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Partial Purification and Characterization of a Heat-Labile Enterotoxin of *Escherichia coli*

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A partially purified enterotoxin was obtained from the growth medium of *Escherichia coli* strain 711 (P307), a derivative of *E. coli* K-12, by ultrafiltration, precipitation with ammonium sulfate, molecular sieving, and anion exchange column chromatography. The active moiety, which is heat-labile, behaved like a protein particle of 180,000 to 200,000 daltons during molecular sieving and ultracentrifugation. During polyacrylamide gel electrophoresis in sodium dodecyl sulfate (SDS-PAGE), it dissociated into two subunits with apparent molecular weights of 68,000 to 70,000 and 14,000 to 15,000. SDS-PAGE after heating in SDS changed the larger subunit to an apparent molecular weight of about 40,000; the smaller subunit did not change. The intact particle induced rounding of the cells in Y-1 mouse adrenal tumor cells used for assay. The detergent-dissociated molecules were not active. Proteolysis of the purified toxin by tolylsulfonyl phenylalanyl chloromethyl ketone-trypsin appeared to enhance its activity. The addition of serum to the assay medium resulted in partial depression of the activity. Activity was also abolished by preincubation of the toxin with either a rabbit antiserum to it or solutions containing GM_1 ganglioside. The length of time needed to evoke a response in the assay system by fractions from different stages in the purification of the enterotoxin was a useful parameter in the evaluation of specific activity.

Two types of enterotoxin have been found to be produced by strains of *Escherichia coli*, one heat labile (LT) and the other heat stable (ST) (9, 21-23). Genes for the control of formation of these toxins are located on plasmids, called Ent plasmids. The genetics of one such plasmid, Ent P307, which carries genes for LT and ST, is being studied (10, 20). This plasmid was first described in strain P307, isolated from a case of porcine diarrhea, and was transferred by S. Falkow into strain 711, a genetically marked derivative of *E. coli* (10). The strain carries only one plasmid, Ent P307, making it a favorable candidate for the isolation and study of LT.

Several papers on the purification of LT have been published (2, 5-7, 13, 16, 24), but the results obtained so far have been variable with regard to chemical composition and molecular weight. There is agreement that LT is a protein.

We have developed a simple and reproducible procedure for the isolation of LT from *E. coli* K-12, strain 711 (P307). The method gives in good yield a toxin of apparent high purity and specific activity. The material should prove useful for further investigation of the toxin, especially for studies dealing with the genetics of toxin production. Here we describe some chemical, immunological, and biological characteristics of the material.

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MATERIALS AND METHODS

Source. Crude enterotoxin was prepared by the Upjohn Co., Kalamazoo, Mich., from a 250-liter culture of strain 711 (P307) grown for 18 h at 37 C in Evans medium. Ten hours after the start of growth, the pH of the medium had dropped from an initial value of 7.4 to 7.1 and was brought back to 7.4 with NaOH. After 18 h, the culture was cooled to 10 C and centrifuged. The supernatant was filtered under pressure through a membrane with a cut-off point of about 50,000 molecular weight to reduce the volume to 25 liters. This material was then freeze-dried (80 g, dry weight), packed in dry ice, and shipped to our laboratory. We are very grateful to James Punch of the Upjohn Co. for his cooperation, and to that company for the gift of crude material.

Bioassay. Fractions were assayed for enterotoxic activity in the Y-1 mouse adrenal tumor cells system of Donta et al. (2). Cells were maintained in culture in 250-ml Falcon flasks with F-10 (Ham) medium (GIBCO) supplemented with 16% horse serum and

2.5% fetal calf serum at 37 C in a humidified atmosphere of 95% air and 5% CO_2. For the purpose of assay, cells were washed three times with Puck saline and treated for 10 min with 4 ml of trypsin (0.5 g/liter)-ethylenediaminetetraacetic acid (0.2 g/liter) to detach them from the monolayer. The cell suspension was then diluted to a concentration of 10,000 cells/ml and plated (1 ml/well) in Falcon microtest plates. After 3 to 4 days, the cells reached 30 to 50% of confluency and were ready for use.

For most assays, culture medium was replaced with 1 ml of fresh medium containing 0.1 ml of the fraction to be tested (final concentration of test protein, 1 to 100 μg/ml). Plates were then incubated under growth conditions and observed at hourly intervals for 8 to 10 h. Scoring was as percentage of cells rounded. For dose-response tests, enterotoxin preparations were serially diluted in medium before assaying. For studies of the apparent binding of the toxin to the cell, the cells were incubated with toxin, which was then removed by aspiration of the medium at short time intervals (0 to 15 min). The cells were then washed twice and reincubated with toxin-free medium. Activity was scored as above.

Polyacrylamide gel electrophoresis (PAGE). Samples containing 0.3 mg of protein per ml were prepared by overnight dialysis against 1% sodium dodecyl sulfate (SDS)-0.1 M sodium phosphate, pH 7.0. Aliquots (250 μl) in 20% glycerol were then layered onto gels (120 by 6 mm) and electrophoresed at 15 mA per gel until the marker dye (bromophenol blue) had run 7.5 cm. Gels were stained in amido Schwarz (0.5% in 7% acetic acid-15% methanol) and/or methylene blue (0.2% in 0.4 M sodium acetate-acetic acid buffer, pH 5.3). Standards of bovine serum albumin and egg white lysozyme were run in parallel, and molecular weights were calculated by plotting relative mobility versus log molecular weight (15).

Two types of gel systems were used: (i) 5% acrylamide-0.2% SDS-0.5 M urea, and (ii) 7.5% acrylamide-0.2% SDS. For both, the running buffer used was 0.1 M sodium phosphate (pH 7.0)-0.2% SDS. The effect of boiling (15 min at 100 C in 0.2% SDS) on the mobility of both standard proteins and the purified enterotoxin was studied in the 5% gel system. Destaining of all gels was for 2 to 3 days in 7% acetic acid-15% methanol for amido Schwarz-stained gels and in distilled water for methylene blue-stained gels.

Immunodiffusion and immunoelectrophoresis. Immunodiffusion studies were done in commercial Ouchterlony plates (Hyland), using rabbit antisera (see below). Immunoelectrophoresis slides were prepared with 1% Noble agar in the running buffer, sodium acetate-sodium barbital, pH 8.6 (3.2 g of sodium acetate, 5 g of sodium barbital, 32 ml of 0.1 N HCl per liter). After running for 2 h at 4 mA per slide, antiserum was added to the troughs. Slides were incubated overnight at room temperature and then at 4 C.

Antisera. Antisera to both crude and purified toxin were raised in rabbits by footpad injection (0.1 ml/pad) of the fractions (containing approximately 1 mg of protein per ml) in Freund complete adjuvant (1:1, vol/vol). Animals were boosted after 1 month and bled at approximately 1-month intervals.

Neutralization studies with these antisera were done by incubating 20 μg of purified toxin in 100 μl of culture medium with serial dilutions of the antisera for 30 min at room temperature. At the end of incubation, 0.8 ml of culture medium was added and the samples were assayed.

Digestion with trypsin. Purified toxin preparations (10 mg/ml) were brought to pH 8.0 by the addition of 0.25 ml of 0.1 M $NaHCO_3$ with a trace amount of phenol red to serve as an internal indicator. Tolysulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (700 μg/ml) was added to 2% the amount of toxin (wt/wt). Incubation was for 16 to 18 h, either at room temperature or at 37 C.

Suitable aliquots of tryptic digests were chromatographed for 20 h on Whatman 3MM paper with a butanol-acetic acid-water-pyridine (7.5:1.5:6:5) solvent system. High-voltage electrophoresis (3,000 V, 150 mA) was carried out in the second dimension for 1.5 h in 0.1 M pyridine-acetate buffer at pH 3.5. Spots were visualized with ninhydrin reagent (3% in acetone) or by first treating the paper with 0.1 M sodium borate-boric acid, pH 8.5, followed by spraying with Fluram (fluorescamine, Roche Diagnostics), 30 mg/100 ml in dry acetone. The Fluram peptides were located under ultraviolet light.

Amino acid analysis. Suitable aliquots were hydrolyzed for 21 h in 6 N HCl (constant boiling) under reduced pressure at 110 C and were analyzed on a Beckman 120C automatic analyzer.

Ultracentrifugation. Runs were done in a Spinco model E analytical ultracentrifuge at 52,000 rpm at 20 C. The partial specific volume ($\bar{V}$) was assumed to be 0.74 for the calculation of molecular weights.

Protein. Protein was determined by calculation from the absorbances at 280 and 260 nm (17) or by the method of Lowry et al. (18).

Molecular sieving. Molecular weights were estimated from the relevant elution volumes obtained on a column of Sephadex G-200 (40 by 2.5 cm) equilibrated with 0.01 M tris(hydroxymethyl)aminomethane (Tris), pH 7.5. Bovine gamma globulin, bovine serum albumin (BSA), egg white lysozyme, and tosyl arginine-methyl ester (TAME) served as standards for the plot of elution volume versus molecular weight.

Heat, acid, and base treatment. Toxin in 0.01 M Tris, pH 7.5, was heated at various temperatures for 10 to 20 min in a water bath, allowed to cool, and assayed. For studies of lability to acid or base, 0.1 ml of either 12 N HCl or 12 N NaOH was added to purified toxin (0.2 mg in 0.1 ml); the mixtures were allowed to stand at room temperature for 1 h, neutralized, and assayed.

Neutralization by GM_1 ganglioside: Toxin (20 μg in 100 μl of Tris buffer [0.01 M, pH 7.5]) was incubated for 30 min under sterile conditions at room temperature with 100 μl of a solution containing GM_1 ganglioside in 0.1 M Tris (pH 7.5)-0.2% gelatin. At the end of the incubation, 0.8 ml of culture medium was added and the samples were assayed.

Final concentrations of the ganglioside ranged from 30 μg to 10 ng per assay.

RESULTS

Purification. The purification of LT proceeded from the lyophilized material that had been stored in the cold (Materials and Methods). Two to 10 g was taken up in 200 to 300 ml of distilled water. The mixture was brought to 90% saturation with respect to ammonium sulfate by the slow addition of the solid salt with stirring at room temperature. After standing at 2 to 4 C for 12 to 48 h, the copious precipitate was collected by centrifugation at 10,000 $\times$ g for 1 h in the cold. The pellet was taken up in water to a protein concentration of 10 to 40 mg/ml and dialyzed exhaustively against water in the cold. The biological activity of this material and that of each of the subsequent steps was monitored by the adrenal tumor cell assay. In previous pilot experiments, it had been ascertained that there was no biological activity in either the membrane filtrate of the culture supernatant or in the ammonium sulfate supernatant. A suitable aliquot of the dialysate (about 50 ml for a column of dimensions 5 by 80 cm) was loaded onto a column of Sephadex G-200 equilibrated with 0.01 M Tris, pH 7.5, and filtered slowly (0.2 to 0.5 ml/min). The material containing biological activity emerged at the V_0 in a fairly sharp and symmetrical peak (Fig. 1). It was pooled and added to a column of diethylaminoethyl (DEAE)-Sephadex A-25, thoroughly equilibrated with 0.01 M Tris, pH 7.5. A stepwise gradient of NaCl in the buffer was applied after running the original eluant for 2 to 3 column volumes. The material emerging with 0.5 M NaCl had the biological activity (Fig. 2). On occasion there were two poorly separated peaks instead of one. The reason for this is not clear. The biologically active material was pooled, dialyzed, and stored after lyophilization. A marginal further increment in specific activity could be obtained by filtering the 0.5 M NaCl material through a column of Sephadex G-75 in the same buffer; however, significant losses of biologically active material resulted.

Yields. The yields from this procedure are shown in Table 1. It can be seen that the 0.5 M NaCl DEAE material represented 10% of the protein in the G-200 material and 1% of the protein of the starting material. Protein concentrations, calculated either by the Lowry method or from the absorptions at 280 and 260 nm using the Layne equation, can only be considered as estimates since the active fraction retained a high 260-nm absorption value throughout the purification steps. This reduces the accuracy of the determination by either method.

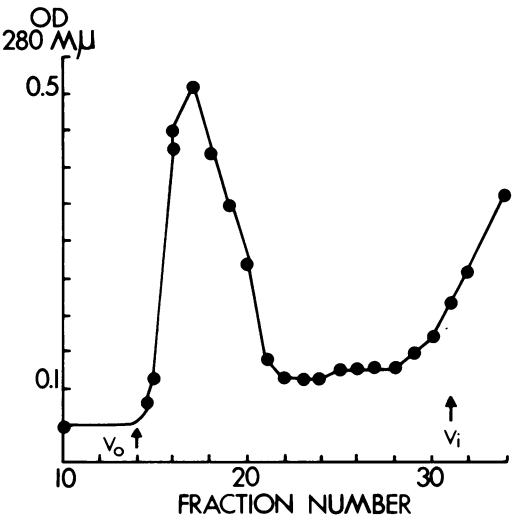

FIG. 1. *Optical density (OD) profile at 280 nm of the relevant fractions from a column of Sephadex G-200 equilibrated with 0.01 M Tris buffer at pH 7.5. The material loaded was an aliquot of the dialysate of the 90% ammonium sulfate step.*

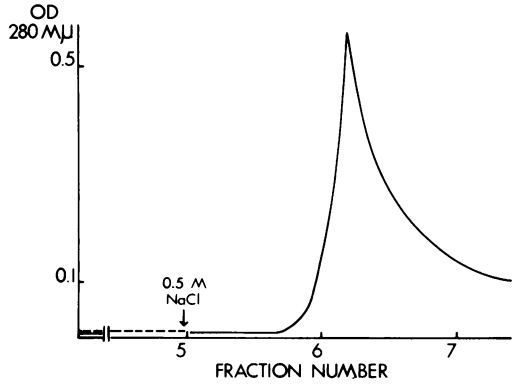

FIG. 2. *Optical density (OD) profile at 280 nm of the fractions eluted with 0.5 M NaCl from a column of DEAE-Sephadex that had been loaded with the biologically active fraction from the Sephadex G-200 column. The elution program consisted of 0.01 M Tris buffer, pH 7.5, followed by a stepwise gradient of 0.1 and 0.5 M NaCl in the buffer.*

Characteristics. (i) Heat and acid-base lability. Exposure of the purified toxin to 60 C for 10 min abolished all biological activity. Sixty to 80% of the activity was lost upon keeping the material at 37 C for 12 h. The crude lyophilized powder appeared to be stable in the cold (2 to 4 C) for at least 6 months. The lyophilized purified toxin was stable for 6 to 8 weeks under these conditions; however, in solution (0.01 M

Tris, pH 7.5), the biological activity slowly decayed even when stored in the cold. Exposure to acid or base for 1 h (see Materials and Methods) resulted in total loss of activity.

(ii) **Ultracentrifugation.** Ultracentrifugation was done at 2.5 and 8.0 mg/ml. At 20 C the *S* values were 10.1 and 10.3, respectively. The single Schlieren peak was symmetrical but showed some trailing, indicating some polydispersity with higher-molecular-weight material (Fig. 3). Assuming a value for the partial specific volume of 0.74, the calculation yielded a molecular weight in the range of 180,000 to 200,000.

PAGE. PAGE in SDS was performed at all stages of purification in order to detect the number of components and obtain an estimate of their molecular weights. The multiplicity of bands in the crude fraction was reduced to two in the purified material. Extensive dialysis of the preparations against SDS (1% in buffer) was found to be necessary for clear separation of these components.

TABLE 1. *Yields of protein during purification of LT*

Purification step	Protein (mg)
Amicon retentate	8,000
Ammonium sulfate	3,800
Sephadex G-200	800
DEAE	80–100
G-75	45–50

In both the 5% and 7.5% gel systems, two stainable bands could be seen with the purified fraction. From their position, the apparent molecular weights of 68,000 to 72,000 and 14,000 to 16,000, respectively, could be calculated (Fig. 4a). In all cases some amido Schwarz-staining material remained at the top of the gel. The lower-molecular-weight band also stained heavily with methylene blue, possibly indicating the presence of an intrinsic or contaminating nucleic acid-like component (18).

Using the 5% gel system, we examined the effect on the apparent molecular weight of boiling the toxin in 0.2% SDS before the electrophoretic run. Marker proteins (BSA and lysozyme) underwent the same treatment. The results showed no measurable difference in mobility of either BSA or lysozyme. However, the larger of the two bands from the purified toxin showed a significantly higher mobility, from which a mo-

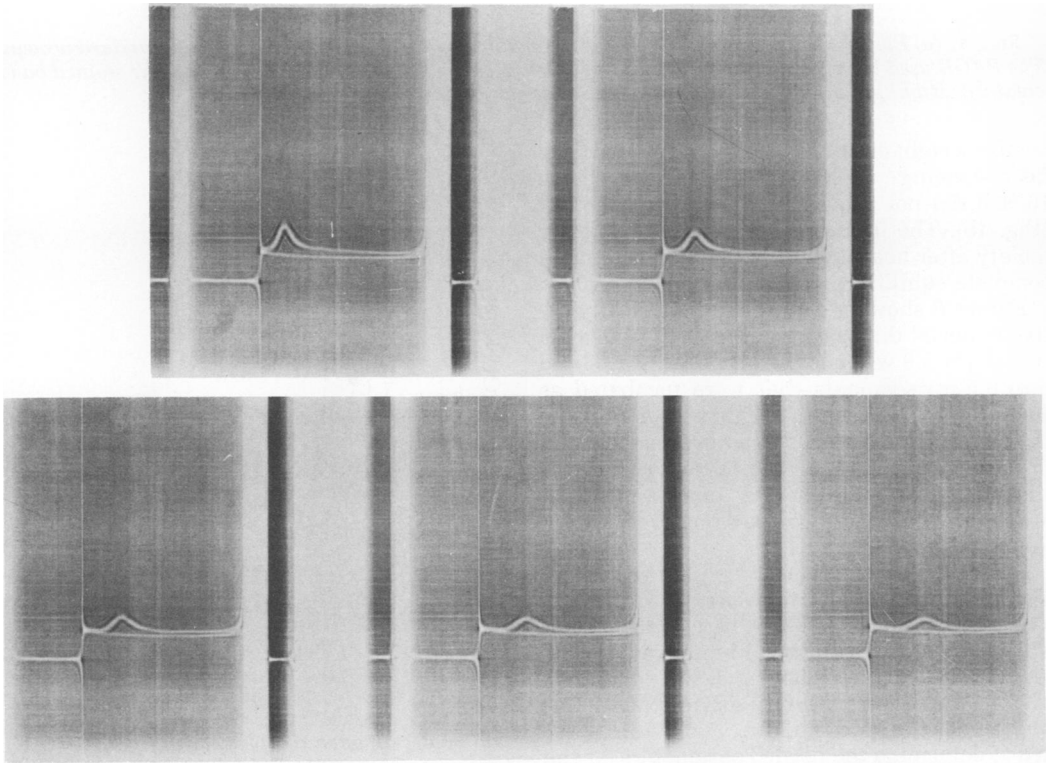

FIG. 3. *Ultracentrifugation of the DEAE 0.5 M NaCl fraction at a concentration of 2.5 mg/ml. Sedimentation is from left to right.*

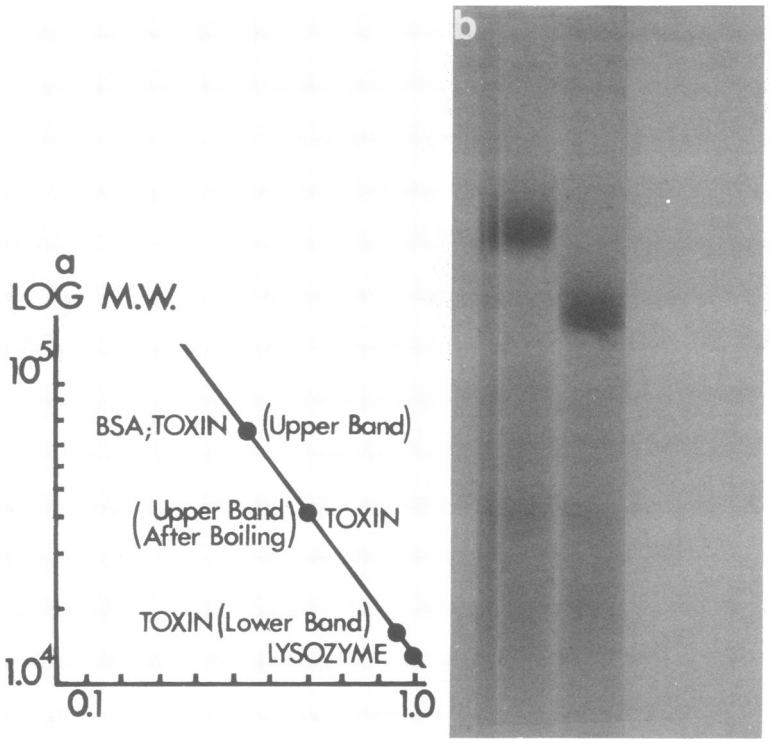

FIG. 4. *(a) Plot of relative mobility versus log molecular weight of the purified toxin and standard proteins. The PAGE used the 5% gel system described in Materials and Methods. (b) Photograph of the stained bands obtained. Right gel: After boiling in 0.2% SDS; left gel: before boiling.*

lecular weight of 40,000 could be estimated. The faster-moving component (e.g., 14,000 to 16,000) did not show any changes in mobility (Fig. 4b). The increased mobility of the larger moiety after heating was probably due to more complete equilibration with SDS.

Figure 5 shows a plot of the biologically active material that could be recovered from a gel run at pH 9.0 without SDS. The gel was sliced into 0.5-cm segments that were incubated as such in the usual assay. It can be seen that the biological activity was spread over some three to four segments. Staining of gels run under similar conditions showed one single but broader-staining zone in the same area (five to seven segments).

Gel filtration. Twenty milligrams (dry weight) of DEAE material was filtered through a column of Sephadex G-200, using 0.01 M Tris, pH 7.5, as eluant. Bovine immunoglobulin G (molecular weight, 165,000), BSA (molecular weight, 68,000), egg white lysozyme (molecular weight, 14,000), dextran blue, and TAME were ued to determine the needed parameters for the estimation of molecular weight. Figure 6 shows the elution pattern. The purified toxin eluted in

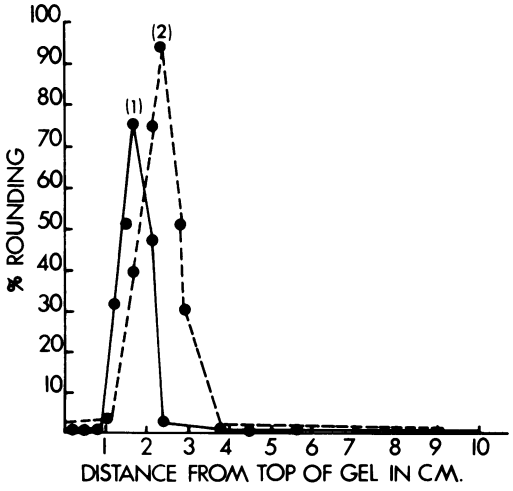

FIG. 5. *Plot of biologically active material from DEAE material run in the 5% acrylamide gel system without SDS. The solid line represents the purified toxin, and the dotted line represents the position of that material after treatment with TPCK-trypsin. Gels were sliced into 5-cm segments and used as such. (1) Purified toxin; (2) purified toxin after trypsin treatment.*

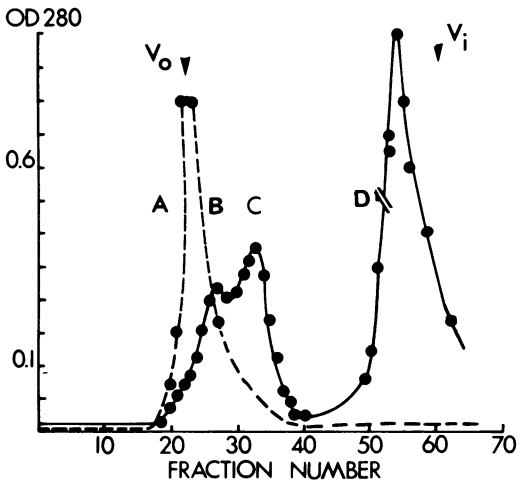

FIG. 6. *Gel filtration of the purified toxin and standard proteins on a column of Sephadex G-200. A, Toxin; B, bovine immunoglobulin G; C, BSA; and D, egg white lysozyme. Dextran blue (V_0) and TAME (V_i) are indicated by arrows. OD280, Optical density at 280 nm.*

the void volume, indicating a molecular weight on the order of 180,000 to 200,000.

Since estimates of molecular weight by SDS-PAGE (see above) had given two stainable bands with values of approximately 70,000 and 15,000 daltons, respectively, it became of interest to examine the effect of detergents on purified toxin in gel filtration experiments. Three to 5 mg of the purified toxin was dissolved in 3 N guanidinium hydrochloride. (Suitable amounts of dextran blue, BSA, and lysozyme served as markers.) The mixture was filtered as above through a column of Sephadex G-200 that had been fully equilibrated with 3 N guanidinium hydrochloride. Examination of the eluates (1-ml fractions) by double immunodiffusion with antisera to either the crude or purified toxin revealed that the toxin emerged around the V_0 of the column, indicating that under these conditions no significant change in molecular weight had taken place.

Another aliquot (4 mg) of purified toxin was taken up in 1 ml of 0.01 M Tris (pH 7.5)-0.2% SDS solution and filtered through a similar column that had been equilibrated with 0.01 M Tris-0.2% SDS. Immunological examination of fractions obtained in this case showed that some of the material had emerged around the V_0 of the column but that a substantial amount had undergone a change in molecular weight, since it coeluted with the BSA added as molecular weight marker.

Amino acid analysis. The amino acid composition of the toxin was determined on several different preparations. The molar yields and calculated residues are given in Table 2. It can be seen that the numbers of residues per mole are close to integers for most residues. Tryptophan was not determined. Remarkable is the total absence of half-cystine and methionine, though this has been reported previously for other exoproteins from *E. coli* (19) as well as more recently for the exfoliative toxin from *Staphylococcus aureus* (14). The absence of cysteine or cystine was confirmed by subjecting the material to exhaustive reduction and alkylation with [^{14}C]iodoacetamide (8). Filtration of the treated material through a column of Sephadex G-25 indicated a virtual quantitative recovery of the toxin at the V_0, devoid of radioactivity, that was recovered in high yield in the V_i of the column (Fig. 7).

The sum of acidic residues (Glu and Asp) was 16; the sum of basic residues (Lys and Arg) was 7. This confirms our observation with isoelectric precipitation (unpublished experiments), which indicated the pH of 3.5 to 4.5 to be one of poor solubility; it is also consistent with two runs using isoelectric focusing that resulted in precipitation of the bulk of the material in the acidic portion of the sucrose-stabilized ampholine gradient.

Tryptic digests. We studied the effect of TPCK-trypsin on the purified toxin by following the production of peptides and by monitoring biological activity. Peptide mapping, as well as high-voltage paper electrophoresis, showed the appearance of six to seven peptides

TABLE 2. *Amino acid composition*

Amino acid	Amt (nmol)	No. of residues
Lysine	6.372	4.01
Histidine	1.570	1.00
Arginine	4.519	2.92
Aspartic acid	14.180	9.03
Threonine	6.822	4.34
Serine	7.803	4.97
Glutamine	11.230	7.10
Proline	4.10	2.68
Glycine	12.631	8.07
Alanine	12.910	8.20
Half-cystine	Not detected	
Valine	6.326	4.02
Methionine	Not detected	
Isoleucine	3.654	1.97
Leucine	6.622	4.01
Tyrosine	Estimate	2
Phenylalanine	Estimate	3
Tryptophan	Not determined (acid hydrolysis)	

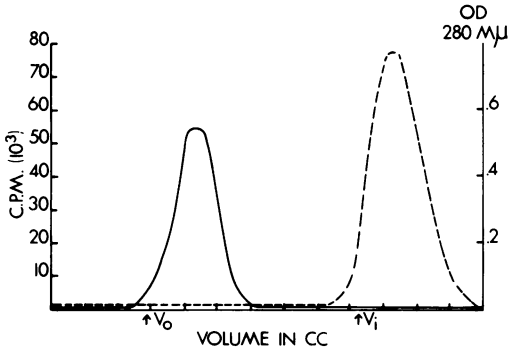

FIG. 7. *Filtration on a column of Sephadex G-25 of toxin that had undergone reduction with dithiothreitol, followed by alkylation with [¹⁴C]iodoacetamide. The solid line represents optical density (OD) at 280 nm. The dotted line gives counts per minute of suitable aliquots counted in a Beckman L-250 scintillation counter.*

not present in the undigested toxin. Visualization of the peptides was done by staining with ninhydrin or Fluram. A schematic diagram of the paper electrophoresis is shown in Fig. 8.

Assay of the whole tryptic digest, to our surprise, showed a significant increment in biological activity, i.e., shortening of the lag period for attainment of 50% rounding of cells (see above). Neither trypsin alone nor trypsin added to the enterotoxin preparation at the time of assay showed this effect. Work is presently in progress that is designed to isolate the tryptic peptides and assay them singly or in combination. It should be mentioned here that the tryptic peptide map showed a large ninhydrin-positive spot that stayed at the origin, i.e., did not move during chromatography or electrophoresis on paper, a feature well known for large peptides. This might indicate that a core protein was present which, under the conditions for digestion used, was not attacked by trypsin, yet retained high biological activity. Possibly supporting this idea was the observation (see Fig. 5) that both the staining pattern and the elution of active material from an acrylamide gel (run without SDS) of a tryptic digest of purified toxin showed a pattern similar to that of the original material, although with a slightly higher mobility toward the positive electrode.

Immunology. The purified toxin gave a single arc in immunoelectrophoresis when challenged with antisera against crude or purified material (Fig. 9a), though the purified antigen sometimes showed a higher mobility. Ouchterlony double-immunodiffusion studies of crude and purified toxin showed multiple precipitin lines with antiserum to crude toxin but a single line with antiserum to purified toxin (Fig. 9bI). The reason for the appearance of multiple lines with the antiserum to crude toxin is at present unknown. It may be due to contaminants in our partially purified toxin preparation.

It was of interest to look for possible cross-reactions with antisera obtained against different toxin preparations. (We are grateful to R. Finkelstein for his kind gift of these sera.) Figure 9bII shows the reaction of partial identity given by Finkelstein's antiserum to "bluing factor" with one of the lines elicited by our antiserum (to crude material) with our crude fraction. Finkelstein's antigen was isolated from human strain 339t5 grown on modified syncase medium (personal communication). Finkelstein's antiserum to the porcine toxin isolated by Dorner (4) did not result in precipitin lines when tested with either our crude or purified toxin.

The ability of antisera to neutralize the biological activity of toxin was tested under the conditions described in Materials and Methods. Anti-crude serum had but scant neutralizing activity against purified toxin. In contrast, the antiserum to the purified fraction completely abolished the activity of both crude and purified LT. Fifty percent inhibition of the activity of 20 μg of toxin was obtained with 100 μl of an antiserum that was diluted 200-fold with saline. The antisera to the toxin isolated by Finkelstein to human strain 339t5 had a similar neutralizing activity when used undiluted.

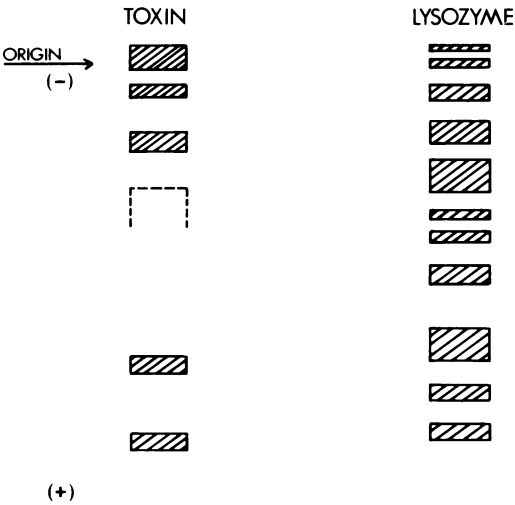

FIG. 8. *Schematic diagram of the location of TPCK-tryptic fragments of the purified toxin after high-voltage paper electrophoresis at pH 3.5. A lysozyme digest done under similar conditions was run in parallel.*

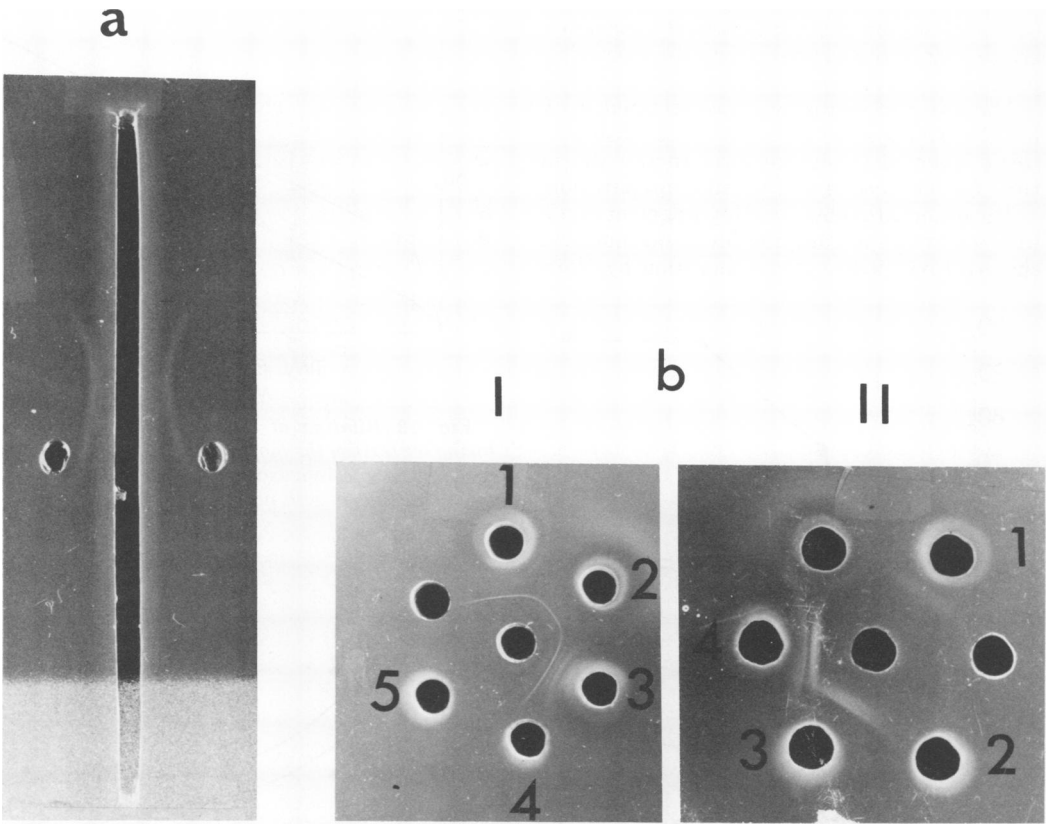

FIG. 9. *(a) Precipitin lines after immunoelectrophoresis of crude and purified toxin with rabbit antiserum against the purified toxin fraction. (b) Ouchterlony double-immunodiffusion plates of both crude and purified material and their antisera. (I) Center well: partially purified toxin. Outer wells: 1 and 2, antisera to purified toxin (two bleedings); 3, antiserum to crude toxin; 4, Finkelstein anti-bluing factor; 5, Finkelstein anti-Dorner enterotoxin. (II) Center well: crude toxin. Outer wells: 1, antiserum to purified toxin; 2, anti-Dorner serum; 3, anti-bluing factor; 4, anti-crude toxin.*

Bioassay. Y-1 murine adrenal tumor cells in monolayer culture respond to enterotoxin by increasing membrane adenyl cyclase activity, leading to increased intracellular cyclic adenosine 5'-monophosphate levels and steroidogenesis, which has been correlated to rounding up of the flattened, fibroblast-like cells (3, 12, 15, 25). We used this marked morphological change as a simple visual assay for enterotoxic activity. The response was linear for toxin concentrations from 1 to 100 μg/ml. Below that range, little or no rounding could be seen, even after 24 to 36 h of incubation; above it, there was an apparent saturation and 100% of the cells rounded up.

Under the usual conditions of assay (enterotoxin added to complete cell medium, containing serum and incubated with cells) there is a lag of 2 to 6 h before a significant effect can be observed. This lag time (which we define as the time required for 50% of the cells to become rounded) is a function of toxin concentration.

Using these parameters (i.e., dose response and lag time) it can be seen (Fig. 10) that: (i) purified toxin gave a 50% response at 10 μg, whereas crude material gave it at 35 μg (when both were observed at 6 h), and (ii) as shown in Fig. 11, when equivalent protein concentrations (16 μg/ml) of pure and crude toxin were assayed, the pure toxin showed a significant reduction in lag time, i.e., from 8 to 10 h for crude to 3.5 to 4 h for purified.

In other experiments, we investigated the effects of short-term incubation of purified toxin with cells (see Materials and Methods). We observed, as have others, that toxin molecules seem to bind rapidly, causing the same rounding of cells as in the continuous incubation. It can also be seen (Fig. 12) that for equivalent amounts of protein, the purified toxin

caused a significantly increased response over crude material for the given time periods of preincubation studied.

The effect of serum was investigated in the usual assay system (Fig. 13). The absence of serum seemed to significantly reduce the lag time for both crude and purified preparations.

The observation by Van Heyningen et al. (25) that a mixture of gangliosides could inactivate cholera toxin led to the observation by Holmgren et al. (12) that the sialydase-resistant ganglioside Gm fixed and inactivated the cholera toxin. Inactivation studies with crude enterotoxin preparations from *E. coli* were less conclusive (11). We have studied the effect of the Gm_1 ganglioside on our purified preparation of enterotoxin (see Materials and Methods). A titration study revealed that the ganglioside from 30 μg down to 10 ng completely inactivated the biological activity of 20 μg of toxin.

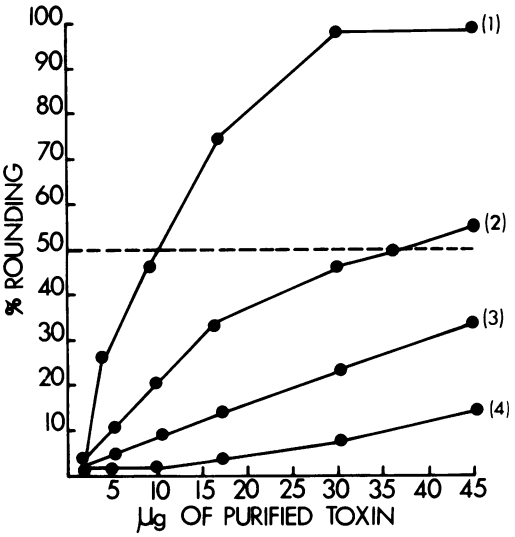

FIG. 10. *Graphs of dose-response relationships of crude and purified toxin in the mouse adrenal tumor cell assay system. (1) Purified (6 h); (2) crude (6 h); (3) purified (3 h); (4) crude (3 h).*

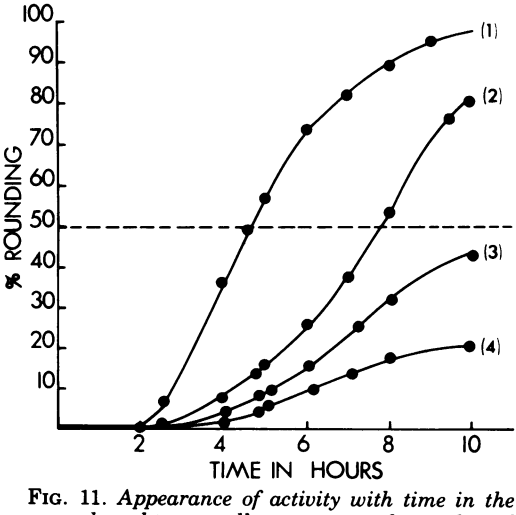

FIG. 11. *Appearance of activity with time in the mouse adrenal tumor cell assay system for crude and purified toxin. (1) Purified (16 μg/ml); (2) crude (16 ug/ml; (3) purified (2 μg/ml); (4) crude (2 μg/ml).*

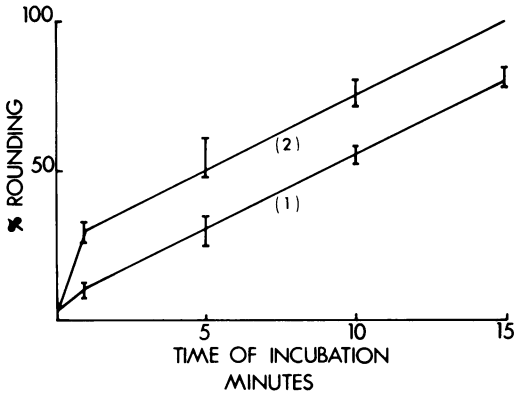

FIG. 12. *Binding of crude (1) and purified (2) toxin (see text) in the mouse adrenal tumor cell assay system. Cells were washed twice after preincubation and reincubated with normal medium.*

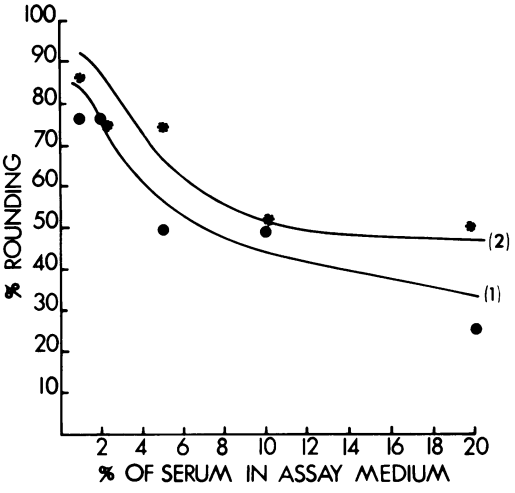

FIG. 13. *Effect of increasing amounts of serum added to assay medium on the biological activity using purified toxin (3 to 6 μg/ml). (1) 12 h; (2) 19 h.*

DISCUSSION

The method described here for the isolation of the heat-labile enterotoxin from *E. coli* strain 711 (P307) gave a preparation highly active in the mouse adrenal tumor cell assay system.

During ultracentrifugation and molecular sieving on Sephadex G-200, it behaved as an aggregate (180,000 to 200,000 daltons). The detergent SDS induced dissociation into two subunits with apparent molecular weights of 70,000 and 15,000. Heating in SDS lowered the larger moiety to an apparent molecular weight of 40,000. Amino acid analysis of the undissociated material revealed the absence of half-cystine and methionine. For half-cystine this was confirmed by the failure to obtain a radioactive material after reduction and alkylation with [^{14}C]iodoacetamide acid.

It is thus tempting to describe the LT as consisting of two different subunits (40,000 and 14,000) that are tightly held together by noncovalent forces. A stoichiometry cannot be deduced from these data, but the 70,000-dalton moiety detected during SDS-PAGE may consist of a single subunit of 40,000 and two subunits of 14,000. The larger aggregate (i.e., 180,000 to 200,000) found during molecular sieving and ultracentrifugation (where no detergents are present) would then represent a "trimer" of the 68,000 particle. Alternatively, the larger subunit may well have an intrinsic molecular weight of about 70,000. Heating in SDS followed by acrylamide gel electrophoresis would then so alter its conformation and coordination with detergent molecules as to give an apparent change in molecular weight, since calculations are based on mobility.

These data are at variance with those published by Dorner (4) for the LT from *E. coli* strain P263 (also a porcine enteropathogen) as well as with those of Evans et al. (6), who have reported on an LT with a molecular weight of 20,000 from *E. coli* strain H10407. The latter toxin was released from the cells by the antibiotic polymyxin B. The possibility arises that these differences are but reflections of strain differences. Proteolysis of an identical or similar larger-molecular-weight toxin molecule present in different strains to give fragments that are active in the various assays used cannot be ruled out completely, but is unlikely, at least in our hands. We have repeatedly tested toxin preparations for proteolytic activity, both before and after membrane filtration, using TAME, BAME, TLME, and *p*-nitrophenyl acetate as substrates, and have not found any measurable hydrolysis.

Of interest is the partial cross-reactivity of the antiserum to the toxin isolated by Finkelstein from a human strain with one of the antigens in our crude material, but not with our purified fractions. Further studies are needed to elucidate the meaning of this finding.

Our purified material represents approximately 1% of the protein of the starting material (i.e., the lyophilized material obtained from the Upjohn Co.). The increment in specific activity was more difficult to establish since the starting material contained substances that interfere with protein determinations. The measurement of lag period was used as an indicator of the progress of purification. We observed a significant shortening of this lag period as a result of purification when equivalent amounts of protein were compared.

Digestion of the intact particle with trypsin results in an increase in specific activity, confirming observations made by others (see especially the recent publication on this activation by trypsin [19a]). Work designed to isolate and study the tryptic fragments is in progress.

The biological activity is abolished by treatment of the active fraction with solutions of GM_1 ganglioside as well as by a rabbit antiserum against the purified toxin. The observed "serum effect," i.e., the apparent increment in specific activity observed when the assay is done without addition of serum to the culture medium, is also being investigated further. The possibility arises that the effect is due to the presence of albumin in the serum, which would counteract the osmotic derangement caused by the enterotoxin. A major effort is directed toward the isolation of the two subunits reported in order to study their role in the biological assay as well as their composition and structure.

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