INFECTION AND IMMUNITY, May 1978, p. 552-558
0019-9567/78/0020-0552$02.00/0

Vol. 20, No. 2
Printed in U.S.A.

Purification of Cholera Toxin and Its Subunits: New Methods of Preparation and the Use of Hypertoxinogenic Mutants

JOHN J. MEKALANOS, R. JOHN COLLIER,* AND W. R. ROMIG

Department of Bacteriology, University of California, Los Angeles, California 90024

Received for publication 6 February 1978

Cholera toxin was obtained in pure form by fractionation on two phosphocellulose columns successively. Cholera toxin and choleragenoid were quantitatively and selectively adsorbed to the first column in 10 mM phosphate buffer, pH 7.0, and were subsequently eluted with buffer of high ionic strength. The toxin was then separated from choleragenoid on the second column by chromatography at pH 8.3. The toxin obtained was highly active and pure as judged by electrophoresis, isoelectric focusing, and various immunological and chemical tests. Pure choleragenoid was a by-product of the procedure. The A_1 chain of the toxin was obtained in pure form by treating phosphocellulose-bound toxin with urea and a reducing agent. The anionic A_1 peptide was thereby released, leaving a complex of the B and A_2 chains ($A_2$5B) bound to the resin. The latter was then eluted and further purified to obtain nontoxic antigen. The overall yields of cholera toxin and choleragenoid were increased two- to threefold by the use of hypertoxinogenic mutants of *Vibrio cholerae*.

Within less than a decade since the initial purification of cholera toxin (7), much has been learned about the structure and activity of this molecule. The toxin (molecular weight, 84,000) consists of a single A subunit containing two polypeptide chains, A_1 (molecular weight, 24,000) and A_2 (molecular weight, 9,700), linked by a disulfide bridge, and a B subunit containing five B chains (molecular weight, 11,590), perhaps in the form of a ring (8, 12). Binding of the B moiety to a cell surface receptor, probably ganglioside GM_1, is apparently the first step in the interaction of the toxin with its target cell (3). After a short lag, the activity of the adenylate cyclase system of the cell is markedly enhanced as a result of the action of the A subunit of the toxin. It has been shown in vitro that the activation of adenylate cyclase by the A subunit is an oxidized nicotinamide adenine dinucleotide (NAD^+)-dependent event (9). Furthermore, the A subunit catalyzes both attachment of the adenosine diphosphate ribose moiety (ADP ribose) of NAD^+ to arginine and hydrolysis of NAD^+ (NAD^+-glycohydrolase activity, EC 3.2.2.5) (14). By analogy with the activities of diphtheria toxin (10) and exotoxin A from *Pseudomonas aeruginosa* (1), it seems likely that the A subunit of cholera toxin (specifically the A_1 chain) catalyzes ADP ribosylation of some component of the adenylate cyclase complex, resulting in activation of the latter.

Although many methods (6) have been reported for purifying cholera toxin, there is a need for improved methodology in this area. Here we report three significant advances: (i) the use of hypertoxinogenic mutants of *Vibrio cholerae* to increase the overall yield of toxin; (ii) a simple procedure for preparing the toxin in pure form by means of chromatography on cation-exchange resins; and (iii) a method for rapidly isolating pure A_1 chain from whole toxin. These methods, together with a simple procedure reported earlier for obtaining choleragenoid (pentamer of B chains) free from toxin, now enable us to prepare cholera toxin, choleragenoid, and the A_2 chain in higher yields and with greater ease than is possible by other published procedures.

MATERIALS AND METHODS

Reagents. All chemicals used were of analytical grade. Sodium hexametaphosphate was purchased from Fisher Scientific Co. (Pittsburgh, Pa.), dithioerythritol was from Pierce (Rockford, Ill.), and urea was from Schwarz/Mann (Orangeburg, N.Y.). Cyanate ion was removed from aqueous urea solutions by treatment with mixed-bed ion-exchange resin AG50-X8 (Bio-Rad Laboratories, Richmond, Calif.). Phosphocellulose P11 (Whatman) was purchased from Reeve Angel (Clifton, N.J.) and cycled as described previously (13).

Analytical techniques. Carbohydrate and phosphate were determined as described (13). Protein was determined by the method of Sedmak and Grossberg (17) with crystalline bovine serum albumin as the standard. Methods for obtaining antiserum to cholera toxin and choleragenoid have been reported (13). The

serum vibriocidal titer was determined by using strain 569B, and the vibriocidal inhibition assay was performed as described (13). Isoelectric focusing and polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate (SDS) were also performed as previously described (13).

Toxin assays. Cholera toxin antigen was quantified by radial diffusion (7) with rabbit antiserum to purified choleragenoid. Choleragenoid purified by phosphocellulose chromatography (13) was used as the standard in this assay. One radial diffusion unit was defined as that amount of antigen giving a ring diameter equal to that produced by 0.5 μg of purified choleragenoid under standard conditions. Phosphocellulose-purified cholera toxin had a specific activity in this assay of about 1 radial diffusion unit per μg of protein.

ADP ribosylation activity of cholera toxin was measured by incorporation of radioactivity from [U-^{14}C]-adenine NAD into trichloroacetic acid-precipitable material in the presence of synthetic poly-L-arginine. [U-^{14}C]adenine NAD was prepared as described (11) and had a specific activity of 493 mCi/mmol. The reaction was performed at 37°C in 0.15 ml of 25 mM sodium phosphate buffer, pH 7.0, containing 20 mM dithioerythritol, 2 μM [^{14}C]adenine NAD, and 1 mg of poly-L-arginine (type II-B, Sigma Chemical Co., St. Louis, Mo.) per ml. Trichloroacetic acid precipitation on paper was performed as described (1) and was used to stop the reaction. Incorporation of radioactivity into poly-L-arginine under these conditions was absolutely dependent on the presence of cholera toxin or the A_1 peptide of the A subunit. Incorporation of label in the presence of 200 μg of cholera toxin per ml was linear for 20 min under these conditions. Only the initial rates of ADP ribosylation were used in computing specific ADP ribosylation activities and in monitoring the fractionation of the toxin and its subunits on phosphocellulose.

Activity of cholera toxin in vivo was measured with the rabbit skin test as described by Craig (2). The minimum bluing dose was determined for test samples diluted in phosphate-buffered saline containing 0.1% gelatin and used to calculate the specific toxicity in terms of bluing doses per milligram of protein.

Toxin production. Three hypertoxinogenic mutants (Htx-1, Htx-2, and Htx-3) derived from *V. cholerae* strain 569B (RV501) were used in the production of cholera toxin and its subunits (13a). The use of these hypertoxinogenic mutants, as well as the high-density growth conditions described below, allowed us to prepare routinely culture supernatants containing 30 to 45 μg of cholera toxin per ml in as little as 3 to 4 h of growth.

Briefly, the cells from 4 liters of overnight shaker culture were isolated by centrifugation (10,000 × g, 40 min), resuspended, and inoculated to an initial cell density of 2×10^9 to 3×10^9 cells per ml in a Kluyver culture flask containing 5 liters of CYE broth (we have found that addition of NaCl to CYE broth [13] was unnecessary for growth and toxin production and therefore no longer include this in our medium). The culture was incubated at 30°C and aerated vigorously with compressed oxygen until stationary phase was reached as judged by the optical density at 600 nm (about 3 to 4 h). The cells were recovered by centrifugation and used to inoculate 5 liters more of fresh CYE broth to the same initial cell density. Another cycle of growth was completed, and finally the supernatant fluids from the two cycles were pooled and cooled to 4°C.

The protein fraction of the culture supernatant was concentrated by any of several methods before dialysis and ion-exchange chromatography. These include ultrafiltration (13), adsorption onto aluminum hydroxide (18), and co-precipitation with sodium hexametaphosphate (16).

The last of these methods, a modification of a procedure described by Rappaport et al. (16), was the easiest and most effective. Sodium hexametaphosphate was added to the culture supernatant to a final concentration of 2.5 g/liter, and after adjustment of the pH to 4.5 with concentrated HCl, the mixture was stirred for 2 h at room temperature. The precipitate was then collected by centrifugation (10,000 × g, 15 min) and redissolved in 0.05 volume of 0.1 M sodium phosphate, pH 8.0. This concentrate was dialyzed against at least two changes of 10 mM sodium phosphate, pH 7.0, and after clarification by centrifugation, it was stored at 4°C in the presence of 0.02% sodium azide.

RESULTS

Production of toxin by hypertoxinogenic mutants. We have recently isolated several hypertoxinogenic mutants of *V. cholerae* strain 569B by means of a new affinity filter screening method (13a). To date, we have successfully employed three independently isolated mutants, Htx-1, Htx-2, and Htx-3, for large-scale production of cholera toxin. All three mutants produce 30 to 45 μg of toxin per ml of culture medium when grown in either shake culture or under high-density growth conditions described above. These concentrations are two to three times higher than those produced by the parental strain, and commensurately high yields of purified toxin are obtained. The toxins produced by these mutants are identical to that obtained from the parental strain as judged by various criteria, and the strains are therefore well suited to toxin production.

Purification of cholera toxin and choleragenoid on phosphocellulose. We reported earlier that choleragenoid adsorbs to cation-exchange resins at pH's well above its isoelectric point (pI) of 7.8 (13). Adsorption was found to occur at pH 8.0 in 10 mM sodium phosphate buffer, and we have recently extended this pH to 8.3 in the same buffer. This behavior suggests that choleragenoid contains a cationic surface which gives the molecule an affinity for anionic resins even within a certain pH range in which the molecule as a whole has a net negative charge.

The same cationic surface appears to be ex-

posed in the whole cholera toxin molecule (pI 6.6) as judged by its behavior on phosphocellulose. Figure 1 shows the elution profile obtained when a crude mixture of cholera toxin and choleragenoid, concentrated from culture fluids with hexametaphosphate, was chromatographed on a phosphocellulose column equilibrated with 10 mM sodium phosphate, pH 7.0. After addition of the sample, the column was washed with two column volumes of initial buffer and was then eluted with 0.2 M sodium phosphate, pH 7.4. Virtually all of the toxin antigen and activity in ADP-ribosylating polyarginine were retained on the column in the initial buffer and were eluted at the elevated ionic strength (peak II, Fig. 1; Table 1). Electrophoresis on polyacrylamide gels in the presence of SDS (Fig. 2) showed that the detectable contaminants present in the hexametaphosphate concentrate did not adsorb to phosphocellulose under the conditions used (peak I, Fig. 1; Table 1) and that peak II contained only the A and B chains of the toxin.

We assumed that peak II (Fig. 1) contained a mixture of cholera toxin and choleragenoid and employed phosphocellulose chromatography at higher pH to separate the one from the other (13). The pooled fractions from peak II were dialyzed against 10 mM sodium phosphate, pH 8.3, and applied to a column of phosphocellulose equilibrated with the same buffer. After the column had been washed with two volumes of initial buffer, it was eluted with 0.2 M sodium phosphate, pH 7.4. The first peak of the elution

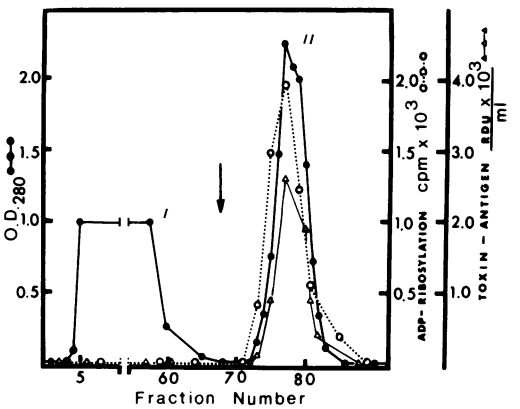

FIG. 1. *Phosphocellulose chromatography, pH 7.0. A 50-ml column of phosphocellulose was equilibrated in 10 mM sodium phosphate, pH 7.0, at room temperature (24°C). Hexametaphosphate concentrate (570 ml) was passed through the column at a flow rate of 90 ml/h, and after washing with two column volumes of initial buffer, the column was eluted (↓) with 0.2 M sodium phosphate, pH 7.4. Fractions (10 ml) were collected and assayed for toxin-antigen and ADP ribosylation activity as described in the text. RDU, Radial diffusion units; OD_{280}, optical density at 280 nm.*

TABLE 1. *Analysis of fractions*

Determination	Vol (ml)	A_{280}[a]	Protein (mg/ml)[b]	Ag[c]	ADPR[d]	Specific toxicity[e]
Phosphocellulose, pH 7.05[f]						
Sample[g]	570	0.96	1.0	200	3.9	1×10^5
Peak I	580	0.74	0.75	<2	0.9	1.3×10^2
Peak II	86	1.4	1.5	1,800	9.0	6.6×10^6
Phosphocellulose, pH 8.30[h]						
Sample[i]	90	1.32	1.5	1,500	10	ND[j]
Peak I	95	0.7	0.78	700	21	1.3×10^7
Peak II	20	1.0	0.93	1,850	0	1×10^3
Phosphocellulose, pH 7.0[k]						
Sample[l]	100	1.9	1.5	370	0.5	ND
Elution 1	60	0.65	0.30	<2	272	3.3×10^3
Elution 2	45	0.52	0.63	625	0	1.6×10^5

[a] A_{280}, Absorbance at 280 nm.
[b] Determined by the method of Sedmak and Grossberg (17), with crystalline bovine serum albumin as the standard.
[c] Antigen concentration in radial diffusion units per milliliter.
[d] Specific ADP ribosylation activity (ADPR) in units of nanomoles of ADPR incorporated per minute per milligram of protein.
[e] Rabbit skin toxicity in units of bluing doses per milligram of protein.
[f] See Fig. 1.
[g] Hexametaphosphate concentrate of RV501 Htx-2 culture supernatant.
[h] See Fig. 2.
[i] Dialyzed peak II from phosphocellulose, pH 7.05 (Fig. 1).
[j] ND, Not determined.
[k] Batch preparation of A_1 and A_2 5B; see text.
[l] Hexametaphosphate concentrate of RV501 Htx-1 culture supernatant.

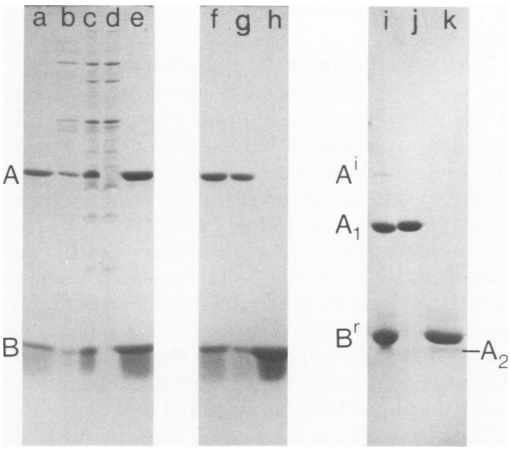

FIG. 2. *SDS-polyacrylamide gel electrophoresis. All samples were heated at 100°C for 3 min before electrophoresis. Samples a through h were heated in the absence of reducing agent, whereas samples i through k were heated in the presence of β-mercaptoethanol. (a) Purified cholera toxin; (b) unconcentrated culture supernatant of hypertoxinogenic mutant Htx-1; (c) sample (hexametaphosphate concentrate) phosphocellulose chromatography, pH 7.0 (Fig. 1); (d) peak I, Fig. 1; (e) peak II, Fig. 1; (f) sample (dialyzed peak II, Fig. 1) phosphocellulose chromatography, pH 8.3 (Fig. 3); (g) peak I, Fig. 3; (h) peak II, Fig. 3; (i) reduced cholera toxin; (j) elution 1 (A_1 subunit, see text); (k) elution 2 ($A_2$5B, see text). We have observed that some batches of phosphocellulose-purified cholera toxin contain a small fraction of A subunit which does not breakdown to the A_1 and A_2 subunits after reduction. We believe that this band represents uncleaved intact A subunit which retains the connecting peptide between A_1 and A_2. During production and purification of the toxin, this precursor is usually processed by limited proteolysis to give the disulfide bridged A_1A_2 type of A subunit characteristic of cholera toxin purified by other methods. We have shown (unpublished data) that limited trypsin digestion of toxin composed of this uncleaved A subunit results in its rapid conversion to the A_1-A_2 complex, thus indicating it is indeed the intact precursor of the A subunit. Previous investigators and ourselves (13) have observed that the B subunit of cholera toxin decreases in mobility upon reduction (B'). Likewise, we have observed as have others (12) that the A_1 subunit of cholera toxin stains only weakly with Coomassie brilliant blue in the presence of SDS and/or is lost from the gel during destaining.*

profile (peak I, Fig. 3), which emerged unretarded in the initial buffer, contained both toxin antigen and ADP ribosylation activity (Table 1). The second peak, which emerged after application of the higher-ionic-strength buffer, contained toxin antigen but no ADP ribosylation activity. Electrophoresis on SDS-polyacrylamide gels showed that peak I contained both A and B chains of the toxin, whereas the second peak contained only the B chain (Fig. 2). These data gave a preliminary indication that purification of cholera toxin and choleragenoid from a crude concentrate had been achieved in two successive column chromatographic steps. The properties of the purified toxin are further described below.

Isolation of the A_1 chain from cholera toxin adsorbed to phosphocellulose. A simple means of removing the A_1 peptide from cholera toxin is desirable not only as a means for obtaining this peptide in pure form, but also as a possible means of deriving nontoxic antigen from the toxin. We have found that the A_1 chain may be released from cholera toxin bound to phosphocellulose by treatment with urea and a reducing agent. The remainder of the toxin molecule remains bound to the resin and may be subsequently isolated by elution with high-ionic-strength buffer. The adsorption and elutions may be performed either by a batchwise procedure or on a column; the former is described below.

A preparation of crude cholera toxin and choleragenoid concentrated from culture fluid with hexametaphosphate and equilibrated with 10 mM sodium phosphate, pH 7.0 (total volume, 100 ml), was mixed with phosphocellulose (packed resin volume, 15 ml) equilibrated with the same buffer. The mixture was stirred at room temperature for 1 h, and the resin was washed free from unbound material by repeated suspension in fresh equilibration buffer and cen-

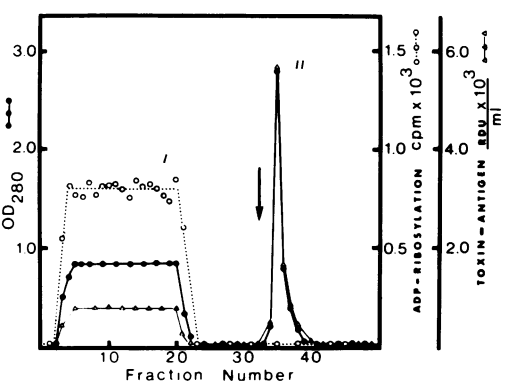

FIG. 3. *Phosphocellulose chromatography, pH 8.3. Peak II from phosphocellulose, pH 7.0 (Fig. 1), was dialyzed against 10 mM sodium phosphate, pH 8.3, and applied at a flow rate of 45 ml/h to a 20-ml column of phosphocellulose equilibrated in the same buffer at room temperature (24°C). After washing with two column volumes of initial buffer, the column was eluted (↓) with 0.2 M sodium phosphate, pH 7.4. Fractions (5 ml) were collected and assayed for toxin-antigen and ADP ribosylation activity as described in the text. RDV, Radial diffusion units; OD_{280}, optical density at 280 nm.*

trifugation. The washed resin was then resuspended in 40 ml of 10 mM sodium phosphate, pH 7.0, containing 6 M urea and 20 mM dithioerythritol. Most of the A_1 peptide was released within 20 min at room temperature (elution 1, Table 1). Finally, the resin was washed with 10 mM sodium phosphate, pH 7.0, until it was free from detectable thiol (4) and was eluted with 40 ml of 0.2 M sodium phosphate, pH 7.4 (elution 2). The material eluted in the presence of urea and dithioerythritol appeared to be pure A_1 chain as judged by electrophoresis on SDS-polyacrylamide gels (Fig. 2).

The major component of the fraction which eluted at elevated ionic strength was B chain. This was in the pentameric form; it migrated on SDS gels according to a molecular weight of 54,000 when the heating step was omitted before electrophoresis. Significant amounts of A_2 chain were also present in this fraction, and we believe this peptide is probably associated with the B-chain pentamer in the absence of SDS, based on evidence presented below. We have given the major product of this fraction the tentative designation $A_2$5B, which distinguishes it from choleragenoid (5B) and cholera toxin ($A_1A_2$5B).

Properties of purified cholera toxin and its subunits. (i) Isoelectric focusing and electrophoresis under nondenaturing conditions. Cholera toxin purified by phosphocellulose chromatography (peak I, Fig. 3) gave only a single band on flat-bed isoelectric focusing in Sephadex G-75. The pI obtained, 6.65, was almost identical to values reported in the literature for the toxin (7). Choleragenoid gave a major band at pI 7.8 and a faint band at pI 7.6. The latter coincided with the major band of the $A_2$5B fraction described above and may have corresponded to traces of this complex. A pI of the $A_2$5B complex slightly lower than that of choleragenoid (5B) would be predicted from the association of the anionic A_2 peptide with the basic B-chain pentamer (pI 7.8).

When subjected to electrophoresis in 10% polyacrylamide gels under nondenaturing conditions, the purified cholera toxin, choleragenoid, and $A_2$5B migrated at different rates and gave band patterns consistent with those described above for the isoelectric focusing.

(ii) Biological properties. The rabbit skin test was used to measure the specific toxicity of the various fractions obtained as described above. A positive skin reaction was obtained with as little as 80 pg of phosphocellulose-purified toxin (peak I, Fig. 3), which is in good agreement with the skin reactive dose reported for cholera toxin purified by more complex methods (5). The A_1 peptide also showed significant activity in the skin test (reactive dose, 0.3 μg), which is consistent with the data of other workers (15).

Purified choleragenoid (5B; peak II, Fig. 3) was less than 10,000-fold as active as the toxin in the skin test. The fraction containing $A_2$5B eluted from phosphocellulose after removal of the A_1 peptide with urea and dithioerythritol exhibited significant toxicity (skin reactive dose, 6 ng) but could be rendered less toxic by rechromatography on phosphocellulose at pH 8.0 (skin reactive dose, 6 μg). The residual toxicity of this fraction and choleragenoid is presumably due to traces of active toxin, but we have been unable to achieve separation of these traces from choleragenoid and $A_2$5B by repeated chromatography on phosphocellulose at pH 8.0.

Cholera toxin, choleragenoid, and the $A_2$5B all retained the capacity to bind ganglioside GM_1 as judged by the ganglioside filter assay (13a). Each of these proteins was spotted on filter papers covalently substituted with mixed gangliosides, and the papers were then treated with ^{125}I-labeled anti-choleragenoid immunoglobulin G. Radioactive antibody was strongly bound to the filters and could not be removed by extensive washing with phosphate-buffered saline, thus indicating a strong affinity of the proteins for the filter. Preincubation of the proteins with a fivefold molar excess of unbound, mixed gangliosides before spotting on the filters resulted in a 70% decrease in radioactive antibody bound.

(iii) Immunological properties. Cholera toxin purified by chromatography on phosphocellulose showed a degree of immunological purity similar to that of choleragenoid purified by the same method (13). No somatic antigen could be detected in the toxin as assayed by the highly sensitive vibriocidal inhibition assay; immunization with the toxin produced no enhancement in original vibriocidal titer of the sera of the animals. The antisera obtained produced only a single band against crude, hexametaphosphate-concentrated toxin, and the band showed immunological identity with the precipitin band formed against purified toxin (peak I, Fig. 3).

(iv) Chemical properties. Cholera toxin isolated by phosphocellulose chromatography was free from carbohydrate (less than 0.5% dry weight). No phosphorus could be detected (less than 0.3% dry weight), indicating the absence of phospholipid and nucleic acid. The ultraviolet absorption spectrum of the toxin was that expected for a highly purified protein; the absorbance at 280 nm was two times that at 260 nm.

DISCUSSION

The ionic properties of cholera toxin and choleragenoid permit these molecules to be purified free from contaminants normally found in cul-

ture fluids by chromatography on cation-exchange resins. In the procedure described here, we have subjected a crude mixture of toxin and choleragenoid to chromatography on two successive phosphocellulose columns at room temperature. In the first, cholera toxin and choleragenoid are adsorbed to phosphocellulose at pH 7.0 under conditions in which contaminating macromolecular materials pass unretarded through the column. After elution with high-ionic-strength buffer and dialysis, cholera toxin and choleragenoid are separated from one another by re-chromatography on phosphocellulose at pH 8.3. This procedure has the great advantage of yielding both proteins in pure form and essentially free from one another. We routinely obtain final yields of 15 to 30 mg of cholera toxin and 2 to 4 mg of choleragenoid per liter of CYE broth.

The basic procedure described here may be advantageously altered in various ways depending upon the circumstances. For example, it is possible to eliminate the step of precipitation and concentration of the toxin and choleragenoid from culture fluids with hexametaphosphate by adsorbing these proteins directly onto phosphocellulose. Both will adsorb at neutral pH and without prior dialysis if the ionic strength of the medium is reduced sufficiently by dilution with distilled water (about eightfold for CYE medium). The resin and bound proteins may then be isolated and either packed into a column or treated batchwise for further fractionation. Large-scale production of cholera toxin and choleragenoid might be more efficient with this modification.

It may be possible to simplify the described procedure so that only one column instead of two must be employed. After adsorption of the toxin and choleragenoid to the resin at pH 7.0, raising the pH to 8.0 or 8.3 should elute the toxin specifically. Because of the high buffering capacity of phosphocellulose in this pH range, it is impossible to raise the pH by this magnitude without either using an inordinately large volume of equilibration buffer or raising the ionic strength of the buffer to the point at which elution of both proteins occurs. However, such a change of pH should be possible with other cation-exchange resins which have average pK_a's well below 7 (for example, carboxymethyl-cellulose or sulfopropyl-Sephadex). The use of this modified procedure is presently under study.

Phosphocellulose is also useful in isolating pure A_1 from the toxin. Dissociation of the toxin to release A_1 is dependent upon reduction of the disulfide bridge linking the A_1 and A_2 peptides. Under the conditions described, reduction of this bridge and release of the anionic A_1 chain from phosphocellulose-bound toxin occur rapidly, leaving a complex of the A_2 peptide and B-chain pentamer attached to the resin. The A_1 chain is thereby obtained in pure form in a single step. The other product of this reaction, apparently an $A_2$5B complex, may be eluted at high ionic strength.

This method of obtaining the A_1 chain is significantly simpler than procedures previously described and should facilitate isolation of this chain for further study of its activity and structure. The method also provides an easy means of isolating nontoxic antigen in high yield from crude mixtures of toxin and choleragenoid. If such a mixture is adsorbed to phosphocellulose and treated to release the A_1 peptide, the resin would then contain a mixture of choleragenoid (5B) and $A_2$5B complex, which may be eluted and freed from traces of toxin by re-chromatography at pH 8.3. The mixture thus obtained may be useful for immunization or other purposes for which nontoxic, ganglioside-binding antigen is desired. These proteins would not be expected to revert to the toxic state, as is possible with some toxoids prepared by chemical inactivation. Furthermore, the capacity of these proteins to bind gangliosides may make them more useful as potential immunogens for administration by the oral route than chemically inactivated toxins which have lost receptor binding activity.

ACKNOWLEDGMENTS

J. J. Mekalanos is a predoctoral trainee supported by Public Health Service grant GM07104 from the National Institute of General Medical Sciences. This investigation was supported by Public Health Service grants AI07877 and AI08024 from the National Institute of Allergy and Infectious Diseases.

We thank Patricia D. Bernard for performing the photography.

LITERATURE CITED

1. **Chung, D. W., and R. J. Collier.** 1977. Enzymatically active peptide from the adenosine diphosphate-ribosylating toxin of *Pseudomonas aeruginosa*. Infect. Immun. **16**:832-841.
2. **Craig, J. P.** 1965. A permeability factor (toxin) found in cholera stools and culture filtrates and its neutralization by convalescent cholera sera. Nature (London) **207**:614-616.
3. **Cuatrecasas, P.** 1973. *Vibrio cholerae* choleragenoid. Mechanism of inhibition of cholera toxin action. Biochemistry **12**:3577-3781.
4. **Ellman, G. L.** 1959. Tissue sulfhydryl groups. Arch. Biochem. Biophys. **82**:70-77.
5. **Finkelstein, R. A.** 1972. Properties of cholera exoenterotoxin (choleragen) and its natural toxoid (choleragenoid). Toxicon **10**:441-450.
6. **Finkelstein, R. A.** 1973. Cholera. Crit. Rev. Microbiol. **2**:553-623.
7. **Finkelstein, R. A., and J. J. LoSpalluto.** 1970. Production, purification and assay of cholera toxin. Production of highly purified choleragen and choleragenoid. J. Infect. Dis. **121**(Suppl.):S63-S72.
8. **Gill, D. M.** 1976. The arrangement of subunits in cholera toxin. Biochemistry **15**:1242-1248.
9. **Gill, D. M.** 1976. Multiple roles of erythrocyte supernatant

in the activation of adenylate cyclase by *Vibrio cholerae* toxin in vitro. J. Infect. Dis. **133**(Suppl.):S55–S63.

10. **Kandel, J., R. J. Collier, and D. W. Chung.** 1974. Interaction of fragment A from diphtheria toxin with nicotinamide adenine dinucleotide. J. Biol. Chem. **249**:2088–2097.
11. **Kornberg, A.** 1950. Reversible enzymatic synthesis of diphosphopyridine nucleotide and inorganic pyrophosphate. J. Biol. Chem. **182**:779–793.
12. **Kurosky, A., D. E. Markel, B. Touchstone, and J. W. Peterson.** 1976. Chemical characterization of the structure of cholera toxin and its natural toxoid. J. Infect. Dis. **133**(Suppl.):S14–S24.
13. **Mekalanos, J. J., R. J. Collier, and W. R. Romig.** 1977. Simple method for purifying choleragenoid, the natural toxoid of *Vibrio cholerae*. Infect. Immun. **16**:789–795.

13a. **Mekalanos, J. J., R. J. Collier, and W. R. Romig.** 1978. Affinity filters, a new approach to the isolation of *tox* mutants of *Vibrio cholerae*. Proc. Natl. Acad. Sci. U.S.A. **75**:941–945.

14. **Moss, J., V. C. Manganiello, and M. Vaughan.** 1976. Hydrolysis of nicotinamide adenine dinucleotide by choleragen and its A protomer: possible role in the activation of adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. **73**:4424–4427.
15. **Ohtomo, N., T. Muraoka, A. Tashiro, Y. Zinnaka, and K. Amako.** 1976. Size and structure of the cholera toxin molecule and its subunits. J. Infect. Dis. **133**(Suppl.):S31–S40.
16. **Rappaport, R. S., B. A. Rubin, and H. Tint.** 1974. Development of a purified cholera toxoid. I. Purification of the toxin. Infect. Immun. **9**:294–303.
17. **Sedmak, J. J., and S. E. Grossberg.** 1977. A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. Anal. Biochem. **79**:544–552.
18. **Spyrides, G. J., and J. C. Feeley.** 1970. Concentration and purification of cholera exotoxin by absorption on aluminum compound gels. J. Infect. Dis. **121**(Suppl.):S96–S100.