

Properties of Homogeneous Heat-Labile Enterotoxin from *Escherichia coli*

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Recently, the heat-labile enterotoxin (LT) of *Escherichia coli* has been purified to homogeneity and partially characterized (Clements and Finkelstein, *Infect. Immun.* 24:760-769, 1979). This study extends our observations on the physicochemical properties of LT. The toxin has an isoelectric point of pH 8.0, as compared with cholera toxin and cholera toxin B, which have isoelectric points of pH 6.75 and 7.75, respectively. Sedimentation equilibrium measurements established an approximate molecular weight for LT of 91,440. LT had an even more marked affinity than cholera toxin for agarose-containing matrixes in gel filtration. Of several mono- and disaccharides tested, only galactose and lactose were highly efficient in removing ¹²⁵I-labeled LT from agarose-containing columns. LT dissociated into subunits (designated A and B) during gel filtration in the presence of 5 M guanidine. These subunits were immunologically distinct and possessed unique and shared antigenic determinants to the corresponding A and B subunits of cholera toxin. During gel filtration of LT at pH 6.5 and room temperature, a spontaneously occurring toxoid of LT, analogous to cholera toxin B, was discovered and designated "coligenoid." This product contains only the B subunits of the toxin. A partial amino acid sequence of the B subunit of LT revealed a remarkable homology to the primary structure of cholera toxin B. Within the first 20 amino acids of the two chains, only 5 differ, and these differences may be attributable to single base substitutions.

Since the first observations more than 10 years ago that *Escherichia coli* strains elaborate a cholera-like enterotoxin(s), investigators in laboratories all over the world, including our own, have attempted to isolate and characterize the responsible protein(s). An immunological relationship between the heat-labile enterotoxin (LT) of *E. coli* and the enterotoxin (cholera toxin) of *Vibrio cholerae* has been firmly established. More recently, it was demonstrated that LT has antigenic determinants in common with each of the isolated subunits (A and B) of cholera toxin (2, 3). It also appears that LT and cholera toxin produce diarrhea by the same basic mechanism, namely, activation of adenylate cyclase, followed by increases in intracellular levels of cyclic adenosine monophosphate (6).

We have recently isolated homogeneous LT which, when activated by protease treatment, has a specific activity equivalent to that of pure cholera toxin in several biological assays (4). The two enterotoxins have remarkable physicochemical similarities, as well as unique features.

This study extends our previous observations on the molecular nature of LT.

MATERIALS AND METHODS

Bacterial strain. The bacterial strain used, which we have employed previously (2-4), was *E. coli* 711 (F1LT) (*phe trp pro his Nx⁺ lac*), a transformed K-12 derivative bearing an LT gene(s) of the *Ent* plasmid from porcine strain P307 (kindly provided by S. Falkow).

Culture conditions and purification of *E. coli* LT. The culture conditions and purification of *E. coli* LT were as described previously (2).

Isoelectric focusing. Cholera toxin, cholera toxin B (both isolated as described previously [9]), and LT derived from a whole-cell lysate (4) were subjected to analytical electrofocusing on polyacrylamide gels (LKB ampholine polyacrylamide gel plates; LKB-Produkt AB, Bromma, Sweden) in the pH range from 3.5 to 9.5. Isoelectric focusing of 15 µg of each protein was begun at 50 mA and continued for 2 h at 4°C. The pH gradient was determined, and the gel was stained with 0.001% Coomassie brilliant blue R-250 (Sigma Chemical Co., St. Louis, Mo.). After destaining, the gel was sectioned and scanned at 595 nm with a Quick Scan densitometer (Helena Laboratories, Beaumont, Tex.).

Sedimentation equilibrium. Sedimentation equilibrium measurements were kindly performed by J. J. LoSpalluto in a model E analytical ultracentrifuge. Sedimentation equilibrium molecular weight deter-

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minations were carried out by the high-speed meniscus depletion method of Yphantis (24). Partial specific volume was calculated from the amino acid analysis (4).

Molecular weight determination by gel filtration. The molecular weight of LT also was estimated by gel filtration on a column (1.5 by 80 cm) of Ultrogel AcA 34 (LKB-Produktor AB) at 4°C. The AcA 34 column, which was equilibrated with TEAN (2) buffer (pH 7.5), was calibrated with proteins of varying molecular weights. Apoferritin (molecular weight, 480,000), aldolase (molecular weight, 158,000), bovine serum albumin (molecular weight, 68,000), and cytochrome *c* (molecular weight, 12,400) served as markers. LT and cholerae were chromatographed in the presence and absence of 0.2 M galactose. The molecular weight markers eluted at identical volumes when chromatographed in the presence and absence of 0.2 M galactose.

Amino acid analyses and sequencing. Amino acid analyses and sequencing were performed as described previously (14).

Preparation of antiserum to LT derived from whole-cell lysate. Goat antiserum to LT derived from whole-cell lysates (4) was prepared by immunization with 1.0 mg of purified antigen suspended in 5 ml of Freund complete adjuvant. The antigen was administered in each hindquarter subcutaneously on a single occasion. After 17 days, sera were collected weekly for 6 months and pooled. This antiserum had activity against both A and B subunits of LT, as demonstrated by both immunodiffusion and radioimmunoassay.

Preparation of *E. coli* enterotoxin subunits. The *E. coli* enterotoxin subunits (*E. coli* A and *E. coli* B) were isolated from LT derived from whole-cell lysate preparations by gel filtration at room temperature under dissociating conditions (14). Guanidine hydrochloride was added to 30 mg of LT (in 8 ml of TEAN) to a final concentration of 5 M. This preparation was placed on a Sephadex G-75 (Pharmacia Fine Chemicals) column (2.5 by 80 cm) equilibrated and eluted with 5 M guanidine hydrochloride. The *E. coli* A and B subunits were pooled separately and dialyzed against diminishing concentrations of urea in TEAN buffer.

Bioassay. Assays in mouse Y-1 adrenal cells were performed essentially as described by Sack and Sack (21). Specific activity is defined as the smallest amount of protein required for a clearly observable rounding response.

Preparation of ¹²⁵I-labeled LT. Radioiodinated LT was prepared by a modification of the method of Hunter (13), as described by Robertson and Cebra (20).

Immunodiffusion. Immunodiffusion experiments were performed in 1% Noble agar (Difco Laboratories) with 1% sodium azide added as a preservative. Immunodiffusion reactions were allowed to develop overnight at room temperature.

Protein determinations. Protein determinations were made by the method of Bradford (1). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was as previously described (4).

RESULTS

Isoelectric focusing of cholerae, cholerae-noid, and LT derived from whole-cell lysate was performed to determine the isoelectric point of each product (Fig. 1). The isoelectric points of cholerae (pH 6.75) and cholerae-noid (pH 7.75) were similar to previously reported values (19). LT derived from whole-cell lysate electro-focused at pH 8.0.

Sedimentation equilibrium measurements were performed to establish an approximate molecular weight for the *E. coli* holotoxin. By using the partial specific volume (\bar{v} 0.737) calculated from amino acid analysis, the molecular weight of LT derived from whole-cell lysate was determined to be 91,440. A plot of the log of the concentration as a function of the square of the distance from the center of rotation yielded a straight line (data not shown) consistent with the line expected for a homogeneous globular protein with no aggregate formation. Since this value is based on a calculated partial specific volume in which tryptophan is not recorded and alanine/cystine percentages are estimated, it can be regarded as a careful approximation only.

It was of interest to determine whether a similar molecular weight approximation could be obtained by gel filtration. LT has a remarkable affinity for agarose-containing gels, including AcA 34, to which it is tightly bound. It can be eluted with galactose, the monosaccharide component of agarose. When a column of AcA 34 was equilibrated with TEAN buffer containing 0.2 M galactose, LT eluted as a single 280-

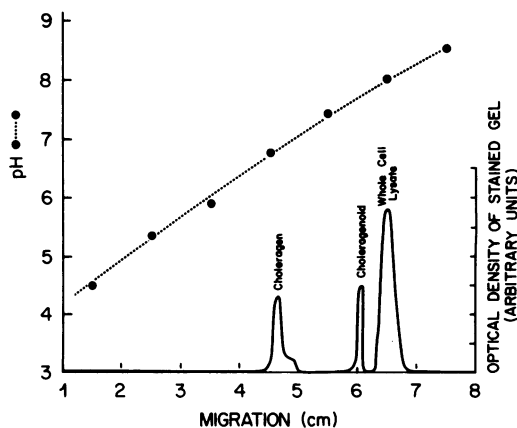


FIG. 1. Isoelectric focusing in polyacrylamide gels of cholerae, cholerae-noid, and LT derived from whole-cell lysate. The isoelectric points of cholerae (pH 6.75) and cholerae-noid (pH 7.75) were similar to previously reported values. LT derived from whole-cell lysate has an isoelectric point of pH 8.0.

nm-absorbing peak, consistent with a protein of molecular weight 25,000 when compared with several proteins of known molecular weights (see above). Under identical conditions of gel filtration, cholera toxin eluted just behind LT, at a molecular weight of 22,000, whereas on the same column in the absence of galactose, it eluted at a molecular weight of less than 12,000. These molecular weight values are considerably lower than those obtained by sedimentation analyses for LT and cholera toxin (19).

To investigate the specificity of the LT-agarose interaction, attempts were made to elute LT bound to agarose with a variety of sugars. As Table 1 shows, 96% of the bound radiolabeled LT eluted with 0.2 M galactose, and 87% eluted with 0.2 M lactose, a galactose-containing disaccharide. Other mono- and disaccharides tested (maltose, sucrose, glucose, and ribose) and bovine serum albumin released only approximately 30% of the bound toxin under identical conditions.

During gel filtration in the presence of guanidine (Fig. 2), LT dissociated into two peaks, designated A and B. The peaks were pooled separately, dialyzed against diminishing concentrations of urea in TEAN buffer, and then analyzed by immunodiffusion (Fig. 3). Antiserum to LT derived from whole-cell lysate (anti-LT) (Fig. 3A and B, wells 2) formed a single precipitin band with either *E. coli* A (Fig. 3A, center well) or *E. coli* B (Fig. 3B, center well). This antiserum also recognized LT holotoxin (Fig. 3A and B, wells 1) with a precipitin reaction that spurred

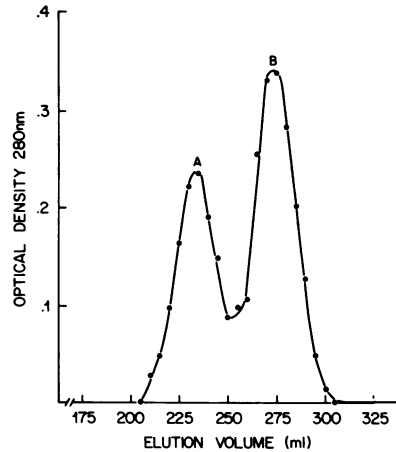


FIG. 2. Gel filtration elution profile of LT on a Sephadex G-75 column (2.5 by 80 cm) equilibrated with 5 M guanidine hydrochloride. Peak A represents the A subunit, and peak B represents the B subunit chain.

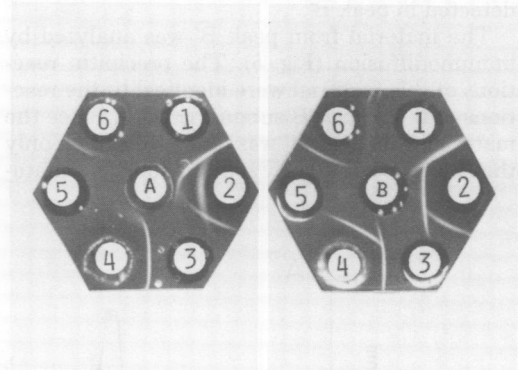


FIG. 3. Reactions of the isolated LT subunits *E. coli* A (A, center well) and *E. coli* B (B, center well) with monospecific antiserum to LT (wells 2), antiserum to cholera B (wells 4), and antiserum to cholera A (wells 6). Wells 1, 3, and 5 contained 10 μ g of LT, cholera B, and cholera A, respectively. (The reactions between anti-cholera A and *E. coli* LT and *E. coli* A were rather weak and did not photograph well.)

TABLE 1. Elution of radioiodinated LT from an agarose matrix with various mono- and disaccharides

Elution sugar ^a	% Of ¹²⁵ I-labeled LT released from the column ^b
Galactose	96.4
Lactose	87.2
Sucrose	33.1
Maltose	32.3
Ribose	30.7
Glucose	29.9

^a Concentration of each sugar, 0.2 M in TEAN buffer.

^b A 300-ng amount of ¹²⁵I-labeled LT (10⁶ cpm/ μ g of protein) in TEAN buffer was applied to separate columns (5 by 0.4 cm) of AcA 34. The columns were washed with five void volumes of TEAN buffer and essentially no radioactivity was eluted. The percentage of LT eluted by each sugar was calculated from the following formula: percent released = [(counts per minute eluted)/(counts per minute eluted + counts per minute remaining)] \times 100. Bovine serum albumin (1 mg/ml) also eluted approximately 30% of the radioactivity applied.

over the *E. coli* A-anti-LT (Fig. 3A) and *E. coli* B-anti-LT (Fig. 3B) reactions. The reaction between anti-LT and *E. coli* A (Fig. 3A) was clearly not identical to the reaction between anti-LT and the isolated B subunit of cholera toxin (Fig. 3A and B, wells 3), whereas the anti-LT-*E. coli* B reaction (Fig. 3B) showed partial identity with the reaction between anti-LT and cholera B. Antiserum to cholera B (Fig. 3A and B, wells 4) recognized cholera B (Fig. 3A and B, wells 3) but not cholera A (Fig. 3A and B, wells 5). It also showed a reaction of partial identity with

E. coli B (Fig. 3B) and did not recognize *E. coli* A in immunodiffusion (Fig. 3A). Antiserum to cholera A (Fig. 3A and B, wells 6) clearly recognized the homologous antigen (cholera A; Fig. 3A and B, wells 5) and recognized *E. coli* LT and *E. coli* A (Fig. 3A), although these reactions were rather weak. Anti-cholera A did not react visibly with *E. coli* B (Fig. 3B).

All of our previous purifications of LT were performed at 4°C at pH 7.5. However, after gel filtration of LT on Sephacryl S-200 in TEAN buffer (pH 6.5) at room temperature, two optical density peaks were observed (Fig. 4A). When the material from each peak was pooled and rechromatographed separately on the same column (Fig. 4B and C), the first peak (peak A') was shown to be holotoxin in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4) (Fig. 4, insert). The second peak (peak B') was found to contain a single protein-staining band that comigrated with the B region of LT in both unheated (Fig. 4, insert) and heated samples. No band corresponding to the *E. coli* A subunit was detected in peak B'.

The material from peak B' was analyzed by immunodiffusion (Fig. 5). The precipitin reactions of this material were identical to the reactions of the *E. coli* B subunit (Fig. 3). Since the material from peak B' was found to contain only the *E. coli* B subunit by sodium dodecyl sulfate-

polyacrylamide gel electrophoresis and showed a high degree of antigenic homology with the holotoxin and the B subunit, this material probably represents a spontaneously formed toxoid, which we here designate "coligenoid," analogous to cholera genoid, which is formed during purification of cholera toxin (11).

After trypsin treatment for maximal biological activation (4), the *E. coli* A and B subunits, coligenoid, and holotoxin were assayed for biological activity in the mouse Y-1 adrenal cell assay. Coligenoid and the B subunits were 3,000- to 1,000-fold less active than the holotoxin; the specific activities of these proteins were 136 ng, 39 ng, and 38 pg, respectively. The activities of coligenoid and the B subunits were at a level that could be consistent with trace contamination with holotoxin. The *E. coli* A subunit at levels up to 10 µg/well had no biological activity detectable in the mouse Y-1 adrenal cell assay.

The amino acid compositions of *E. coli* A, *E. coli* B, and coligenoid were determined and compared with the previously published compositions (14, 16, 17) of the A and B subunits of cholera toxin (Table 2). There were some notable differences in the compositions of the A subunits of the two toxins; for example, cholera A had a higher content of the acidic amino acids aspartic acid and glutamic acid and was lower in the hydrophobic amino acids valine, isoleucine, and

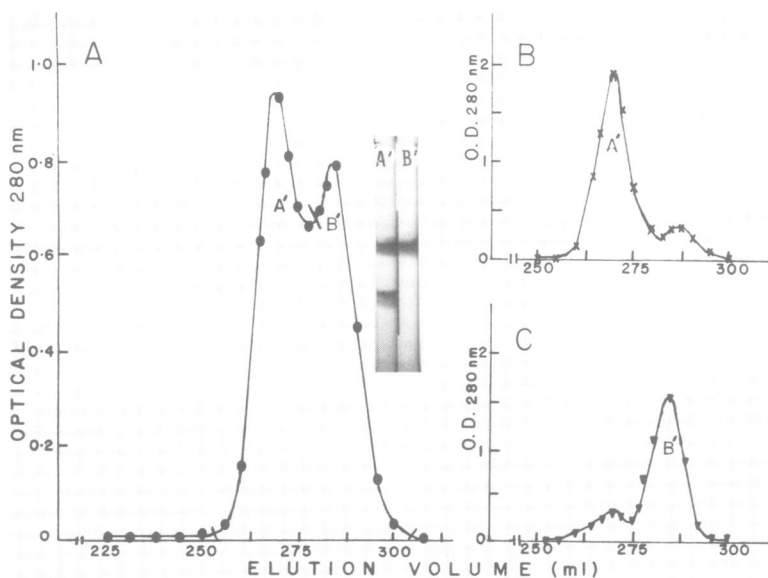


FIG. 4. Gel filtration profile of LT on a Sephacryl S-200 column (2.5 by 80 cm) in TEAN buffer (pH 6.5) at room temperature. (A) Profile of LT holotoxin. (B and C) Material from peaks A' and B', respectively, which was pooled as indicated (slashes in A) and rechromatographed separately on the same column. The inserts are the sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns (unheated samples) of the rechromatographed material from peaks A' and B'. O.D., Optical density.

leucine than *E. coli* A. It also had a lower content of tyrosine and methionine. As expected from the data shown above, the amino acid compositions of coligenoid and *E. coli* B were

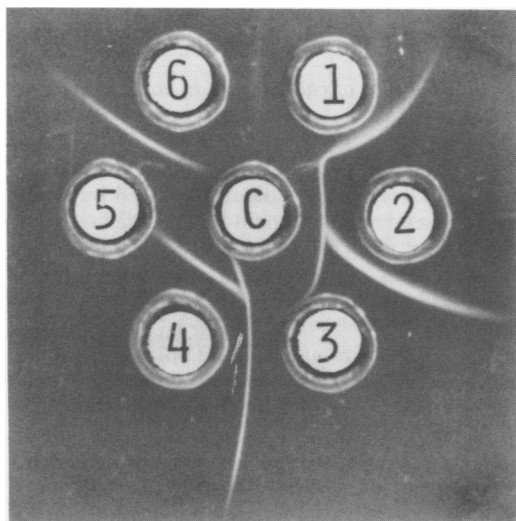


FIG. 5. Reaction of 10 µg of coligenoid (center well) with monospecific antiserum to LT (well 2), antiserum to cholera B (well 4), and antiserum to cholera A (well 6). Wells 1, 3, and 5 contained 10 µg of LT, cholera B, and cholera A, respectively.

identical, and these were remarkably similar to the composition of the cholera B subunits, except for the alanine/cystine content.

A comparison of the partial sequence of the B chain of *E. coli* LT with the primary structure of the cholera toxin B chain (14, 16) reveals remarkable similarities (Fig. 6). Of particular significance are the cystine found in position 9 of both molecules and the observation that in the first 20 amino acids of the two chains, there are only 5 amino acid differences. Each of these differences may be attributable to a single base substitution.

DISCUSSION

We have recently described the first purification to homogeneity and some of the physicochemical properties of *E. coli* LT with high specific activity (4). The present study more completely characterizes the *E. coli* holotoxin and further examines the molecular nature and subunit structure of the molecule, with particular regard to the relationship of the subunits of *E. coli* LT to the subunits of cholera toxin.

LT was shown to have a pI of 8.0 by isoelectric focusing and to have an approximate molecular weight of 91,440 by sedimentation equilibrium determination.

As reported previously (4), LT interacts firmly

TABLE 2. Amino acid compositions of the subunits of *E. coli* LT from whole-cell lysate and cholera toxin

Amino acid	No. of residues per molecule in: ^a				
	Cholera A ^b	<i>E. coli</i> A	Cholera B ^c	<i>E. coli</i> B	Coligenoid
Aspartic acid/asparagine	36	21	11	9	9
Threonine	10	11	10	11	11
Serine	24	15	5	6	7
Glutamic acid/glutamine	29	8	12	11	11
Proline	15	17	3	3	3
Glycine	29	29	3	3	3
Alanine/cystine	17	21	13	5	5
Valine	6	11	4	3	3
Methionine	6	14	3	4	4
Isoleucine	8	17	10	10	10
Leucine	17	22	6	5	5
Tyrosine	16	22	3	4	4
Phenylalanine	8	9	3	2	2
Histidine	11	9	4	2	2
Lysine	6	4 ^d	9	9	9
Arginine	19	24 ^d	3	5	5
Tryptophan	ND ^e	ND	1	ND	ND

^a Numbers of residues per molecule are given to nearest whole number. With *E. coli* subunits, calculations were based on the assumption that the unit molecular weight was nearly equivalent to the size as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4). The molecular weights, excluding tryptophan (except in case of cholera B) were as follows: cholera A, 28,571; *E. coli* A, 28,720; cholera B, 11,590; *E. coli* B, 10,652; coligenoid, 10,739.

^b From Klapper et al. (14).

^c From Kurosky et al. (16).

^d Calculated from the amino acid composition of the holotoxin (4) (assuming a formula of A₁B₆).

^e ND, Not determined.

		1									10
Cholera B:	NH ₂	Thr	Pro	Gln	Asn	Ile	Thr	Asp	Leu	Cys	Ala
<i>E. coli</i> B:	NH ₂	<u>Ala</u>	Pro	Gln	<u>Thr</u>	Ile	Thr	<u>Glu</u>	Leu	Cys	<u>Ser</u>
		11									20
Cholera B:		Glu	Tyr	His	Asn	Thr	Gln	Ile	His	Thr	Leu
<i>E. coli</i> B:		Glu	Tyr	His	Asn	Thr	Gln	Ile	His	Thr	<u>Ile</u>

FIG. 6. Comparison of partial amino acid sequences of the B chains of cholera and *E. coli* enterotoxins. Underlined *E. coli* B residues differ from cholera B residues.

with gels containing agarose, a polymer of galactose. In the present study, only galactose and lactose (a disaccharide containing galactose) were effective in eluting LT from agarose gels; other sugars tested (maltose, sucrose, glucose, and ribose) did not release bound LT as effectively. Other investigators have shown that, like cholera, LT interacts with GM1 ganglioside (22). The binding of LT to agarose may reflect the natural affinity of the B (binding) region of LT for that galactose-containing glycolipid. Our present observations offer reasonable explanations for previous reports of low-molecular-weight LT (7, 8, 23). It is clear that size approximations by gel filtration may be quite erroneous. In the present study, LT with a molecular weight of 91,440 eluted from agarose, even in the presence of 0.2 M galactose, as a protein of 25,000 molecular weight. It is also not unlikely that under different elution conditions, the B region may remain firmly attached to the gel matrix while allowing dissociation of the non-covalently associated A subunit, which might express some biological effects with low specific activity in some systems. The low-molecular-weight LT isolated by Takeda et al. (23), for example, appears to be similar, if not identical, to our A subunit.

Like cholera, LT can be dissociated into its A and B regions by a chaotropic reagent, such as guanidine. Antiserum against LT holotoxin recognized both A and B subunits of LT with reactions of nonidentity. The isolated subunits are immunologically related but not identical to the corresponding subunits of cholera. These antigenic differences probably reflect differences in the amino acid compositions and primary structures between the analogous subunits of the two toxins.

Finally, like cholera, *E. coli* LT can, under relatively mild conditions, spontaneously liberate an aggregate of its B subunits (coligenoid), which appears to be analogous to cholera, the "natural toxoid" of cholera enterotoxin, which was first discovered in our laboratory a little over 10 years ago (10).

Although evidence has been presented that

isolated cholera and cholera, it has been suggested by Lai et al. (18) and others, including ourselves, that cholera as synthesized by *V. cholerae* may actually have six B subunits and that one (or more) B subunit may spontaneously dissociate and reaggregate to form cholera. Consideration of the size of the LT B subunits (molecular weight, 10,700, by amino acid composition) and the variance in the size of the holotoxin (molecular weight, 91,440 [$\pm 5\%$; J. J. LoSpalluto, personal communication]) as determined by ultracentrifugation in the present study does not resolve conclusively the question of whether the molecular formula of *E. coli* LT is A₁B₅ or A₁B₆, but it is suggestive of the latter. Likewise, the pronounced similarities in the primary structures of the B subunits of LT and cholera do not answer the evolutionary question of whether the two toxins arose through genetic transfer followed by limited divergence or by extensive convergence from different proteins.

A subunit structure similar to that reported here and previously (4) was observed for radio-labeled LT produced in minicells (5). The LT described by Kunkel and Robertson (15), although having a similar subunit structure, differs in some respects (size, immunological reactivity, and reported amino acid composition) from that described here and previously (4). The differences may be attributable to the different strains used and raise the possibility that there may be some variation among LTs from different sources, although other explanations (e.g., post-translational modifications or a different number of B subunits) may be applicable.

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