PATHOGENESIS OF EXPERIMENTAL CHOLERA

PREPARATION AND ISOLATION OF CHOLERAGEN AND CHOLERAGENOID*

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Earlier reports (1–5) have described the partial purification and some of the properties of choleragen, an antigenic, diarrheagenic protein enterotoxin elaborated by certain strains of cholera vibrios. Although the biological activity of choleragen in promoting fluid loss via the intestinal tract is usually demonstrated in experimental animal systems, such as the infant rabbit or the ligated intestinal loop of the adult rabbit, a choleragen-containing culture filtrate has been reported to produce choleraic manifestations in human subjects (6) thus confirming its activity and pertinence to the human disease. In addition, similar biologic activities have been found in filtrates of the liquid stool of cholera patients (7, 8). Recently, rises in titer of serum antibody capable of neutralizing one of the activities associated with choleragen, its skin reactivity (9), have been reported to occur in cholera convalescents (10). The fact that choleragen is immunogenic (11), as well as antigenic, is of considerable interest since currently used conventional cholera vaccines offer only limited protection against the disease in man (12). Although partially purified preparations have been demonstrated to contain potent vascular permeability activity (9), the precise mode of action of choleragen in producing choleraic diarrhea has yet to be defined. Moreover, the question of whether choleragen is itself a permeability factor or whether the permeability enhancing activity is associated with a contaminant has not been resolved.

The accumulation of data relating to the chemical composition and structure of choleragen, its mode of action, and its immunogenic potential has been impeded by the lack of sufficient amounts of material of suitable purity to carry out these studies. Accordingly, we have attempted to escalate the laboratory scale production of choleragen and to develop methods for its purification. During this investigation, which relied on double diffusion immune precipitation techniques for recognition of choleragen antigen, a new moiety was detected. This protein is apparently identical with choleragen antigenically, but differs in size and charge. We propose that this substance, which has little, if any,

choleragenic or permeability activity, be called "choleragenoid". It may prove useful as a nontoxic immunogen. In addition, it was found that highly purified choleragen retains its permeability enhancing activity, thus offering additional evidence that choleragenicity and permeability enhancement are associated phenomena.

Materials and Methods

Choleragen was produced in 9 or 10 liter volumes of syncase medium (4) in a Virtis Model 40-100 fermenter of 12 liter capacity inoculated with approximately 10^5 viable cells/ml of *Vibrio cholerae* strain 569 B Inaba. Antifoam A (Dow Corning Corp., Midland, Mich.) was added at 8 hr. After incubation at 30°C for 20-24 hr with vigorous aeration (7.6 liter/min.) and stirring by means of a magnetic agitator at 500-700 rpm, the temperature was raised to 37°C and the bulk of the organisms killed by the addition of 0.05 to 0.1% beta-propiolactone (BPL, Betaprone, Fellows-Testagar, Div. Fellows Medical Mfg. Co., Detroit, Mich.) and further incubation for 2 hr. The cells were removed by continuous flow centrifugation at 16,000 rpm and a flow rate of approximately 100 ml/min. in a Sorvall RC2B refrigerated centrifuge fitted with the Szent-Gyorgi and Blum system. The choleragen and other proteins were precipitated by the addition of ammonium sulfate (70 g/100 ml), which also served effectively to kill any residual viable vibrios. The precipitate was harvested, after standing overnight at room temperature, by continuous flow centrifugation using a "basket" centrifuge (Virtis Co., Inc., Gardiner, N.Y.) with fine grade filter paper. A second precipitate, which formed in the supernate on further standing for a day, was harvested by conventional filtration through paper and added to the first. After dialysis in the cold vs. distilled water, the preparation was lyophilized. When four such preparations had been accumulated, the powders were pooled by suspension in approximately 200 ml of 0.01 M PO₄ buffer, pH 7.5, and added to 1 kg wet weight of diethylaminoethyl cellulose (DEAEC, Whatman microgranular DE 52) which had previously been equilibrated with the same buffer. After stirring for 1 hr, the excess fluid was removed by filtration through a sintered glass funnel and choleragen was eluted by washing with 0.1 M PO₄ buffer, pH 6.0. This procedure leaves a considerable amount of unrelated protein and brown pigment absorbed to the DEAEC (which was then recycled for subsequent use). At first, the choleragen-containing eluate was concentrated by (NH₄)₂SO₄ precipitation, dialysis, and lyophilization. In later experiments it was concentrated by ultrafiltration through Diaflo UM-10 membranes (Amicon Corp., Lexington, Mass.). The concentrate was then subjected to gel filtration on a 5 × 125 cm column of Agarose (Bio-Gel A-5m, 200-400 mesh, Bio-Rad Laboratories, Richmond, Calif.) using Tris-EDTA buffer pH 7.5 (0.05 M tris-hydroxymethylaminomethane, 0.001 M ethylenediaminetetraacetic acid disodium salt, 0.003 M NaN₃, 0.2 M NaCl) as eluant at a flow rate of approximately 60 ml/hr. The choleragen-containing fractions, identified by Ouchterlony agar gel double diffusion tests with specific anti-choleragen rabbit serum, in a late appearing component as indicated by its absorbance at 280 mμ, were pooled, concentrated by ultrafiltration, and rechromatographed through two 2.5 × 90 cm columns of Sephadex G-75 (Pharmacia Fine Chemicals, Piscataway, N.J.) connected in series. In the latter instance, 0.4 M ammonium formate containing 0.003 M NaN₃ was employed as eluting buffer at a flow rate of 20 ml/hr. Fractions containing choleragen antigen were again identified by Ouchterlony precipitation, pooled, and concentrated by ultrafiltration. Gel filtration columns were calibrated periodically by determination of elution volume of known molecular weight markers, according to methodology adapted from Andrews (13).

Immunoelectrophoresis with LKB apparatus (LKB Instruments Inc., Rockville, Md.) was performed on glass slides prepared with 1% Noble agar using 0.1 M Tris buffer, pH 8.6, and

5 μl of antigen preparation at a concentration of 10 mg/ml. Current was set at 50 ma and 250 v for 1 hr, after which 0.1 ml of antiserum was added to the trough. Results were photographed after incubation overnight in a humid chamber at room temperature. Disc electrophoresis using Canalco equipment (Canal Industrial Corp., Rockville, Md.) was performed according to the procedure of Ornstein and Davis (14). Protein concentrations were determined by the biuret colorimetric procedure as well as by optical density readings at 280 mμ in a Beckman DU spectrophotometer employing an extinction coefficient (1% solution) of 10.5. Ultracentrifugation studies were conducted in a Spinco Model E ultracentrifuge in the standard cell or in double sector cells.

Tests for biologic or immunologic activity, namely, choleragenicity in suckling rabbits, enterosorptive effects in ligated intestinal loops of adult rabbits, skin reactivity in adult rabbits, and Ouchterlony precipitin tests, were performed essentially as described in earlier publications (see above). Variations considered significant were the introduction of a system of scoring choleragenicity in infant rabbits, which makes interpretations of activity more quantitative, and a micro-Ouchterlony technique for assay of smaller amounts of antigen or antibody. As described previously, groups of four gastrically lavaged infant rabbits approximately 10 days old were fed dilutions of test material in 1 ml of 2% K_2HPO_4, 2% Na_2HPO_4. After 16–18 hr, surviving animals were sacrificed. The relative amount of fluid in the intestinal tract at autopsy was observed and scored on a 0 to 4 + basis. If the animals had overt diarrhea, this was also scored on a relative 0 to 4 + basis according to the proportion of the distance from anus to neck, on the ventral surface, which was stained or wet with liquid feces.[1] Animals found dead with excessive intestinal fluid and/or diarrhea were given an additional two points; if moribund, one point. The total score for each animal was obtained and a mean determined for the dosage group. Thus, if all four animals succumbed, the mean score for that group was 10. If no animals died, but three out of four had 2 + diarrhea, one had none, and all had 4 + excessive fluid, the mean score would be 5.5 derived as follows:

$$\frac{\text{(score of fluid)} \quad + \quad \text{(score of diarrhea)}}{(4) \text{ No. of animals}} = 5.5$$

$$\frac{(4 + 4 + 4 + 4) + (2 + 2 + 2 + 0)}{(4) \text{ No. of animals}} = 5.5$$

The micro-Ouchterlony technique employed "immuno-plates" (Hyland Laboratories, Los Angeles, Calif.) which were filled, in this laboratory, with 2.5 ml of melted and cooled 0.5% Ionagar No. 2 (Oxoid) in 0.85% saline containing 1:10,000 merthiolate. Wells were cut with a specially designed and constructed stainless steel gel cutter and the agar was removed by suction through a sawed-off hypodermic needle. The wells were 2mm in diameter; the distance from the edges of the perimeter wells to the edges of the central wells was 2.5 mm; four patterns of six peripheral wells and a central well were made simultaneously in each plate; and the wells were filled with 0.004 ml by means of a micropipette. Distinct precipitin bands appeared as early as 1 hr but were generally observed after overnight standing at room temperature in a sealed polyethylene bag.

A radial diffusion technique was also employed for precipitin assay of submicrogram quantities of choleragen and choleragenoid. The technique, an adaptation of the method of Fahey and McKelvey (15), was similar to that described above, except that antiserum was incorporated in the agar, at 0.2 ml per 10 ml, and a more appropriate linear pattern was used. These assays were performed in quadruplicate and the diameters of the zones of precipitation were determined with the aid of a stereoscopic dissecting microscope fitted with an optical reticle. The amount of serum incorporated in the agar menstruum was determined by prior titration to attain the highest sensitivity consistent with optimal visibility of the precipitin zone.

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[1] We are indebted to Dr. George Grady for suggesting this method of scoring the diarrhea.

Antisera to partially purified choleragen were prepared as described previously (11) and treated with living vibrios of the homologous strain to remove anti-vibrio antibodies. The reference antiserum pool formed a visible precipitin band with undiluted culture filtrate in the macro-Ouchterlony test at a dilution of 1:16 to 1:32. When highly purified choleragen and choleragenoid were obtained, additional rabbits were immunized in the same manner, with the exception that only 2 mg of antigen were used per animal instead of 5. Absorption of the latter sera was not required.

RESULTS

Characteristics of Fermenter Cultures and Fermenter Culture Filtrates.—The viable count of fermenter cultures ranged between 1×10^{10} and 2×10^{10} vibrios per ml prior to the addition of BPL. Filtrates sterilized by passage through Millipore membranes (Millipore Corp., Bedford, Mass.), prepared before addition of BPL, caused marked choleraic manifestations (a mean choleragenic score of 5) at a dilution of 1:71 in groups of gastrically lavaged infant rabbits administered 1.0 ml *per os* into the stomach (Fig. 1). After the addition of BPL, there appeared to be some reduction of the choleragenic activity as indicated by somewhat more erratic bioassay results. In a representative assay (Fig. 1), the reduction of activity was found to be approximately 2.5-fold, so that a 1:28.5 dilution of BPL-treated filtrate was required to produce the same effect in infant rabbits. Treatment with BPL, however, had no detectable effect on the precipitating antigen content of the filtrates: a visible precipitin band was formed with 0.05 ml anti-choleragen serum when 0.1 ml of 1:4 to 1:8 dilution was applied to peripheral wells of macro-Ouchterlony plates (Fig. 2). When injected intradermally, 0.1 ml of a 1:6400 to 1:12,800 dilution of untreated filtrate was sufficient to cause the characteristic delayed, sustained, erythematous, edematous, indurated local reaction in adult rabbits, whereas 2- to 4-fold larger amounts of BPL treated filtrates were required to give the same reaction.

Isolation of Choleragen and Choleragenoid.—Initially, choleragen was separated from the bulk of contaminating material present in the $(NH_4)_2SO_4$ precipitate of culture supernate by chromatography on 5×50 cm columns of DEAEC. The dialyzed, lyophilized precipitate was resuspended in 0.01 M PO_4 buffer, pH 7.5, and applied to the DEAEC column. Elution was carried out in a stepwise fashion, employing the initial buffer followed by 0.1 M phosphate, pH 6.0, and finally 1.0 M NaCl. The fractions were tested for the presence of choleragen antigen by the Ouchterlony technique. As shown in Fig. 3a, the bulk of the antigen was eluted by the 0.1 M buffer at pH 6 and separate pools, B_1, B_2, and B_3, were concentrated by ultrafiltration and applied to Agarose A-5m. Fractions constituting the peak of antigen activity (Fig. 3b) were again pooled, concentrated, and subjected to gel filtration on Agarose A-5m (Fig. 3c). The peak of antigen activity was then applied to a column of Sephadex G-75. The latter procedure resulted in the partial separation of three protein peaks (Fig. 3d); of these, the first two were found to contain antigen detectable by the Ouchterlony

method with anti-choleragen serum. On the basis of these observations, the procedure was modified to the batch method of DEAEC adsorption and elution described in Materials and Methods; a larger Agarose column was employed (Fig. 4a); and better final separation was achieved by using two Sephadex G-75 columns connected in series. The results, (Fig. 4b), indicated the presence of at least two protein peaks. In testing immunologic activity of fractions eluting from the double G-75 columns by the micro-Ouchterlony technique, it was observed that the distance from the antigen well to the nearest edge of the precipitin band followed a diphasic curve (Fig. 4b) corresponding to the diphasic optical density profile. The two major protein antigen-containing peaks were pooled separately and concentrated by ultrafiltration. Choleragenicity tests in infant
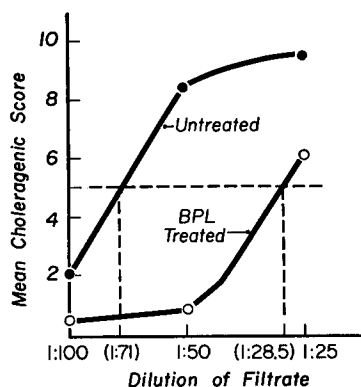

FIG. 1. Choleragenicity assay of untreated and BPL-treated fermenter filtrate.

rabbits indicated that the first peak, choleragen, was extremely active in producing diarrhea in infant rabbits (see below). The second peak, which gave strong precipitin reactions with anti-choleragen serum, was relatively inactive in producing cholera in infant rabbits. The latter peak contains the protein we have designated as "choleragenoid".

Estimations of Molecular Size of Choleragen and Choleragenoid.—Adaptation of the method of Andrews (13) permitted an approximation of the molecular size of choleragen and choleragenoid based upon their elution volumes from double Sephadex G-75 columns calibrated with known molecular weight "marker" proteins, bovine serum albumin (BSA), ovalbumin, and myoglobin (Mann Research Laboratories, Inc., New York, N.Y.). The results (Fig. 5) indicated that choleragen, with an elution volume of 388 ml (Fig. 4b), behaved like a protein of 61,000 mol wt and choleragenoid, elution volume of 446 ml (Fig. 4b), like one of 42,000. The third, inactive smaller component, mol wt = 33,000, was not characterized further. These observations have been reproduced

repeatedly. On the other hand, results derived from Agarose A-5m gel filtration on similarly calibrated columns have indicated, again repeatedly, that these molecules behave in that system as if their mol wt was below 20,000. On ultra-
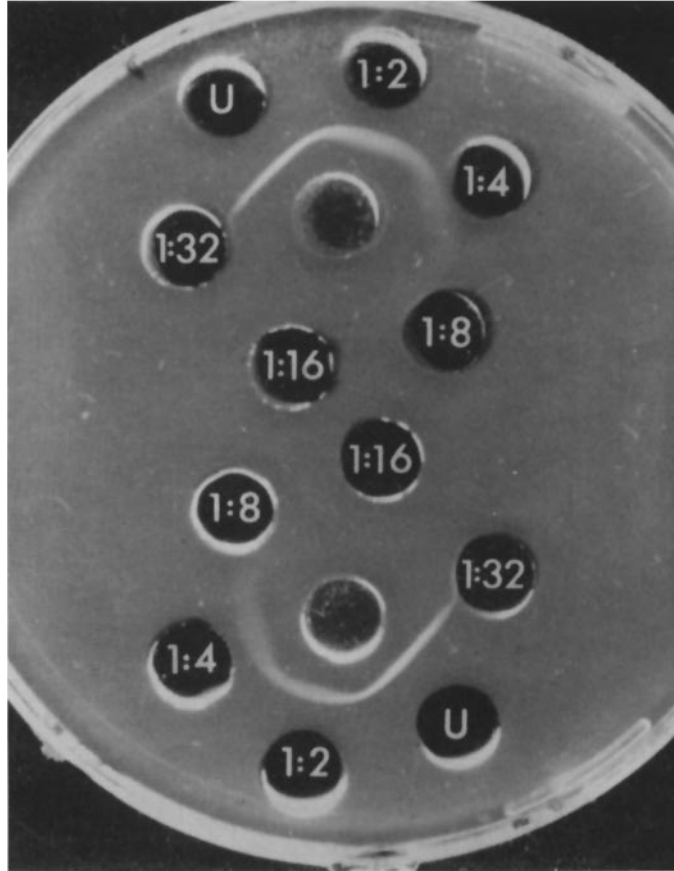

FIG. 2. Ouchterlony precipitin assay of untreated (upper pattern) and BPL-treated fermenter filtrate (lower pattern). Center wells: rabbit anti-choleragen serum; peripheral wells: dilutions of filtrate as indicated (U = undiluted).

centrifugation in saline (Fig. 6), the sedimentation coefficient of purified choleragen was calculated to be 5.6 S while that of choleragenoid was 4.2 S. Although both proteins sedimented as single symmetrical peaks indicative of homogeneous preparations, the sedimentation coefficients were suggestive of larger molecular sizes. An attempt was made to resolve the enigma presented by these apparently conflicting observations by means of filtration with Diaflo mem-
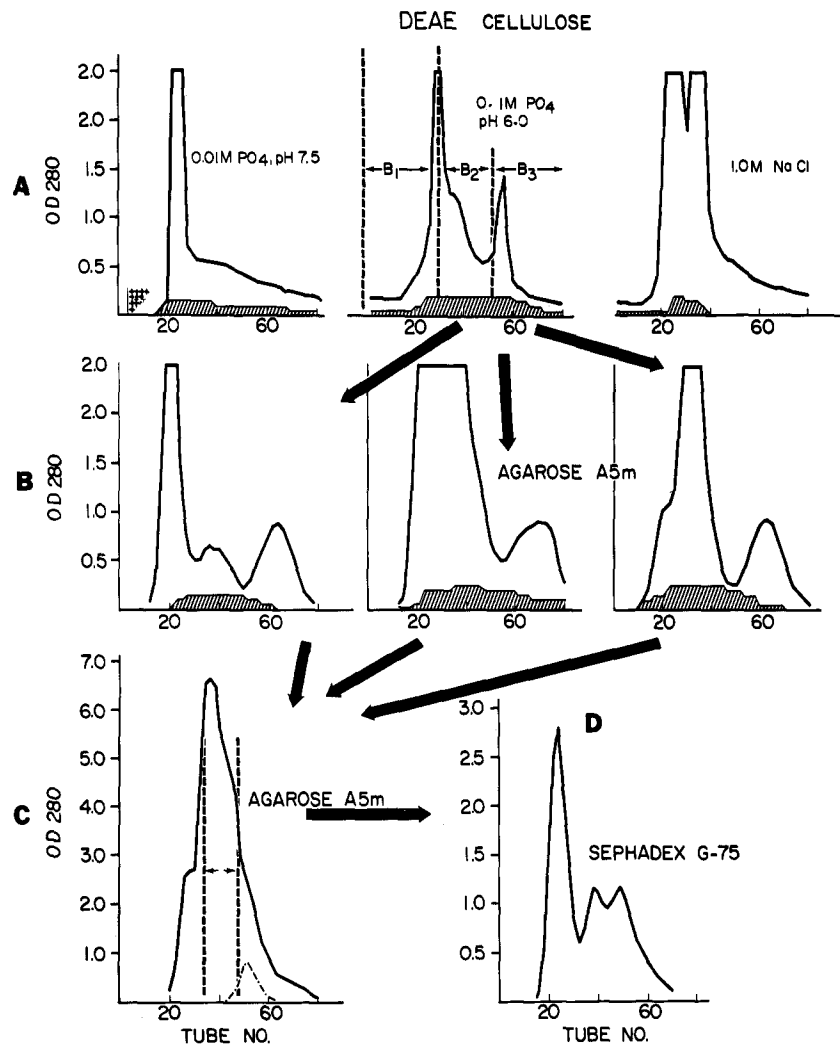
FIG. 3. Isolation of choleragen by sequential chromatography on columns of DEAEC (A), Agarose A-5m (B and C) and Sephadex G-75 (D). A. Stepwise elution, with indicated buffers, from 5 × 50 cm column of DEAEC, 20 ml per fraction. B. Gel filtrations of pools, B_1, B_2, and B_3 (from A), on Agarose A-5m, 2.5 × 87 cm, Tris-EDTA buffer, flow rate 20 ml/hr, discard first 280 ml, 2 ml per fraction. C. Gel filtration of active fractions from B on Agarose A-5m. Conditions similar to B. Interrupted dash curve delineates a component with absorption maximum at OD 415. Vertical dash lines indicate fractions pooled for further purification. D. Gel filtration of active peak (dash lines, C) on Sephadex G-75, 2.5 × 85 cm, 0.4 M ammonium formate, flow rate 20 ml/hr, discard first 150 ml, 2 ml per fraction. Cross hatched areas under curves in A and B represent estimates of relative antigen content by intensity of precipitin band observed in Ouchterlony test.

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branes of different porosity. The activity, both antigenic and choleragenic, of crude fermenter culture supernatants, was found to be fully retained by UM-10 and UM-20E membranes rated by the manufacturer to retain proteins of sizes
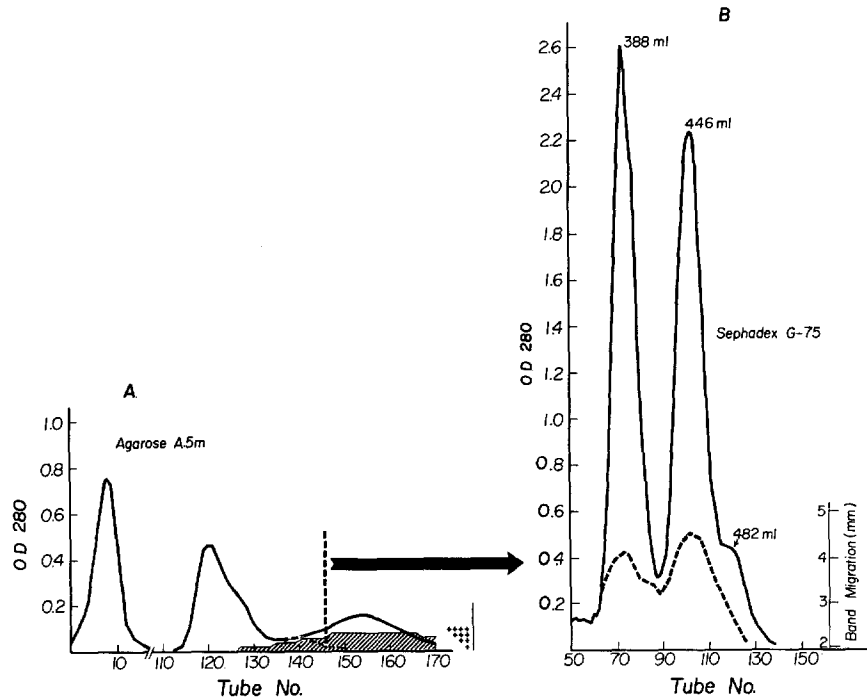

FIG. 4. Gel filtration of DEAEC eluate of $(NH_4)_2SO_4$ precipitate from four fermenter supernates on Agarose A-5m (A) followed by Sephadex G-75 (B). A. Gel filtration on Agarose A-5m, 5 × 125 cm, Tris-EDTA buffer, flow rate 60 ml/hr, discard first 912 ml, 10 ml per fraction. Fractions to right of vertical dash line pooled for further purification. B. Gel filtration on Sephadex G-75, two 2.5 × 90 cm columns in series, 0.4 M ammonium formate buffer, flow rate 20 ml/hr, discard first 242 ml, 2 ml per fraction. Dash curves represent distance in mm from antigen well to precipitin band in micro-Ouchterlony test. (In this case, an earlier pattern with 5 mm distances from peripheral wells to central wells was used.)

larger than 10,000 and 20,000, respectively. XM-50 membranes, with a cutoff stated to be 50,000 mol wt, retained approximately 50% of the antigen activity. However, gel filtration studies of the XM-50 retentate indicated that it was partially enriched in the larger, choleragen, moiety whereas the effluent, which passed through the XM-50 membrane, was found to be somewhat enriched in the smaller-sized, choleragenoid species. The XM-50 membrane, when tested

with known protein markers, was found to retain BSA (mol wt = 45,000), but allowed passage of chymotrypsinogen (mol wt = 25,000). Thus, the membrane filtration results tend to support the molecular size approximations obtained by Sephadex G-75 gel filtration. Apparently, the moieties behaved anomalously on Agarose A-5m and studies are in progress to determine whether reversible aggregation and disaggregation takes place.

Radial Diffusion Immunoassay of Choleragen and Choleragenoid.—The radial diffusion immunoassay technique was found to be a sensitive, precise, and re-
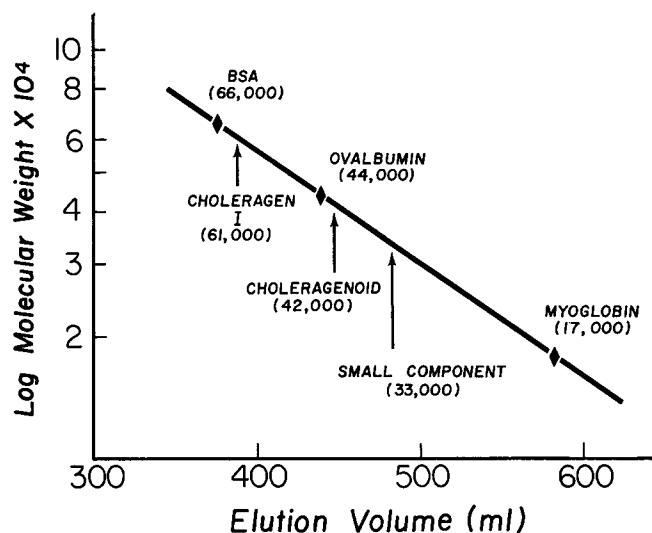

FIG. 5. Molecular size estimation of choleragen and choleragenoid by gel filtration on Sephadex G-75 double columns in comparison with known molecular weight marker proteins.

producible method of detecting and quantitating choleragen and choleragenoid. Patterns resulting from a typical assay are depicted in Fig. 7 and zone diameters resulting from assays of a choleragen and choleragenoid preparation performed in quadruplicate, are summarized in Table I. The relationship between concentration of antigen, plotted logarithmically, and precipitin ring diameter was linear over greater than a 4-fold range of concentrations, as illustrated in Fig. 8. Statistical analysis did not reveal the regression curves to differ significantly although results practically identical to these were obtained with different choleragen and choleragenoid preparations assayed on different occasions. As might be expected from its smaller molecular size, choleragenoid produced larger zone diameters than choleragen at the same concentration.

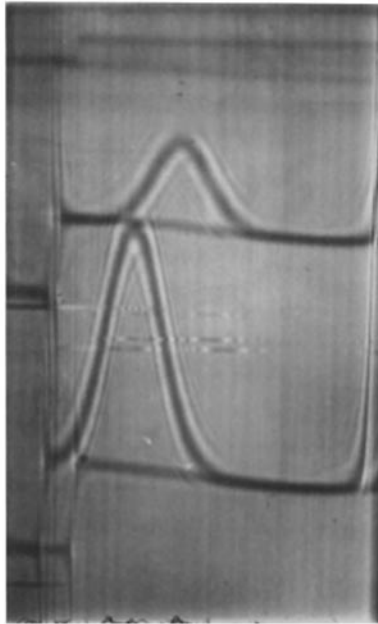*Immunoelectrophoresis of Choleragen and Choleragenoid.*—Immunoelectro-

FIG. 6. Ultracentrifugation of choleragen (upper pattern) and choleragenoid (lower pattern) in double sector cell. Choleragen, 4.4 mg/ml; choleragenoid, 9.0 mg/ml; 39,460 rpm; 116 min, elapsed time.

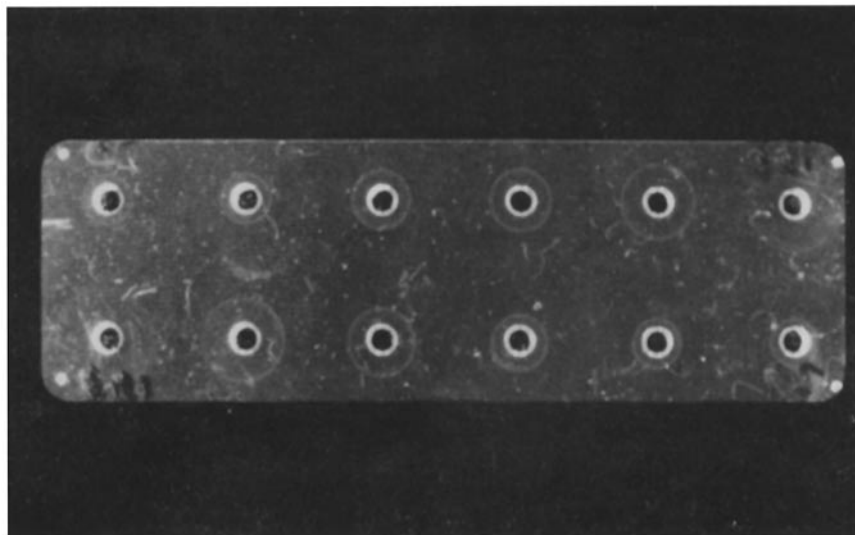
FIG. 7. Radial diffusion immune precipitation of choleragen. Top row, l. to r., 20, 30, 40, 50, 75, 100 μg choleragen per ml. Bottom row, same concentrations, r. to l. 4 μl per well.

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phoretic analysis of choleragen, choleragenoid, and artificial mixtures of the two isolated components (Fig. 9) revealed that choleragen migrated faster than did choleragenoid. In mixture, the two formed a continuous precipitin band with the anti-choleragen serum. Similar tests with unadsorbed sera gave identical results: there was no evidence of other components reactive with serum prepared against crude choleragen or live vibrios. It should also be mentioned that older preparations of partially purified choleragen gave a pattern identical with that of the artificial mixture of the two components. Neither BPL nor $(NH_4)_2SO_4$ had been used in their preparation.

Antigenic Identity of Choleragen and Choleragenoid.—Macro-Ouchterlony tests

TABLE I

RADIAL DIFFUSION IMMUNOASSAY OF CHOLERAGEN AND "CHOLERAGENOID"

CHOLERAGEN						CHOLERAGENOID					
ANTIGEN CONC.* μg./ml.	ZONE DIAMETER (mm.)					ANTIGEN CONC.* μg./ml.	ZONE DIAMETER (mm.)				
	1	2	3	4	Mean ± 2 s		1	2	3	4	Mean ± 2 s
20.0	4.83	4.92	4.98	4.83	4.89 ± 0.14	10.0	4.05	3.99	3.90	3.78	3.93 ± 0.23
40.0	6.18	6.18	6.03	6.03	6.11 ± 0.17	14.7	4.77	4.65	4.68	4.71	4.70 ± 0.32
60.6	7.20	7.20	7.23	7.41	7.26 ± 0.20	20.0	5.70	5.43	5.52	5.28	5.48 ± 0.37
81.0	8.25	8.10	7.89	8.25	8.12 ± 0.34	30.1	6.60	6.18	6.30	6.15	6.31 ± 0.22

*Note: Since volumes of 4λ were used in making these determinations, the actual amount of antigen is given by multiplying the concentration by 0.004. Thus the system detects amounts of antigen of the order of 4×10^{-8} g.

(Fig. 10) also showed the two components to be immunologically identical, at least by their reaction with our standard antiserum. Because of the possibility that the standard antiserum, which was prepared against what now appears to be a mixture of the two components, might have been directed primarily against the choleragenoid component, it was conceivable that the antiserum could not detect antigens unique to the larger, more highly charged, choleragen. For this reason, additional antisera were prepared in rabbits, against each of the isolated components. Each antiserum gave reactions of complete cross-identity with both choleragen and choleragenoid. In addition each of the antisera gave only a single precipitin line when tested against crude culture filtrate (except for the serum of 1 of 4 rabbits immunized with choleragenoid). The exception, which produced only a single band when tested with isolated choleragen and choleragenoid, gave a second minor band when tested with crude cholera culture filtrate. In contrast to previous experience (11), none of the antisera gave any evidence of agglutinating antibody when tested against live antigens of the

homologous culture. Rises of vibriocidal antibody activity of the sera tested by an exquisitely sensitive technique (16) were inconsequential. Some of the immunized rabbit sera exhibited transient rises to titers as high as 10^2 (two tubes) declining to 10^1 by 4 wk postinoculation while the precipitin titer continued to rise. Others failed to show any rises.

Electrophoresis of Choleragen and Choleragenoid.—Choleragen was found to give a single, broad, protein-staining band after electrophoretic separation in polyacrilamide gel. This was in marked contrast with previous experience (5) in which partially purified material was found to contain multiple protein-staining
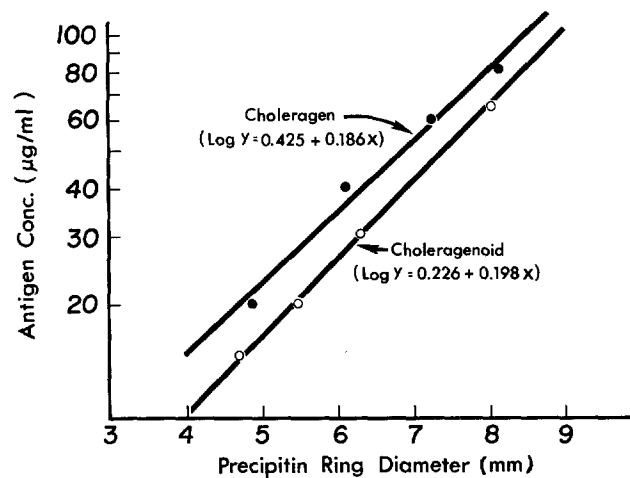

FIG. 8. Least squares regression lines relating antigen concentration to precipitin ring diameter for choleragen and choleragenoid.

constituents. Choleragenoid, on the other hand, exhibited four or five very closely spaced bands which had migrated further than the choleragen band (Fig. 11).

Protein Determinations on Choleragen and Choleragenoid.—Repeated protein determinations, by the biuret technique, on lyophilized preparations of choleragen and choleragenoid indicated protein contents between 85–92%. No carbohydrate could be detected by periodic acid-Schiff staining after disc electrophoresis. No lipid could be detected when the proteins were each extracted with 3:1 $CHCl_3$–methanol and the extracts applied to thin layer plates containing Dichlorofluorescein. The technique was sufficiently sensitive to recognize lipid contamination at the 1% level.

Biological Activity of Choleragen and Choleragenoid.—Tests in infant rabbits of isolated choleragen indicated that a mean choleragenic score of 5 was produced by doses of different preparations ranging between 0.25 and 0.5 μg. Approxi-
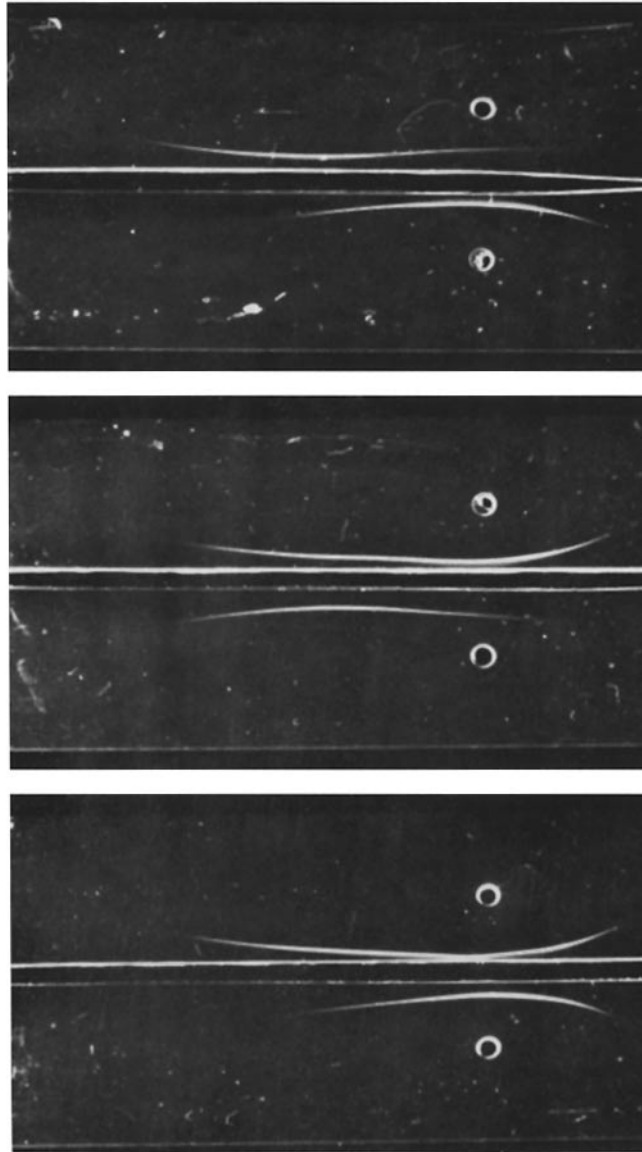
FIG. 9. Immunoelectrophoresis of choleragen, choleragenoid, and an artificial mixture of the two. Top slide: choleragen (upper well) and choleragenoid (lower well). Middle slide: artificial mixture (upper well) and choleragen (lower well). Lower slide: artificial mixture (upper well) and choleragenoid (lower well).
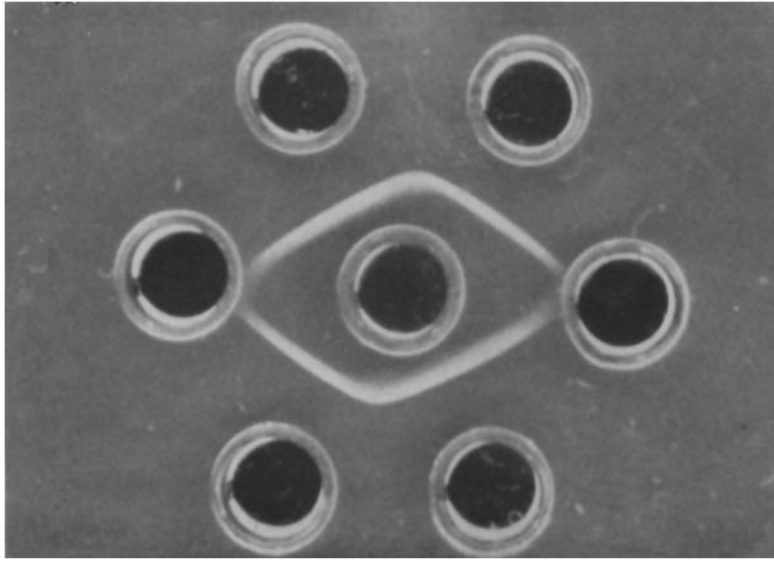
Fig. 10. Ouchterlony precipitin patterns of choleragen and choleragenoid. Choleragen (upper right and lower left); choleragenoid (upper left and lower right); approximately 100 μg per well; side wells are empty.

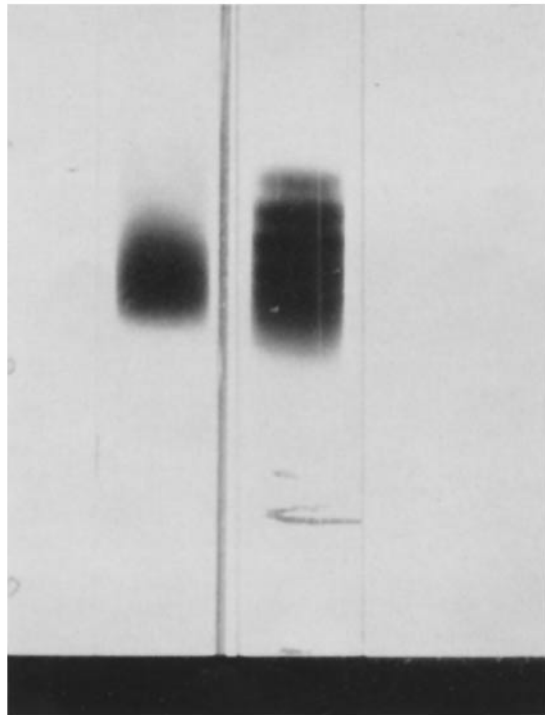Fig. 11. Polyacrilamide gel (disc) electrophoresis of choleragen (left) and choleragenoid (right).

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mately 50 μg or more of choleragenoid was required to produce the same effect. Choleragen in doses as small as 0.0004 μg produced typical reactions in rabbit skin; the minimal skin test dose of choleragenoid ranged between 0.015 and 0.125 μg. Preliminary tests using the ligated rabbit intestinal loop assay have indicated that amounts of choleragen less than 1 μg are sufficient to produce a positive reaction.

DISCUSSION

The results presented above indicate that choleragen may be obtained in a relatively high state of purity and high specific activity by the procedures described. The procedure also entails considerable losses, in part attributable to our discarding of active fractions which have relatively high levels of unrelated proteins for the sake of ultimate purification, and possibly in part to inactivation and losses inherent in the procedures. Preliminary observations indicate that these losses may largely be circumvented by using selective ultrafiltration steps in lieu of the $(NH_4)_2SO_4$ precipitation and DEAEC absorption and elution. The isolated protein appears to be homogeneous by all available criteria (immunoelectrophoresis, disc electrophoresis, ultracentrifugation, and immunologic) and behaves as a pure, unconjugated protein, with molecular size of 61,000 determined by Sephadex gel filtration and supported by membrane filtration experiments. The apparent peculiarity of its retention on Agarose A-5m was most useful in its isolation since, by Agarose gel filtration, it was effectively separated from unrelated proteins, which presumably behave normally in that system, and would otherwise accompany choleragen isolated only by Sephadex gel filtration. The sedimentation coefficient of purified material was higher than one would expect suggesting that aggregation may occur in normal saline or other low molarity solutions. It is of interest that the sedimentation coefficient corresponded more closely with previous estimates of size (5) obtained with impure material isolated by other techniques.

In a recent report, Coleman et al (17) claim to have purified a cholera enterotoxin, the so-called "ileal loop reactive type 2 toxin" of Burrows (18), from cell lysates and from peptone broth cultures. We have found both of these to be relatively poor sources of choleragen (5, and unpublished studies). Although their product was derived from the same vibrio strain, its reported properties are so different from those observed in our studies that the question is raised as to whether it is the same substance. If their ileal loop reactive toxin is choleragen, then the high lipid content and relatively low specific activity of their product (approximately $\frac{1}{10}$ of that reported herein) would suggest over 90% contamination with unrelated materials. The authors indicated that, for preparative purposes, their "fraction I" was eluted directly from DEAE-Sephadex with deionized water without preliminary or subsequent treatment. That being the case, our observations (Fig. 3a–d) would suggest that an extremely impure prod-

uct would result and this factor could explain some of the discrepancies in the two studies. The authors did acknowledge the possibility that toxicity might reside in a component of "the fraction I complex".

The finding of an antigenically identical moiety, choleragenoid, which seems to be devoid of choleragenic or skin-reactive activity, (the low levels of specific biologic activity found in our choleragenoid preparations could readily and likely be attributable to a 1% contamination with choleragen which precedes the choleragenoid in eluting from the Sephadex G-75 columns) is of considerable interest from both the standpoint of its relationship to choleragen and its potential value as a nontoxic immunogen. Our studies have provided no information as to whether it is a precursor to, or a degradation product of, choleragen. The choleragen molecule is apparently larger and more highly charged and one might speculate that in these differences lies the faculty of causing the diarrhea of cholera. It is also of interest that, even with its larger size and different charge, the choleragen moiety gains no antigenic feature to distinguish it from choleragenoid. It should be pointed out that choleragenoid does not appear to be an artifact of the isolation procedure since both moieties have been isolated from culture filtrates not treated with BPL and subjected to only gentle separation by physical methods involving only Diaflo and gel filtration (to be published, present authors).

Both moieties are antigenic. If choleragenoid is a precursor of choleragen, it might be possible to isolate a stable mutant, which one might predict would be incapable of causing cholera, and which could potentially provide the ideal immunizing agent—a living, attenuated vibrio, capable of multiplication in the gut and stimulation of both antibacterial and antitoxic immunity at the important local level.

Of paramount interest with regard to the pathogenic mechanism of cholera is the observation that apparently pure choleragen in minute amount retains the capacity to produce alterations of permeability of vascular endothelium as demonstrated in the skin test. Thus, it now seems more likely that choleragenicity and increased vascular permeability are intimately associated phenomena. This does not *prove* that the diarrhea of cholera is the *result* of increased vascular permeability. Rather, the observations make it more probable that *the same basic biochemical-physiological lesion, initiated by this single substance, may be "expressed" as an alteration of vascular permeability in skin or an outpouring of fluid in gut,* and suggest the potential value of experimental inquiry into a common mechanism.

The availability of purified components should facilitate more definitive studies on the chemical composition and nature of choleragen, its mode of action, and its potential role in the production of immunity. Additional observations on biologic activities of choleragen were summarized recently (19).

SUMMARY

Choleragen, a diarrheagenic protein enterotoxin elaborated by *Vibrio cholerae*, has been isolated from the supernate of fermenter cultures by steps involving ammonium sulfate precipitation, DEAE cellulose, Sephadex G-75, and Agarose A-5m chromatography. The resulting product appears to be pure according to immunoelectrophoretic, disc electrophoretic, ultracentrifugal, and immunologic criteria. Sephadex gel filtration and membrane filtration studies suggest a molecular size of 61,000. The isolated product is highly active in inducing experimental cholera in infant and adult rabbit models. It also elicits, in small dosage, an increased vascular permeability in skin. These observations indicate that choleragenicity and increased vascular permeability are intimately associated phenomena and may be manifestations of the same basic mechanism.

An additional, antigenically identical, protein has also been isolated by the same procedures. The latter substance, termed "choleragenoid", lacks the permeability effect and choleragenicity of the choleragen moiety. Its size (estimated from Sephadex gel filtration at 42,000) is smaller than that of choleragen and it also differs in charge. Choleragenoid may prove useful as a nontoxic immunogen to protect against pathologic effects of *V. cholerae* infection.

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