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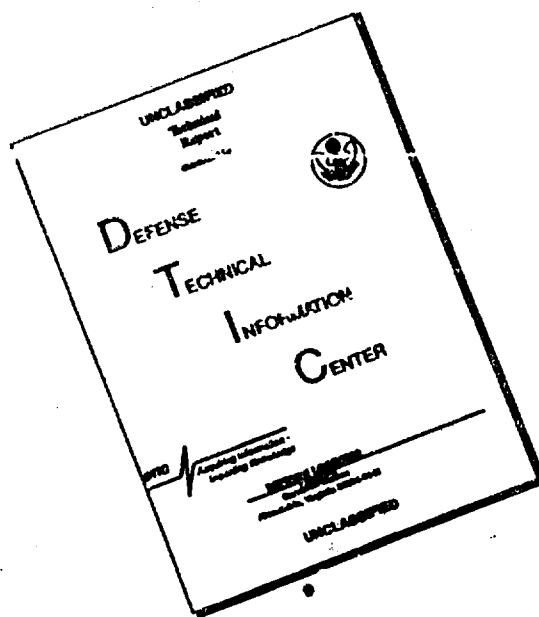
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EXAMINATION OF TYPE F BOTULIN TOXIN WITH THE AID OF
SEPHADEX GEL FILTRATION

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The type F causative agent of botulism, first isolated in 1958, has since been more thoroughly studied by a number of investigators (Jensen, Hannemann, 1959; Koller, Scheibel, 1960; Dolman, Murakami, 1961; Mikhaylova, 1965). However, in the available literature we encountered no data on the molecular mass of this toxin type. The monograph by Vorob'yev et al. (1965) specifies the following molecular mass values for botulin toxins: 1 million for types A, C and D and 500,000 (according to other findings, 70,000) for type B; the molecular mass of type E botulin toxin is more than 200,000 (Sakaguchi et al.).

We investigated molecular mass by means of filtration through the gel sephadex. This filtration is based on the molecular-sieve principle. During the passage of the mixture of molecules through a sephadex-filled column first the largest molecules appear in the effluent and later the others, in the order of decrease in smallness (Flodin, 1962; Porath, 1962). Hence, the gel-filtration method may be used to determine the molecular mass of albumins and polysaccharides (Bagdasar'yan et al., 1964; Squire, 1964; Auricchio, Bruni, 1964).

The method is also used in research into bacterial proteins. Thus, Kaldor and Gitlin (1954), on investigating the neuraminidase of *Vibrio comma* with the aid of filtration through the gel sephadex G-75, established that its molecular mass is close to 90,000. Jungwirth and Bodo (1964) estimated the molecular mass of the interferon at 32,000-35,000 by comparing the findings on the chromatography of this protein on sephadex G-100 with the findings for other proteins with known molecular mass.

We used chromatographic columns measuring internally 500x22 mm for the G-100 sephadex gel and 600x22 for the G-200 sephadex gel. Porous plates covered with a layer of filter paper were placed on the bottom of the columns. Five cc of the investigated solution was poured into the columns. Elution was performed with isotonic borate buffer having a pH of 8.2 for the columns with G-100 gel and a pH of 7.32 for the columns with G-200 gel. The filtration rate was 25 cc/hr in the first case (the volume of fractions collected was 10 cc each) and 15 cc/hr in the second case (volume of fractions collected 5 cc each). The content of nitrogenous substances in the collected fractions was determined by the photolorimetric method (using an FEK-M57 instrument) with the Louri-Folin reagent and the toxicity was determined (in Dlm) on mice. Fraction count was commenced after the first 10 cc was obtained.

To calibrate the columns with sephadex G-100 under standard conditions, we successively carried out filtration of gamma-globulin and albumin obtained by fractionating the serum albumin of a horse with ammonium sulfate, egg albumin, Spofa-grade trypsin, and freshly distilled crystalline phenol. The molecular mass of serum gamma-globulin is normally considered 160,000; of serum albumin, 65,000; of egg albumin, 46,000; of trypsin, 24,000; and of phenol, 94.1.

On filtration through sephadex G-100, gamma-globulin was not trapped within the column and it emerged freely in bulk with a maximum in the 5th or 6th fractions; the maximum elution of serum albumin was recorded for the 9th fraction; of egg albumin, for the 10th fraction; of trypsin, for the 12th-13th fractions; and phenol, 18th-19th fractions.

Columns containing sephadex G-200 were calibrated according to the albumins of the rabbit serum pursuant to Nezlin's (1964) recommendations. The albumins having a molecular mass of about 1 million (19S-globulins) emerged in the 12th-14th fractions, while the albumins having the molecular mass of 160,000 (7S-globulins) and 65,000 (4.1S-globulins) reached their maximum in the 19th and 26th fractions, respectively.

According to Gaurovits (1965), bacterial exotoxins are typical representatives of globular albumins, and hence the use of serum albumins with a known molecular mass to calibrate the column is justified.

Botulin toxins of the F type, produced on a liver nutrient medium, were subject to separation. Native toxins having the activity of 3000-5000 Dlm/cc and toxins salted-out in ammonium sulfate with subsequent dissolution in native toxins

were employed; the activity of the final preparation in this case reached 20,000-60,000 Dlm/cc. The load per cc of nitrogen was $0.7-1 \cdot 10^3$ Dlm in the native toxins and $0.2-1.2 \cdot 10^4$ Dlm in the concentrated toxins.

In the columns with sephadex gel G-100, both native and concentrated toxins were separated into two peaks. The first peak included the 5th-7th fractions, i.e., corresponded to albumins with a molecular mass of more than 100,000; the entire toxic activity of the preparation was associated with the albumins of this peak. The second peak corresponded to low-molecular compounds with a concentration maximum in the 18th-19th fractions; toxic activity was not displayed by compounds of this peak (Fig. 1).

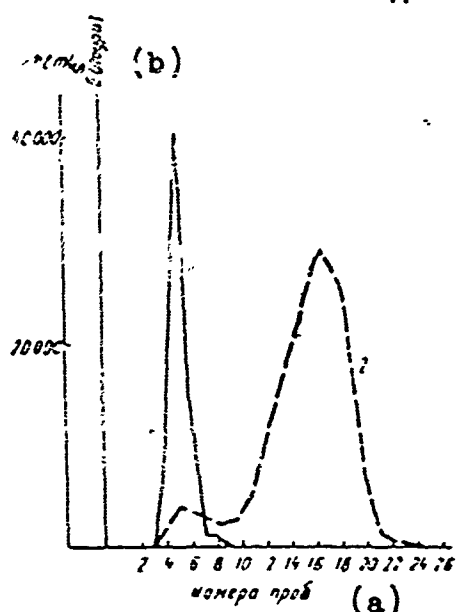


Fig. 1. Decomposition of Concentrated F Type Botulin Toxin by Means of Filtration Through G-100 Sephadex Gel (500x22 mm column). 1 -- Number of Dlm per cc; 2 -- Extinction with Louri-Folin reagent.

a -- Number of sample; b -- E(Louri).

In the columns with the gel G-200, the bulk of the nitrogenous substances present in type F botulin toxin, as detected by the Louri-Folin reaction, emerged in the 39th-44th fractions. In the fractions corresponding in molecular mass to serum albumins (12th-30th fractions), an insignificant amount of the substances was eluted. This may be attributed to the fact that in type F botulin toxins produced on liver

nutrient media, the content of albumin nitrogen is insignificant (less than 3 mg% compared with 450-500 mg% of total nitrogen). Nevertheless, the entire toxic activity was contained within the 19th-21st fractions (Fig. 2).

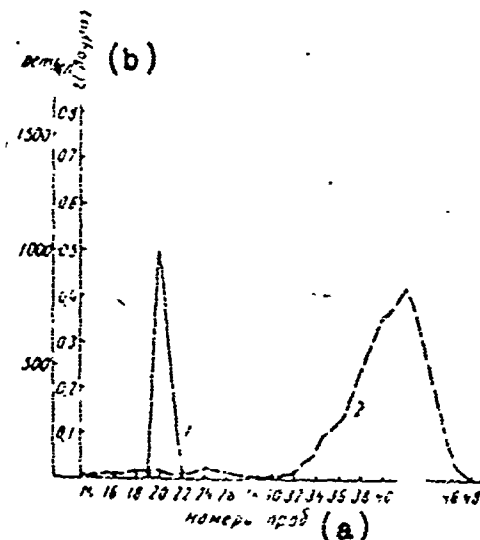


Fig. 2. Separation of Native Type F Botulin Toxin by Filtration Through Sephadex Gel G-200 (600x22 mm column). Notation the same as in Fig. 1.

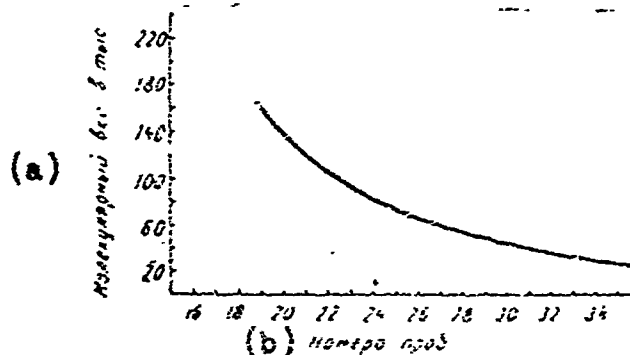


Fig. 3. Curve for determining the Molecular Weight of Albumins by Means of Filtration Through Sephadex Gel G-200 (600x22 mm column).

a -- Molecular weight, thousands; b -- Number of sample.

The molecular weight of type F botulin toxin was found from the calibration curve (Fig. 3) derived with respect to proteins with a known molecular mass (gamma-globulins, serum and egg albumins, trypsin). It is of a curvilinear shape which, according to Andrews (1964), is normally observed in cases of

this kind. Type F botulin toxin emerged from the column with G-200 sephadex gel in the 19th-21st fractions, with the activity maximum in the 20th fraction, which corresponded to a molecular mass of approximately 140,000. Thus, type F botulin toxin differed markedly in molecular mass from the other types of toxins. Its filtration through the gel resulted in the elimination of nitrogenous ballast compounds from the active fractions. Thus, in some cases it proved possible to purify the toxin until it contained $3.0 \cdot 10^6$ Dlm per mg of nitrogen. It is worth noting that the obtained purified preparations of type F botulin toxins rapidly lost their activity.

CONCLUSIONS

1. By means of filtration through the sephadex gels G-100 and G-200 it is possible to separate type F botulin toxins into fractions differing in their biologic activity.

2. It is established that, as determined by the gel filtration method, the molecular mass of type F botulin toxin is close to 140,000.

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