INFECTION AND IMMUNITY, Dec. 1972, p. 934-944
Vol. 6, No. 6

Properties of the Cholera Exo-Enterotoxin: Effects of Dispersing Agents and Reducing Agents in Gel Filtration and Electrophoresis

RICHARD A. FINKELSTEIN, MICHAEL K. LaRUE, AND JOSEPH J. LoSPALLUTO

Departments of Microbiology and Biochemistry, The University of Texas Southwestern Medical School, Dallas, Texas 75235

Received for publication 24 July 1972

Initial studies, by using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS), led to the attractive hypothesis that the cholera exo-enterotoxin (choleragen) consisted of two noncovalently linked peptides of 56,000 and 28,000 molecular weight. The spontaneous toxoid (choleragenoid) appeared to be identical with the 56,000 molecular weight piece. The results appeared to be very similar to those obtained recently with diphtheria toxin. However, they did not agree with earlier studies which indicated that both the toxin and the toxoid consisted of subunits of approximately 14,000 molecular weight. More extensive investigation, by using gel filtration with ^{3}H-labeled toxin, ultracentrifugation, and immunologic studies, failed to support the observations of SDS-gel electrophoresis, suggesting the existence of a 28,000 molecular weight fragment which is unique to the toxin. The results were more compatible with our earlier observations regarding subunit composition. It is concluded that these proteins are relatively resistant to dissociation by SDS and need to be unfolded by prior treatment for more complete dissociation into subunits. Indirect evidence suggests that the 28,000 fragment may be a dimer, or larger aggregate, of a smaller subunit which can be integrated in the formation of choleragenoid. It is also evident that caution should be exercised in the interpretation of results, however pleasing, obtained only by SDS-gel electrophoresis.

We have previously reported (11) that the purified cholera exo-enterotoxin, designated choleragen (3, 4, 5), is a homogeneous simple protein with a molecular weight of approximately 84,000. The toxin is both choleragenic and skin reactive (5). At *p*H 3.5 to 3.7, it can readily and reversibly be dissociated into subunits with molecular weights of approximately 14,000 to 15,000 (11). The spontaneously formed cholera toxoid, choleragenoid (3, 4, 5), isolated concurrently from fermentor-grown *Vibrio cholerae* culture filtrates, is also dissociable into similarly sized subunits (11). Although choleragenoid is apparently immunologically identical to the toxin, it is a smaller protein, molecular weight of approximately 56,000 to 58,000, and lacks the toxicity of the parent toxin. Choleragenoid is apparently a mixture of at least three isomers which differ slightly in charge (4). That choleragenoid is derived from choleragen, and not vice versa, was clearly demonstrated by using ^{131}I-labeled proteins (7). The toxin can be converted into choleragenoid by shaking (7) and by heating (2). In the latter case, the reaction proceeds through an intermediate polymer of high molecular weight (29 to 31*S*), which was designated procholeragenoid. Procholeragenoid is relatively nontoxic. However, it is highly immunogenic (2, 8) and appears to stimulate intestinal immunity, after parenteral administration, better than the other related antigens (8). It should also be mentioned that choleragen and choleragenoid behave somewhat peculiarly in gel filtration chromatography. For example, from chromatography on calibrated columns of Sephadex G-75 (3), one would judge the two proteins to have molecular sizes of 61,000 and 42,000, respectively, instead of the values given above which were determined by sedimentation velocity-diffusion and sedimentation equilibrium (11). On columns of agarose (Biogel A5M), both proteins behave identically as if their molecular size were approximately 25,000 or less. This peculiarity has been invaluable in separating these proteins from other substances present in supernatant fluids which behave normally in

molecular sieving. Both proteins also interact with natural membranes such as those at the surface of intestinal villi (Peterson, LoSpalluto, and Finkelstein, J. Infect. Dis., *in press*) and untanned erythrocytes (6, 9).

In an effort to learn more about the differences between the toxin and the toxoid which may be related to the capacity of the toxin to produce disease, the effects of various chemical treatments were investigated and provide, in part, the substance of this report.

The technique of electrophoresis in polyacrylamide gel in the presence of the anionic detergent sodium dodecyl sulfate (SDS-gel electrophoresis) (10, 12) has been widely used as a means of estimating the molecular weights of polypeptide chains. However, in earlier preliminary experiments in this laboratory (*unpublished data*), the results obtained with SDS-gel electrophoresis of choleragen and choleragenoid differed significantly from the findings provided by more direct physical methods, most notably with regard to the subunit composition. Accordingly, the subject was investigated more extensively as described herein.

MATERIALS AND METHODS

Choleragen and choleragenoid were isolated and purified from supernatant fluids of fermentor-grown *V. cholerae* 569B Inaba as described previously (2). Although not crystallized for this study, they are equivalent to crystalline material (5). Procholeragenoid was prepared by heating purified choleragen at 60 C (2) and was reisolated in the void volume of Sephadex or polyacrylamide (Biogel P-150) columns. ^{3}H-labeled choleragen, prepared previously (Peterson, LoSpalluto, and Finkelstein, J. Infect. Dis., *in press*) by the Wilzbach procedure (New England Nuclear), was repurified extensively by column chromatography. It is presumed that the Wilzbach tritium exchange results in a uniform distribution of label. In various experiments, the labeled protein was added to cold carrier.

The following proteins were used as markers for gel chromatography or SDS-gel electrophoresis, or both: bovine serum albumin (BSA, Schwarz-Mann); ovalbumin (OA, Worthington); cytochrome *c* (Cyt *c*), myoglobin (Myo) and chymotrypsinogen (CT) (Mann Research Laboratories, Inc.); and an additional sample of CT (Sigma). Dithiothreitol (DTT), 2-mercaptoethanol (ME), tracking dye (bromophenol blue), and sodium lauryl sulfate (sodium dodecyl sulfate, SDS) were also purchased from Sigma Chemical Company. The latter was reprecipitated from ethanol prior to use. Acrylamide and *N*-*N*′-methylene bisacrylamide were obtained from Eastman. *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and aniline blue black were from Canalco; ammonium persulfate, reagent grade, from Baker; and Coomassie blue (brilliant blue R) was from K & K Laboratories.

Conventional electrophoresis was conducted at *p*H 9.5 in 7% polyacrylamide gels at 4 C with iced buffer in Canalco apparatus by using only slight modifications of the procedure recommended by the manufacturer. Electrophoresis was continued 30 min after the tracking dye left the gel to allow more complete separation between choleragen and choleragenoid. The gels were stained with aniline blue black and destained electrophoretically. SDS (0.1%) electrophoresis was conducted in 10% polyacrylamide gels essentially as described by Weber and Osborn (12) and their formula was used for calculation of mobility. The gels were stained with Coomassie blue in methanol-acetic acid and destained electrophoretically. Both photopolymerized and chemically polymerized gels were tested with representative samples in both the above procedures. Although the results were similar, chemically polymerized gels were used in the work reported. When tritium-labeled protein was used, the gels were sliced, dissolved in 30% H_2O_2, and "counted" in Aquasol (New England Nuclear) in either a Nuclear-Chicago or Beckman liquid scintillation counter. Fractions obtained after gel chromatography (on 2.5 by 90 cm columns [3]) were counted in Bray solution. Ultracentrifugal studies were performed in a model E analytical ultracentrifuge.

Toxicity (choleragenicity) was determined in mice fed solutions of (modified) proteins in bicarbonate as we have described recently (8) and by tests of skin reactivity in rabbits (3, 4, 5).

RESULTS

Conventional gel electrophoresis. As illustrated in Fig. 1 (column A, no. 1), choleragen forms a single major band, in conventional gel electro-

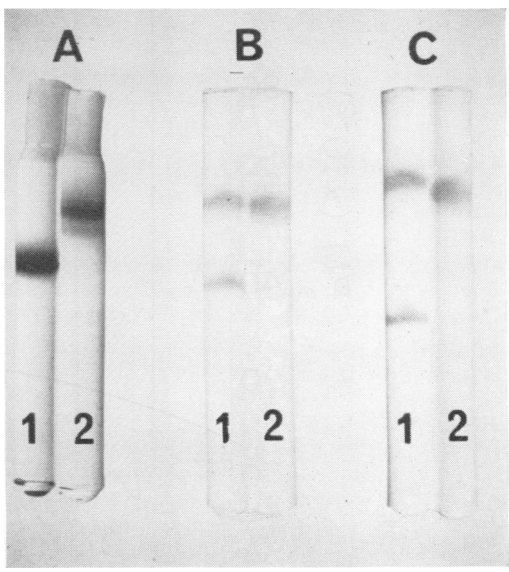

FIG. 1. *Conventional (A) and SDS electrophoresis (B, C) of choleragen (1) and choleragenoid (2). Gels in C were run in the presence of ME.*

phoresis, moving somewhat faster than choleragenoid which appears (Fig. 1, column A, no. 2) in two or three closely spaced bands (4).

SDS-gel electrophoresis. In 0.1% SDS-gel electrophoresis, as shown in Fig. 1 (column B, no. 1), choleragen gives rise to two major bands. The slower moving band corresponded to the mobility expected of a protein of approximately 54,500 molecular weight (based upon the mean of 19 determinations, range 44,000 to 68,000, in parallel with marker proteins, BSA, OA, CT and Cyt *c*). The second, faster moving band corresponded to that of a polypeptide of molecular weight approximately 28,000. Choleragenoid, on the other hand, gave rise to only a single major band (Fig. 1, column B, no. 2) nearly equivalent (mean of 16 determinations, 51,340; range, 41,000–63,000) to that of the slower moving band found with the toxin. These results, which have been confirmed independently by Michael Gill and A. M. Pappenheimer, Jr. (*personal communication*) by using choleragen and choleragenoid supplied by us, suggested the presence of a fragment or piece which was unique to the toxin and not found in the toxoid. Furthermore, the results were very much in accord with our previous determinations of the molecular weights of the two parent proteins and, combined with other evidence, suggested that choleragenoid might be the carrier protein and the 28,000 piece might be the active segment of the toxin in a manner analogous to diphtheria toxin (A. N. Pappenheimer, Jr., T. Uchida, and A. A. Harper, Immunochemistry, *in press*). However, these experiments did not support the observation of 14,000 molecular weight subunits in each of the two proteins on acid dissociation (11). Addition of thiol reducing agents (ME or DTT) to the samples prior to SDS electrophoresis did not significantly affect the mobility of choleragenoid (Fig. 1, column C, no. 2). The mobility of the second band observed with choleragen was increased (Fig. 1, column C, no. 1) so that it now behaved as if it represented a protein of approximately 23,000 molecular weight, perhaps by the excision of a peptide which migrated out of the gel during electrophoresis. Similar results were obtained by Gill and Pappenheimer (*personal communication*). The behavior of choleragen and choleragenoid was unaffected by incubation at 37 C for 22 hr.

When similar experiments were performed by using ^{3}H-choleragen and the SDS gels were cut and counted (Fig. 2), approximately 16% of the total radioactivity recovered was in the area of the 28,000 piece. Following inclusion of DTT, the radioactivity of the 28,000 piece appeared to be divided between the now faster moving stained band (Fig. 2) and an even faster moving fraction which is barely visible in the stained gel.

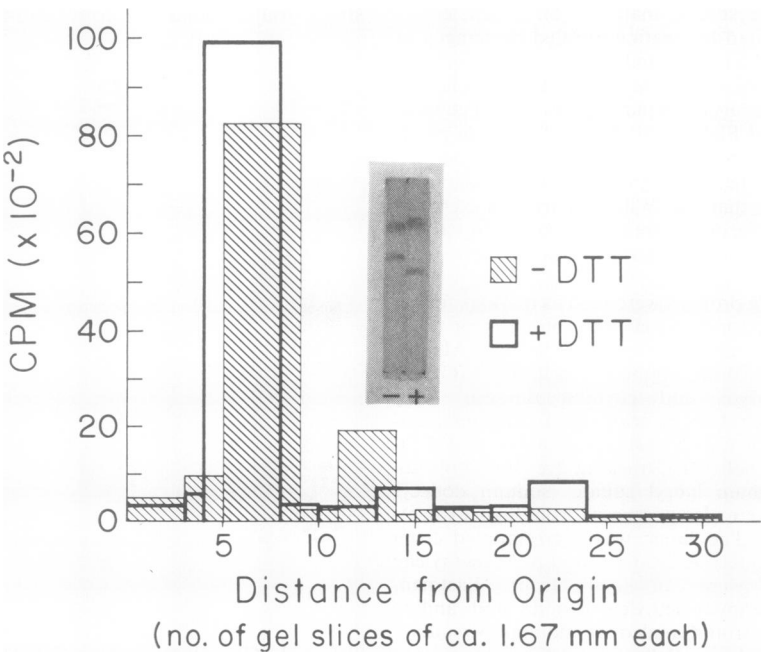

FIG. 2. *SDS electrophoresis of ^{3}H-choleragen. Three gels identical to those depicted in the inserts were sliced; similar slices were pooled, dissolved in H_2O_2, and counted in Aquasol.*

Gel filtration on polyacrylamide (Biogel P-150). To isolate the 28,000 piece in amounts sufficient for further study, column chromatography on polyacrylamide P-150 was carried out in the presence of SDS. On chromatography through a calibrated 2.5 by 90 cm column of Biogel P-150 (Bio-Rad Laboratories) in tris(hydroxymethyl)-aminomethane-ethylenediaminetetraacetic acid (Tris-EDTA) buffer (3), choleragen (elution volume, 185 ml) behaved as if its moleculer size was approximately 49,500, and choleragenoid (elution volume, 205 ml) emerged only slightly behind with an approximate molecular size of 44,000. The marker proteins used in the calibration, BSA (molecular weight, 67,000), OA (molecular weight, 45,000), and CT (molecular weight, 25,000), provided a reasonably linear relationship between their molecular weights, plotted on a logarithmic scale, and their elution volumes. After equilibration of the column with 0.1% SDS, the marker proteins, BSA and OA, emerged at the void volume of the column (113–115 ml), and CT eluted in a fairly broad region with elution volume of 148 ml. Choleragen emerged in three peaks with elution volumes of approximately 121, 163, and 177 ml, whereas choleragenoid formed a single major heterogeneous peak covering the area encompassed by the last two peaks of choleragen. Results of such a chromatographic separation by using ^{3}H-labeled choleragen are shown in Fig. 3. The fractions comprising three areas were pooled. Approximately 16% of the label was found in pool I, suggesting that only one-sixth of the choleragen molecule appeared in this region. Subsequent SDS-disc electrophoresis of the pools (not shown) indicated that pool I apparently corresponded with the 28,000 piece, whereas pool III corresponded with the slower moving 56,000 fragment observed previously. Pool II gave rise to both bands. These results were unexpected in that normally the faster eluting components in gel filtration are of larger, not smaller, molecular size. The behavior of the pools in conventional electrophoresis is shown in the figure. Pool I migrated very rapidly whereas pools II and III appeared in the area between choleragen and choleragenoid.

When the same column was equilibrated with 0.14 M ME in 0.1% SDS in the Tris-EDTA buffer, choleragen emerged in a single symmetrical peak, with elution volume of 172 ml, with a slightly heterogeneous leading edge (Fig. 4). On subsequent SDS electrophoresis, the major peak was found to be predominantly the 56,000 fragment, but a slight amount of the smaller piece was also observed. The leading edge corresponded with the 28,000 fragment. In conventional electrophoresis (Fig. 4), the major peak appeared just slightly behind the normal choleragen band, whereas the leading edge was similar to that observed previously.

On a similar column of P-150 equilibrated with ME, but without SDS, ^{3}H-labeled choleragen emerged as a single peak at 190 ml (Fig. 5), corresponding with choleragen passed through the same column without ME. All the radioactivity emerged within the peak. There was no evidence of a smaller labeled fragment. On subsequent SDS electrophoresis of the fractions comprising the peak, the two characteristic bands were again

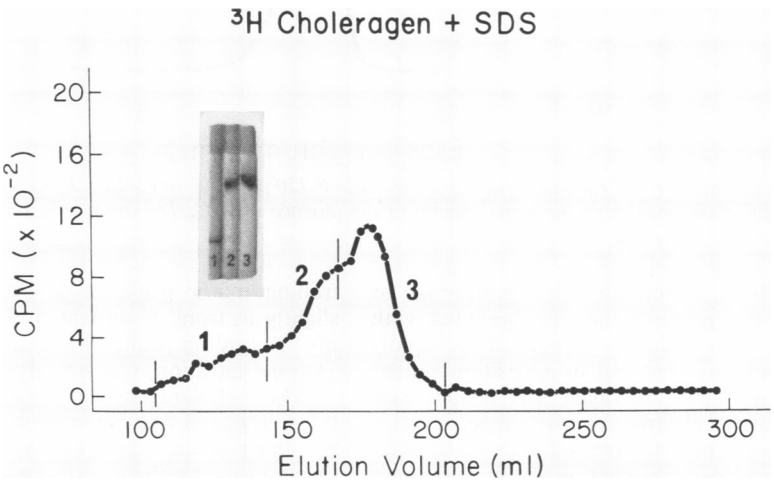

FIG. 3. *Gel filtration of ^{3}H-choleragen on polyacrylamide P-150 in SDS. Fractions included between vertical hash marks were pooled. Behavior of the pools in conventional gel electrophoresis is depicted in the inserts. The first gel had to be removed prior to elution of the tracking dye to demonstrate the band observed.*

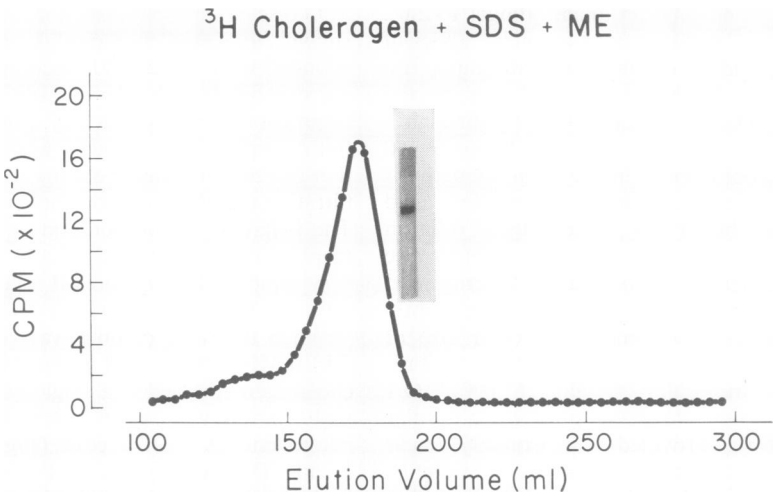

FIG. 4. *Gel filtration of ^{3}H-choleragen on polyacrylamide P-150 in SDS plus ME. The insert shows the behavior of the major peak in conventional electrophoresis. The leading edge was similar to that of the preceding figure.*

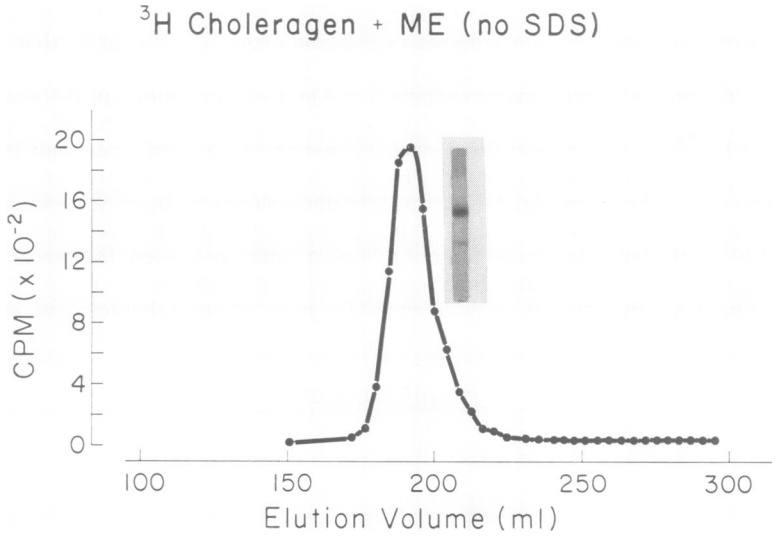

FIG. 5. *Gel filtration of ^{3}H-choleragen on polyacrylamide P-150 plus ME (no SDS). This elution pattern is identical with that of choleragen on the same column in the absence of ME. The insert illustrates conventional gel electrophoresis of the pool.*

observed. In conventional electrophoresis, as illustrated, the major band corresponded with choleragen. In addition, a minor, more rapidly moving band was observed in a position similar to that seen when choleragen was treated with ME just prior to electrophoresis (*see below*).

When choleragen was treated with 0.1% SDS, dialyzed, and then subjected to gel filtration on a P-150 column, two peaks were observed (data not shown). The major peak emerged, at 178 ml, slightly ahead of untreated choleragen, and the trailing minor peak, at 208 ml, emerged very slightly in front of untreated choleragenoid (215 ml). A leading peak, similar to that obtained in SDS-gel filtration, was not present.

When choleragen was treated directly with 0.1% SDS prior to conventional electrophoresis, two bands were found (Fig. 6, column A, no. 1). One appeared to be identical with choleragen and one was slightly slower moving, similar to the products isolated from the SDS columns. When ME was included in the SDS, only one band was

observed (Fig. 6, column A, no. 2) in an area between the two bands observed in Fig. 6 (column A, no. 1). Treatment of choleragen with ME prior to conventional electrophoresis produced two bands (Fig. 6, Column A, no. 3), one minor faster moving band and the major band moving just slightly behind choleragen. On treatment of choleragen with 0.01 M DTT at *p*H 7.5 (the *p*H of all the previous conditions), three bands were observed (Fig. 6, column A, no. 4). Of these, two were very similar to those found on treatment with ME, and one appeared to be similar to unaltered choleragen. However, when the treatment was performed at *p*H 8.0, only a single major band (Fig. 6, Column A, no. 5), similar to that of untreated choleragen, resulted. Multiple bands were not observed after treatment of the marker proteins.

It should also be pointed out that procholeragenoid, the large-molecular-weight aggregate formed by heating choleragen, behaved in a manner similar to choleragen in SDS electrophoresis, but is too large to penetrate the gels in conventional electrophoresis.

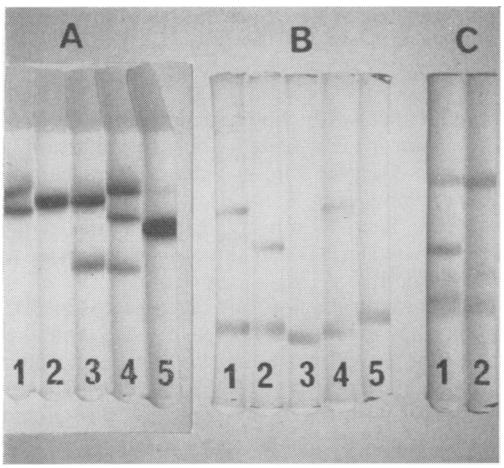

FIG. 6. *Conventional (A) and SDS (B, C—described later in the text) gel electrophoresis of the following. Column A: no. 1, choleragen treated with SDS; no. 2, choleragen treated with SDS plus ME; no. 3, choleragen treated with ME; no. 4, choleragen treated with DTT at pH 7.5; no. 5, choleragen treated with DTT at pH 8.0. Column B: no. 1, choleragen heated in SDS for 3 min at 100 C; no. 2, choleragen heated in SDS plus ME for 3 min at 100 C; no. 3, choleragenoid heated in SDS for 3 min at 100 C; no. 4 and 5, choleragen and choleragenoid, respectively, acidified prior to addition of SDS. Column C: no. 1 and 2, choleragen and choleragenoid, respectively, in SDS electrophoresis in 8 M urea. Note: The above electrophoretic analyses were performed on different occasions, hence the minor differences in mobility of similar components.*

Reduction and alkylation of choleragen. ^{3}H-labeled choleragen (0.2 mg) diluted with 20 mg of cold carrier was treated with 0.14 M ME at *p*H 8.0 and was then alkylated with 0.2 M iodoacetamide at *p*H 8.0. After dialysis, a trace of precipitate was removed, and the soluble product was chromatographed on P-150. The optical density elution profile (data not shown) consisted of a diphasic peak in the choleragen-choleragenoid region, which contained virtually all of the radioactivity (Fig. 7), and a slower minor peak, elution volume 268 ml, which contained only a trace of radioactivity. The heterogenous major peak gave rise to three bands on electrophoresis (Fig. 7, gels 2–4), of which two were apparently variants of choleragenoid and the third corresponded to choleragen. The minor peak (Fig. 7, no. 5), which was not radioactive, corresponded to the faster moving component observed when choleragen was treated with ME and subjected to electrophoresis (see Fig. 6, Column A, no. 3). Fig. 7 (no. 1) illustrates the preparation prior to gel filtration.

Electrophoresis after heating in SDS, in SDS and 8 M urea, and in 8 M urea. Since the results of SDS electrophoresis indicated fewer and larger subunits than those observed in our previous work (11), more rigorous treatments were tried. After heating of choleragen and choleragenoid in SDS for 3 min at 100 C (10), the 56,000 band was no longer prominent in SDS electrophoresis (Fig. 6, column B, no. 1–3). Instead, a band migrating somewhat faster than Cyt *c* appeared which, by extrapolation of the calibration curve obtained by using BSA, OA, CT and Cyt *c*, represented a peptide of approximately 10,000 molecular weight (mean of eight determinations, range 8,000–15,000). Neither the 28,000 piece of choleragen nor the marker proteins, BSA and Cyt *c*, appeared to be altered by this procedure. Virtually identical results were obtained when choleragen and choleragenoid were adjusted to *p*H 3.25, the SDS was added, and the proteins were subjected to electrophoresis in SDS after neutralization (Fig. 6, column B, no. 4 and 5). On SDS electrophoresis in the presence of 8 M urea, the results tended to be similar. Although there was still a residual 56,000 band, a band also appeared in the region of 10,000 molecular weight (Fig. 6, column C, no. 1 and 2). After electrophoresis in 8 M urea, without SDS, choleragen and choleragenoid appeared as single discrete bands, with choleragenoid migrating somewhat further (Fig. 8, no. 1 and 2); treatment of choleragen with ME had no effect on its mobility in this medium and there was no evidence of another band (Fig. 8, no. 3). Of the three pools isolated previously from the SDS P-150 column (see Fig. 3), the first

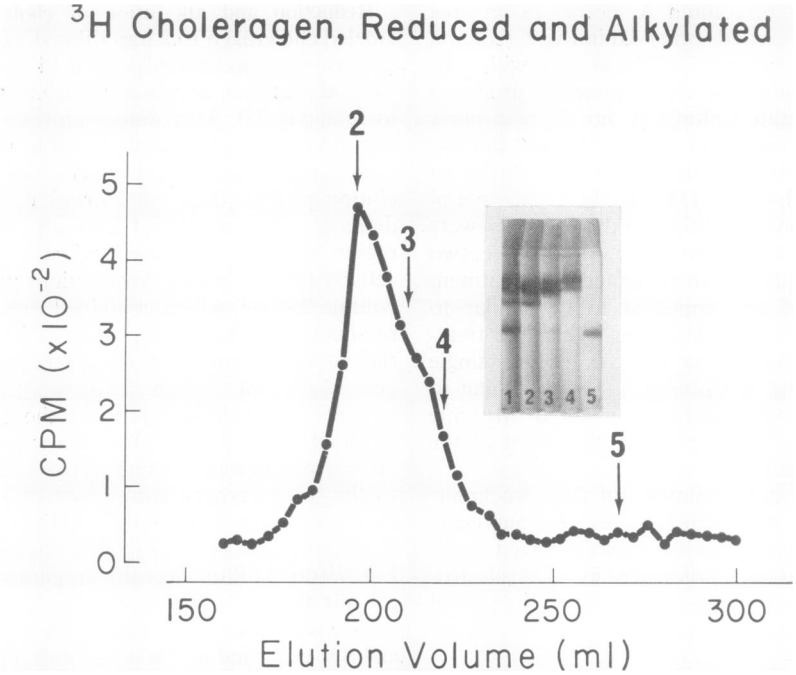

FIG. 7. *Gel filtration of reduced and alkylated ^{3}H-choleragen on polyacrylamide P-150. Insert 1 depicts the conventional electrophoretic behavior of the mixture prior to gel filtration; inserts 2 to 5, the behavior of the indicated fractions.*

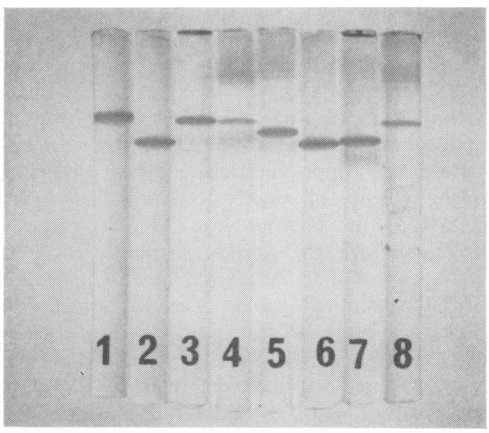

FIG. 8. *Electrophoresis in 8 M urea. 1, Choleragen; 2, choleragenoid; 3, choleragen plus ME; 4–6, consecutive peaks from SDS-P-150 gel filtration; 7, major peak from SDS-ME-P-150 gel filtration; 8, leading edge of SDS-ME-P-150 gel filtration.*

migrated like the toxin in urea gel electrophoresis, but also exhibited a series of slow moving bands (Fig. 8, no. 4). The major band of the second pool was intermediate between the toxin and the toxoid (Fig. 8, no. 5) and also had some slower moving material, whereas the third pool appeared to be identical with choleragenoid (Fig. 8, no. 6). The major peak isolated after gel filtration in SDS plus ME (see Fig. 4) behaved identically to choleragenoid (Fig. 8, no. 7), whereas the leading edge (Fig. 8, no. 8) appeared similar to the leading edge isolated in SDS in the absence of ME (see Fig. 8, no. 4).

SDS-gel filtration of choleragen heated in SDS. When choleragen was heated in SDS and then subjected to gel filtration in SDS, two major peaks were obtained (Fig. 9). The first appeared to contain both the 28,000 and 23,000 fragments and a trace of the 56,000 species. The major peak contained residua of the 28,000 and 23,000 pieces, but consisted primarily of the approximately 10,000 molecular weight subunit described above. Interestingly, its elution volume was similar to those observed in earlier separations of unheated choleragen in the same SDS column. These fractions, however, did not stain with the usual procedures after conventional gel electrophoresis.

Ultracentrifugation studies. The sedimentation coefficients of choleragen and choleragenoid, corrected for concentration dependence, were previously reported to be 5.5 and 4.35, respectively (11). When ultracentrifuged in the presence of 0.1% SDS (Fig. 10, column A, top), choleragen

exhibited a major component of 4.9*S* and a minor component of approximately 3.6*S*. In the presence of SDS and DTT (Fig. 10, column A, bottom), a single homogeneous peak of 4.2*S* was found. After ultracentrifugation, samples were examined by electrophoresis. The preparation treated with SDS behaved like choleragen in subsequent SDS electrophoresis but, in conventional electrophoresis, it exhibited, in addition to the choleragen band, a slower moving band similar to choleragenoid. Material treated with SDS and DTT behaved precisely like choleragenoid in conventional and SDS electrophoresis.

In contrast to the results obtained with the reducing agent DTT in electrophoresis, ultracentrifugation in the presence of DTT yielded only a single symmetrical 5.1*S* peak at both *p*H 7.5 (Fig. 10, column B) and 8.0 (Fig. 10, column C). Material removed from the ultracentrifuge was subjected to electrophoresis with results precisely as reported above (Fig. 6, column A, no. 4 and 5): three bands at *p*H 7.5 and one band at *p*H 8.0.

Attempts were made to evaluate the effects of boiling cholera toxin in SDS and of acidification prior to SDS treatment in the ultracentrifuge. However, the results were uninterpretable be-

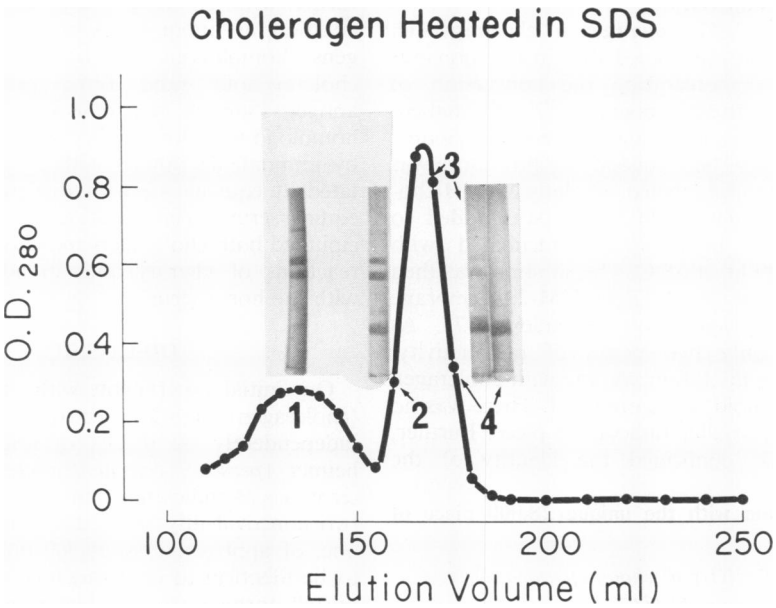

FIG. 9. *SDS-gel filtration of choleragen pretreated by boiling in 1% SDS. Inserts show the behavior of respective fractions in SDS-gel electrophoresis.*

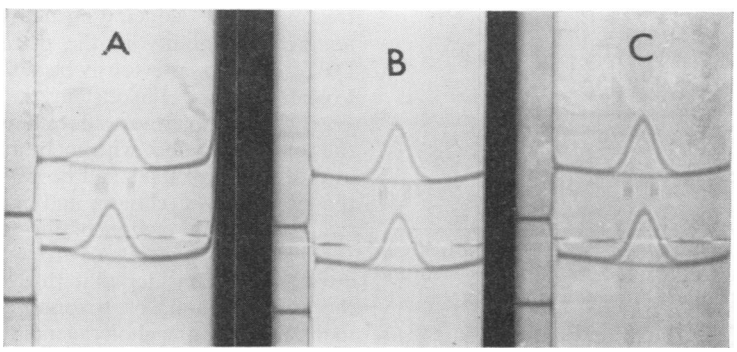

FIG. 10. *Ultracentrifugation of choleragen in: column A(top), SDS; A(bottom), SDS plus DTT; column B(top), in Tris buffer (pH 7.5); B(bottom), in Tris buffer (pH 7.5) plus DTT; column C(top), in Tris buffer (pH 8.0) plus DTT; C(bottom), in Tris buffer (pH 8.0). After 80 min at 56,100 rev/min.*

cause of sedimenting material in similarly treated diluent.

Toxicity studies: choleragenicity and skin reactivity. Results of toxicity tests, summarized in Table 1, indicated, in accordance with our previous observations, that both choleragenicity and skin reactivity occur in parallel with each other even using modified proteins. Choleragen retained both activities following reduction with DTT at both *p*H 7.5 and *p*H 8.0 and upon treatment with SDS. Following treatment with both SDS and DTT, both skin reactivity and choleragenicity were significantly lowered. Treatment with ME alone had no detectable effect on toxicity. Toxicity was still evident following reduction and alkylation.

Conversion of ^{3}H-choleragen to choleragenoid. Because it might be argued that in our previous experiments demonstrating the conversion of ^{131}I-choleragen to choleragenoid (7), a fragment low in tyrosine content, and hence also in iodine label, might have been missed, the procedure was repeated with ^{3}H-labeled choleragen. ^{3}H-choleragen mixed with cold carrier was added to sterile syncase medium and incubated with shaking for 20 hr at 35 C. The solution was then concentrated on an Amicon UM-2 membrane and chromatographed on Sephadex G-75. As before (7), only two peaks of radioactivity, corresponding in elution volume with choleragen and choleragenoid, were observed. There was no evidence of a smaller radioactive piece. Further, electrophoresis confirmed the identity of the two peaks.

Immunization with the unique 28,000 piece of choleragen. To test the hypothesis that the leading peak, isolated from choleragen after gel filtration on P-150 in SDS (which in subsequent SDS electrophoresis behaved like the 28,000 piece), might contain antigens unique to choleragen and not present in choleragenoid, two rabbits were immunized by inoculation in multiple sites with 2.5 mg of the protein in complete Freund adjuvant. Their antisera, tested in gel diffusion precipitin tests, gave reactions of identity with choleragen, choleragenoid, and the peak I used in immunization. Further, no differences among the antigens could be detected by using anticholeragenoid horse serum (11), antitoxic goat serum, and a battery of sera from rabbits immunized earlier with choleragen and choleragenoid. In addition, an attempt was made to "block" antigens homologous to both choleragen and choleragenoid (and thus, perhaps, unmask antigens unique to choleragen to which the homologous region might be immunodominant) by immunizing rabbits with choleragen precipitated at equivalence with the anticholeragenoid equine serum. The resulting antisera again precipitated both choleragen and choleragenoid with reactions of identity and, in addition, reacted with the horse serum.

TABLE 1. *Toxicity of choleragen and derived products*

Product	Choleragenicity[a]	Skin reactivity[b]
Choleragen	+	+
Choleragenoid	−	−
Choleragen + SDS	+	+
Choleragen + SDS + DTT	−	−
Choleragen + ME	+	+
Choleragen + DTT, *p*H 7.5 and 8.0	+	+
Choleragen, reduced and alkylated	+	+

[a] Caused experimental cholera, manifested by excessive fluid in the gut 5 to 6 hr after feeding 50 μg with bicarbonate, in groups of four adult mice.

[b] Elicited a typical erythematous, edematous, indurated skin reaction evident 24 hr after intracutaneous inoculation of 0.0003 to 0.0016 μg in two rabbits.

DISCUSSION

Our initial experiments with the cholera toxin (choleragen) in SDS electrophoresis, supported independently by results of Gill and Pappenheimer (*personal communication*), could be interpreted as indicating that the toxin consists of two noncovalently associated peptides. Of these, one, of approximately 56,000 daltons, appeared to be identical to choleragenoid, the "spontaneously" formed toxoid. The other, of approximately 28,000 molecular weight, was ostensibly unique to the toxin and appeared to contain an even smaller piece which could be detached by treatment with reducing agents (as evidenced by its greater mobility in the presence of ME or DTT). As it has previously been shown (Peterson, LoSpalluto, and Finkelstein, J. Infect. Dis., *in press*) that choleragenoid retains the ability of the toxin to interact with membranous structures at the surface of the small bowel without causing the symptoms of cholera and can even competitively inhibit the action of the toxin (N. Pierce, *personal communication*), it was immediately attractive to consider that the 28,000 piece was the active region, or toxophore group, of the toxin, and that the choleragenoid portion was the carrier responsible for the recognition of host cell receptors. The appeal of this hypothesis was enhanced by its similarity to the diphtheria model.

The diphtheria toxin has recently been shown to consist of two peptides: an enzymatically active portion, fragment A, which by itself is not toxic as it cannot enter cells, and a carrier portion, fragment B, which is essential for penetration (A. M. Pappenheimer, Jr., T. Uchida, and A. A. Harper, Immunochemistry, *in press*).

However, despite the attractiveness of the hypothesis generated by the results of SDS electrophoresis with choleragen, little, if any, independent support developed from other methods of investigation. In an attempt to isolate the 28,000 piece by P-150 gel filtration in the presence of SDS, the first peak to emerge from the column had the characteristic mobility of the 28,000 piece in subsequent SDS electrophoresis, but it contained only 16% of the radiolabel instead of an expected one-third. Further, this peak, used to immunize rabbits, was found to stimulate the production of antibodies identically reactive with both choleragen and choleragenoid. When a reducing agent, ME, was included with the SDS used in gel filtration, the protein emerged in essentially a single peak with an elution volume similar to that of choleragenoid. It appeared to be similar to the 56,000 molecular weight portion on subsequent SDS electrophoresis and had greatly reduced toxicity. In other words, it appeared that a very effective transformation of choleragen to choleragenoid had occurred. In this case, however, there was only a trace of the 28,000 piece whereas, according to the previous results of SDS electrophoresis with reducing agent, the 28,000 piece should still be present in significant amount but should only migrate further.

On ultracentrifugation in the presence of SDS and DTT, choleragen again appeared as a single symmetrical peak with a sedimentation coefficient similar to that of choleragenoid. These results thus tended to agree with the gel filtration experiments rather than with the results of SDS electrophoresis. On ultracentrifugation in SDS alone, there was some evidence for a minor smaller fragment. However, the sedimentation coefficient of the minor component (3.6*S*) in SDS, alone, does not permit the conclusion that its size is 28,000, as was predicted from the SDS electrophoresis.

Other anomalies were revealed when the direct effects of reducing agents were examined in systems other than electrophoresis. After treatment of choleragen with ME or DTT, two bands (or three in the case of treatment with DTT at *p*H 7.5) were observed in electrophoresis. However, no evidence for significant fragmentation of the molecule was observed in the ultracentrifuge or by gel filtration in the presence of reducing agent. In addition, ^{3}H-choleragen treated with DTT and then alkylated with iodoacetamide retained its toxicity although some choleragenoid was formed, perhaps as a result of the manipulations. It is significant that the trailing peak isolated from this mixture, which formed a prominent band in electrophoresis, was not radioactive.

We examined further the transformation of choleragen to choleragenoid which is brought about by shaking at slightly elevated temperatures (7), by using, in the present effort, a tritium instead of an iodine label, and again we could find no evidence of a labeled piece of 28,000 or any other molecular size. This transformation thus appears to conserve all of the protein in the toxin. On the other hand, when choleragen was previously converted to aggregate (procholeragenoid) and thence to choleragenoid by heating (2), there were significant losses in the form of insoluble denatured protein. This selective process could then account for the reported amino acid differences (11) between choleragen and choleragenoid. One would thus predict that some choleragenoid species would have amino acid composition identical with the toxin. For example, in the present study, choleragen appeared to be converted to choleragenoid most effectively by treatment with SDS in the presence of a reducing agent. This process most likely would be a rearrangement perhaps involving disulfide interchange.

Under usual conditions of treatment with SDS, there was no evidence for dissociation into the 14,000 molecular weight subunits demonstrated earlier (11) by mild acid treatment. However, when more rigorous treatment, such as heating in SDS or addition of 8 M urea, was applied, both choleragen and choleragenoid did apparently give rise to subunits whose molecular weights were approximately 10,000 according to the results of SDS electrophoresis. Inasmuch as the reliability of the method in providing accurate molecular weight approximations in this range is admittedly questionable these may, in fact, correspond with the 14,000 molecular weight subunits reported previously. In the case of choleragen, however, these treatments did not affect the 28,000 piece. Interestingly, following boiling, the major component which had an apparent molecular size of 10,000 emerged from the SDS P-150 column in the same position as material which had not been heated and had an apparent size of 56,000.

Our observations thus indicate that both choleragen and choleragenoid are relatively resistant to dissociation by SDS unless previously unfolded by low *p*H (*p*H 3.25–3.7), heat, or by treatment with 8 M urea (which results in only

partial unfolding). Procholeragenoid is apparently partially dissociable and behaves like choleragen when treated with SDS.

It is extremely difficult to reconcile the apparent size of the 28,000 fragment with the observation that it contains only 1/6 of the radioactivity of parent choleragen. A more reasonable explanation, in the light of the present experimental evidence and the previously demonstrated aberrant behavior of these proteins in molecular sieving, is that the 28,000 piece actually represents a dimer or larger aggregate of a smaller subunit. This would be compatible with the observation that choleragenoid may be formed from choleragen with total conservation of protein. In view of the observation that the 28,000 piece stimulates the formation of antibody which is equally reactive with both the toxin and the toxoid, it is highly unlikely that it represents a component entirely unique to the toxin. Apparently, in the transformation of toxin to toxoid, this piece is so integrated that it is no longer detectable by the techniques thus far applied. However, the presence, in this fragment, of an immunologically inactive labile region which is unique to the toxin could well account for the experimental observations.

The evidence also suggests that the multiplicity of bands observed in conventional electrophoresis of choleragen treated with reducing agents may be artifacts of the procedure rather than fragments actually produced by reduction of disulfide bonds. No evidence could be generated to support a significant reductive cleavage either by ultracentrifugation, gel filtration, urea-gel electrophoresis, or toxicity studies. However, it is conceivable that a fragment, or fragments, too small to be detected in these procedures, could have been split off. However, one such fragment, isolated following reduction and alkylation of ^{3}H-choleragen, had essentially no radioactivity.

The applicability of these observations to the results of studies involving other proteins of bacterial origin merits further consideration. In our estimation, our present results illustrate the hazards potentially involved in the interpretation of data, however pleasing, on the basis of only one parameter, the estimation of size of "subunits" by SDS electrophoresis.

The reasons for the toxicity of choleragen and the lack of toxicity of choleragenoid remain to be elucidated by further investigation. They may yet be found to involve the "labile region" of the "28,000 piece."

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service research grant no. AI-08877 under the United States-Japan Cooperative Medical Science Program administered by the National Institute of Allergy and Infectious Diseases. We appreciate the unpublished observations graciously provided by Michael Gill and A. M. Pappenheimer, Jr.

LITERATURE CITED

1. Finkelstein, R. A. 1970. Monospecific equine antiserum against cholera exo-enterotoxin. Infect. Immunity **2**:691–697.
2. Finkelstein, R. A., K. Fujita, and J. J. LoSpalluto. 1971. Procholeragenoid: an aggregated intermediate in the formation of choleragenoid. J. Immunol. **107**:1043–1051.
3. Finkelstein, R. A., and J. J. LoSpalluto. 1969. Pathogenesis of experimental cholera: preparation and isolation of choleragen and choleragenoid. J. Exp. Med. **130**:185–202.
4. Finkelstein, R. A., and J. J. LoSpalluto. 1970. Preparation of highly purified choleragen and choleragenoid. J. Infect. Dis. **121**(Suppl.):S63–S72.
5. Finkelstein, R. A., and J. J. LoSpalluto. 1972. Crystalline cholera toxin and toxoid. Science **175**:529–530.
6. Finkelstein, R. A., and J. W. Peterson. 1970. In vitro detection of antibody to cholera enterotoxin in cholera patients and laboratory animals. Infect. Immunity **1**:21–29.
7. Finkelstein, R. A., J. W. Peterson, and J. J. LoSpalluto. 1971. Conversion of cholera exo-enterotoxin (choleragen) to natural toxoid (choleragenoid). J. Immunol. **106**:868–871.
8. Fujita, K., and R. A. Finkelstein. 1972. Antitoxic immunity in experimental cholera: comparison of immunity induced perorally and parenterally in mice. J. Infect. Dis. **125**: 647–655.
9. Hochstein, H. D., J. C. Feeley, and S. H. Richardson. 1970. Titration of cholera antitoxin levels by passive hemagglutination tests using fresh and formalinized sheep erythrocytes. Proc. Soc. Exp. Biol. Med. **133**:120–124.
10. Lenard, J. 1970. Protein components of erythrocyte membranes from different animal species. Biochemistry **9**: 5037–5040.
11. LoSpalluto, J. J., and R. A. Finkelstein. 1972. Chemical and physical properties of cholera exo-enterotoxin (choleragen) and its spontaneously formed toxoid (choleragenoid). Biochim. Biophys. Acta **257**:158–166.
12. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem. **244**:4406–4412.