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Lincomycin Increases Synthetic Rate and Periplasmic Pool Size for Cholera Toxin

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Increased enterotoxigenicity of *Vibrio cholerae* 569B grown with low concentrations of lincomycin, previously described in terms of increased extracellular biological activity (capillary permeability factor and fluid accumulation in ligated rabbit ileal loops), was further characterized. Polyacrylamide gel electrophoresis and single radial immunodiffusion showed that lincomycin-stimulated cells produced increased molar quantities of cholera toxin (CT) both extra- and intracellularly. The intracellular CT was released in comparable amounts by sonication, deoxycholate extraction, and polymyxin B treatment. Polymyxin B release of CT was nearly complete under conditions wherein only 6% of total cellular β-galactosidase was released, implying a periplasmic pool of CT in stimulated cells. No intracellular choleragenoid (CT subunit B) was found in stimulated cells by polymyxin B release. No proteolysis of ^{14}C-labeled CT was detected after prolonged incubation with sonicated nonstimulated cultures or sonicated concentrated cells. These data support the conclusion that the stimulatory effect of lincomycin involves an increase in the rate of synthesis of the CT molecule, and argue against alternative models involving inhibition of putative normal degradation of CT, increased release of otherwise cell-bound CT, or activation of inactive, or less active, forms of CT.

Cholera toxin (CT) is a protein enterotoxin consisting of subunits A and B. The A portion has been shown to catalyze the intracellular ADP ribosylation and activation of adenylate cyclase (8), and it contains two polypeptide chains, A_1 and A_2, linked by a disulfide bond (6). It appears that subunit A is synthesized as a single polypeptide chain which is nicked extracellularly to produce A_1 and A_2 (9). The B portion, which binds to receptors of CT target cells (7), is composed of four or five identical polypeptide chains (6, 13), here denoted B_m (for monomer). The molecular weights of A_1, A_2, and B_m have been reported to be 23,000, 6,000, and 11,000, respectively (6).

Cellular mechanisms which regulate the synthesis of cholera toxin in *Vibrio cholerae* are of particular interest because of both the relevance of the toxin to human health and its apparent irrelevance to the producing organism. Largely because of this absence of any known function of CT in the growth of *V. cholerae*, little is known about the regulation of its synthesis. As a possible means of probing this regulation, we have been examining the increased enterotoxigenicity caused by lincomycin.

We have previously described large increases in extracellular CT in *V. cholerae* 569B when cells are grown with low concentrations of lincomycin, an inhibitor of protein synthesis (14), as measured either by capillary permeability factor or by fluid accumulation in ligated rabbit ileal loops. Heat-labile *Escherichia coli* enterotoxin (LT) was also shown to be increased by lincomycin (14). Data are presented here which strongly support the conclusion that lincomycin stimulates an increase in the rate of synthesis of the CT molecule, and which argue against several alternative models involving reduced turnover of active CT, increased release of cell-bound CT, or activation of otherwise inactive, or less active, forms of the toxin.

The data also show that lincomycin-stimulated *V. cholerae*, relative to untreated cells, contain large amounts of cell-associated CT which can be released by sonication or by treatment of cells with either sodium deoxycholate (DOC) or polymyxin B. The kinetics of release by polymyxin B of CT and β-galactosidase, a cytoplasmic enzyme, suggest that intracellular CT is localized in a periplasmic pool, as also seems to be true for *E. coli* LT (4).

MATERIALS AND METHODS

Media and growth conditions for CT production. *V. cholerae* 569B was grown overnight at 37°C on 5-ml TRY agar slants and then suspended in 5 ml of TRY medium (14a). These cells were diluted at least 100-fold in TRY medium, and cultures were incubated with reciprocal shaking in 500-ml baffled

flasks at 30°C for various times. Intracellular toxin was maximal at about 22 h. Cultures grown with lincomycin showed increased yields of extracellular CT per cell by this time, but increases in total extracellular CT per milliliter of culture filtrate were more reliably obtained after 48 h.

Preparations of CT and subunit B. CT was concentrated and partially purified from cell-free filtrates of *V. cholerae* 569B by precipitation with sodium metaphosphate at pH 4.6 (21). To prepare subunit B, precipitation was carried out on 100 g of lyophilized culture filtrate which had been dissolved in 200 ml of water and dialyzed at 4°C against four changes of phosphate-buffered saline (PBS) (0.01 M phosphate-0.15 M NaCl, pH 7.0) (1 liter each). The precipitated material was dissolved in 0.15 M Na_2HPO_4 (pH 8.0), concentrated further by dialysis against Ficoll, and finally dialyzed against 0.07 M Tris-hydrochloride, pH 6.8. After addition of 2% (wt/vol) sodium dodecyl sulfate (SDS) and 10% (vol/vol) glycerol, a 3-ml sample containing 8 mg of protein was subjected to preparative SDS-polyacrylamide gel electrophoresis (PAGE), using a Canalco Prep-Disc (Canal Industrial Corp., Rockville, Md.) containing a 3-cm stacking gel (4% acrylamide) and a 1.5-cm separating gel (9% acrylamide). Elution was carried out with electrode buffer, and fractions containing pure subunit B, as determined by analytical SDS-PAGE, were pooled, dialyzed versus 0.05 M NH_4CO_3, and lyophilized. The preparation showed the same electrophoretic mobility under nondenaturing conditions as did trace amounts of subunit B found in various toxin preparations. Purified CT was kindly provided by R. Rappaport, and was also purchased from Schwarz/Mann, Orangeburg, N.Y.

PAGE. (i) SDS-gels. Separating gels contained 0.38 M Tris-hydrochloride (pH 8.8), 13% (wt/vol) acrylamide, 0.35% (wt/vol) *N,N'*-methylenebisacrylamide (BIS), 0.1% (wt/vol) SDS, 0.033% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine (TEMED), and 0.05% (wt/vol) ammonium persulfate. Stacking gels contained 0.11 M Tris-hydrochloride (pH 6.8), 5% (wt/vol) acrylamide, 0.13% (wt/vol) BIS, 0.1% (wt/vol) SDS, 0.05% (vol/vol) TEMED, and 0.1% (wt/vol) ammonium persulfate. Electrode buffer contained 0.3% (wt/vol) Tris base, 1.44% (wt/vol) glycine, and 0.1% (wt/vol) SDS. Samples were applied in 0.05 M Tris-hydrochloride (pH 6.8), 2% (wt/vol) SDS, and 10% (v/v) glycerol, with bromophenol blue as the tracking dye. Heated and reduced samples contained 5% (vol/vol) 2-mercaptoethanol and were incubated in boiling water for 2 min immediately before electrophoresis. Slab gels (145 by 100 by 1.5 mm) were run at 5-W constant power at room temperature until the tracking dye was about 1 cm from the bottom of the gel. Gels were stained with 0.1% (wt/vol) Coomassie brilliant blue, 10% (vol/vol) methanol and 7% (vol/vol) acetic acid, and destained in 10% (vol/vol) methanol and 7% (vol/vol) acetic acid.

(ii) Nondenaturing gels. All recipes were as described by Davis (3). Cylindrical gels (diameter, 5 mm) were used here rather than slabs, because in our hands the latter gave poor resolution without SDS. Gels contained 6% and 2.5% (wt/vol) acrylamide in separating gel (1.0 ml) and stacking gel (0.2 ml), respectively. They were run at 3 mA/gel, at room temperature, for 30 min after the tracking dye (bromophenol blue) reached the bottom of the gel.

Immunoassay of CT. Antigen concentrations were determined by single radial immunodiffusion. Gel slabs (1-mm thick) containing 1% (wt/vol) SeaKem agarose (type ME, Marine Colloids, Rockland, Maine), 0.01 M NaN_3, and 0.5% (vol/vol) goat antiserum prepared against purified CT (obtained from R. Rappaport), in PBS, were used with 4.5-μl samples placed in 3-mm-diameter wells. This antiserum precipitated CT and subunit B, but no activity against purified subunit A was detected. After incubation for 18 to 20 h at room temperature in a humid atmosphere, gels were pressed under Whatman 3M chromatography paper with an evenly applied pressure of approximately 40 g/cm^2, soaked in 0.1 M NaCl for 15 min, rinsed in distilled water, pressed again, dried at 80°C, and stained for protein as described above for SDS-gels. After destaining with tap water and drying again at 80°C, ring diameters were measured with a calibrating viewer (Transidyne, Ann Arbor, Mich.). Purified CT was run as a standard at four different concentrations on each gel, and values for experimental samples, which were run in duplicate, were obtained by linear regression analysis of diameter squared versus concentration, using the method of least squares. Zwittergent 314 (Calbiochem, La Jolla, Calif.) was added to all samples (3%, wt/wt) just before immunodiffusion because it eliminated a diffuse haze associated with cell extracts which partially obscured immune precipitate rings. Zwittergent (3%), lincomycin (50 μg/ml), and polymyxin B (2 mg/ml) had no effect on ring diameters.

RESULTS

Increased concentration of extracellular CT after growth with lincomycin. Cultures of *V. cholerae* 569B were grown in TRY (14a) medium at 30°C for 46 h with 0 or 10 μg of lincomycin per ml. After removal of cells by centrifugation and filtration, CT was concentrated and partially purified from the filtrates by precipitation with sodium metaphosphate (21). Analysis of the concentrated material by PAGE showed a large increase in CT yield for the lincomycin-containing cultures (Fig. 1A). SDS-PAGE of the same material showed large increases in the CT subunits A_1 and B_m (Fig. 1B). (Subunit A_2 stains poorly and is not evident here.)

Increased cell-associated CT induced by lincomycin. Cultures of *V. cholerae* 569B grown with and without lincomycin were centrifuged, and the washed pelleted cells were then either sonicated, treated with polymyxin B, or extracted with DOC. Cells and debris were then removed by centrifugation and filtration, and the filtrates were assayed for released CT by single radial immunodiffusion (see Materials and Methods). Little detectable CT was released

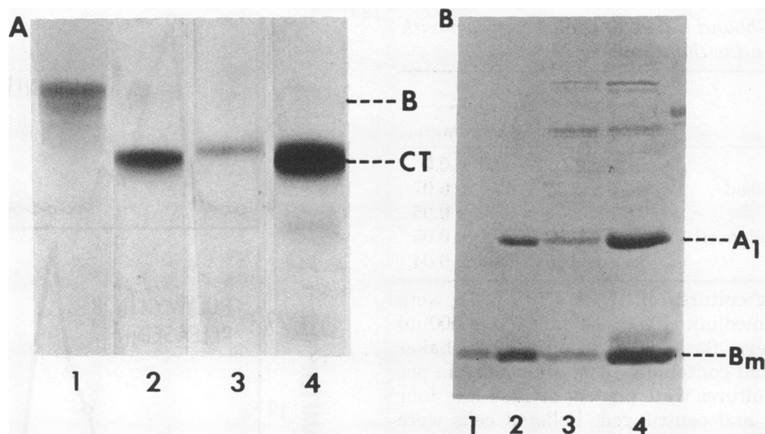

FIG. 1. *Increased yield of extracellular CT from cells grown with lincomycin. Triplicate cultures of V. cholerae 569B were grown in TRY medium (200 ml) with or without 10 μg of lincomycin per ml for 46 h and then pooled. After removal of cells by centrifugation and filtration of supernatants (nitrocellulose filters; 0.2-μm pore diameter), CT was concentrated and partially purified from the filtrates by the method of Rappaport et al. (21). Recovery of extracellular CT from filtrates, measured by immunodiffusion, was 48 and 76% for cells grown with and without lincomycin, respectively. PAGE was carried out as described in Materials and Methods. (A) Nondenaturing conditions; (B) SDS slab gel. (1) Purified subunit B; (2) purified CT (Schwarz/Mann); (3) partially purified CT from cells grown without lincomycin; (4) partially purified CT from cells grown with lincomycin.*

when washed cells were incubated with PBS at 30°C (Table 1). However, sonication, DOC extraction, and polymyxin B treatment all released significant, and very similar, amounts of CT. Cells grown with lincomycin released about four times as much antigen as cells grown without lincomycin.

The antiserum used in the immunoassay of Table 1 reacted with purified CT subunit B as well as with whole toxin. To determine whether the released cell-associated antigen was in fact whole toxin, preparations from the lincomycin-stimulated cells were subjected to PAGE, along with purified CT and purified subunit B. Material eluted from the sliced gels was then assayed by immunodiffusion. Whole toxin and subunit B could be distinguished in this way, and polymyxin B treatment released cell-bound antigen which comigrated with whole toxin and not with subunit B (Fig. 2). Antigen released by sonication or by DOC extraction also comigrated with whole toxin (data not shown).

Polymyxin B preferentially releases periplasmic constituents of *E. coli* and probably *V. cholerae*, but may also produce cell lysis and release of cytoplasmic material (1, 17). To assess whether polymyxin B-released CT was in fact periplasmic, we compared the kinetics of release of CT and β-galactosidase, an enzyme known to be cytoplasmic in *E. coli*. As shown in Table 2, 95% of the total soluble CT of *V. cholerae* grown with lincomycin was released by polymyxin B under conditions where only 6% of the total β-galactosidase was released. We therefore concluded that the released CT was periplasmic.

Absence of detectable turnover of CT. Since increased CT associated with growth in the presence of lincomycin might reflect an inhibition by lincomycin of normal proteolytic degradation of CT, with a consequent increased accumulation of toxin, we examined noninduced cells for this putative proteolytic activity. ^{14}C-labeled CT, prepared as described for Fig. 3, was added either to sonicated whole *V. cholerae* 569B cultures or to sonicated cell concentrates. After incubation at 30°C for up to 3.5 h, the mixtures were subjected to gel electrophoresis. The amount of ^{14}C migrating with CT subunits was measured (Table 3). No detectable loss of ^{14}C-labeled subunits A_1 and B_m occurred during the 3.5-h incubation.

DISCUSSION

We have previously described a large increase (at least fourfold) in extracellular CT which results from growth of *V. cholerae* 569B in low concentrations of lincomycin, an inhibitor of bacterial protein synthesis (14). These data were based on increased biological activity, as measured by either rabbit skin permeability factor or capacity to elicit fluid accumulation in ligated rabbit ileal loops. Several possible mechanisms for the stimulatory effect of lincomycin are feasible: (i) an increased rate of CT synthesis; (ii) unchanged synthesis but enhanced release of normally cell-bound CT; (iii) unchanged syn-

TABLE 1. *Cell-bound CT in V. cholerae grown with and without lincomycin*[a]

Prepn	CT (μg/ml)	
	+Lincomycin	−Lincomycin
Culture filtrate	4.4 ± 1.0	2.0 ± 0.5
Sonication released	0.73 ± 0.15	0.14 ± 0.07
DOC released	0.66 ± 0.15	0.20 ± 0.05
Polymyxin B released	0.66 ± 0.10	0.21 ± 0.05
PBS released	0.19 ± 0.06	0.06 ± 0.04

[a] Four to six cultures of *V. cholerae* 569B were grown in TRY medium (100 to 200 ml each in 500-ml baffled Erlenmeyer flasks) at 30°C on a linear shaker for 22 h, with half containing 10 μg of lincomycin per ml. Replicate cultures were pooled, divided into four equal portions, and centrifuged. Pelleted cells were then washed with cold PBS and pelleted again, and the four pellets were then suspended and treated as follows: (i) suspended in 2 ml of PBS and shaken at 30°C for 30 min; (ii) suspended in 2 ml of PBS and sonicated (four times in ice bath, 1 min each, 60 W, Sonicator with microtip [Heat Systems-Ultrasonics, Inc., Plainview, N.Y.]); (iii) suspended in 2 ml of PBS containing 2 mg of polymyxin B sulfate per ml (Sigma Chemical Co., St. Louis, Mo.) and shaken at 30°C for 30 min; (iv) suspended in 2 ml of PBS containing 0.1% (wt/vol) DOC and 20 μg of DNase per ml (Worthington Biochemicals Corp., Freehold, N. J.; RNase-free) (to reduce viscosity) and shaken at 30°C for 30 min. After further centrifugation and filtration (nitrocellulose filters; 0.2-μm pore diameter), released CT was assayed by immunodiffusion (see Materials and Methods). Values are given in micrograms of CT per milliliter of original culture volume per optical density unit at 550 nm (OD_{550}) of the original cultures. Typical values of OD_{550} at 22 h were 4.0 and 2.0 for cells grown without and with lincomycin, respectively. Data represent the mean and standard error of the mean from six experiments.

thetic rate and release but increased biological activity per molecule of CT; (iv) proteolytic degradation of CT by noninduced cells which is somehow inhibited by lincomycin, leading to increased accumulation and release of CT.

The data presented here strongly favor the first of these possibilities. PAGE of sodium metaphosphate-concentrated culture filtrates (Fig. 1) showed large increases in extracellular CT, confirmed by increased amounts of CT subunits seen in SDS-PAGE, when *V. cholerae* 569B was grown in medium containing 10 μg of lincomycin per ml. Thus, no change in biological specific activity is needed to explain the increase in total activity seen in cultures grown with the drug (14).

In addition, cells grown with lincomycin contained more cell-associated CT than did control cells, as indicated by the amount of CT which could be released by sonication, polymyxin B treatment, or DOC extraction (Table 1). The released material was identified as CT on the basis that it was precipitated by anti-CT antibody and it comigrated with CT in PAGE under nondenaturing conditions (Fig. 2). Hence, the lincomycin effect is not a matter of increased cellular permeability with CT synthesis at the usual level. To the contrary, there is a large pool of cellular toxin associated with stimulated cells, relative to control cells, and this difference in pool size is even larger than the difference seen extracellularly, suggesting that, if anything, lincomycin decreases the proportion of intracellular toxin which is released per unit of time.

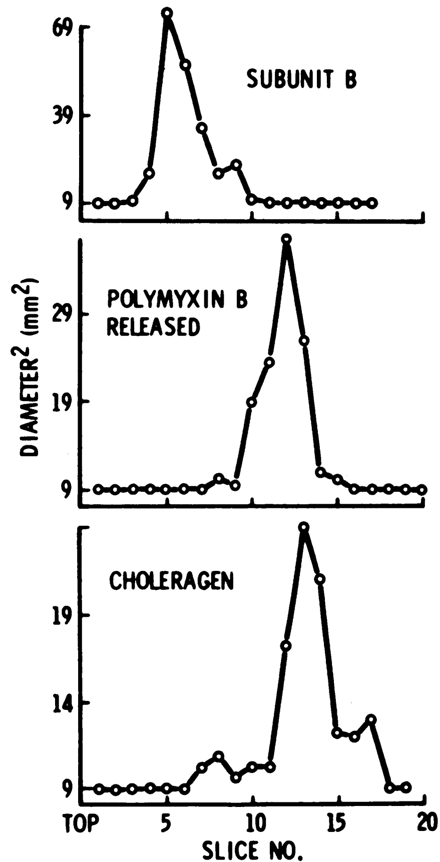

FIG. 2. *Identification of polymyxin B-released antigen as CT. Cells grown in medium containing 10 μg of lincomycin per ml were washed and treated with polymyxin B as described for Table 1. Released material was subjected to PAGE in nondenaturing conditions (see Materials and Methods), with purified CT and subunit B run in parallel gels. Two-millimeter slices of the gels were minced and agitated overnight in 100 μl of PBS at room temperature. Eluted material was then assayed by immunodiffusion (see Materials and Methods). Value of 9 mm² on the ordinate axis indicates absence of detectable antigens.*

TABLE 2. *Polymyxin B release of CT and β-galactosidase from V. cholerae*[a]

Time (min) with polymyxin B	CT released (%)	β-Galactosidase released (%)
0.5 (0.1 mg/ml)	12.2	2.5
0.5 (2.0 mg/ml)	94.8	6.5
30 (2.0 mg/ml)	97.8	24.4

[a] *V. cholerae* 569B cells grown for 23 h at 30°C in TRY medium with lincomycin (10 μg/ml) were chilled, pelleted, washed with PBS, and resuspended in 1/30 the original volume of cold PBS. Portions were either sonicated as in Table 1 or incubated at 30°C with 0.1 or 2.0 mg of polymyxin B sulfate per ml. All portions were then chilled and centrifuged, and the cell-free supernatants were filtered. Filtrates were assayed for CT by immunodiffusion and for β-galactosidase by the method of Kennell and Magasanik (12). Values are expressed as percentages of those obtained for filtrates of sonicated cells: 0.61 μg of CT/ml per OD_{550} and 0.314 U of β-galactosidase/ml per OD_{550}. Filtrates of sonic extracts from parallel cultures grown without lincomycin contained 0.25 μg of CT/ml per OD_{550} and 0.266 U of β-galactosidase/ml per OD_{550}. One unit of enzyme activity hydrolyzed 1 μmol of *o*-nitrophenyl-β-D-galactoside per min. Sonic extracts of cells grown with and without lincomycin contained 0.103 and 0.060 U per mg of protein, respectively. (For comparison, it can be deduced from data in reference 15 that *E. coli* contains about 3 U per mg of protein when fully induced by growth with glucose.)

Furthermore, we were unable to demonstrate any degradation of CT in cells grown without lincomycin (Table 3). The method used would not have detected nicking of subunit A to produce fragments A_1 and A_2, nor would it have detected small changes in molecular weight, but the data do argue against proteolysis by intra- or extracellular enzymes of a magnitude sufficient to account for the large differences in concentration of subunits A_1 and B_m seen in Fig. 1 for stimulated and control cells. It therefore seems unlikely that lincomycin inhibits normal degradation of CT, either intra- or extracellularly, leading to increased accumulation of the toxin.

We conclude that the primary effect of lincomycin is an increase in the rate of synthesis of CT per cell. Earlier, we reported that *E. coli* LT, which is very similar both structurally (2) and functionally (23) to CT, is also increased by growth of toxinogenic cells with lincomycin (14). The gene coding for LT was carried on a plasmid, and it was shown that lincomycin did not cause any change in copy number of this plasmid. Assuming that enhanced synthesis of LT and CT involves a common mechanism, it appears that growth with lincomycin results in altered regulation of CT synthesis at either the transcriptional or the translational level.

Others have reported that the synthesis by gram-negative bacteria of proteins destined for export is different from the synthesis of cytoplasmic proteins. Hirashima et al. (10) described differential inhibition of the synthesis of *E. coli* envelope proteins, compared with synthesis of cytoplasmic proteins, by a variety of ribosome-specific antibiotics. The average half-life of envelope protein mRNA's was also found to be significantly greater than that of cytoplasmic protein mRNA's. Randall and Hardy (20) found that exported proteins of *E. coli* were synthesized on membrane-bound polysomes which were more resistant to puromycin than free polysomes, and which were associated with mRNA of greater stability than the mRNA of free polysomes. It appears that CT is a periplasmic protein (see below), and lincomycin is ribosome specific (18), so there is a clear similarity between lincomycin stimulation and these other observations. However, the large increases seen for CT may well reflect a very different mechanism than the differential inhibition of synthesis of these other proteins, particularly since the latter effects are short-term ones seen under conditions strongly inhibitory to growth, whereas lincomycin stimulation is a long-term effect of low concentrations of the drug which alter growth kinetics only slightly.

From Table 1, about 13% of the total CT in lincomycin-stimulated cultures at 22 h was cell associated and could be released by sonication, extraction with DOC or digestion by polymyxin B. That this antigen was CT and not subunit B was demonstrated by PAGE (Fig. 2). We infer that most or all of this bound toxin was periplasmic, since it was released much more quickly by polymyxin B than was β-galactosidase, which appeared to be cytoplasmic as in *E. coli* (Table 2). This would further extend the analogy between CT and LT, which is also thought to be periplasmic and which can also be released by polymyxin B (4). With control cells, released material antigenically related to CT was also detected after sonication or after DOC or polymyxin B treatment, but at about a fourfold-lower concentration than was seen with stimulated cells (Table 1).

Holmes et al. have also reported the release by sonication of low concentrations of CT-related antigen from *V. cholerae* 569B (11), and Fernandes and Smith described the extraction of biologically active CT with DOC, although this was found only for aerobic growth at 37°C and not 30°C (5). However, Ohtomo et al. (16) found by immunoassay that sonication of *V. cholerae* 569B released large amounts of CT subunit A but less than 6% as much, by weight, of subunit B. Extraction of insoluble debris from

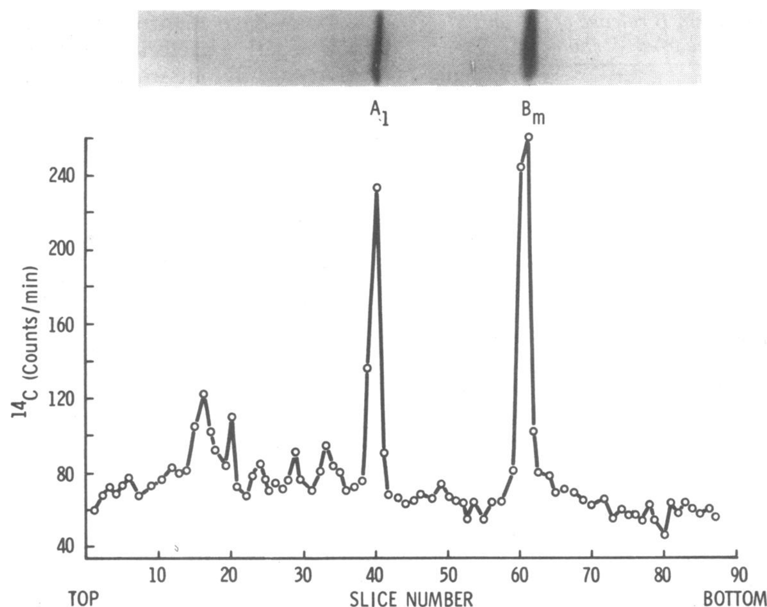

FIG. 3. Preparation of ^{14}C-labeled CT. V. cholerae 569B was grown in TRY medium containing 0.25 μCi of ^{14}C-labeled L-amino acid mixture (approximately 2 mCi per mg of amino acid) (New England Nuclear Corp., Boston, Mass.) per ml for 72 h at 30°C. Cells were removed by centrifugation and filtration, and ^{14}C-labeled CT was partially purified from the filtrate by precipitation with sodium metaphosphate (21). A portion of the ^{14}C-labeled CT was reduced, heated, and subjected to SDS-PAGE (see Materials and Methods), with purified unlabeled CT in an adjacent lane. The lane containing ^{14}C-labeled CT was then cut into 1-mm slices, which were agitated in Soluene (Packard Instrument Co., Rockville, Md.) for 4 h at 37°C and then counted in Instagel (Packard) in a scintillation spectrophotometer. The adjacent lane was stained as usual (inset). Specific activity of the ^{14}C-labeled CT was estimated to be 0.14 μCi/mg.

TABLE 3. *Absence of detectable degradation of ^{14}C-labeled CT by noninduced V. cholerae*[a]

^{14}C-labeled CT incubated with:	Time of incubation (h)	^{14}C recovery (cpm)	
		A_1	B_m
Sonicated whole culture	0	180	461
	1.0	173	454
	3.5	185	459
Sonicated cells	0	152	360
	1.0	160	402
	3.5	173	398

[a] *V. cholerae* 569B was grown at 30°C in TRY medium, with linear shaking, for 22 h. Duplicate cultures were pooled, and a portion was then sonicated as described for Table 1. The rest of the pooled cultures was centrifuged, and the pelleted cells were resuspended in 1/50 of the original volume and sonicated. One-fifth volume of ^{14}C-labeled CT (see Fig. 3) was then added, and the mixtures were incubated at 30°C for 1 or 3.5 h. Additional nonradioactive CT was then added (50 μg/ml) to each sample as an electrophoretic marker, and samples were heated, reduced, and subjected to SDS-PAGE. Portions of the stained gels corresponding to the positions of CT subunits A_1 and B_m were cut out, and radioactivity was measured as for Fig. 3. Data have been corrected by subtraction of a background of 50 cpm.

sonicated cells with SDS yielded about three times as much subunit B as did the soluble fraction, and no more subunit A, although it seems possible that any subunit A might have been rendered nonantigenic by SDS. The total mass of cell-associated antigen was nevertheless at least 90% subunit A. Because of our low yields of intracellular antigen from cells grown without lincomycin, we have not yet characterized it to the same extent as the intracellular CT from lincomycin-stimulated cells, and it may be that the intracellular data of Table 1 for unstimulated cells reflect subunit B or other CT-related antigen rather than whole CT, as shown for stimulated cells (Fig. 2).

Preliminary estimations of periplasmic subunit A concentrations by immunodiffusion with purified anti-subunit A antibody, generously provided by N. Ohtomo, show that, in contrast to whole CT, there is little or no change in the concentration of subunit A for lincomycin-stimulated cells compared with parallel cultures grown without drug (data not shown). These data include both free subunit A and subunit A in CT. It is possible that subunit A is normally present in much greater quantities than is sub-

unit B, that synthesis of B but not A is normally at a repressed rate which is somehow derepressed by lincomycin, and that essentially all subunit B is quickly converted intracellularly to CT by the excess of subunit A.

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LITERATURE CITED

1. **Cerny, G., and M. Teuber.** 1971. Differential release of periplasmic versus cytoplasmic enzymes from *Escherichia coli* B by polymyxin B. Arch Mikrobiol. **78**:166-179.
2. **Dallas, W. S., and S. Falkow.** 1979. The molecular nature of heat-labile enterotoxin (LT) of *Escherichia coli*. Nature (London) **277**:406-407.
3. **Davis, B. J.** 1964. Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. **121**:404-427.
4. **Evans, D. J., Jr., D. G. Evans, and S. L. Gorbach.** 1974. Polymyxin B-induced release of low-molecular-weight, heat-labile enterotoxin from *Escherichia coli*. Infect. Immun. **10**:1010-1017.
5. **Fernandes, P. B., and H. L. Smith, Jr.** 1977. The effect of anaerobiosis and bile salts on the growth and toxin production by *Vibrio cholerae*. J. Gen. Microbiol. **98**: 77-86.
6. **Gill, D. M.** 1976. The arrangement of subunits in cholera toxin. Biochemistry **15**:1242-1248.
7. **Gill, D. M., and C. A. King.** 1975. The mechanism of action of cholera toxin in pigeon erythrocyte lysates. J. Biol. Chem. **250**:6424-6432.
8. **Gill, D. M., and R. Meren.** 1978. ADP-ribosylation of membrane proteins catalyzed by cholera toxin: basis of the activation of adenylate cyclase. Proc. Natl. Acad. Sci. U.S.A. **75**:3050-3054.
9. **Gill, D. M., and R. S. Rappaport.** 1979. Origin of the enzymatically active A_1 fragment of cholera toxin. J. Infect. Dis. **139**:674-680.
10. **Hirashima, A., G. Childs, and M. Inouye.** 1973. Differential inhibitory effects of antibiotics on the biosynthesis of envelope proteins of *Escherichia coli*. J. Mol. Biol. **79**:373-389.
11. **Holmes, R. K., M. L. Vasil, and R. A. Finkelstein.** 1975. Studies on toxinogenesis in *Vibrio cholerae*. III. Characterization of nontoxinogenic mutants *in vitro* and in experimental animals. J. Clin. Invest. **55**:551-560.
12. **Kennell, D., and B. Magasanik.** 1964. The control of the rate of enzyme synthesis in *Aerobacter aerogenes*. Biochim. Biophys. Acta **81**:418-434.
13. **Kurosky, A., D. Markel, B. Touchstone, and J. Peterson.** 1976. Chemical characterization of the structure of cholera toxin and its natural toxoid. J. Infect. Dis. **133**(Suppl.):S14-S22.
14. **Levner, M., F. P. Wiener, and B. A. Rubin.** 1977. Induction of *Escherichia coli* and *Vibrio cholerae* enterotoxins by an inhibitor of protein synthesis. Infect. Immun. **15**:132-137.

14a. **Lewis, A. C., S. H. Richardson, and B. Sheridan.** 1976. Biochemistry of *Vibrio cholerae* virulence: purification of cholera enterotoxin by preparative disc electrophoresis. Appl. Environ. Microbiol. **32**:288-293.

15. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 354. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Ohtomo, N., T. Muraoka, and K. Kudo.** 1977. Observations on intracellular synthesis of cholera toxin subunits, p. 414-423. *In* Proceedings of the 13th Joint Conference of the U.S.-Japan Cooperative Medical Science Program, Cholera Panel. DHEW publication no. (NIH)78-1590.
17. **Parton, R., and M. H. Jeynes.** 1975. Spheroplasts of *Vibrio cholerae* induced by polymyxin B and other membrane-active agents in the absence of lysozyme. Microbios **14**:103-119.
18. **Pestka, S.** 1974. The use of inhibitors in studies of protein synthesis. Methods Enzymol. **30**:261-282.
19. **Peterson, J. W., K. E. Hejtmancik, D. E. Markel, J. P. Craig, and A. Kurosky.** 1979. Antigenic specificity of neutralizing antibody to cholera toxin. Infect. Immun. **24**:774-779.
20. **Randall, L., and S. Hardy.** 1977. Synthesis of exported proteins by membrane-bound polysomes from *Escherichia coli*. Eur. J. Biochem. **75**:43-53.
21. **Rappaport, R. S., B. A. Rubin, and H. Tint.** 1974. Development of a purified cholera toxoid. I. Purification of toxin. Infect. Immun. **9**:294-303.
22. **Richardson, S. H.** 1969. Factors influencing in vitro skin permeability factor production by *Vibrio cholerae*. J. Bacteriol. **100**:27-34.
23. **Sack, R. B.** 1975. Human diarrheal disease caused by enterotoxigenic *Escherichia coli*. Annu. Rev. Microbiol. **29**:333-353.