A STUDY OF BOTULIN TOXINS AND TOXOIDS BY FILTRATION THROUGH GEL

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A STUDY OF BOTULIN TOXINS AND TOXOIDS BY FILTRATION THOUGH GEL

Report 1. Examination of Botulin Toxins and Toxoids of the A and C Types by Filtration Through Sephadex G-100

[Following is the translation of an article by V. B. Freyman and V. K. Golshmid, Moscow Institute of Vaccines and Sera imeni Mechnikova, published in the Russian-language periodical Zhurnal Mikrobiologii, Epidemiologii i Immunobiologii (Journal of Microbiology, Epidemiology and Immunobiology) No 5, 1966, pages 52--58. It was submitted on 1 Oct 1965. Translation performed by Sp/7 Charles T. Ostertag Jr.]

Native botulin toxins and toxoids, used for the purpose of active immunization, are complex mixtures of protein and peptide compounds and free amino acids. A study of the composition of these preparations has great significance for the further improvement in their quality.

One of the methods for studying complex protein mixtures is filtration through dextran gel (sephadex), proposed in 1959 by Porath and Flodin. With the help of these gels it is possible to carry out the separation of serum proteins (Fireman et al., 1964; Johansson, 1964; Killander, 1963; Feinstein et al., 1963; Nezlin and Kulpina, 1964; Nezlin, 1964), enzymes (Scandurra, 1963; Yevtikhina et al., 1963), sugars (Davidson et al., 1963; Miettinen, 1963) and bacterial proteins (Phillips and Batty, 1963; Shemanova et al., 1963, Khavkin et al., 1963; Tikhomirova, 1964).

In the available literature we did not find any papers dealing with the study of botulin and toxoids of types A and C by means of filtration through dextran gel, therefore we considered it expedient to conduct work in this direction. These types of toxins and toxoids were selected as the objects of study because they differ sharply in their proteolytic activity: Type A possesses an expressed proteolytic activity, and type C is weakly active. Differences in proteolytic activity may also condition differences in the chemical composition of native preparations.

Toxins and toxoids, obtained on vegetable casein nutrient media were subjected to the investigation. The type A toxins contained 50,000--150,000 Dlm in 1 ml and their antitoxin-binding activity fluctuated within 100-200 EC; the antitoxin-binding activity of toxoids equaled 20 EC. Type C toxins contained 100,000--500,000 Dlm in 1 ml and 800--3200 EC; the antitoxin-binding activity of the toxoids fluctuated within 80--260 EC. The charge of EC per 1 mg of total nitrogen comprised 10 for type A toxoids and 14--50 EC for type C toxoids.

For the separation of the botulin toxins and toxoids we used columns with dimensions of 600 x 24 or 500 x 22 mm. On the bottom we placed a porous plate, covered with filter paper, and carefully filled the column with a suspension of Sephadex G-100 (series No 2946 from the "Pharmacia" firm, Sweden) in a buffer solution with pH 8.2 (boric acid 5.25 g, borax, 3.56 g, sodium chloride 6.18 g, water up to 1 liter).

For the purpose of thickening the layer of Sephadex the filled column was washed with a buffer solution for no less than a day, after which the column was ready for use.

On the day of the experiment the buffer solution was filtered in the column to the surface of the gel and slowly and carefully 10 ml of toxin or toxoid was added to the surface of the gel. After filtration of the toxin or toxoid the walls of the column were rinsed several times with buffer solution (in a total volume up to 6 ml) and elution began. The liquid flowing out of the column was subjected to investigation. 10 ml samples were taken (the flow rate equaled 10 ml in 18--20 minutes). A 15--20 cm high layer of buffer solution was maintained constantly over the upper layer of gel.

In samples of the liquid, we determined the content of total nitrogen (Conway method), protein (Loury method, reaction with sulfosalicylic acid, and based on optical density at 280 mμ), extinction (on a FEK) [FEK = photoelectrocolorimeter], and biological activity (on mice). In some of the tests we determined the antigenic structure in the fractions by counter immunoprecipitation according to Okli or Ouchterlony.

Particular attention was given to the method for determining protein in the fractions. Most often of all, during filtration through Sephadex the quantity of protein is determined on a spectrophotometer based on the absorption of solutions at 280 mμ (Khavkin, 1963; Shemanova et al., 1963; Perelmutter et al., 1963). However, as Ginodman (1964) points out, this method is not strictly quantitative since the extinction coefficients for proteins vary considerably depending on the content of aromatic amino acids in them. Besides this, the capability for absorbing light at 280 mμ is possessed by tyrosine, tryptophan and peptides containing them (Doyti and Geydushek, 1956). Therefore the peaks of optical density obtained on the spectrophotometer show not only the content of protein, but also the content of many compounds possessing the property of absorbing light at 280 mμ .

The content of protein in fractions is also determined by the method of Loury-Folin (Shemanova et al., 1963; Nezlin and Kulpina, 1964; Nezlin, 1964), which is based on the complex application of the biuret method and a phenol reagent. However, the Loury-Folin method is not strictly specific, since during the interaction with the Folin reagent a positive reaction is caused by the aromatic amino acids and their peptides. With polypeptides, beginning with the tripeptides, a positive biuret reaction is obtained.

When working with serum proteins or concentrated toxins the influence of low molecular compounds can be ignored, since their relative contant is at a minimum. When working with nutrient media and native unconcentrated toxins and toxoids in which the content of general nitrogen exceeds the content of protein nitrogen by 30--100 times, the determination of the protein in the fractions just based on optical density at 280 mμ and the Loury method may be insufficient.

Therefore we additionally used the reaction with sulfosalicylic acid. The latter is a sufficiently sensitive reagent for protein, exposing it in a concentration of 0.015 0/00. Depending on the amount of protein, the formation of a flocculent precipitate, turbidity or a light opalescence is observed. Sometimes high molecular compounds may also cause a positive reaction, but the cloudiness caused by the presence of high molecular peptides disappears during heating (Predtechenskiy et al., 1960).

We used the following method: To 2 ml of fraction we added 6 drops of a 30% solution of sulfosalicylic acid, agitated it, and after an hour subjected it to photometry on an FEK-57 with a green light filter (590 mμ); the amount of protein in the samples was judged according to the magnitude of extinction. The absolute amount of protein can be determined by means of comparison with a standard curve, but as a rule the greatest interest lies in the relative content of protein. This is guided by the desire for the most rapid selection of samples for biological investigation.

We established the extinction in the separate samples for judging on the rapidity of filtration of the pigments on the FEK-57 with a green light filter (530 mμ) in 10 or 5 mm wide cuvettes depending on the pigmentation of the initial toxins and toxoids.

By filtration of botulin toxins and toxoids through Sephadex G-100 gel these preparations can be separated into fractions. As can be seen from figures 1 and 2, during the calculation of optical density at 280 mμ and by the Loury--Folin reaction 2 peaks appear: In 600 x 24 columns the first peak was small with a maximum in the area of disposition of fractions 6--8, and the second peak - many times higher with the maximum in the area of fractions 19--22. When determining the content of total nitrogen by the method of mineralization we established the presence of one peak with a maximum in the area of fractions 19--22. When the reaction with sulfosalicylic acid is set up 2 peaks of turbidity are apparent. One peak was located in the area of fractions 6--8 and conformed with the first peak of optical density at 280 mμ and the Loury-Folin reaction. All the antitoxin-binding activity of the toxoids and the basic toxicity of the toxins were developed in the samples which caused this peak. The second peak of turbidity with sulfosalicylic acid was found in the area of fractions 10--13 and matched up with the lower ascending part of the second peaks of optical density at 280 mμ and the Loury-Folin reaction. It should be pointed out that during the separation of type A botulin toxins on Sephadex G-100, the toxicity for mice, which however comprises less than 10% of the

total toxicity exposed after separation, corresponded to the second peak. The relative dimensions of the first and second peaks of turbidity with sulfosalicylic acid, obtained by filtration, were different in the type A and C toxins and toxoids; for type C toxins and toxoids the first and second peaks of turbidity with sulfosalicylic acid were approximately the same, and for type A toxins and toxoids the second peak was considerably lower than the first.

For the purpose of refining the approximate molecular weight of protein, eluted with the various fractions in the columns used (600 x 24 mm), we successively carried out the filtration of albumin and globulin, obtained by fractionation of horse serum protein with the help of ammonium sulfate. Gammaglobulin (molecular weight around 160 000) came out freely from the column and corresponded to fractions 6--8, that is, to the first peak of optical density at 280 mμ, the Loury--Folin reaction, and to the first peak of turbidity with sulfosalicylic acid. Albumin (molecular weight 65 000) was eluted with fractions 9--11 and corresponded to the second peak of turbidity with sulfosalicylic acid.

Thus it can be roughly considered that when using 600 x 24 mm columns, proteins with a molecular weight greater than 100 000 will be eluted with fractions 6--8, proteins with a molecular weight close to 65,000 - with fractions 9--11, and further the low molecular proteins, peptides and free amino acids will be eluted successively. In 500 x 22mm columns proteins with a molecular weight greater than 100 000 came out with fractions 5--6, and proteins with a molecular weight close to 65,000 - in fractions 8--10.

In analyzing the mutual disposition of peaks, exposed by the various methods of calculation (spectrophotometry at 280 mμ, determination of total nitrogen, Loury-Folin reaction, extinction on a spectrophotometer, turbidity with sulfosalicylic acid), it can be noted that the first peak, corresponding to proteins with a molecular weight greater than 100 000, and almost all the toxic and antitoxin-binding activity for mice were revealed both by spectrophotometry at 280 mμ and the Loury-Folin method and by turbidity with sulfosalicylic acid. Other peaks were not revealed by all the tests. The second peak, located in the area of fractions 9--11 and made up of proteins with a molecular weight close to 65,000, was detected usually only by the reaction with sulfosalicylic acid. This peak corresponded to the beginning of the ascending sector of the peaks revealed with the help of the Loury-Folin reaction, by means of determining the peaks of optical density at 280 mμ and total nitrogen by the mineralization method. We consider that under the stated conditions of separation on Sephadex G-100 there was no clear separation of proteins with a molecular weight on the order of 65,000 from lower molecular compounds, the content of which exceeded the content of protein by many times. Therefore it was all the more expedient to use the reaction with sulfosalicylic acid. This made it possible to get an idea of the nature of the compounds corresponding to the area of disposition of fractions 9--11.

The coincidence of the large peaks of optical density at 280 mμ, the Loury Folin reaction and the content of total nitrogen with a maximum for all

three indices corresponding to fractions 19--22, testified that the main mass of low molecular compounds are included in the latter. The maximum in the area of fractions 16--18 corresponded to pigments determined by extinction on the FEK-57.

In summarizing the results of studying the different fractions with the help of the various tests, it should be noted that during separation of type A and C botulin toxins and toxoids on Sephadex G-100 it is possible to obtain 4 peaks, formed by compounds with different molecular weights. On 600 x 24 mm columns proteins with a molecular weight greater than 100 000 were isolated first and formed a peak in the area of fractions 6--8. This peak mainly characterizes the toxic and antigenic activity of toxins and toxoids of these types.

The second peak, corresponding to fractions 10-- 3, was formed by proteins with a molecular weight close to 65,000. In some of the tests a toxic activity, making up less than 10% of the toxicity of the first protein peak, was revealed in it. In order to resolve the problem of whether or not this toxicity is inherent to proteins with a molecular weight close to 65,000, or whether the partial entry of high molecular proteins into the second protein peak took place, it is necessary to measure the molecular weights of the botulin toxins by the method of filtration through gel.

The third peak with a maximum in the area of fractions 16--18 corresponded to pigments, determined during the determination of extinction in the visible spectrum. The fourth peak, formed by low molecular nitrogen compounds, had the maximum in the area of fractions 19--22.

The borders between the second, third and fourth peaks are unclear, diffuse, and the peaks may be captured only under the condition that various tests are used.

The initial preparation and the fractions obtained during filtration through gel were investigated in the precipitation reaction in agar with purified concentrated (diaferm-3 method) antibotulin serum. When carrying out verticle diffusion by the Okli method it was shown that the antigenic composition of fractions was heterogeneous; usually 2--3 precipitation bands were detected in each fraction. It should be noted that the rings in the various fractions appeared at a various height; this permitted the proposal that the antigenic structure of the fractions is different, and that the fractions include proteins with an equal molecular weight but a different antigenic structure. The antigenic spectrum of the fractions was reconstructed on the basis of the results (fig. 3).

For the purpose of establishing the identity of antigenic structures we carried out a counter immunoprecipitation in agar by the Ouchterlony method (figure 4). The fractions, corresponding to the first and second peaks of

turbidity with sulfosalicylic acid, were concentrated by dialysis against gum arabic and placed in the wells. The counter immunoprecipitation observed here testified to the nonidentity in the antigenic structures of the first and second protein peaks.

The fractions bearing the main antigenic load contained a very small amount of nitrogenous substances. The load (in units of binding per 1 mg of total nitrogen) in active fractions was up to 1700 EC for the toxoids of type A and C, with a load per 1 mg of total nitrogen in the initial type C toxoids equal to 14--50 EC, and in the type A toxoids - equal to 10 EC.

This fact points to the expedience of studying the feasibility of obtaining highly purified preparations by means of filtration through Sephadex gel.

Conclusions

1. By filtration thorugh Sephadex G-100 dextran gel it is possible to separate type A and C botulin toxins and toxoids into fractions.

2. The first fraction isolated by filtration through gel contained proteins with a molecular weight greater than 100,000; it corresponded to the antitoxin-binding activity of the toxoids and the partial lethal activity of toxins.

3. Filtration through gel makes it possible to separate botulin toxins and toxoids into fractions which are different in their antigenic spectrum.

4. By filtration of botulin toxoids through Sephadex gel it is possible to purify the active fractions from ballast low molecular compounds. ~~It is~~ expedient to make a further study of problems related to the purification of botulin toxoids by filtration through gel.

Literature

Ginodman, L. M., In the book: Modern Methods in Biochemistry, Moscow, 1964, vol 1, p 37.

Yevtikhina, Z. F., Orekhovich, V. N., Shpikiter, V. O., Vopr. med. khimii, 1963, No 6, p 626.

Nezlin, R. S., Biokhimiya, 1964, No 3, p 548.

Nezlin, R. S., Kulpina, L. M., Vopr. med khimii, 1964, No 5, p 543.

Predtechenskiy, V. Ye., Smirnova, L. G., Kost, Ye. A., Handbook on Clinical Laboratory Investigations, Moscow, 1960.

Tikhomirova, G. V., In the book: Use of New Methods in the Study and Preparation of Vaccines and Toxoids, Moscow, 1964, p 36.

Khavkin, Yu. A., Morozova, M. A., Kristallinskaya, N. S., Vopr. med. khimii, 1963, No 5, p 526.

Shemanova, G. F., Vlasova, Ye. V., Shamrayeva, S. A., et al., In the book: Problems of the Biology of Microorganisms and Applied Immunology. Kishinev, 1963, p 93.

Davidson, W. D., Sackner, M. A., Davidson, M. H., J. Lab. clin. Med., 1963, vol 62, p 501.

Doti, P., Geydushek, Ye., In the book: Proteins, Moscow, 1956, vol 2, p 363.

Feinstein A., Gell, P. G., Kellus, A. S., Nature, 1963, v 200, p 653.

Fireman, P., Vannier, W. E., Goodman, H. C., Proc. Soc. exp. Biol., (N.Y.), 1964, v. 115, p. 845.

Johansson, B. G., Rymo, L., Acta chem. scand., 1964, v. 18, p 217.

Killander, J., Hogman, C. F., Scand. J. clin. a. Lab. Invest., 1963, v 15, Suppl. 69, p 130.

Miettinen, T. A., Clin. Chim. Acta, 1963, v 6, p 693.

Perelmutter, L., Devlin, W., Stephenson, N. R., Canad. J. Biochem., 1963, v 41, p 2493.

Phillips, A. W., Batty, J., Nature, 1963, v 198, p 1200.

Porath, J., Flodin, P., Ibid., 1959, v 183, p 1657.

Sakaguchi, G., Sakaguchi, S., Imai, N., J. Bact., 1964, v 87, p 401.

Scandurra, R., J. Biochim., 1963, v 12, p 159--168.

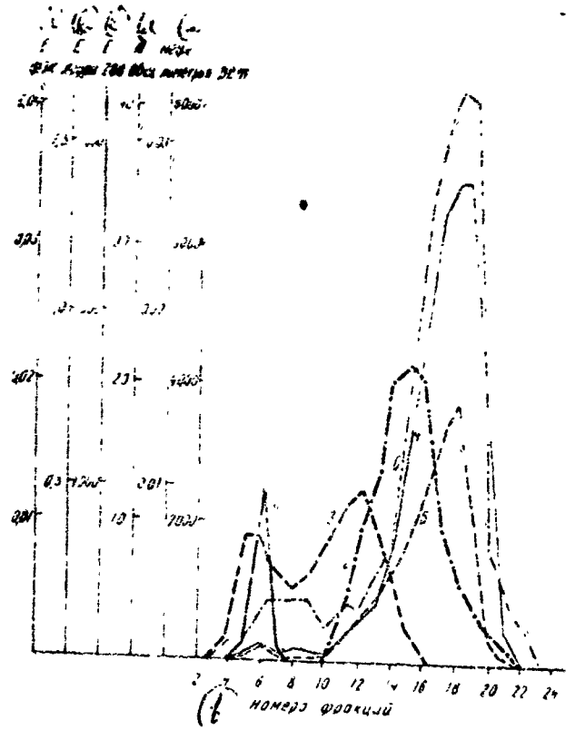

Figure 1. Separation of botulin toxoid type C (series I) by filtration through Sephadex G-100 gel.

1 - Dlm determined on mice; 2 - extinction on FEK-M; 3 - nephelometry with sulfosalicylic acid; 4 - extinction at 280 mμ; 5 - extinction with Loury-Folin reagent; 6 - total nitrogen (in mg %).

a - E-FEK; b - E-Loury; c - E-280; d - Total nitrogen; e - Nephelometry; f - Number of fraction.

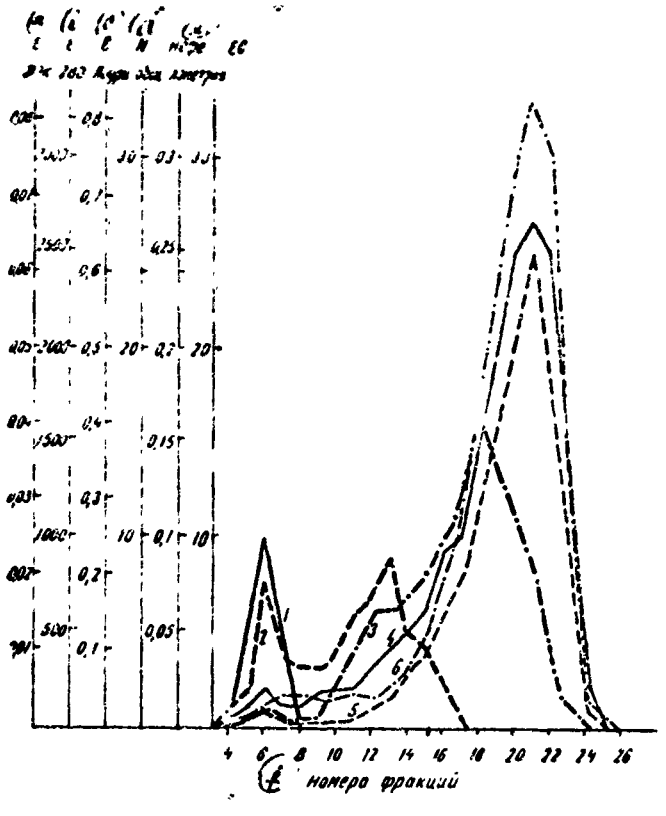

Figure 2. Separation of botulin toxin type C (flask 38) by filtration through Sephadex G-100 gel.

1 - EC determined on mice; 2 - nephelometry with sulfosalicylic acid; 3 - extinction on FEK-M; 4 - extinction at 280 mμ ; 5 - extinction with Loury-Folin reagent; 6 - total nitrogen (in mg%).

a - E-FEK; b - E-280; c - E-Loury; d - Total nitrogen; e - Nephelometry; f - Number of fractions.

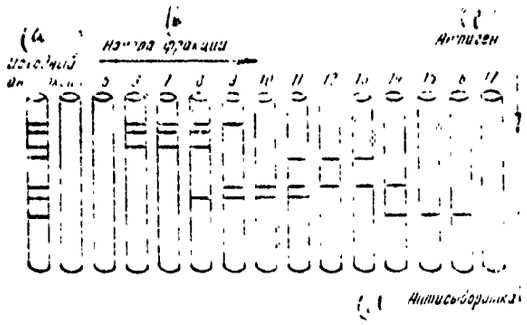

Figure 3. Scheme for the antigenic spectrum of native botulin toxoid type A (flask 31) and the fractions obtained from it on a column with Sephadex G-100 gel.

a - initial toxoid; b - number of fraction; c - antigen; d - antiserum.

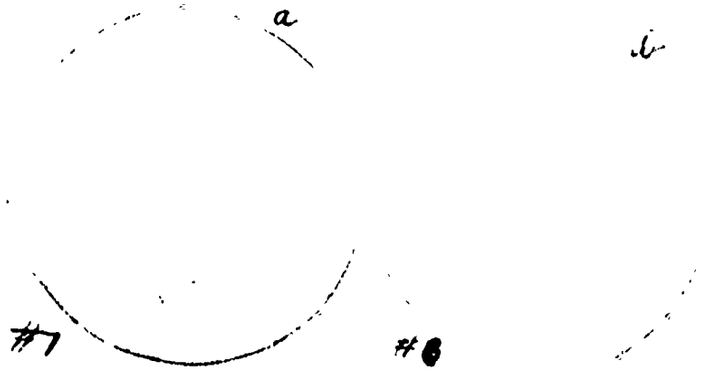

Figure 4. Immunoprecipitation in agar by fractions of botulin toxins.

a - toxin type C (test No 7); b - toxin type A (test No 6). Upper hole - specific serum, left hole - fractions of the first peak, developed by sulfosalicylic acid, right hole - fractions of the second peak, developed by sulfosalicylic acid.