Biochemistry of *Vibrio cholerae* Virulence

I. Purification and Biochemical Properties of PF/Cholera Enterotoxin

STEPHEN H. RICHARDSON, DOLORES G. EVANS, AND JOHN C. FEELEY

Bowman Gray School of Medicine of Wake Forest University, Winston-Salem, North Carolina 27103, and Division of Biologics Standards, National Institutes of Health, Bethesda, Maryland 20014

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A simple method for concentrating, isolating, and purifying PF/cholera enterotoxin from culture supernatant fluids is presented. Precipitation with dextran sulfate and ammonium sulfate, followed by gel filtration and ion-exchange chromatography, yielded PF/enterotoxin of high biological potency. Although not completely free from traces of vibriocidal antibody-stimulating activity, the resultant purified toxic immunogen appeared homogeneous as judged by immunoelectrophoresis, immunodiffusion, and disc electrophoresis. Advantages and disadvantages of this purification method are discussed in comparison with those of previously published techniques.

Evidence has been mounting which indicates that the agent responsible for the profuse diarrhea of cholera is an extracellular enterotoxin elaborated in vivo and in vitro by *Vibrio cholerae*. Recently, Coleman et al. (1) isolated a "type 2" ileal loop toxin from peptone culture filtrates and showed that the semipurified material caused massive fluid accumulation in rabbit ligated ileal loops in doses as small as 10 μg. Finkelstein and LoSpalluto (4), employing a series of chromatographic procedures for purification of cholera, demonstrated that submicrogram levels of the resultant single homogeneous protein evoked fluid accumulation in ligated intestinal loops and a cholera-like syndrome in infant rabbits. The same protein elicited strong permeability factor (PF) activity when assayed by the skin test procedure of Craig (2).

We present here a relatively simple technique for obtaining usable quantities of high-purity cholera enterotoxin from semisynthetic media which surmounts some of the difficulties imposed by the use of complex growth media or repeated chromatographic procedures. In a forthcoming paper, the immunogenic and biological properties of the purified toxin are described. To be consistent with our earlier publications, toxin will be designated PF which we consider, in view of the accumulated data, to be identical to and synonymous with cholera enterotoxin, "type 2" ileal loop toxin, and choleraagen.

MATERIALS AND METHODS

**Microorganisms.** All of the vibrio strains (569B, VC12, and B1307) employed in this study were from the collection of one of us (J.C.F.). The methods used for maintenance of stock cultures and preparation of inocula have been described previously (8).

**Culture media.** Depending on the requirements of the experiment, the Tri-Casamino Acids-yeast (TCY) medium defined earlier (8) was employed as such or with the modifications described in the text. The standard medium for large-scale toxin production was TRY, which contained, in grams per liter of 5 mN tris(hydroxymethyl)aminomethane (Tris)-maleate buffer: NaCl, 2.5; KCl, 2.5; Na2HPO4, 0.2; yeast extract, 0.05; and glycerol, 0.5. TRY was supplemented with 1.0 ml of the following trace salts mixture per liter: 5% MgSO4, 0.5% MnCl₂·4H₂O, and 0.5% FeCl₃ in 0.001 N H₂SO₄. After all of the ingredients were dissolved, the medium was adjusted to pH 8.0 with NaOH and then back-titrated to pH 7.5 with 1 M maleic acid. For strains other than 569B (3), the pH was brought directly to 6.5 by the addition of maleic acid.

Other modified media in which the Casamino Acids of TRY were replaced by equivalent amounts of mixtures of amino acids are described in the appropriate places below.

**Toxin production.** Various numbers of 300-ml nephelometer flasks, each containing 80 ml of medium, were inoculated with 0.01 their volume (0.8 ml) of a saline suspension (10⁶ colony-forming units/ml) of cells from overnight Columbia agar (BBL) slants incubated at 38 C. The inoculated flasks were incubated at 25°C for up to 24 hr at 250 rev/min on a rotary shaker.

**Toxin assays.** PF titers were determined in rabbits and guinea pigs as described previously (8). Each titration was carried out at least in duplicate, and the test materials were injected in a randomized pattern. In most cases, the gelatin-saline diluent employed in the titrations contained 0.005% ethylenediaminetetraacetate (EDTA). Titers are expressed as blueing

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doses (BD), i.e., the reciprocal of the dilution eliciting a reaction 8 mm in average diameter.

**Immunological methods.** Antiserum against semi-purified PF was prepared in two rabbits by use of dialyzed TCY filtrates which had been batch-treated with diethylaminoethyl (DEAE)-Sephadex (Sigma Chemical Co., St. Louis, Mo.) and concentrated by dextran sulfate precipitation followed by ammonium sulfate fractionation to remove the dextran sulfate (see below). This material (>200,000 BD/ml) was mixed (in a Waring Blender) with an equal volume of Freund's complete adjuvant, and 0.5-ml portions were injected proximal to each axillary, inguinal, and cervical lymph node. Three booster injections (0.3 ml each) of the same antigen were injected near the cervical site at 3-day intervals; the animals were rested for 1 week and then were test bled from the ear vein. After another 7 days of rest, the animals were killed by exsanguination, and the serum obtained was stored frozen at −20°C. The immunized animals showed no signs of localized Schwartzman or other reactions, and no ill effects were seen in a control animal given 1.5 ml of the antigen intravenously.

Immunoelctrophoresis (5) and Ouchterlony double-diffusion assays (7) were done according to standard techniques.

**PF purification.** The procedure adopted for PF purification was as follows (all operations carried out at 5°C unless stated otherwise). The supernatant fluid from centrifuged 16-hr TRY cultures (or any other PF-containing crude culture supernatant fluid) was mixed with 0.01 volume of 1 M Tris-hydrochloride buffer (pH 8) containing 5% disodium EDTA. The solution was then passed through a prewashed 0.45-μm membrane filter (Millipore Corp., Bedford, Mass.) and dialyzed with constant stirring against at least 20 volumes of 1 M Tris-hydrochloride buffer (pH 8) containing 0.005% disodium EDTA (TE buffer). Dialysis required 18 to 48 hr, depending on the complexity of the growth medium.

The dialysate was made 0.05 M with CaCl₂ and 1.5 mg (per ml) of solid dextran sulfate 2000 (Pharmacia Fine Chemicals, Piscataway, N.J.) was added. After being stirred in the cold for 30 min, the turbid suspension was centrifuged at 20,000 × g for 20 min. The supernatant fluid was discarded, and the pellet and viscous layer which remained at the bottom of the tube were resuspended in 40 to 50 ml of neutralized 0.05 M CaCl₂. The turbid suspension was recentrifuged at 20,000 × g for 20 min, and the clearly separated viscous layer deposited in the bottom of the tube was dissolved in about 4% of the original filtrate volume of 1 M NaCl in TE buffer. Material insoluble in the salt solution was removed by centrifugation at 20,000 × g for 20 min.

The clarified supernatant solution (dextran sulfate-toxin complex, DST) was brought to 90% saturation by the slow addition of solid ammonium sulfate and was allowed to stand in the cold with gentle stirring for 30 min. The toxin, freed from most of its associated dextran sulfate, was recovered as a pellet after centrifugation at 20,000 × g for 20 min. The centrifuge tubes were carefully washed free from residual ammonium sulfate, and the pellet was dissolved in 8 ml of 1 M NaCl in TE buffer.

The solution was then applied to a column (1.5 × 80 cm) of Bio Gel P150 (Calbiochem, Los Angeles, Calif.) equilibrated with the same buffer. Fractions of 1.5 ml were collected with an automatic fraction collector at room temperature. The effluent was monitored by determining the absorbancy of each fraction at 260 and 280 nm. The fractions were tested for dextran sulfate by placing a drop of each fraction on a filter paper which was dipped into a dilute solution of Toluidine Blue O; fractions containing dextran sulfate stained purple. Those fractions which gave a negative test for dextran sulfate and which yielded a single band when tested for the presence of antigen by immunodiffusion were combined. The pooled toxic fractions were diluted to a final NaCl concentration of 10 mM with TE and were concentrated to a final volume of 2 to 5 ml in a Diaflo apparatus with a UM 10 membrane (Amicon Corp., Lexington, Mass.)

The concentrated P150 pool was applied to a column (0.9 × 10 cm) of DEAE-Sephadex A25 equilibrated with 0.01 M NaCl in TE. The material eluted with the starting buffer contained nearly all of the detectable toxin. The immunochromatographic and biochemical properties of this fraction will be described below.

**Biochemical assays.** Protein was estimated by the procedure of Lowry et al. (6) with five times crystallized bovine serum albumin as a standard. Total carbohydrate was determined by the anthrone method (10) with glucose as a standard.

**RESULTS**

**Preparation and preservation of culture filtrates.** Studies of the physical and chemical parameters influencing in vitro production of PF (8) showed that supernatant fluids with maximum PF titers minimally contaminated by vibrio autolysis products were obtained after low-temperature (25°C) growth for 12 to 16 hr. In addition, it was found that incorporation of 50 mg of disodium EDTA per liter preserved the initial PF activity of filtrates throughout the extensive dialysis or ultrafiltration steps employed to remove low molecular weight metabolites and salts. Because of this protective effect, EDTA was subsequently added to crude supernatant fluid immediately after removal of the bacteria and was routinely included in all buffer and diluent solutions.

**PF precipitation by dextran sulfate.** Because the studies of Kaur and Burrows (personal communication) and Coleman et al. (1) suggested that PF contained lipid, attempts were made to employ dextran sulfate precipitation (a well-established procedure for isolating lipoproteins) as a simple means of concentrating PF. We subsequently showed that PF from two strains of vibrios could be precipitated with a reasonable recovery (10 to 20%) of activity by reaction with dextran sulfate 500 [molecular weight, 10⁴ (9)]. Substitution of dextran sulfate 2000 (average molecular weight, 2 × 10⁶) as the precipitating
agent made the procedure more reproducible and the recovery of PF virtually quantitative.

To test the general applicability of this technique to other crude toxins, a TRY filtrate and a peptone-based toxin supplied by the National Institutes of Health (lot 001, Wyeth) both were subjected to dextran sulfate treatment. Optimal quantities of CaCl₂ and dextran sulfate were determined in each case by plotting turbidity (i.e., DST formed) as a function of CaCl₂ concentration at a constant dextran sulfate level and then by reversing the variables. At the concentrations employed for TRY filtrates (1.5 mg/ml and 0.05 M), the DST formed from the peptone toxin rapidly dissolved after its formation (Fig. 1). At the optimal reagent concentration for the latter toxin (0.6 mg/ml and 0.07 M), recovery of PF in the resultant DST was essentially quantitative. These results stress the need to determine optimal reagent concentrations whenever DST formation from a previously untried source of PF is contemplated.

A further example of the applicability of this technique to PF concentration is indicated by the results of experiments summarized in Table 1. In these studies, three different vibrios, 569B, VCV12, and B1307, were grown in four different media ranging from complex (peptone) to simple (TAY). Preparations of DST were made from each filtrate by the arbitrary addition of 1.5 mg of dextran sulfate per ml and 0.05 M CaCl₂. The large variation in recovery of total PF activity among these filtrates again underlines the necessity for establishing the optimal precipitating conditions for each new preparation in order to achieve maximum yields. Note that the original titers of the filtrates from the completely synthetic (TAY) medium are as good as those obtained with more complex media, and that the most potent DST was derived from 569B grown in TRY.

Once washed and redissolved in TE buffer containing 1 M NaCl, DST preparations are quite stable. Several preparations have been stored for as long as 3 months with no decline in PF titer. DST preparations can be washed free from contaminating low molecular weight substances on ultrafilters or in dialysis bags, or by lowering the salt concentration (to 0.01 M), making the dilute solution 0.05 M with CaCl₂, and repeating the original sedimentation and redissolving process. As an additional purification step, several DST preparations have been reprecipitated as many as four times with no decline in PF activity.

Dissociation of the DST. Several chemical procedures for separating PF from dextran sulfate were tried with little success. In each instance in which the PF was freed from residual dextran, toxicity was lost. Ultimately, a simple ammonium sulfate precipitation (see Materials and Methods) was adopted for routine use. This process accomplished two purposes: the high salt concentration neutralized the ionic interactions between the dextran sulfate and the PF, and at the same time precipitated protein moieties from the mixture, leaving the bulk of the dextran sulfate behind in the supernatant fluid. This step is essentially quantitative in that the total PF activity of the DST is recoverable in the ammonium sulfate insoluble pellet.

Gel filtration. Exploratory experiments showed that, among the various techniques applicable to further purification of PF activity, molecular seiving was the most efficient process. Bio-Gel P150 (Bio-Rad Laboratories, Los Angeles, Calif.), which fractionates in the molecular weight range of 50,000 to 150,000, was found to be ideal for separating residual dextran sulfate, lipopolysaccharides, PF, and salt. The samples are run in TE containing 1 M NaCl to obviate reassociation of PF and residual dextran sulfate and to avoid the excessive swelling and packing of the gel which occurs at low ionic strengths. Figure 2 illustrates the results obtained in a typical experiment. An ammonium sulfate pellet dissolved in 8 ml of TE-buffered 1 m NaCl

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**Fig. 1. Determination of optimal dextran sulfate and CaCl₂ concentrations for precipitation of PF/enterotoxin as dextran sulfate-toxin complex (DST).** Optical density at 700 nm directly proportional to DST formed. TRY, ○; Wyeth lot 001, △.
TABLE 1. Comparison of activities of dextran sulfate-toxin complex (DST) derived from vibrio strains 569B, VC12, and B1307 grown in complex and synthetic media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Titer (BD/ml)</th>
<th>569B</th>
<th>VC12</th>
<th>B1307</th>
<th>DST</th>
<th>Potency (BD/μg of Lowry protein)</th>
<th>Recovery&lt;sup&gt;b&lt;/sup&gt;</th>
<th>569B</th>
<th>VC12</th>
<th>B1307</th>
</tr>
</thead>
<tbody>
<tr>
<td>TCY&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>4,280</td>
<td>—</td>
<td>171</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>TRY&lt;sup&gt;a&lt;/sup&gt;</td>
<td>24 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>4 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>8 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>5,720</td>
<td>1,820</td>
<td>3,750</td>
<td>44.5</td>
<td>4.6</td>
<td>30.4</td>
<td></td>
</tr>
<tr>
<td>Peptone&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>24 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>24 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>1,415</td>
<td>875</td>
<td>1,128</td>
<td>49.3</td>
<td>11.4</td>
<td>18.6</td>
<td></td>
</tr>
<tr>
<td>TAY&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>32 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>16 × 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>895</td>
<td>1,380</td>
<td>2,040</td>
<td>84.5</td>
<td>10.6</td>
<td>73.5</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> All cultures grown for 16 hr at 25°C. Initial pH 7.5 for 569B, 6.5 for others. All DST preparations were made by the standard procedure.

<sup>b</sup> Percentage of total BD present in original filtrate.

<sup>c</sup> Lactate carbon source.

<sup>d</sup> Glycerol, potassium, and phosphate added to TCY.

<sup>e</sup> Peptone (2%) plus 0.5% NaCl, buffered with 5 mM Tris-maleate.

<sup>f</sup> Casamino Acids replaced by glutamate, serine, aspartate, and arginine, 0.25% each.

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**Fig. 2.** Bio Gel P150 fractionation of ammonium sulfate-precipitated DST.

was mounted on the column, and 1.5-ml fractions were collected at the rate of 0.5 ml/min. The effluent was monitored by determining the optical density at 280 and 260 nm. Two distinct peaks of approximately equal absorbance were eluted. However, when each fraction was assayed for protein content by the Lowry procedure, quite a different pattern emerged. The second peak (Fig. 2) contained about four times as much protein as the first, and, when the fractions were tested for dextran sulfate, only the first peak (fractions 13 through 21) was positive. Each fraction was checked for the presence of PF antigen by immunodiffusion against PF-specific antisera and unabsorbed antisera. Fractions 13 through 21 gave a weak diffuse band with the unabsorbed antiserum, and anti-PF serum showed no detectable reaction towards these fractions. Both antisera yielded lines of identity with fractions 20 through 34.

To elucidate further the position of PF among the eluates, each fraction was diluted 1:500 and injected into guinea pigs. The resultant areas of bluing were determined and are plotted in Fig. 2 as the area of bluing in square millimeters. Fractions 13 through 21 were negative in the skin test when dilutions as low as 1:10 were employed. In fractions 22 through 34, the area of bluing roughly paralleled the protein curves.

It was observed that the ratio of the absorbance at 280 nm to that at 260 nm reversed between the first and second peaks; i.e., in fractions 13 through 19 the ratio was less than 1.0, whereas in 20 through 36 the ratio was well above 1.0. The cause of this change can be seen by inspection of Fig. 3. The curves are ultraviolet absorption spectra of representative fractions from a P150 column. Fractions from the first peak showed a shoulder in the 250 to 300 nm region, whereas those from the second peak had a discrete maximum around 280 nm with a minimum at 250 to 255 nm. When the ratio of absorbance at 280 nm to that at 260 nm for each
fraction was plotted coordinately with the other parameters, as in Fig. 2, the highest ratios coincided with the protein and area of bluing curves.

**DEAE chromatography.** The fact that the absorbance ratios varied throughout the PF-containing peak indicated that further purification steps were necessary. It was demonstrated by Coleman et al. (1) that type 2 toxin is a weakly charged molecule that can be separated from contaminating substances by passage through DEAE-Sephadex at pH 8 in low ionic strength buffer. To see whether this technique could be applied to PF, dialyzed filtrates were batch-treated with DEAE-Sephadex before dextran sulfate precipitation. At pH 8 in TE buffer, PF was not absorbed by the ion exchanger but most of the contaminating materials were, a result which is consistent with the selective precipitation of PF by the polyanion dextran sulfate under identical conditions. But, since it was found that this technique resulted in large losses of PF activity and marked instability of the remaining PF, the batch process was abandoned in favor of the column method.

Antigen-containing P150 fractions which were negative for dextran sulfate were pooled, and their ionic strength was reduced to 10 mM NaCl by repeated dilution and concentration in an ultrafiltration apparatus. The final concentrate was applied to a column (0.9 × 30 cm) containing DEAE-Sephadex A25 equilibrated with 10 mM NaCl buffered with TE. Three broad peaks eluted with the starting buffer (Fig. 4), and the sequential addition of 0.5 and 1.0 mM NaCl in TE buffer eluted two more peaks. Fractions from each peak were pooled and concentrated to 4 ml. Each pool was assayed for PF activity, antigen, protein, and total carbohydrate. Nearly all of the PF activity and antigen were recovered in peak A; the other peaks showed very little PF activity (Table 2). No detectable carbohydrate was found in peak A, whereas peaks B and D contained considerable quantities of anthrone-positive materials. When the pooled fractions composing peak A were chromatographed on P150, the resultant materials (one sharp peak) exhibited equal absorbance ratios (280 to 260 nm), indicating that the PF is relatively homogeneous.

**General applicability of the method.** To assess the usefulness of the entire method, a TRY filtrate and the National Institutes of Health toxin (lot 001, Wyeth) described earlier were purified in parallel. Figure 5 summarizes the results of these experiments. The first peak (lipopolysaccharide) of the Wyeth toxin was more than three times as high as the analogous TRY peak when the P150 column fractions were compared. This was not an unexpected result because the Wyeth toxin was derived from a 48-hr culture whereas the TRY was harvested at

![Fig. 3. Ultraviolet absorption spectra of peaks I and II from Bio Gel P150 fractionation. I is equivalent to fraction 17, Fig. 2; II is equivalent to fraction 28, Fig. 2.](http://iai.asm.org/)
16 hr when autolysis was negligible. The DEAE-Sephadex elution patterns, although identical in position, indicate that the yield from the TRY preparation was approximately twofold higher than that from the Wyeth material. The specific activity of the TRY preparation (7.7 × 10^4 BD/µg) was also higher than that of the other product (5.0 × 10^4 BD/µg).

**Summary of PF purification.** After at least 10 large-scale preparations were processed and it became clear that the entire technique was reproducible, we carried out an analysis on the recovery of protein and PF during each step of the procedure. The biological assays (Table 3) were carried out approximately 30 days (storage at 4°C) after the samples had been prepared, which probably accounts for the relatively low (6.6 × 10^4 BD/ml) PF activity of the crude filtrate and the apparent loss of activity in the ammonium sulfate fraction.

Based on the Lowry protein value for the filtrate, a slight increase in specific activity was achieved with dextran sulfate precipitation whereas the ammonium sulfate step (mentioned above) showed an inordinate drop in activity. After the P150 step, 70% of the PF activity and 100% of the protein were recovered. These results are congruent with the data presented in Fig. 2.

**Table 2. Composition and biological activity of DEAE fractions**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein (µg/ml)</th>
<th>Carbohydrate (µg/ml)</th>
<th>Antigen</th>
<th>Absorbance ratio (avg)</th>
<th>BD/µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>75</td>
<td>—</td>
<td>++++</td>
<td>1.50</td>
<td>10^4</td>
</tr>
<tr>
<td>B</td>
<td>15.2</td>
<td>12.0</td>
<td>±</td>
<td>0.65</td>
<td>330</td>
</tr>
<tr>
<td>C</td>
<td>5.8</td>
<td>3.0</td>
<td>—</td>
<td>1.00</td>
<td>0</td>
</tr>
<tr>
<td>D</td>
<td>8.9</td>
<td>22.0</td>
<td>—</td>
<td>0.95</td>
<td>110</td>
</tr>
</tbody>
</table>

*These fractions are the same as those shown in Fig. 4.

**Table 3. Summary of PF purification: recovery of PF activity and protein**

<table>
<thead>
<tr>
<th>Material</th>
<th>Vol (ml)</th>
<th>BD/ml</th>
<th>Total protein (mg)</th>
<th>BD/µg</th>
<th>Δ (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUP</td>
<td>1,720.00</td>
<td>6.58 × 10^4</td>
<td>245.00</td>
<td>461</td>
<td>(1.00)</td>
</tr>
<tr>
<td>DST</td>
<td>17.20</td>
<td>197.00 × 10^4</td>
<td>46.10</td>
<td>735</td>
<td>1.59</td>
</tr>
<tr>
<td>AS</td>
<td>7.74</td>
<td>114.00 × 10^4</td>
<td>17.50</td>
<td>504</td>
<td>1.09</td>
</tr>
<tr>
<td>P150</td>
<td>27.50</td>
<td>86.70 × 10^4</td>
<td>18.50</td>
<td>1,290</td>
<td>2.80</td>
</tr>
<tr>
<td>DEAE</td>
<td>5.00</td>
<td>780.00 × 10^4</td>
<td>6.75</td>
<td>5,780</td>
<td>12.50</td>
</tr>
</tbody>
</table>

*SUP, crude filtrate; DST, dextran sulfate-toxin complex; AS, ammonium sulfate-treated DST; P150, AS after treatment with Bio Gel P150; DEAE, P150 pool after treatment with DEAE-Sephadex A25.
Although not tabulated here, the absorbed protein could be recovered from the column by elution with salt-containing buffer. As before (Fig. 4), the latter material contained little PF activity or demonstrable antigen. The final product (eluted from DEAE-Sephadex with the starting buffer) had 100% of the total PF activity of the DST and was 7.9 times as toxic on a microgram of protein basis. Fifteen per cent of the protein of the DST was recovered in the active DEAE-Sephadex eluate. On the basis of the final protein value and assuming complete PF recovery throughout the procedure, the filtrate was estimated to contain about 4 μg of toxin per ml.

Immunochromatographic properties of PF. Samples of each of the fractions described in Table 3 were subjected to immunoelectrophoresis (Fig. 6). To render the precipitation patterns visible, the filtrate sample was reduced to 2% of its original volume by ultrafiltration. The samples were allowed to react with two different antisera after electrophoresis at pH 8.6 for 1 hr. The antisera were (i) the antiserum preparation described in Materials and Methods and (ii) antisera formed against crude syncase filtrates in which 569B had been grown. PF-specific (absorbed with whole cells) antiserum was run as a control but is not shown in Fig. 6. In each test system, all of the fractions showed multiple precipitin lines through the P150 stage. After DEAE-Sephadex treatment, only a single line could be seen, even when the diffusion step employed crude antisera. The elimination of trace amounts of antigenic material by DEAE-Sephadex treatment was compatible with the observations (Fig. 4; Table 2) that several peaks could be eluted from the DEAE-Sephadex after the removal of PF. These data suggest (but certainly do not prove) that PF at the DEAE-Sephadex stage is an antigenically homogeneous material.

Homogeneity of PF. To investigate the possibility that immunodiffusion and immunoelec-
trophoresis were leaving some additional protein component undetected, each fraction was tested for chemical homogeneity by disc electrophoresis. In line with the chromatographic and immunochromical data, several distinct bands could be seen (Fig. 7) in each fraction prior to DEAE-Sephadex treatment. After the ion-exchange process, only one line remained. When an identical PF-containing gel (unfixed and unstained) was tested in a modified immunodiffusion procedure for the presence of antigen by use of a monospecific antiserum, the position of the resultant single sharp band coincided exactly with that of the protein detected by staining.

**DISCUSSION**

From the foregoing data, we conclude that dextran sulfate precipitation is an ideal initial step for purifying PF. The technique is selective, gentle, and easy to perform, and it results in a stable concentrated form of PF which is amenable to a variety of subsequent manipulations. The selectivity of the procedure is illustrated by the observation that DST repeatedly reprecipitated by dilution and readdition of CaCl₂ contained only one major antigenic component (PF) compared with the half-dozen found in once-precipitated DST or crude filtrates.

The obvious disadvantages of having to predialyze large volumes of filtrate can be overcome by absorbing the PF (and other proteins) from crude filtrates with alumina gel (G. Spyrides and J. C. Feeley, unpublished data), eluting into a small volume, and desalting the concentrated crude toxin by ultrafiltration. Crude alumina gel concentrates purified by the precipitation and chromatographic techniques described above yield PF comparable to that derived from dialyzed filtrates (Richardson, unpublished data).

In another publication (Richardson and Nofl, in press), we have shown that PF derived from the four media discussed in Table 1 can be treated by our technique to yield preparations of PF with identical immunochemical, biochemical, and biological properties. This suggests that crude PF, whatever the source, can be concentrated and purified without lyophilization, flash evaporation, or sequential chromatographic procedures such as those employed by Coleman et al. (1) and Finkelstein and LoSpalluto (4).

Since the ammonium sulfate and gel filtration steps do not appear to increase PF specific activity (at least on a protein basis), we have recently investigated other means of separating PF from dextran sulfate. By precipitating the dextran sulfate from DST preparations with barium salts, it is possible to eliminate these intermediate steps and proceed directly to ion-exchange chromatography. This modification is in the small-scale analytical stage at the present time, but appears to yield PF identical to that produced by the more complex process.

In spite of the detection of a single band in immuno-electrophoresis and disc electrophoresis, some batches of purified PF are apparently still contaminated with trace amounts of somatic antigen, as indicated by a low but consistent vibriocidal antibody titer rise in rabbits vaccinated with them (Richardson, Bacteriol. Proc., p. 89, 1969). Efforts are now being made to achieve higher degrees of purification without concomitant losses in PF activity.

Based on PF assays carried out in this and several other laboratories (by Nate Pierce and William Greenough, Johns Hopkins University, and John Craig, Downstate Medical Center, Brooklyn, N.Y.), the dose of purified material required to cause a positive bluing response (7 mm in rabbits, 8 mm in guinea pigs) lies between 0.1 and 2 ng. These values are of the same order of magnitude as those reported (0.4 ng) by Finkelstein and LoSpalluto for pure choleragen (4).

After considerable experience in applying our technique to a wide variety of crude preparations, we conclude that it is the simplest method available at the present time for obtaining high-purity PF. In another report, we will present data which we believe shows beyond reasonable doubt that PF and cholerenterotoxin are biologically and immunologically identical.

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