Development of a Purified Cholera Toxoid

I. Purification of Toxin

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The enterotoxin from Vibrio cholerae is selectively concentrated from cell-free culture supernatant by co-precipitation with hexametaphosphate and is further purified by adsorption on aluminum hydroxide powder. The bulk of residual somatic antigen becomes insoluble upon lyophilization of the toxin preparation and is removed by centrifugation of the rehydrated material. Other contaminants are eliminated by treatment with activated carbon. Preparations of toxin, purified by this method, have been characterized by: (i) a single immunoprecipitin line against polyvalent antisera; (ii) homogeneity on acrylamide gels; (iii) specific activities on the order of 22 limit-of-bluing doses/μg; (iv) ultraviolet spectra characteristic of pure protein; and (v) overall yields on the order of 50%, irrespective of purification scale. Such preparations, however, have been shown to contain trace amounts of somatic antigen when they are intensively tested either for their ability to elevate serum vibriocidal antibody titers in immunized rabbits or for their ability to increase resistance of immunized mice to live vibrio challenge. In the latter test system, the level of residual somatic antigen per 50 μg of toxin (toxoid) antigen generally did not exceed 0.025% of the Division of Biological Standards reference vaccine, V. cholerae Inaba IN-12. Methods for elimination of this small amount of somatic antigen have been investigated and are discussed. The particular combination of purification steps which are presently described have been easily and reproducibly applied on a production scale to prepare gram amounts of toxin with a high degree of purity, even under a variety of initial conditions.

At present, a convincing body of evidence supports the view that the symptoms of clinical cholera are caused by an enterotoxin secreted into the gut by proliferating Vibrio cholerae (2, 18). On the basis of this view and because conventional bacterial vaccines do not provide adequate long-term protection (1, 24), interest has been aroused in determining whether antitoxic immunity alone can provide effective protection against the clinical manifestations of cholera.

The availability of V. cholerae strains, most notably Inaba 569B, that can produce toxin in vitro, together with reliable methods for its production (8, 13, 20), purification (12, 21, 22), and routine assay (3–5), have greatly advanced the possibility of testing this hypothesis.

Further, the development of experimental cholera models (2, 18) has made it possible to study the efficacy of antitoxic immunity in preventing or controlling symptoms of the disease. In the canine model, for example, parenteral immunization with crude cholera toxin, which stimulates the production of both antibacterial and antitoxic antibody, protected dogs against subsequent intraintestinal challenge with viable V. cholerae (6). This protection correlated more significantly with serum antitoxin titers than with serum vibriocidal titers (6). More recently, dogs immunized parenterally with pure cholera toxin (plus Freund adjuvant) exhibited elevated serum antitoxin titers and were protected against challenge with viable V. cholerae for at least 10 months (19). In the same study, parenteral immunization with formalinized toxoid (without adjuvant), although resulting in lower antitoxin titers, gave rise to significant protection which endured for a minimum of 5 months (19). This result suggested that antitoxic antibody elicited by chemically detoxified toxoid (toxoid) may also correlate with protection in experimental canine cholera. It remains to be determined, however, whether or not appropriate parenteral immunization with a suitable cholera toxoid will provide effective protection in experimental
human cholera, and such studies have already been initiated (R. B. Hornick, personal communication).

To determine unambiguously whether antitoxic antibodies alone can provide effective and prolonged protection in the field, a pure toxin, possessing little or no ability to induce vibriocidal antibodies, is required. To produce purified toxin in sufficient quantity for potential use as a prophylactic against cholera, a simple, large-scale purification scheme has been developed. The details of this method are described in this report. The problems surrounding the preparation of a stable, antigenic toxoid are the subject of a subsequent communication.

MATERIALS AND METHODS

Production of toxin. V. cholerae serotype Inaba, strain 568B, was obtained from John C. Feeley of the National Institutes of Health, Bethesda, Md. Approximately 250 liters of TRY medium (20) was inoculated with vibrios from an overnight nutrient agar slant, and the medium was incubated with aeration at 26 C for 48 h. In later studies, incubation time was changed from 48 to 24 h to minimize components arising from lysis of the vibrios. After incubation, the organisms were harvested by centrifugation and discarded, and the supernatant, containing toxin, was filtered through a membrane filter (0.22 μm pore size, Millipore Corp.) and held at 4 C.

Purification of toxin. The procedure adopted for purification was as follows. Sodium hexametaphosphate (2.55 g/liter) (Fisher Scientific Co.) was added at room temperature to cell-free toxin supernatant precleared to 4 C. When the metaphosphate was dissolved, the pH of the solution was adjusted slowly, with stirring, to pH 4.6 by the addition of concentrated HCl. The solution was stirred under these conditions until optical density readings at 640 nm indicated no additional precipitation. When equilibration was complete, filter aid (0.5 g/liter) (Celite-Johns-Manville) was added, and the mixture was stirred for an additional 10 min. The precipitate was collected on a filter bed (Whatman no. 1 filter paper plus filter aid) by suction filtration, and the filtrate was discarded. The precipitate was washed on the filter with 20% of the initial volume (of toxin supernatant) of cold 0.2 M NaCl, pH 4.5 to 4.6, and the wash was discarded. The washed precipitate plus filter aid were resuspended in one-half of the desired final volume (that volume which concentrated toxin to a value of approximately 3,000 limit-of-bluing [Lb] doses/ml) of 0.15 M Na₃HPO₄, pH 8.0; the precipitate was dissolved by stirring at room temperature for 1 h. The solution was collected by suction filtration, and the filtrate was set aside. The filter aid was resuspended in the remaining 0.5 volume of buffer, stirred for 1 h, and collected as before. The first and second filtrates were combined. This solution was designated metaphosphate concentrate and was held at 4 C until further use.

The metaphosphate concentrate, at or near room temperature, was adjusted to pH 5.6 by the dropwise addition, with stirring, of concentrated HCl. To each 100 ml, 30 g of Al(OH)₃·3H₂O (Mallinkrodt Chemical Works) was added and dispersed uniformly by stirring at room temperature for 2 h. The adsorbent was collected by suction filtration with Whatman no. 42 filter paper. The filtrate, designated aluminum hydroxide supernatant, was discarded, and the adsorbent was washed on the filter with a volume of distilled water, pH 5.3 to 5.5, equal to twice the initial volume of metaphosphate concentrate. The adsorbent was resuspended in one-half to three-quarters of the initial volume (metaphosphate concentrate) of 0.2 M NH₄HCO₃, pH 7.9 to 8.3, and the suspension was stirred for 1 h. The suspension was suction-filtered, as before, and the adsorbent was discarded. Alternatively, toxin could be eluted from the adsorbent in two sequential extractions by using one-half of the desired final volume of buffer for each extraction. The filtrate, designated bicarbonate eluant, was held at 4 C until further use.

The bicarbonate eluant was lyophilized and then redissolved in a volume of 0.067 M phosphate-buffered saline, pH 7.8 to 8.0, so that the final concentration was approximately 1 mg/ml. Acid-washed activated carbon (Pfanstiehl Laboratories, Inc.) was added to a final concentration of 1 mg/ml and dispersed uniformly throughout the solution by stirring for 1 h at room temperature. The carbon was removed by suction filtration, and the concentrated toxin solution was centrifuged either in a Spinco 30 rotor at 78,140 × g for a minimum of 6 h or in a Spinco 21 rotor at 44,330 × g for a minimum of 16 h. The supernatant was removed from the centrifuge tubes by aspiration, filtered aseptically through a membrane filter (0.22 μm pore size, Millipore Corp.) and stored at 4 C. The sediment was discarded.

Preparation of antisera. Antiserum was prepared in goats. Toxin preparations were mixed 1:2 with Amphogel (Wyeth Laboratories, Inc.), and 2.5 ml of the mixture was injected intramuscularly into each of four legs. Boosters were given 2 and 4 weeks after the initial injection by using 1.25 ml of the mixture in each of four legs. The goats were bled 2 weeks after the last booster injection. Antisera against two preparations of toxin were obtained. One preparation, containing approximately 3,000 Lb doses/ml, was a 10 × Amicon PM-10 concentrate of the antigens in crude cell-free culture fluid. The second preparation, containing approximately 1,300 Lb doses/ml, was concentrated from cell-free supernatant by precipitation with ammonium sulfate (60% saturation), adsorbed onto and eluted from aluminum hydroxide, and filtered through an Amicon XM-100 membrane. The latter preparation contained some sedimentable somatic antigen as determined retrospectively by analysis of both the immune sera and the antigen preparation. Antiserum to the first preparation was labeled Wyeth goat no. 1 antitoxin, and antiserum to the second preparation was labeled Wyeth goat no. 2 antitoxin.

Measurement of toxin potency. Toxin content of preparations was estimated by the limit of bluing method in rabbit skin as described by Craig (3, 4),

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with the exception that 1% Evans Blue dye (Allied Chemical Corp.) in saline, given intravenously in a dose of 0.5 ml/lb (about 0.45 kg), was substituted for Pontamine Sky Blue 6BX for visualization of zones of increased vascular permeability. For titration of Lb potency of the toxin, 1 volume of provisional standard cholera antitoxin (Swiss Serum and Vaccine Institute-purified cholera antitoxin [horse] with an assigned value of 4,470 antitoxin units [AU] per ml, supplied by John Seal, National Institute of Allergy and Infectious Diseases) containing 1 AU/ml was incubated with an equal volume of appropriate serial 0.15 log toxin dilutions (in 0.067 M phosphate buffer, with 0.1% gelatin, pH 7.4) for 1 h at 37 C before quadruplicate intracutaneous inoculations of 0.1 ml into each of two rabbit skins. The results were based on the Lb end point as redefined by Craig (4). One Lb is that amount of toxin which, in the presence of 1 AU/ml, gives a 4-mm bluing lesion in rabbit skin (4). Unless otherwise specified, all Lb titrations were carried out at the Lb/20 level, and potencies are expressed in Lb doses per milliliter.

Toxoid units. To quantify preparations of inactivated toxoid, toxoid units (TU), a measure of antitoxin combining power, were determined according to a method devised by Craig (4; personal communication). Based on the ability of toxoid to combine with antitoxin in vitro, the assay measures the apparent decrease in neutralizing capacity of a known amount of antitoxin after it has been incubated with toxoid. A series of tubes containing equal volumes of antitoxin (2 AU/ml) and an appropriate dilution of toxoid were incubated at 37 C for 30 min. Another series of tubes containing equal volumes of antitoxin (2 AU/ml) and buffer were incubated under the same conditions. After incubation, an equal volume of serial 0.15 log dilutions of toxoid were added to each series of tubes, and the assay was carried out in a manner identical to the Lb assay described above. The displacement of the bluing end point was used to calculate the amount of free and bound antitoxin in the antitoxin-toxoid mixture. One TU was that amount of toxoid which could effectively bind 1 unit of antitoxin.

Mouse protection test for determination of somatic antigen. Protective activities of residual somatic antigen in various toxin preparations against Inaba NIH 35A-3 challenge were determined relative to the DBS reference vaccine, V. cholerae Inaba IN-12, by the mouse protection test described by Feeley and Pittman (9). For immunization, toxin was converted into a Formalin toxoid by a standard procedure (see Results). Results were determined as the ratio of the mean effective doses (in milliliters) of the reference to the toxoid preparation per 1,000 TU (approximately 50 μg of toxoid antigen).

Vibrioidal assay. Vibrioidal activity of various immune rabbit sera was determined by using Feeley's modification (personal communication) of the bac- tericidal assay described by Muschel and Treffers (16). Inaba VC-13, obtained from Dr. Feeley, was the vibrio strain used. Sera were obtained from rabbits 4 or 6 weeks after primary intramuscular inoculation with 100 μg of Formalin or glutaraldehyde toxoid (see Results) and 2 weeks after identical booster inco-

Immunodiffusion and immunoelectrophoresis. Ouchterlony-type immunodiffusion tests were performed at room temperature in IDF-I cells obtained from the Cordis Corp. (Miami, Fla.). For quantitative analysis of sedimentable somatic antigen, a radial immunodiffusion technique similar to that described by Mancini et al. (15) was used. Uniform agar layers (1% Noble agar in 0.1 M barbiturate buffer, pH 8.6), 1-mm thick and containing a final dilution of 1:64 Wyeth goat no. 2 antitoxin, were prepared on glass slides (83 by 102 mm). A single immunodiffusion plate accommodated 36 wells, each of which had an internal diameter of 2.8 mm and held 5 slits. Serial twofold dilutions of antigen were assayed in sextuplicate, and zones of precipitation were allowed to develop at room temperature for 24 h. Each immunoprecipitin plate was photographed with a Cordis camera, and the diameters of the precipitin rings, magnified 1.9 times by photography, were measured directly from the photograph.

Immunoelectrophoresis was performed with a Shandon electrophoresis apparatus according to the procedure described by Finkeinstein and LoSpalluto (12) with the exceptions that: (i) electrophoresis was performed for 2 h at 4 C; and (ii) polyvalent antiserum (Wyeth goat no. 1 antitoxin) was used for development of the immunoprecipitin pattern. Samples were concentrated to approximately 10 mg/ml by dialysis against Ficoll (Pharmacia, Piscataway, N. J.) before electrophoresis.

Acrylamide gel electrophoresis. Acrylamide gel electrophoresis was performed at pH 9.3 by using the standard two-gel system described by Ornstein (17) and Davis (7), and by using Canalco equipment (Canal Industrial Corp., Rockville, Md.). Before electrophoresis, samples were dialyzed against 0.025 M tris(hydroxymethyl)aminomethane, 0.19 M glycine, pH 8.3. Between 100 and 200 μg of protein in 0.2 to 0.3 ml was layered on top of the spacer gel with a drop of glycerol. Current was 1 mA per gel until the tracking dye entered the separating gel; then the current was increased to 2 mA per gel for the remainder of the run. In some instances, electrophoresis was allowed to proceed 30 min beyond the clearance of the tracking dye from the gel.

Electron microscopy. Microdroplets of purified toxin (90 μg/ml) were deposited on carbon-coated copper specimen grids and negatively stained with 2.0% uranyl acetate, pH 4.1. Specimens were observed at 50 kV with an RCA EMU-3H electron microscope equipped with double condenser illumination.

Protein determinations. Protein was determined by the Lowry modification of the Folin-Ciocalteau method (14) with crystalline bovine serum albumin as standard.

RESULTS

Purification. Table 1 summarizes the results of purification in a step-by-step analysis of the recovery of protein and toxin activity in a
representative large-scale preparation. In the first step of the procedure, toxin was concentrated from cell-free culture filtrate by co-precipitation with sodium hexametaphosphate at pH 4.6. Figure 1 shows the distribution of precipitating species as a function of pH in the presence and absence of metaphosphate. The addition of metaphosphate to the culture filtrate enhanced precipitation throughout the pH gradient and partitioned the precipitating species into three distinguishable zones. Activity assays and immunodiffusion tests showed that toxin precipitated in the first zone between pH 5.4 and 4.6. When toxin was concentrated by the metaphosphate procedure, increases in specific activity on the order of 15-fold or greater and recoveries of 90% or more were reproducibly obtained in four consecutive large-scale preparations.

In the second step of the procedure, toxin was further purified by adsorption onto, and elution from, aluminum hydroxide powder. Adsorption was pH dependent, with maximal binding of toxin occurring at or near pH 5.6. Elution appeared to be independent of the buffer system employed as long as the pH was alkaline. In this procedure, toxin was eluted from the adsorbent with 0.2 M ammonium bicarbonate, pH 8.0.

Under conditions of maximal binding, toxin recoveries were governed by the relative concentration of toxin and adsorbent. Figure 2 shows the dependence of recovery on percent concentration of adsorbent suspension for the case in which the amount of toxin exceeded the adsorption capacity of the powder. When conditions were such that the ratio of total Lb units per gram of adsorbent was on the order of $10^4$, toxin recoveries ranging from 60 to 72% were reproducibly obtained in four consecutive large-scale preparations.

When examined by agar gel double-diffusion assays, toxin preparations at this stage of purification exhibited a single immunoprecipitin line upon diffusion against antiserum containing antibodies against toxin and several somatic antigens (Wyeth goat no. 1 antitoxin). Such preparations, however, were able to protect immunized mice against live vibrio challenge, thus indicating the presence of residual somatic antigen. Further studies showed that the level of somatic antigen could be significantly reduced if toxin preparations were centrifuged at high speed.

Table 2 gives representative data showing the

**Table 1. Summary of purification: specific activity and recovery**

<table>
<thead>
<tr>
<th>Stage of purification</th>
<th>Vol (liters)</th>
<th>Lb/ml</th>
<th>Total Lb $\times 10^5$</th>
<th>Protein (µg/ml)</th>
<th>Total µg $\times 10^5$</th>
<th>Sp act (Lb/µg)</th>
<th>Fold increase in sp act</th>
<th>Recovery (%)</th>
<th>OD$_{260}$-abs$^*$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell-free, culture filtrate</td>
<td>255</td>
<td>282</td>
<td>71.9</td>
<td>829</td>
<td>211.4</td>
<td>0.34</td>
<td>1</td>
<td>100</td>
<td>0.8</td>
</tr>
<tr>
<td>Metaphosphate concentrate</td>
<td>30</td>
<td>2,692</td>
<td>80.8</td>
<td>175</td>
<td>5.25</td>
<td>15.4</td>
<td>45</td>
<td>112</td>
<td>1.2</td>
</tr>
<tr>
<td>Bicarbonate eluant, from aluminum hydroxide</td>
<td>21</td>
<td>2,291</td>
<td>48.1</td>
<td>121</td>
<td>2.54</td>
<td>18.9</td>
<td>56</td>
<td>67</td>
<td>1.4</td>
</tr>
<tr>
<td>After lyophilization</td>
<td>2.4</td>
<td>18,620</td>
<td>44.7</td>
<td>1000</td>
<td>2.40</td>
<td>18.6</td>
<td>55</td>
<td>62</td>
<td>1.4</td>
</tr>
<tr>
<td>After activated carbon</td>
<td>2.4</td>
<td>18,410</td>
<td>44.2</td>
<td>840</td>
<td>2.02</td>
<td>21.9</td>
<td>64</td>
<td>61</td>
<td>1.8</td>
</tr>
<tr>
<td>After centrifugation</td>
<td>2.4</td>
<td>17,990</td>
<td>43.2</td>
<td>820</td>
<td>1.97</td>
<td>21.9</td>
<td>65</td>
<td>60</td>
<td>1.8</td>
</tr>
<tr>
<td>After 0.22-µm filtration</td>
<td>2.4</td>
<td>19,720</td>
<td>47.3</td>
<td>800</td>
<td>1.92</td>
<td>24.6</td>
<td>72</td>
<td>66</td>
<td>1.8</td>
</tr>
</tbody>
</table>

$^*$ Optical density at 280 nm/optical density at 260 nm.

**Fig. 1. Distribution of precipitating species in crude culture filtrates as a function of pH in the presence (●) and absence (○) of metaphosphate (2.55 g/liter).**

**Fig. 2. Dependence of toxin recovery on concentration of aluminum hydroxide suspension at pH 5.6 when the total Lb doses per gram of adsorbent were greater than or equal to $3.2 \times 10^4$, i.e., for the case in which the toxin concentration exceeded the capacity of the adsorbent.**
results of mouse protection tests performed on an aluminum hydroxide-purified toxin preparation before and after centrifugation. The harvested sediment, after concentration by centrifugation, was identified by immunodiffusion with one of the somatic antigens present in crude culture fluids and was found to be highly effective in protecting mice against challenge with live vibrios. The same material also gave rise to high vibriocidal antibody titers in immunized rabbits.

Conditions for sedimenting optimal amounts of somatic antigen were determined by centrifuging samples of crude culture filtrate (from which all detectable toxin had been removed by metaphosphate precipitation) and determining by radial immunodiffusion the amount of somatic antigen sedimented as a function of time. For this purpose, antiserum specific for both this particular somatic antigen and toxin (Wyeth goat no. 2 antitoxin) was employed. Since toxin had been removed from the culture filtrate, only precipitin rings due to the interaction of somatic antigen with its antibody were observed. The amount of somatic antigen which could be sedimented reached a maximum after centrifugation at 78,410 × g for 6 h in a Spinco 30 rotor (Fig. 3). In scaling up the procedure to accommodate larger volumes, equivalent results were obtained when the Spinco 21 rotor was substituted for the 30 rotor and sedimentation was performed at 44,330 × g for a minimum of 16 h.

To achieve additional purity, aluminum hydroxide-purified toxin preparations were lyophilized, dissolved in a reduced volume of buffer, treated with activated carbon, and centrifuged under the conditions described above. Lyophilization was employed to reduce volumes for centrifugation but, more importantly, because residual somatic antigen tended to become insoluble after lyophilization. Activated carbon removed variable (from lot to lot) amounts of yellow-brown contaminant(s), which became apparent only after concentration. After carbon treatment, toxin preparations were generally water-clear and they exhibited ultraviolet spectra characteristic of pure protein (Fig. 4 and Table 1).

Finally, purified toxin was filtered aseptically through a membrane filter (0.22 μm, Millipore Corp.) and stored at 4°C without loss in activity for periods of at least 6 months. Overall toxin yields on the order of 50% or more, and specific activities on the order of 22 Lb doses/
μg, were reproducibly obtained in four consecutive large-scale preparations.

**Immunodiffusion and immunoelectrophoresis.** Ouchterlony-type double diffusion was used as a qualitative method for monitoring purification. Figure 5 shows immunoprecipitin lines which were observed when various fractions obtained during the first two steps of the purification procedure were diffused against Wyeth goat no. 1 antitoxin. With this antiserum, it was possible to distinguish reproducibly at least four antigens in cell-free culture filtrates, three of which were identified as somatic in origin and one of which was identified as toxoid. The three somatic antigens were invariably observed in the metaphosphate supernatant fraction, and a fourth, very weak, unidentified antigen, which was not apparent in crude culture filtrates, was generally observed along with toxin antigen in the metaphosphate concentrate fraction. (The weak, unidentified antigen may not be clearly visible in Fig. 5; its location in wells no. 2, 4, and 5 is between the toxin-antitoxin immunoprecipitin line and the antiserum well.) Occasionally, some proportion of one of the three somatic antigens described above (second precipitin line from center well) also appeared in the metaphosphate concentrate fraction. The weak unidentified antigen and the somatic antigen, when present, did not bind to aluminum hydroxide under the conditions that were employed and therefore appeared in the aluminum hydroxide supernatant fraction. The bicarbonate eluant from aluminum hydroxide exhibited a single immunoprecipitin line, which was identified as toxoid and which could be monitored quantitatively by radial immunodiffusion throughout subsequent steps of the purification procedure. Purified toxin preparations, containing on the order of 1 mg of toxin per ml, continued to exhibit a single immunoprecipitin line when they were diffused against the aforementioned polyvalent antiserum. The somatic antigen nearest to the antigen wells in the cell-free culture filtrate and metaphosphate supernatant fractions (see Fig. 5), respectively, was identical to the antigen which was subsequently removed from toxin preparations by sedimentation.

The discovery and isolation by Finkelstein and LoSpalluto (12, 13) of a degradation product of toxin (11), which they named choleragenoid and which they showed was electrophoretically distinguishable but immunologically indistinguishable from toxin (12), prompted an investigation of its possible presence in preparations of toxin purified by the present procedure. Immunoelectrophoretic analysis comparing two representative toxin preparations with a preparation of choleragenoid provided by R. A. Finkelstein revealed that toxin purified by the present method does not contain significant amounts, if any, of choleragenoid (Fig. 6). Polyvalent antiserum (Wyeth goat no. 1 antitoxin) was used in the development of the immunoprecipitin pattern because Ouchterlony-type immunodiffusion tests had shown that choleragenoid and the toxin preparations exhibited a single, identical immunoprecipitin line when reacted against this antiserum.

**Acrylamide gel electrophoresis.** After electrophoresis and staining, toxin appeared as a broad, somewhat diffuse band which could be resolved into one major and one (or two) minor bands (Fig. 7). The possibility that the minor band(s) were contaminants was dismissed when the major band, after excision from unstained gels, exhibited the same electrophoretic pattern (as before) upon re-electrophoresis. In addition, when toxin was preincubated at 37 C in the presence of 0.035% ammonium persulfate (the catalyst for polymerization of the separating gel), a change in the distribution of proteins from the major to the minor species was observed. Whether or not the minor bands occurred exclusively as a result of electrophoresis (because of oxidation due to excess persulfate) or occurred during production and/or purification as well, has not been determined.

Efforts to distinguish between the major and minor species either by immunological techniques or by electron microscopy proved unsuccessful. When unstained gels were sliced into

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**Fig. 5.** Agar gel double immunodiffusion of various fractions obtained during purification. Clockwise from the top: well 1, cell-free culture fluid; well 2, metaphosphate concentrate; well 3, bicarbonate eluant; well 4, aluminum hydroxide supernatant; well 5, same as well 2; and well 6, metaphosphate supernatant. Center well contained polyvalent antiserum (Wyeth goat no. 1 antitoxin).
immunization and into either a Formalin or glutaraldehyde toxoid for rabbit immunization. The conditions surrounding the preparation of these toxoids are presented in the subsequent report.

With regard to the mouse protection test, it should be emphasized that, unless mice were immunized with dilutions of toxoid containing at least 10,000 TU (approximately 500 μg of toxoid antigen) in the lowest dilution, most preparations did not exhibit statistically significant protection. Even at these doses, survival curves were often erratic or nonlinear; it is noteworthy that mice immunized with placebo (phosphate-buffered saline) also exhibited survival curves similar to those observed with various toxoid preparations. Nevertheless, an estimate of the amount of residual somatic antigen in representative toxoid preparations was obtained, and it generally did not exceed 0.025% of the DBS reference vaccine, V. cholerae Inaba IN-12, per 1,000 TU.

As determined by the microtiter vibriocidal assay, geometric mean serum titers from groups of rabbits immunized with representative Formalin toxoids ranged from 10 to 40 vibriocidal antibody units per ml 4 weeks after primary inoculation with approximately 2,000 TU (100 μg of toxoid antigen). Like antitoxin titers, vibrio-

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**Fig. 6.** Comparison of immuno-electrophoretic pattern of two representative purified toxin preparations (top and bottom wells) with a preparation of Finkelstein's choleragenoid (center well). Precipitin lines were developed with polyvalent antiserum (Wyeth goat no. 1 antitoxin).

2-mm disks and the disks were placed on agar containing antitoxin, immunoprecipitin rings were observed surrounding gel slices from regions corresponding to both the major and minor species. Similarly, when protein was eluted from such slices and assayed for activity, a symmetrical profile (of activity) with a shoulder in the region occupied by the minor bands was observed. Further, electron microscopy of excised fractions revealed similar particle types in the eluants associated with both the major and minor species.

**Electron microscopy.** Examination of a representative toxin preparation by electron microscopy revealed a quasi-crystalline array of uniform, closely packed particles (Fig. 8). Individual particles appeared to be hollow with mostly rectangular, but sometimes circular, outlines. The diameter of the particles ranged from 5 to 6 nm.

**Somatic antigen analyses.** Because the immunological and biophysical results presented thus far did not reveal the presence of trace amounts of extraneous somatic antigen, it was necessary to use sensitive in vivo assays to demonstrate its presence. In general, the level of somatic antigen was determined in two ways: (i) by measuring resistance of immunized mice to live vibrio challenge in a mouse protection test; and (ii) by measuring vibriocidal antibody titers in the sera of immunized rabbits. Because the concentration of toxin which would have been required to reliably determine residual levels of somatic antigen was either lethal or caused reactions at the site of inoculation, toxin was converted into a Formalin toxoid for mouse

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**Fig. 7.** Acrylamide gel electrophoretic patterns of purified toxin. Electrophoresis was performed until the tracking dye reached the bottom of the gel (left) and 30 min beyond the clearance of the dye from the gel (right) using approximately 100 μg of toxin per gel.
cidal titers rose 2 weeks after booster inoculation, achieving levels approximately one-third of the NIH reference (convalescent) antiserum; but they declined towards the titers cited above within several weeks (Table 3). However, rabbits twice immunized with 100 μg of glutaraldehyde toxoid (with a 6-week interval between inoculations) exhibited vibriocidal titers which were significantly reduced relative to titers elicited by Formalin toxoid (Table 4). Even when this toxoid was administered with adjuvant (Table 4), post-booster titers were approximately 100-fold less than the NIH reference antiserum.

**DISCUSSION**

An effective and comparatively simple method for the large-scale production of purified cholera toxin has been developed. Starting with cell-free culture filtrates, the method relies entirely upon batch procedures, each of which results in a reduction in volume and a concomitant increase in specific activity. The method has proven reliable regardless of whether the initial volume of culture filtrate was 1 or 250 liters.

The metaphosphate precipitation technique employed in the first step of the procedure was developed some years ago by one of us (H. Tint, U.S. patent 2,772,201, 1956) for the concentration and purification of tetanus and diphtheria toxoids. Involving the formation of complexes between the phosphate polymers and proteins and the subsequent precipitation of these complexes at their respective isoelectric pHs, the method was designed to purify as well as concentrate. In its original application, all of the protein complexes having an isoelectric pH equal to or higher than that of the desired protein were precipitated together and dis-

### Table 3. Serum antitoxin and vibriocidal titers after immunization of rabbits with 100 μg of Formalin toxoid at 0 and 4 weeks, respectively

<table>
<thead>
<tr>
<th>Weeks post-immunization</th>
<th>Antitoxin titer (AU/ml)*</th>
<th>Vibriocidal titer (VU/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prepn 1</td>
<td>Prepn 2</td>
</tr>
<tr>
<td>0</td>
<td>&lt;2 (6)*</td>
<td>&lt;2 (8)</td>
</tr>
<tr>
<td>4</td>
<td>367 (6)</td>
<td>283 (8)</td>
</tr>
<tr>
<td>6</td>
<td>1,392 (6)</td>
<td>1,011 (7)</td>
</tr>
<tr>
<td>10</td>
<td>469 (6)</td>
<td>341 (7)</td>
</tr>
<tr>
<td>16</td>
<td>268 (6)</td>
<td>93 (6)</td>
</tr>
<tr>
<td>NIH reference re-agent (convalescent antiserum)</td>
<td>362</td>
<td>256</td>
</tr>
</tbody>
</table>

* AU: Antitoxin units; VU, vibriocidal antibody units.
* Numbers represent geometric means. Numbers in parentheses indicate number of rabbits.

### Table 4. Serum vibriocidal titers after immunization of rabbits with 100 μg of glutaraldehyde toxoid at 0 and 6 weeks, respectively

<table>
<thead>
<tr>
<th>Weeks post-immunization</th>
<th>Vibriocidal titer (VU/ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prepn 1</td>
</tr>
<tr>
<td>0</td>
<td>1.0 (8)*</td>
</tr>
<tr>
<td>6</td>
<td>1.5 (8)</td>
</tr>
<tr>
<td>8</td>
<td>3.7 (7)</td>
</tr>
<tr>
<td>NIH reference re-agent (convalescent antiserum)</td>
<td>1,024.0</td>
</tr>
</tbody>
</table>

* VU, Vibriocidal antibody units.
* Adjuvant was protamine-aluminum phosphate. Details of its preparation and use are given in the subsequent report.
* Prepared from the same toxin as preparation 2 in Table 3.
* Numbers represent geometric means. All serum titers < 2 were arbitrarily assigned the value of 1 VU/ml. Numbers in parentheses indicate number of rabbits.
solved in an appropriate buffer, and the desired protein was then fractionated according to its solubility characteristics. In this particular system, the toxin-metaphosphate complexes were among the first species to precipitate as the pH was lowered to 4.6, so that concentration as well as a substantial increase in specific activity was accomplished in a single step. It may be of interest that the somatic antigens remaining in the metaphosphate supernatant fraction (see Fig. 5) could also be concentrated and purified by selective precipitation below pH 4.6.

In the purification of toxin by aluminum hydroxide adsorption, the powder form of the adsorbent was used exclusively. This was because the powder form proved to have greater selectivity for toxin than did aluminum hydroxide gels such as those used by Spyrides and Feeley (22) in their original observations. The one disadvantage of the powder adsorbent was that approximately 2 g of adsorbent was used to purify as little as 1 mg of toxin. A possible explanation for this observation may be that other ions in the medium are competing with toxin for binding sites on the adsorbent.

Although the specific activity of toxin preparations was relatively high after aluminum hydroxide purification, subsequent lyophilization and centrifugation consistently resulted in a substantial reduction in the amount of residual somatic antigen. In a few instances where residual somatic antigen levels were higher than usual (as, for example, when the starting material was 48-h culture filtrate), recycling the purified toxin preparation through the lyophilization and centrifugation steps resulted in removal of approximately 70% of the residual somatic antigen. Also, in cases where the amount of pigment contamination was greater than usual, a repetition of the carbon treatment resulted in the complete removal of these contaminants.

On the basis of mass, the amount of residual somatic antigen recovered by sedimentation was small. Although no detailed chemical analyses were performed, this antigen exhibited characteristics similar to the protein-lipopolysaccharide complex isolated from Inaba serotypes by Watanabe et al. (23), since it could be described as a polydisperse, fine suspension which tended to become insoluble upon freezing and lyophilization. The observation that toxin preparations continued to exhibit very low levels of somatic antigen after lyophilization and centrifugation suggested that, even when present in trace amounts, this antigen was either highly immunogenic or its immunogenicity was greatly enhanced in the presence of toxin or toxoid.

In terms of specific activity (of toxin), the preparations described here compare favorably with other purified preparations described by the most often cited investigators, Finkelstein and LoSpalluto (12) and Richardson et al. (21), in that the amount of toxin required to produce a 7-mm bluing lesion in rabbit skin is on the order of 0.1 to 0.5 ng. With regard to content of somatic antigen, it is more difficult to compare our material with that of other investigators because either insufficient data are available or different methods of analysis have been employed.

Richardson et al. (21) reported the presence of "low but consistent vibriocidal antibody titer rise" in rabbits immunized with some of their preparations, and Finkelstein and LoSpalluto employed an in vitro neutralization test (10) to demonstrate the absence of somatic antigen from their toxin preparations (13). Recently, a group of rabbits immunized in this laboratory with 100 µg of a Formalin toxoid prepared from a toxin preparation purified by R. A. Finkelstein (lot 1071; prepared under contract for the National Institute of Allergy and Infectious Diseases essentially according to procedures described in reference 12) showed no significant rise in serum vibriocidal antibody after primary and secondary immunizations.

Although the ultimate objective of this investigation was to develop a reliable production-scale method for the preparation of purified toxin (toxoid) with no ability to elicit vibriocidal antibody, the data clearly show that representative toxoid preparations stimulate low levels of vibriocidal antibody in mice and rabbits when they are suitably immunized with a higher than anticipated human dose (100 to 200 µg). The reason that glutaraldehyde toxoids elicited significantly less vibriocidal antibody than did Formalin toxoids is thought to be related more to the different stability characteristics of the toxoids (the subject of the subsequent manuscript) than to a direct effect of glutaraldehyde on somatic antigen.

In addition, it has been demonstrated that residual somatic antigen can be eliminated by molecular sieve chromatography (using Sephadex G-150, Sephadex G-200, or Bio-Gel A-5M gels) or, alternatively, by affinity chromatography (using purified vibriocidal antibody bound to Sepharose 4B); these techniques are presently being evaluated on a production scale.

Whether or not material of the quality presently achieved can be utilized to determine, unambiguously, the efficacy of antitoxic immunity in endemic areas is the subject of continuing investigation. Irrespective of the outcome of such investigations, this material should be
useful in studying the protein chemistry and mechanism of action of cholera toxin in various biological systems. Ultimately, material of the quality presently described should, without further purification, be a suitable candidate for large-scale immunization, provided that antitoxic efficacy is established.

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LITERATURE CITED