INFECTION AND IMMUNITY, Nov. 1974, p. 1010-1017
Vol. 10, No. 5

Printed in U.S.A.

Polymyxin B-Induced Release of Low-Molecular-Weight, Heat-Labile Enterotoxin from *Escherichia coli*

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Received for publication 3 September 1974

Polymyxin B-induced release of enterotoxin from *Escherichia coli* strain H-10407 was demonstrated. Incubation of *E. coli* cells derived from 6-h cultures with polymyxin caused the rapid release of enterotoxin with a molecular weight of approximately 20,000, as estimated by the gel filtration technique. The rapidity of the release of enterotoxin indicates that it probably resides in the periplasmic space of the cell. The low-molecular-weight enterotoxin possessed vascular permeability factor and diarrheagenic activities, both of which were found to be heat-labile. The permeability factor activity of this enterotoxin was neutralized by antisera prepared against crude *E. coli* enterotoxin, *Vibrio cholerae* enterotoxin (choleragen), and *V. cholerae* toxoid (choleragenoid), respectively. Supernatant fluids of 6-h *E. coli* cultures did not contain this molecular form of enterotoxin but did contain very high-molecular-weight, heat-labile enterotoxin. Incubation of cells derived from older (18 h) cultures with polymyxin caused the release of both low- (20,000) and high-molecular-weight forms of enterotoxin. We concluded that either the 20,000-dalton form of heat-labile enterotoxin is not released by *E. coli* under in vitro growth conditions or that enterotoxin released in this form is rapidly destroyed or inactivated.

Enterotoxigenic *Escherichia coli* associated with acute diarrhea in man and animals produce heat-stable and heat-labile enterotoxins (13, 36). The relationship between these forms of enterotoxin is unclear, but these are generally recognized as separate and independent entities because of dissimilarities in their physical (20, 36) and antigenic (13) characteristics and in their biological (13, 27) activities. There is considerable evidence that the *E. coli* enterotoxin (hereafter referred to as ECT) which is heat-labile is a protein with biological activities similar to those of the enterotoxin (choleragen) of *Vibrio cholerae*. The diarrheagenic activity of both toxins is mediated via stimulation of intestinal adenyl cyclase (9, 19, 26, 31) and both exhibit delayed vascular permeability factor (PF) activity in the skin of experimental animals (5, 11). The diarrheagenic and PF activities of ECT are neutralized by anticholeragen antibody and those of choleragen are neutralized by anti-ECT antibody (12, 22, 23, 35).

Attempts to define the molecular size and composition of ECT have met with limited success. Jacks et al. (25) reported ECT to have a molecular weight greater than 5×10^6 and to be inseparable from endotoxin. These workers postulated that the enterotoxic activity resides in the protein moiety of the endotoxic carbohydrate-lipid-protein complex. Although several investigators have demonstrated ECT activity in cell lysates, Gyles and Barnum (22) reported that ECT activity could not be associated with cell wall or capsular preparations of *E. coli*. Lariviere et al. (28) investigated ECT derived from whole-cell lysates and from broth culture supernatants and also found ECT activity associated with very high-molecular-weight fractions. These workers suggested that ECT is produced as a large molecule and may give rise to somewhat smaller active fractions; however, attempts to disaggregate ECT into smaller units resulted in inactivation.

Our efforts to define the molecular characteristics of ECT have also met with mixed success, and our previous results (unpublished) are in general agreement with those described above. Therefore, we sought an alternative approach to the problem of defining the molecular nature of ECT with the ultimate goal of purifying the active moiety to homogeneity. The phenomenon of induced release of enzymes and other proteins from *E. coli* by the antibiotic polymyxin B, as described in detail by Cerny and Teuber (2), and the availability of the PF assay for ECT (11) provided such an alternative. This report

describes results obtained by the induced release of ECT from intact *E. coli* cells by polymyxin B. We report here the existence, in *E. coli*, of a low-molecular-weight form of ECT which has the characteristics of the heat-labile enterotoxin.

MATERIALS AND METHODS

Organism. *E. coli* strain H-10407 (serotype O78:H11) was used. This strain has been used in previous investigations into the biological activities of *E. coli* enterotoxin(s) (9, 11, 13, 34). Stock cultures were maintained on slants composed of 2.0% peptone (Difco), 0.5% NaCl, and 2.0% agar.

Preparation of E. coli cells for polymyxin B-induced release of enterotoxin. Cultures were loop-inoculated from stock slants and grown as follows: 80 ml of the Casamino Acids (Difco)-yeast extract-salts medium employed for enterotoxin production (11) was placed in 500-ml Erlenmeyer flasks, inoculated, and agitated in a rotary incubator shaker at 135 to 140 rpm for 18 h at 37 C. This culture was used as inoculum for the growth of cells to be treated with polymyxin B. Twelve 500-ml flasks containing 50 ml of the medium, prepared with 0.15% yeast extract (Difco) instead of 0.6%, were inoculated with 5.0 ml of the 18-h culture and were incubated for 6 h under the same conditions. Cells were harvested from 640 ml of the young culture by centrifugation at 12,000 × *g* for 20 min. Cells were then washed three times by resuspension and centrifugation, as above, in 320, 160, and 80 ml, respectively, of 0.02 M tris(hydroxymethyl)-aminomethane (Tris)-chloride buffer, pH 8.0.

Polymyxin B-induced release of enterotoxin. The toxin-release buffer was composed of 2.0 mg of polymyxin B sulfate per ml (Aerosporin, Burroughs Wellcome Co., Research Triangle Park, N.C.) and was dissolved in 0.15 M Tris-chloride, pH 6.6, containing 0.9% NaCl. For the release of ECT, the cells were prepared as described above and resuspended in 1/16th volume (40 ml) of toxin-release buffer. Several methods of incubation were used during the course of this work. In the first method, the cell suspension was placed in a 500-ml Erlenmeyer flask and shaken at 100 rpm for 1 h at 37 C. After incubation, the cells were removed from the toxin-release buffer by high-speed centrifugation (15 min at 16,000 × *g*). In one experiment, the suspension was incubated for 5 min, and the cells were removed by centrifugation at 12,000 × *g* for 20 min, resuspended in fresh toxin-release buffer (40 ml), reincubated for another 30 min, and finally removed by high-speed centrifugation. The second method of incubation involved more control over time and temperature, since it is based on the differential release of periplasmic versus cytoplasmic proteins by polymyxin B and the effect of temperature on protein release (2). Immediately after the final wash, the cells were resuspended in toxin-release buffer at 0 to 4 C by using an ice bath. The flask was gently shaken by hand in a 37-C water bath, incubating the suspension for 7 min after the temperature had risen to 15 C. This was immediately followed by removal of the cells by high-speed centrifugation.

After centrifugation, the ECT-containing release buffer was decanted into a flask containing 0.1 ml of 2.0 M Tris-chloride buffer, pH 8.0. Storage of ECT preparations was at −40 C.

Preparation of materials for gel filtration. ECT in the toxin-release buffer was concentrated by ammonium sulfate (AMS) precipitation. The fluid was brought to 80% saturation by the slow addition of solid AMS at room temperature with gentle stirring. The precipitate was collected by centrifugation at 16,000 × *g* for 15 min, and the pellet was dissolved in 4 to 5 ml of 0.02 M Tris-chloride, pH 8.0, containing 2.0 mM ethylenediaminetetraacetate. This concentration procedure caused no detectable loss of ECT activity. The same method of concentration was employed for P-60 gel filtration analysis of culture supernatant fluids.

Gel filtration. A column (2.6 by 100 cm; Pharmacia K26/100) was employed for gel filtration in conjunction with an LKB-7000 fraction collector. Fractionation was carried out at 4 C. Fractions were monitored by measuring optical density at 280 nm with a Perkin-Elmer (Coleman 124) double-beam spectrophotometer. Bio-Gel A-1.5M agarose and P-60 polyacrylamide gels were obtained from Bio-Rad Laboratories, Richmond, Calif. Flow rates were 12.4 ml/h for agarose and 4.4 ml/h for polyacrylamide gel filtration. The elution buffer was composed of 0.1 M Tris-chloride, pH 8.0, containing 0.2 M NaCl and 2.0 mM ethylenediaminetetraacetate.

Enterotoxin assays. The vascular PF assay, or skin test, employed here was recently described in detail (11). In this technique, test or appropriate control materials are injected intradermally, 0.1 ml per injection site, into the back of depiliated rabbits. After 18 h, the vascular permeability response is visualized by intravenous injection of Evans blue dye. The PF reaction was determined by measuring the diameter of the zones of intense bluing to the nearest millimeter in two different directions. The score was derived by squaring the average of the two values. Elution buffer served as negative control when testing column fractions. Dilutions were prepared with 0.01 M phosphate-buffered saline, pH 7.2, plus 0.02% bovine serum albumin.

The adult rabbit intestinal ligated loop assay (6) for ECT diarrheagenic activity was performed as previously described (13) by using a 6-h exposure time. One milliliter of test or appropriate control material was injected intraluminally into 4- to 5-cm ligated segments of small intestine; the response was determined as milliliters of fluid accumulated per centimeter of intestine.

Antibody neutralization of polymyxin B-released enterotoxin. The preparation of hyperimmune rabbit anti-ECT and anticholeragen sera has been described in detail elsewhere (12). A partially purified preparation of ECT derived from culture filtrates, 10-fold concentrates of crude polymyxin B-released ECT, and purified (15) choleragen, respectively, were employed as antigens. Pure choleragen and monospecific horse anticholeragenoid serum (14) were prepared under contract for the National Insti-

tute of Allergy and Infectious Diseases by R. A. Finkelstein, The University of Texas Southwestern Medical School, Dallas.

Samples of crude polymyxin-released ECT (7-min method) diluted 1:25 in phosphate-buffered saline were combined and equal volumes of anti-ECT sera (diluted 1:500), anticholeragen serum (1:25), or anticholeragenoid serum (1:25), respectively. Mixtures were incubated at 37 C for 30 min prior to injection of 0.1 ml for PF determination. The control mixtures consisted of ECT without serum (final dilution 1:50) or ECT plus normal rabbit serum (1:50).

RESULTS

Gel filtration analysis of ECT released by polymyxin B. *E. coli* strain H-10407 cells were grown, washed, resuspended in toxin-release buffer, and incubated for 1 h at 37 C according to the procedures described above (first method of incubation). The supernatant fluid recovered after high-speed centrifugation contained significant ECT activity, producing a PF response of 37.4 $mm^2 \pm 6.6$ (standard error of the mean) ($n = 6$) at a dilution of 1:250. This activity was entirely destroyed by treatment at 100 C for 20 min.

An experiment was designed to determine the approximate molecular weight of polymyxin-released ECT by using agarose gel filtration. Agarose A-1.5M was chosen for this purpose because it has been shown (25, 28) that the heat-labile ECT derived from culture supernatant fluids elutes at or near the void volume of agarose columns. A typical polymyxin-released ECT preparation was concentrated by the AMS precipitation method and submitted to A-1.5M gel filtration as described above. The elution profile (Fig. 1) shows that the ECT activity was associated with material eluting near the beginning of the third column volume of eluate. This result indicated that the test preparation of ECT contained a molecular form of enterotoxin considerably smaller than that found in concentrates of culture supernatant fluids (see below).

The molecular weight of polymyxin-released ECT was estimated by the gel filtration technique of Andrews (1), which uses several proteins of known molecular weight as reference points. For this purpose, the active fractions from four separate agarose filtration experiments were combined, concentrated by the AMS precipitation method, and subjected to filtration through a polyacrylamide P-60 gel column (Fig. 2). The ECT activity eluted at the very base of the first, large peak and before the second, small peak. Several reference proteins were then eluted through the same column under identical conditions. Assuming normal

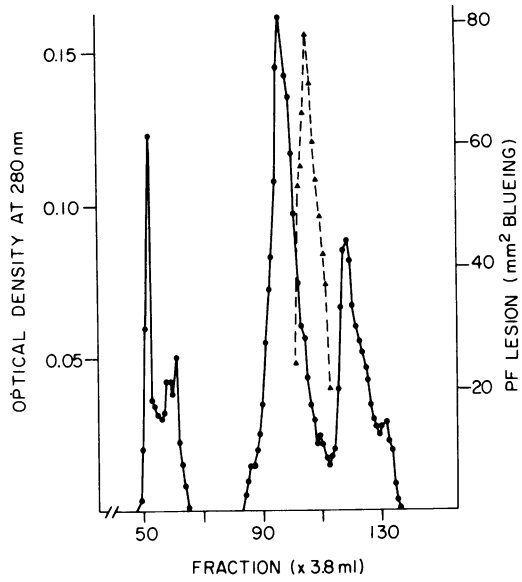

FIG. 1. *Agarose A-1.5M elution profile; test material was a concentrate of material released from E. coli by a 1-h treatment with polymyxin. The solid line represents absorbence at 280 nm; the broken line represents PF (ECT) activity determined with undiluted samples, n = 6.*

behavior of ECT during polyacrylamide gel filtration, the results (Fig. 2, inset) indicate that polymyxin-released ECT possesses a molecular weight of approximately 20,000.

Effect of incubation time on polymyxin-induced release of ECT. Cerny and Teuber (2) demonstrated that the release of proteins from *E. coli* is biphasic, with rapid release of periplasmic proteins within 2 min and the slow release of cytoplasmic proteins during subsequent incubation, presumably by lysis. They also demonstrated that polymyxin B-induced release did not occur at temperatures below 15 C. These observations were the basis for an experiment designed to determine whether a more preferential release of ECT could be achieved by decreasing the time of exposure of the cells to polymyxin. *E. coli* cells were grown, washed, resuspended in ice-cold toxin-release buffer, and incubated in a 37-C water bath for 5 min after the temperature of the suspension had risen to 15 C. Cells were recovered by centrifugation at 4 C, resuspended in fresh toxin-release buffer, and incubated, as above, for an additional 30 min. After removal of the cells by high-speed centrifugation, the 5- and 30-min preparations were assayed for PF activity. More ECT (PF) was released during the initial 5-min incubation time than during the second, longer

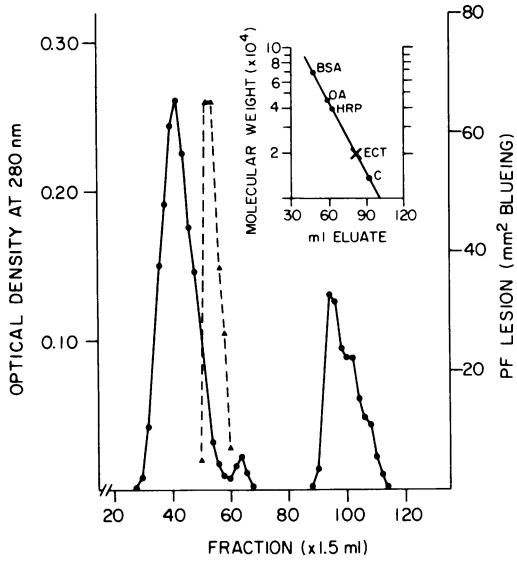

FIG. 2. *Polyacrylamide P-60 elution profile; test material was a concentrate of A-1.5M fractionated ECT. The solid line represents absorbence at 280 nm; the broken line represents PF (ECT) activity determined at 1:5 dilution, n = 6. Inset: plot for estimation of molecular weight of ECT. BSA, Bovine serum albumin; OA, ovalbumin; HRP, horse radish peroxidase; ECT, E. coli enterotoxin; C, cytochrome c.*

incubation (Table 1). Based on these experimental results, subsequent release of ECT from *E. coli* by polymyxin was performed with a 7-min exposure time, starting at 15 C. This is described above as the second method of incubation.

Characterization of ECT procured by the polymyxin B-release technique. In previous studies using ECT derived from culture filtrates of *E. coli* strain H-10407 (11), it was demonstrated that the PF activity of *E. coli* is a function of the heat-labile diarrheagenic toxin and that ECT PF activity is neutralizable by anti-ECT and anticholeragen sera. In the present study, the standard (7 min) polymyxin-release technique was employed to procure ECT from *E. coli* H-10407 to test this form of enterotoxin for behavior during gel filtration and for diarrheagenic activity, heat-lability, and neutralization by antibody. The combined product from two such preparations was subjected to P-60 gel filtration, monitoring each fraction for ultraviolet absorbence (optical density at 280 nm) and for PF activity. Figure 3 shows the results of a typical P-60 elution experiment. A single peak of ECT (PF) activity is observed at approximately the same elution position as that previously observed (Fig. 2).

TABLE 1. *Effect of incubation time on polymyxin B-induced release of ECT from intact cells*

Incubation[a]	Dilution	PF activity[b]
First (5 min)	1:10	75.75 ± 7.95
	1:50	47.83 ± 5.97
	1:250	20.44 ± 2.24
Second (30 min)	1:10	50.17 ± 4.90
	1:50	23.06 ± 4.32
	1:250	0.0

[a] *E. coli* H-10407 cells were incubated 5 min at 15 to 37 C in toxin-release buffer, recovered by centrifugation, and reincubated in the same buffer for 30 min at 15 to 37 C.

[b] PF activity is expressed as diameter of bluing, in square millimeters; mean of nine values ± 1 (standard error of the mean).

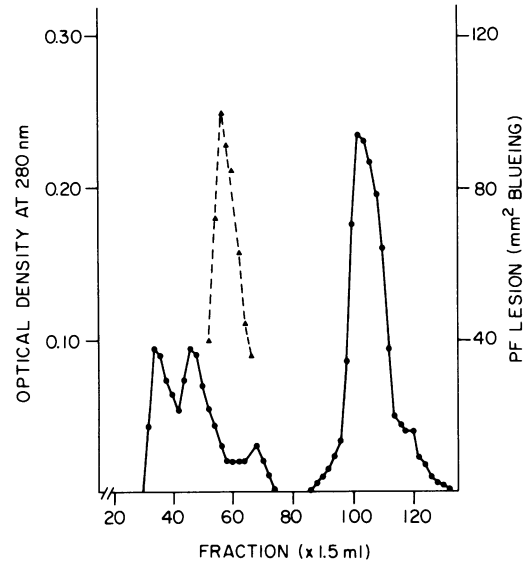

FIG. 3. *Polyacrylamide P-60 elution profile; test material was a concentrate of material released from E. coli by a 7-min treatment with polymyxin. The solid line represents absorbence at 280 nm; the broken line represents PF (ECT) activity determined at 1:2 dilution, n = 6.*

Figure 4A shows the absorption profile of a crude preparation of ECT-containing toxin-release buffer, and Fig. 4C shows the absorption profile of a preparation derived by pooling P-60 column fractions (Fig. 3) with PF activity and concentrating this material 4.7-fold by the AMS precipitation method. Most of the absorbency of the crude material (Fig. 4A) is apparently due to nucleotides which are rapidly released from *E. coli* cells by polymyxin B (2). A significant amount of this material was precipitable by AMS (Fig. 4B) and was associated with the

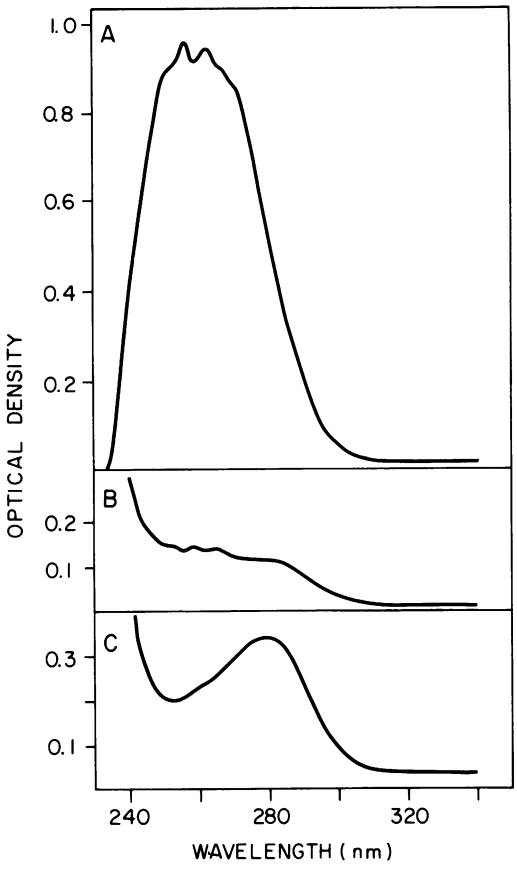

FIG. 4. *Ultraviolet absorption spectra. Samples: (A) Supernatant fluid recovered after polymyxin treatment of E. coli; (B) redissolved material after precipitation from supernatant fluid by ammonium sulfate; (C) pooled PF-positive fractions (Fig. 3) after concentration (4.7 times) by ammonium sulfate precipitation.*

second large peak of material (fraction 102, Fig. 3), which eluted through P-60 gel. The ECT-containing fractions (Fig. 4C) showed a single major absorption peak centered at 280 nm and increased absorbency below 240 nm.

The P-60 fractions containing PF activity (Fig. 3) were combined and tested for heat-labile diarrheagenic activity in the adult rabbit ligated intestine (ileal loop assay). This ECT preparation produced a typical positive response (n = 8, volume/length ratio = 0.841 ± 0.124 [standard error of the mean] ml/min), and this activity was completely destroyed by heat (100 C for 20 min).

A crude preparation of polymyxin-released ECT was tested for neutralization by anti-ECT, anticholeragen, and anticholeragenoid sera according to the procedures described above. The results (Table 2) show that the low-molecular-weight form of ECT released from intact *E. coli* cells was immunogenic and was neutralized by specific antibody. Also, this form of ECT carried the antigenic sites responsible for the immunological cross-reactivity of ECT and *V. cholerae* enterotoxin.

Effect of culture age on polymyxin-induced release of ECT. Cerny and Teuber (2) reported that polymyxin B-induced release of proteins was influenced by the strain of *E. coli* tested and by the conditions employed for growth of the cells. Thus, an experiment was performed to analyze the ECT released by polymyxin from *E. coli* H-10407 harvested at 18 h instead of at 6 h. An 18-h culture was grown as described above, and the cells were subjected to the standard (7 min) polymyxin-release treatment. The resultant ECT preparation was concentrated by the AMS precipitation method and eluted through a P-60 gel column employing previously described conditions. Figure 5 shows the resultant elution profile. Although the 20,000-dalton form of ECT was detectable, a significant amount of the PF activity appeared to be associated with higher molecular weight material eluting at or near the void volume of the column. Interestingly, the second major elution peak of PF activity (fraction 42, Fig. 5) represents material with a molecular weight of approximately 40,000, indicating that dimerization and higher polymerization of ECT might account for the observed results. Also, the 20,000-dalton form of ECT appeared to be the smallest active form discernible by the methods employed here.

Gel filtration analysis of naturally released ECT. Attempts to demonstrate low-molecular-weight ECT in supernatant fluids of 18-h cultures of *E. coli* H-10407 failed. Therefore, an

TABLE 2. *Neutralization of polymyxin-released ECT by anti-enterotoxin sera*

Incubation mixture[a]	PF activity[b]
ECT	53.94 ± 1.97
ECT plus anti-ECT (1:500) prepared against culture filtrate ECT	0.0
ECT plus anti-ECT (1:500) prepared against polymyxin B-released ECT	0.0
ECT plus anticholeragen (1:25)	0.0
ECT plus anticholeragenoid (1:25)	0.0

[a] Incubation mixtures were composed of equal volumes of crude polymyxin-released ECT (1:25) and either standard diluent (phosphate-buffered saline) or immune serum; incubation (30 min, 37 C) was immediately followed by PF assay.

[b] PF activity is expressed as in Table 1; mean of 9 values ± 1 (standard error of the mean).

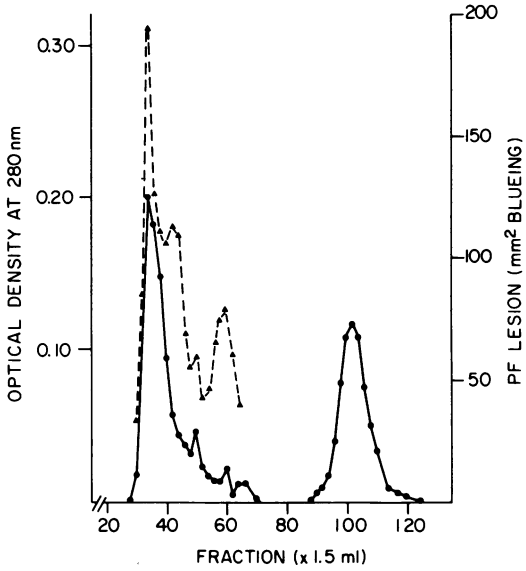

FIG. 5. *Polyacrylamide P-60 elution profile; test material was a concentrate of material released from E. coli, derived from an 18-h culture by a 7-min treatment with polymyxin. The solid line represents absorbence at 280 nm; the broken line represents PF (ECT) activity determined with undiluted samples, n = 6.*

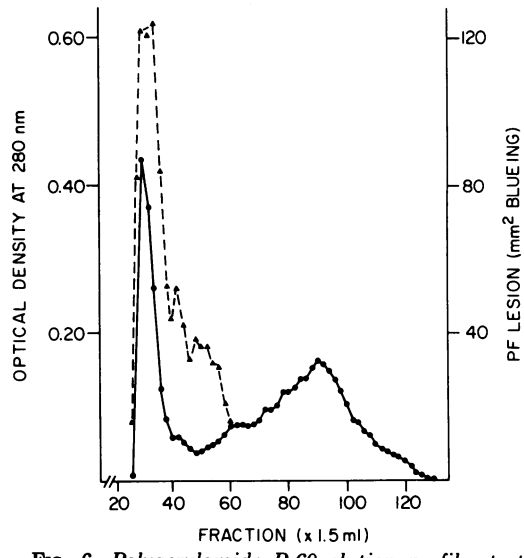

FIG. 6. *Polyacrylamide P-60 elution profile; test material was a concentrate of material derived from the supernatant fluid of a 6-h E. coli culture. The solid line represents absorbence at 280 nm; the broken line represents PF (ECT) activity determined with undiluted samples, n = 8.*

attempt was made to demonstrate this form of ECT in the supernatant fluids of young (6 h) cultures. Cultures were grown as for the preparation of cells for polymyxin treatment and ECT preparations derived by the AMS precipitation method. Three milliliters of an ECT concentrate (72 times with respect to the original culture fluid) were subjected to P-60 gel filtration. Figure 6 shows the resultant elution profile. Virtually all of the ECT (PF) activity derived from the supernatant fluid of the young (6 h) culture was associated with material eluting at or near the void volume of the column. The 20,000-dalton form of ECT seen in Fig. 3, representing intracellular enterotoxin, is not seen in Fig. 6, which represents the extracellular enterotoxin present at 6 h under identical culture conditions.

DISCUSSION

It is apparent from the results presented here that the heat-labile enterotoxin of *E. coli* exists in numerous molecular forms, as indicated by the work of Lariviere et al. (28). The smallest detectable active form of ECT exhibited a molecular weight of approximately 20,000 and was found only intracellularly. The rapid release by polymyxin B of this low-molecular-weight form of ECT indicates that it probably resides in the periplasmic space of *E. coli*. Cells in young (6 h) cultures retain the 20,000-dalton ECT while selectively excreting ECT, which has apparently undergone polymerization or binding to high-molecular-weight cell components. Cells in older (18 h) cultures retain both the 20,000-dalton and the higher molecular weight forms of ECT. It is also possible that the 20,000-dalton form of ECT is released but destroyed or inactivated in the culture medium, since inactive ECT would not have been detected in this study.

The higher molecular weight forms of ECT appear to be a natural product of *E. coli* and not an artifact due to nonspecific binding after natural release from the cell. Indeed, this observation is consistent with the evidence that ECT resides in the periplasmic space. According to Costerton et al. (4), the periplasmic space can be visualized as a functional continuum in which the location of specific enzymes, binding proteins, and pigments appears to depend on binding to specific structural components. For example, the lipopolysaccharide of Gram-negative bacteria resides in the periplasmic area (33), and the enzyme alkaline phosphatase occurs as a lipopolysaccharide-enzyme complex (3, 29). This lipopolysaccharide-enzyme complex can be released intact under growth conditions, as in "periplasmic leaky" mutants (29), or artifically, as shown by Ingram et al. (24).

An intriguing question is that of the relationship between ECT and *V. cholerae* enterotoxin (choleragen) and its natural toxoid choleragenoid. Choleragen possesses a molecular weight of 84,000 and one of its subunit components polymerizes to form inactive choleragenoid (30). This transformation is not mediated by *V. cholerae* but occurs extracellularly as the result of agitation (16). Choleragenoid possesses the tissue-binding site but not the active site of choleragen (31). The 20,000-dalton form of ECT reported here possessed both biological activity (presumably via specific binding sites) and immunological cross-identity with both choleragen and choleragenoid. This is somewhat difficult to reconcile. The general observation that the degree of immunological cross-identity between ECT and choleragen is very small (12, 21) suggests that only a small region or only a very few antigenic sites are involved. Thus, our results indicate that it is unlikely that ECT and choleragen share an entire subunit, unless that subunit is indeed very small. A final conclusion will depend upon the purification of the low-molecular-weight form of ECT to homogeneity and peptide analysis of both ECT and choleragen.

The various gel filtration analyses reported here demonstrate that the lowest molecular weight form of ECT possessing full activity elutes in a narrow, sharp band. This type of elution behavior has not been observed with ECT derived from culture fluids. Therefore, it should now be possible to apply standard purification techniques to ECT using polymyxin B-released ECT as the starting material, although the polymyxin B method does possess inherent limitations. One limitation is the apparent low yield of enterotoxin obtained by a "one-hit" technique of artifical release, as compared to the yield obtained by natural release into the culture fluid which is a cumulative process. However, large-scale production is feasible using fermenter technology. Another limitation of the polymyxin B-release technique is the time-consuming washing procedure. Preliminary results, not reported here, indicate that this procedure can be eliminated without decreasing enterotoxin yield. Other possibilities for improvement include the use of high-yield strains of *E. coli* and simplified culture media.

Enterotoxigenic *E. coli* have been associated with severe human diarrhea in numerous parts of the world (7, 10, 17, 18). Although these *E. coli* isolates include countless different serotypes (8, 12, 17), immunological studies have demonstrated only one antigenic form of heat-labile ECT (8, 12, 13, 22, 37). Therefore, the possibility of developing a vaccine based on a toxoid of ECT must be investigated. Sack (32) has recently demonstrated a protective antibody response in the rabbit employing crude preparations of ECT. The availability of homogeneous, purified ECT preparations would fill an obvious need and stimulate progress towards the development of a protective vaccine directed against enterotoxigenic *E. coli* diarrhea. It is indeed encouraging that crude preparations of the 20,000-dalton form of ECT have elicited, in rabbits, neutralizing antibody comparable to that obtained with crude high-molecular-weight ECT preparations.

ACKNOWLEDGMENTS

The technical assistance of Dorothy Sidney, Ronald Koller, and Flor Lei is gratefully acknowledged.

This investigation was supported by Public Health Service grant A1-11365 from the National Institute of Allergy and Infectious Diseases and by U.S. Army Medical Research and Development Command contract no. MIPR-4952.

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