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Factors Affecting Release of Heat-Labile Enterotoxin by Enterotoxigenic *Escherichia coli*

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Various conditions affecting the release of heat-labile enterotoxin (LT) by enterotoxigenic *Escherichia coli* have been examined. The pH of a defined medium containing three amino acids, M-9 salts, and 0.5% glucose decreased to less than 7.0 in early log phase of growth, and no extracellular LT was detected. Adjustment of the pH at 8 h from 6.0 to 8.0 resulted in a concomitant increase in LT activity in culture supernatants. The release of cell-associated LT was significantly reduced by preincubation with protease inhibitors and increased by preincubation with trypsin. Cell-associated LT was not released by pH adjustment of cells grown at 21°C; however, polymyxin B treatment released a toxin species active in only the pigeon erythrocyte lysate (PEL) assay system. As the growth temperature was increased, polymyxin B released toxin species which exhibited both PEL and Y-1 adrenal tumor cell activity. Polymyxin B extracts of enterotoxigenic *E. coli* in early log phase grown at 37°C possessed only PEL activity, whereas extracts from cells in late-log and stationary phases had biological activity in both assay systems. Also, LT released by pH adjustment from mid-log to stationary phase was active in both PEL and Y-1 adrenal tumor cell assays. Gel electrophoresis of polymyxin B extracts revealed at least three molecular weight species active in either the PEL (22,000 daltons and 30,000 daltons) or both the PEL and the Y-1 adrenal tumor cell assay (72,000 daltons), depending on the growth temperature. These observations may help to explain the chemical and biological heterogeneity of most LT preparations and facilitate purification of LT by increasing the yield of enterotoxin.

Recently, enterotoxigenic (ENT^+) *Escherichia coli* have been implicated as one of several kinds of gram-negative bacteria which produce a heat-stable enterotoxin (ST) and/or a heat-labile extracellular enterotoxin (LT), both of which can cause a watery diarrhea in humans and neonatal animals (27). The ST, a polypeptide of 5,100 daltons, has been purified to homogeneity (1). Several laboratories have described the purification and characterization of LT; however, the molecular weight ranged from 23,000 to 180,000 (6, 8, 10, 11, 15, 28). Most of the studies on LT have been complicated by both the inability to produce significant amounts of biologically active extracellular toxin and by a large molecular-weight heterogeneity in what were considered to be purified preparations (8, 11, 28).

Studies on the release of LT could prove to be especially useful not only in understanding the mechanism of protein processing and/or release by gram-negative bacteria, but also a release step might be incorporated into a purification scheme to increase yields of LT. We have examined various chemical and physical parameters affecting the release of LT from intact cells and cell wall preparations. From these studies, some insight into the biosynthesis, location, and factors controlling release of LT was gained.

MATERIALS AND METHODS

Cultures. The ENT^+ *E. coli* strains of porcine origin and human ENT^+ strain H-10407 used in this study were supplied by Harley Moon of the National Animal Disease Center, Ames, Iowa. Other strains of human origin were supplied by R. Bradley Sack of the Johns Hopkins University School of Medicine, Baltimore, Md. Non-ENT^+ *E. coli* strain O111B4 was obtained from Clarence Buller of this department and was used as a negative control throughout this study.

Media and growth conditions. The minimal salts medium containing 10 mM tricine, 0.5% glucose, and three amino acids (methionine, lysine, and either aspartic acid or glutamic acid) was prepared as described by P. H. Gilligan and D. H. Robertson (13a). Cultures were grown aerobically in either 250-ml Erlenmeyer flasks containing 50 ml of defined medium or Fernbach flasks containing 1 liter of medium. The flasks were incubated at 37°C on a New Brunswick rotary shaker (300 rpm). Starter cultures were grown in M-9 minimal medium (20) for 8 h, and each experimental flask was inoculated with the starter culture, using a volume to

give an initial absorbance at 620 nm of 0.05. Cells were removed by centrifugation at 23,000 × *g* for 30 min, and the supernatant was assayed for LT activity. When necessary, culture supernatants were stored at −20 or −70°C until assay.

LT assays. Two biological assay systems were used to examine preparations for LT activity: (i) the Y-1 adrenal tumor cell assay of Donta et al. (7), and (ii) a slight modification of the pigeon erythrocyte lysate assay (PEL) system described by Gill (12) and Gill and King (13). The adrenal cells were maintained on F-10 medium (Grand Island Biological Co., Grand Island, N.Y.) supplemented with 15% horse serum, 2.5% fetal calf serum, and 50 μg of gentamicin per ml. Assays for enterotoxin activity were performed in 24-well cluster dishes (Costar). Each well contained 1 ml of medium and was seeded with 10^5 cells. After 2 days of growth at 37°C in a humidified atmosphere of 95% air and 5% CO_2, 0.05 ml of sample was added to each well. Toxin activity was determined by estimating the amount of rounding at 18 h and, in some experiments, by extraction of steroids. Under these assay conditions, 1 ng of either purified LT (S. L. Kunkel and D. C. Robertson, manuscript in preparation) or cholera toxin induced greater than 90% rounding, and 5 to 10 pg induced 20 to 30% rounding. Samples for PEL assay were preincubated in 0.15% sodium dodecyl sulfate (SDS)–0.02 M sodium phosphate, pH 7.0, for 15 min before the PEL assay (P. H. Gilligan and D. C. Roberston, in press).

Release of periplasmic LT. The osmotic shock technique described by Nossal and Heppel (22) was used to effect release of periplasmic proteins, as was the polycationic antibiotic polymyxin B (4) with a slight modification of the original procedure. At the end of each incubation period (1 to 8 h), the cells were harvested, washed once at 4°C with 0.14 M NaCl (pH 7.2), and suspended in 0.14 M NaCl (pH 7.2) containing 2 mg of polymyxin B per ml. The suspension was warmed from 4 to 15°C in a 37°C water bath and removed 5 min after reaching 15°C. Cells were removed immediately by centrifugation at 4°C (10,000 × *g*), and the supernatant was assayed for LT activity.

The method of Malamy and Horecker (19) was used to convert log-phase ENT^+ *E. coli* to spheroplasts. When conversion was complete, as measured by diluting the suspension 1:10 (vol/vol) in distilled H_2O and noting the absorbance at 620 nm, the spheroplasts were stabilized by addition of 20 mM $MgCl_2$. The suspension was centrifuged at 10,000 × *g*, and the supernatant was assayed for LT activity. Large quantities of stationary-phase cells were treated by the procedure of Wilholt et al. (29), which converted 85 to 90% of the cells to spheroplasts.

Release of LPS. The procedure of Leive (16) was employed for the release of lipopolysaccharide (LPS) from the outer membrane of both enterotoxigenic and nonenterotoxigenic *E. coli*.

Other factors affecting release of LT. To examine the effect of pH on cellular elaboration and release of LT, the pH of the culture was adjusted after 8 h of growth from 6.5 to 8.0 with 5 N NaOH. In some experiments, the pH was adjusted at various times during the growth cycle of the bacteria.

The effect of temperature on the release of LT was tested by growing ENT^+ *E. coli* at temperatures ranging from 12 to 42°C. The cells were divided evenly for pH and polymyxin B treatment when late-log to early-stationary phase of growth was reached. The influence of temperature on the structure and/or release of LT was determined by polymyxin B extraction of bacteria (4) grown to the same absorbance of 3.2 (620 nm) at 22, 28, and 37°C. The extract obtained from about 18 g of cells grown at each temperature was dialyzed, lyophilized, and dissolved in 0.01 M tris(hydroxymethyl)aminomethane (Tris)-chloride (pH 8.0) at a protein concentration of 4.8 mg/ml. Approximately 150 μg of extract protein incubated in either neutral SDS at room temperature or heated at 90°C for 1 h was fractionated by slab gel electrophoresis using the basic procedure of Ames (2) with a modified gel composition (17). After electrophoresis, the gel was first cut into strips parallel to the direction of migration and the strips were sliced into 5-mm sections. Each section was extracted with 1 ml of 0.01 M Tris-chloride (pH 8.0), and the biological activity eluted from the gel was measured by using both the PEL and Y-1 adrenal tumor cell assays.

Glucose and two glucose analogs, 2-deoxyglucose and β-methyl-glucoside were added at a final concentration of 50 mM to separate flasks after 8 h of incubation at 37°C. Portions were removed at 0-, 15-, 30-, and 60-min intervals, centrifuged, and assayed for LT activity.

Cell suspensions were adjusted to pH 8.0 and incubated with trypsin (25 μg/ml) at 37°C for 30 min. Trypsin activity was terminated by the addition of 50 μg of soybean trypsin inhibitor per ml.

The effect of protease inhibitors on the release of LT from whole cells was examined by incubating with either phenylmethyl sulfonyl fluoride (50 μg/ml) or soybean trypsin inhibitor (50 μg/ml). After the addition of protease inhibitors, the cell culture was adjusted to pH 8.0 and incubated at 37°C for 30 min. Cells were removed by centrifugation, and the supernatant was assayed for LT activity.

Preparation of crude cell wall fragments. A 20% (wt/vol) suspension of washed cells was prepared in 0.12 M Tris-hydrochloride (pH 6.5), and 2.5 g of glass beads (0.17 to 0.18 mm) per g of cells was added. The cells were homogenized for 2 min, using a Bronwill cell homogenizer. After homogenization, the glass beads were allowed to settle, and the crude cell walls were aspirated off and centrifuged at 10,000 × *g* for 15 min.

β-Galactosidase assay. ENT^+ *E. coli* strains were induced for β-galactosidase by using isopropyl-β-D-thio-galactopyranoside (final concentration, 1.1×10^{-4} M). β-Galactosidase activity was determined by using the colorimetric assay based on the enzymatic hydrolysis of *o*-nitrophenyl-β-galactoside (25).

Reagents used. All medium components and chemical reagents were purchased from Sigma Chemical Co., St. Louis, Mo. unless otherwise indicated.

RESULTS

Factors affecting release of LT. Factors affecting the release of LT were studied as follows. (i) The effect of pH on LT release was

examined by using ENT$^+$ *E. coli* strains of both human and porcine origin. Results obtained with a representative porcine strain, 1362, are shown in Fig. 1. After adjusting the pH from 6.0 to 8.0, the appearance of LT activity measured by either the PEL or Y-1 adrenal tumor cell assay was rapid and nearly complete in 20 min. The PEL assay was the more quantitative of the two assays and was used to detect a toxin fragment devoid of a binding component with biological activity only in a broken cell preparation as well as the holotoxin form of LT. The Y-1 adrenal cell assay was used to detect a LT species with both a binding component and a subunit involved in the activation of adenylate cyclase. Low-molecular-weight fragments detected by the PEL assay (22,000, 30,000) were active in the Y-1 assay only at 10- to 100-fold higher amounts than used in the PEL assay and probably reflected decreased efficiency due to lack of a binding component. This conclusion was further supported when purified holotoxin and purified A-subunit of LT became available (S. L. Kunkel and D. C. Robertson, manuscript in preparation). The holotoxin and isolated A-subunit of LT were active at a level of 100 pg in the PEL assay, compared with the Y-1 assay in which 5 to 10 pg of holotoxin and 10 ng of the A-subunit induced 20 to 30% rounding. The data points in Fig. 1, 2, and 4 are the absolute values obtained from at least three PEL assays and the percent founding data normalized to facilitate presentation of data. Two lines of Y-1 adrenal tumor cells were used during the course of these experiments which explains the apparent differences in the PEL and Y-1 adrenal tumor cells responses for a given sample (e.g., Fig. 1 and Fig. 3). No LT activity was detected until the pH of the culture was increased to 7.0, and maximum toxin activity was observed when the pH of the

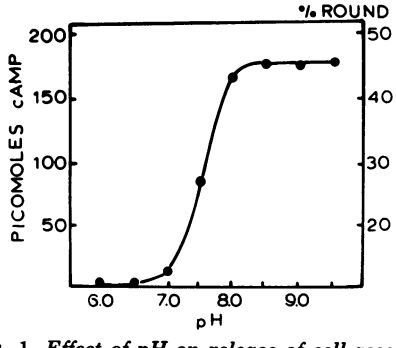

FIG. 1. *Effect of pH on release of cell-associated LT by ENT$^+$ E. coli strain 1362. Each point reflects both PEL and Y-1 activity as the pH was increased. The equivalent of 2 μl of culture supernatant was used in the PEL assay.*

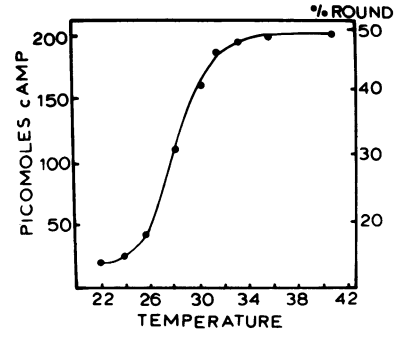

FIG. 2. *Effect of growth temperature on release of cell-associated LT by ENT$^+$ E. coli strain 1362. Each point reflects both PEL and Y-1 activity as the growth temperature increased. The PEL assay was performed as described in the legend to Fig. 1.*

culture was adjusted to 8.0. The level of extracellular β-galactosidase in ENT$^+$ *E. coli* induced for the enzyme was measured to give an indication of the release of intracellular proteins due to cell lysis. No β-galactosidase was found in the culture supernatant after pH adjustment, and this suggested that pH-released LT was exterior to the cytoplasmic membrane.

(ii) Human and porcine strains of ENT$^+$ *E. coli* were grown at temperatures ranging from 22 to 42°C, and the culture supernatant was assayed for pH-released LT (Fig. 2). The results show the critical role that temperature plays in toxin synthesis, processing, and/or release. No LT was detected by either assay system until the cells were grown above 26°C.

(iii) In an attempt to further understand the release process, osmotic shock and polymyxin B treatment were used to release LT from the periplasmic space of ENT$^+$ *E. coli*. If cells grown below 26°C were osmotically shocked or treated with polymyxin B, an active species of LT was released which stimulated only the PEL assay but not Y-1 adrenal tumor cells, a whole cell assay (Fig. 3). No toxin species active in the Y-1 adrenal cell assay was pH released until the cultures were grown above 26°C, but polymyxin B did release toxin active in the PEL assay, even from cultures grown at temperatures as low as 12°C.

(iv) Since glucose was required for synthesis and release of LT (13a), the effect of glucose and two glucose analogs, 2-deoxyglucose and β-methyl-glucoside, on LT release by stationary-phase cells was examined by using ENT$^+$ *E. coli* strain 1362. Glucose at 50 mM and the glucose analogs were added to separate cultures, and samples were removed at timed intervals (Fig. 4). An increase in LT activity was observed almost immediately after the addition of either

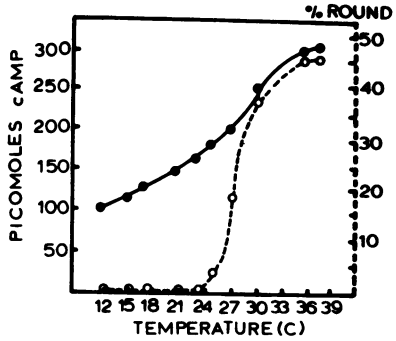

FIG. 3. *Effect of temperature on the biological activity of polymyxin B extracts of ENT⁺ E. coli strain 1362. Symbols: ●, LT activity in PEL assay; ○, LT activity in Y-1 adrenal tumor cell assay.*

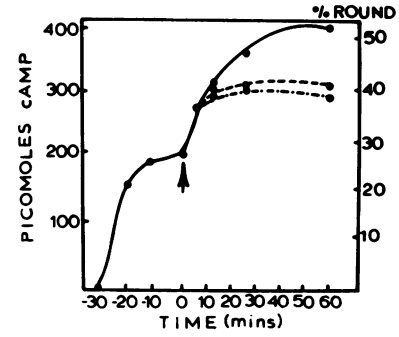

FIG. 4. *Release of LT by stationary-phase ENT⁺ E. coli strain 1362 supplemented with glucose and glucose analogs. The culture was adjusted to pH 8.0 for 30 min before addition of 50 mM glucose (●——●), 50 mM 2-deoxy-glucose (●— —●) or 50 mM β-methyl-glucoside (●-·-·-●) to separate incubation mixtures noted by the arrow at time 0.*

glucose or the analogs. Within 15 min, the activity of the released LT had reached a maximum with the glucose analogs; however, the LT activity from the glucose-treated cells increased up to 60 min. The continued LT release from the glucose-treated cells is probably due to toxin synthesis and not to a release phenomenon.

Effect of temperature on the molecular weight of LT released by polymyxin B. In an attempt to determine how temperature affected structure and function of LT, molecular weight activity scans were conducted in SDS-polyacrylamide gels of LT released by polymyxin B. The samples were either taken up in neutral SDS at room temperature or heated in neutral SDS at 95°C for 1 h. After completion of electrophoresis, the gel was sliced, and the toxin was eluted in 1 ml of 0.01 M Tris-hydrochloride (pH 8.0), for 12 h. Both bioassay systems were used to detect toxin in the eluted samples. A typical scan of LT species released by polymyxin B treatment from cells grown at 22, 28, and 37°C is shown in Fig. 5. All samples were incubated in neutral SDS at room temperature before polyacrylamide gel electrophoresis. Only a 30,000-dalton species with PEL activity was detected at 21°C, whereas 22,000- and 30,000-dalton species with PEL activity were released from cells grown at 28°C. Those cells grown at 37°C and treated with polymyxin B released three species with PEL activity, corresponding to molecular weights of 22,000, 30,000, and 72,000. The 72,000-dalton species was active in both the PEL assay and Y-1 adrenal tumor cell assay yet lost biological activity upon boiling. A summary of the molecular weight species with LT activity as the temperature was varied and the effect of heating is shown in Table 1. The data show that heating had no effect on the biological activity of either the 30,000- or 22,000-dalton species with PEL activity but destroyed

TABLE 1. *Effect of temperature on the molecular weight and biological activity of LT released by polymyxin B treatment of ENT⁺ E. coli strain 1362*

Temp (°C)	Molecular wt ($\times 10^3$) active species			
	Neutral SDS (23°C)		Neutral SDS (95°C)	
	PEL Activity	Y-1 Activity	PEL Activity	Y-1 Activity
21	30	—[a]	30	—
28	22, 30	—	22, 30	—
37	22, 30, 72	72	22, 30	—

[a] —, No activity.

Y-1 adrenal tumor cell activity associated with the 72,000-dalton molecule.

Effect of trypsin and protease inhibitors on LT release. Since a 22,000-dalton toxin appeared as the temperature was raised (Table 1) and both temperature and pH caused LT to be released from cells, it seemed that a protease could be involved in LT processing and/or release. Therefore, the effect of trypsin and protease inhibitors was examined for either stimulation or inhibition of LT activity released by pH adjustment. Enterotoxigenic *E. coli* strain 1362 grown at 37°C for 8 h was treated with trypsin, phenylmethyl sulfonyl fluoride, or soybean trypsin inhibitor and the cellular supernatant examined for LT activity. The amount of LT in the supernatant of whole cells treated with trypsin increased 1.5-fold with the PEL assay over pH release treatment alone. Treatment of whole cells with phenylmethyl sulfonyl fluoride or soybean trypsin inhibitor resulted in a 70% decrease in the stimulation of the PEL assay as compared with the amount of toxin released by pH adjustment.

Kinetics and release of cell-associated LT. Cell cultures of *E. coli* strain 1362 were either pH adjusted or treated with polymyxin B in lag, log, and stationary phases of growth. To detect toxin species with different biological activities, undiluted culture supernatant from each time period was assayed in both the Y-1 adrenal tumor cell and the PEL assay systems. The results in Fig. 6 show the rate of release of LT by pH adjustment and polymyxin B treatment from cells grown at 37°C. Polymyxin B treatment released toxin active in the PEL assay from cells in early log phase, whereas pH adjustment did not release active toxin in either the Y-1 adrenal cell or the PEL systems until the fourth hour of growth (mid-log phase) (Fig. 6).

Release of LT from cell wall fragments. Since no intracellular proteins were released by pH adjustment, as determined by induction and assays for β-galactosidase in culture supernatants, pH-released LT was likely associated with the cell exterior to the cytoplasmic membrane. To further examine this possibility, cell wall fragments were first treated with trypsin and protease inhibitors, and then were pH adjusted. A similar effect of pH on release of LT was observed by using cell wall preparations. Also, trypsin caused a stimulation of pH-released LT, and the protease inhibitors caused an inhibition of pH-released LT from the cell wall fragments, again similar to whole cells.

Comparison of LT released by spheroplasting, osmotic shock, polymyxin B, and EDTA. To aid in detecting the location of cellular LT and to determine whether LT could be released in quantities appreciable for purification, ENT$^+$ *E. coli* were converted to spheroplasts, osmotically shocked, and treated with polymyxin B and ethylenediaminetetraacetic acid (EDTA). A comparison of LT released by each method is shown in Table 2. Samples were diluted, if necessary, relative to unconcentrated culture supernatants so that LT activity was due to an equivalent amount of bacteria in each experiment. Since no toxin was released by pH adjustment at 21°C, it is not surprising that EDTA treatment did not release any active species from cells grown at this low temperature. An active species in the periplasmic space of cells grown at 21°C was released by spheroplasting, osmotic shock, and polymyxin B treatment. A low level of toxin active in the Y-1 adrenal tumor cell assay and the PEL assay was released by pH adjustment or EDTA treatment of cells grown at 28°C. An intermediate level of toxin activity in the two bioassays was observed when the cells grown at 28°C were converted to spheroplasts, osmotically shocked, or treated with polymyxin B. At 37°C, significant LT was re-

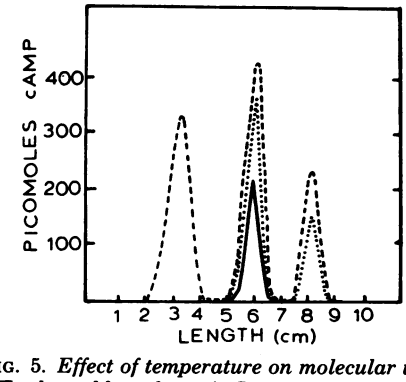

FIG. 5. *Effect of temperature on molecular weight of LT released by polymyxin B treatment of ENT$^+$ E. coli strain 1362. Symbols:* (——) *PEL activity from cells grown at 22 C;* (·····) *PEL activity from cells grown at 30°C; and* (---) *PEL activity from cells grown at 37°C. Approximately 150 μg of protein was applied to each gel, and the biological activity was determined.*

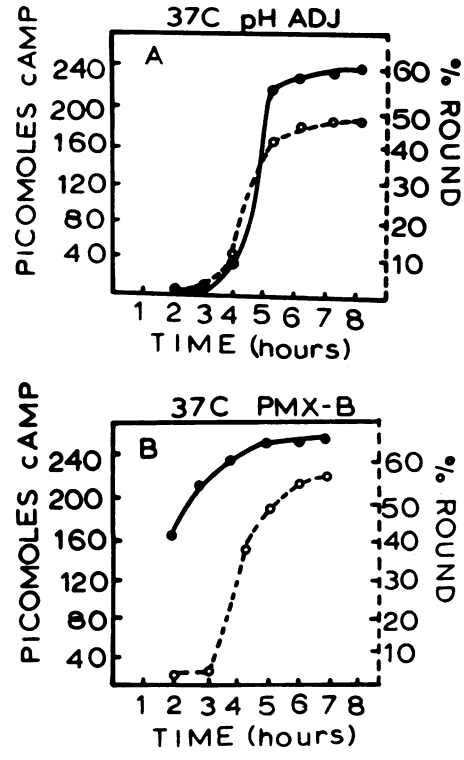

FIG. 6. *Kinetics of LT release from ENT$^+$ E. coli strain 1362 by pH adjustment (ADJ) and polymyxin B (PMX-B) treatment. Symbols:* (●——●) *LT activity in PEL assay;* (○——○) *LT activity in Y-1 adrenal tumor cell assay.*

TABLE 2. *LT activity released by different treatments from ENT+ E. coli strain 1362 grown at various temperatures*

Procedure	Temp[a] (°C)	PEL activity[b]	Y-1 activity[c]
pH Adjustment	21	5	0
	28	70	10
	37	200	40
EDTA treatment	21	5	0
	28	80	20
	37	280	40
Formation of spheroplasts	21	160	5
	28	230	35
	37	340	50
Osmotic shock treatment	21	130	0
	28	180	10
	37	220	40
Polymyxin B treatment	21	180	0
	28	250	10
	37	300	40

[a] Cells were grown to an absorbance at 620 nm of 3.2 for each of the temperatures indicated.

[b] Values indicate picomoles of cAMP generated by LT sample diluted 1:10 in 0.15% SDS–0.02 M PO_4^{3-} (pH 7.0).

[c] Values determined by estimating the percent cells with altered morphology.

leased by each of the procedures, although converting cells to spheroplasts released more toxin than any of the other procedures.

DISCUSSION

During nutritional studies to optimize conditions for synthesis of LT, it was observed that ENT+ *E. coli* strains produced large amounts of acid with glucose as the carbon source. Consequently, the pH of fermentor cultures was adjusted manually during growth in an attempt to keep the pH above 7.0. Only trace amounts of LT activity were observed below pH 7.0, and LT levels increased as the pH was raised in 0.5-U intervals from 6.0 to 10.0. Most cell-associated LT was released between pH 7.5 and 8.0. The LT released by pH adjustment apparently was located exterior to the cytoplasmic membrane, since no extracellular β-galactosidase, an intracellular enzyme, was observed in culture supernatants under the same pH release conditions.

Temperature was another physical parameter which greatly influenced toxin processing and/or release (Fig. 2). At least three molecular weight species of LT with one or more biological activities were detected by polymyxin B treatment of cells grown between 22 and 37°C. A 30,000-dalton fragment which stimulated only the PEL assay, a broken cell preparation with no requirement for a binding component, was released by polymyxin B treatment of cells grown between 12 and 22°C. As the growth temperature was raised above 22°C, an additional species of 22,000 daltons with only PEL activity was observed. It is possible that above 22°C, a protease nicks the intact 30,000-dalton species (analogous to the $A_1 + A_2$ polypeptides of cholera toxin) to form the 22,000-dalton fragment. As the growth temperature increased from 28 to 37°C, the amount of cell-associated LT released by pH adjustment increased from minimal to normal levels, and a 72,000-dalton species with both PEL and Y-1 adrenal tumor cell activities was released by polymyxin B treatment. The 72,000-dalton species is likely the holotoxin of LT for several reasons: (i) the same molecular weight species is released by pH adjustment of whole cells, (ii) biological activity in the Y-1 adrenal cell assay requires both a binding component and a fragment which activates adenylate cyclase, and (iii) boiling in SDS destroyed the biological activity associated with the higher molecular weight of 72,000, and only low-molecular-weight species with PEL activity were detected. These latter observations suggested that the binding component was being dissociated as is the B component of cholera toxin under the same conditions (13).

Orskov et al. (23) observed that K88 fimbriae, the proteinaceous antigen responsible for adhesion, did not develop when ENT+ *E. coli* were grown at or below room temperature, but the antigen was fully expressed when the bacteria were grown at body temperature. It is interesting that the effect of temperature on the appearance of K-88 and LT is so similar, since they are both coded by plasmid genes.

The ability of ENT+ *E. coli* to synthesize and process LT at lag, log, and stationary phases of growth was examined. During the first 4 h of growth, only polymyxin B treatment released active toxin, and the biological activity was limited to the PEL assay. These data indicated the selective release from the periplasmic space of the toxin fragment involved in activation of adenylate cyclase. After 4 h, intact toxin, as determined by Y-1 adrenal tumor cell activity, appeared to be synthesized and released by either polymyxin B treatment or pH adjustment. Similar observations on the time-dependent release of cholera toxin and its subunits have been made (N. Ohtomo, T. Muraoka, and K. Kudo, Abstr. 13th Joint Conf. Cholera, p. 27, 1977). As with the fragment of LT with enzymatic activity, the free A-subunit of cholera toxin was found to be synthesized within cells during early stages of growth. On the other hand, the binding component (B protomer) and the holotoxin from *Vibrio cholerae* were found in limited amounts throughout the lag and log phases of growth. It was proposed that the A and B protomers of

cholera toxin were synthesized independently at different sites in the cell, and assembly to form whole toxin occurred only when the two were released from the cell (Ohtorno et al. Abstr. 13th Joint Conf. Cholera, 1977). The synthesis and processing of cholera toxin by *V. cholerae* and LT by ENT$^+$ *E. coli* appear to be remarkably similar.

Since the release of LT appeared to be affected by both temperature and pH, two parameters also known to effect protease activity, it was of interest to determine if a protease was involved in toxin processing and/or release. Protease inhibitors reduced the amount of pH-released LT by as much as 70%; however, trypsin caused an increase in the amount of pH-released LT. Rappaport et al. (24) observed that treatment of culture filtrates with trypsin and rabbit intestinal fluid increased LT activity about twofold. A protease may be involved in processing a protoxin of LT analogous to the iota toxin from *Clostridium welchii* (26) and the neurotoxin of *C. botulinum* type E (9). Lampen (14) observed that release of the exopenicillinase of *Bacillus licheniformis* was influenced by temperature and pH. Also, trypsin treatment of whole cells and cell wall fragments converted the cell wall-bound penicillinase to a free penicillinase form. Another possible role for a protease in the synthesis of LT may be in release of a membrane-bound form of the components of the holotoxin into the periplasmic space for assembly and insertion into the outer membrane. The LT molecule may be released from the outer membrane or, more likely, remains associated with LPS or large outer membrane aggregates and complicates attempts at purification.

A comparison can be drawn between the release of LT and cholera toxin and the release of degradative enzymes localized either on the cell surface (4, 18) or in the periplasmic space of a number of gram-negative bacteria (3, 5). Specifically, alkaline phosphatase is located in the periplasmic space (18) and associated with the outer membrane, which enables the enzyme to be sequestered in a microenvironment of LPS and protected from chemical and physical agents (18). The sequestering effect afforded by LPS may also protect LT from environmental extremes. Dorner (8) demonstrated that incubation of excess LPS with LT yielded a high-molecular-weight, heat-stable complex which no longer possessed many of the physical characteristics of native LT. The different fragments of the LT are probably synthesized at different sites in association with the inner surface of the cytoplasmic membrane. The nascent polypeptides are extruded through the cytoplasmic membrane and fold into their tertiary structures in the periplasmic space or on the outer surface of the cytoplasmic membrane. The intact holotoxin then transverses the outer membrane with newly synthesized LPS and becomes a component of the outer surface of *E. coli*. The association of LT with LPS provides a microenvironment that affords protection to the labile protein from chemical and physical agents.

ACKNOWLEDGMENTS

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