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# Structure and Function of Cholera Toxin and the Related *Escherichia coli* Heat-Labile Enterotoxin†

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## INTRODUCTION

The disease we know today as Asiatic cholera was unknown in Europe before the early 19th century but was, in fact, known quite well to British colonists, sailors of many nations, and the population of southern India, for whom it was epidemic following each pilgrimage to the Ganges River (186). Cholera made its way across Turkey, into the Russias, and thence to Poland and central Europe in the late 18th and early 19th centuries. The disease entered western Europe through Marseilles in 1830 and spread to Paris, where an epidemic appeared with frightening intensity among all classes of people. Epidemics subsequently occurred throughout mainland Europe, Britain, and North and South America, spread by sailors and colonists. Cholera generated as much horror and revulsion among Europeans as bubonic plague had before it, in part because of the blue-black shriveled appearance of its victims and in part because it could strike anyone without warning and kill in 4 to 6 h.

Cholera remains an important problem in Third World nations and any area where water supplies can become

† This review is dedicated to the memory of Michael Gill. His untimely death cut short a career filled with seminal contributions to the field of toxin research.

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contaminated; we are, for example, currently witnessing a major ongoing epidemic in South America (108). In the United States, three or four cases are reported each year, some of which are caused by an endogenous *Vibrio* strain native to the southeastern United States (177). Although cholera is treatable with antibiotics and oral rehydration therapy (fluid and electrolyte replacement) (113), it is nevertheless an extremely debilitating and sometimes fatal disease. The severe dehydration and cramps symptomatic of the disease are a consequence of the rapid, extreme loss of fluid and electrolytes during the course of the infection. The diarrhea is caused by the action of cholera toxin (CT), secreted by the bacterium *Vibrio cholerae* (46, 59, 79), although in some cases it may be caused by the related *Escherichia coli* heat-labile enterotoxin (LT) (111, 238, 240). The nucleotide sequences of the two toxins are approximately 80% homologous (43, 193). *V. cholerae* and CT, however, are associated with severe, cramping diarrhea, whereas the *E. coli* enterotoxin designated LTh-I is responsible for a much milder disease in adult humans, known as traveler's diarrhea, "Montezuma's revenge," and other, more colorful epithets (238). A similar LT designated LTp-I causes diarrhea in pigs (119). The disparity between the severe, sometimes fatal symptoms of cholera and the relatively mild *E. coli*-associated disease may be related to differences in secretion and processing of the two toxins by the respective bacteria (223). In terms of human morbidity on a global scale, however, the *E. coli* diarrheas are many orders of magnitude more significant. As many as 50 to 90% of those who develop nonfebrile diarrhea on arrival in the Middle East, Southeast Asia, and South America can be affected by LT (238). There are several variations in primary structure (Fig. 1) between CT and LTh-I (43) and between LTh-I and LTp-I (90, 296), with consequent differences in antibody-binding interactions (154) and receptor specificities (87), but the three-dimensional structures of the toxins are believed to be essentially the same (255). The high degree of similarity among these toxins permits discussion of LTh and LTp together as "LT," and they will therefore be considered together in this article unless otherwise specified. Much of the description of CT is equally valid for LT as well. Van Heyningen (288) has provided a brief comparison of the structure and function of CT and LT, and Finkelstein (72, 73) has reviewed CT and LTs together.

CT and the related *E. coli* LT are heterohexameric proteins ($M_r$, 84,000) (193) produced in the intestinal lumen by the respective bacteria. The toxins consist of an A subunit separated from the plane of a ring formed by five smaller, identical, B subunits (93, 99, 164, 219, 255) (Fig. 2 and 3). The B pentamer binds ganglioside $GM_1$ in the membrane of intestinal epithelial cells or any other cell that contains $GM_1$ (286). Subunit A is inserted into the cytosol, activated by reduction of a disulfide bond (104, 192, 274), and associated with one or more cytosolic factors (103, 148, 150, 152, 205, 210, 277–280). The A1 fragment released by reduction is capable of binding NAD (28, 89) and catalyzing the ADP-ribosylation of $G_{s\alpha}$, a GTP-binding regulatory protein associated with adenylate cyclase. The result is a sharp increase in cyclic AMP (cAMP) production (29, 68, 95, 105). The consequence of this or other factors, such as increased synthesis of prostaglandins (226, 227) by the intoxicated cell, is excessive accumulation of salt and water in the intestinal lumen and cell death (68, 95). The symptoms are manifested as severe cramp and the copious "rice-water" diarrhea characteristic of the disease. Neither subunit A nor the binding oligomer (B pentamer) is cytotoxic alone. Although subunit A, suitably activated, is enzymatically functional in the absence of B pentamer (287), the pentamer is required for receptor binding and association with the target cell (286). Once in the cytosol, subunit A cannot infect another cell and is therefore lost when the cell dies and is sloughed. The B pentamer alone has no toxic activity (77), and the disease is therefore self-limiting as a result of the nature of the structure and function of the toxin. Symptoms are pepetuated by the production and secretion of toxin into the intestinal lumen by the bacteria until they are washed out of the gut.

This article reviews recent information about CT and LT structure and physical properties, interactions with membranes, receptor binding, translocation, enzymology, and immunology. It also discusses effects of CT on cells and substrates not involved in the disease process. My purpose is to describe the physical and biochemical properties of CT and LT and relate those properties to molecular structure. I hope this information will provide a framework for those wishing to understand the role of the toxins in the disease process and provide necessary information for those who use CT as a tool for studies of other systems.

## OVERVIEW OF MICROBIAL TOXINS

*E. coli* produces several other toxins, including a heat-stable peptide toxin (ST) known to cause diarrhea (47, 256), but these are not related to CT and are therefore not described here. The bacterium also produces other LTs, LT-IIa and LT-IIb (228, 229), that are not neutralized by antisera against the type I toxins CT, LTh-I, and LTp-I (130). The nucleotide sequence of LT-IIa (229) was found to be only 57% homologous with that of the A subunit of LTh-I and 55% homologous with the A gene of CT. Significantly, the B gene of LT-IIa was not homologous with that of LT-I or CT. Thus LT-IIa and LT-IIb are probably not closely related to LT-I and CT, and they are therefore not considered further in this review.

A new enterotoxin has recently been isolated (67) from attenuated strains of *V. cholerae* specifically mutated in genes encoding CT. This new toxin affects intestinal tight junctions, causing a mild diarrhea. It is unrelated to CT.

Structurally similar protein toxins are produced by a diverse group of bacteria (96, 194). Purification and assay protocols for many of these toxins have been reviewed (121). Table 1 lists some of the more common members of this group and briefly outlines their structures and functions. There are, in addition to those listed and *E. coli* ST, several streptococcal and staphylococcal toxins (273) and the ADP-ribosylating toxin botulinum C3 (4, 231, 239), whose functional domains are less well characterized. A toxin from *Campylobacter jejuni* (197) has been reported to be similar to CT and LT, but these observations remain to be confirmed. Ricin, a plant-derived toxin with A-B structure, is functionally and structurally similar to Shiga toxin and the Shiga-like toxins of bacterial origin (63, 64, 220). The toxins in this group are composed of discrete subunits or domains: a subunit or domain (A) with a specific enzymatic function and a binding domain, subunit, or oligomer (B) that interacts with a cell membrane receptor. Some of the toxins are plasmid borne, whereas others are integrated into the bacterial genome. It is clear that there is considerable overlap among the various toxins. Their common features give rise to speculation about their origins and evolutionary relationships, while their differences, some subtle and others more obvious, present an opportunity to extract information about

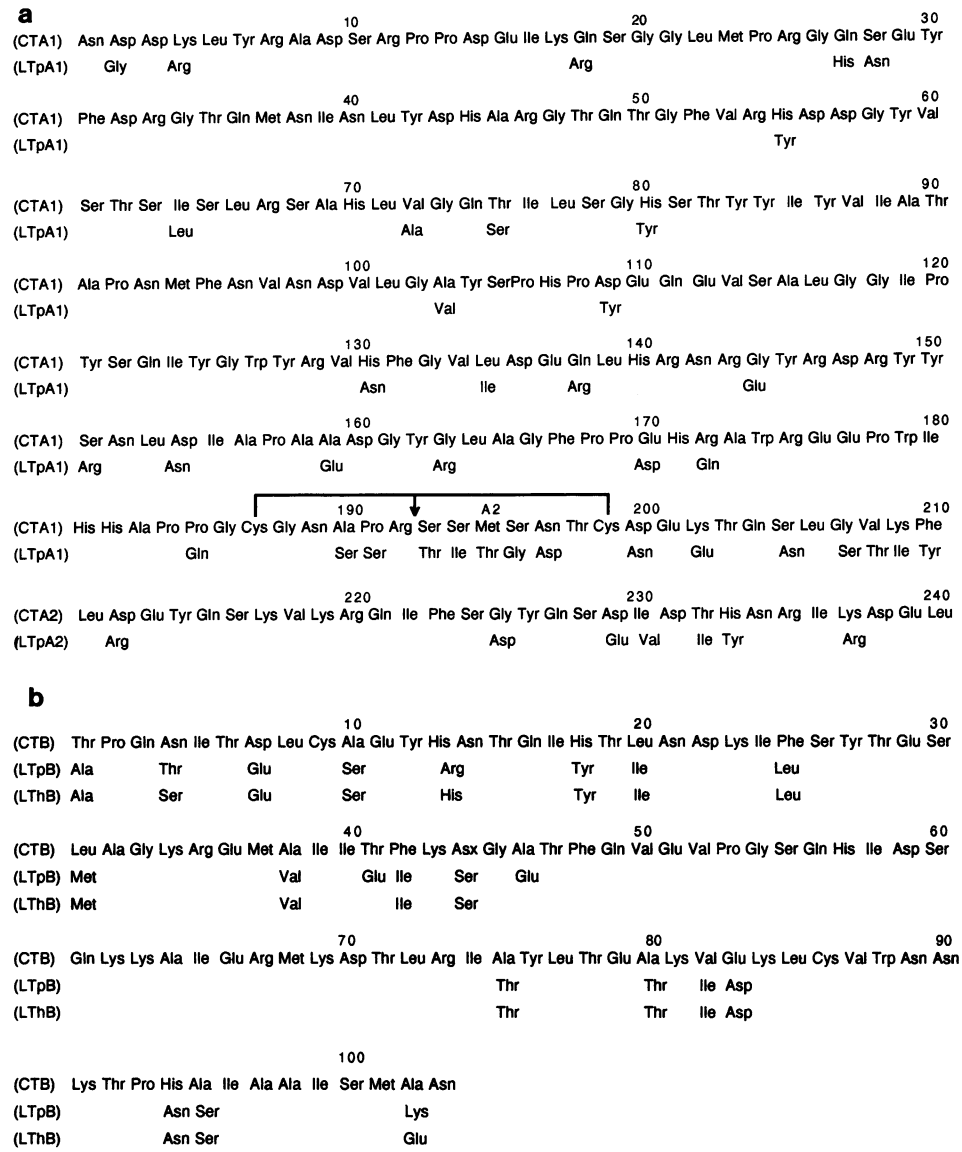


FIG. 1. (a) Amino acid sequence of CTA strains 2125 and 569B, which have identical sequences (73, 165, 176, 193). Amino acid substitutions found in porcine LTpA are given beneath the CTA sequence (62, 90, 295). The arrow indicates the tryptic cleavage site. (b) Amino acid sequences of CTB (162, 163, 193, 270). The sequences of strains 569B and 62746 are identical. Strain 3083 has B:Gly-54. Strain 2125 substitutes B:Tyr-18, Ile-47, and Gly-54 (45a, 69a, 270). B:Asx-44 is 50% deamidated; B:Asn-20 and B:Asn-70 in the nucleotide sequence are Asp in the peptide sequence of 569B (270). Amino acid substitutions found in porcine (LTp) and human (LTh) LTs are given beneath the CTB sequence.

the relationship between structural variations and functional modifications. Analysis of their three-dimensional structures will provide additional information about protein folding, subunit interactions, and interactions with ligands, as well as hints about evolutionary relationships and recombinations (Table 1).

## History and Genetics of Cholera

During a localized London epidemic in 1854, John Snow, the queen's physician, demonstrated that all the cases of cholera in a district of central London could be traced to a single contaminated well (186), marking one of the earliest epidemiological studies. The causative organism, *V. cholerae*, was described by Pacini in 1854 (137, 221) and isolated in pure culture from an Egyptian stool sample by Robert Koch 30 years later (137, 159, 159a). It was not until 1959, in Calcutta, that a cell-free culture filtrate was shown to be capable of producing massive accumulation of characteristic "rice-water" fluid in the ligated ileal loops of adult rabbits (46). In the same year, Dutta and colleagues (59) in Bombay described the production of diarrhea in infant rabbits by a crude protein isolate from *V. cholerae* culture filtrate.

The vibrio presents several serotypes, which have been reviewed by Marchlewicz and Finkelstein (183) and Finkelstein et al. (75). Epidemic cholera is caused by two biotypes of *V. cholerae* O group 1, the El Tor biotype and the classical biotype, each containing two serotypes, Ogawa and Inaba.

FIG. 2. Ribbon plot showing the $AB_5$ complex of LT. Note the free end of the small helix and the tail of the C terminus of the A1 subunit (center, base of pentamer). This reaches the surface of the B pentamer and may interact with the membrane surface. Residues 189 to 195 are missing in the top left corner of the figure, as they have not been clearly resolved in the structure determination. Reprinted by permission from *Nature* (**351**:371–377, 1991, copyright © 1992 Macmillan Magazines Ltd.) (255).

Epidemics after 1961 have been caused by El Tor vibrios, whereas epidemics before that time were associated with the classical biotype strains, especially Inaba 569B (97). The latter biotype is exceptionally good for production of the cholera enterotoxin. Strain 569B is therefore the vibrio routinely used in research laboratories and as a source of toxin.

CT is encoded by chromosomal genes in *V. cholerae* (193, 195, 289). *V. cholerae* strains of the classical biotype (e.g., strain 569B) contain a nontandem chromosomal duplication of the *ctxAB* operon that is structurally identical in all strains (187). In contrast, most of the El Tor strains, such as strain 3083 (75), have only a single copy of *ctxAB*, the remainder having two or more copies present on a tandemly repeated genetic element (193). The first two nucleotides of the *ctxA* translation termination signal are the last two nucleotides of the *ctxB* translation initiation triplet. Although this type of coupling is found between cistrons whose gene products interact in a one-to-one stoichiometry, CT has one A and five B subunits. Fusion of the *ctxB* gene to various *E. coli* promoters, however, allows high expression of the B subunit, suggesting that translation of *ctxB* relies on independent initiations promoted by its own ribosome-binding site. The association of the *ctxB* mRNA with ribosomes was reported to be ninefold higher than the association of *ctxA* mRNA with ribosomes (193). Studies of mutant and wild-type biotypes (196) indicate that synthesis of CT is controlled by regulation of transcription of the CT operon and suggest that differing efficiencies of initiation of transcription of the mRNA sequences coding for the A and B subunits may also contribute to the appropriate 1:5 molar ratio required for assembly of holotoxin. Taken together, these data may also account for the excess B pentamer secreted by the vibrio.

Cholera vibrios elaborate a substantial number of virulence factors in addition to toxin, such as pili, a polar sheathed flagellum (170), and several hemagglutinins which could serve as adhesins (76). They produce a protease (*V. cholerae* HA/protease) capable of nicking, and thus activating, CT (16) and other factors, such as fimbriae, associated with virulence (188). The genomic *toxR* gene in the vibrio encodes a protein (ToxR) that coordinately controls the expression of more than 17 of these virulence genes, including the *ctxAB* gene encoding CT (50). The regulatory cascade activated by ToxR has recently been extended to include several other associated genes (49).

### History and Genetics of *E. coli*-Associated Diarrhea

Certain serotypes of *E. coli* cause diarrhea in swine, and the responsible toxin, LT, was identified in 1969 (119). Shortly thereafter, during a major diarrheal epidemic in Calcutta, Gorbach et al. (111) and Sack et al. (240) isolated a single specific serotype of *E. coli*, rather than the expected cholera vibrio, from the bowels of many patients. An epidemiologic study (238) demonstrated that *E. coli* O148K/H28 was responsible for the disease known as "travellers' diarrhoea" or the "Aden trot" among British soldiers in Aden. These pathogenic *E. coli* serotypes were found to elaborate an enterotoxin with activity very similar to that of CT (37, 105). The two toxins are closely related immunologically, and cholera antitoxin neutralizes the ability of LT to cause fluid accumulation in the ileal loop test (119).

In contrast to CT, the gene encoding LT in *E. coli* is plasmid borne (44, 257). Many of the enterotoxin plasmids found in *E. coli*, including the LT and the $ST_A$-LT plasmids of human origin, also code for production of adhesive factors such as colonization factor antigens (256). By analysis of deletion mutants (44) the genes for the LT subunits, LTA and LTB, termed *eltA* and *eltB*, were found to be transcribed into a single mRNA. Sequencing of the LT region (261) demonstrated an overlap of *eltA* and *eltB*. As with the CT gene products, expression of excess B over A subunits might be the result of the LTB portion of the mRNA having a more efficient rRNA-binding site. A strain with cloned LTB alone produced more B subunit than did a strain carrying a plasmid with the cloned LTA and LTB genes (241), giving some support to this hypothesis.

The remarkable structural and functional similarities between LT and CT suggest that they may be evolutionarily related (98). An examination of synonymous nucleotide sequence substitutions in strains of CT, LT, and ST (295) provides evidence to postulate that the LT gene is a foreign one acquired by *E. coli* and transmitted by species-to-species transfer of pathogenic determinants from the *V. cholerae* CT gene ancestor (295).

Recent structural data have demonstrated an intriguing and striking similarity between the three-dimensional structure of the B oligomer of verotoxin (264) and that of the B oligomer of LT (255), despite a complete lack of primary sequence homology. Further analysis of the relationship between structure and function and perhaps a reevaluation of the relationship between primary sequence and three-dimensional structure may be in order, especially as more toxin structures become available.

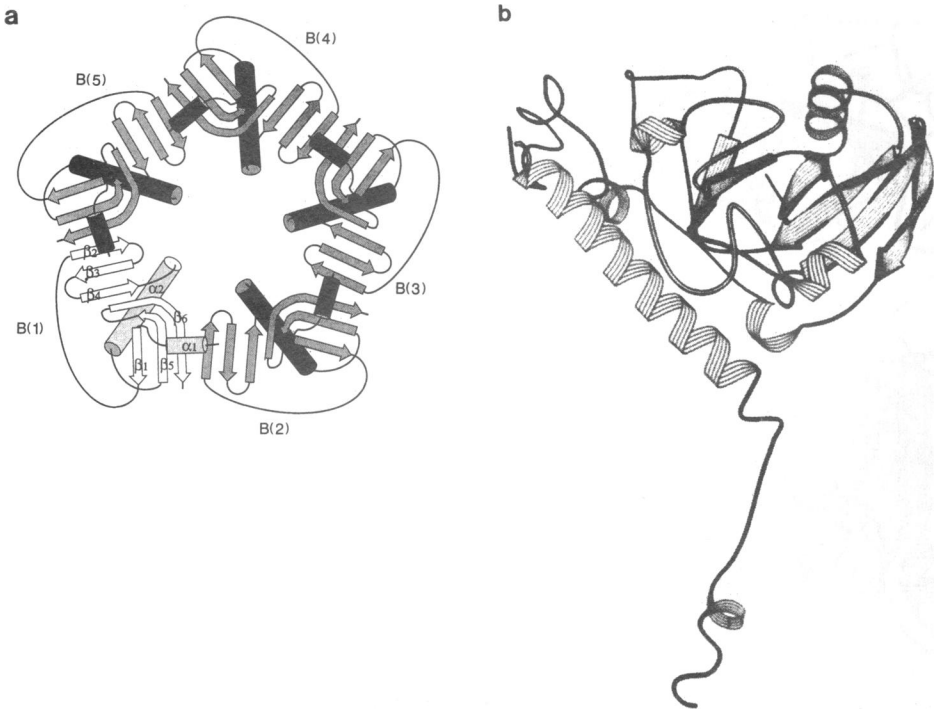


FIG. 3. (a) Schematic secondary-structure diagram (projection) of the B pentamer. A single monomer is depicted in light grey. Reprinted by permission from *Nature* (**351**:371–377, 1991, copyright © 1991 Macmillan Magazines Ltd.) (255). (b) Ribbon diagram of the A subunit showing the A1 subunit folds and the long helix of A2, terminated by a small single helix and tail. Residues 189 to 195 at the upper left corner are not shown, as they are not clearly defined by the crystallographic data. These residues span the proteolytic cleavage site between A1 and A2. Reprinted by permission from *Nature* (**355**:561–564, 1992, copyright © 1992 Macmillan Magazines Ltd.) (254).

## ASSEMBLY AND SECRETION

CT is an oligomeric protein toxin which is secreted across the bacterial outer membrane into the extracellular environment. In contrast, its homologous counterpart, LT, remains within the *E. coli* periplasm (126). Expression in *E. coli* of a plasmid-borne CT gene resulted in the assembled CT holotoxin remaining within the *E. coli* periplasm (91, 126, 128, 223), while expression of recombinant plasmid-borne LT in a genetically engineered *V. cholerae* strain resulted in secretion of the LT holotoxin (126, 215). These observations suggest that a requirement for specific secretory machinery is present in *V. cholerae* but absent from *E. coli*. Genetic evidence for this secretory apparatus in *V. cholerae* has been provided by Holmes et al. (131), who found a mutant of *V. cholerae* which was defective in CT secretion and which accumulated the holotoxin within the periplasm (125).

The secretion of enterotoxin subunits from the periplasmic compartment of *V. cholerae* was found to be specific and was not accompanied by the release or leakage of other periplasmic proteins (124, 125). The vibrio normally secretes both holotoxin and pentameric B subunit alone, known as choleragenoid (77). Hirst et al. (126) showed that expression of the B-subunit pentamer of either CT or LT by an $A^-B^+$ strain of *V. cholerae* resulted in the efficient secretion of pentamer into the medium, although all of the A subunits synthesized by an $A^+B^-$ strain of *V. cholerae* remained cell associated (126). This finding suggests that the differences in amino acid sequence between CT and LT do not result in an inability of the LT B oligomer to engage the secretory apparatus. The observation that A is not secreted in the absence of B implies that although the B oligomer can be secreted independently of A, assembly into holotoxin may be required for the translocation of A. Inquiry into the mechanism of secretion may enhance our understanding of the mechanism of binding and translocation of toxin into a target cell. A more detailed discussion of the export, assembly, and secretion of oligomeric toxins, particularly CT and LT, can be found in an extensive review by Hirst (123).

## ISOLATION AND PURIFICATION

The pandemics of the 1960s and early 1970s in Southeast Asia initiated an interest in the pathogenesis of the disease. Finkelstein et al. (77–79) isolated and purified a protein that was secreted by the vibrio and showed that the protein enterotoxin induced fluid accumulation in isolated ileal loops of guinea pigs. Initial purification involved an ammonium sulfate precipitation and ion-exchange chromatography. Membrane filtration steps followed by gel filtration first on Sephadex G-75, then on agarose and then on Sephadex G-75 again were subsequently substituted.

Purification led to the identification of two factors with differing biological activities (77). One, the active principle (denoted choleragen), is in fact the $AB_5$ form of the toxin, containing both the enzymatically active cytotoxic A subunit and a pentamer ($B_5$) of identical subunits that binds membrane receptors. The second factor, $B_5$ oligomer, is known as choleragenoid because it is not cytotoxic in itself.

TABLE 1. Some bacterial protein toxins having A-B structure

| Toxin (bacterial source) | Structure and function | References |
|---|---|---|
| Cholera toxin (*V. cholerae*) | Heterohexamer ($AB_5$); A = 27 kDa; B = 11.6 kDa; ADP-ribosylation of 42-kDa $G_{s\alpha}$ subunit of adenylate cyclase and other $G_\alpha$; elevated cAMP; severe watery diarrhea | |
| Heat-labile enterotoxin (*E. coli*) | Heterohexamer ($AB_5$); A = 27 kDa; B = 11.6 kDa; ADP-ribosylation of 42-kDa $G_{s\alpha}$ subunit of adenylate cyclase; elevated cAMP; watery diarrhea | |
| Pertussis toxin (*Bordetella pertussis*) | Heterohexamer (A = S1; B = S2–S5); S1 = 26 kDa; S2 = 22 kDa; S3 = 22 kDa; S4 (×2) = 12 kDa each; S5 = 11 kDa; ADP-ribosylation of 41-kDa $G_{i\alpha}$ subunit of adenylate cyclase and other $G_\alpha$; elevated cAMP in previously activated cells; variety of cellular effects | 22, 212, 244, 271 |
| Diphtheria toxin (*Corynebacterium diphtheriae*) | A-B polypeptide; 60 kDa; A = N-terminal domain; ADP-ribosylation of EF2; inhibition of protein synthesis; necrosis | 26, 31 |
| *Pseudomonas* exotoxin A (*Pseudomonas aeruginosa*) | A-B polypeptide; 66 kDa; A = C-terminal domain; ADP-ribosylation of EF2; inhibition of protein synthesis; necrosis | 5, 18, 27 |
| Shiga toxin (*Shigella dysenteriae*, *S. flexneri*, *S. boydii*, *S. sonnei*) | Heterohexamer ($AB_5$); A = 32 kDa; B = 7.7 kDa; removal of $Ad_{4324}$ in 28S rRNA; inhibition of protein synthesis; cell death; watery diarrhea; dysentery | 19, 64, 218 |
| Shiga-like toxins (verotoxins) (*E. coli*) | Heterohexamer ($AB_5$); A = 32 kDa, B = 7.7 kDa; removal of an adenine in 28S rRNA; inhibition of protein synthesis; cell death; watery diarrhea; renal disease | 19, 64, 218, 264 |
| Tetanus toxin (*Clostridium tetani*) | A-B polypeptide; 150 kDa; activation of protein kinase C; inhibition of presynaptic transmission in spinal cord motor neurons; paralysis and death | 237, 248, 251 |
| Botulinum toxin C1 (*Clostridium botulinum*) | A-B polypeptide; 150 kDa; blocks release of acetylcholine in peripheral nerves; death | 248–250 |
| Botulinum toxin C2 | ADP-ribosylation of nonmuscle actin; death | 3, 250 |
| Botulinum toxins A, B, and E | Inhibition of $Ca^{2+}$-dependent catecholamine secretion | 13 |
| Anthrax toxin (*Bacillus anthracis*) | Three independent proteins; A = LF = 89 kDa, A′ = EF = 83 kDa; B = PA = 82.7 kDa; elevated cAMP; EF is an adenylate cyclase; hemorrhagic effects, edema; death | 167, 168, 252 |
| Bt toxins (*Bacillus thuringiensis*) | Three or more independent proteins including one cytotoxic 28-kDa protein; death of specific insect larvae | 9, 292 |

The hypertoxinogenic strain 569B is universally used for the production of toxin. The vibrios are cultured in CYE medium (189), and crude CT is recovered from the culture supernatant by coprecipitation with hexametaphosphate. Following dialysis against phosphate and centrifugation to remove insoluble material, the toxin and choleragenoid are purified in a single step by ion-exchange chromatography on Whatman P11 phosphocellulose (190). If toxin substantially free of choleragenoid is required, the protein may be dialyzed and rechromatographed (189).

The isoelectric point (pI) of the purified choleragenoid (B oligomer) is 7.8 (78, 190, 260), whereas that of the holotoxin is 6.6 (78) or 6.9 (260). The molar absorptivity ($\varepsilon_{280}$) of cholera toxin is $9.6 \times 10^4$, and the $A^{1\%}$ is 11.41 in a 1-cm cell at 280 nm. The molar absorptivity of choleragenoid is $1.43 \times 10^4$, and the $A^{1\%}$ is 9.56. Measurements were made with the substances in 200 mM Tris-HCl (pH 7.5) (70).

LT was purified to homogeneity (36, 161) because, unlike CT and the B pentamer, which are merely retarded in their elution from agarose gel filtration columns, LT binds firmly to agarose. LT can then be eluted in nearly pure form by galactose or lactose (36), providing a single-step affinity procedure for LT based on its ability to be bound by the oligosaccharide receptor analogs. LTp (136) and LTh (90) were isolated from additional strains of *E. coli*. A one-step isolation of LT with $GM_1$ cellulose affinity chromatography was described by Hirst et al. (126).

## STRUCTURE AND PHYSICAL PROPERTIES

### Sequences

The complete amino acid sequence of the CT A subunit (193) is given in Fig. 1 for strains 2125 and 569B, shown with the differences found in LTp. The complete amino acid sequence of the B subunit is given for CT from strain 569B (162, 163, 270) and for LTp and LTh (based on nucleotide sequences) (43, 90, 261, 262). The original designation of methionine at position 43 in LTB was later found to be in error (73). As Fig. 1 demonstrates, there are variations between strains of CT and some heterogeneity between LTh and LTp. Analysis by fast-atom bombardment mass spectrometry, coupled with peptide isolation and Edman degradation of CT from strain 569B (270), confirmed the sequence of the B subunit, except for the amino acid residues at positions 22 and 70, which were identified as aspartic acid (Asp), not asparagine (Asn). The Asn at position 44 was partially deamidated to Asp: some peptides examined contained Asp, and others contained Asn. This observation may explain the isoelectric heterogeneity found in preparations of CT used for crystallization (see below). On the basis of the amino acid sequence derived from the nucleotide sequence, the A subunit has a molecular mass of 27,234 Da. The monomeric B subunit has a molecular mass of 11,677 Da; the pentameric binding oligomer therefore has a total molecular mass of 58,387 Da. The total molecular mass of the holotoxin, $AB_5$, is 85,620 Da. The A subunit genes from strains

2125 and 569B were completely sequenced and found to be identical; the B subunit genes were closely similar (193).

The plasmid-borne *E. coli* LT-I gene shares considerable structural homology with the chromosomal CT gene found in *V. cholerae* (Fig. 1). At the nucleotide level, A and B cistrons of the LT-I and CT operons are 75 and 77% homologous, respectively (43, 193). The amino acid sequences overall have been largely conserved between the two, with the differences scattered throughout the sequence except for the region around the cleavage site between subunits A1 and A2 (residues 192 and 195), where the homology drops to 33% between amino acids 189 and 212 (43, 62, 193, 261, 262, 295, 296).

### Crystallization and Heterogeneity

CT was crystallized for X-ray diffraction studies in 1977 (246), but neither Sigler and his group nor workers in other laboratories were able to reproduce the quality and size of those first crystals, despite efforts over a period of almost 10 years. The cause of their difficulty was found to be isoelectric heterogeneity in all available preparations of CT (259, 260), owing to deamidation in the B subunit monomers. Deamidation leads to two charge variants, B and B′. The toxin becomes heterogeneous over time, especially during storage in solution, and this results in six isoelectric species, $AB_5$, $AB_4B'$, $AB_3B'_2$, $AB_2B'_3$, $ABB'_4$, and $AB'_5$. These charge variants were visualized by isoelectric focusing of intact CT, which showed six bands, the primary species having a pI of 6.9. Focusing of the B oligomer demonstrated a similar pattern around pH 7.8, whereas focusing of acid-dissociated B subunits resulted in the observation of two variants. The A subunit may also have more than one isoelectric variant, especially in toxin that has been stored in solution for long periods. Although lyophilized toxin is stable when stored at 4°C, long-term storage of CT in solution at 4°C or room temperature storage results in extensive isoelectric heterogeneity. Severe isoelectric heterogeneity may lead to some decrease in cytotoxicity, and it is most probably responsible for disorder in the crystals. Well-ordered crystals suitable for diffraction studies were produced by isolation of isoelectrically pure CT by small-zone ion-exchange chromatography (259, 260). Choleragenoid was crystallized independently by Maulik et al. (185), and structure determinations of both CT and choleragenoid are in progress.

LT was crystallized (233), and preliminary studies were done, but the crystals proved to be difficult to obtain and the structure determination was seriously hampered by nonisomorphism even between native crystals, making it necessary to use the same crystal twice, first for a native data set collection and again for the derivative data set. The situation improved with changes in the crystallization buffer (233) and protein isolation, enabling a complete structure determination of the holotoxin to 2.3-Å (0.23-nm) resolution, with significant accuracy (255). An additional structure determination, that of the LT holotoxin complexed with lactose, is discussed in detail below.

### Oligomeric Structure, Physical Properties, and Interactions

Some initial confusion about the size and number of the subunits in CT and the relationship between the toxin and choleragenoid was clarified by a careful purification of the holotoxin and its subunits (74, 161, 287) and clarified entirely by an elegant series of cross-linking experiments (93) showing that the toxin is composed of five similar B subunits and one A subunit consisting of two peptides linked by a single disulfide bond. Gill (93) suggested that the B subunits were probably arranged in a ring with A on the axis; that A2, the smaller of the two A peptides, was required for reassembly; and, in agreement with van Heyningen (287), that the B oligomer is required for entry of subunit A into the cytosol.

A pH of 3.2 is required for complete disaggregation of holotoxin at room temperature in the absence of denaturing agents (93). The toxin can also be completely dissociated into monomeric subunits by heating in 0.1% sodium dodecyl sulfate (SDS) (287). In contrast, addition of SDS without heating, followed by immediate electrophoresis in a denaturing (SDS-containing) gel, results in dissociation of subunit A from intact pentameric B (93, 260). Heating to 65°C for 5 min in the absence of denaturing agent also dissociates the intact B pentamer from the A subunit. Dissociation by treatment with low pH or heat in the absence of denaturing agents results in rapid precipitation of the A subunit (287), leaving the B pentamer in solution. The precipitation of isolated A subunit in aqueous buffer and the fact that it is solubilized in 0.1% SDS led several investigators to assume (incorrectly) that it was a hydrophobic subunit or that it contained a hydrophobic patch on the surface. The model inspired by these observations was a hydrophobic or partially hydrophobic A subunit surrounded by a doughnut composed of B subunits that protected it from precipitation in aqueous solvents. This model was not substantiated by the three-dimensional structure of LT, however.

The crystallographic structural model of LTp (255) contains all 515 residues of the B pentamer and 230 residues of the A subunit. LTA ($M_r$ = 27,232) and, by analogy, CTA can be described as a triangular or wedge shape in one view and a V shape when rotated by 90° (Fig. 2). The A subunit is actually composed of two functionally different subunits, an enzymatic subunit (A1) and a short connector (A2) that mediates contact between A1 and the B pentamer. The A1 strand is a single-domain subunit with a complicated topology, consisting of many short stretches of secondary structure containing both α-helices and β-strands. The A2 subunit is an extended α-helix, beginning at residue 200 of the A polypeptide. The helix begins on one of the triangular sides of A1 (Fig. 2), associating with A1 through extensive van der Waals interactions, and then continuing into the center of the central pore formed by the B pentamer. The N-terminal residues of A1, the C-terminal eight residues of A2, and the residues A:186 through A:196 at the junction of A1 and A2 are not well defined in the LT structure, even after repeated attempts at refinement. Thus, there is some suggestion that these residues may be inherently flexible in the intact A chain.

Subunit A is synthesized as a single polypeptide in both *V. cholerae* and *E. coli*. CTA is proteolytically "nicked" (104) between residues 192 and 195 during secretion from the vibrio by *V. cholerae* hemagglutinin/protease (16), giving rise to two polypeptides, A1 ($M_r$ = 21,826) and A2 ($M_r$ = 5,407), covalently linked through a disulfide bridge between residues 187 and 199. In contrast, LT remains in the *E. coli* periplasm and is not nicked. Introduced into a genetically engineered strain of *V. cholerae* (126), LT remained unnicked, although it was secreted in the same manner as CT. Proteolytic processing is therefore not a prerequisite for secretion. Purified LTh can, however, be nicked in vitro (16, 76), suggesting that the mutant vibrio used by Hirst et al. (126) contained insufficient soluble hemagglutinin to catalyze nicking, rather than indicating an inability of LTA to be

nicked. CT, when introduced via an engineered plasmid into *E. coli* (223), remains unnicked and cell associated in *E. coli*. Therefore, the defect in processing of CT and LT in *E. coli* is related to the failure of *E. coli* to nick and secrete either toxin. This defect may explain the reduced severity of *E. coli*-induced enteric disease when compared with cholera (223). In both CT and LT, the disulfide bond linking A1 to A2 remains unreduced and the toxin is therefore essentially inactive, until it enters a cell.

The ring configuration postulated by Gill (93) was verified by electron microscopy (179, 234, 235) and is clearly seen in the X-ray crystallographic structure of LT (Fig. 3a). The inner surface of the ring of B subunits is hydrophilic, having a total of not fewer than 25 positive and 15 negative charges lining the central wall. The B-subunit monomers as described by Sixma et al. (255), and presumably those of CT as well, are tightly packed around the central pore. The subunits themselves have a novel topology, the main features in the B monomer being two triple-stranded antiparallel β-sheets, a small N-terminal helix, and a large central helix (Fig. 3). The monomers making up the pentamer form a six-stranded antiparallel β-sheet with a sheet from the next subunit, giving the ring the appearance of a smooth outer surface, while long α-helices form a helical barrel in the center, creating a pore 30 Å (3 nm) long, with a diameter ranging from 11 Å (1.1 nm) near the A surface to approximately 15 Å (1.5 nm) near the surface of the pentamer facing away from the A subunit. The pentamer has an overall diameter of approximately 64 Å (6.4 nm) and a height of 40 Å (4 nm).

When viewed looking down the fivefold axis, into the central pore (Fig. 3), the monomer backbones are tightly packed to form a pentamer of interlocking subunits. The surface area of the monomer, when pentamer is formed, accounts for 39% of the total accessible surface, one of the largest percentages of buried surface found in protein multimers (255). These structural features may account for the very high stability of the pentameric form (110, 267). Residue B:Ala-64 is in contact with the completely buried B:Met-31 of the adjacent subunit, which explains why mutation of B:Ala-64 to valine interferes with pentamer formation (139).

Ludwig et al. (179) produced two-dimensional crystals of CT bound to ganglioside $GM_1$, and, using image processing of electron micrographs of the resulting 15-Å (1.5-nm)-resolution crystals, revealed the doughnut shape formed by the B pentamer lying flat on the membrane surface, directly confirming the arrangement postulated by several workers who had used indirect methods (60, 93). The dimensions of the pentamer calculated from the three-dimensional structure of LT (255) are consistent with those measured by electron microscopy and image enhancement methods (179, 235). In addition to the fivefold symmetry, a hexagonal crystal lattice has been reported (179), together with evidence of a hexamer of B subunits from cross-linking experiments, although X-ray crystallographic evidence for CT (297) and LT (255) is consistent with a fivefold rotational axis. Reed et al. (234) measured lateral diffusion coefficients in supported monolayers of CT bound to $GM_1$-lipid monolayers, and, using electron microscopy and image enhancement methods, confirmed the B-subunit pentameric doughnut shape with central aqueous channel.

The crystal structure of LT shows the proximal surface of the B pentamer, facing subunit A, to be remarkably flat, with a high concentration of charged residues (35 charges in all). The distal surface of the ring is covered with 45 charged side chains, almost all with their functional groups pointing away from the surface.

There is little biochemical evidence for a hydrophobic surface on CTA (53), and the distribution of amino acids on the surface of LTA does not indicate either a hydrophobic surface or any large hydrophobic patch on the surface (255). A1 is, in fact, held away from the B pentamer and appears to have minimal contact with the ring. It is unlikely that the A1 subunit could reside within the pore and equally unlikely that it would pass through the pore as part of a membrane translocation process. The electron microscopy of the CT holotoxin associated with a lipid-$GM_1$ monolayer (235) is consistent with the absence of A in the pore when it is associated with a receptor-bearing membrane.

Interaction of subunit A with the B pentamer is mediated almost entirely by the A2 fragment, as previous biochemical studies (93) had suggested. The extended portion of A2 continues through the pore and appears to turn back into the pore at its C terminus. The five C-terminal residues of A2 are not clearly defined in the 2.3-Å (0.23-nm) resolution structure (255), leading to the suggestion that they may be flexible. This situation is clarified in a crucial report describing the crystal structure of LT complexed with lactose (254), an oligosaccharide receptor analog of the terminal galactose in $GM_1$, whose ganglioside structure is Galβ1→3GalNAcβ1 (NeuAcα2→3)→4Glc-ceramide (Fig. 4).

The 2.3-Å-resolution structure of the complex (254) contains well-defined density for the five C-terminal residues of A2. Residues A:232 through A:235, the portion of A2 that turns back into the pore, adopt a 1.5-helical-turn conformation. Temperature factors derived from the refinement of the residues (254) are high. A large temperature factor indicates that the atoms in these residues have a high degree of vibrational movement about their rest points and suggests that they are held less firmly by their covalent bonds (265). This portion of the helix may therefore be poised for conformational change (254). Since the C-terminal helix is clearly resolved by more detailed 1.95-Å (0.195-nm) data and refinement (253), it may be an intrinsic feature of LT and CT and should have functional relevance in membrane-binding interactions.

## INTERACTIONS WITH THE MEMBRANE RECEPTOR

### Receptor Recognition

The interaction of CT and LT with membranes and membrane receptors is perhaps the most extensively studied and well characterized function of the toxins (40, 45, 81–83, 87). The toxin receptor was identified by King and van Heyningen (158), who observed that ganglioside $GM_1$ prevented CT from increasing the capillary permeability of rabbit skin (skin bluing test), prevented CT from inducing the accumulation of fluid in ligated ileal loops of rabbit intestine, and inhibited the action of CT on the adenylate cyclase system in the small intestine of the guinea pig. Several groups quickly showed that $GM_1$, but not other gangliosides, had this effect on CT (40, 41, 135). Exogenously incorporated $GM_1$ was shown to be functionally equivalent to endogenously induced $GM_1$ receptors in cell membranes (83, 85); CT activation of adenylate cyclase was inhibited by $GM_1$ but not by other gangliosides (135). There is an increase in fluorescence (blue shift) in the spectrum of the B pentamer upon binding to $GM_1$ but not by addition of other gangliosides to CTB (200), and $GM_1$ is protected from labeling by preincubation with CT (81). Taken together,

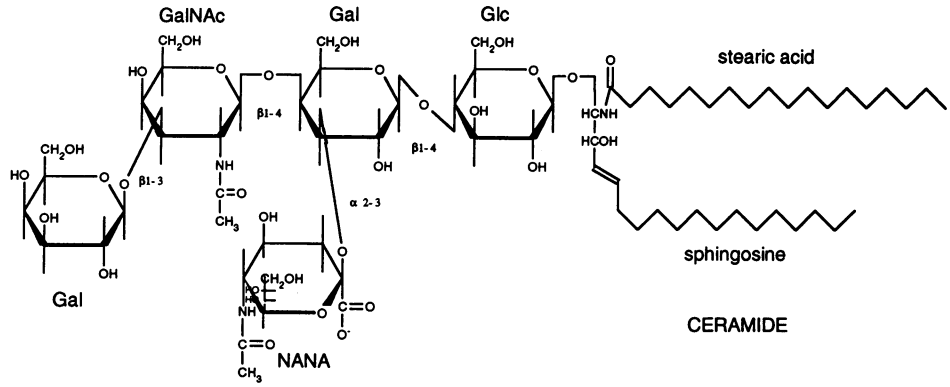


FIG. 4. Ganglioside $GM_1$. This may also be referred to as Galβ1→3GalNAcβ1(NeuAcα2→3)→4Glc-ceremide, where Gal is galactose, GalNAc is *N*-acetylgalactosamine, Glc is glucose, and NeuAc (NANA) is *N*-acetylneuraminic acid (sialic acid).

these observations defined the nature of the specific cell membrane receptor for CT. Studies in which quasi-elastic light-scattering techniques were used (61) to demonstrate CT-mediated agglutination of $GM_1$-containing phospholipid vesicles and $GM_1$-coated polystyrene spheres and data from photolabeling experiments (294) established that $GM_1$ is the membrane receptor.

The ability of CT to agglutinate $GM_1$-containing phospholipid vesicles (61) depends on the lipid composition of the vesicles, with only those composed of short chains ($C_{14}$, $C_{16}$) being appreciably agglutinated, perhaps as a result of poor packing of the $GM_1$ within the membrane bilayer and the consequent restriction of lateral movement of the glycolipid within the plane of the membrane. The ceramide (lipophilic) portion of the receptor is presumably required to anchor the oligosaccharide-sialic acid moieties in a membrane, although there is some evidence (184) to indicate that it may be involved more directly in intoxication. It is possible to generate $GM_1$-neoganglioproteins on the surface of rat glioma cells deficient in ganglioside $GM_1$ by cross-linking oligo-$GM_1$ ($GM_1$ lacking the ceramide portion) to cell surface proteins (222). These synthetic receptors were highly responsive to toxin but were nonfunctional, as evidenced by the fact that the cells did not show increased production of cAMP or activation of adenylate cyclase. The results of these experiments suggest that $GM_1$ does not act as a passive acceptor for CT but may play a more active role in CT insertion, translocation, or action.

Both CT and LT require the free carboxyl group of sialic acid for optimum binding (243), since neither binds asialo-$GM_1$ effectively. They do not require C-8 and C-9 of the sialosyl moiety or the acetyl groups associated with sialic acid and galactosamine (243). In fact, although other structural features do contribute to effective binding and intoxication by CT and LT, nuclear magnetic resonance data (247) suggest that a conformational change occurs in the oligosaccharide portion of the ganglioside when CT binds oligo-$GM_1$. This observation and other binding data (87, 135, 200, 243) demonstrate that it is the oligosaccharide portion of the receptor glycolipid which is specifically recognized by the toxins. CT and LT bind the terminal sugar sequence Galβ1→3GalNAcβ1(NeuAcα2→3)→4Gal. . . (87, 135), and LT can interact directly with lactose, binding the galactose portion of that sugar (254).

Both CT and LT-I bind ganglioside $GM_1$. CT interacts very weakly, approximately 1,000-fold less effectively (135), with $GD_{1b}$, but it does not bind any other related ganglioside, such as $GM_2$, $GM_3$, $GD_{1a}$, $GT_{1b}$, or $GQ_{1b}$ (85, 87, 115, 135, 200). On the other hand, LT-I can interact with a second class of receptors not recognized by CT (115, 133, 134). These additional interactions include weak binding to $GM_2$ and to asialo-$GM_1$ (87), which may account for the specificity of the agarose affinity purification technique for LT. Thus, although CT recognizes the terminal sequence GalNAc-Gal, the minimum requirement for LT-I binding is the terminal Gal alone (87, 256). Although this makes CT more selective than LT, a recent study of *V. cholerae* neuraminidase (88), which catalyzes the conversion of higher-order gangliosides to $GM_1$, concludes that this bacterial enzyme may play a subtle but significant role in the binding and uptake of CT by susceptible cells by creating additional accessible receptor sites.

## Binding Kinetics

The observation that clustered oligo-$GM_1$ bound CT very effectively (243), although CT recognizes oligo-$GM_1$ less readily than it does $GM_1$ (135, 263), may be due to the fact that the cluster is a multivalent ligand. Gangliosides in high concentration, or when manipulated to do so, form micelles in aqueous solutions (283). These multivalent micellar ligands can interact with CT, which itself is pentavalent, producing extensive cross-linking and aggregation of $GM_1$ micelles or $GM_1$-containing vesicles in solution (61). Determining the stoichiometery of the CT-ganglioside interaction is complicated by the self-association of gangliosides in solution, but a number of studies indicate that there is one binding site per monomer, and therefore there are five potential binding sites per CT molecule. One approach to quantitation of this interaction (84) made use of circular dichroism to demonstrate oligosaccharide-binding sites. The multivalent nature of the CT binding was also shown by its ability to produce patching and capping of receptors on binding to lymphocytes (39, 156). Fishman and Atikkan (81, 83) have reviewed the conditions necessary for binding of CT to various cells. The results are consistent with a model in which CT must bind several $GM_1$ molecules on the cell surface to subsequently activate adenylate cyclase. The surface phenomena such as phase changes, capping and patching, and lectinlike qualities observed by Goins and Freire (109), Richards et al. (236), and Kellie et al. (156) may be required only to achieve multivalent binding in cells with few or scattered receptors.

A quantitative molecular interpretation based on an anal-

ysis involving equilibrium dialysis and the Hill equation (242) indicated positive cooperativity and provided affinity constants for binding of each of the five subunits to $GM_1$ in the range of $2.0 \times 10^6$ to $3.0 \times 10^6$ $M^{-1}$. Analysis by quasi-elastic light-scattering techniques (61) based on observations of the lectinlike agglutination capability of CT provided an estimated minimum value of $4.5 \times 10^4$ $M^{-1}$, considered consistent with the calculated estimate of Schafer and Thakur (242), although Dwyer and Bloomfield (61) assumed no cooperativity.

### Structure of the Binding Site

The B pentamer of LT or CT (115, 286) binds $GM_1$, and that interaction causes a blue shift of approximately 12 nm in the fluorescence emission maximum of tryptophan (200, 211). That observation is of particular importance because there is only one tryptophan (Trp-88) in each B monomer, and the observation therefore specifies the position of the receptor-binding site in the toxin. The involvement of the unique tryptophan was further indicated by a $^{13}C$ nuclear magnetic resonance study (247), in which a resonance shift was observed and attributed to the ε2 carbon of Trp-88 on binding of $GM_1$ by the B subunit, and by fluorescence studies and chemical modification with nitrophenyl derivatives (51, 52) or formic acid (178), which resulted in disruption of the interaction between $GM_1$ and CT.

Because of the presence of the unique tryptophan residue in each of the B monomers, a study of its fluorescence properties in the presence or absence of $GM_1$ provided an opportunity to obtain additional convincing evidence of its involvement in binding to the receptor. Complex formation between CT and $GM_1$ results in an energy transfer between the indole moiety of the tryptophan residue and a dansyl derivitive of $GM_1$ (52), indicating that the residue is in or near the receptor-binding site. These studies suggested that the tryptophan residue was located on each B monomer adjacent to a second monomer, on a β-turn, and there was positively charged residue, perhaps Arg-35, on the adjacent monomer in its microenvironment. In the crystal structure of LT (255) B:Trp-88 is found at the bottom of a small cavity surrounded by several loops, including one containing B:Gly-33 from the adjacent monomer. Mutation of Gly-33 by substitution of Asp was found to abolish receptor binding by CT (281). The residue is, as predicted, part of a β-turn in LT, and the mutation can be expected to alter the conformation of the backbone, perhaps changing the shape of the binding site. The positions of the B:Trp-88 and the adjacent monomer B:Gly-33 strongly suggest that the cavity is part of the ganglioside-binding site and imply that $GM_1$ binding requires two B subunits, as proposed by De Wolf et al. (52).

The use of chemical modification for structural studies has limitations, primarily owing to the nonspecificity of the modification and the possibility that the reaction conditions have denatured the protein. Specific substitutions at critical positions in the B subunit have provided interesting insights about the functional prerequisites for binding. Several variants of CTB have been identified (75), and LTs from swine or humans differ from CTB at approximately 25% of the positions. Although all of these bind $GM_1$, the type I LTs bind several other glycoprotein receptors to which CT does not bind (87, 115). Thus it becomes important to distinguish precisely the role of specific residues involved in receptor recognition.

The recent analysis of the three-dimensional structure of LT complexed with lactose (Galβ1→4Glc), part of the oligosaccharide portion of $GM_1$ (254), may have helped to clarify the observed binding differences between the two toxins. The specificity of the galactose binding stems from the fact that almost all hydrogen bond donors and acceptors of galactose are engaged in hydrogen bonding. The hydrogen bonding involves the side chains of residues LT B:Glu-51, B:Gln-61, B:Asn-90 and B:Lys-91, as well as the carboxyl oxygen of residue 56 and two well-defined water molecules. There is extensive van der Waals association between the hydrophobic groups of galactose and B:Trp-88. B:His-57 is also in van der Waals contact with the C-6 atom of galactose, and, in agreement with previous proposals for the role of lysine in binding (178), B:Lys-91 is also involved in van der Waals contact with galactose. All residues directly interacting with galactose in this LT-lactose complex are conserved in CT, leading to the assumption that $GM_1$ binding is the same in CT and LT. The difference between CT and LT occurs only in the second shell, where position 95 is Ser in LTB and Ala in CTB. The change may influence the binding of the several additional glycolipids that are recognized by LT but not CT.

Substitution of Asp into LT B:Gly-33 prevented $GM_1$ binding (281). A more extensive substitution series in CT (146) produced mutants in which several amino acids were able to substitute for CT B:Gly-33 without affecting $GM_1$-binding ability, indicating that Gly-33 per se is not required for binding of CT. Various substitutions at that position demonstrated that a negative charge or a large hydrophobic residue interfered with binding and abolished toxicity.

All substitutions at residue B:Trp-88 drastically decreased the amount of immunoreactive toxin. This dramatic effect suggests that Trp-88, like Cys-9 and Cys-86, is crucial for the establishment or maintenance of native conformation of the B subunit. Both positively charged and negatively charged substitutions at position 88 abolished receptor-binding activity in the material tested. However, substitution of neutral amino acids (Ile, Leu, Asn, or Gln) or the basic His led to $GM_1$-binding activity similar to that of the wild type. Although substitution of acidic residues for Arg-35 interfered with the formation of holotoxin, indicating that it too is involved with conformational maintenance, it is remarkable that none of the observed substitutions for Lys-34 or Arg-35 affected the binding of CT to $GM_1$. It appears that, in this case, the specific amino acid may not be as important as the chemical properties of the residue for binding interactions or structural integrity.

### Entry of Toxin into the Cytosol

The B pentamer binds with its plane parallel to the membrane (179, 234, 235), but these electron-microscopic observations do not clarify whether the binding of LT and CT occurs with the A subunit directed away from or toward the membrane. In the LT structure, the B-subunit-binding pocket, including Trp-88, is distal from the position of subunit A, making it difficult to deduce whether the toxin binds with subunit A facing toward or away from the membrane. In the crystal structure of the LT-lactose complex (254), however, the orientation of the bound galactose portion of lactose is such that binding with the A subunit facing the membrane would not only force the A subunit into the lipid bilayer but also necessitate the insertion of the hydrophilic B pentamer into the bilayer by at least 8 Å (0.8 nm), in contradiction to the biochemical data that place the B pentamer on the outside of the lipid bilayer (234, 274, 294). The conclusion drawn here is that the initial binding of LT or

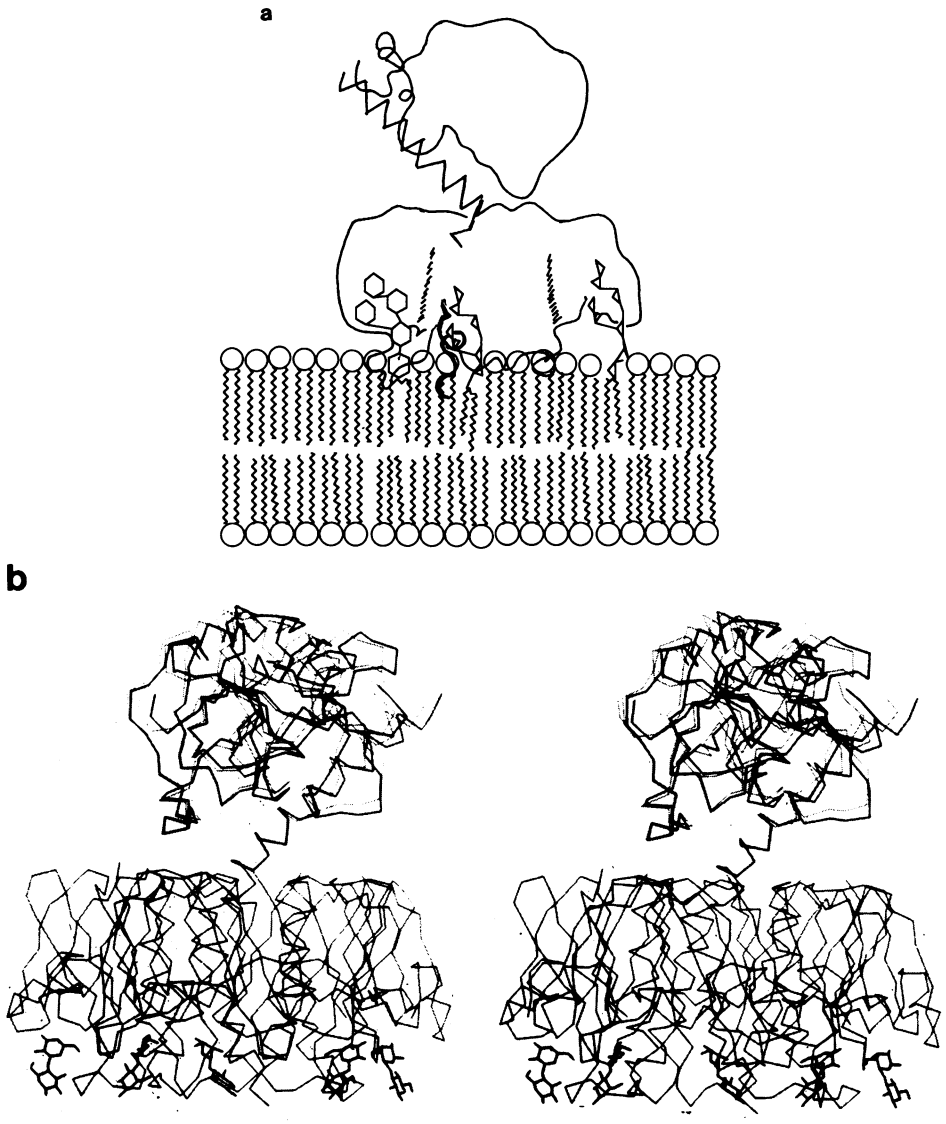


FIG. 5. (a) Cartoon representation of LT positioned on a membrane in the orientation suggested by the three-dimensional structure (254) of LT complexed with lactose. (b) Stereo view of LT (Cα trace) with lactose bound to all five B subunits. Lactose is the small disaccharide clearly visible at the lower left corner of the B pentamer and oriented at intervals around the structure. The Cα trace of the native A subunit is shown superimposed on the LT-lactose complex after direct superposition of the B pentamers of each structure. There is a slight change in the relative orientation of the A1 fragment with respect to the B pentamer. Panel b reprinted by permission from *Nature* (**355**:561–564, 1992, copyright © 1992 Macmillan Magazines Ltd.) (254).

CT to $GM_1$-containing membranes occurs with the A subunit facing away from the membrane (Fig. 5), with all five $GM_1$-binding sites occupied (254). In this orientation, the C terminus of the A2 subunit could interact with the membrane. Nevertheless, some evidence has been interpreted to produce a model with A pointing toward the membrane (60, 235, 294).

Hydrophobic photolabeling experiments with photoreactive lipids (274) indicate that the specific reduction of the disulfide bond between A1 and A2 (A:Cys-187 through A:Cys-199) on the external surface of the membrane is a prerequisite for penetration of the A subunit into the bilayer, although these studies did not establish that A alone actually penetrates the bilayer. Additional photolabeling (294), light-scattering (60), and electron-microscope (235) data support a model in which the B pentamer remains intact and on or within the surface of the membrane and subunit A1 is inserted into the bilayer.

However, there is evidence that in rat liver hepatocytes, both subunits (A and B) may sequentially associate with plasma membrane, endosomes, and lysosomes (144). A pH-dependent increase in the generation of the A1 peptide, coupled with membrane translocation of A or A1 across the endosomal membrane, has also been observed (145). Although internalization of CT via endocytosis could account for entry into a cell, the A subunit must still be inserted into and translocated out of the endosomal compartment, and the mechanism for those processes remains unclear.

CT is capable of inducing channels in lipid bilayers (275) and leakage of glucose entrapped in liposomes (198, 201).

Phase transitions in the membrane which lead to disruption of the membrane integrity and packing arrangement of the lipid molecules (109) have also been demonstrated. Ionic channels with an effective diameter of about 2.1 nm have been induced by CTB in planar lipid bilayers (160), leading to the suggestion that at acid pH, the B subunit does enter the membrane. There is some evidence that CTB may possess mitogenic activity (181) and that this activity may be mediated by an increase in the $Ca^{2+}$ concentration resulting from an influx of extracellular $Ca^{2+}$ into isolated jejunal enterocytes (182) or rat hepatocytes (54). The apparent activation of a transmembrane signaling system is based on fluorescence changes of intracellular probes and measurement of membrane potential changes in response to exposure of the cells to subunit B. Treatment of isolated jejunal enterocyte cells with CT caused significant elevation of $Ca^{2+}$ concentrations, indicating that CT is an effective $Ca^{2+}$ ionophore in isolated mammalian cells.

Fourier transform infrared spectroscopy is a very sensitive technique for the measurement of conformational changes, or disorder. By this method, Surewicz et al. (267) found that there were no significant changes in the percentage of β-sheet or α-helix on binding of the B pentamer to the receptor. On the other hand, in the presence of $GM_1$, the B pentamer showed a marked increase in thermal stability. Interestingly, oligo-$GM_1$ (lacking the ceramide tail) contributed somewhat less to the increased stability of the pentamer. In the absence of the receptor, the B pentamer began to cooperatively unfold between 66 and 78°C, whereas in the presence of $GM_1$ the thermal transition was not observed until 87°C and was not complete even at 92°C (45, 267) or 94°C (110). Subunit A, however, was much less well ordered in the native state, and it unfolded at 46°C. Subunit A does not contribute to the stability of the B pentamer (110) since the unfolding thermal stability of the holotoxin is essentially the same as that of the B pentamer. Other calorimetric measurements (45) corroborate the conclusion that there is little significant conformational change in either the holotoxin or the CTB pentamer on binding to the receptor. In fact, the primary effect of $GM_1$ appears to be to increase the stability of the pentamer. Little, if any, direct interaction could be observed between subunit A and $GM_1$ (110). The evidence from a variety of experimental approaches supports the notion that there is very little conformational change in the B pentamer on binding (45, 60, 61, 178, 247, 267). Recent studies by Bhakuni et al. (12) performed at low pH or in the presence of guanidine hydrochloride, however, suggest the existence of two nearly equal folding domains in the B subunit of CT, providing a highly cooperative unfolding process on binding of the CTB pentamer to $GM_1$.

The three-dimensional structure of the native LTB pentamer can be directly superimposed on the three-dimensional structure of the LT complexed with lactose (Fig. 5b). There is, however, a 5° change in relative orientation of the A1 fragment with respect to the B pentamer. Many atoms of the A subunit, particularly those located some distance away from the B pentamer and near the enzymatic active site, are shifted by more than 3 Å (0.3 nm) between the two structures (254). The color figure in the original article (254) shows this quite clearly. Binding to a receptor analog therefore results in a change in the relative orientation of the subunits rather than in extensive conformational change.

CT induces a well-known elongation of cultured CHO cells, presumably as a result of changes in the cytoskeleton (116, 122), but this phenomenon is related to increased cAMP activity and is thus a function of subunit A. Although the morphological changes in CHO cells have not been observed in the presence of subunit B preparations, it is possible that B oligomer preparations obtained from mixtures of holotoxin and choleragenoid contain very small amounts of holotoxin. A recent, more sensitive assay with HT29 cells (30), described subsequently, has been used to detect the apparent presence of residual CT holotoxin representing about 0.0003% of the total protein sample. Morphological changes occurred in cultured HT29 cells, but not in CHO cells, when high concentrations of CTB (greater than 6 ng/ml) were assayed. It is not known whether this very low level of residual enzymatic activity would be sufficient to affect results in other cell systems. The use of subunit B prepared from mutants lacking the gene for subunit A (151, 193) might clarify these apparently contradictory results.

## ENZYMATIC FUNCTION

### Subunit Activation and Structure of the NAD-Binding Site

Both the intact A subunit and the holotoxin are relatively inactive ADP-ribosyltransferases compared with the A1 polypeptide (192). Catalytic activity requires the reduction of the disulfide bond (A1:Cys-187—A2:Cys-199) linking A1 to A2 (104, 192, 274). The cleavage (nicking) between residues A1:Arg-192 and the start of the A2 polypeptide at A2:Met-195 takes place during secretion of CT from the vibrio. Tryptic digestion serves the purpose in vitro for LT. Reduction, which releases CTA1 from CTA2, may be accomplished by a variety of agents, usually dithiothreitol or 2-mercaptoethanol in vitro or a thiol:protein oxidoreductase. The endogenous reducing agent and mechanism of reduction are not known. An observed lag time of about 16 min between apparent binding of the toxin to the membrane receptor and the first appearance of the modified substrate intracellularly may be related to the time required for this step to occur following or during insertion and translocation (80). The A1 fragment, isolated following alkylation of the cystine with iodoacetamide, exhibits both ADP-ribosyltransferase activity and NAD-glycohydrolase activity, indicating that the cysteine residue itself is not critical for enzymatic activity (203). Although the bond is not necessary for activity, an extensive analysis (146) of mutants at several critical sites in the CTB oligomer, including B:Cys-9 and B:Cys-86, indicated that substitutions at positions preventing formation of the disulfide link between residues 9 and 86 effectively blocked the formation of stable and immunoreactive CTB. The enzymatic activity of CT is reported to be enhanced in the presence of detergents and phospholipid (217, 290), which appear to be prerequisite for enhancement of CT activity by ADP-ribosylation factors (15).

The A subunit of LT does not contain the classical NAD-binding βαβ-fold found in dehydrogenases that bind NAD (255). This is not unexpected, since the enzymatic association of toxins with NAD, resulting in the transfer of ADP-ribose, differs from the reduction of NAD by the dehydrogenases. To identify and compare the structure of the LT active site, Sixma et al. (255) superimposed on the LTA1 structure the enzymatic domain of *Pseudomonas* exotoxin A (ETA) (15, 18), an NAD-dependent ADP-ribosyltransferase with an entirely different acceptor substrate (138). The active site of ETA showed significant structural similarity to LT in that 44 residues could be superimposed. The equivalent residues roughly coincide with a helix and two β-strands that form the central part of both enzymes.

There is, however, almost no sequence homology between the two, with the exception of LT residues A1:Tyr-6, A1: Ala-69, and A1:Glu-112. A1:Glu-112 in LT corresponds to Glu-553 in the *Pseudomonas* ETA molecule, which is an active site capable of being covalently linked to NAD (27, 58). Since residue A1:Glu-112 in LT has been shown by mutagenesis studies to be important for activity (282) and since it is hydrogen bonded to A1:Ser-61, also shown to be essential for activity by mutagenesis studies (120), one may surmise that the environment around Glu-112 is the active site for NAD-binding activity in both CT and LT. The structure reveals several additional residues (His-44, His-107, Ser-114, and Arg-54) that form an elongated crevice under the $\beta_3$-strand. Although there is structural similarity, the active sites of the two proteins contain very different residues, which is consistent with the difference between the affinity constants of LT and *Pseudomonas* ETA for NAD (89).

Pertussis toxin (PT), CT, and LT do share a region of nearly identical amino acid sequence near the N termini of their respective enzymatic subunits. The sequences for some of the active-site residues are shown below.

| | | |
|---|---|---|
| CTA1:5–10 | Lys Tyr Arg Ala Asp Ser | |
| LTA1:5–10 | Lys Tyr Arg Ala Asp Ser | |
| PTS1:7–12 | Val Tyr Arg Tyr Asp Ser | (20,32,175) |
| ETA:438–443 | Gly Tyr His Gly Thr Phe | (5,18) |
| DT:19–24 | Ser Tyr His Gly Thr Lys | (31,112) |

Substitution of a Lys residue for Arg-9 in the PT catalytic subunit (S1) resulted in substantial elimination of enzyme activity (20, 175). Burnette et al. have recently shown (21) that substitution of Lys for CTA1:Arg-7 abolishes all detectable ADP-ribosyl transfer activity. A similar substitution in LTA1 (Lys for LTA1:Arg-7) also substantially eliminates ADP-ribosyl transfer activity (174). Arg-7 in CT and LT is adjacent to a Tyr that is conserved in the active sites of CT, LT, PT, diphtheria toxin (DT), and *Pseudomonas* ETA. Computer modeling of the NAD-binding sites of the toxins (55) indicates an NAD-binding site geometry common to CT, LT, PT, ETA, and DT.

The A1 subunit of CT and LT catalyzes several different reactions on the basis of its ability to activate the ribosyl-nicotinamide bond of NAD and transfer ADP-ribose to a variety of acceptors (28, 199, 204, 205, 276). The enzymatic activity of CT has been extensively reviewed (208, 209), especially in terms of its relationship to G-protein function. The reader should consult the reviews cited for additional, detailed information.

## ADP-Ribosyl Transfer

The characteristic rice-water stool symptomatic of both cholera and LT-caused diarrhea may be associated with adenylate cyclase activation in intestinal mucosa cells, although other factors, such as ion flux (68, 69, 113) and prostaglandin production (226, 227), may also play a role (see the following section). Toxin-catalyzed transfer of ADP-ribose (reaction 1) from NAD to $G_{s\alpha}$ modifies this guanine nucleotide protein responsible for stimulation of the cyclase system, rendering it incapable of dissociating from the active cyclase complex (Fig. 6) (23, 29, 95, 192):

$$\text{NAD} + G_{s\alpha} \xrightarrow{\text{CTA1}} [\text{ADP-ribosyl } G_{s\alpha}] + \text{nicotinamide} + H^+ \quad (1)$$

Both CTA1 and LTA1 are arginine-specific ADP-ribosyl transferases, capable of ADP-ribosylating a number of artificial substrates, including poly(L-arginine), L-arginine methyl ester, *N*-guanyltyramine, and a variety of membrane and soluble proteins (101, 191, 202, 206). Both toxins are capable of hydrolyzing NAD to free nicotinamide and ADP-ribose in the presence or absence of an acceptor (199, 207). The kinetic mechanism is random sequential (166, 191), with a $K_m$ of 5.6 mM for NAD and 39 mM for the artificial acceptor substrate L-arginine methyl ester, measured by using a high-performance liquid chromatography (HPLC) assay (166). These values are consistent with values previously determined when $^{125}$I-*N*-guanyltyramine was used as the artificial acceptor substrate (191).

## Autocatalysis

CT has autocatalytic activity, and although at least three modification sites can be identified on the A1 peptide, the automodified toxin retains catalytic activity (204, 276). However, the guanidinium group of A1:Arg-146, the site of one of the autoribosylation sites in LT (165), is 30 Å (3 nm) from A1:Glu-112, a crucial part of the NAD-binding site (265). The sites are quite far apart, suggesting that the toxins probably carry out "cross" rather than "self" auto-ADP-ribosylation (255).

## NAD-Glycohydrolase Activity

CT and LT are also capable of using water as an ADP-ribose acceptor (199) (reaction 2). This NAD glycohydrolase activity is considerably slower than the ADP-ribosylation of guanidino compounds (206).

$$\text{NAD} + \text{HOH} \xrightarrow{\text{CTA1}} \text{ADP-ribose} + \text{nicotinamide} + H^+ \quad (2)$$

## Cellular Factors That Promote CT Activity

Although CTA1 is capable of ADP-ribosylation of substrate analogs in vitro, a number of membrane proteins and intracellular factors promote or augment the enzymatic activity in vivo (65, 94, 100, 103, 148, 150, 152). GTP is required for activation of adenylate cyclase, and there is evidence that CTA1 has little activity in the absence of GTP (65). The GTP binds S, a membrane-bound protein (103), or ADP-ribosylation factor (ARF) (150), and the [S · GTP] (or [ARF · GTP]) promotes ADP-ribosylation of $G_{s\alpha}$ by CTA1. The fact that $G_{s\alpha}$-independent auto-ADP-ribosylation of CTA1 was stimulated by ARF (279) and that S was required for ADP-ribosylation of secondary substrates (see below) is consistent with the hypothesis that the cofactor acts directly on the toxin rather than on $G_{s\alpha}$ (217).

The ARFs have been described in recent reviews (147, 205, 210), but details of their functions in eukaryotic cells have been reported subsequently (213, 280). The ARFs, which number at least six to date (213), are approximately 20-kDa monomeric proteins related to the oncogene product *ras* (100, 148, 150). They have been localized to Golgi organelles (290) and are found predominantly in brain tissue rather than in nonneural tissues (278).

One of the purified cofactors, sARFII, requires $Mg^{2+}$, phospholipid, and sodium cholate or a low concentration of SDS for maximal stimulation of toxin activity (14). There is also some indication (278) that in the presence of either SDS or dimyrstoylphosphatidylcholine-cholate, ARF and toxin may exist as multiple species or aggregates which exhibit different substrate specificities. ARF binding to phosphatidylserine was found to be coincident with stimulation of

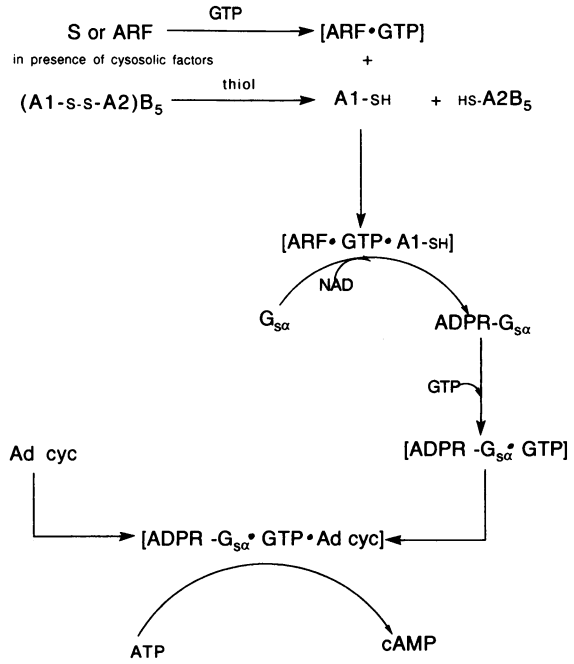


FIG. 6. ADP-ribosylation scheme including cofactors required for activation of CTA1 in the cytosol. Once A1 is released from the holotoxin by nicking and reduction, it forms a complex with the activated [ARF · GTP] complex, to become activated A1 complex. This activated form of A1 is then able to bind NAD and transfer ADP-ribose from NAD to $G_{s\alpha}$. The modified $G_{s\alpha}$ can bind GTP and form an active ternary complex with adenylate cyclase (Ad cyc), which catalyzes the production of cAMP from ATP. However, ADP-ribosylation inhibits the hydrolysis of GTP to GDP, and the complex is therefore maintained in its active form. Adapted with permission from the *Journal of Biological Chemistry* (279).

CT-catalyzed ADP-ribosylation (290), suggesting a mechanism involving cycling between soluble and membrane-bound forms. A model scheme for the stimulation of adenylate cyclase by CT is shown in Fig. 6.

It is probable that S and CF, an additional cytosolic factor (65), are the same proteins as specific members of the ARF family. Purified ARFs have an $M_r$ of 21,000 (148, 150), as does purified S (100), and the proteins seem to have comparable characteristics. Both S and ARF bound GTP maximally in the presence of $Mg^{2+}$ when membrane when bound (100) or phospholipid (150) vesicles were added to purified protein. An additional cytosolic factor, CF (65), the soluble (cytosolic) form of S, appears to be required for binding of S to GTP and subsequent association with CTA1 (100). It should be noted, however, that all experiments with S, ARF, sARF, and CF were performed with purified components or washed membranes and should therefore be considered in vitro observations.

### Alternate Substrates for ADP-Ribosylation by CT

Although the primary cellular substrate for ADP-ribosylation by CTA1 is $G_{s\alpha}$, several other proteins have been found to be less rapidly modified "secondary substrates" (100, 102). The ADP-ribosylation requires activation of ARF/S. One of these proteins has been identified in pigeon erythrocyte ghosts as the anion-transporting protein which is the major component of band III, an erythrocyte membrane protein, and a second has been identified as coincident with the avian erythrocyte-specific high-mobility group nuclear protein ($M_r$, 28,000). Neither of these are GTP-binding proteins. They were ADP-ribosylated under conditions that selectively rendered $G_s$ unsuitable as a toxin substrate while permitting the S-dependent ADP-ribosylation of minor substrates (100). More recently (140) the α subunit of $G_{i\text{-}2}$ in HL-60 cells was modified by CT-catalyzed ADP-ribosylation when membrane receptors were stimulated by agonists, resulting in various functional modifications of the signal-coupling protein. The CT target amino acid, possibly an Arg on the $G_{i\alpha}$ subunit, is different from the target amino acid that is ADP-ribosylated by PT. This result is similar to that seen in the modification of the transducin α subunit by both toxins (see following section).

### Role of Toxin in Pathogenesis

In the normal cyclase system, $G_{s\alpha}$ is active when GTP is bound to it. The ternary complex formed by $G_{s\alpha}$, GTP, and adenylate cyclase catalyzes the conversion of ATP to cAMP (reactions 3a and 3b).

$$G_{s\alpha} + GTP \longrightarrow [G_{s\alpha} \cdot GTP] \quad (3a)$$

$$[G_{s\alpha} \cdot GTP] + \text{adenylate cyclase} \longrightarrow [G_{s\alpha} \cdot GTP \cdot \text{adenylate cyclase}] \quad (3b)$$


When GTP is hydrolyzed to GDP by the intrinsic GTPase activity of $G_{s\alpha}$, the complex dissociates and is therefore inactivated (107).

ADP-ribosylation increases the sensitivity of the regulatory protein, $G_{s\alpha}$, to the activator GTP (94, 173) and decreases its GTPase activity (29), resulting in preservation of the active ternary complex. It has been found that ADP-ribosylation promotes dissociation of the α and β subunits of $G_s$ (149) and facilitates the release of GDP (23), thus permitting reactivation of the ternary complex by further GTP binding. Both consequences of ADP-ribosylation result in an enhancement of cAMP production. This enhancement is several hundred-fold (116, 122) in CHO cells treated with CT 3 h prior to measurement.

Adenylate cyclase is in the basolateral membrane of the columnar epithelial cells lining the intestinal lumen (291), and CT (or LT) binds on the brush border at the lumen side of the cell. The adenylate cyclase is therefore opposite, not adjacent, to the CT receptor. There is little evidence that A1 itself traverses the cytoplasm to reach the adenylate cyclase. Rather, it is the $G_{s\alpha}$ that is localized in the brush border membrane of rabbits (56), where it can be ADP-ribosylated by A1 and then diffuse through the cytosol to complex with adenylate cyclase. Little or no $G_s$ is found in the brush border of rat intestinal cells, and this observation may explain why rats are less sensitive than rabbits to the effects of CT (69). Excess fluid and electrolyte secretion may be related to impaired absorption of sodium by villus cells that line the intestinal lumen. Thus, the consequent flux of fluid into the lumen is induced to maintain osmolarity (68, 69, 113). That fluid, normally returned to the blood across the intestinal wall, would be lost in watery stool, causing the blood volume to decline. The effects of the dehydration may be fatal, particularly in infants and the aged.

The role of enhanced cAMP production in the pathogenesis of experimental cholera is not entirely clear-cut, however. Not only cAMP but also prostaglandins may regulate

water and electrolyte secretion in cholera (226, 227), and there is evidence that prostaglandin E is released into the intestinal lumen of rabbits after injection of CT into the lumen. CT was found to stimulate both cAMP and arachidonic acid metabolism in CHO cells, evoking the synthesis of prostaglandin $E_2$ and other prostaglandins (227). Pathways other than or in addition to those associated with cAMP may therefore be involved in the pathology of the disease. A more extensive examination of all the metabolic pathways affected by the activity of the toxin may clarify the detailed mechanism(s) of pathogenesis. It has also been suggested (188) that CT may be implicated in providing nutrients for the bacterium or in aiding bacterial colonization.

## ASSAYS

All of the following methods may be used with LT, provided that it is suitably activated by both tryptic cleavage and thio-reduction. CT requires only reduction of the disulfide bond.

The earliest biological assay for CT activity was described by S. N. De, of the Calcutta Medical College, Calcutta, India, who reported in a note to *Nature* (46) that fluid secretion into the lumen of the gut could be elicited by injection of culture filtrates of *V. cholerae* into the ligated ileal loop of a rabbit. A segment of small intestine was isolated with two silk ligatures, and the sample to be tested was inoculated into the lumen of the isolated loop. The rabbit was sacrificed after 24 h, and the fluid in the distended isolated segment was aspirated and measured. This method was used to demonstrate the presence of a bacterium-free factor (toxin ) in *V. cholerae* culture filtrates. The presence of a filterable agent, or toxin, was demonstrated almost simultaneously in Bombay by Dutta et al. (59), who measured diarrhea produced in infant rabbits fed multiple doses of a cell-free lysate of suspensions of *V. cholerae* 569B. This assay and the bluing assay (below) are included as historical information. There are a variety of more sensitive and specific assays available that do not require the use of live animals.

Another method to estimate CT activity in crude and purified preparations made use of the increased capillary permeability induced by CT in the skin of a rabbit, as revealed by subsequent intravenous injection of pontamine blue dye (38). One bluing dose is defined as that amount of CT which, when injected intracutaneously into a rabbit in a 0.1-ml volume, will bring about an area of increased capillary permeability 7 mm in diameter.

### Bioassays

Cell culture bioassays have been developed for use with both CT and LT; they are based on the observation of morphological changes associated with ADP-ribosyltransferase activity. CHO cells treated with CT change from a normal confluent state to a distinctly elongated, spindle morphology (116, 122), and the degree of alteration is dependent on concentration. Very small amounts of toxin (in the nanogram range) are required to obtain a positive reading. In a more recently described assay (30), vacuole formation in HT29 cells was found to be dependent on the ADP-ribosyltransferase activity of CTA, and this vacuole formation occurred at lower concentrations of CT than did CT-induced elongation of CHO cells. LT induced similar changes in HT29 cells.

[$^{32}$P]ADP-ribosylation of proteins can be monitored in tissue samples after a prior exposure of membranes to CF and a nonhydrolyzable analog of GTP such as GTPγS, Gpp(NH), or Gpp($CH_2$)p. Membranes are incubated with [$^{32}$P]NAD, and then the membrane proteins are electrophoresed on polyacrylamide gels. The labeled proteins are identified by their migration patterns (106). There are, however, several tissue enzymes that catalyze nonspecific ADP-ribosylation of proteins in broken cells, and these can mask toxin-dependent labeling. Residual [$^{32}$P]ADP-ribose may also interact with the polyacrylamide gel itself during electrophoresis. These counts generate a radioactive smear toward the bottom of the gel. The smear is not evident when the tissue samples contain ADP-ribose phosphodiesterase and magnesium ions (106). ADP-ribosylation can also be monitored by using the artificial acceptor agmatine (207). CT (10 mg/ml) gives an incorporation of 5 to 15 pmol of ADP-ribose, about 10 times the background level in trichloroacetic acid-precipitated proteins from brain particles (106).

The most sensitive activity assay involves measuring the rise in adenylate cyclase activity as a consequence of ADP-ribosylation, for example in freshly lysed pigeon erythrocytes. Enhanced production of cAMP as a result of CT activity may be measured by using a commercial kit (Amersham TRK.432) which provides a rapid, simple, and specific method for cAMP determination in the range of 0.2 to 16 pmol (4 to 320 pmol/ml of extract). The method is based on the competition between unlabeled cAMP and a fixed quantity of [$^3$H]cAMP for binding to a protein which has a high specific affinity for cAMP. In this assay the amount of labeled cAMP-protein complex measured is inversely related to the amount of unlabeled cAMP present in the sample.

### In Vitro Assays

Although any of several proteins or poly(L-arginine) can be used with [*adenylate*-$^{14}$C]NAD as a donor (206, 208), providing a means of measuring the appearance of product, the method is not ideal because of the heterogeneity of potential acceptor groups. An alternative is to use a synthetic low-molecular-weight acceptor, such as $^{125}$I-*N*-guanyltyramine, which can be specifically ADP-ribosylated by thiol-activated CT or by CTA1 in the presence of β-NAD (191). ADP-ribosyl-$^{125}$I-*N*-guanyltyramine can be quantified after separation from unreacted $^{125}$I-*N*-guanyltyramine after batch adsorption of the unreacted material to cation-exchange columns (207).

A more recent method developed by Larew et al. (166) makes use of commercially available L-arginine methyl ester as the acceptor. The ADP-ribosylated L-arginine methyl ester is separated from the reaction mixture on a reversed-phase HPLC column following derivatization with an *ortho*-phthaldialdehyde–2-mercaptoethanol reagent. The fluorescence of the product is monitored, thereby eliminating the need for a radiolabel. The method is suitable for a variety of kinetic experiments.

The transfer of ADP-ribose from [*carbonyl*-$^{14}$C]NAD to an acceptor such as water, guanidino compound, or protein may also be used to monitor enzymatic activity (207). The transfer is accompanied by release of the [*carbonyl*-$^{14}$C]nicotinamide, which is eluted from a Bio-Rad AG1-X2 resin. This assay measures the NAD glycohydrolase activity of the toxin (reaction 2).

## IMMUNOLOGY

### Immunological Relationships

Immunological relationships among the various isolates of CT reveal at least three distinct serotypes, designated CT-1, CT-2, and CT-3. These isolates can be distinguished by specific monoclonal antibodies (72, 154). There are three human LT strains, LTh-I, LTh-IIa, and LTh-IIb (117, 130), as well as one porcine LT (LTp) (36, 43, 90). All of these isolates cross-react with one another to various degrees, and their immunological relationships have been extensively characterized (11, 75, 117, 129, 154).

### Epitope Mapping

The B pentamer is immunodominant, and numerous antibodies, both polyclonal (155) and monoclonal (11, 129, 154, 155, 178), have been generated for use in epitope-mapping and -binding studies that have compared CT, CTA, CTB, LTh-I, LTp, and LT-II. Many fewer antibodies have been found to react with subunit A, and it should be noted that these have generally been elicited by isolated, denatured CTA. The anti-A antibodies were used to detect differences between CTA and LTA (11, 75, 178). A variety of solid-phase assays have been used for this purpose, including a novel adsorption assay (checkerboard immunoblotting) (154), which involves the use of dot blots on nitrocellulose, and an antigen capture method (129), in which $GM_1$ is adsorbed to microtiter plate wells followed by toxin or B pentamer. In both methods, the bound toxin or subunit is probed with antibody and the complex is visualized by standard enzyme-linked immunosorbent assay (ELISA) methods. The most protective antibodies, based on cell culture neutralization studies (129), were found to be against CTB. In contrast, only some of the monoclonal antibodies against CTA had neutralizing activity, and that activity was low. There appeared to be no correlation between the ability of the monoclonal antibodies to neutralize CT and to cross-react with LTh or LTp.

Checkerboard immunoblotting was applied (154) to a wide variety of monoclonal and polyclonal antibodies against both CT and LT that demonstrated various degrees of reactivity and cross-reactivity among a number of antigenic variants of both CT and LT. Reactivity of the monoclonal antibodies appeared to be directly primarily against conformational epitopes. The study defined the importance of several specific residues in antigenic determinants, confirming an earlier study, using more traditional ELISA methods (75), that placed CTB:Ala-46 at or near the $GM_1$-binding site. This residue is separated from a flexible loop containing several residues that interact with the galactose portion of lactose (254), and its importance is thus anomalous.

Several monoclonal antibodies assayed by the checkerboard immunoblotting method (154) bound to adsorbed CT alone but not to adsorbed CT bound to $GM_1$. A similar inhibition of specific antibody binding was found in a previous study when CT was bound to $GM_1$ (178). That antibody, when bound to CT, produced fluorescence changes in the CTB:Trp-88. Although both antibodies bound CT poorly, the studies indicated that the specific monoclonal antibodies bound an epitope at or near the $GM_1$-binding site.

The receptor-binding site of CT has been the target of a large number of epitope-mapping attempts, since vaccines capable of blocking this site would be most efficacious. Chemical and immunochemical studies in which a panel of monoclonal antibodies was used to monitor the conformational integrity of modified CT (178) indicated the importance of arginyl and lysyl residues in addition to CTB:Trp-88. LTB:Asn-90 and LTB:Lys-91, found in the binding pocket of LT, are in fact involved in hydrogen bonding with the galactose portion of the lactose receptor analog (254).

### Antipeptide Monoclonal Antibodies

Antipeptide monoclonal antibodies against various segments of CT have been used in several types of studies, including some directed at vaccine development. Peptide-antibody interactions were studied by nuclear magnetic resonance methods (7, 8, 172), which defined contact interactions between antibodies and a highly immunogenic peptide, CTB-3 (143), corresponding to residues CTB:50 through CTB:64 in the holotoxin. Aromatic amino acids of two of the elicited antibodies (TE32 and TE33) were found to interact with Val, Pro, Gly, Gln, His, and Asp in the peptide, homologous to CTB residues 50, 53, 54, 56, 57, and 58, respectively, all of which are part of an exposed flexible loop near the binding pocket of LT (255). In solid-phase assays with holotoxin, however, the antipeptide monoclonal antibodies TE32 and TE33 reacted weakly with CT, and in solution-phase competition assays and solution-phase interaction studies, neither reacted with native CT (258). These results and similar observations (92) suggest that the antibodies recognize an extended, denatured, or deformed epitope in the native protein.

Peptide fragments from PT enzymatic subunit S1 have been used to look at cross-reactivity between PTS1 and CTA (232). Anti-PTS1 peptide polyclonal antibodies were elicited against the fragment from PTS1:8 to PTS1:18, a portion of the PT sequence associated with NAD binding (32) and having homology to CTA:6 through CTA:16. CTA was found to interact with the polyclonal antibodies against PTS1:8 through PTS1:18, as expected, although anti-CT antibodies did not recognize the PTS1:8 through PTS1:18 peptide. S1:Tyr-8, LTA1:Tyr-6, and the equivalent Tyr residues in *Pseudomonas* ETA (Tyr-439) and DT (Tyr-20), are part of the NAD-binding site in each of the respective toxins, and by analogy, CTA:Tyr-6 should be part of the NAD-binding site in CT. The homology and immunological cross-reactivity therefore represent similarities in NAD-binding-site structure among several ADP-ribosylating toxins.

## VACCINES AND VACCINE DEVELOPMENT

Observations that choleragenoid (B pentamer) blocked toxin activity by binding specifically to the membranes of intestinal microvilli in the same way as does holotoxin suggested a receptor-blocking therapy (34, 57) that was only partially successful. Antibiotics can eliminate the etiologic agents and oral rehydration therapy can alleviate the symptoms of diarrheal disease, but prevention and control of spread are the most effective means of eliminating the problem. These measures require good sanitation and an effective vaccine.

While the disease itself provides effective lifelong immunity to cholera, attenuated whole-cell vaccines administered parenterally provide little protection, and that protection is very short-lived (about 3 months). The extensive historical and epidemiological background describing the development and efficacy of CT vaccines has been reviewed in several excellent articles (71–73, 171a), and these authors, as well as

Holmgren (132), have reviewed current progress. There are two candidate vaccines currently being tested on volunteers and in field trials in Bangladesh and Pakistan. One is a mixture of killed whole bacterial cells and B subunit (14), and the other is based on a live attenuated strain of *V. cholerae* (169, 170). Both have been effective in the tests, but both have limitations that require further development to resolve (171a).

### Whole-Cell and Antitoxin Vaccines

Considerable direct and indirect evidence from epidemiologic studies, animal models, and studies of experimental cholera in volunteers indicates the existence of both antibacterial and antitoxic immunity. Nevertheless, the earliest efforts, in 1884, to provide immunity to cholera by using attenuated broth cultures were not effective (17), although more recent formulations of killed whole-cell vaccines administered parenterally or orally do provide significant protection against cholera (72, 73, 132, 171a). The most effective immunity, however, involves both whole-cell and toxin antigens (14, 225, 230, 268). Oral or enteral administration of B pentamer can also produce a protective intestinal secretory (immunoglobulin A [IgA]) response (269), as could oral administration of a peptide corresponding to the sequence CTB:50 through CTB:75 (48, 118, 224). Although the first field trials of various combinations of CT with B pentamer and killed whole vibrios were not entirely satisfactory (73, 171a), a combined killed whole vibrio and subunit vaccine evaluated in adults in Bangladesh did provide promising antibody response and memory responses more recently (14, 268).

A substantial serum IgG response occurs and persists for several years following clinical or experimental cholera, and there is considerable evidence that antitoxin can neutralize the effect of CT in vivo. However, previous vaccines consisting of killed whole vibrios or purified toxoid (B pentamer) have provided very poor short-term protection (170). Administration of B pentamer alone has been used but is less effective than administration of killed holotoxin, possibly because the A subunit may exhibit an adjuvant effect (73). Long-term immunological memory for CT after oral immunization of mice can be carried on by B lymphocytes (180). The memory cells could be isolated 1 year after priming immunization with CT and could be transferred to naive recipient mice, which then adopted the ability to respond with strong specific antitoxin antibody when challenged with CT orally.

In a large-scale (almost 90,000 individuals) field trial in Bangladesh (34), one oral dose of the combined B pentamer/killed whole-cell vaccine produced an initial 64% protection (14). More recent tests in volunteers have been even more successful (171a). Responses remained protective for 3 years after initial vaccine administration in more than 40% of those who received the whole-cell vaccine in a large-scale field trial in Bangladesh (35). In addition, the B subunit/killed whole-cell vaccine gave substantial short-term protection against LT-associated diarrhea (33). Although they are not as effective in young children and have drawbacks, including a requirement for multiple doses and a high cost of manufacture, the B subunit/killed whole-cell vaccines nevertheless represent an improvement over any vaccines previously tested.

### Live Attenuated Vaccines

Much of the difficulty in providing effective protection may be due to the ineffective generation of an in situ intestinal immunological response, as well as the necessity for using severely denatured protein antigen (170, 171a). An oral vaccine that delivers detoxified, conformationally near-native antigen to the gut in a simple, inexpensive manner is desirable. Longer exposure times in the intestine may also be beneficial. These considerations led to several genetically based strategies for producing immunogen, such as deletion of the genes that encode subunit A from pathogenic *V. cholerae* O1 strain 569B (151, 193) to provide an attenuated strain of vibrio.

Recombinant deletion mutants of *V. cholerae* provided protection in 9 of 10 volunteers vaccinated (171). This vaccine consisted of live *V. cholerae* cells capable of colonization in the gut but genetically unable to produce subunit A. In more recent field trials in Bangladesh with a mutant vibrio containing resistance to $Hg^{2+}$ (so that the strain could be distinguished from the wild type when cultured) (169), very effective rises in antibody titer were obtained: 91% of 144 subjects vaccinated showed rises in serum IgG antitoxin titer and very high vibriocidal titers. Ingestion of a single oral dose of the genetically engineered vibrio provided initial protection in 95% of the vaccinated volunteers. The higher single dose required makes the engineered material an easier oral vaccine to deliver, and the reports of mild diarrhea following doses of an earlier strain have been reduced with later strains (169). Tests in young children are in progress, and field trials are planned.

### Antipeptide Vaccines

There has been considerable interest in the use of synthetic peptides as vaccine immunogens because of the low potential for adverse reactions and the ease of synthesis. CT is an immunologically well-characterized protein with high immunogenicity, and it has been used in several studies along these lines. Oral or intraperitoneal administration of a peptide (CTB:50 through CTB:75) (118) elicited serum polyclonal antibodies capable of neutralizing CT activity in the rabbit skin bluing assay. In solid-phase ELISAs, however, the peptides used did not inhibit binding of anti-CT antibodies to intact CT, but the peptides did compete with CT for binding to antipeptide antibodies. Antipeptide antibodies to residues CTB:42 through CTB:62 had weak but detectable neutralization activity and reacted at low affinity with CT holotoxin in solid-phase assays.

Peptides homologous to residues CTB:50 through CTB:64 and CTB:50 through CTB:75 elicit protective responses in mice (118, 143, 224), and peptides with sequences corresponding to residues CTB:50 through CTB:64 and CTB:8 through CTB:20 were capable of eliciting antibodies capable of neutralizing the effect of CT on adenylate cyclase (143) and conferring immunity to LT as well (142). A synthetic oligonucleotide specifying a peptide (CTB:50 through CTB:64) inserted into a *Salmonella* flagellin gene (216) evoked polyclonal antibody that recognized CT in solid-phase assays. This sequence is part of the exposed flexible loop adjacent to the binding cleft in LT, and several of the residues included in it interact with receptor analog, which may account for its effectiveness as a protective epitope.

## CT AS A PROBE OF BIOLOGICAL FUNCTION

In addition to studies of the disease process itself, CT has been used in investigations of a variety of other systems. Work by Shen et al. (245), for example, made use of the receptor specificity of CT to demonstrate that glycolipids inserted into lipid vesicles retained their biological function and orientation, showing by gel filtration that labeled CT coeluted with a $GM_1$-containing vesicle-rich fraction. Other uses include studies of immune system stimulation (6, 114, 157), nuclear magnetic resonance or HPLC studies of antibody diversity and antibody-antigen interactions (8, 172, 259), and studies of vaccine development, in which it is used as a model (216). Hybrid proteins consisting of B subunit and foreign antigens for immunoprotection have generated some recent interest (42). This work is based on the observation that CT and CTB are potent enteric immunogens in humans and that the B pentamer can therefore serve as a carrier or adjuvant for other antigens. CT has also provided considerable insight into fundamental processes of cellular regulation as a result of its functional association with GTP-binding proteins and the adenylate cyclase complex in a large variety of systems, many of which are described in recent reviews (107, 141, 206, 266, 272). The literature describing the biological functions of CT in cell systems other than intestinal epithelium is enormous, meriting a review on its own. The reader is advised to consult the various citation indices (keyword, cholera toxin) for further information. Two examples that exploit the exquisite specificity of toxin molecular recognition are briefly described, however.

### Elucidation of Transducin Functional Domains

Transducin is a heterotrimeric GTP-binding protein consisting of subunit $T_\alpha$ and the combined subunit $T_{\beta\gamma}$, which does not dissociate physiologically. It is a signal-transducing G protein that mediates the signal coupling between rhodopsin and a membrane-bound cyclic GMP phosphodiesterase in retinal rod outer segments (127). The CT-catalyzed ADP-ribosylation of the $T_\alpha$ subunit results in a reduced rate of GTP hydrolysis (1, 214). Use of CT-catalyzed and PT-catalyzed ADP-ribosylation, as well as other chemical modifications, led to the identification of the functional domains of the transducin $T_\alpha$ subunit (127). Interestingly, PT catalyzes ADP-ribosylation of a Cys (Cys-347) in the C-terminal region of $T_\alpha$ (293), blocking the hydrolysis of GTP to GMP (285), whereas CT catalyzes the ADP-ribosylation of Arg-174 (284) near the middle of the $T_\alpha$ subunit. The form of the substrate also differs: PT recognizes $T_{\alpha\beta\gamma}$, and CT can ADP-ribosylate only $T_\alpha$-GTP. The differences in substrate fine-structure recognition between the two toxins may be comparable to the differences between their different physiological targets, $G_{s\alpha}$ and $G_{i\alpha}$. The recent observation that the CT-catalyzed modification of $G_{i\alpha}$ under certain specific conditions does not inhibit PT-catalyzed ADP-ribosylation (140) provides further insight into the mechanism for modulation of substrate specificity.

### Retrograde Labeling of Neurological Pathways

CT is used extensively for cytochemical and immunohistochemical studies on the basis of its ability to bind preferentially to ganglioside $GM_1$, which is found in high concentration in the membranes of neurons (2). The toxin is used in several different forms. B pentamer, for example, may be injected into specific areas of the rat brain and, for retrograde labeling, into specific organs or nerves (10). The subunit can then be detected along sections of the neuronal pathway and localized in brain tissue sections by using polyclonal anti-CTB goat antiserum visualized with lissamine rhodamine antiserum to goat IgG (24, 86) or other labeling techniques (66). Alternatively, the CT may be conjugated to peroxidase or biotin and injected into the hypothalamus of animals to trace neurological connections (153). CT-peroxidase conjugates have also been used directly on chromatograms as a stain (25).

## CONCLUSION

Since the last review of CT and LT literature was completed, there have been major breakthroughs in the solution of the most pressing and important problems. The three-dimensional structure of LT has permitted us to relate many of the biochemical and biophysical properties of both toxins to structural features. The structure of the complex between lactose and LT has apparently answered the question of toxin orientation on the cell membrane while intensifying the mystery of how the A1 subunit is inserted and translocated into the cytosol. Although the experimental observations of interaction between the toxins and receptor-bearing membranes are extensive and the X-ray structure provides much information for speculation, there is as yet no unequivocal model for the insertion or translocation of holotoxin or enzymatic subunit across the plasma membrane of intestinal epithelial cells. It would appear that there is no significant conformational change associated with receptor binding by CT or LT under conditions resembling those in the native state, and there is some evidence of alternative mechanisms in cells other than enterocytes.

Researchers may now write the long-awaited chapter detailing the success of vaccines against cholera and the *E. coli*-associated diarrhea that were devised and tested throughout the 1980s. These latest results indicate that the tools and knowledge assembled since 1959 have led to vaccines that could have as much as 95% efficacy in the short term and perhaps 50% efficacy after 3 years. The vaccines should go far toward reducing the morbidity and mortality caused by these diarrheal diseases and, by so doing, may allow sufficient economic recovery to make possible the improved sanitation that is necessary to prevent disease.

There are still unanswered questions. A great deal of CT and LT research has been directed toward understanding receptor binding, and the three-dimensional structure makes that work much more meaningful. It should now be possible to delineate the structural requirements for specific binding to receptor ligands and to make use of these data to design drugs that will specifically inhibit binding to ganglioside $GM_1$. The data accumulating on the role of cytosolic factors in toxin activation could be the basis for an expansion of our knowledge of the role these factors play in normal cellular metabolism. We need to clarify the precise mechanisms involved in the excess fluid and electrolyte secretion caused by the toxins. The relationship between ADP-ribosylation and the morphological changes observed in cultured cells requires explanation. The mechanism by which CT apparently stimulates the immune system is just beginning to be explored, and the results of these investigations have consequences for many other disease conditions.

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