Properties of the Enterotoxic Component in
Escherichia coli Enteropathogenic for Swine

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The enterotoxic component in sterile syncase broth filtrates of Escherichia coli strains 340 (O9:K_:NM) and P307 (O8:K87,88a,b:H19) was studied. The enterotoxic activity in both strains was retained by an ultrafiltration membrane with a molecular weight retention of 100,000 (XM-100A) and eluted from a Sephadex G-200 column in the void volume. The enterotoxic activity in strain 340 was resistant to heating at 75 C for 30 min, but the activity in strain P307 was destroyed by heating at 60 C for 30 min. The P307 Sephadex G-200 column eluate possessing the enterotoxic activity, when desalted, contained 45.8% carbohydrate and 9.3% protein, and had an EDT₅₀ of 2.2 mg/rabbit ileal loop. Immunodiffusion studies showed that this material contained both endotoxin and acid-polysaccharide capsular material. The enterotoxic activity was acid-labile and was destroyed by Pronase, but was resistant to trypsin and eluted as a single peak in the void volume of a 4% agarose column. The enterotoxic component could not be separated from the endotoxin; in fact, the data indicated that the two components are closely associated and that the enterotoxic activity resides in material of a protein nature.

Enterotoxigenic Escherichia coli strains isolated from both humans (5, 19, 22) and animals (9, 13, 15, 23) have been reported to produce an enterotoxin, of which two forms are generally recognized. The heat-stable toxin has been isolated from the supernatant fluid of broth cultures and from fluid expressed from soft-agar cultures of enterotoxigenic E. coli. The heat-labile toxin has been isolated from lysates of enterotoxigenic E. coli and also from the supernatant fluid of broth cultures. It has been postulated that these apparently dissimilar enterotoxins are probably two different forms of what is essentially the same enterotoxin (21).

Vibrio cholerae enterotoxin (cholera) has been shown to be free from endotoxin (7), whereas all studies on E. coli enterotoxin have been done with endotoxin-containing preparations. Since endotoxins are known to cause a multitude of physiological disturbances, any reaction attributed to an enterotoxin should be shown to occur in the absence of endotoxin. The object of this study was the isolation and purification of the enterotoxic component in E. coli. The main goal was to separate the enterotoxic component from the endotoxin. This report presents our findings on the enterotoxic component in E. coli strains 340 (O9:K_:NM) and P307 (O8:K87,88a,b:H19).

MATERIALS AND METHODS

Organisms. E. coli strain 340 (O9:K_:NM), a porcine enteropathogen (12), was obtained from E. R. Kohler of the Ohio Agricultural Research and Development Center, Wooster, Ohio. In this publication, S340 will represent strain 340. E. coli strain P307 (O8:K87,88a,b:H19), a porcine enteropathogen (10), and E. coli strain M432 (O139:K82:H1), described as not enteropathogenic for swine (10), were obtained from D. A. Barnum, Ontario Veterinary College, University of Guelph, Guelph, Ontario, Canada. The E. coli O8:K8:H4 strain was obtained from P. J. Glantz, Pennsylvania State University, University Park.

Production of sterile broth (SB) filtrates. A 9.5-liter volume of syncase medium (6) with glucose substituted for sucrose was inoculated with 500 ml of a 6-hr syncase broth culture of strain S340 or P307. Growth was for 8 or 18 hr under high aeration in a 28-liter fermentor (Microferm, New Brunswick Scientific Co., Inc.). A plate count was performed on the 8- or 18-hr culture. On certain batches, plate counts were performed hourly for 9 hr to determine the time needed for optimal growth. The cells were removed by centrifugation at 15,000 x g for 30 min, and the supernatant fluid was filtered through a 0.45-µm membrane filter (filter-sterilized). The pH of the filtrate was recorded, and the filtrate was checked for sterility by streaking it on blood agar plates and noting the presence or absence of growth.

Ultrafiltration. A 1,100-ml amount of S340 or P307 SB filtrate was diluted with 500 ml of distilled
water and placed in an ultrafiltration system (model 202 cell with a reservoir, Amicon Corp.) fitted with an XM-100A membrane. Ultrafiltration was continued until 60 ml of fluid remained above the membrane. This material (the S340 or P307 XM-100A retentate) was washed with 3 volumes of 0.01 M phosphate buffer (pH 7.0), filter-sterilized, and lyophilized. The S340 retentate was tested in pig jejunal loops at 40 to 125 mg/loop and in rabbit ileal loops at 5 to 40 mg/loop. The P307 retentate was tested in rabbit ileal loops at 7.5 and 15 mg/loop. Amounts of 600 ml of the S340 XM-100A filtrate were concentrated to 15 ml with a PM-10 membrane or to 90 ml with a UM-2 membrane. These concentrates were filter-sterilized, lyophilized, and tested in pig jejunal loops at 100 mg/loop for the PM-10 concentrate and 250 mg/loop for the UM-2 concentrate. A 1,500-ml amount of P307 XM-100A filtrate was concentrated to 15 ml with a PM-10 membrane or a 300-ml amount was concentrated to 15 ml with a UM-2 membrane. These 15-ml concentrates were filter-sterilized, lyophilized, and tested at 7.5 and 15 mg/rabbit ileal loop.

An additional 1,100-ml amount of P307 SB filtrate was diluted with 500 ml of distilled water and placed in the same ultrafiltration system fitted with a PM-10 membrane. Again, ultrafiltration was continued until 60 ml of fluid remained above the membrane. The retentate was washed with 3 volumes of 0.01 M phosphate buffer (pH 7.0), filter-sterilized, and lyophilized. This retentate (P307 PM-10 retentate) was tested at 1.25 to 30 mg/rabbit ileal loop. The PM-10 filtrate was discarded. Strain M432 SBP was also ultrafiltered according to this procedure, and the retentate (M432 PM-10 retentate) was tested at 5 to 30 mg/rabbit ileal loop.

**Gel filtration chromatography.** A 250-ml amount of the S340 XM-100A retentate or the P307 PM-10 retentate was placed on a column (90 by 2.5 cm) of Sephadex G-200 equilibrated with 0.05 M phosphate buffer (pH 7.0). The material was eluted from the gel at a flow rate of 10 ml/hr. The eluate was collected in 3-ml fractions and monitored at 274 nm on a spectrophotometer (model DB, Beckman Instruments, Inc.). Fractions comprising each of the first and second peaks and the eluate between the two peaks were pooled and concentrated to 15 ml via ultrafiltration. A PM-10 membrane was used for the first peak fractions and the eluate between the peaks; a UM-2 membrane was used for the second peak. Each 15-ml retentate was filter-sterilized and lyophilized. The S340 retentates were tested at 50 to 200 mg/pig jejunal loop, and the P307 retentates, at 1.65 to 26.4 mg/rabbit ileal loop. Blue Dextran 2000 (Pharmacia Fine Chemicals) was used to determine the void volume of the Sephadex G-200 column.

A portion (50 mg) of the P307 Sephadex G-200 first peak material was placed on a column (2.5 by 40 cm) of 4% agarose (Seapharose 4B, Pharmacia Fine Chemicals) equilibrated with phosphate buffer. The enterotoxic material was eluted at a flow rate of 10 ml/hr, and the eluate was monitored at 274 nm. The void volume of the column was determined by placing live *E. coli* cells on the column and monitoring their appearance in the eluate at 274 nm.

**Ion-exchange chromatography.** A 125-mg portion of S340 XM-100A retentate, or 31 mg of the first peak of the Sephadex G-200 fractionation of the S340 XM-100A retentate, dissolved in 3 ml buffer, was placed on a column (2.5 by 45 cm) containing CM Sephadex C-25 (Pharmacia Fine Chemicals) equilibrated with 0.05 M phosphate buffer, pH 6.3. The sample was eluted at a flow rate of 22 ml/hr with the phosphate buffer by use of a continuous gradient to 0.2 M NaCl. Fractions were collected in 3-ml volumes, and the eluant was monitored at 274 nm. Fractions comprising each of the peaks were pooled, filter-sterilized, and lyophilized. They were tested at 175 to 200 mg/pig jejunal loop. Fractionation of the S340 and P307 Sephadex G-200 first peak materials was also attempted on other cationic and anionic exchange resins by use of batch and column chromatographic procedures.

**Chemical analysis.** Carbohydrate was estimated by the phenol-sulfuric acid technique (3); dextran with an average molecular weight of 200,000 to 300,000 (J. T. Baker Chemical Co.) was used as the standard. Protein was estimated by the method of Lowry et al. (14), with bovine serum albumin as the standard.

**Effect of heat on enterotoxic materials.** A 120-mg amount of the S340 XM-100A retentate was dissolved in 5 ml of distilled water and heated at 75 C for 30 min. These samples were tested at 4.0 ml (100 mg/pig jejunal loop.

A 35-mg amount of P307 PM-10 retentate was dissolved in 3.5 ml of distilled water and heated at 75 C for 30 min. The solution was tested in two rabbit ileal loops at 1.5 ml/loop. Amounts of 33 mg of the P307 Sephadex G-200 first peak material were each dissolved in 3.5 ml of distilled water and heated at 40, 50, 60, and 80 C for 30 min. These heated solutions along with an unheated solution were tested in two rabbit ileal loops at 1.5 ml/loop.

**Effect of pH on enterotoxic material.** Amounts of 35 mg of P307 Sephadex G-200 first peak material (enterotoxic material) were dissolved in 3.5 ml of distilled water in each of five tubes. The contents of the tubes were adjusted to pH 1, 3, 5, 7, and 9 with concentrated HCl or 5 N NaOH. The tubes were incubated at room temperature for 4 hr, and then each tube was adjusted to pH 7. No apparent increase in volume was noticed. The contents of each tube were tested in rabbit ileal loops at 1.5 ml/loop, and 0.1 ml of a 1:4 dilution of each tube prepared with saline was tested for endotoxic activity via the local Schwartzman reaction as described by Nowotny (16).

**Effect of proteolytic enzymes on enterotoxic material.** P307 Sephadex G-200 first peak material (enterotoxic material) in an amount of 33 mg (approximately 1.120 μg of protein) was dissolved in 3.5 ml of distilled water, and 0.045 ml of 0.1% Pronase (Calbiochem) in 0.03 M phosphate buffer, pH 7.4 (approximately 45 μg of protein), was added. The ratio of protein substrate to enzyme was approximately 25:1. The mixture (pH 7.0) was incubated at 37 C for 24 hr. Toluene (0.05 ml) was added to prevent microbial contamination. Substrate without...
envelope and enzyme without substrate also were incubated under the same conditions to serve as controls. These materials were tested at 1.5 ml/rabbit ileal loop. This procedure was repeated, and 3.5 ml (approximately 33 mg) of Pronase-treated enterotoxic material was chromatographed on Sephadex G-200 as previously described. This chromatography was used to determine whether this reagent caused dissociation of the enterotoxic material.

The enterotoxic material was treated with trypsin via two different procedures. In the first experiment, 33 mg of material was dissolved in 3.3 ml of distilled water, and 100 μg of trypsin (twice crystallized-salt free, Nutritional Biochemicals Corp.) dissolved in 0.2 ml of 0.001 M HCl was added. The ratio of protein substrate to enzyme was approximately 11:1. The mixture (pH 7.0) was incubated at 25 C for 3 hr. Tryptic activity was confirmed with p-toluene-sulfonfonyl-l-arginine methyl ester (TAME; Calbiochem; 11). Substrate without enzyme and enzyme without substrate also were incubated under the same conditions. These materials were tested at 1.5 ml/rabbit ileal loop.

In the second trypsin experiment, 125 mg of enterotoxic material was dissolved in 20 ml of distilled water and placed in an ultrafiltration cell fitted with a PM-10 membrane. This material was washed five times with 20 ml of 0.046 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, pH 8.1. The final wash was concentrated to 11 ml, producing a solution containing approximately 11.3 mg of the original enterotoxic material/ml. To 4.25 ml of this solution, 160 μg of trypsin dissolved in 0.16 ml of 0.001 M HCl and 0.13 ml of 0.035 M CaCl2 were added. The final Ca2+ concentration was approximately 0.001 M and the reaction pH was 8.0. The ratio of protein substrate to enzyme was approximately 10:1. The mixture was incubated at 37 C for 5 hr. Tryptic activity was confirmed with TAME. Substrate without enzyme and substrate without substrate also were incubated under these same conditions. These materials were tested at 2.0 ml/rabbit ileal loop. This procedure was repeated, and 3.5 ml (approximately 39 mg) of trypsin-treated enterotoxic material was chromatographed on Sephadex G-200.

Effect of chemical reagents on enterotoxic material. The P307 Sephadex G-200 first peak material was treated with a series of reagents in an effort to separate the enterotoxic component from the endotoxin. After each treatment, the solution was tested for enterotoxic activity in rabbit ileal loops and for endotoxic activity by the Shwartzman reaction. Except for Triton X-100-treated samples, which were not diluted prior to testing, 0.1 ml of a 1:4 dilution of each solution was used for the Shwartzman reaction.

Enterotoxic material (45 mg) was added to 5 ml of a solution of 4 M urea and 0.05 M sodium acetate (25). The mixture was incubated at 30 C for 2 hr after which it was dialyzed with 100 ml of 0.05 M phosphate buffer, pH 7.0, via ultrafiltration with a PM-10 membrane. The final dialysand (5 ml) was tested in rabbit ileal loops at 1.5 ml/loop (approximately 2 units/loop).

Enterotoxic material was treated with guanidine hydrochloride and tested for enterotoxic activity in exactly the same manner as described for urea; 6 M guanidine hydrochloride was used. The procedure was repeated twice, and 5 ml of the final dialysand was chromatographed on Sephadex G-200.

Solutions of enterotoxic material were reduced with mercaptoethanol and alkylated according to procedures used for immunoglobulins (20). A 120-mg amount of enterotoxic material was dissolved in 15 ml of 0.01 M phosphate-buffered saline, pH 7.0 (PBS). Two 5-ml portions were each dialyzed against 1 liter of 0.1 M mercaptoethanol in PBS for 3 hr and then against 1 liter of 0.02 M iodoacetamide for 4 hr to prevent reassociation of reduced molecules. This was followed by dialysis against PBS overnight. The other 5 ml of the original 15 ml served as a control and was treated in the same manner except that mercaptoethanol was omitted from the dialysis buffer. Both the control and mercaptoethanol-treated enterotoxic solutions were tested in rabbit ileal loops at 1.7 ml/loop. In addition, 5 ml of the mercaptoethanol-treated material was chromatographed on Sephadex G-200. These procedures were followed on two different occasions.

A mixture composed of 90 mg of enterotoxic material added to 10 ml of a solution of 0.04 M Cleland's reagent (dithiothreitol, J. T. Baker Chemical Co.) in 0.5 M Tris-hydrochloride buffer, pH 8.0 (2), was incubated at 30 C for 1 hr; 150 mg of iodoacetamide was then added. This mixture was incubated at 30 C for 2 hr, after which it was dialyzed with 200 ml of phosphate buffer via ultrafiltration with a PM-10 membrane. This procedure was repeated with a separate sample in which Cleland's reagent was omitted. Both final products (10 ml each) were tested in rabbit ileal loops at 1.5 ml/loop, and 5 ml of Cleland's reagent-treated material was chromatographed on Sephadex G-200.

An 80-mg amount of enterotoxic material was added to 13 ml of phosphate buffer containing 0.1% Triton X-100 (W. R. Grace Inc.). The mixture was placed in a centrifuge tube (1.6 by 7.6 cm; Beckman Instruments, Inc.) and centrifuged at 100,000 x g for 2 hr. The contents of the tube were split into three fractions of approximately 4 ml each. This procedure was repeated with phosphate buffer without Triton X-100 and with phosphate buffer plus Triton X-100 but no enterotoxic material. All fractions were tested in rabbit ileal loops at 1.75 ml/loop. A 50-mg amount of enterotoxic material was dissolved in 3 ml of phosphate buffer containing 0.1% Triton X-100 and placed on a column (2.5 by 40 cm) of 4% agarose equilibrated with the phosphate buffer containing 0.1% Triton X-100. The enterotoxic material was eluted at a flow rate of 10 ml/hr, and the eluate was monitored at 245 nm. The fractions comprising each of the two peaks were pooled and concentrated via ultrafiltration with a PM-10 membrane to 5 ml. They were tested in rabbit ileal loops at 1.5 ml/loop.

Ammonium sulfate was added to solutions of enterotoxic material (11 mg of enterotoxic material/ml) in amounts to form final concentrations of 10 to 100% saturation. The solutions were observed for...
visible precipitates and then centrifuged at 30,000 × g for 60 min.

Desalting of enterotoxic material. The P307 Sephadex G-200 first peak material was exhaustively desalted by washing it five times with distilled water in an ultrafiltration cell fitted with a PM-10 membrane. The final product was lyophilized and tested in rabbit ileal loops at 0.55 to 8.8 mg/loop.

Assays for enterotoxic materials. Enterotoxic activity in live cultures and cell-free preparations of strain S340 was assayed in the jejunal loops of pigs 6 to 12 weeks old and ranging in body weight from approximately 4.5 to 28 kg. The operative procedure was performed according to described methods (10, 23). Starting approximately 1 meter distal to the pyloric end of the stomach, silk ligatures were placed around the jejunum to form loops 10 to 12 cm long. Alternate loops were inoculated with test materials. Live cultures were tested in a 1-ml volume; other test materials were in a 4-ml inoculum. For positive controls, two loops in each pig were inoculated with 1 ml of a 6-hr syncase broth culture of S340, one loop was inoculated with 1 ml of a 6-hr syncase broth culture of strain M432, and one loop was inoculated with 150 mg of syncase medium. The pigs were used in pairs; all test materials were given in duplicate to two pigs. The pigs were necropsied at 18 hr, and a volume to length ratio was calculated for each loop. Data were accepted only if the five control loops in each pig gave the appropriate responses.

Enterotoxic activity in S340 and P307 cell-free preparations was assayed in rabbit ileal loops by the method of Burrows and Musteikis (1). Live cultures were not tested in rabbit ileal loops. Locally obtained albino rabbits weighing 1.0 to 2.0 kg were used. Six experimental loops, each 10 to 12 cm long and separated by double ties, were created in each rabbit. One loop served as the negative control and was injected with 14 mg of M432 PM-10 retentate dissolved in 1.5 ml of a neomycin sulfate water solution (0.33 μg of neomycin base/ml of water). Another loop served as the positive control and was injected with 14 mg of P307 PM-10 retentate or 14 mg of P307 Sephadex G-200 first peak material dissolved in 1.5 ml of the neomycin sulfate water solution. The remaining four loops were used for test materials or for titrations of the enterotoxic materials. All materials were tested in a 1.5-ml inoculum except for certain treated materials which were tested in a 1.7- to 2.0-ml inoculum. Each loop received 500 μg of neomycin base. The rabbits were used in pairs; all test materials were given in duplicate to two rabbits. The rabbits were autopsied at 18 hr, and a volume (milliliters) to length (centimeters) ratio was calculated for each loop. Data were accepted only if the two control loops in each rabbit gave the appropriate responses.

Immunodiffusion assay. An immunodiffusion assay was developed to detect O and K somatic antigens in strain P307 enterotoxic preparations. Double-diffusion (Ouchterlony) discs (Miles Laboratories, Inc.) were used. Rabbit O and OK antisera to P307 were produced by conventional procedures (4). An extract of P307 was prepared by suspending the growth from overnight brain heart agar (Difco) plates in saline to obtain 10¹⁰ to 10¹¹ cells/ml. This suspension was heated at 100 C for 1 hr, and the cells were removed by centrifugation at 27,000 × g. Bovin antigen was prepared according to described procedures (24). The O antibody was removed from P307 OK antiserum with an O8:K8:H4 E. coli strain according to procedures described by Orskov and Orskov (18). The plates were incubated for 3 days at room temperature in a moisture chamber. Similar procedures were employed in the development of an immunodiffusion assay to detect O antigen in S340 preparations.

RESULTS

P307 enterotoxic component. Viable-cell counts on the 18-hr fermentor cultures of strains P307 and M432 ranged from 1.10 × 10¹⁰ to 1.35 × 10¹⁰ cells/ml, and the pH of the SB filtrates from these 18-hr cultures ranged from 8.0 to 8.8. Live cultures of P307 were not tested for enterotoxic activity. The enterotoxic activity in P307 cell-free preparations was always assayed in rabbit ileal loops.

The enterotoxic activity in the P307 SB filtrate was retained by the XM-100A membrane. In eight rabbit ileal loops, the XM-100A retentate produced a mean volume to length ratio ± standard error (SE) of 1.69 ± 0.3. In seven of eight ileal loops, the XM-100A filtrate did not cause fluid accumulation; the remaining loop had a volume to length ratio of 1.5. Thus, the mean volume to length ratio for the XM-100A filtrate was 0.18 ± 0.2. The 50% effective dose (ED₅₀), or 1 unit of toxin as defined by Burrows and Musteikis (1), for the P307 PM-10 retentate was 7.8 mg/loop (Fig. 1A), and the activity per milligram of protein was 1.2 units (Table 1). The M432 PM-10 retentate did not cause fluid accumulation in any of the 30 rabbit ileal loops in which it was tested.

The fractionation of the P307 PM-10 retentate on Sephadex G-200 is shown in Fig. 2. The first peak appeared in the void volume of the column, and the second peak was in the total bed volume of the column. The enterotoxic activity was confined to the first peak; the eluate between the two peaks and that contained in the second peak did not cause fluid accumulation in rabbit ileal loops. Figure 1B is a titration (dose-response) curve for the first peak material. The maximal mean volume to length ratio obtained was 2.75. This value was used to determine the ED₅₀ levels for the P307 enterotoxic materials. The ED₅₀ or 1 unit of toxin for the first peak material was 7.3 mg/loop, and the activity per milligram of protein was 4.0 units (Table 1).
The enterotoxic activity of the P307 PM-10 retentate was entirely destroyed by heating at 75 C for 30 min. The Sephadex G-200 first peak material was heat-labile in that the activity was affected at 50 C and almost entirely destroyed at 60 C (Table 2).

Figure 3 shows the immunodiffusion studies with P307. At well 2 in Fig. 3A, two lines were evident. The outer, bolder line was the K antigen (acid-polysaccharide), and the inner line was the O antigen (endotoxin). The reaction at well 1 proves this. Here, the O antibody but not the K antibody was removed from the antiserum, and thus the O antigen line was absent while the K line was still present. At well 3, both lines were apparent, but the K line was absent at well 4. When antiserum was made against P307 cells heated at 100 C, some sera contained K antibody and others did not. In Fig. 3B, the center well contained OK antiserum made against heated (100 C) P307 cells. This was the same serum contained in well 3 of Fig. 3A. The reactions at all three wells in Fig. 3B were identical and resembled those of Fig. 3A. Here, however, the inner bold line was the K antigen and the outer line was the O antigen. These reactions showed that the Sephadex G-200 first peak material contained both O antigen (endotoxin) and K antigen (acid-polysaccharide). Also, the reaction at well 3 showed that the bovin antigen was contaminated with K antigen.

The composition of the Sephadex G-200 first peak material (enterotoxic material) is shown in Table 1.

<table>
<thead>
<tr>
<th>Determination</th>
<th>PM-10 retentate</th>
<th>Sephadex G-200 first peak material</th>
<th>Desalted first peak material</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer salts (%)</td>
<td>ND</td>
<td>67</td>
<td>&lt;5</td>
</tr>
<tr>
<td>Carbohydrate (%)</td>
<td>ND</td>
<td>21.8</td>
<td>45.8</td>
</tr>
<tr>
<td>Protein (%)</td>
<td>10.5</td>
<td>3.4</td>
<td>9.3</td>
</tr>
<tr>
<td>ED50 (1 unit) dose/loop (mg)*</td>
<td>7.8</td>
<td>7.3</td>
<td>2.2</td>
</tr>
<tr>
<td>One unit as mg of protein</td>
<td>0.82</td>
<td>0.25</td>
<td>0.20</td>
</tr>
<tr>
<td>Units/mg of protein</td>
<td>1.2</td>
<td>4.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Dry weight (mg/ml)</td>
<td>6.1</td>
<td>10.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Total volume (ml)</td>
<td>60</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Total dry weight (mg)</td>
<td>366*</td>
<td>159</td>
<td>54</td>
</tr>
<tr>
<td>Total protein (mg)</td>
<td>38.4</td>
<td>5.4</td>
<td>5.0</td>
</tr>
<tr>
<td>(26.3)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total units</td>
<td>46.1</td>
<td>21.6</td>
<td>25.0</td>
</tr>
<tr>
<td>(31.6)*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>(100)</td>
<td>68.4</td>
<td>79.1</td>
</tr>
</tbody>
</table>

* Not determined.
* Determined in rabbit ileal loops.
* Only 250 mg of the PM-10 retentate was used for further purification. The yields are calculated on the basis of 250 mg of starting material.

**Table 1. Compositions and activities of the enterotoxic materials isolated from strain P307 sterile broth filtrate**
Figure 4 shows the effect of pH on both the enterotoxic and endotoxic activities of the Sephadex G-200 first peak material. The enterotoxic activity was highly acid-labile, whereas the endotoxic activity was only slightly affected at the acidic pH points. A skin lesion area of <0.30 cm² was considered a negative Shwartzman reaction, and a lesion area >3.10 cm² was considered a maximal reaction.

Table 2. Effect of heat on the enterotoxic activity in 2 units* of strain P307 Sephadex G-200 first peak material in rabbit ileal loops

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume to length ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unheated</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Heated for 30 min at 40°C</td>
<td>2.0 ± 0.2</td>
</tr>
<tr>
<td>50°C</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>60°C</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>80°C</td>
<td>0</td>
</tr>
</tbody>
</table>

*One unit is that amount of material which gave 50% of the maximal response (ED₅₀) in rabbit ileal loops.

* Mean ± SE for six loops.

in Table 1. The action of two proteolytic enzymes on the enterotoxic activity is shown in Table 3. The enterotoxic activity was destroyed by Pronase but was not affected by trypsin (the difference between 1.9 ± 0.2 and 1.8 ± 0.2 is not statistically significant, P > 0.10). The two trypsin procedures produced almost identical results and therefore the data were combined. In the experiments employing enterotoxic material washed with Tris buffer, quantities containing the equivalent of 3 units of original material had to be placed in the rabbit ileal loops. When 2 units were used, the positive control loops which contained enterotoxic material but no trypsin did not consistently cause fluid accumulation. Also, the activity of the enterotoxic material in Tris buffer was reduced by incubation at 37°C for 21 to 24 hr. Positive control loops containing 3 units of material which had been incubated at 37°C for 21 to 24 hr did not consistently cause fluid accumulation.

Table 3. Effect of trypsin and Pronase on the enterotoxic activity of P307 Sephadex G-200 first peak material in rabbit ileal loops

<table>
<thead>
<tr>
<th>Conditions*</th>
<th>Volume to length ratio*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Trypsin experiment</strong></td>
<td></td>
</tr>
<tr>
<td>Enterotoxic material alone</td>
<td>1.9 ± 0.2</td>
</tr>
<tr>
<td>Enterotoxic material + trypsin</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td><strong>Pronase experiment</strong></td>
<td></td>
</tr>
<tr>
<td>Enterotoxic material alone</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Enterotoxic material + Pronase</td>
<td>0.2 ± 0.1</td>
</tr>
</tbody>
</table>

*One unit of enterotoxic material is that amount which gave 50% of the maximal response (ED₅₀) in rabbit ileal loops. In the trypsin experiment 2 or 3 units were used, and in the Pronase experiment 2 units were used.

* Mean ± SE for 10 loops in the trypsin experiment and for 4 loops in the Pronase experiment.
Fig. 4. Effect of pH on the enterotoxic activity (●) and endotoxic activity (×) of the P307 Sephadex G-200 first peak material. Each point on the solid line represents the mean ± standard error volume-length ratio for seven rabbit ileal loops. Each ⨯ on the broken line represents the mean skin lesion area for six sites. A skin lesion area <0.30 cm² was considered a negative Shwartzman reaction; a lesion area >3.10 cm² was considered a maximal reaction.

Fig. 5. Elution pattern of the P307 Sephadex G-200 first peak material from a 4% agarose column (2.5 by 40 cm). The solid line represents the enterotoxic material. The broken line represents the live E. coli cells used to mark the void volume of the column.

The behavior of the enterotoxic material on 4% agarose is shown in Fig. 5. There was one peak which was at the void volume slightly ahead of the live E. coli cells.

The effects of the chemicals and enzymes on both the enterotoxic and endotoxic activities of the Sephadex G-200 first peak material are shown in Table 4. None of the reagents affected the endotoxic activity, but half of the reagents destroyed the enterotoxic activity. The elution pattern of the enterotoxic material on Sephadex G-200 was not altered in any of the solutions tested. No precipitates were obtained with ammonium sulfate at final concentrations of up to 70%. At 70 to 100% saturation, precipitates were obtained; however, the precipitates contained both endotoxin and K antigen.

Figure 6 shows the elution pattern of the enterotoxic material from the 4% agarose column equilibrated with 0.1% Triton X-100. The appearance of two peaks was evidence that this reagent caused dissociation of the enterotoxic material. Enterotoxic activity was found in both peaks. However, endotoxic activity was also found in both peaks, showing that dissociation of endotoxin also took place. Table 5 shows a similar dissociation of the enterotoxic material as revealed by ultracentrifugation.

Table 4. Effects of chemicals and enzymes on the enterotoxic and endotoxic activities of the P307 Sephadex G-200 first peak material

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Enterotoxic activity</th>
<th>Endotoxic activity</th>
<th>Elution pattern on Sephadex G-200 after treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanidine HCl</td>
<td>-</td>
<td>+</td>
<td>No change</td>
</tr>
<tr>
<td>(6 m)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea (4 m)</td>
<td>-</td>
<td>+</td>
<td>Not tested</td>
</tr>
<tr>
<td>Cleland's reagent</td>
<td>(0.04 m)</td>
<td>-</td>
<td>No change</td>
</tr>
<tr>
<td>Mercaptoethanol</td>
<td>(0.1 m)</td>
<td>+</td>
<td>No change</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>(0.1%)</td>
<td>+</td>
<td>Tested on 4% agarose</td>
</tr>
<tr>
<td>Trypsin (10:1)</td>
<td>+</td>
<td>+</td>
<td>No change</td>
</tr>
<tr>
<td>Pronase (25:1)</td>
<td>-</td>
<td>+</td>
<td>No change</td>
</tr>
</tbody>
</table>

* Symbols: -, activity was destroyed; +, activity was present after treatment.

* Enterotoxic material normally elutes from a Sephadex G-200 column in the void volume. Alteration of this elution pattern would be considered evidence of dissociation.

* Substrate to enzyme ratio.

Fig. 6. Fractionation of the P307 Sephadex G-200 first peak material on a 4% agarose column (2.5 by 40 cm) equilibrated with 0.1% Triton X-100 (broken line). The solid line represents the elution pattern of the enterotoxic material from the same column with the Triton X-100 omitted. The column eluates were monitored at 245 nm because the Triton X-100 absorbance was minimal at this wavelength.
Endotoxic activity paralleled enterotoxic activity, and both activities accompanied the movement of the carbohydrate material.

Table 1 gives the composition of the Sephadex G-200 first peak material after desalting. Desalting reduced the dry weight from 10.6 to 3.6 mg/ml. This was approximately a two-thirds reduction and can be attributed to the removal of the buffer salts. The protein content correspondingly increased approximately three times, and the carbohydrate content roughly doubled. Figure 1C is a titration curve for the desalted enterotoxic material. Desalting reduced the ED₅₀ from 7.3 to 2.2 mg/loop. This was also approximately a two-thirds reduction. Thus, desalting produced a lower ED₅₀ which quantitatively paralleled the reduction in dry weight. The activity per milligram of protein of the desalted enterotoxic material was 5.0 units, compared with 4.0 units before desalting. Seventy-nine percent of the original activity in the PM-10 retentate was recovered in the first desalted product.

S340 enterotoxic component. For the 8-hr fermentor cultures of S340, the viable-cell counts ranged from 9.1 × 10⁷ to 1.24 × 10⁸ cells/ml, and the pH of the SB filtrate ranged from 7.3 to 7.8. For the 18-hr fermentor cultures, the viable cell counts ranged from 9.0 × 10⁸ to 1.31 × 10⁹ cells/ml, and the pH of the SB filtrate ranged from 7.9 to 8.5. The plate counts taken at 1-hr intervals showed that optimal growth occurred within 8 hr. There was no apparent difference in the enterotoxic activity of the 8- and 18-hr SB filtrates.

The enterotoxic activity in live cultures and cell-free preparations of S340 was assayed mostly in pig jejunal loops. Live cultures of S340 in 52 pig jejunal loops produced a mean volume to length ratio ± SE of 4.03 ± 0.3. The M432 live cultures did not cause fluid accumulation in 37 of 38 pig jejunal loops.

The enterotoxic activity in the S340 SB filtrate was also retained by the XM-100A membrane. Figure 7A is a dose-response curve for the XM-100A retentate in pig jejunal loops. The ED₅₀ or 1 unit of toxin was 73 mg/loop. The XM-100A filtrate in six pig jejunal loops produced a mean volume to length ratio ± SE of 0.2 ± 0.1. The S340 XM-100A retentate did not cause fluid accumulation in any of the eight rabbit ileal loops which contained 5 to 40 mg/loop.

The elution pattern for the S340 XM-100A retentate on Sephadex G-200 was almost identical to that of P307 PM-10 retentate. Again, the enterotoxic activity was confined to the first peak. Figure 7B shows the activity of the first peak material in pig jejunal loops. The CM Sephadex C-25 fractionation of the S340 XM-100A retentate is shown in Fig. 8. There were two peaks. The first peak appeared in the void volume of the column, and the second immediately followed. The first peak material caused fluid accumulation in two of three pig jejunal loops, producing a mean volume to length ratio ± SE of 1.33 ± 0.2; the second peak material did not cause fluid accumulation in any of three pig jejunal loops. Endotoxin was detected only in the first peak. The S340 Sephadex G-200 first peak material eluted in the void volume of the CM Sephadex C-25 column. All attempts at fractionating the S340 or the P307 Sephadex G-200 first peak materials on other ion-exchange resins were unsuccessful.

The enterotoxic activity of the S340 XM-100A retentate was reduced by heat at 75 C for 30 min. In the six pig jejunal loops, the mean volume to length ratio ± standard error for the unheated controls was 2.72 ± 0.4, and for the heated samples it was 1.56 ± 0.5. An immunodiffusion assay similar to the one described for P307 showed that all of the S340 preparations contained endotoxin.

The large amounts of S340 cell-free materials required to cause fluid accumulation in pig jejunal loops would readily exhaust our supplies, and approximately 20% of the pigs were unreactive to cell-free preparations; that is, live enteropathogenic cultures caused fluid accumulation but accompanying cell-free preparations did not. Thus, we decided to switch to

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**Table 5.** Effect of Triton X-100 on the ultracentrifugational pattern of the P307 Sephadex G-200 first peak material

<table>
<thead>
<tr>
<th>Centrifuge tube fraction</th>
<th>Per-cent CHO*</th>
<th>Ileal loop reaction†</th>
<th>Shwartzman reaction (cm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enterotoxlic material</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top</td>
<td>10.0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Middle</td>
<td>13.3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Bottom</td>
<td>76.7</td>
<td>2.4 ± 0.3</td>
<td>3.46</td>
</tr>
<tr>
<td><strong>Enterotoxlic material + Triton X-100</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Top</td>
<td>22.8</td>
<td>0.9 ± 0.4</td>
<td>0.95</td>
</tr>
<tr>
<td>Middle</td>
<td>26.5</td>
<td>1.3 ± 0.3</td>
<td>1.77</td>
</tr>
<tr>
<td>Bottom</td>
<td>50.7</td>
<td>1.8 ± 0.1</td>
<td>3.14</td>
</tr>
</tbody>
</table>

*Note: top, middle, and bottom fractions of tubes containing phosphate buffer plus 0.1% Triton X-100 only did not have any carbohydrate content, enterotoxic, or endotoxic activity.

†Carbohydrate.

‡Volume to length ratio (mean ± SE) for six gut loops.

§Mean skin lesion area for four sites.
Fig. 7. Dose-response curves for the S340 enterotoxic materials in pig jejunal loops. A represents the XM-100A retentate and B represents the Sephadex G-200 first peak material. The ratios of fluid volume in the loop to length of the loop were plotted against the logarithm of concentration of the enterotoxic material in the loop. The numbers in parentheses indicate the number of loops assayed to establish each point on the curve. The vertical line at each point indicates the standard error of the mean ratio. The curves were fitted by inspection.

Fig. 8. Fractionation of S340 XM-100A retentate on a CM Sephadex C-25 column (2.5 by 45 cm). The solid line represents optical density at 274 nm; the broken line represents molarity of NaCl.
the rabbit ileal loop assay. Since cell-free preparations of S340 were unreactive in rabbit ileal loops, we could no longer use this strain.

**DISCUSSION**

Gyles and Barnum (10) detected enterotoxic activity in SB filtrates of 7-hr peptone dialysate cultures of strain P307, and Kohler (12) found that the qualitative and quantitative enterotoxic activity of SB filtrates of syncase cultures of S340 was the same with incubation periods of 6 to 24 hr. Our observation that there was no apparent difference in the enterotoxic activity of SB filtrates from 8- and 18-hr cultures agrees with these findings. However, these data contrast with results reported by Sack et al. (19), who obtained maximal enterotoxic activity at 18 hr but could not detect enterotoxic activity in SB filtrates of syncase cultures during the first 12 hr of growth. Our viable-cell counts for all three strains at 8 and 18 hr were similar and were slightly higher than counts reported by other investigators for 18- to 20-hr enterotoxigenic E. coli cultures (15, 19). The pH range for the 18-hr cultures was similar to that reported by Moon et al. (15) for 20-hr enterotoxigenic E. coli cultures. The increase in pH between 8 and 18 hr reflects the increased use of amino acids as a carbon source.

The retention of the enterotoxic activity in the S340 and P307 SB filtrates by the XM-100A membrane was surprising. Kohler (12, 13) had described the enterotoxigenicity in S340 as heat-stable, since SB filtrates caused diarrhea after being heated at 100 C for 1 hr. Also, Gyles and Barnum (10) reported that P307 produced a heat-stable form of enterotoxin in addition to a heat-labile form. The heat-stable form of E. coli enterotoxin was reported to be dialyzable and to pass through a PM-10 membrane (9). Thus, we anticipated that the enterotoxic activity would readily pass through the XM-100A membrane while most of the endotoxin would be retained. This retention by the XM-100A membrane of the enterotoxic activity in SB filtrates from both S340 and P307 showed that there was no readily isolated low-molecular-weight (dialyzable) enterotoxic component in either of these two strains. The ED_{50} of 73 mg/pig jejunal loop for the S340 XM-100A retentate was approximately five times the ED_{50} (14.4 mg/pig jejunal loop) for enterotoxigenic E. coli strain 263 (O8:K87,88a,b:H19) reported by Moon et al. (15). The failure of the S340 XM-100A retentate to cause fluid accumulation in rabbit ileal loops agrees with results described by Gyles (9) for the heat-stable form of E. coli enterotoxin. Gyles found that comparatively large doses were required to elicit dilatation in rabbit ileal loops, and at these high dose levels there was a high incidence of apparently nonspecific reactions. Although the P307 enterotoxic activity was retained by the XM-100A membrane, we found that PM-10 retentates displayed slightly greater enterotoxic activity than XM-100A retentates. Thus, we used PM-10 membranes to concentrate the P307 SB filtrate prior to gel filtration. The ED_{50} of the PM-10 retentate (7.8 mg/loop) was similar to the ED_{50} levels reported by Moon et al. (15) and Sack et al. (19) for SB filtrates or dialyzed SB filtrates of enterotoxigenic E. coli.

The Sephadex G-200 fractionation of the S340 XM-100A retentate and the P307 PM-10 retentate was simply a separation of the high-molecular-weight components from the low-molecular-weight components. There was no fractionation of the high-molecular-weight components. We hoped to isolate the enterotoxic component from the endotoxin, which we knew would appear in the void volume. However, the P307 Sephadex G-200 first peak material did represent a partial purification and resulted in an active enterotoxigenic preparation. Although its ED_{50} was similar to the ED_{50} of the PM-10 retentate, its activity per milligram of protein was 3.3 times that of the PM-10 retentate. Also, the P307 Sephadex G-200 first peak material produced greater volume to length ratios than the PM-10 retentate at the two highest concentrations tested (19.8 and 26.4 mg/loop for the Sephadex G-200 first peak material and 20 and 30 mg/loop for the PM-10 retentate). This first peak material was not further desalted at this time because column eluates desalted to 15 ml always displayed high enterotoxic activity. Also, the ED_{50} or 1 unit of toxin for this material (7.3 mg/loop) meant that a 2-unit dose was well tolerated by the young rabbits. Thus, there was no need for further desalting at this time, and this material was used for further experimentation. When we desalted this material later, the desalted material was approximately 3.5 times as active on a dry weight basis (ED_{50} of 2.2 versus 7.3 mg/loop), and the activity per milligram of protein increased 25%. The percentages of the analyzed carbohydrate (45.8%) and protein (9.3%) in the desalted Sephadex G-200 first peak material were comparable with the amounts of these two substances in endotoxin. Carbohydrates usually amount to a maximum of 40 to 50% and protein to 10% in endotoxin (17).

The approximate molecular weight exclusion limit for polysaccharides on 4% agarose is 5 x
10^4. Since the P307 Sephadex G-200 first peak material was largely carbohydrate and appeared in the void volume of the 4% agarose column, it can be concluded that this material probably has a molecular weight > 5 x 10^4. The S340 XM-100A retentate fractionated on the CM Sephadex C-25 column, but, unfortunately, the enterotoxic activity was in the first peak along with the endotoxin.

Approximately 60% of the activity of the S340 XM-100A retentate remained after heating at 75 C for 30 min. This was in contrast to the heat lability of the enterotoxic component in P307. The P307 PM-10 retentate was completely inactivated at 75 C, and the Sephadex G-200 first peak material was inactivated at 60 C. The heat-lability of the P307 Sephadex G-200 first peak material is comparable to that of the heat-labile form of *E. coli* enterotoxin found in whole-cell lysates of enterotoxigenic *E. coli* (9). The heat lability of our P307 preparations isolated from SB filtrate contrasts with findings of Gyles and Barnum (10), who reported that the enterotoxic activity in P307 SB filtrate was heat-stable in that the activity was not completely destroyed by heating at 100 C for 30 min. Various degrees of heat resistance for the enterotoxic component in SB filtrates of enterotoxigenic *E. coli* have been reported (5, 10, 13, 15, 23, 29). These variations may be due to individual differences in the strain used, to differences in the media and methods used to prepare the SB filtrates, or to a combination of these factors.

The action of the proteolytic enzymes on our enterotoxic material was expected. Other characterized enterotoxins are resistant to trypsin but are destroyed by Pronase (8). *E. coli* enterotoxic preparations have been shown to be resistant to trypsin (10).

We were unable to separate the enterotoxic activity from the endotoxic activity as evidenced by the results obtained with the chemical reagents, enzymes, and chromatography. In fact, we showed that there was a very close association between the two activities. Even when we achieved dissociation of the enterotoxic material with Triton X-100, dissociation of the endotoxic component also occurred, and endotoxic activity followed enterotoxic activity. The destruction of the enterotoxic activity by Pronase and the acid and heat lability of this activity indicated that the enterotoxic activity resides in material of a protein nature. These findings contrast to endotoxic activity, which is heat-stable, stable to mild acid treatment, and not affected by Pronase. Endotoxic activity is attributed to a glycolipid moiety (17). Since endotoxin is a carbohydrate-lipid-protein complex and since the enterotoxic and endotoxic activities in *E. coli* have not been separated, it may be that the same molecule is responsible for both activities, with a glycolipid moiety accounting for the classical endotoxic activity and a protein moiety responsible for the enterotoxic activity.

Strain P307, as shown by its antigenic formula, contains two K antigens. The K88 antigen has been characterized as a protein (26), and the K87 antigen has been shown to be an acid-polysaccharide (27). This K88 is destroyed by heating at 80 C, and therefore antiserum prepared against P307 cells heated at 100 C will not contain K88 antibody. Thus, our immunological assay employing OK P307 antiserum prepared against cells heated at 100 C was detecting the acid-polysaccharide K87 antigen in our enterotoxic preparations. According to classical *E. coli* serology, antiserum prepared against heated (100 C) cells containing a B-type antigen such as K87 should not contain K antibody (4). On five different occasions, we prepared rabbit antiserum against P307 cells heated at 100 C and found that the higher the O antibody titer was, the more K antibody the serum contained. In our work on the development of the immunodiffusion assays for P307 and S340, no evidence suggesting that the enterotoxic component is antigenic was found. We hoped to extend our immunological assay to detect the enterotoxic component, but were unable to do so. In a recent study, Truszczynski and Ciosek (28) reported that enterotoxic *E. coli* preparations contained O and probably K antigen. These investigators also concluded that the enteropathogenic factor contained in the enterotoxic preparations was not antigenic.

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**LITERATURE CITED**

6. Finkelstein, R. A., P. Atthasampunna, M. Chulasa-


