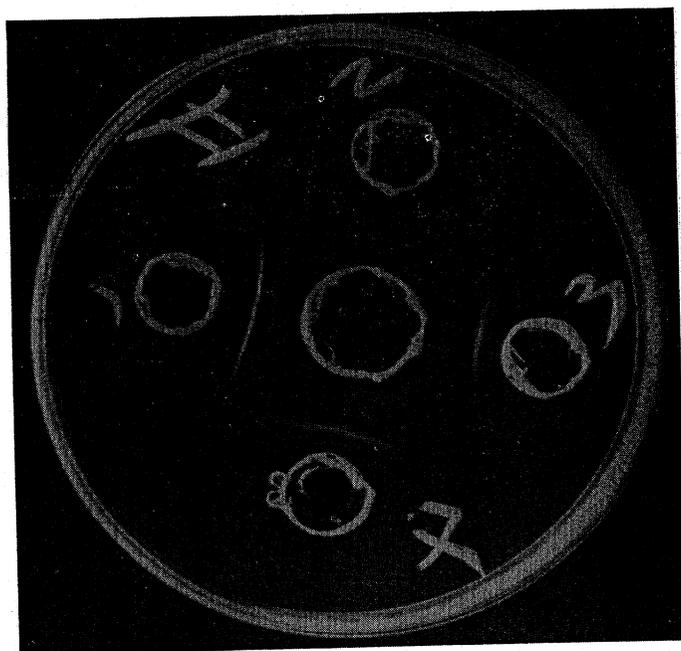


Fig. 2. Ouchterlony gel double diffusion test. Central well: antiserum to crystalline toxin of *C. botulinum* type A. Lateral wells: 1, crystalline toxin; 2, toxic Fraction  $\alpha$ ; 3, nontoxic Fraction  $\beta$ ; 4, mixture of  $\alpha$  and  $\beta$ .