CHROMATOGRAPHIC FRACTIONATION OF THE CRYSTALLINE TOXIN OF CLOSTRIDIUM BOTULINUM TYPE A

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Crystalline toxin of Clostridium botulinum has been characterized as a simple homogeneous protein of molecular weight 900,000 (Lamanna et al., 1946), (Putnam et al., 1946) and is thought to be an aggregate of smaller molecular weight units. On the basis of cysteine and cystine content the minimum molecular weight of crystalline toxin was calculated as 45,000 (Buehler et al., 1947). In an analytical ultracentrifuge at pH 9.2 this toxin sediments as two components: one S_{20w} 7.3 and the second S_{20w} 13.7 (Wagman, 1963). From this study Wagman suggested that these components are products of dissociation of the large aggregate. To date, to our knowledge, there are no reports in the literature of successful chromatographic isolation and study of these components of the crystalline toxin.

Observations made in the course of our study of this substance cast some doubt as to the correctness of the suggested homogeneity, as well as the molecular weight of this preparation. This prompted an attempt to examine crystalline Type A toxin in regard to these two parameters. In the following we present evidence that crystalline botulinum toxin fractionated on DEAE-Sephadex column yielded at least two components differing in molecular weight, physical-chemical, toxic and serological properties.

The toxin used throughout the experiments was a crystalline preparation obtained through the courtesy of Dr. E. J. Schantz, Fort Detrick, Maryland. This toxin resembled in all respects preparations described in reports of the original authors. The crystals in 0.1 M Na phosphate buffer pH 6.8 and 0.9 M $(NH_4)_2 SO_4$ were removed from suspension by centrifugation and dissolved in 0.07 M Tris (Hydroxymethylaminomethane)-HCl buffer pH 7.2 at room temperature. The dissolved toxin was dialyzed against 100 volumes of the same buffer at 4° for 16 hrs and freed from residual turbidity by centrifugation. The water clear supernatant was used as stock solution in the studies. The buffer was prepared by titrating 0.07 M Tris against 0.07 M HCl to pH 7.2. The best preparation of three times crystallized botulinum toxin was reported by Schantz (1964) to yield ratios of absorption at 260 and 278 mμ of 0.5 - 0.52. The ratio of the stock solutions, at these wave lengths, ranged between 0.49 - 0.50. The protein content, as determined from absorbance at 278 mμ (absorbance/1.7 = mg of protein per ml, Schantz (1964)) was 5 mg/ml.

DEAE Sephadex A-50 was equilibrated for 5 days in 0.07 M Tris-HCl buffer pH 7.2 and washed 3 times during this period with the same buffer. The column, 2 cm I.D., was packed to 25 cm height. After about 500 ml of the buffer was passed through the column at 30 ml/hr flow rate to achieve proper packing, the gel bed was reduced to 22 cm (column bed volume approximately 69 ml). All chromatography was performed at room temperature. The eluate was collected in 3 ml amounts and the protein content was monitored by fluorescent intensity (F.I.) in an Aminco-Keirs Spectrophosphorimeter at 285 mμ excitation and 350 mμ fluorescent wave length, and protein concentration determined from absorbance at 278 mμ in a Zeiss Model PMQ II Spectrophotometer. The toxicity was measured in LD_{50} by intraperitoneal injections of serially diluted portions of the eluate into mice (Wagman and Bateman, 1953). Concentration of Cl^- was determined by titration of Cl^- against standard $AgNO_3$ solution, K_2CrO_4 being used as an indicator.

In a typical experiment 4.5 ml of the stock solution was placed on the column and washed with 70 ml of the buffer. It was established

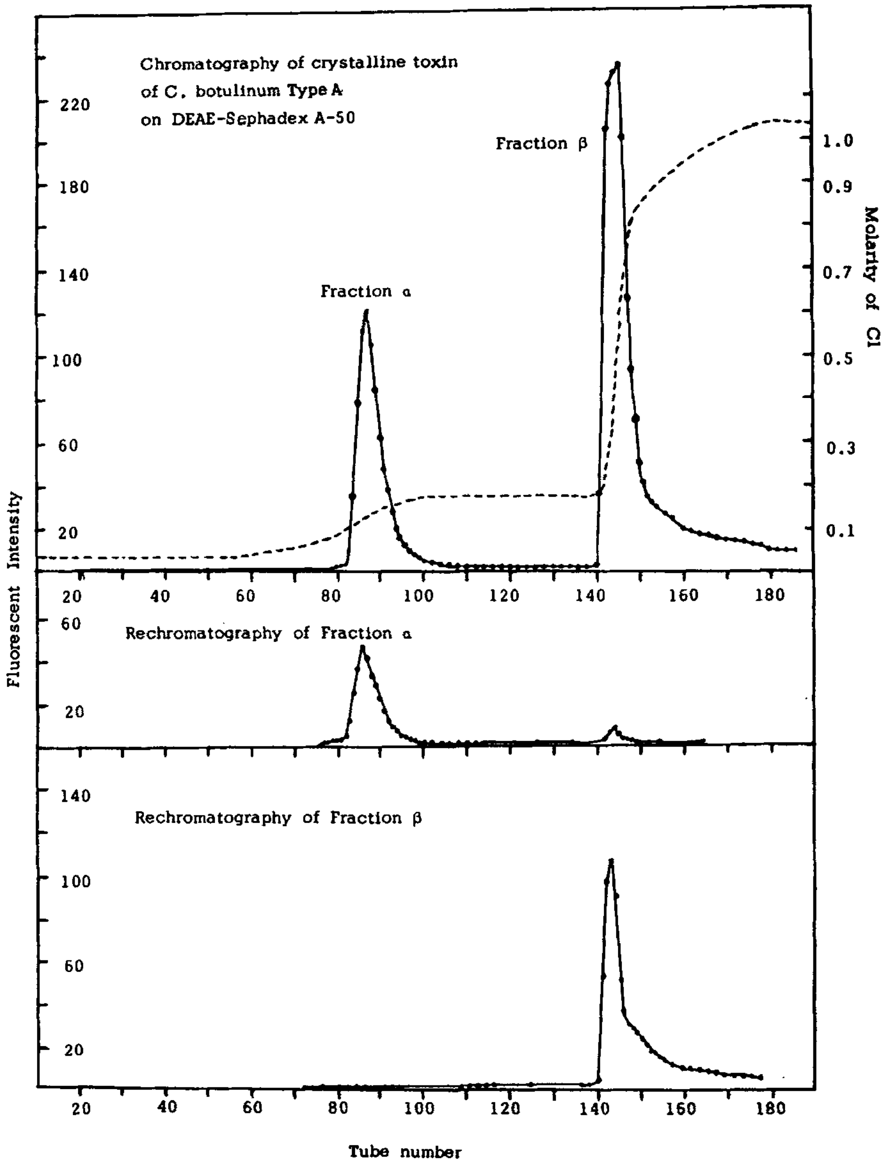

FIGURE 1

Chromatography of crystalline toxin of Clostridium botulinum Type A on DEAE-Sephadex A-50. Top section-fractionation of crystalline toxin. Middle section- rechromatography of a portion of pooled α fraction obtained from 1st run. Bottom section-rechromatography of a portion of pooled β fraction obtained from 1st run.

earlier that unless the column was overloaded with toxin, no protein emerged, even after washing with 200 ml of the Tris-HCl buffer. The elution of protein was commenced by generating a gradient with 0.2 M NaCl added to the buffer. After the concentration of Cl^- in the eluate reached 0.17 M this molarity of the salt was maintained for the next 111 ml of the eluate, the gradient was then resumed but with 1.0 M of NaCl in the buffer.

By this procedure two distinct components (Figure 1) were obtained, one (α) emerging when the concentration of Cl^- reached about 0.1 M above that contributed by Cl^- of the HCl in the buffer. No more protein emerged until the Cl^- in the eluate reached 0.16 above that in the buffer. At this concentration of the salt component β appeared in the eluate.

Component α contained 1.86×10^8 LD_{50} toxicity per mg protein, about 5 times greater than that of the best preparation 3.87×10^7 LD_{50}/mg in the unfractionated toxin (Schantz, 1964). The protein concentration in α was approximately 20% of the original, β component contained the rest of the original protein but was only feebly toxic. For rechromatography a portion of the eluate of each of the components was dialyzed against Tris-HCl buffer to remove the excess NaCl and recycled under identical conditions used in the original procedure. Both components emerged at their previously observed elution volume and Cl^- concentration (Figure 1). A small peak in the region of β emergence, which appeared on rechromatography of α, was no more detectable on subsequent recycling.

The isolated fractions were used to determine molecular weight on Sephadex G-200 column by the method developed by Andrews (1965), the validity of which has been confirmed by Siegal and Monty (1965) and Sorof et al., (1966). By this method (Figure 2) components α and β appeared to have 150,000 mol. wt. and 500,000 mol. wt. respectively. By the same procedure unfractionated crystalline toxin was of 74,000 mol. wt. These components, when examined in the analytical ultracentrifuge at

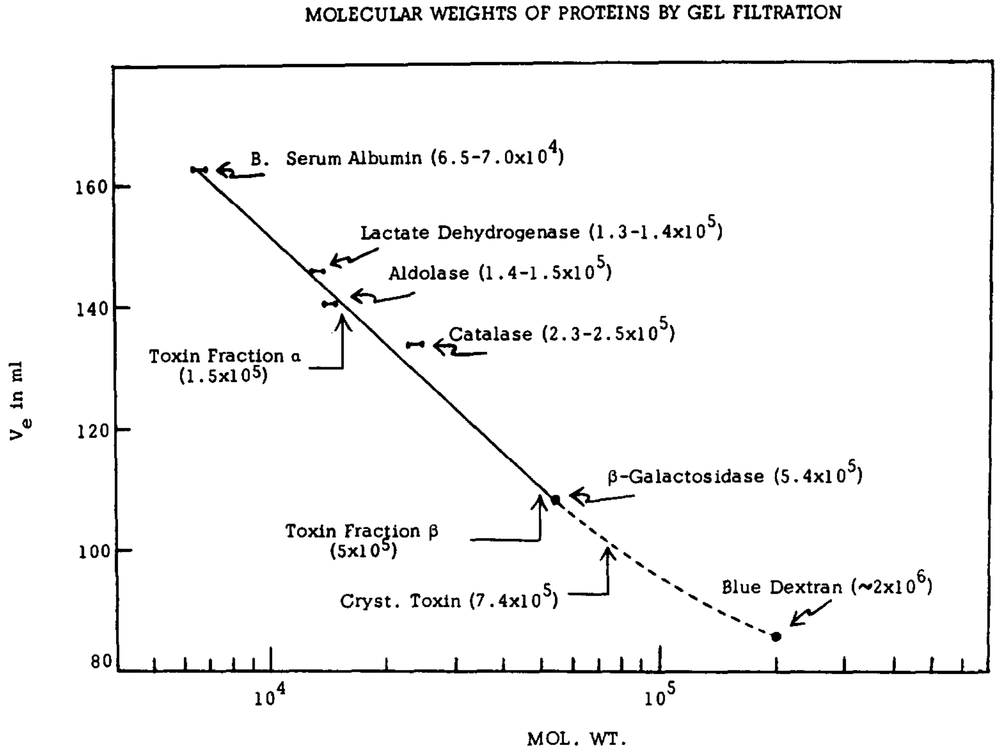

FIGURE 2

Molecular weight of protein as determined by gel filtration on Sephadex G-200. Ve is the elution volume, ploted against log (mol. wt.). Column (2.5 cm x 50 cm) equilibrated and eluted with 0.05 M Tris-HCl buffer pH 7.5 containing o.1 M KCl. Molecular weight of proteins, indicated in parenthesis, in this experiment are according to Andrews (1965) except for β galacto sidase, which is from Craven et. al. (J. Biol. Chem. 240: 2468 (1965). α and β components were from tubes with the highest concentrations of these components obtained after rechromalography.

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pH 9.5, sedimented with S_{20w} 7.2. for α and 13.1 for β. The details of ultracentrifuge studies will be the subject of a separate report. As mentioned above, the mol. wt. of crystalline toxin based on S_{20w} 17.3 (Putnam et. al. 1946) was established as 900,000. Wagman (1963) found the S_{20w} for the toxin at various pH and ionic concentration to be no more than 16.3. Thus the mol. wt. for the unfractionated crystalline toxin of 740,000 is not inconsistent with the values ascribed to it by previous authors.

On storage for as long as 30 days, either at 4° or at -28°, α retained its molecular weight and toxicity as before storage.

By the Ouchterloney gel double diffusion technique, each component reacted in a single precipitation line when tested against rabbit anti-crystalline toxin serum. Each of the lines corresponded to one of the two precipitation lines obtained when this serum was reacted with solution of the crystalline toxin. On electrophoresis in 1% Noble agar (Difco) at pH 8.6 Tris-perchloric acid buffer 0.05 M for 7200 sec. at 8V/CM. and 4° α and β components exhibited different electrophoretic mobilities.

Although at present it appears that crystalline toxin of *Clostridium botulinum* Type A consists of at least two fractions, the possibility of further resolution of the crystalline toxin cannot yet be ruled out. Rechromatography showed that α and β components are not artifacts and that α is stable was further evidenced by the retention on storage of its mol. wt. of 150,000 and high toxicity. By the criteria applied (chromatography, molecular weight, electrophoretic mobility, toxicity and serological reactivity) α and β components appear to be different. Whether β is the same as the inactive subunit proposed by Wagman (1963), or the inactive precursor postulated by Bonventre and Kempe (1960) is not yet clear. Further characterization of these components is presently in progress.

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REFERENCES

Andrews, P., Biochem. J., 96, 595 (1965).
Bonventre, P.F. and Kempe, L.L., J. Bact., 79, 24 (1960).
Buehler, H.J., Schantz, E.J. and Lamanna, C., J. Biol. Chem., 169, 295 (1947).
Lamanna, C., McElroy, D.E. and Eklund, H.W., Science, 103, 613 (1946).
Putnam, F.W., Lamanna, C. and Sharp, D.G., J. Biol. Chem., 165, 735 (1946).
Schantz, E.J., in "Botulism" (ed. Lewis, K.H. and Cassel, K.). Public Health Service Publication No. 999-FP-1, pp 91-104 (1964).
Siegal, L.M. and Monty, K.J., Biochem. Biophys. Res. Comm., 19, 494 (1965).
Sorof, S., Young, E.M., McBride, R.A. and Coffey, C.B., Arch. Biochem. Biophys., 113, 83 (1966).
Wagman, J. and Bateman, J.B., Arch. Biochem. Biophys., 45, 375 (1953).
Wagman, J., Arch. Biochem. Biophys., 100, 414 (1963).