ISOLATION OF CHROMATOGRAPHICALLY PURE TOXIN OF *CLOSTRIDIUM BOTULINUM* TYPE B

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The toxin of *Clostridium botulinum* type B of high activity first isolated by Lamanna and Glaesman (1947) was found to be of 500,000 mol. wt. by Wagman and Bateman (1951). Thereafter Duff *et al.* (1957) further purified the toxic protein which in the analytical ultracentrifuge at pH 6.0 resolved into two components of S_{20w} 14.9 and 10.9. With some modifications in the purification procedures these authors obtained the toxin with a single boundary of S_{20w} 12.7. This preparation, regarded as highly purified toxin, was not further examined for homogeneity.

In this communication we demonstrate that the type B toxin purified by the method of Duff *et al.* (1957) consists of more than one component. We also describe a method of isolation and purification of the toxin of *C. botulinum* type B of mol. wt. 165,000 and show that it is homogeneous by anion exchange chromatography and by gel filtration.

Preparations of buffers of pH 7.2, 8.0 and 9.0, all of 0.05 M Tris-HCl containing 0.1 M KCl; 0.067 M citrate-phosphate pH 5.6; 0.2 M Tris-HCl pH 7.3; Cl^- titration method; determination of protein by fluorescent intensity and optical density measurements in 10 mm cells, packing and operation of gel filtration, DEAE-cellulose and DEAE-Sephadex columns were described before (DasGupta and Boroff, 1968; DasGupta *et al.*, 1966). Chromatography was done at 25 ± 2°.

Type B toxin, isolated and purified by the method of Duff *et al.* (1957), was analyzed on a DEAE-cellulose column according to the chromatographic

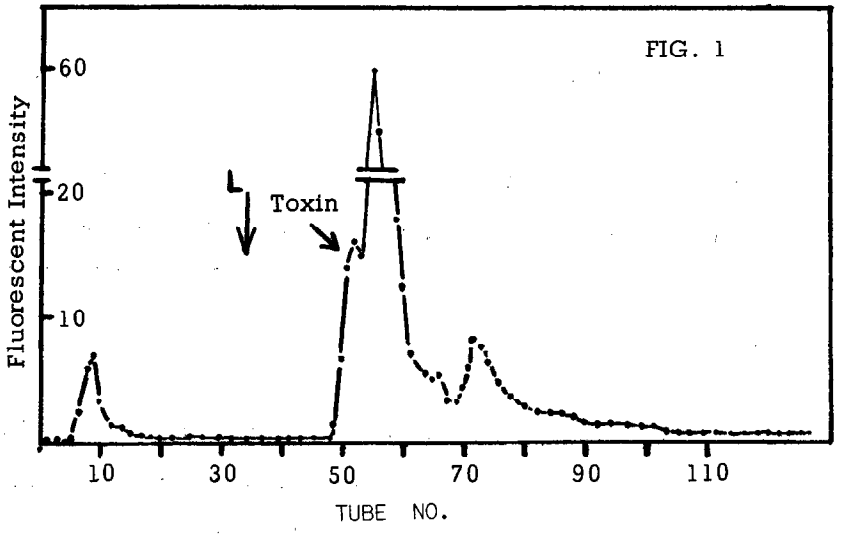

Fig. 1: Type B toxin, Duff et al. (1957), 8.4 mg/4 ml applied on a 0.9x30 cm DEAE-cellulose column equilibrated with 0.15 M Tris-HCl pH 8.0 buffer was chromatographed with a linear gradient elution according to DasGupta and Boroff (1968). Gradient started at arrow marked L.

conditions applied to type A toxin (DasGupta and Boroff, 1968). Fig. 1 shows the dissociation of this toxin into at least 4 components. The shoulder of the main peak was most toxic. The other peaks were either non-toxic or of very little toxicity.

C. botulinum type B, strain Lamanna, obtained from Dr. J. Gerwing, (U. British Columbia, Vancouver), and stored in frozen culture, was grown in a dialyzing sac (Boroff et al., 1968) containing 400 ml of 0.85% NaCl. The sac was immersed in 600 ml of medium (Proteose peptone No. 2, 4%, N-Z-Amine type B, 2%, and yeast extract 2%) adjusted to pH 7.3 before autoclaving. One ml of the seed culture and 10 ml of sterile glucose, 10 gr, were added to the sac. Incubation of the culture, precipitation of the toxin from the bacterial culture filtrate at 50% $(NH_4)_2SO_4$ saturation, preparation of the crude toxin and toxicity determinations were according to Boroff et al. (1968). The crude toxin was dialyzed against 15 X volume of pH 5.6 buffer at 4° for 16 hrs. with one change of the

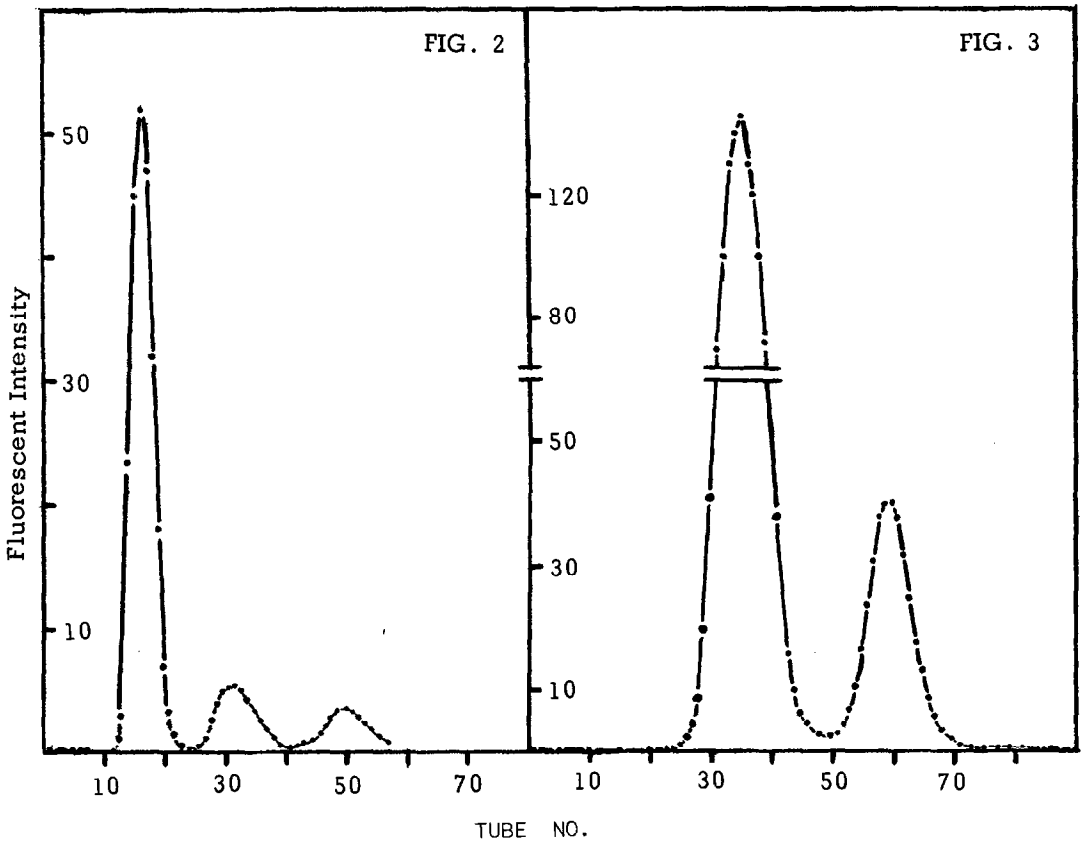

Fig. 2: Elution of 10.0 ml pH 5.6 toxin through a 2x40 cm Sephadex G-100 column operated with pH 5.6 buffer at a flow rate 20 ml/hr with 3.0 ml fractions.

Fig. 3: Elution of 1.0 ml of precipitate II through a 2.5x90 cm Sephadex G-200 column operated with pH 7.3 buffer at a flow rate 10 ml/hr. Fractions were of 3.0 ml.

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buffer. A sediment which appeared upon dialysis was removed by centrifugation. DEAE-cellulose columns, 0.9x30 cm, equilibrated with pH 5.6 buffer, were loaded with 4.0 ml portions of the toxic supernatant. The chromatographic procedure and the results obtained were as previously described (Boroff et al., 1968). The eluted peak, referred to as pH 5.6 toxin, obtained from a number of columns were combined. Ten ml of the pH 5.6 toxin were applied on a 2x40 cm Sephadex G-100 column and eluted with pH 5.6 buffer. The material emerged as a major peak at the void

volume, followed by two small peaks (Fig. 2). Most of the toxicity of the material applied on the column was found in the first peak, which will be referred to as the G-100 toxic peak. The toxic eluates thus obtained from a number of Sephadex G-100 columns were combined and solid $(NH_4)_2SO_4$, 39 gr/100 ml, was added and left for 36-48 hrs. at 4°. The precipitate formed contained the toxin. This was collected by centrifugation at 18,000 rpm for 1 hr. and suspended in pH 7.3 buffer, 2.5% of the original volume of pooled G-100 toxic peak. Although most of the precipitate dissolved, some white shreds remained insoluble. After removing the insoluble material by centrifugation at room temperature, 1.0 ml aliquots of the clear supernatant (precipitate II) were applied on several 2.5x90 cm Sephadex G-200 columns eluted with pH 7.3 buffer. As shown in Fig. 3, a large peak containing only trace toxicity emerged followed by a small peak with high toxic activity. Portions of the eluate containing the second peak were pooled and 90 ml of the pooled eluate was applied on a 0.9x30 cm DEAE-Sephadex A-50 column equilibrated with pH 7.3 buffer. After washing the column with this buffer a linear gradient was generated with 130 ml of the pH 7.3 buffer containing NaCl to give net 0.23 M Cl^- and 130 ml of the equilibrating buffer. One sharp symmetrical peak (B_α fraction) eluted with the apex of the peak emerging at 0.12 M Cl^- in the eluate. After the passage of 260 ml of the eluate the column was washed with pH 7.3 buffer containing 0.5 M NaCl which resulted in the elution of a second peak (Fig. 4A). The specific toxicity of the eluate in the first peak was uniform across the peak with 5.9×10^7 MLD/1.0 absorbance at 278 mμ. The second peak had trace amount of toxicity. For rechromatography 25 ml of the eluate from the first peak was dialyzed against pH 7.3 buffer to remove NaCl and rechromatographed under identical conditions described above. Only one peak emerged at the same Cl^- concentration and the same B_α position (Fig. 4B). The homogeneity of B_α fraction was further examined by gel filtration on 1.5x60 cm

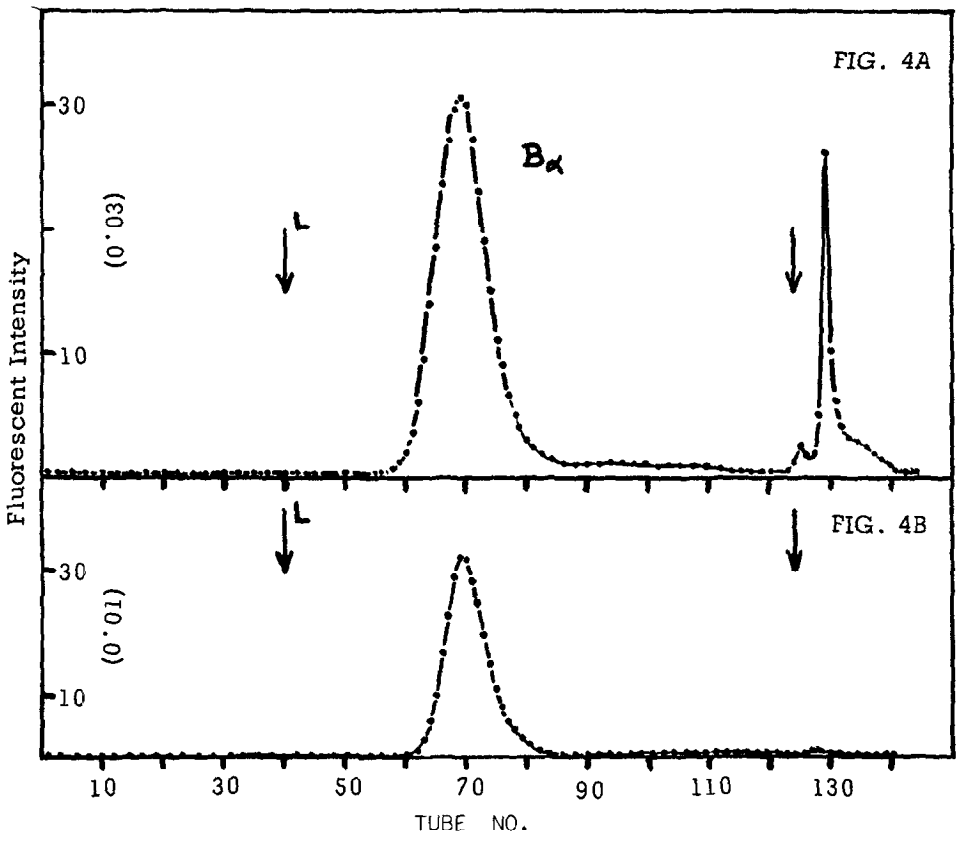

Fig. 4A: Chromatography of 90 ml of toxin eluate (the second peak) obtained from Sephadex G-200 column on a 0.9x30 cm DEAE-Sephadex A-50 column equilibrated with pH 7.3 buffer. Linear gradient started at arrow marked L. Buffer with 0.5 M NaCl was added at the second arrow. Specific toxicity of the eluate in tubes 66, 68 and 71 expressed as MLD/1.0 absorbance at 278 mμ were 57 million, 61.5 million and 60 million respectively.

Fig. 4B: Rechromatography of 25 ml of B_α fraction under identical conditions of the original run. The instrument sensitivity for recording fluorescent intensity in Fig. 4B was 3 X more than in Fig. 4A. Fractions of 3.0 ml were collected at a flow rate 20 ml/hr.

Sephadex G-200 columns eluted with a buffer of either pH 7.2, 8.0 or 9. In all cases one symmetrical peak eluted with no sign of further dissociation (Fig. 5). The mol. wt. of the B_α fraction determined by the gel filtration method of Andrews (1965) was found to be 165,000. The procedure employed was described by DasGupta and Boroff (1968) differing only in the use of a 1.5x60 cm instead of 2.5x50 cm Sephadex G-200

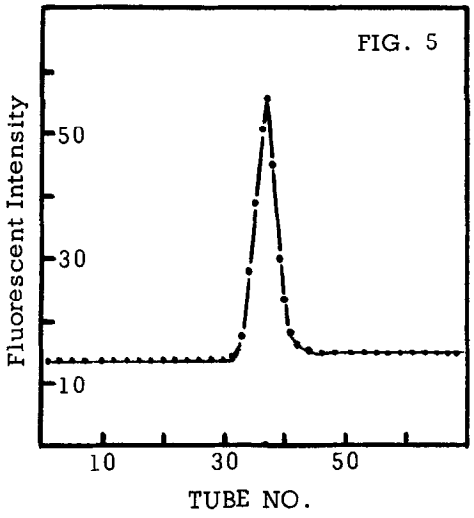

Fig. 5: Elution profile of B_α fraction, 1.0 ml, from a 1.5x80 cm Sephadex G-200 column operated with 0.05 M Tris-HCl pH 8.0 buffer containing 0.1 M KCl. Apex of the toxin peak at tube 37. Apex of blue dextran peak, not shown here, was at tube 21. Fractions of 1.95 ml/tube were collected. Columns operated with pH 7.2 or 9.0 buffer of above composition gave similar results.

column. By all tests used B_α appeared to be a simple protein. The method reported here resulted in 500 fold purification of the toxin with a 13 - 15% yield of the total toxic activity present in the bacterial culture.

The specific toxicities of types B and A, expressed as MLD/1.0 absorbance at 278 mμ, were 5.9×10^7 and 9×10^7 respectively. Besides the serological difference between types A and B toxins, the mol. wt. of B_α appears to be higher than the α fraction of type A toxin, 150,000 (DasGupta and Boroff, 1968). The former also behaves as a more anionic protein on DEAE-Sephadex column than the latter.

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