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Immunochemical Studies of Two Cholera Toxin-Containing Standard Culture Filtrate Preparations of *Vibrio cholerae*

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Two crude toxin preparations of *Vibrio cholerae*, labeled lot 4493 G (Inaba) and lot 001 (Ogawa), consisting of freeze-dried culture filtrate, were studied with regard to toxicity, precipitinogenicity, and chemical composition. Lot 4493 G contained much more carbohydrate and less protein than lot 001, but both of the preparations contained three precipitinogens which were identical. One of these factors was identified as cholera exotoxin. In addition, both contained a fourth cross-reacting precipitinogen related to lipopolysaccharide. The toxicity of the lots was very similar. The toxin active as permeability factor in the intradermal test, identified completely in comparative double-diffusion analyses with the toxin causing cholera-like symptoms in experimental animals, strongly indicated that the same toxin molecule was active in the two different model systems. The isoelectric point of the toxin was about *p*H 7, but some toxic activity focused toward a *p*H of 9. The toxicity was completely retained by a UM-10 membrane (cut at a molecular weight of 10,000), practically retained by a PSED membrane (cut at a molecular weight of 25,000), but not retained by an XM-50 membrane (cut at a molecular weight of 50,000). By gel filtration through agarose, it was possible to separate and purify more than 1,000-fold the toxin from the other, more rapidly filtering three precipitinogens. With lot 4493 G, the toxicity of the agarose gel filtration fractions was restricted to an elution volume similar to that of globular proteins with a molecular weight of 25,000 to 38,000, whereas gel filtration experiments through Sephadex G-75 indicated a size of the toxin corresponding to a molecular weight of about 55,000 to 60,000.

The essential features of cholera can be produced in experimental animals by cell-free culture filtrates of *Vibrio cholerae*. The available data recently reviewed by Burrows (2) indicate heat-labile, nondialyzable type 2 toxin(s) is responsible for the profuse diarrhea and excessive fluid loss so characteristic for the disease. Toxic activity is demonstrable in animal models as movement of fluid from the tissues into the gut lumen. The same toxic substance, tentatively called choleragen, is presumably responsible for this effect in all the three model systems employed, i.e., the infant rabbit (8), the dog (16), and the ligated ileal loop of the adult rabbit (7). These tests for toxicity are, however, laborious in comparison with the intradermal toxicity test elaborated by Craig (4). The toxin active in this test has been designated permeability factor (PF), and its relation to choleragen has not yet been definitely settled (2). Benenson et al. (1) demonstrated in humans an increase in PF-neutralizing antibodies in response to infection with *V. cholerae*. The role of these antibodies for protection in man remains to be clarified, but support for a protective effect of toxin-neutralizing antibodies has been obtained from canine cholera (6).

Recently, two preparations of freeze-dried culture filtrate containing toxin have been made available for investigation by the National Institutes of Health (NIH), Bethesda, Md. This report concerns the immunochemical characterization of these preparations and their derivates, and aims to identify and study the toxin as it appears as PF. The latter task seemed essential since two research groups (3, 10), claiming to have prepared choleragen in a highly purified state, reported such different properties of the toxic substance that "the question is raised as to whether it is the same substance" (10).

MATERIALS AND METHODS

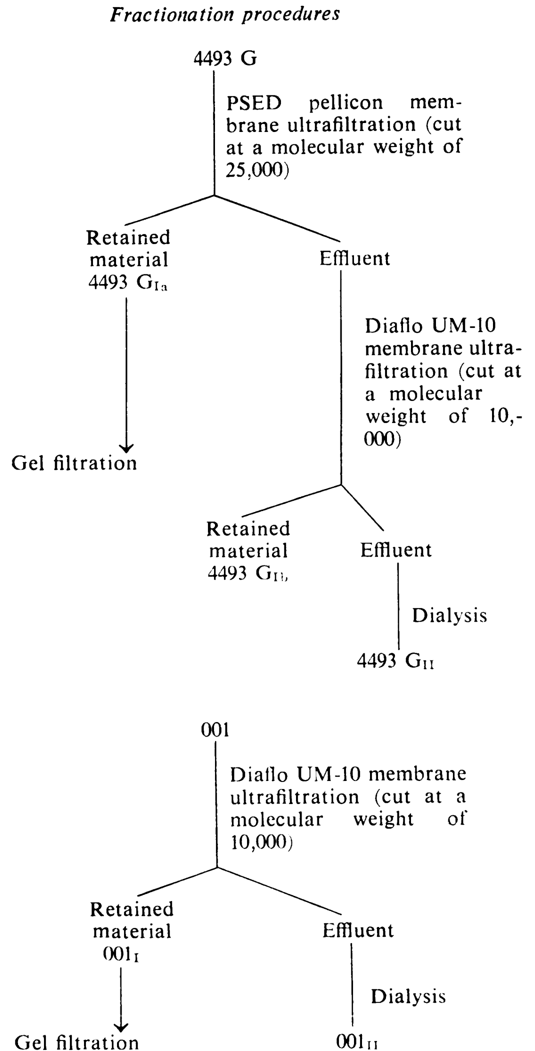
Starting materials. Freeze-dried filtrate from cultures of *V. cholerae* strain 569 B serotype Inaba,

grown in a modified syncase medium (9) with sucrose content reduced to 0.1% at 30 C for 24 hr under aeration and with 1.0% beta lactose added as a stabilizer during drying, was obtained from NIH. This material, NIH label "cholera toxin (Finkelstein method) lot 4493 G," is referred to with its lot number. The same designation principle will be followed for the other toxin preparation distributed by NIH with the label "cholera toxin (Craig method) lot 001." The latter preparation consisted of freeze-dried filtrate from cultures of *V. cholerae* strain B 1307 serotype Ogawa, grown in a medium containing 2% peptone broth (Difco) and 0.5% NaCl, adjusted to *p*H 7.3 with NaOH (5). Data provided by the distributer on the toxicity of the two preparations reveal that the filtrates have been active in the canine model, in the ligated rabbit ileal loop model, as well as in the intradermal toxicity test, with lot 4493 G probably being slightly more toxic on a weight basis than lot 001.

For reference purposes, purified choleragenoid (10), i.e., low-toxic substance having identical antigenic determinants with the highly toxic purified choleragen, and its corresponding antiserum anticholeragenoid were used. These preparations were kindly provided by R. Finkelstein, Dallas, Tex. Also, lipopolysaccharide, prepared by means of a phenol-water extraction process from vibrios of the serotype Inaba and purified as described for *Escherichia coli* lipopolysaccharides (15), was employed, together with rabbit hyperimmune serum against Inaba vibrios (Holmgren, Svennerholm, and Ouchterlony, Acta Pathol. Microbiol. Scand. Sect. B, *in press*).

Fractionation by ultrafiltration through defined membranes. For fractionation (*see* also schematic diagram), 10.0 g of lot 4493 G was dissolved in 200 ml of distilled water and ultrafiltered under stirring at a positive pressure of 7 kg/cm² through a PSED pellicon membrane (Millipore Corp., Bedford, Mass.), which is reported by the manufacturer to retain spherical proteins with a molecular weight higher than approximately 25,000. Tris(hydroxymethyl)aminomethane (Tris)-ethylenediaminetetraacetic acid (EDTA) buffer (*p*H 7.5; 0.05 M Tris, 0.001 M EDTA disodium salt, 0.003 M NaN_3, 0.04 M NaCl) was added repeatedly during the ultrafiltration process for washing of retained material, which was then lyophilized and designated 4493 G_{Ia}. The ultrafiltrate was refiltered under the same conditions through a Diaflo UM-10 membrane (Amicon Corp., Lexington, Mass.), stated to cut at a molecular weight of about 10,000. The retained material was labeled 4493 G_{Ib} after lyophilization. The ultrafiltrate was put into a dialysis bag, concentrated against polyethylene glycol 20 M (Kebo AB, Stockholm, Sweden), and finally dialyzed against 0.5% NaCl, lyophilized, and designated 4493 G_{II}. In later experiments with lot 4493 G, the filtration through the PSED membrane was excluded, and this modified procedure was also used for the fractionation of lot 001, where the material retained by the UM-10 membrane was labeled 001_I and the dialyzed freeze-dried ultrafiltrate, 001_{II}.

In some experiments, 4493 G_{Ia} was filtered during



stirring through a Diaflo XM-50 membrane (Amicon, Co.) at a positive pressure of 1.8 kg/cm² or in some experiments 7 kg/cm², and the toxicity was estimated in the retained and in the filtered portion as described below.

Fractionation by gel filtration. For further fractionation of components, 1.0 g of 4493 G_{Ia} or 001_I was dissolved in 20 to 30 ml of Tris-EDTA buffer, *p*H 7.5 (0.05 M Tris, 0.001 M EDTA, 0.003 M NaN_3, 0.2 M NaCl), and the solution was subjected to gel filtration on a column (5 by 125 cm) of Agarose (Biogel A-5m, 200 to 400 mesh; Bio-Rad Lab., Richmond, Calif.) by using the Tris-EDTA buffer for elution. Flow rate was kept at 60 ml/hr, and the transmission through the eluate was measured continuously at 254 nm. The eluate was collected in 25-ml fractions which were then pooled and designated as

shown in Fig. 5. For the immunodiffusion studies, it was concentrated 30- to 60-fold against polyethylene glucol. The elution volumes from the agarose column of human serum immunoglobulin (Ig)M, IgA, IgG and albumin, ovalbumin, pepsin, and lysozyme were also determined for preparation of a plot curve for elution volume/molecular weight.

Also, 4493 G_{Ia} was subjected to filtration through a column (2.5 by 90 cm) of Sephadex G-75 (Pharmacia, Uppsala, Sweden) in two different buffers (Tris-EDTA buffer with 0.2 M NaCl or 0.04 M NaCl, *p*H 7.5). Flow rate was 20 ml/hr, and transmission through the eluate was continuously registered at 254 nm. The eluate was collected in 6-ml fractions. For calculation of the molecular size of the PF, the elution volume of the PF activity was compared with the curve drawn through the plotted values of elution volume against the molecular weight for the marker substances of human serum albumin, sheep hemoglobin, ovalbumin, and ribonuclease A filtered through the Sephadex G-75 column.

Isoelectric focusing. Isoelectric focusing was performed in polyacrylamide tubes in a chemical polymerization modification (19). The cylindrical gel was cut in 15 pieces which were eluted with distilled water in the cold, overnight. The *p*H was estimated and adjusted to 7 to 8 before determination of toxicity.

Chemical methods. Nitrogen content was estimated by a semi-micro Kjeldahl method (12) and carbohydrate by the alpha-naphthol method (20). Protein content was determined by a modified Folin-Ciocalteau reaction with human IgG as standard (13). Due to lack of material, only the standard and a few of the samples could be tested also by the biuret method.

Toxicity determination. Intradermal toxicity tests were performed in rabbits as described by Craig (4). The smallest amount of material in a 0.1-ml volume, required to produce a blue area with a diameter of 7 mm, was determined in duplicate or triplicate experiments in each of two animals and is referred to as minimal blueing dose (1 BD).

Preparation of antisera. Antisera were prepared in rabbits against lots 4493 G and 001 (three animals per antigen), respectively, by three subcutaneous injections of 5 mg of the preparations in Freund's complete adjuvant, followed after a test-bleeding by two intravenous injections of 5 mg of the lots without adjuvant. The injections were given once a week, all of them in a 2-ml volume. Eight days after the last injection, the rabbits were sacrificed by heart puncture under barbiturate anesthesia. After clotting of the blood at room temperature, serum was dispensed in 0.5-ml samples and kept without preservative at −30 C until use. The antisera are designated anti-4493 G and anti-001, respectively.

Immunodiffusion methods. Double diffusion in gel was performed in a microplate modification (17), with the wells in the plexiglass matrices taking about 25 μliters of reactant. For quantitation, dilutions of material were tested, and positive reactions were registered as deviation of a reference antigen-antibody precipitation line (14). This is a very sensitive method compared to other immunodiffusion methods, revealing C-reactive protein at concentrations of 0.5 μg/ml (14) and albumin at 1.0 μg/ml (*unpublished results*). Immunoelectrophoresis was performed as described by Wadsworth and Hanson (18).

PF-neutralizing antibody determination. Titration of PF-neutralizing antibodies was performed as described by Benenson et al. (1).

Vibriocidal antibody determination. Titration of vibriocidal antibodies was performed by means of a spot agar plaque method of Holmgren, Svennerholm, and Ouchterlony (Acta Pathol. Microbiol. Scand., Sect. B, *in press*), employing 35 A3 Inaba vibrios and NIH 41 Ogawa vibrios.

RESULTS

Studies on lot 4493 G and its ultrafiltration fractions. The protein content of lot 4493 G was 2.2%, the nitrogen content was 2.3%, and carbohydrate was 26.7%. The 1 BD was estimated at 7.5 μg. In double-diffusion experiments employing anti-4493 G, four precipitates (numbered 1 to 4 in order from the antigen well) were registered (Fig. 1). Lot 4493 G dissolved in Tris-EDTA buffer was separated into fractions according to retention of material by various filters. Recovery and some properties of the obtained fractions from 10 g of starting material are given in Table 1. Apparently, only 30% of the starting material was recovered, the rest being dialyzable. The fraction 4493 G_{Ia}, retained by the pellicon membrane (cutting at a molecular weight of 25,000), contained all the four precipitinogens present in the starting material, whereas 4493 G_{Ib}, passing this membrane but retained by the UM-10 filter (cutting at a molecular weight of about 10,000), only contained the precipitinogens forming the precipitates 2 and 4 (Fig. 1). Fraction 4493 G_{II} passing also the UM-10 filter was nonprecipitinogenic and showed very low toxicity in comparison with the other fractions (Table 1).

The retention of toxicity by XM-50 membranes was also tested employing 4493 G_{Ia}. Different results were obtained when the ultrafiltration was performed at different pressures. Although at low pressure (1.8 kg/cm²) practi-

TABLE 1. *Recovery, chemical properties, and toxicity of membrane filtration fractions of lot 4493 G in comparison with the starting material*

Material	Dry weight (g)	Nitrogen content (%)	Protein content (%)	Carbohydrate content (%)	BD[a] (μg)
4493 G	10.0	2.3	2.2	26	7.5
4493 G_{Ia}	2.0	2.6	6.6	17	0.5
4493 G_{Ib}	0.05	1.1	6.5	10	0.5–2.5
4493 G_{II}	1.0	0.2	0.2	1.5	800–8,000

[a] Blueing dose.

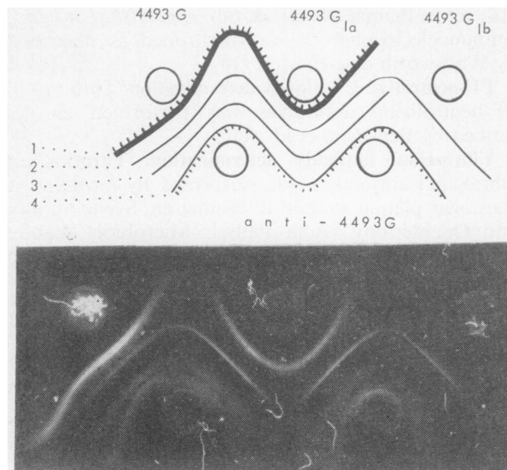


FIG. 1. *Comparative double-diffusion analysis of 4493 G, 4493 G_{Ia}, and 4493 G_{Ib} with anti-4493 G.*

cally no toxic material was retained, most of the toxicity was retained at high pressure (7 kg/cm^2). This initiated a more detailed study of the influence on the permeability of XM-50 of the parameters pressure, stirring intensity, and concentration of material employing ovalbumin (molecular weight of 46,000) as test substance. From these experiments it can be concluded that, to avoid undue effects at the membrane caused by a solute layer, the concentration and pressure should be kept low and the stirring intensity kept high.

The purified choleragenoid used as a reference gave one, single precipitate with anti-4493 G diluted down to 1/250. This precipitate fused in comparative double-diffusion analyses with the number 2 precipitate formed by the same antiserum and 4493 G (Fig. 2). Since the choleragenoid is antigenically identical to choleragen (10), it can be concluded that the antigen involved in precipitate 2 (precipitinogen 2) is identical to choleragen with respect to antigenic determinants. About 25 μg of 4493 G_{Ia} was the lowest detection level for precipitinogen 2 in comparative double-diffusion analyses as compared to 25 ng for choleragenoid, and 1,000 times more was needed of 4493 G_{Ia} than of choleragenoid to inhibit the PF-neutralizing capacity of anti-4493 G.

After isoelectric focusing of 4493 G_{Ia}, the bulk of the toxicity appeared at about *p*H 7, but some activity was also noted at *p*H 9. The latter activity, however, differed in various batches of 4493 G_{Ia}.

Studies on lot 001 and its ultrafiltration fractions. Table 2 gives the results obtained on some properties of 001 and the fractions 001_I, consisting of UM-10 (cut at a molecular weight of 10,000) retained material, and 001_{II}, constituting the nondialyzable UM-filtrable material.

With its homologous antiserum, lot 001 gave four precipitates (labeled 1 to 4 in order from the antigen well) the second of which fused with the precipitate formed by choleragenoid and anti-001 (Fig. 3). Fraction 001_I contained the same four precipitinogens demonstrated in 001 and, in addition, gave an inconsistently appearing precipitate with anti-001 located between the number 2 and 3 precipitates (Fig. 3). In 001_{II} no precipitinogens could be demonstrated.

After isoelectric focusing of 001_I, most of the toxicity appeared at *p*H 7, but some toxic activity, different in various batches, also was registered at about *p*H 9.

Comparison of lots 4493 G and 001. In comparative double-diffusion analyses, the precipitinogens 2 to 4 in lot 001 identified with the correspondingly numbered precipitinogens in lot 4493 G or in the 4493 G_{Ia} fraction (Fig. 4). Also,

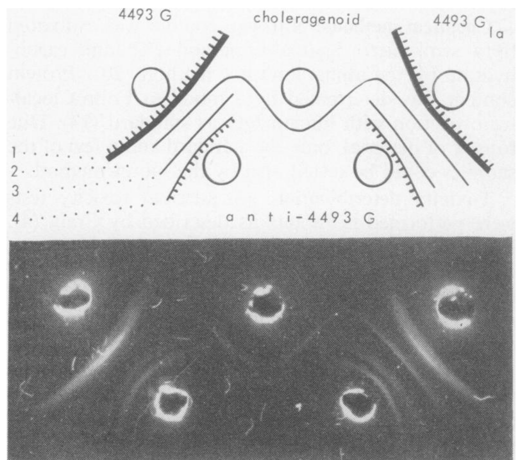


FIG. 2. *Comparative double-diffusion pattern of purified choleragenoid 4493 G and 4493 G_{Ia} developed with anti-4493 G.*

TABLE 2. *Recovery, chemical properties, and toxicity of membrane filtration fractions of lot 001 in comparison with the starting material*

Material	Dry weight (g)	Nitrogen content (%)	Protein content (%)	Carbohydrate content (%)	BD[a] (μg)
001	10.0	12.0	29.2	0.6	9.0
001_I	2.1	14.0	50.4	0.7	0.5
001_{II}	5.2	0.7	1.9	0.3	200–2,000

[a] Blueing dose.

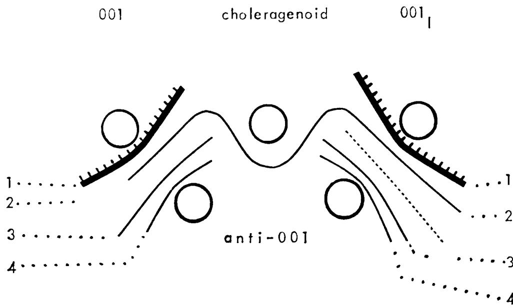


FIG. 3. *Purified choleragenoid, compared in double-diffusion experiments with 001 and 001_I against anti-001. Dotted line represents on inconsistently appearing precipitate.*

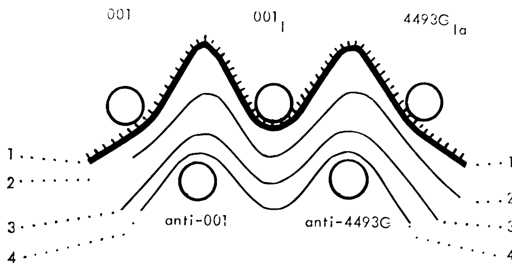


FIG. 4. *Comparative double-diffusion analysis of 001, 001_I, and 4493 G_{Ia} with anti-001 and anti-4493 G.*

the number 1 precipitinogens in the two preparations had at least one antigenic determinant in common (Fig. 4). The poor diffusibility of these antigens, together with the fuzzy precipitates they formed with antiserum, did, however, not allow the exclusion of unshared determinants. The antisera against the two lots neutralized the PF activity of the heterologous lot in the same titer as the homologous one (this titer being 1/1,250 to 1/5,000 in antisera from six rabbits). These antisera also contained vibriocidal antibodies in titers exceeding 1/250,000 against the homologous serotype (Inaba or Ogawa) and in three- to ninefold lower titers against the heterologous serotype.

Agarose gel filtration studies. To separate the antigenic components and thereby purify the toxin and the endotoxin, 4493 G_{Ia} was subjected to gel filtration through agarose. The fractions, pooled as indicated in Fig. 5, were then tested for PF content in the intradermal toxicity test and after 30- to 60-fold concentration were analyzed with regard to precipitinogen content by comparative double diffusion. The data from one of three experiments giving very similar results are listed in Fig. 5. The intradermal toxicity was confined to fractions 6 to 8 (1 BD = 10^{-3} to 3×10^{-3} μg in fraction 7) with an elution volume similar to that of proteins with molecular weight of 25,000 to 38,000. Precipitinogen 2 was demonstrated by means of comparative double diffusion as the only demonstrable precipitinogen in fractions 6 and 7 and in trace amounts in fraction 8 (Fig. 6) and retained full determinant identity with purified choleragenoid in comparative double-diffusion analyses with anti-4493 G or anticholeragenoid. Incubation of the fractions 6 to 8 with anti-4493 G or with anticholeragenoid removed the precipitinogenicity and resulted in disappearance of the PF activity of the fractions. Incubation of these fractions at 56 C for 30 min also destroyed their toxic activity. The number 1 precipitinogen was found in fractions 1 and 2 with trace amounts also in fraction 3, and the remaining two antigens (3 and 4) were demonstrated in fractions 4 and 5 (Fig. 5 and 6).

Indication was obtained that precipitinogen 1 was related to lipopolysaccharide by the finding that incubation of the concentrated fraction 1 with anti-Inaba antiserum or anti-4493 G at 37 C for an hour completely inhibited the vibriocidal antibody activity of these antisera. Furthermore, precipitinogen 1 was resistant to boiling for an hour, gave a reaction of identity with purified Inaba lipopolysaccharide in comparative double-diffusion analyses, and showed an immunoelectrophoretic pattern very similar to that of the purified lipopolysaccharide when tested against anti-Inaba or anti-4493 G serum.

In later experiments, the initial PSED membrane filtration was omitted, and the fractions of lot 4493 G, nonfiltrable through UM-10 (cut at a molecular weight of 10,000), was subjected to agarose gel filtration. The transmission elution curve was identical to that of 4493 G_I and so were the toxicity elution values, with the exception that some toxicity (30 to 90 BD/ml as compared to 3,000 to 10,000 BD/ml in the peak fractions) appeared later than the bulk of toxicity and extended into an elution portion similar to that of lysozyme.

Also, 001_I was subjected to agarose gel filtration, and the fractions were tested in a similar way. The data from one of three experiments, giving very similar results, are shown in Fig. 5. Although the transmission curve was similar to that of 4493 G_I, the toxicity, as well as the precipitinogen 2, was spread over a considerably larger elution volume, but it was still possible to obtain fractions containing the precipitinogen 2 as the only demonstrable antigen.

Sephadex gel filtration studies. Fraction 4493

G_{Ia} was also filtered through a column of Sephadex G-75 employing varying ionic strengths of the elution buffer. The eluate was tested for PF activity and for presence of precipitinogens. It was found that the transmission curves were markedly different in two buffers (Fig. 7), but the elution volume of PF was unaffected by the buffer change, with the activity peak similar to that of a globular protein of a molecular weight of 55,000 to 60,000 (Fig. 7). In this elution region,

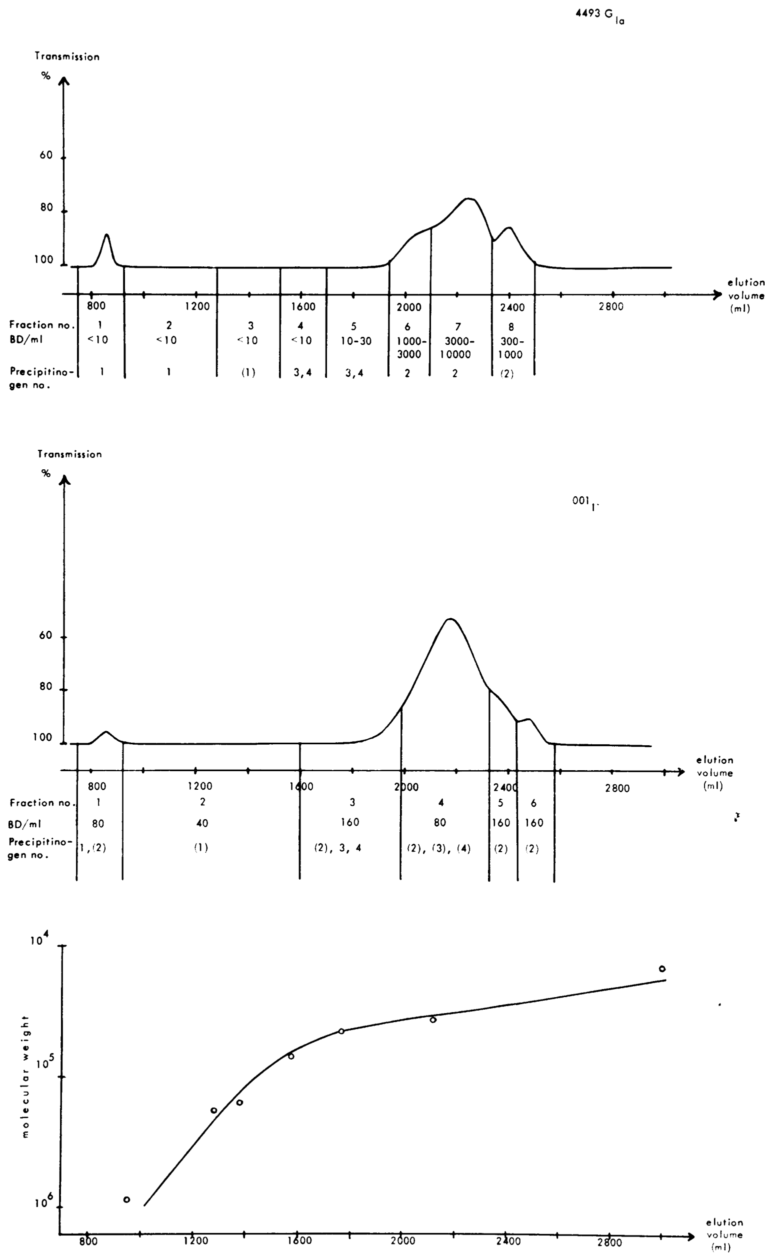


FIG. 5. *Elution curves of agarose gel filtration of 4493 G_{Ia} and 001I with precipitinogenicity and toxicity [blueing doses (BD)/ml] of the fractions indicated. Lower curve, dots indicate the elution volume plotted against molecular weight for the marker proteins from left to right: human serum immunoglobulin (Ig)M, IgA, IgG and albumin, ovalbumin, pepsin, and lysozyme.*

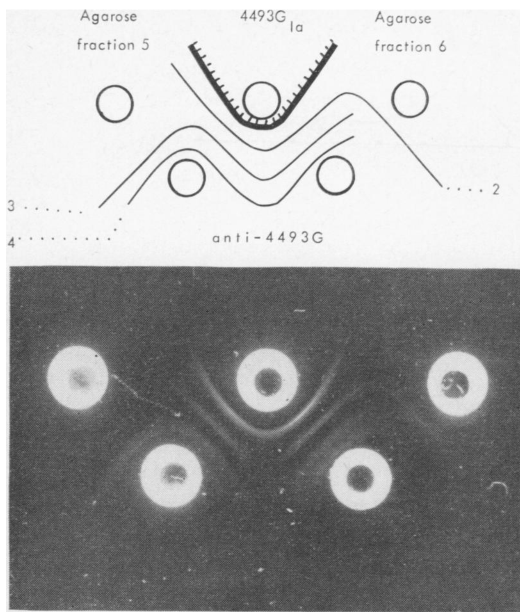


FIG. 6. *Comparative double-diffusion pattern of 4493 G_{Ia} and the agarose column fractions (see Fig. 5 and 6) developed with anti-4493 G.*

precipitinogen 2 was the only demonstrable precipitinogen.

DISCUSSION

The results presented show that the two lots of freeze-dried culture filtrate preparations, lot 4493 G and lot 001 distributed by NIH, each contain at least four precipitinogens, one of which was identified as toxin active in the intradermal skin test. Although originating from strains of different serotypes, the double diffusion, as well as neutralization, experiments indicated that the toxin is identical in the two preparations. Also, from a chemical point of view the preparations are inhomogeneous, [e.g., lot 4493 G contained as much as 70% dialyzable material probably mainly originating from the medium (9), which consists of dialyzable low-molecular-weight constituents]. About one-third of the nondialyzable part of lot 4493 G was filtrable through a Diaflo UM-10 membrane (stated to cut at a molecular weight of 10,000) and was found to be practically nontoxic in the intradermal test as well as nonprecipitinogenic. Of the material nonfiltrable through this membrane, 98% was retained also by a pellicon membrane stated to retain spherical proteins with molecular weight more than about 25,000, indicating that this fraction (4493 G_{Ia}) was of large molecular size.

Fraction 4493 G_{Ia} was toxic in a 0.5-μg amount, which was roughly a 10-fold concentration of the toxicity of the starting material and in the same range as the toxicity of the very small (in weight) fraction 4493 G_{Ib}, filtrable through the pellicon membrane but retained by the UM-10 filter. Both of these toxic fractions contained a precipitinogen which had identical antigenic determinants with the purified choleragen of Finkelstein and LoSpalluto (10), supporting the notion of these authors that PF and choleragen activities reside in the same molecule. About 1,000 times more material was needed of 4493 G_{Ia} than of purified choleragenoid for inhibition of the PF-neutralizing capacity of antiserum as well as for formation of a toxin (toxoid)-antitoxin precipitate. These findings indicate that approximately 0.1% of 4493 G_{Ia} consisted of toxin or toxoid. The finding of the BD of 4493 G_{Ia} being 0.5 μg, which is roughly 1,000-fold the value given by Finkelstein and LoSpalluto (10) for purified choleragen, is in accordance with this notion and indicates that the ratio of toxoid to toxin in 4493 G_{Ia} is low. In addition, toxins 4493 G_{Ia} and 4493 G_{Ib} contained at least three and one precipitinogens, respectively. One of these antigens, not present in 4493 G_{Ib}, was related to bacterial lipopolysaccharide and was responsible for the high vibriocidal antibody titers found in antiserum against lot 4493 G. The nature of the other two antigens remains to be clarified, although it can be concluded from gel filtration experiments on an agarose column that they, in similarity with the lipopolysaccharide-related antigen, are not associated with PF activity. In the latter experiments it was possible to obtain PF from 4493 G_{Ia} free from the other precipitinogens and active in the intradermal toxicity test in amounts down to 1 to 3 ng.

Molecular-size estimations of the PF gave inconclusive results. With lot 4493 G, on which the main work was done, no PF activity was retained by an XM-50 membrane when the ultrafiltration was performed at optimal conditions concerning pressure, stirring intensity, and concentration of material. This membrane is stated to cut at a molecular weight of about 50,000, but, from data presented by the manufacturer (21), it is evident that the permeability is dependent on molecular shape as well as size, since 80% retention is reported for pepsin (molecular weight of 35,000, low axial ratio) but only 10% retention is reported for dextran 110 (molecular weight of 110,000, high axial ratio). In the agarose gel filtration experiments all of the PF activity of 4493 G_{Ia} appeared in an elution volume corresponding to that of globular

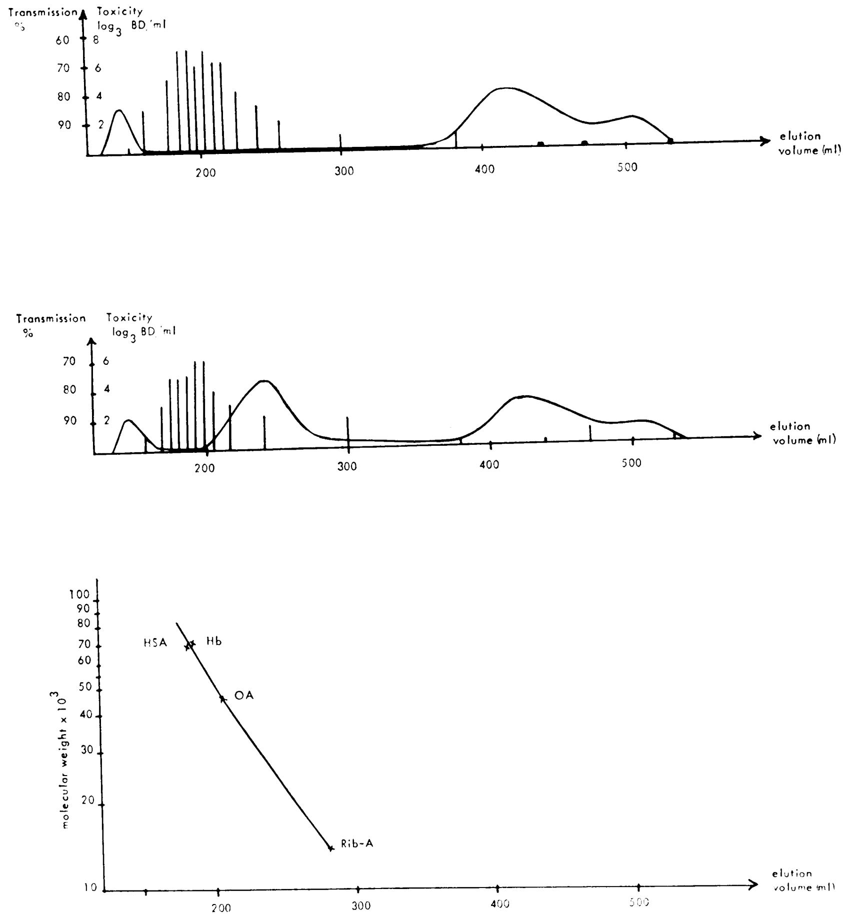


FIG. 7. *Elution curves with toxicity [blueing doses (BD)/ml] indicated as staples for filtration of 4493 G_{Ia} through Sephadex G-75 employing Tris-EDTA buffer with 0.04 M NaCl (upper curve) or 0.2 M NaCl (middle curve). Lowest is shown the elution volume-molecular weight curve, identical with both buffers, obtained with human serum albumin (HSA), human hemoglobin (Hb), ovalbumin (OA), and ribonuclease A (Rib-A).*

proteins with a molecular weight of 25,000 to 38,000, whereas the results from gel filtration experiments through Sephadex G-75 indicated a size of PF corresponding to a molecular weight of about 55,000 to 60,000. The filtration rate of the PF through Sephadex G-75 appeared unaffected by variations of the molarity of the buffer, but a change of this parameter caused an appreciable change of the protein elution curve employing 4493 G_{Ia} (Fig. 7). At low ionic strength, PF was eluted free from light (254 nm) absorbing material which might be considered for further purification of PF. Our experimental findings on the size of PF are very similar to those reported by Finkelstein and LoSpalluto (10) on choleragen, who on the basis of gel filtration experiments through Sephadex G-75 stated a molecular weight of 61,000 for the toxin, thereby neglecting the much lower value provided by their gel filtration experiments through agarose. The discrepant results on the molecular size of the toxin obtained by filtration through the two types of gel could be due to an ion exchange effect in the agarose, delaying the elution of the toxin, or to different aggregation states of the toxin. These hypotheses are, however, opposed by the finding that the filtration rates of PF through Sephadex G-75 and agarose were unaffected by wide changes of ionic strength of the eluting buffers.

The available data on the molecular size of PF could be consistent with a protein (10) molecule with a high axial ratio, but obviously the size and nature of the cholera toxin will need further studies to be definitely settled. This includes extended work on the electrical charge heterogeneity indicated from the isoelectric

focusing experiments. Most of the toxic activity focused toward a *p*H of 7, i.e., similar to the value reported for choleragen protein (11), but some activity was also found about *p*H 9. The relation, if any, between the toxic molecules studied by, on the one hand, Finkelstein and LoSpalluto and us and, on the other hand, by Coleman et al. (3) is not clarified. It would be interesting to study, by means of comparative double diffusion, the purified toxin of the latter authors (stated to have a molecular weight of about 12,000) with the antigenically identical toxin of Finkelstein and LoSpalluto and us to see whether the substances are identical according to the criteria of this technique.

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