Purification and Biological Characterization of Shiga Toxin from *Shigella dysenteriae* 1

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Shiga toxin has been purified in milligram quantities to near homogeneity from cell lysates of *Shigella dysenteriae* 1 strain 3818-0. Purification involved an initial ultracentrifugation, ammonium sulfate fractionation, chromatography on DEAE-cellulose and carboxymethyl cellulose, gel filtration, and preparative isoelectric focusing in sucrose gradients. The purified toxin was resolved by discontinuous polyacrylamide gel electrophoresis into a major cytotoxic protein band and a closely migrating, cytotoxic protease-nicked minor band. Antiserum generated by immunization with glutaraldehyde-inactivated toxin was shown to be monospecific against *S. dysenteriae* cell lysates. This highly purified toxin was cytotoxic to HeLa cells, enterotoxic in rabbit ileal loops, and lethal to mice. Monospecific antiserum to the toxin neutralized completely these toxin activities in both purified toxin preparations and crude shigella cell lysates.

*Shigella dysenteriae* 1, a causative agent of shigellosis, has been known for about 80 years to produce a protein toxin (6). The role of Shiga toxin in pathogenesis is still not understood. Nonetheless, involvement of toxin has been proposed both in the diarrheal (13) and in the invasive aspects of this disease (11). Bacterial invasion of the colonic epithelium is a recognized feature in the pathogenesis of shigellosis (15). We considered that further understanding of the role of toxin in disease required its purification and characterization.

Several biological activities have been associated with Shiga toxin. It is: (i) lethal to rabbits (20) and mice (25), (ii) cytotoxic to several cell lines (9, 12, 14, 26), (iii) enterotoxic in that it causes fluid accumulation in rabbit ileal loops (13), and (iv) able to inhibit cell-free protein synthesis in both mammalian (24) and bacterial (19) systems. Inhibition of protein synthesis appears to be the primary cytotoxic event in whole cells (2). Such inhibition of cell-free protein synthesis is apparently catalytic and involves an inhibition of the elongation phase of translation (3).

Olitsky and Kliger first demonstrated Shiga toxin to be distinct from endotoxin by using a combination of ion-exchange chromatography (18). These attempts provide microgram quantities of toxin, physically detectable only after radioiodination during the purification procedure.

We have purified Shiga toxin to near homogeneity in milligram amounts from cell lysates of *S. dysenteriae* 1 by using a combination of non-exchange chromatography, gel filtration chromatography, and preparative isoelectric focusing. A preliminary report of this purification procedure has been made (J. E. Brown, M. K. Gentry, D. E. Griffin, S. W. Rothman, W. J. Cabillane, B. P. Doctor, and M. R. Thompson. Abstr. Annu. Meet. Am. Soc. Microbiol. 1979, B42, p. 22). The results clearly demonstrate that all of the biological activities associated with Shiga toxin are present in the purified protein.

**MATERIALS AND METHODS**

Preparation of initial cell lysate. *S. dysenteriae* 1 strain 3818-0 (11) was grown and processed at the New England Enzyme Center to yield a lysate which served as the initial toxin extract of our purification procedure. The lysate was prepared as follows. A 500-liter fermentor containing modified syn case medium (2) was inoculated with a 1% inoculum, and bacterial growth was allowed to continue for 18 h at 37°C with maximum aeration. After the cells were harvested in a Sharples centrifuge (size 16), the cell pellet was suspended at 4°C in 1.5 parts of lysis buffer (50 mM Tris-hydrochloride–50 mM KCl–10 mM magnesium acetate [pH 8] containing 10 μg of phenylmethylsulfonyl fluoride per ml). The suspension was passed twice through a Manton-Gaulin model 15M8TBA laboratory homogenizer. Between passages the suspension was cooled on ice to 15°C. This suspension was centrifuged again in the Sharples centrifuge, and the resulting superna...
Several buffers were used during purification and characterization. These common buffers are referred to as follows. Buffer 10/80 consisted of 0.020 M Tris-hydrochloride (pH 8.0) containing 0.10 M NaCl; buffer 20/80 consisted of 0.020 M Tris-hydrochloride (pH 8.0) containing 0.25 M NaCl; buffer 10/80 consisted of 0.010 M sodium phosphate (pH 6.0) containing 0.10 M NaCl; buffer 10/30 consisted of 0.10 M sodium phosphate (pH 6.0) containing 0.35 M NaCl; buffer 10/80 consisted of 0.13 M glycine-NaOH (pH 8.0).

Cytotoxicity assay. Cytotoxic activity was used to monitor Shiga toxin enrichment during purification. The extent of HeLa cell detachment was employed as the indicator of cytotoxicity for Shiga toxin (12). HeLa cells (line CCL2; Flaw Laboratories, Rockville, Md.) were maintained at 35°C in growth medium consisting of Eagle minimum essential medium with Earle salts supplemented with 10% heat-inactivated fetal bovine serum, 2.0 mM glutamine, 1.0 mM penicillin per ml, and 0.18 mg of streptomycin per ml in a 5% CO₂ atmosphere (HEM Research, Rockville, Md.). To establish monolayers, freshly trypsinized cells were suspended at a concentration of 1.6 x 10⁶ cells per ml in growth medium, and 0.10-ml samples were dispensed into 96-well microtiter plates (Costar, Cambridge, Mass.). Cells were allowed to attach for 18 to 20 h before experimental use. Serial dilutions of samples were added (0.10 ml), and plates were incubated for an additional 18 h. The endpoint of toxin activity was determined by fixing and staining with crystal violet-formaldehyde solution. Stained cell monolayers were incubated sequentially in buffer containing BSA and antiserum (1:50), several washes to remove unbound antibody, buffer containing BSA and 125I-labeled protein A (4 x 10⁵ cpm/ml), and final washes to remove unbound 125I-protein A. Autoradiography was performed at -70°C with X-Omat AR film (Eastman Kodak Co., Rochester, N.Y.).

### TABLE 1. Purification of Shiga toxin from cell lysates

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Vol (ml)</th>
<th>Cytotoxicity/d (CD₅₀/ml)</th>
<th>Protein (mg/ml)</th>
<th>Sp act (CD₅₀/mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture broth</td>
<td>250,000</td>
<td>5.7 x 10⁴</td>
<td>0.46</td>
<td>1.2 x 10⁴</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Cell lysate</td>
<td>3,800</td>
<td>170 x 10⁴</td>
<td>2</td>
<td>3.3 x 10⁵</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Centrifugation, type 21 rotor</td>
<td>2,650</td>
<td>33 x 10⁴</td>
<td>28</td>
<td>1.2 x 10⁵</td>
<td>0.36</td>
<td>13</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 28 to 50% saturation</td>
<td>1,225</td>
<td>190 x 10⁴</td>
<td>56</td>
<td>3.4 x 10⁵</td>
<td>1.0</td>
<td>35</td>
</tr>
<tr>
<td>DEAE-Sephacel</td>
<td>3,230</td>
<td>60 x 10⁴</td>
<td>2.45</td>
<td>32 x 10⁵</td>
<td>9.7</td>
<td>39</td>
</tr>
<tr>
<td>CM-52</td>
<td>355</td>
<td>220 x 10⁴</td>
<td>1.0</td>
<td>110 x 10⁵</td>
<td>33.3</td>
<td>12</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ 70% saturation</td>
<td>18.4</td>
<td>4,600 x 10⁴</td>
<td>31.3</td>
<td>147 x 10⁵</td>
<td>44.5</td>
<td>13</td>
</tr>
<tr>
<td>Sephacryl S-200</td>
<td>59</td>
<td>90 x 10⁵</td>
<td>2.81</td>
<td>352 x 10⁵</td>
<td>107</td>
<td>8.9</td>
</tr>
<tr>
<td>Isofocusing, pH 5 to 8</td>
<td>24</td>
<td>980 x 10⁵</td>
<td>1.2</td>
<td>820 x 10⁵</td>
<td>250</td>
<td>3.6</td>
</tr>
</tbody>
</table>

*CD₅₀ is the amount of toxin activity required to reduce dye staining to half the control values, as described in the text.*
FIG. 1. DEAE-cellulose chromatography of toxin-containing ammonium sulfate fraction. Four columns were run simultaneously, each loaded with 17 g of protein. Elution was carried out as described in the text. The eluant was monitored for absorbance at 280 nm. Every sixth fraction was assayed for cytotoxicity in the HeLa cell microtiter assay (fivefold serial dilutions). Toxin-containing fractions, as indicated by the bar, were collected from each column and pooled for further processing. Fraction size was 12 ml.

Neutralizing activity was determined by measurement of that dilution of antiserum which would completely neutralize the cytotoxicity of a constant amount of Shiga toxin. Serial dilutions of antiserum were prepared and mixed with an equal volume of toxin solution. After incubation for 1 h at 37°C, 0.1 ml of each mixture was added to duplicate HeLa cell monolayers. After overnight incubation, the monolayers were fixed and stained as described above. Endpoints were determined visually and by measurement with the microtiter plate colorimeter. The endpoint was defined as that dilution of antibody which completely neutralized the toxin.
2. The dialyzed solution was divided into four equal portions of 300 ml and each was applied simultaneously to one of four separate DEAE-cellulose columns (DEAE-Sephacel; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated with 20T8/100 buffer. The columns were eluted with 20T8/100 buffer. A typical elution profile is shown in Fig. 1, with cytotoxic activity shown below the absorbance profile. Cytotoxicity was not detected in the initial absorbance peak, but was retained slightly and eluted as a broad band after the initially eluted protein. After elution of 6 column volumes, the buffer was changed to 0.25 M NaCl in 20T8 buffer. No further cytotoxin was detectable in this eluate. The toxin-containing fractions were pooled and dialyzed overnight against 10P6/50 buffer.

Neutralization of enterotoxic activity was tested by incubation of toxin-antiserum mixtures into ligated sections of rabbit ileum. Toxin-containing solutions were mixed with antiserum and incubated at 0°C for 30 min. Ileal loops were injected with 1-ml volumes. Toxin controls were diluted with normal rabbit serum or 20T8/100 buffer. Neutralization of mouse lethality was tested by intraperitoneal injection of toxin-antiserum mixtures. Toxin-containing solutions were mixed with antiserum and incubated at 0°C for 30 min. Mice were injected with 0.2-ml volumes. Diluted antiserum and 20T8/100 buffer alone were used as controls.

RESULTS

Initial enrichment. Cell lysates served as the starting material in our purification, since these lysates contained more total cytotoxic activity and more activity per milligram of protein than did culture filtrates (Table 1). Approximately 4 liters of thawed lysate was subjected to centrifugation at 20,000 rpm overnight in Beckman type 21 rotors. The supernatant fluid was collected, and ammonium sulfate (Ultrapure; Schwartz/Mann, Orangeburg, N.Y.) was added to 28% saturation. After 2 h at 4°C, the suspension was centrifuged in a GSA rotor (Sorvall) at 10,000 rpm for 30 min, and ammonium sulfate was added to the supernatant fluid to achieve 50% saturation. After 4 h, the precipitate was collected by centrifugation in a GSA rotor at 10,000 rpm for 60 min. The pellet was suspended and dialyzed at a flow rate of 3.5 ml/min. The dialyzed solution was divided into four equal portions of 300 ml and each was applied simultaneously to one of four separate DEAE-cellulose columns (DEAE-Sephacel; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated with 20T8/100 buffer. The columns were eluted with 20T8/100 buffer.

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DEAE-cellulose chromatography. Chromatography was performed in 15- by 5-cm columns at a flow rate of 3.5 ml/min. The dialyzed solution was divided into four equal portions of 300 ml and each was applied simultaneously to one of four separate DEAE-cellulose columns (DEAE-Sephacel; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated with 20T8/100 buffer. The columns were eluted with 20T8/100 buffer. A typical elution profile is shown in Fig. 1, with cytotoxic activity shown below the absorbance profile. Cytotoxicity was not detected in the initial absorbance peak, but was retained slightly and eluted as a broad band after the initially eluted protein. After elution of 6 column volumes, the buffer was changed to 0.25 M NaCl in 20T8 buffer. No further cytotoxin was detectable in this eluate. The toxin-containing fractions were pooled and dialyzed overnight against 10P6/50 buffer.

Carboxymethyl cellulose chromatography. Chromatography was performed using carboxymethyl cellulose (CM-52; Whatman Ltd., Clifton, N.J.) equilibrated with 10P6/50 buffer in a 9.6- by 5-cm column at a flow rate of 10 ml/min. The toxin-containing fraction recovered from DEAE-cellulose chromatography (>3 liters) was pooled, dialyzed, and applied to a single column. All cytotoxic activity was found to be retained by the column, whereas most (90%) of the protein was eluted (Fig. 2). After the column was washed with one sample volume of 10P6/50 buffer, the toxin was eluted with 10P6/350 buffer. The toxin-containing eluant was pooled, dialyzed, and ammonium sulfate was added to 70% saturation. After 4 h at 4°C, the precipitate was collected by centrifugation in a GSA rotor at 10,000 rpm for 60 min. The pellet was suspended and dialyzed at a flow rate of 3.5 ml/min. The dialyzed solution was divided into four equal portions of 300 ml and each was applied simultaneously to one of four separate DEAE-cellulose columns (DEAE-Sephacel; Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) equilibrated with 20T8/100 buffer. The columns were eluted with 20T8/100 buffer.
with 130G8 buffer overnight and centrifuged at 10,000 rpm for 30 min in a Sorvall SS-34 rotor to remove insoluble material.

**Gel filtration.** Sephacryl S-200 equilibrated with 130G8 buffer was poured into a column (3.3 by 95 cm). The flow rate was adjusted to 2 ml/cm² per h, and the concentrated toxin sample was applied. The elution profile (Fig. 3) indicates two major protein peaks. The cytotoxic activity eluted as a narrow band with the second absorbance peak. Calibration of this column with aldolase, hexokinase, BSA, ovalbumin, and myoglobin gave an Mₚ of 70,000 for the partially purified toxin. Those fractions containing cytotoxic activity were pooled for subsequent isoelectric focusing.

**Preparative isoelectric focusing.** Since glycine is isoelectric and does not disturb the pH gradient, the pooled sample (60 ml) from gel filtration was applied directly to an electrofocusing column. The toxin sample in 130G8 buffer was mixed into a 0 to 20% sucrose gradient (110 ml) containing 1.0% Ampholine (pH 5.5 to 8.5; LKB) in an LKB 8100 Ampholine column. Isoelectric focusing was carried out with constant voltage for 24 h at 10°C, after which current had dropped to 2 mA. Collected samples (2 ml) were assayed for cytotoxicity, absorbance (280 nm), and pH (Fig. 4). The toxin activity was found as a distinct peak at pH 7.2 in the pH gradient. No cytotoxic activity was observed elsewhere in the pH gradient. Toxin-containing fractions were applied to a Sephadex G-25 column (2.5 by 30 cm) equilibrated with 20T8/100 buffer to remove ampholytes and sucrose. Purified toxin was frozen at -70°C.

**Purity of the isolated protein.** The procedure described here provided 25 mg of purified Shiga toxin from approximately 4 liters of cell lysate. Cytotoxic activity was enriched about 250-fold at an overall recovery of about 3% (Table 1). Recovery and purification factor can vary considerably between batches, depending on starting toxin concentrations and on timely processing during the purification. To ascertain the homogeneity of the isolated toxin, discontinuous buffer PAGE was carried out in 5, 8, and 10% gel rods (Fig. 5A). In each case Coomassie blue staining showed two closely migrating protein bands. Densitometric analysis of the stained gel indicated that 82% of the protein was in the slow band and 17% was in the faster-migrating band. To determine which band possessed cytotoxic activity, companion gels were sliced into 1-mm slices which were eluted overnight for cytotoxicity assays. Each of the bands detected by protein staining showed cytotoxic activity. A slight contaminant, reflecting less than 1% of the staining intensity (RF = 0.51, 8% gel), did not contain cytotoxic activity. Other preparations of Shiga toxin could be separated by discontinuous PAGE into three or four closely migrating bands, suggesting that limited proteolysis of a single native toxin molecule might cause the multiple banding pattern observed. This possibility was tested by incubation of purified toxin with *Streptococcus griseus* protease (Fig. 5B). Clearly, the toxin is susceptible to proteolysis.

![Graph](image-url)
B. dysenteriae cell lysates. When assayed against purified toxin, the antiserum (0.1 ml) at a 1:8 dilution completely neutralized 780 ng of toxin protein. The preimmune serum did not neutralize cytotoxicity (data not shown). To assess the specificity of the antiserum, immunochemical analysis was performed using a Western blot immunoblot technique (4). Toxin samples were separated by PAGE, transferred to nitrocellulose, and analyzed with serum collected at 0 and 6 weeks (Fig. 7A). As early as 2 weeks post-immunization, specific antibody was detected (data not shown). At 6 weeks post-immunization, the antibody response had increased dramatically (lanes 4 and 8): both of the closely migrating toxin bands observed by Coomassie blue staining are readily detectable. The slight contaminant in the preparation can be detected when the autoradiograph is overexposed (lane 8). To determine whether this antiserum could be employed as a monospecific reagent, the banding pattern detected for pure toxin was compared with that of S. dysenteriae cell lysates (lanes 3 and 7). The two closely migrating toxin bands were readily detectable in cell lysates. However, several other protein bands reacted with the immune serum. With the exception of one band, these proteins in the cell lysate were also detectable with preimmune serum (lane 5). Serum from a number of other unimmunized rabbits was examined by this immunoblot technique. In each serum, antibodies against various bacterial cell lysate proteins could be detected (data not shown). Therefore, the antibodies to these additional proteins were not generated by immunization with purified toxin. The one unidentified protein (Rf = 0.32) detected in cell lysates by immune serum migrated to the same position as the fastest moving of the three toxin species generated by treatment of purified toxin by S. griseus protease (Fig. 7B). Therefore, this band appears to be a proteolytic product of the native toxin present in the cell lysate. Thus, when tested against S. dysenteriae cell lysates, this antiserum generated by immunization with purified toxin behaved as a monospecific reagent.

Toxicity of purified Shiga toxin. Purified toxin was assayed for enterotoxic activity and mouse lethality. The results in Table 2 demonstrate that purified Shiga toxin possesses all three biological toxicities of shigella lysates. In the cytotoxicity assay, toxin caused 50% cell detachment at doses in the range of 1 pg per well. Fluid accumulation in rabbit ileal loops was observed consistently at a dose of 1 μg of toxin per loop. To obtain a value indicating a 50% dose level, we defined a 50% enterotoxic dose so that the data could be analyzed by the method of Reed and Muench (22). A response was considered...
FIG. 6. Cytotoxicity neutralization by rabbit antiserum. The curves on the left demonstrate cytotoxicity assays of purified toxin (■) or crude cell lysates (■■). Purified toxin was adjusted to 7.8 μg/ml, and cell lysates were adjusted to 720 μg/ml. Each was serially diluted (fivefold) with medium, and 0.05-ml volumes were applied to cell monolayers. The curves on the right are neutralization assays with rabbit antiserum collected 6 weeks post-immunization. In these assays, 0.13 ml of serially diluted (twofold) serum was mixed with an equal volume of purified toxin (○) or crude lysate (●); 0.1 ml of this mixture (390 ng of pure toxin or 36 μg of lysate protein) was applied to each HeLa cell monolayer. Values are the mean of three determinations with the standard deviations indicated by the bars.

positive if fluid accumulation was >0.5 ml/cm of the loop. By this procedure, purified toxin had a 50% enterotoxic dose value of 0.02 μg. For cell lysates, the 50% enterotoxic dose value was 2 μg. In addition to its enterotoxicity, purified toxin was lethal to mice when injected intraperitoneally. The 50% lethal dose was 0.2 μg, whereas heat-inactivated toxin (100°C, 30 min) was not lethal as high as 0.9 μg. In contrast, crude cell lysates had a 50% lethal dose of 2 μg, whereas heat-inactivated lysate (0.6 mg) was not lethal.

Neutralization of biological activity. Monospecific antibody for purified Shiga toxin was used to determine whether the toxic activities observed in cell lysates of S. dysenteriae were due solely to the presence of the protein purified here. This antiserum (13 μl) neutralized the cytotoxic activity of cell lysates (36 μl) as shown in Fig. 6. Furthermore, the antiserum neutralized enterotoxic activity and mouse lethality of both purified toxin and cell lysates. Antisera (0.75 ml) prevented fluid accumulation in rabbit ileal loop segments treated with 5.0 μg of purified toxin; for cell lysates, 0.20 ml of antisera prevented fluid accumulation in loops injected with 60 μg of lysate protein. In mice, antiserum (0.20 ml) prevented lethality of 0.90 μg of purified toxin injected intraperitoneally in five of five animals. For cell lysates, 0.15 ml of antiserum neutralized lethality of 10 μg of crude lysate (five of five mice). In the absence of antiserum or in the presence of normal rabbit serum, the amounts of either toxin or cell lysate used were strongly toxic in both the ileal loop and the mouse lethality assay.

DISCUSSION

Through the use of gentle biochemical techniques, we have developed a procedure which allows recovery of milligram amounts of purified Shiga toxin from bacterial lysates. As shown in Fig. 5, purified toxin behaved as a near-homogeneous preparation, containing 1% contaminant. Although our procedure should minimize chemical or biological insults to the purified protein, the toxin occasionally displayed multiple bands after PAGE. Limited proteolysis during purification is the most likely explanation of this behavior since the treatment of purified toxin with proteases also yielded multiple species. Additional support for the purity of the toxin was obtained by the observation that monospecific
Antiserum was produced when toxin was used to immunize rabbits.

Previous reports have suggested that the toxic activities of Shiga toxin extracts can be resolved into different proteins. On the basis of our findings, this conclusion is unlikely. As shown in Table 2, the purified protein possessed all of the biological activities attributed to Shiga toxin. The protein displayed cytotoxicity at picogram levels, enterotoxicity at nanogram levels, and was lethal to mice in submicrogram quantities when injected intraperitoneally. Furthermore, all three activities were completely neutralized by monospecific antiserum. Cell-free protein synthesis inhibition was also neutralized by immunoglobulin isolated from the antiserum by protein A-Sepharose chromatography (unpublished observation). In addition, monospecific antibody was able to neutralize all toxic activities of crude cell lysates, including inhibition of cell-free protein synthesis. It is unlikely therefore that such cell lysates contain other toxins possessing the classic toxicities observed in Shiga toxin extracts. Our conclusion is supported by the finding that the protein purified by O'Brien et al. also contained all of the toxic activities of Shiga toxin (18).

Our observations indicate that the Shiga toxin purified here differs in some respects from toxins isolated by others. When subjected to preparative isoelectric focusing during purification, the toxin focused as a sharp peak at pH 7.2. In contrast, the material obtained from culture filtrates by McLver et al. contained two toxic moieties that were resolved by preparative isoelectric focusing (17). Both of these were cytotoxic, but one had no enterotoxicty or mouse lethality. The purity of their preparation, however, was not established. The cytotoxin purified by Olsnes and Eiklid appeared to be homogeneous, but showed a rather broad isoelectric distribution, focusing between pH 6 and 7 when
TABLE 2. Biological properties of Shiga toxin

<table>
<thead>
<tr>
<th>Prep</th>
<th>Cyto-</th>
<th>Entero-</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>toxicity*</td>
<td>toxicity*</td>
<td>lethality*</td>
</tr>
<tr>
<td></td>
<td>(CD$_{50}$)</td>
<td>(ED$_{50}$)</td>
<td>(LD$_{50}$)</td>
</tr>
<tr>
<td>Cell lysate</td>
<td>4 x 10$^6$</td>
<td>1 x 10$^7$</td>
<td>5 x 10$^5$</td>
</tr>
<tr>
<td>Purified toxin</td>
<td>2 x 10$^6$</td>
<td>5 x 10$^7$</td>
<td>6 x 10$^6$</td>
</tr>
</tbody>
</table>

* Fivefold serial dilutions of toxin were assayed for cytoxicity in triplicate HeLa cell monolayers. After the cells were fixed and stained the dilution causing 50% detachment (CD$_{50}$) was determined from a logarithmic plot of the mean of absorbances of retained dye versus dilution of toxin.

Fluid accumulation of at least 0.5 ml per cm of loop was considered a positive enterotoxic response. Each amount of toxin was assayed in at least three doses. The dose producing a positive response in 50% of loops tested (ED$_{50}$) was calculated by the method of Reed and Muench (22).

Mouse lethality was determined by intraperitoneal injection of each dose into five or more mice. The 50% lethal dose (LD$_{50}$) was calculated by the method of Reed and Muench (22).

analyzed with sucrose gradient isoelectric focusing (21). Enteroxicity and mouse lethality were not tested. The toxin isolated by O'Brien et al. contained all three classical toxicities of Shiga toxin, but molecular weight estimates by Sephacryl S-200 gel filtration gave an $M_r$ of 33,000, suggesting to the authors that dissociation may have occurred during elution at the affinity chromatography step (18). The toxin specified here gave an estimated $M_r$ of 70,000 in the process of purification on Sephacryl S-200, in closer agreement with that of Ofsnes and Eiklid (21). In all cases, previous attempts have produced only microgram quantities of purified toxin.

Toxin purified as described here has been used to establish the biochemical basis of Shiga toxin action. Our studies of toxin action in cell culture have shown that inhibition of protein synthesis is the primary event (22). Additional studies with cell-free protein synthesis indicate that Shiga toxin may function enzymatically through an inhibition of peptide elongation, whereas no evidence of ADP-ribosyl transferase activity was found (3). Preliminary data suggest that the toxin inhibits the amino-acyl tRNA binding stage of peptidyl elongation (J. E. Brown and M. Ussery, Fed. Proc. 40:1580, 1981). This apparently occurs by inactivation of the aminoacyl-tRNA synthetase (23).

The precise role of Shiga toxin in pathogenesis remains unclear. Inhibition of protein synthesis appears to be the basis of all recognized toxic properties of Shiga toxin. The sequence of events leading to pathological sequelae of toxin activity such as fluid secretion in rabbit ileal loops or lethality to mice remains to be elucidated.

Furthermore, in light of our findings, previous reports of electrolyte secretion (8), nonelectrolyte secretion (1), and intestinal adenylate cyclase activation (5) require reevaluation with purified toxin. With the ready availability of purified Shiga toxin, it will be possible to define carefully its biochemical properties and relate these to its function in pathogenesis.

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We thank P. Genski for critical advice, continued interest, and extensive discussions during the course of this work. We thank H. Collins for skilled animal surgery. Excellent technical assistance was provided by M. K. Gentry, M. Barreiro, A. Stone, M. Alvarado, and W. Cahillane.

LITERATURE CITED

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SHIGA TOXIN PURIFICATION AND CHARACTERIZATION

Purification and characterization of Shiga toxin from Shigella dysenteriae 1

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Toxin, Shigella, dysentery, purification, protein synthesis

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