THE CHEMISTRY AND BIOLOGY OF CHOLERA TOXIN

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I. INTRODUCTION

Cholera toxin is a protein isolated from the culture filtrate of *Vibrio cholerae* as the causative agent of diarrhea in experimental animals. This protein has attracted considerable attention in recent years because of its unexpected biological activity; it has been found to stimulate the enzyme adenylate cyclase in diverse types of avian and mammalian cells, and thereby induces cyclic AMP-mediated changes in cell functions. A pure preparation of cholera toxin was first obtained and identified as the diarrheagenic substance in 1969 by Finkelstein and LoSpalluto.[1] In the decade since this discovery, much information about the chemistry and biology of cholera toxin has been accumulated, and will be the subject of this review. Studies on the mode of action of cholera toxin at the molecular level have, in turn, provided clear understanding of the pathogenesis of cholera as well as other diarrheal diseases. Excellent reviews on cholera, cholera toxin as the etiologic agent, and its mechanism of action may be found in References 2 to 5.

In this article, events or findings that in the author's opinion contributed a great deal to our present understanding of cholera toxin will be discussed with some experimental details, and with emphasis on more recent developments. Work in this area has been published in a wide range of scientific journals, and the author apologizes for any unintentional omissions from the review.

II. HISTORICAL PERSPECTIVES

The comma-shaped bacteria, *Vibrio cholerae*, was isolated and identified as the pathogen of cholera by Robert Koch in 1883. The next year Koch suggested that the profuse diarrhea characteristic to cholera was probably due to a poison elaborated by the vibrio in the small intestine. The suggestion was based on the observation that the invading bacteria were found only in the contents of the patient's intestine and not in the epithelial wall or the surrounding tissues. The idea of cholera being a toxicosis was, however, not substantiated because i.p. injection of the culture filtrate of *V. cholerae* failed to produce the symptoms of the disease in experimental animals, and had not been seriously tested for 75 years.[3]

The difficulty in finding suitable animal models for cholera was apparently the main reason for the long lapse in the studies on the pathogenesis of cholera. Oral administration of even a large amount of live vibrio had either failed to induce the infection in laboratory animals or killed the animal through septicemia rather than diarrhea, as seen in human cholera. Metchnikoff reported in 1894 that 25 to 50% of infant rabbits could be infected orally with *V. cholerae* to produce diarrhea and death, but the results

were irregular and unpredictable, and the study was not pursued further. Other factors had also influenced the delay in recognizing cholera as a toxicosis. One of these appeared to be the existence of the dogma that only gram-positive bacteria would produce exotoxins. *V. cholerae,* being a gram-negative bacillus, was unlikely to excrete toxins. A lethal toxicity was seen occasionally with a massive dose of heat-killed bacterial suspension to experimental animals, but the effect was thought to be due to the endotoxin, a lipopolysaccharide constituent of the bacterial-cell wall.[6] The earlier findings of the morbid anatomy of cholera victims that the intestinal epithelium was desquamated and sloughed had further led to the misconception of the etiology of cholera. Thus, the disease state of cholera was generally thought to arise from denudation of the intestinal surface by the invading vibrio, possibly through the action of digestive enzymes and mucinase,[7] resulting in the exudation and loss of body fluid. This view appeared to have prevailed through the first half of the 20th century.

Interestingly, great physicians of the mid-19th century had already noted, even before the discovery of *V. cholerae,* that the characteristic rice-water stool in cholera consisted of water and "blood salts" but contained very little albuminous matter, and suggested a role of poison rather than physical lesion in the disease.[2-4,8] This observation was later confirmed by a few others, but studies on cholera took the general miscourse mentioned above for almost another century. Interest in cholera was apparently rekindled after the great Egyptian epidemic in 1947 and the Thai epidemic in 1958.[2] More refined observations on the composition and dynamics of electrolytes and fluid movements were made and formed the basis of fluid-replacement therapy, which has since saved a great many lives from cholera infection. The rapid recovery of cholera patients under proper treatment indicated again a poisoning as the cause of the illness. These clinical observations seemed to have also prompted reexamination of the pathogenesis in cholera in the late 1950s.

The first successful animal model for cholera was developed by De and Chatterje[9] in 1953. The authors were able to cause accumulation of "rice water" in the ligated segments of the small intestines of rabbits by injection of live vibrio suspensions. Under anesthesia, the small intestines from fasted rabbits were tied in segments into which aliquots of test material were injected. The intestines were then returned to the abdominal cavity, and the animals were killed the following day. Fluid accumulated in the segments and the tissues were examined. In this study, a relatively high content of albumin was noted, which was different from that in the cholera stool. The infant rabbit model of Metchnikoff was revived and successfully used for chemotherapeutic study in 1955 by Dutta and Habbu.[10] Under anesthesia, ten-day-old rabbits were injected with the animal-passed *vibrio* suspensions directly into their small intestine. Profuse diarrhea much like that of human cholera developed in 22 to 52 hr when more than 10^3 vibrio per 100 g body weight was injected. It was also found that rabbits older than 16 days were completely resistant to the infection. This procedure had much greater reproducibility than that originally reported by Metchnikoff, and was later adapted for use in oral administration of test materials.

In 1959 both De[11] and Dutta et al.[12] succeeded in producing experimental cholera in the respective animal model with the cell-free culture filtrate of *V. cholerae,* thus providing strong evidence for the role of the extracellular substance in the pathogenesis of cholera. The infant rabbit model appeared to simulate more closely the natural infection and symptoms of cholera, and was used to demonstrate the presence of a diarrhea-causing, or enterotoxic, substance in the filtrates of human cholera stools.[13] By that time the technique for biopsy of the small intestine had become available, and in 1960 Gangarosa et al.[14] reported that the small intestine of cholera patients showed no evidence of damage during profuse diarrhea. A separate study to test the permea-

bility of intestinal tract to macromolecules by intravenous injection of ^{131}I-polypyrrolidone also indicated no change in the intestinal epithelium during cholera.[15] Thus, it became apparent that desquamation of intestine observed earlier was due to a postmortem artifact, and that the lesion in cholera was at the functional rather than the physical level. Clinical investigations on human cholera as well as experiments with animal models had by then pointed to toxinosis in cholera and the stage was set for the search for the extracellular substance responsible for the symptoms of the disease.

The cell-free culture filtrate of *V. cholerae* was indicated earlier to alter vascular permeability; it caused edema of myocardium on perfusion through an isolated mammalian heart, or increased the outflow of fluid from an isolated intestinal strip immersed in it (Manwaring et al. (1923) and Burrows et al. (1944), as cited in Reference 9). The latter experiment apparently provided the basis for the development of the ligated intestinal loop model for the experimental cholera.[9] The presence of a heat-labile, nondiffusable substance that caused firm swelling in the skin of rabbits or guinea pigs was demonstrated by Craig[16] in filtrates of cholera stools and culture supernates in 1965. Intravenous injection of a blue dye into the animal caused coloration of the indurated sites of inoculation, indicating the increased permeability of local capillaries. The active substance was named vascular permeability factor (PF). The size of the swelling could be measured with reasonable accuracy after the blueing, and was found to be directly proportional to the concentration of PF. Subsequent studies on the production of PF by *V. cholerae,* its properties, and neutralization by cholera antiserum, strongly indicated that PF was identical to the diarrheagenic exotoxin elaborated by the vibrios.[3,17-19] The assay of PF, though requiring considerable skill, has provided a sensitive and accurate measure of cholera toxin.

An extracellular product of *V. cholerae* having enterotoxicity (defined as the ability to cause the net loss of water and electrolytes into gut lumen without tissue destruction) was naturally the subject of studies by many investigators, using either or both the ligated intestinal loop model[20,21] and the infant rabbit model[22] to follow the activity. Quantitation with these assay methods was poor, and refinement of the procedures was necessary. Relying mainly on the assay of diarrheagenic activity in infant rabbits, Finkelstein et al.[1] succeeded in the isolation of cholera enterotoxin in 1969. In the last step of the purification procedure, a protein which cross-reacted with anticholera toxin antibody but was not enterotoxic, was separated from the enterotoxin, and was named choleragenoid. The cholera enterotoxin, or choleragen, was shown to be a protein with a molecular weight of 84,000 that exhibited dose-dependent enterotoxicity in both infant rabbits and ligated ileal loops as well as PF activity. All activities were neutralized by the same antibody, indicating that these were of the same molecular entity.[23] Choleragen and choleragenoid were subsequently obtained in crystalline forms.[24]

Studies on cholera toxin took a dramatic turn at the beginning of the 1970s, following some seemingly unrelated experimental findings. During the in vitro study of ion transport across intestinal membrane, Field et al.[25] discovered in 1968 that cyclic AMP caused reversal in the ion flux, resulting in the net secretion of electrolytes by rabbit ileal mucosa. Since the phenomenon was analogous to that in cholera, the experiment was repeated with cholera toxin, and the same effect on the intestinal chloride secretion was observed.[26,27] Meanwhile, Greenough et al. found that addition of cell-free culture filtrate of *V. cholerae* to a fat-cell suspension caused an increase in glycerol production after a lag period.[28,29] The amount of glycerol released from fat cells was proportional to that of the cholera toxin added. Furthermore, cholera toxin was found to stimulate breakdown of glycogen, both in liver cells and platelets.[30] Lipolysis in fat cells and glycogenolysis in liver cells had recently been recognized as the metabolic functions mediated by cyclic AMP. Thus, stimulation of those functions by cholera toxin indi-

cated the involvement of cyclic AMP.[27] Indeed, the cyclic AMP level in intestinal mucosa was found to increase on treatment with cholera toxin.[31] It was further demonstrated that the rise in the cyclic AMP concentration was due to stimulation of adenylate cyclase by cholera toxin, rather than to inhibition of the breakdown of cyclic AMP.[32,33] Tests with pure cholera toxin available by then strongly indicated that stimulation of adenylate cyclase was the primary biological function of this protein.[1] Studies on various tissues have since provided increased evidence for this view, most notably on the alteration of cytotoxic action of lymphocytes,[34] induction of steroid-genesis in adrenal cell culture,[35] inhibition of histamine production in leucocytes,[36] and elevation of the cyclic AMP level followed by stimulation of amino acid transport in thymocytes.[37]

The pathogenesis of cholera, characterized by profuse diarrhea that leads to severe dehydration and hypovolemic shock, has now been understood to be the derangement of the ion-transport function of the small intestine as a result of the increased production of cyclic AMP in epithelial cells, in response to the exotoxin of *V. cholerae*. Cholera toxin is thus not a toxin in the classical sense, in that it does not cause permanent damage to tissue or cells. Choleragenoid, the cross-reacting protein devoid of enterotoxicity obtained in the purification of choleragen,[1,23] was shown to block the action of choleragen in vitro, suggesting its possible therapeutic use in cholera. Whether choleragenoid is formed *in situ*, or is involved in the rapid recovery of cholera patients under the fluid-replacement therapy, are still questions to be answered.

Cholera toxin has in recent years been used as a reagent for the testing of cyclic AMP-mediated cell functions because of the ubiquity of its action on animal cells (Figure 1). Availability of pure choleragen and its interesting biological activities including its affinity to a wide variety of cell membranes, have further made it an excellent model for studying protein structure-function relationship.

III. PREPARATION AND ASSAYS

In the purification of a biologically active material, a reliable method of measuring the activity must first be established. In the case of cholera toxin, this became possible only when the methods to produce the symptoms of cholera in animals were found. Finkelstein et al.[1] mainly followed the diarrheagenic activity in infant rabbits in their purification of cholera toxin. Others followed the activity to increase vascular permeability in rabbit skin (PF),[17,18] or accumulation of fluid in ileal loops of adult rabbits.[21] Studies using pure cholera toxin have indicated, however, that these activities and the diarrheagenic activity are the function of the same protein.[3] As discussed in the previous section, the primary effect of cholera toxin on the cells appears to be the stimulation of adenylate cyclase, the results of which are expressed differently in different cells. It is thus apparent that any assay which conveniently measures stimulation of adenylate cyclase or its consequences may be used for the assay of cholera toxin. Alternatively, since pure choleragen is available from a commercial source (Schwarz/Mann, Orangeburg, N.Y.), antibody may be prepared and immunoprecipitation or radioimmunoassay may be performed. In this review, the assay methods that the author considers convenient, accurate, and relatively simple are described, as well as those of historic importance.

A. Assays of Cholera Toxin

1. Enterotoxicity Assay

Measurements of the activity to stimulate intestinal fluid secretion in rabbits were originally used to identify the etiological agent in cholera.[9,10] The procedures are cum-

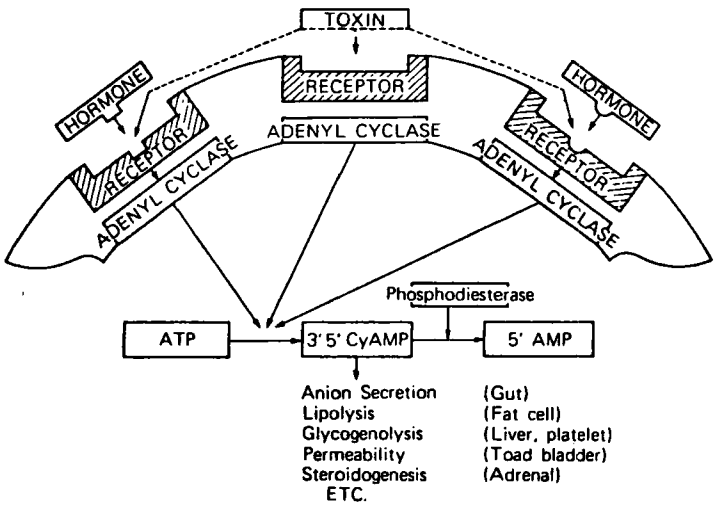


FIGURE 1. Diagram depicting ubiquitous action of cholera toxin on a variety of cells, with modulated cell functions as the consequence of elevated cyclic AMP level. (Courtesy of Carpenter, C. C. J., Jr. and Guerrant, R. I., in *Symp. on Cholera*, U.S.-Japan Cooperative Medical Science Program, Tokyo, 1972, 105.)

bersome and give poor quantitations. Of the two methods (the ligated ileal loop test in adult rabbit[9] and the diarrheagenicity test using infant rabbits[10]), only the latter is given as described by Finkelstein et al.,[1,22] in order to provide perspective on the assay.

Suckling rabbits approximately 10 days old were given gastric lavages with lukewarm water to remove milk and suspended matter. They were then fed with dilutions of test material in 1 m*l* of 2% K_2HPO_4-2% Na_2HPO_4 from a syringe connected to a gastric tube. After 16 to 18 hr surviving animals were sacrificed and the amount of fluid in the intestinal tract and on the abdominal surface were scored. Additional points were assigned to succumbed animals and points averaged for the group which received the same amount of test material. The mean score thus obtained was the measure of relative enterotoxicity (see also Reference 1).

Dog is another species of animal which has been found to be susceptible to experimental cholera.[38] It is to be noted that suckling mice have been used for the assay of *Escherichia coli* enterotoxin[39] in the same manner as the suckling rabbit test for cholera toxin. In view of the similarity in the mode of action[40] and immunological cross-reactivity[41] between *E. coli* heat-labile toxin and cholera toxin, suckling mice might be an economical substitution for infant rabbits for assay of choleragen.

2. Vascular Permeability Assay

This is still the most widely used in vivo assay of cholera toxin because of its reliability, high sensitivity, and accuracy when combined with the use of anticholeragen antibody.[3] However, the procedure is by no means a simple one and requires considerable experience.

The back hair of large-size rabbits is clipped, and a checkerboard pattern of about ½ in. squares drawn on the bared back skin with a felt pen. A serial dilution of the test material in physiological saline is mixed with a constant amount of antitoxin so that 0.1 m*l* contains 0.05 AU (antitoxin unit, measured against the provisional standard cholera antitoxin of Craig[17]) and incubated for 1 hr at 37°. At random sites at the center of the squares 0.1 m*l* of the solution is injected intracutaneously in dupli-

cate or triplicate, making records of the site of injection of each sample. About 18 hr later the rabbit is injected i.v. with 1.2 mℓ/kg of 5% Pontamine Sky Blue, whereupon the firm swellings due to the vascular permeability induced by cholera toxin turn blue. The diameters of the blued indurations are measured 1 hr after injection of the dye, and the values for a given dilution averaged. The amount of the vascular PF of the sample is expressed in Lb (limit of blueing) units; one Lb unit is defined as the amount of toxin neutralized with 1 AU of antitoxin to cause a blueing area 4 mm in diameter for a 0.1-mℓ injection per site. Neutralization of PF activity with antitoxin follows a sigmoid curve with a sharp end point of BD (blueing diameter) of 4 mm, providing greater accuracy than that without the use of antitoxin (Figure 2). In the experiment described above, a 4-mm lesion is made with 4.4 Lb units of toxin per injection site (Figure 2). When the assay is carried out without pretreatment with antitoxin, the amount which causes the 7-mm diameter lesion is used as the unit of measure and designated as 1 BD_7 blueing dose.[3] Approximately 830 BD_7 units correspond to 1 Lb unit (Figure 2). One μg of the purified cholera toxin shows 24.6 Lb units of PF activty, according to Rappaport et al.[42] The sensitivity of detection of cholera toxin (1 BD_7 unit) is therefore calculated to be about 0.2 ng with considerable accuracy.

3. Adenylate Cyclase Stimulation Assay

The activity of cholera toxin to stimulate adenylate cyclase has been measured with many mammalian or avian cells, through the assay of the change in the cyclic AMP concentration, or in the cell function after treatment with the toxin. The unit of activity is defined as the amount which causes 50% of the maximum change in that particular assay system.[29-31,35,37]

For purified cholera toxin, the activity is conveniently assayed by the pigeon erythrocyte lysate system described by Gill and King.[43] In this procedure, washed pigeon ergthrocytes suspended in the 0.01 *M* hydroxyethyl piperazine-2 ethane sulfonate (HEPES) buffer (pH 7.3) containing 0.13*M* NaCl and 0.01% NaN_3 in 1:1 volume ratio, is lysed by freezing-thawing and immediately used for the assay. On addition of cholera toxin pretreated with dithiothreitol (DTT), NAD, and ATP at 1 m*M* each, adenylate cyclase of pigeon erythrocyte is activated as much as tenfold within 15 min at 37°C. After incubation, the mixture (25 to 50 $\mu\ell$ is diluted with 20 volumes of the cold buffer and centrifuged. The pelleted red-cell ghost is suspended in the buffer for the assay of adenylate cyclase activity by any of the available methods.[44,45] As will be explained later, preincubation of the toxin with DTT and addition of NAD and ATP to the reaction mixture are necessary for the stimulatory activity in ruptured cells. Under these conditions, 50% of maximum stimulation was attained with 0.9 μg of purified cholera toxin when incubated with 0.25 mg ghost protein in a 25-$\mu\ell$ reaction mixture[46] (see also Figure 12).

Although the use of pigeon erythrocyte lysate provides a sensitive measure of the cholera toxin activity, it is influenced by substances which affect the effective concentrations of DTT, NAD, and ATP, or the physical state of erythrocyte membrane in the reaction mixture. For the assay of crude toxin preparations, therefore, the use of intact pigeon erythrocytes is recommended. This is carried out by incubation of the red-cell suspension with the test material for 30 min at 37°C in the absence of DTT, NAD, or ATP. Cells are then washed, lysed by freezing and thawing, and the ghosts used in the assay of its adenylate cyclase activity. The maximum stimulation in the intact cells is only about twofold over the basal adenylate cyclase activity (1.4 pmol cAMP per minute per milligram of ghost protein), and 50% maximal activity is obtained with 0.5 μg of pure toxin per milligram of ghost protein.[48]

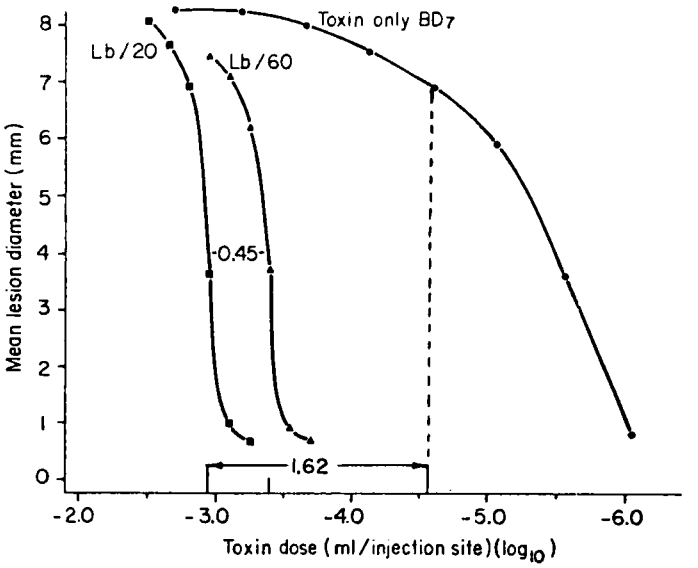


FIGURE 2. Dose-response curves in vascular permeability test. Lb/20 and Lb/60 are, respectively, the values obtained after incubation with 1/20 AU and 1/60 AU of antitoxin. The log distance between Lb/20 and BD_7 is 1.62. Therefore, 1 LB dose is equivalent to 830 ($10^{1.62}$ × 20) BD_7. (From Craig, J. P., *Microbial Toxins*, Vol. 2A, Kadis, S., Montie, T. C., and Ajl, S. J., Eds., Academic Press, New York, 1976, 53. With permission.)

4. Radioimmunoassay

If the antiserum against pure choleragen is available, radioimmunoassay offers the most convenient, accurate, and sensitive measure of cholera toxin. In the author's laboratory, antiserum with good titer was obtained from rabbits immunized by two intramuscular injections at 1-month intervals of 1-m*l* emulsion of purified cholera toxin (12.5 μg/m*l* with complete Freund's adjustment) under the shoulder blades. This was followed by two additional injections with the same amount of cholera toxin in physiological saline at 1-week interval 6 weeks later. The blood was withdrawn 1 month after the last injection. For the assay, the antibody was adsorbed onto polystyrene beads by mixing 10 μ*l* of antiserum with 10 m*l* of a 1%-suspension of beads (# 54225 polystyrene, Dow Diagnostics, Indianapolis, Ind.) in 0.1 *M* $NaHCO_3$ (pH 9.6) for 18 hr at room temperature. The antibody-coated beads are then washed twice and suspended with 10 m*l* of 0.05*M* PO_4 (Na) buffer (pH 7.4) containing 0.9% NaCl and 0.1% bovine serum albumin.[47] Purified cholera toxin is labeled with (^{3}H)−$NaBH_4$ in the presence of formaldehyde,[48] and adjusted to approximately 300 cpm/ng of cholera toxin. In this procedure, a constant amount of (^{3}H)-choleragen (about 3000 cpm) is added to a series of tubes containing 5 to 100 ng choleragen standard and serially diluted unknown samples. Then, fifty μ*l* of the antibody-beaded suspension is added to each tube and incubated for 1 hr at 37°C. The suspension is then diluted with 2 m*l* of the buffer, centrifuged to precipitate the beads, washed with the buffer, and the beads transferred to scintillation vials with 1 m*l* of the buffer for counting. A plot of percentage of radioactivity in the precipitate vs. logarithms of weight of cholera toxin from the standard samples is made on semilog paper, and the weight of cholera toxin from the unknown sample read off the graph (Figure 3).

With the procedure described above, 5 to 20 ng of cholera toxin in a sample has

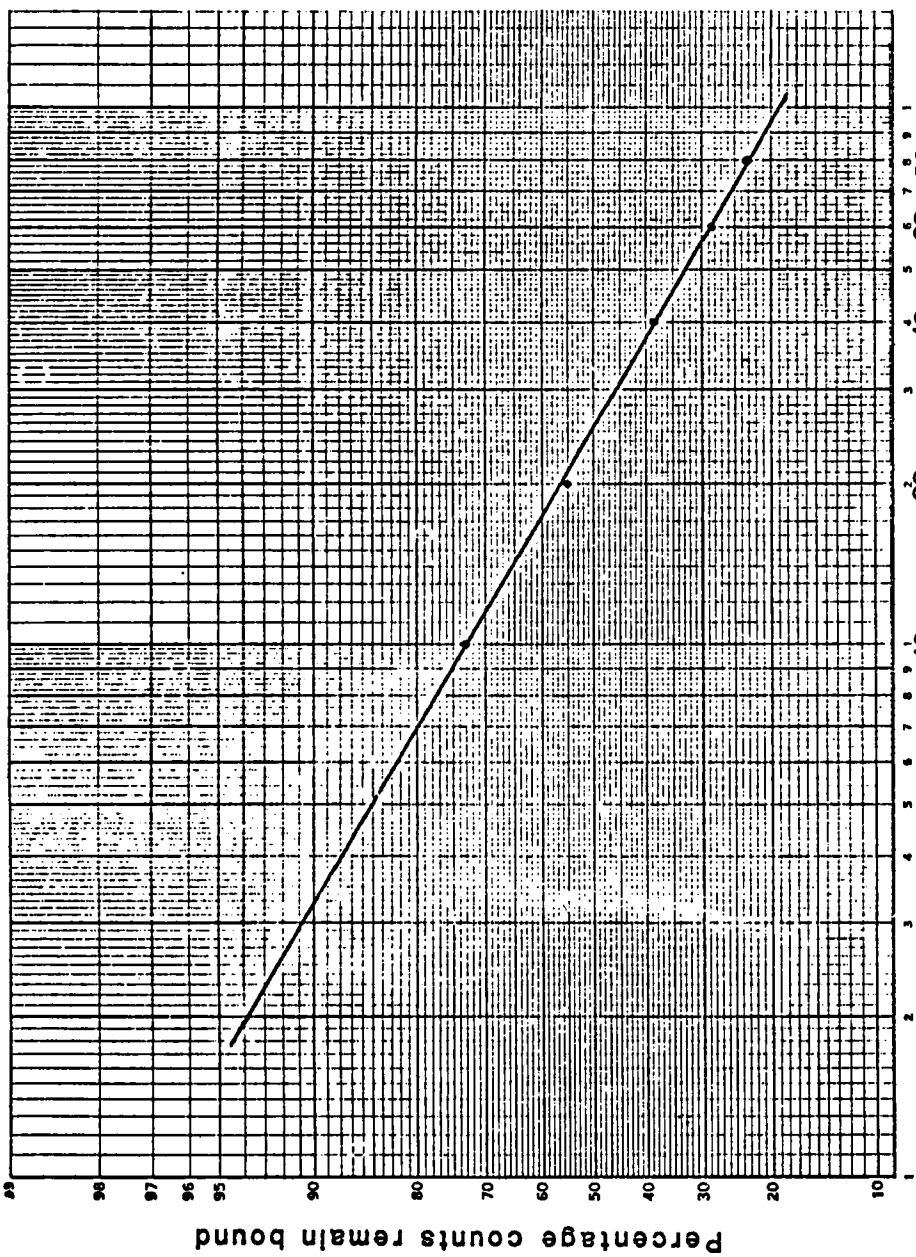


FIGURE 3. A standard curve for the radioimmunoassay. In this example 3000 cpm of the labeled choleragen was mixed with the indicated amount of cholera toxin for reaction with antibody-coated nylon beads.

been quantituated with better than a 10% accuracy. The sensitivity and accuracy may be increased by the use of cholera toxin with higher specific radioactivity and a smaller amount of adsorbed antibody on the polystyrene beads. The disadvantage of the method is that choleragenoid cannot be distinguished from choleragen. The same is true in the assay of cholera toxin by measurement of Lb units stated above. In principle, this problem may be avoided if antiserum specific to the A-subunit (see later section) can be prepared and used in these assays. Such a procedure has thus far not been seen in the literature.

5. Cell biological activity

Chinese hamster ovary (CHO) cell cultures have been used for a sensitive assay of cholera toxin. Guerrant et al.[49] reported that enterotoxins of *V. cholerae* and *E. coli* caused a marked elongation of CHO cells in culture, mimicking the previously known effects of dibutyryl cyclic AMP and testosterone. The change in the cell morphology occurred concomitantly with the rise in the intercellular cyclic AMP level, indicating that the latter was again involved in the process. In this assay, CHO cell suspension (5×10^3 cells in 0.25 m*l*) was incubated with various amounts of toxin in a culture slide for 24 hr, when the morphological response reached maximum. The percentage of cells elongated was determined by counting the cells under a phase-control microscope. Under this condition, 1 ng/m*l* of cholera toxin caused a half-maximal change in cell morphology (20 to 25% of cells elongated), and the effect was abolished by antitoxin serum, choleragenoid, or by heating the toxin.

More recently, Nozawa et al.[50] described a simplified assay method based on the increased adhesiveness of CHO cells in response to cholera or *E. coli* enterotoxin. Confluent monolayers of CHO cells were incubated with varying amounts of cholera toxin in the culture medium for 18 hr, and the cells floating in the medium were counted. The toxin reduced the number of cells floated into the medium. The amount of toxin required to reduce the number of floating cells to 50% of the control (no toxin) was about 80 pg/m*l*, with a monolayer of 10^5 cells per cm^2 in 35-mm plastic culture dishes.[50] Antitoxin and heat treatment abolished the action of cholera toxin.

B. Preparation

1. Formation of Enterotoxin in the Culture of V. cholerae

Practically all cholera toxin preparations used in biochemical studies are purified from the culture filtrate of *V. cholerae* Inaba strain 569B. Dutta and Habbu[10] found that high virulence evolved on passage of the Inaba strain 569B through infant rabbits, and the level could be maintained by animal passage every 6 months.

Maximum yields of enterotoxin were obtained by Finkelstein et al.[22] from agitated cultures of the Inaba strain 569B grown in a medium containing sucrose, inorganic salts, and 1% caseamino acids. This synthetic medium was designated as "Syncase", and the toxin produced in this medium as "Syncase choleragen" before its identity to the vascular PF was established.[3] Following the PF activity, Evans and Richardson[18] found that addition of caseamino acid and yeast extract to a simple medium containing sucrose and ammonium salts enhanced the production of PF by at least tenfold and formulated the TRY medium.[51] The latter was used in the large-scale production (250-*l* fermenter) of cholera toxin by Rappaport et al.[42] at Wyeth Laboratories, Philadelphia which supplied lyophilized culture filtrates for some time to the Geographic Medicine Branch of the National Institutes of Health for investigative use. High yields of cholera toxin have been obtained in either medium. *V. cholerae* Inaba strain 569B from an overnight nutrient agar slant is inoculated to the liquid medium and grown at 24 to 30°C for 24 to 48 hr with vigorous aeration and mechanical agitation. The cells were

removed either by continuous centrifugation or by filtration through a membrane filter to obtain the starting material for toxin preparation.

2. Purification of Cholera Toxin from the Culture Filtrate of V. cholerae

Purification of cholera enterotoxin active in the ligated ileal loop test was described in 1968 by Coleman et al.[21] A preparation showing a single precipitin line with antiserum was obtained by gel-filtration on Sephadex® G-200 and DEAE-Sephadex® chromatography, and was found to contain lipid and no tyrosine, methionine, or tryptophan on alkaline hydrolysis. In retrospect, this preparation must have been heavily contaminated with lipopolysaccharide endotoxin.

A choleragenic protein of well-defined purity was first obtained by Finkelstein and LoSpalluto[1] in 1969. In this procedure, the diarrhea-causing activity was first concentrated by $(NH_4)_2SO_4$ precipitation (70 g/100 m*l*) from the cell-free supernatant of the vibrio strain 569B culture. The precipitate was dialyzed against 0.01 *M* PO_4 buffer (pH 7.5) in the cold, adsorbed on DEAE-cellulose (Whatman DE-52), and the enterotoxic fraction eluted with 0.1 *M* PO_4 buffer (pH 6). Gel filtration on a large agarose (Bio-Gel ® A-5m, Bio-Rad Laboratories, Richmond, A) column, followed by separation of the precipitin-forming fraction on Sephadex ® G-75, yielded two protein peaks. Both formed single and identical precipitin lines with anticholera toxin serum (Figure 4). The peak eluted first from the Sephadex ® G-75 column was found to be enterotoxic in the infant rabbit assay, whereas the other fraction was inactive. These were designated choleragen and choleragenoid, respectively.[1] Both fractions were found to be homogeneous with respect to electrophoretic mobility in polyacrylamide gel, sedimentation velocity, and immunoelectrophoresis. In later studies, the procedure was modified to use ultrafiltration of the culture supernate on XM-100 filters (Amicon Corp., Lexington, Mass.) to remove endotoxin and large molecular species, then on PM-30 to concentrate the toxin fraction. The concentrate was gel-filtered successively on Sephadex ® G-75, agarose A-5m, and finally on Sephadex ® G-75 again to separate choleragen and choleragenoid.[23] The use of agarose A-5m was important since choleragen and choleragenoid were apparently retarded specifically on this column. The average yields of choleragen and choleragenoid in this procedure were 28.3 mg and 44.1 mg, respectively, from 9*l* of culture in 17 experiments.[23] In 1974 Rappaport et al.[42] reported an efficient purification procedure for cholera toxin which provided high yields, with little or no choleragenoid. In this procedure, the choleragen-containing fraction (assayed for rabbit skin vascular permeability [see A2 above]) was concentrated from the cell-free culture filtrate by adsorption on the sodium methaphosphate precipitate. Sodium hexametaphosphate was added in the clear culture filtrate (through Millipore ® filters [pore size 0.22 μm], Millipore Corp., Waltham, Mass.) at 4°C at 2.55 g/*l*, and the pH of the solution slowly adjusted to 4.6 with concentrated HCl, whereby a precipitate formed. The precipitate was collected on a filter with filteraid (Celite, added at 0.5 g/*l*), washed with 0.2 *M* NaCl at pH 4.6, and then suspended in 0.15 *M* PO_4 (Na) buffer, pH 8 (3000 Lb units per milliliter) to extract the toxin from Celite. The metaphosphate concentrate was adjusted to pH 5.6 and adsorbed onto $Al(OH)_3$ (30 g/100 m*l*) by stirring for 2 hr at room temperature. The adsorbant was washed with water at an approximate pH of 5.5, eluted with 0.2 *M* NH_4HCO_3 (pH 8), and lyophilized. The preparation was dissolved in buffered saline, treated with activated charcoal, and then filtered through a membrane filter. The specific activity, calculated as Lb units per μg of protein, increased from 0.34 for the culture filtrate to 24.6 for the purified toxin, and the recovery based on Lb units was 66% in a preparation from a 250-*l* culture.[42] In terms of protein, the yield was 8 mg/*l* or 72 mg/9 *l*, comparable to that obtained by Finkelstein and LoSpalluto for the combined amount

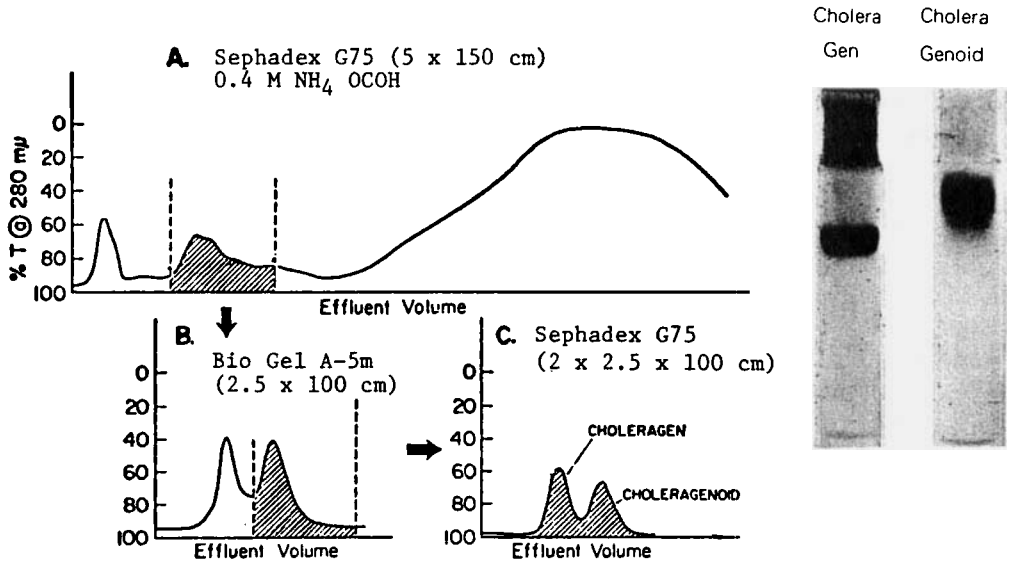


FIGURE 4. Separation of choleragen and choleragenoid. Fractions containing immunoreactivity (against anticholeragen) from Sephadex® G-75 (A) and Bio-Gel® A-5m chromatography (B) were combined and gel-filtered on Sephadex® G-75 (C). Two antigenic peaks emerged, the first with enterotoxicity (choleragen) and the second without activity (choleragenoid). The photograph on the right shows a typical polyacrylamide gel-electrophorograms at pH 9 for the two proteins.

of choleragen and choleragenoid. The homogeneity of the purified choleragen was established with immunoelectrophoresis and acrylamide gel electrophoresis, and the specific BD_7 activity was comparable to that of Finkelstein's purified choleragen. The absence of choleragenoid in the preparation was implicitly shown by its high specific activity.

Purification of cholera toxin using its specific affinity to dextran sulfate was described previously by Richardson and Evans.[19] The dextransulfate toxin (PF) complex was dissociated by ultracentrifugation in concentrated salt solution, and the PF further purified to homogeneity as judged by immunoelectrophoresis. But the yield of dextransulfate precipitation was less than 10%, and the product was suggested to contain a lipoprotein.[19,51]

In the author's laboratory, a procedure modified from those of Finkelstein and LoSpalluto[23] and from Rappaport et al.[42] has been successfully used for purification of choleragen and choleragenoid from the lyophilized culture filtrate of *V. cholerae* Inaba strain 569B. The metaphosphate-toxin complex is precipitated at 4.6 from a dialyzed, concentrated culture filtrate, as in Rappaport's procedure, and collected by centrifugation. The precipitate is dissolved in 0.2 *M* NH_4HCO_3 (pH 8) and gel-filtered on a large Bio-Gel® P-100 column (polyacrylamide gel, Bio-Rad Laboratories) to yield an essentially pure preparation of cholera toxin[52] with 80% recovery, as judged by radioimmunoassay. For studies where contamination of choleragenoid is undesirable, the preparation is gel-filtered on Sephadex® G-75, as in Finkelstein's procedure.

An important advance in the method of cholera toxin production was made recently by Mekalanos et al.[53] These investigators used hypertoxinogenic mutants of *V. cholerae* strain 569B, selected by a novel procedure in which filter paper impregnated with ganglioside-serum albumin conjugates and ^{125}I-labeled anticholera toxin were employed to detect the mutants.[54] These mutants were found to produce two to three times the amount of toxin produced by the wild-type strain and used in the toxin.

Phosphocellulose was used for the purification of cholera toxin after the metaphosphate precipitation step of Rappaport et al.[42]

3. Occurrence of Choleragenoid in the Toxin Preparations

Several lines of evidence suggest that choleragenoid is formed during the toxin purification procedures, rather than by the vibrios in culture. Finkelstein et al.[55] reported that vigorous agitation of a choleragen sample at 30°C for 48 hr, either with or without added *V. cholerae,* caused formation of choleragenoid, detectable by Sephadex® G-75 chromatography or disk-gel electrophoresis. On heating choleragen at 60 to 70°C at pH 7.5 for 30 min, they observed the formation of a large molecular weight "pro-choleragenoid", which subsequently underwent decomposition to choleragenoid.[56] As mentioned above, the procedure of Rappaport yielded preparations essentially free of choleragenoid with recoveries comparable to the combined yields of choleragen and choleragenoid obtained in Finkelstein's procedure,[23] which involved many manipulations through membrane filters and molecular shieves. It became clear in later studies on the subunit structure of cholera toxin that choleragenoid was an aggregate of B subunits, whereas choleragen contained A subunits in addition to the former, and the two types of subunits could be separated under various conditions.[57-59] On repeated freezing and thawing of a choleragen sample over a period of several weeks, we have observed formation of choleragenoid, which could be separated on Sephadex® G-75 chromatography.[52]

IV. CHEMISTRY OF CHOLERA TOXIN

A. Physicochemical Properties

The molecular weights of choleragen and choleragenoid were determined by analytical ultracentrifugation methods as 84,000 and 56,000, respectively, by LoSpalluto and Finkelstein,[60] and general agreement was obtained in later experiments by other investigators.[57,61] Other values were also reported for the molecular weight of choleragen prepared by a different procedure and in different buffer[62] and might reflect the difference in the state of aggregation of subunits in the sample. The frictional coefficients, 1.25 and 1.24, as calculated from diffusion coefficients (determined in the sedimentation velocity experiments), indicated that both choleragen and choleragenoid were typical globular proteins.[60] Isoionic points as determined by isoelectric focusing gels were 6.6 for choleragen and 7.75 for choleragenoid.[23] The solubility curve of choleragen in $2M$ $(NH_4)_2SO_4$ at 22°C showed a sharp break at 1.7 mg of protein per milliliter indicating its homogeneity, but that of choleragenoid in 2.67 M $(NH_4)_2SO_4$ indicated molecular heterogeneity, saturating at approximately 2 mg of protein per milliliter.[24] Choleragenoid often appeared as a broad band in polyacrylamide-disk electrophoresis[1] (see Figure 4).

Electron micrography of choleragen at 398,000 × magnification showed a quasi-crystalline array of closely packed particles, which appeared hollow with rectangular or circular outlines.[23] In the electron micrographs published later by Ohtomo et al.[63] at 825,000 × magnification, choleragen molecules appeared to consist of a ring of five to seven particles surrounding a larger particle, in a similar arrangement as proposed by this author[57] on the basis of chemical analysis. Choleragenoid, on the other hand, appeared as packed particles. These results must, however, be interpreted with caution in view of the known instability of cholera toxin to physical manipulations, which had been employed in the negative staining and drying of the specimen for observation.

B. The Subunit (Quarternary) Structure of Cholera Toxin

It was noted earlier that purified choleragen and choleragenoid showed rather broad

bands in disk-gel electrophoresis (Figure 4). This, and the conversion of choleragen to choleragenoid observed under protein denaturating conditions,[55] suggested a polymeric structure for cholera toxin. Indication of the presence of subunits in both choleragen and choleragenoid was first provided by the observation that the sedimentation coefficient of these proteins decreased dramatically in acid or on succinylation.[60] Subsequently, the toxin was shown to contain two types of subunits, while the toxoid contained only one of these; the evidence was obtained by polyacrylamide-gel electrophoresis in the presence of Na-dodecylsulfate (SDS)[58] by adsorption of cholera toxin onto a ganglioside GM_1-cerebroside complex followed by elution of one type of subunit with 8*M* urea and then the other with 6*M* guanidine-HCl,[64] or by isoelectric focusing in 6*M* urea. Choleragenoid showed as a single band but choleragen resolved into two bands, one of which corresponded to that of choleragenoid.[65] However, there were discrepancies as to the size and number of each subunit. For example, conventional gel electrophoresis in 0.1% SDS of cholera toxin showed two major bands, the one with a larger apparent molecular weight corresponded to that from choleragenoid and exhibited affinity to ganglioside GM_1-Agarose.[66] On heating the samples to 57° in 8*M* urea, 2% SDS and 0.04*M* iodoacetamide, the subunit derived from choleragenoid migrated further as a smaller molecular species.[55]

Separation of the two types of subunits was achieved in a preparative scale by gel filtration on Bio-Gel ® P-60, either in 0.1*M* glycine buffer (pH 3.2) containing 6.5*M* urea[59] or in 0.2*M* Na-formate buffer (pH 3.5) containing 5.2*M* guanidine HCl,[69] or on Sephadex® G-75F in 5% formic acid[57] (Figure 5a). The latter procedure has proved to be the most efficient and the simplest because the solvent may be readily removed for analysis or further manipulations. The two subunits were designated A and B in order of their elution from the column. The high recovery of protein (85 to 95%) in this procedure, as estimated by the optical absorbance at 280 nm, indicated that the two subunits were the only constituents of cholera toxin.[57] Finkelstein et al.[59] were able to reconstitute the functionally and immunologically active holotoxin from subunits A and B separated in urea at pH 3, by neutralization and dialysis of the combined fractions. When purified choleragenoid was chromatographed on the same column, only the protein corresponding to subunit B was obtained. Choleragenoid and subunit A were found to form choleragen on neutralizing the mixture in 6.5*M* urea and slow dialysis. These results strongly indicated that subunit B was the sole constituent of choleragenoid, and suggested that subunit A was responsible for the enterotoxicity of choleragen.[59]

Subunits A and B of cholera toxin in solutions of physiological pH are either insoluble or in a polymeric state, and present a problem in physicochemical measurements. Apparent molecular weights as estimated by electrophoretic mobilty in the presence of 0.1% SDS and 6 to 8*M* urea were 28,000 for subunit A and 9,000 to 11,700 for subunit B.[57,59,61,63] Sedimentation equilibrium measurements in 6 *M* guanidine HCl gave the values 30,000 for subunit A[57] and 10,300 to 14,000 for subunit B.[57,61] On renaturation of the subunit proteins by dialysis against neutral buffer with diminishing urea concentrations, clear solutions of both subunits were obtained. Subunit A was apparently unstable in solution, and formed a precipitate in an attempt to measure the molecular weight by the sedimentation equilibrium method. The renatured subunit B showed homogeneity in molecular weight distribution in such a run with a value of 55,200, in close agreement with that of choleragenoid.[57] From the apparent molecular weights of holotoxin, choleragenoid, and monomeric subunits, it was deduced that choleragen consisted of 1 mol of subunit A and 4[61,69] 5, or 6[57,59,63] mol of subunit B. Lai et al.[57] calculated the molar ratios of subunits B to A in the holotoxin from the known content of cysteine in each subunit, and also by quantitation of the terminal dipeptide Ala-

Asn, released from choleragen on BrCN cleavage, arriving at a value of 5.5 from both analyses; subunit B contains a Met-Ala-Asn sequence at the COOH-terminus (see Figure 7) and would release 1 mol of Ala-Asn per mole of B subunit on treatment with BrCN. Based on these results, it was proposed that choleragen samples used in most studies consisted of a mixture of molecules whose subunit compositions were AB_5 and AB_6[57] (Figure 6). The scheme was also intended to provide an explanation for the formation of choleragenoid from choleragen reported earlier.[55] With the use of dimethyl suberimidate to effect intersubunit cross-linking, and subsequent separation on polyacrylamide-gel electrophoresis in 0.1% SDS, Gill observed five molecular species corresponding to B_1, B_2, B_3, B_4, and B_5 from the treated choleragenoid, and nine bands from choleragen corresponding to A_1, AB_1, AB_2, AB_3, AB_4, and AB_5 in addition to B_1, B_2, and B_3.[67] Gill[67] suggested that choleragenoid was a pentamer of B subunits and that choleragen had the structure of AB_5. The molecular weight of Subunit B calculated from the primary structure (Figure 7) is 11,604.[71-73] This value, in conjunction with the molecular weights of choleragen 84,000 and choleragen 56,000 (as determined by the sedimentation equilibrium method), would support the subunit structures as proposed by Gill for these proteins. In view of the tendency for choleragen to lose subunit A under various conditions[55] as discussed above and the uncertainty of physicochemical measurement of polymeric proteins, existence of choleragen with an AB_6 composition (Figure 6) and of tetrameric and hexameric choleragenoids is still a possibility.

When subunit A of cholera toxin was reduced with dithiothreitol in urea and *S*-carboxymethylated, dissociation of the subunit into two polypeptides occurred. The two component peptides of A subunit were readily separated on Sephadex® G-75 in 5% HCOOH (Figure 5c), and were designated A_1 and A_2 according to the order of their elution. The labeling pattern of SH groups and amino acid analysis of each peptide indicated that A_1 and A_2 were present in equimolar ratio and that a single disulfide bond linked the two polypeptides.[57] The same reaction performed on subunit B resulted in the labeling of SH groups in the protein and a slight forward shift in elution volume, indicating that subunit B was a single-chain polypeptide with an intrachain disulfide bridge (Figure 5d). On polyacrylamide-gel electrophoresis in 0.1% SDS containing 8 *M* urea, each of the isolated polypeptides showed a single band (Figure 5b), indicating its homogeneity. The reduced and *S*-carboxymethylated subunit B showed less mobility than the untreated B, suggesting that unfolding of the molecule occurred on reduction of the intrachain disulfide bond. The apparent molecular weights of A_1 and A_2 polypeptides were estimated to be 20,000 and 7500, respectively,[57] in general agreement with values obtained in other laboratories: 22,000 to 24,000 for A_1 and 5,000 to 7,000 for A_2.[60,68,69] The presence of two component peptides in subunit A had been indicated when the subunit was chromatographed on Bio-Gel ® P-60 in 0.1 *M* glycine buffer (pH 3.2) containing 1 *M* urea and 0.1 *M* mercaptoethanol.[59] Polypeptide A_2 was not detected previously in experiments with the reduced choleragen in SDS-gel electrophoresis because the peptide migrated closely with subunit B.[58] Separation of unmodified A_1 and A_2 has also been achieved by gel-filtration of subunit A on Sephadex ® G-75 in 5% HCOOH containing 1 m*M* dithiothreitol.[46]

C. Chemical Characterization and Primary Structures

In an attempt to understand the relationship between choleragen and choleragenoid and subunits A and B, Finkelstein et al. performed amino acid analyses and peptide mappings on these proteins. The results essentially supported the notion that subunit B was identical to choleragenoid in structure and that subunits A and B were clearly different in composition.[57]

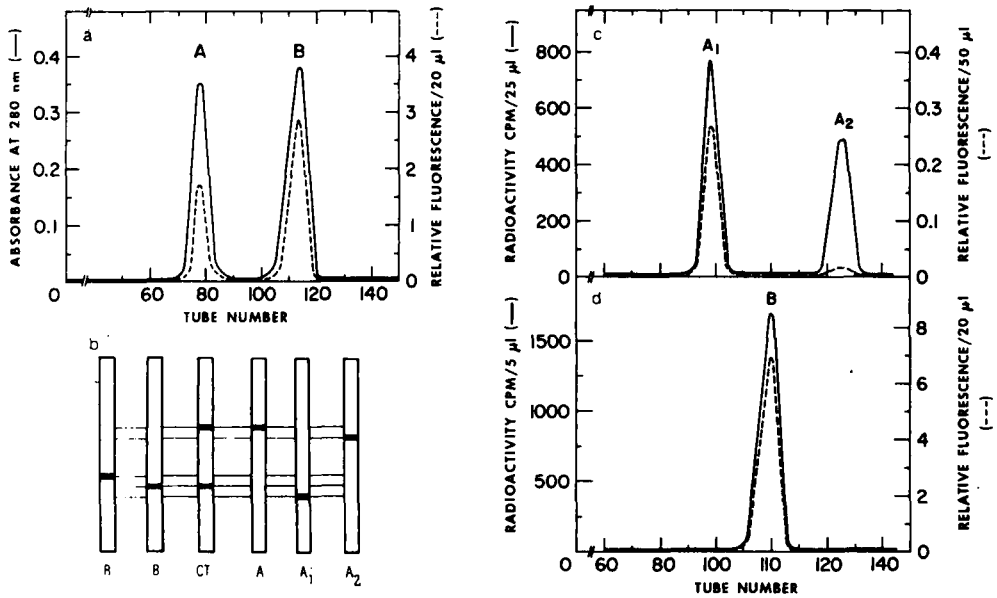


FIGURE 5. Separation of subunits of cholera toxin. (a) Gel-filtration of choleragen on Sephadex G75 in 5% HCOOH. (b) A sketch of electrophoretic pattern of isolated subunits in polyacrylamide gel containing 0.1% SDS and 8 *M* urea. (c) Separation of A_1 and A_2 on Sephadex G75 in 5% HCOOH after reduction and carboxymethylation of subunit A. (d) Gel-filtration of subunit B after reduction and carboxymethylation under the same conditions as in (c).

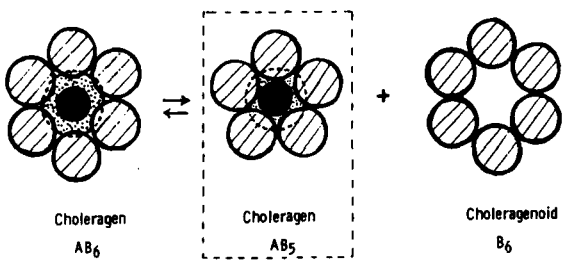


FIGURE 6. Proposed arrangements of subunits in choleragen and choleragenoid. Hatched circles, subunit B; dotted circle, A_1; black circle, A_2. (Reprinted from Lai, C. Y., Mendez, E., and Chang, K., *J. Infect. Dis.*, 133, S23, 1976, by permission of the University of Chicago Press. Copyright 1976 by the University of Chicago. All rights reserved.)

Amino acid compositions of polypeptides A_1 and A_2 and subunit B were reported independently by Lai et al.,[57] Klapper et al.,[70] Kurosky et al[69] and Markel et al.[121] The updated values are listed in Table 1. Polypeptides A_1 and A_2 each contains one cysteine residue, indicating that the peptides are linked through a single disulfide bridge in subunit A. The cysteine residues in subunit B have previously been shown to form a disulfide bridge in the native structure.[57,59,60] The A_1 polypeptide is noted for its high arginine and proline content (16 arginine and 15 proline per chain of 22,000 molecular weight) and A_2 for its lack of proline and possibly alanine. In contrast, subunit B contains only three residues of arginine as opposed to nine lysine residues per mole (Table 1).

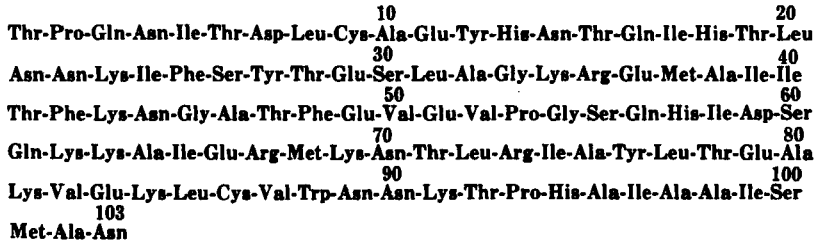


FIGURE 7. Primary structure of subunit B (responsible for the membrane binding) of cholera toxin (71,72).

The amino acid sequence analysis on the intact cholera toxin using an automated sequencer was reported by Jacobs et al.[71] in 1974, but the results could only be taken as tentative, in the absence of knowledge concerning subunit composition of the toxin sample. Nevertheless, the NH_2-terminal sequence of 20 residues in the major component, subunit B, was essentially correct except for two residues in this analysis. Similar experiments were carried out by Kurosky et al.[69] on isolated subunits A and B, where the sequence of as much as 42 residues in subunit B was correctly assigned. Mendez et al.[72] reported earlier the separation and sequence analyses of the tryptic peptides containing cysteine residues in the polypeptides A_1, A_2, and B subunit. One of the two cysteine-containing peptides from subunit B was found to be derived from the NH_2-terminus of the subunit, and its primary structure of 23 amino acids was identical to that proposed by Kurosky et al.[69] The cysteine-containing peptide of A_2 was also found to be derived from the NH_2-terminus of the peptide chain, and has a sequence of Ser-Asn-Thr-Glu-Cys-Asp-Lys. One preparation of this peptide was found to contain 0.6 mol/mol of methionine, and the sequence with methionine at the NH_2-terminus followed by that stated above. This finding suggested that A_2 polypeptide was initially synthesized with methionine as the NH_2-terminus, and under certain conditions, lost the residue. This notion was supported by the findings of Kurosky et al.[69] and Klapper et al.[70] that the NH_2-terminal sequence of A_2 polypeptide was Met-Ser-Asp-Thr-Glu-Cys-Asp-Lys in the toxin preparation they used.

Availability of subunit B in a relatively large quantity, and the interests in the role of this subunit in the binding of cholera toxin to cell membranes as the first step in the intoxication process, prompted the studies to elucidate its complete primary structure. Determination of the sequence of 103 amino acid residues in cholera toxin subunit B (Figure 7) was accomplished independently by two groups of investigators in 1977, with the results in total agreement.[73,74] The structure was deduced basically by procedures in which all peptides from the tryptic digestion of the *S*-carboxymethylated subunit B were separated, purified, and their sequence determined. The alignment of these peptides was determined by isolation and analyses of peptides that contained overlaps among the tryptic peptides, through other types of peptide bond-cleavage reactions. The two cysteine residues are at Positions 9 and 86, thus bringing both ends of the peptide chain to proximity on forming the disulfide bridge. This feature confirms the earlier suggestion that subunit B with intact disulfide bond has a more compact configuration than the reduced and *S*-carboxymethylated one, exhibiting a smaller apparent molecular weight in the SDS-urea gel electrophoresis.[72] Based on the primary structure and the localization of the disulfide bond, a secondary structure prediction has been made for subunit B[75] (Figure 8), according to the method of Chou and Fasman.[76] The molecule is characterized by as much as 35% helical content. The structural relevance to its biological activity will be discussed later.

Table 1
AMINO ACID COMPOSITIONS OF POLYPEPTIDEDE CHAINS OF CHOLERA TOXIN

	Polypeptides		
Amino acids	A_1	A_2	B
Lys	2	6	9
His	9	1	4
Arg	14	3	3
CM-Cys	1	1	2
Asp	22	11	11
Thr	7	4	10
Ser	12	7	5
Glu	20	10	12
Pro	15	0	3
Gly	21	4	3
Ala	15	0	11
Val	9	3	4
Met	3	1	3
Ile	9	4	10
Leu	12	4	6
Tyr	14	3	3
PHe	5	3	3
Trp	2	0	1

Studies on the primary structure of polypeptide A_1, the component of cholera toxin believed to contain the active site for the adenylate cyclase stimulation, are expected to be considerably more difficult than those for subunit B because of its larger molecular size and small content in the holotoxin than the latter; for the protein of 22,000, the amount required for the complete sequence analysis is estimated to be the minimum of 2 μmol or 44 mg, which is contained in 170 mg of pure cholera toxin. The NH_2-terminal sequence of 20 amino acid residues as determined with an automated sequencer has been reported,[70] which is essentially in agreement with the result of an earlier analysis using subunit A, the mixture of A_1 and A_2[69] (Figure 9a). Information on the gross primary structure of A_1 polypeptide was recently obtained by isolation and characterization of the four fragments derived from specific cleavage at methionyl bonds, and their array in A_1 deduced by separation and analyses of methione-containing peptides[77] (Figure 9b). The cysteine residue, which forms the linkage to A_2 polypeptide, is found within the COOH-terminal third of the molecule.

V. MECHANISM OF ACTION OF CHOLERA TOXIN

As mentioned in the previous sections, the principal biological activity of cholera toxin is the stimulation of adenylate cyclase, and the unique feature of it is the broad spectrum of cell types with which the toxin interacts. Adenylate cyclase (EC 4.6.1.1: ATP pyrophosphate-lyase [cyclizing]) has now been implicated in the modulation of various cell functions by many hormones and drugs, and the mechanism by which its activity is controlled has been of considerable biochemical as well as pharmacological interest. The ubiquitous action of cholera toxin on the membrane-bound adenylate

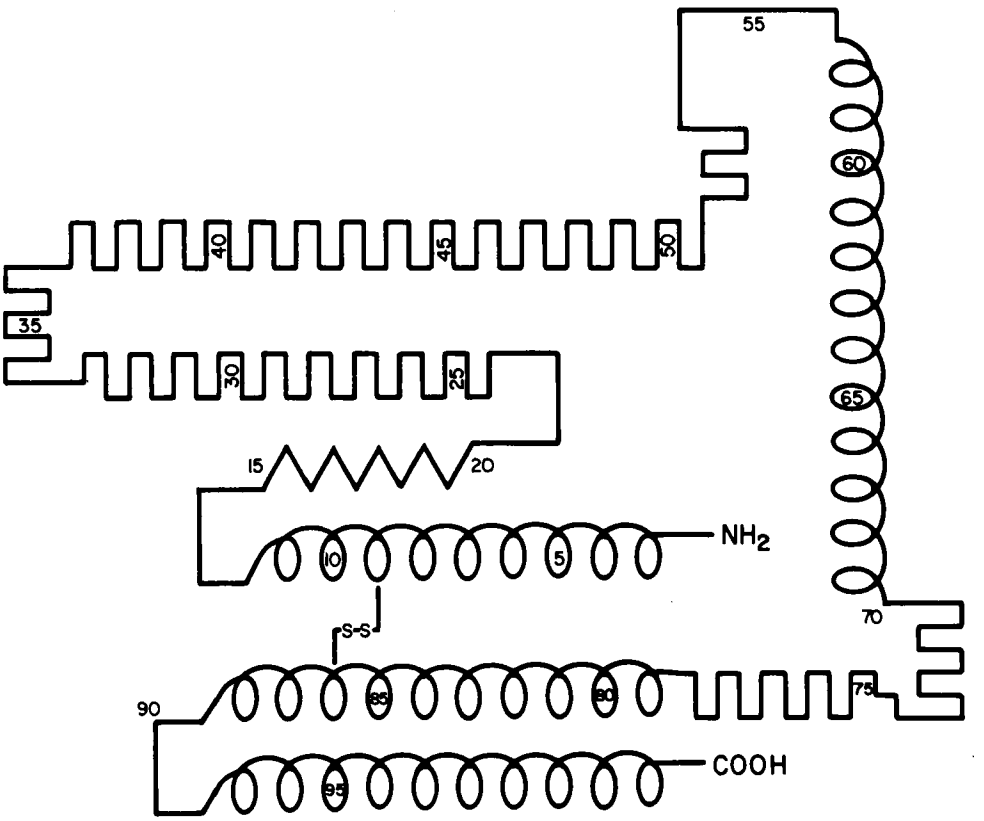


FIGURE 8. Predicted secondary structure of subunit B of cholera toxin. (From Duffy, L. K. and Lai, C. Y., *Biochem. Biophys. Res. Commun.*, 91, 1005, 1979. With permission.)

cyclase in diverse tissues offers an excellent model for such studies. Interaction of cholera toxin with cells occurs basically in two steps: the attachment of the toxin to cell surface membranes and a subsequent event that leads to activation of adenylate cyclase.

A. Binding of Cholera Toxin to Cell Membranes — the Role of Subunit B

It was observed earlier that a brief exposure of intestinal lumen to cholera toxin followed by repeated wash to remove the free toxin caused a normally delayed and sustained secretion of water and electrolytes in the canine jejunal loops, and it was suggested that a rapid fixation of the toxin to intestinal epithelium might be the first step in the intoxication process.[78] The first direct evidence for the attachment of cholera toxin to luminal surfaces of intestine was provided by Peterson et al.[79] in the experiments using the purified toxin and the specific antibody prepared by immunoadsorption. Mice intestinal loops were injected with choleragen, choleragenoid, and the formalin-treated choleragen, respectively. After incubation for 3 hr isolated loops were cut open, washed thoroughly, and the tissue slices examined with immunohistochemical techniques. Choleragen and choleragenoid, but not the formalin-toxoid, were found uniformly adsorbed to the entire mucosal surface of the villi and crypt areas. On electron-micrographic examination, using the horseradish peroxidase-antibody method for the specific staining, the toxin was found localized on the membrane of the microvilli. Uptake of choleragen or choleragenoid into intestinal cells was not ob-

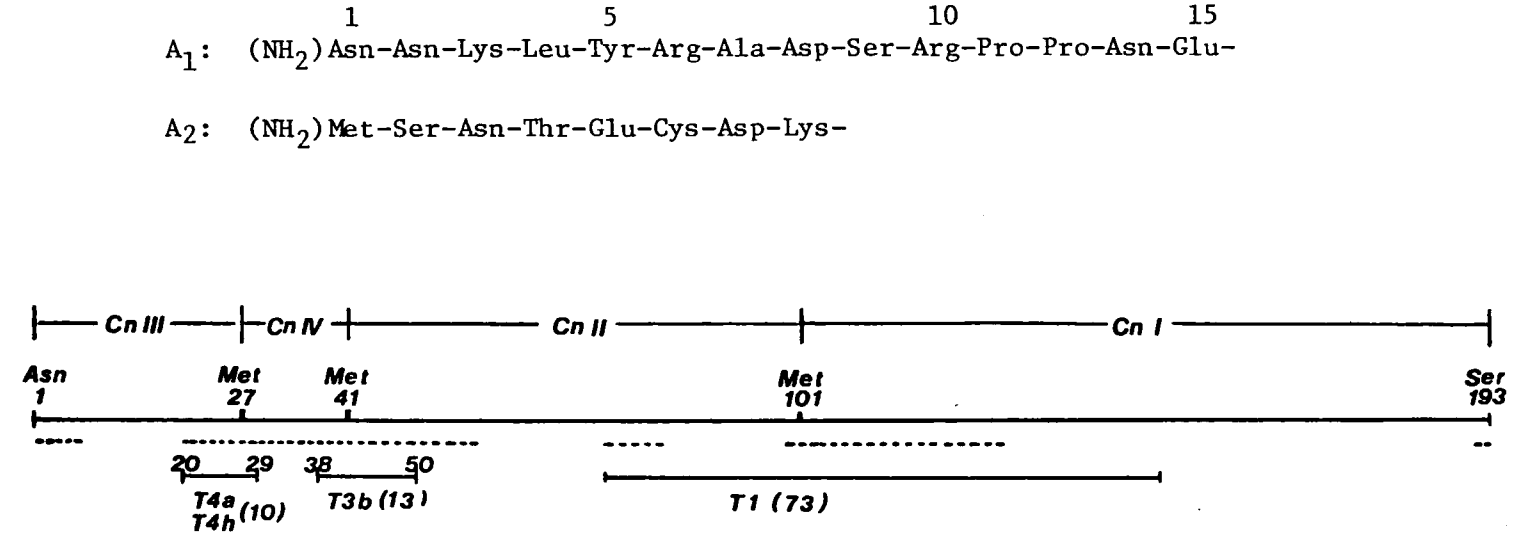


FIGURE 9. Amino acid sequences at the NH_2-termini of polypeptides A_1 and A_2 (top) and the gross primary structure of polypeptide A_1 (responsible for the toxin's activity to stimulate adenylate cyclase). Cn I, II, III, IV are BrCN peptides. T1, T3b, and T4a or T4h are tryptic peptides that overlap BrCN peptides (bottom).

served but it was suggested, correctly in retrospect, that the undetectable "toxophore" component of choleragen could have penetrated the cell.[79]

1. Involvement of Ganglioside GM_1 in Choleragen Binding

Perhaps stimulated by the earlier finding that brain extracts inhibited tetanus toxin,[80] van Heyningen et al.[81] tested the effects of tissue extracts and gangliosides on the ability of cholera toxin to increase skin permeability[3,16], to cause fluid secretion in ligated ileal loop of rabbits,[11] and to stimulate lipolysis in isolated fat cells.[3] The chloroform-methanol extracts of various tissues of rabbit were found to inhibit the activity of cholera toxin, with potencies in the decreasing order from brain (requiring 0.5 μg), gut epithelium (100 μg), and liver, kidney, lung, or heart (>25,000 μg). With purified ganglioside prepared from brain tissue, 25 ng was required to deactivate 20 blueing doses of crude cholera toxin preparation.[81] Precipitation was observed when 2.5 mg ganglioside per milliliter and 5 mg of the crude toxin per milliliter were mixed, and no toxin activity was found in the supernatant, suggesting a complex formation between ganglioside and cholera toxin.[81] Interaction of cholera toxin with various cell membranes was also studied with the use of ^{125}I-cholera toxin of very high specific radioactivity by Cuatrecasas,[82] following the procedure he described earlier for measuring the specific binding of insulin to its receptor. Binding of ^{125}I-cholera toxin to liver or fat cell membranes occurred within 5 min at 24°C and was found to diminish by pretreatment of the membranes with increasing amounts of unlabeled choleragen, indicating specificity of the interaction. Of several galactose-containing sugars tested, only stachyose (Gal_2Glu,Fru) inhibited the toxin binding by 20%; and of 18 glycoproteins, feutin at 0.5 mg/mℓ, and undiluted human serum exhibited near complete inhibition. On preincubation of ^{125}I-choleragen with crude ganglioside at 1 to 10 μ/mℓ, total inhibition of the toxin binding to liver membrane was observed, confirming the previous results of van Heyningen et al.[81]

Evidence for the ganglioside nature of the membrane receptor for choleragen was also obtained independently by Holmgren et al.,[63,84] who further provided information on the structure involved in the ganglioside-choleragen interaction. The complex formation between the glycolipids and the toxin was conveniently detected by the double diffusion in agar, analogous to that used in the immunoprecipitin test, and its effect on the choleragen activity measured by rabbit skin permeability testing. In these studies, ganglioside GM_1, but not other related glycolipids GM_3, GM_2, GM_1-GlcNAc, GD_{1b}, and GT_1, was found to inactivate the toxin with high specificity; only 100 pg was required to inactivate 5 ng of pure toxin (50 blueing doses), even in the presence of other glycolipids.[82] GD_{1a} and globoside GA_1 had the effect with 1000 times less potency, indicating that the portion of the molecule involved in the toxin binding had the structure

$$\begin{array}{l} \text{Gal} \rightarrow \text{GalNAc} \rightarrow \text{Gal} \rightarrow \\ \qquad\qquad\quad\ \uparrow \\ \qquad\qquad\quad\ \text{NAN} \end{array}$$

This structure was postulated as that of the tissue receptor for cholera toxin. Choleragenoid was found to also form a precipitin line with ganglioside GM_1.[81]

In a series of reports, Cuatrecasas[85-87] described experiments which provided further evidence for the involvement of ganglioside GM_1 in the action of cholera toxin, and the role of choleragenoid, or B-subunits, in the toxin-cell interaction. Inhibition of binding of cholera toxin to cell membranes by ganglioside GM_1 was initially observed when cells were treated briefly with the toxin pretreated with GM_1. On a prolonged incubation, however, increased binding as well as stimulation of lipolysis in fat cells was seen, indicating the attachment of the ganglioside-choleragen complex to cell mem-

branes. This was verified by experiments which showed that ganglioside GM_1 could be incorporated into cell membranes. The increased content of GM_1 enhanced the binding of cholera toxin and the subsequent activation of adenylate cyclase.[85] Recently, King et al.[88] made a similar observation using pigeon erythrocytes and obtained evidence indicating that 90% of the extra toxin-binding sites on the GM_1-treated cell were nonproductive with regard to adenyl cyclase activation. The effect of insulin to inhibit the adenylate cyclase activity in fat cells was not altered by cholera toxin, indicating an involvement of separate receptors. Tetanus toxin did not inhibit the binding of ^{125}I-choleragen to liver membrane, suggesting a different ganglioside specificity for its affinity.[86] Interaction of choleragenoid, a polymeric form of subunit B, with ganglioside GM_1 and cell membranes, was shown to occur in the same manner as that with choleragen. Moreover, the binding of ^{125}I-choleragen to cell membranes was specifically and competitively inhibited by choleragenoid, indicating that the same binding site in the membrane was involved in the binding of these proteins.[87] In view of the subunit structure elucidated later for cholera toxin, these results strongly indicated that subunit B provided the vehicle for the initial attachment of the toxin in its intoxication process. Recently, evidence for the presence of ganglioside GM_1 in cell membranes and its attachment to cholera toxin was obtained by labeling the membrane with the galactose oxidase-(^{3}H)$NaBH_4$ procedure and detecting the radioactivity in a chromatograph at the position corresponding to GM_1 after extraction of the toxin-receptor complex for the analysis.[89]

2. The Role of Subunit B in the Binding

Affinity of ganglioside GM_1 to subunit B was evidenced by van Heyningen's[64] experiments in which subunit A was selectively eluted from the precipitate formed between choleragen and ganglioside GM_1. The subunit B separated from choleragen by gel filtration in 5% HCOOH,[57] after the renaturation procedure, has been shown to form the precipitin line with ganglioside GM_1 as choleragen in a gel double-diffusion test (Figure 10), where subunit A is unreactive.[75] A recent study on equilibrium dialysis of cholera toxin or choleragenoid with monosialogangliotetrose, the oligosaccharide moiety of ganglioside GM_1, revealed that there was cooperativity in the binding of oligosaccharide to the proteins and that 1 mol of the toxin or choleragenoid bound 4 mol of the oligosaccharide.[90] Further evidence for the binding of choleragen to the oligosaccharide portion of a glycolipid was provided by studies on the cell-agglutinating activity of cholera toxin.[91] Choleragen was found to cause agglutination of erythrocytes and liposomes containing ganglioside GM_1, and the oligosaccharide isolated from GM_1 specifically inhibited the agglutination.

B. Events Leading to Activation of Membrane-Bound Adenylate Cyclase by Cholera Toxin

In studies of experimental cholera in the canine model,[38] Carpenter et al.[92] made the observation that onset of fluid loss through intestine always followed a lag period of 2 to 3 hr. With the use of catheters inserted into ligated jejunal or ileal loops in dogs, they were able to measure the rate of fluid secretion by small intestine following the intraluminal injection of crude cholera toxin. All of the sterile culture filtrates of *V. cholerae* injected were absorbed normally by jejunal loops within 60 min and fluid secretion started after 2 hr, reaching the maximum rate at the 4th hr and lasting for 4 to 6 hr.[92] Similar lags have also been observed in the stimulation of adenylate cyclase or cyclic AMP-mediated changes of cell functions by purified cholera toxin in isolated tissues and cells.[26,28,32,33,37,43] The period of the lag, the time it takes to detect the effect of cholera toxin on the cell, is 15 to 60 min in isolated cells.

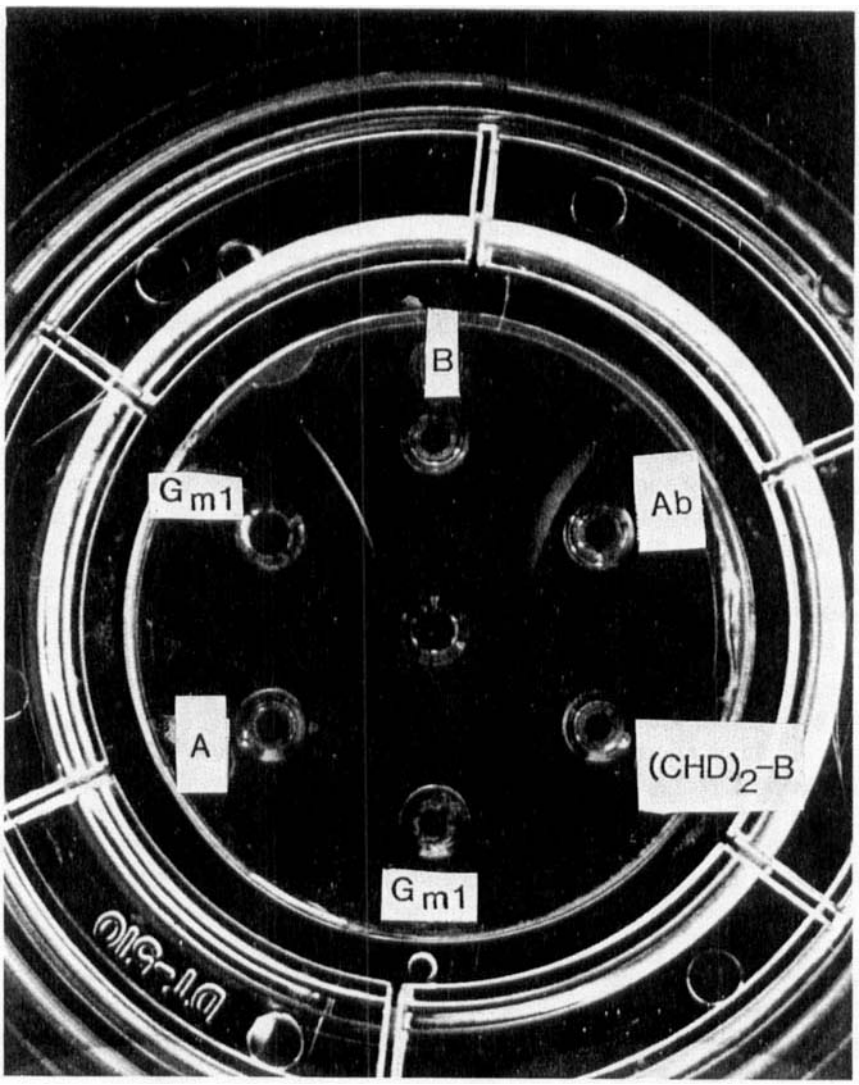


FIGURE 10. Interaction of ganglioside GM_1 with subunits A and B of cholera toxin.[73] Ab: Anti-choleragen; $(CHD)_2$-B: Modified subunit B.

1. The Mobile Receptor Hypothesis for Toxin-Adenylate Cyclase Interactions

The mode of action of cholera toxin and the nature of lag phase was studied by Cuatrecasas[86] using the ^{125}I-labeled toxin and assay of lipolytic response in isolated fat cells. The attachment of choleragen to the cells occurred almost instantaneously, and the toxin-cell complex formed withstood repeated washing. The increase in the rate of lipolysis by the cell was not seen for 30 to 60 min, however, whether or not the unbound cholera toxin was removed by washing, thus suggesting that only the toxin initially bound to the cell was responsible for its activity. Brief treatment of fat cells with dilute detergent, 0.005% Triton ® X-100, in the absence or presence of cholera toxin, did not alter the extent of lipolytic response or the lag period. Based on these observations, Cuatrecasas proposed a mechanism for the action of cholera toxin in which

the toxin-receptor complex formed initially would undergo a spontaneous transformation through a time- and temperature-dependent process (the lag phase) into a form which was active in the stimulation of adenylate cyclase.[84] Choleragenoid was found to bind to the same receptor on the membrane as choleragen and cause specific inhibition of the choleragen action, but only when it was mixed with cells before the addition of the latter. This was interpreted to indicate that the choleragenoid-receptor complex could not undergo the transformation into active form, because of the small difference in its structure from choleragen.[87] In a later modification of this hypothesis, a lateral motion of the toxin-receptor complex to the site of adenylate cyclase in the membrane, instead of the molecular "transformation" of the complex, was proposed as the mechanism of adenylate cyclase activation; a detergent solubilized adenylate cyclase was found to bind to Agarose columns containing an "active" subunit of cholera toxin.[93] The apparent affinity of adenylate cyclase to the "active" subunit observed in the experiment could, however, be due to a presence of ganglioside GM_1 in the detergent-solubilized adenylate cyclase and of subunit B to the "active" subunit preparation.

2. Direct Interaction of Active Subunit With Adenylate Cyclase System

Gill and King[43] reported in 1975 that stimulation of adenylate cyclase by cholera toxin could be observed with concentrated lysates of pigeon erythrocytes and that, in this system, the characteristic lag period seen with intact erythrocytes was absent. The extent of activation of adenylate cyclase increased with the increasing amounts of cholera toxin added, reaching a plateau which was three to four times higher than that attainable with intact cells (Figure 11). It appeared that cholera toxin exerted its effect through intracellular sites, which became more and directly accessible to the toxin on cell lysis. In the erythrocyte lysates, polypeptide A_1, separated by gel electrophoresis in SDS after reduction with a thiol reagent, was also found to activate adenylate cyclase, while the holotoxin was required for the effect in intact cells. Choleragenoid, which blocked action of cholera toxin on intact cells, was found to be ineffective in the broken cells, regardless of the time of addition, indicating that the surface receptors were not involved in the activation of adenylate cyclase. These studies led the authors to postulate that, subsequent to the attachment of cholera toxin to the cell surface, subunit A or polypeptide A_1 penetrated the membrane to cause the effect and that the lag period represented the time for the traverse.[43] It was subsequently demonstrated by van Heyningen and King[94] that a purified subunit A stimulated adenylate cyclase in both intact and lysed pigeon erythrocytes, and that antitoxin was ineffective in blocking its activity. Curiously, similar lag phase in the action of holotoxin on intact cells was observed with subunit A.

3. The Role of Polypeptide A_1

Direct evidence for the essential role of polypeptide A_1 in the stimulation of adenylate cyclase was obtained by Wodnar-Filipowicz and Lai[46] using washed membranes of pigeon erythrocytes and tests on the isolated subunits A and B, polypeptides A_1 and A_2, of demonstrated purity (see Figure 5). For the activation of adenylate cyclase in washed membranes with cholera toxin or subunit A, incubation with dithiothreitol (DTT) in addition to NAD[95] was found to be necessary. In this system, the holotoxin, subunit A and polypeptide A_1, but not subunit B nor peptide A_2, were found to be active (Figure 12). The highest stimulation was attained with polypeptide A_1, and the amount required for the half-maximal stimulation of adenylate cyclase in 1 mg of membrane was 0.2 nm for polypeptide A_1, 0.5 nm for subunit A, and 1.1 nm for cholera toxin. Moreover, with the A_1, addition of DTT in the system was not required

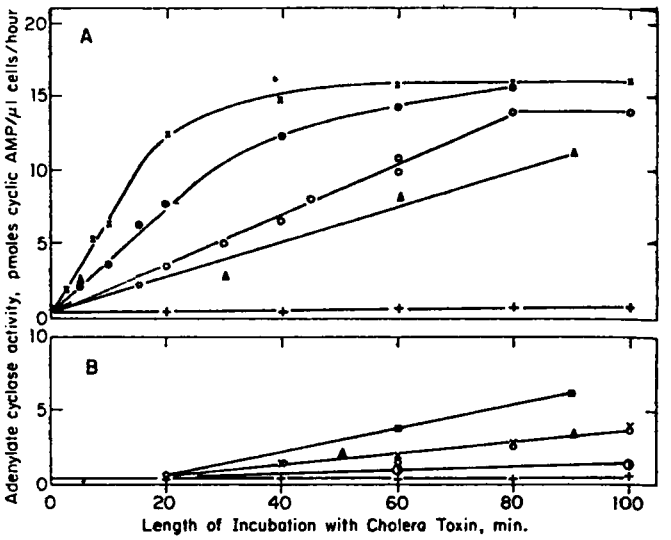


FIGURE 11. Activation of adenylate cyclase by cholera toxin in disrupted (A) and intact pigeon erythrocytes (B). (From Gill, K. M. and King, C. A., *J. Biol. Chem.*, 250, 6424, 1975. With permission.)

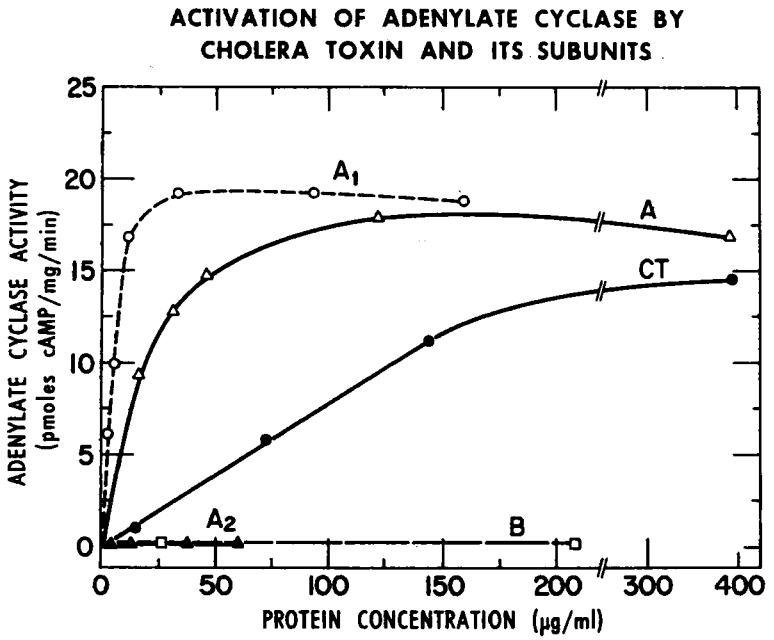


FIGURE 12. Activation of adenylate cyclase in washed membrane of pigeon erythrocytes with cholera toxin, subunits A and B and polypeptides A_1 and A_2. The reaction mixture contained 1 m*M* each of DTT and NAD.[44]

for its activity (Table 2). The results suggested that DTT required for the holotoxin and subunit A served merely to release polypeptide A_1 which then acted upon the membrane. While bound to polypeptide A_2, A_1 was apparently not effective in exerting its activity (see Table 2). These studies confirmed and identifed polypeptide A_1 as the

Table 2
EFFECT OF THIOL ON ACTIVATION OF ADENYLATE CYCLASE IN PIGEON ERYTHROCYTE MEMBRANE BY CHOLERAGEN AND ITS SUBUNITS

Addition	Adenylate cyclase activity (pmol of cAMP produced/min/mg)		
	Choleragen	A	A_1
None	2.7	8.8	23.0
Dithiothreitol (1 m*M*)	10.7	12.7	18.8

active moiety of cholera toxin. The conclusion was in agreement with the notion that following attachment of the holotoxin, a freed A_1 entered the cell to activate adenylate cyclase at the inner surface of the membrane. Polypeptide A_1 was unexpectedly found to act on intact pigeon erythrocytes, but as much as 60-fold molar excess over cholera toxin was required to raise the adenlate cyclase activity to the same level (Figure 13). The dose-response curve suggested that a nonspecific, concentration-dependent permeation of polypeptide A_1 into the cell might occur.

Further support for the mechanisms in which polypeptide A_1 could traverse the membrane to intracellular sites of interaction with adenylate cyclase was provided by van Heyningen's[96] experiments. In these studies, he was able to show activation of adenylate cyclase by cholera toxin and peptide A_1 in the 100,000 g supernatant from the Lubrol PX extract of liver membranes. Only the extracts made in the presence of cytosol, NAD, ATP, and DTT could be activated by A_1 or the holotoxin. Since membrane fragments were not present in the solution of adenylate cyclase, the experiments indicated that the lateral movement of toxin-ganglioside complex in the membrane, as proposed by Bennett et al.,[93] would not be involved in the cyclase activation. The requirement of cytosol, NAD, ATP, and DTT in the solubilization process suggested involvement of these factors in the action of polypeptide A_1.

C. The Activation Process — Roles of NAD and Other Factors in the Interaction of Polypeptide A_1 With the Adenylate Cyclase System

1. Requirement of NAD

It has now been generally accepted that a free polypeptide A_1, not a toxin-receptor complex, is directly involved in the activation of adenylate cyclase at the inner surface of the membrane. In the studies of the mechanism of the activation process with pigeon erythrocyte lysates, Gill[95] discovered that NAD was a necessary cofactor for the cholera toxin action. The requirement of NAD was subsequently confirmed in other systems including liver homogenates,[96,97] turkey erythrocyte lysates,[98] cancer cell membranes,[99,100] fibroblast membranes,[101] and thyroid membranes.[102] The inability of cholera toxin to activate adenylate cyclase in mammalian erythrocyte lysates was considered to be due to the high content of NAD glycohydrolase activity in the lysates, which destroyed the available NAD in the cytoplasm.[95] NADH was almost as effective, presumably for its ready conversion to NAD, but NADP or NADPH were poor substitutes for NAD. The apparent K_m for NAD in the cholera toxin action was reported to be about 10^{-3} *M*, suggesting a relatively weak interaction between the two.

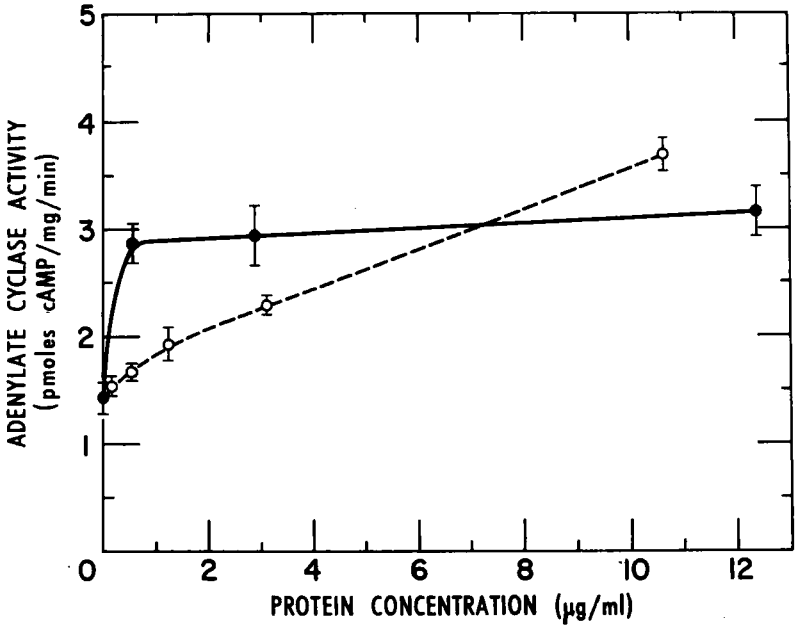


FIGURE 13. Activation of adenylate cyclase in intact pigeon erythrocytes with cholera toxin and polypeptide A_1.[46]

In addition to NAD, Gill[103] made important observations which suggested the involvement of other cytoplasmic substances in the action of cholera toxin to stimulate adenylate cyclase in pigeon erythroctye ghosts. The requirement for NAD and the effects of ATP and unknown cytosol factor(s) in the enhancement of the cholera toxin action are illustrated by the experiment shown in Figure 14. It must be noted that the substances other than NAD are not required, but act merely as effectors in the action of cholera toxin (Figure 14). This has also been demonstrated in studies by other investigators.[46,98,99] In order to obtain an immediate and maximal activity, Gill found it necessary to preincubate cholera toxin with a buffer containing 1 m*M* DTT and 0.1% SDS. It was thought that the treatment would generate polypeptide A_1 from the holotoxin and that A_1 was required for the activity. The notion that A_1 alone was responsible for the stimulation of adenylate cyclase was confirmed and extended by experiments of Wadnar-Filipowicz and Lai[46] (see Figure 12). A macromolecular effector substance was recently purified 100-fold from cytosol of pigeon erythrocytes in the author's laboratory[104] and found to enhance the activity of isolated A_1 polypeptide by about 60% in the presence of NAD and ATP. This cytosol effector appeared to be a heat-labile protein and acted nonenzymatically in the reaction. Isolated polypeptide A_1 was found to lose its activity in 30 min at 37°C, unless 0.1% serum albumin (inert as an effector) or the cytosol effector was added.

2. ADP-Ribosyl Transferase Activity of Cholera Toxin and its Implications

Evidence has been accumulating in the past 2 years indicating that cholera toxin or its active component catalyzes the transfer of the adenosine diphosphate ribose (ADPR) moiety of NAD onto an as yet unidentified substance, which then causes activation of adenylate cyclase in the membrane. The idea of the involvement of ADP-ribosylation reaction in the action of cholera toxin apparently stemmed from the similar requirement of NAD in the cell intoxication by diphtheria toxin. It had been estab-

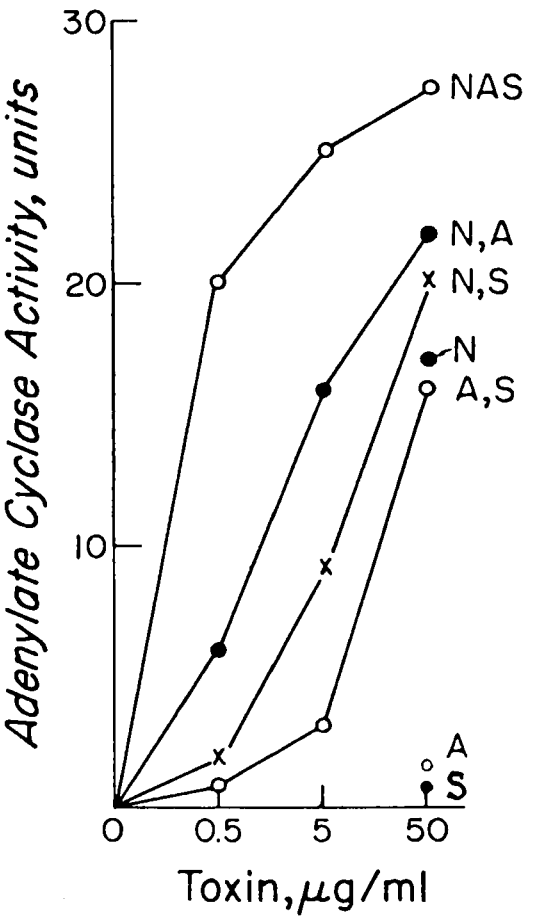


FIGURE 14. Requirement of NAD and enhancing effects of cytosol and ATP in the activation of adenylate cyclase with cholera toxin. Experiments were carried out with washed pigeon erythrocyte membrane. Cytosol was pretreated with activated charcoal to remove nucleotides. N, NAD; A, ATP; S, supernatant fraction (cytosol). (Reprinted from Gill, D. M., *J. Infect Dis.*, 133, S55, 1976, by permission of the University of Chicago Press. Copyright 1976 by the University of Chicago. All rights reserved.)

lished that the active fragment of diphtheria toxin catalyzed ADP-ribosylation of the protein, elongation factor (EF-II), rendering it inactive in the process of peptide-chain elongation on ribosomes. NAD was another substrate of the reaction (Equation 1) which led to inhibition of protein synthesis and cell death.[105,106]

$$\text{NAD} + \text{EF-II} \underset{}{\overset{\text{diptheria toxin}}{\rightleftharpoons}} \text{ADPR} - \text{EF-II} + \text{nicotinamide} \qquad (1)$$

A hint of the analogous reaction in the action of choleragen was first provided by Moss et al.,[107] who observed hydrolysis of NAD to ADPR and nicotinamide in the presence of a large concentration of choleragen. Nearly 50% of NAD (2 m*M*) was hydrolyzed on incubation with choleragen or subunit A (at about 6 μM) in 2 hr at 37°C, and K_m was estimated to be $3.8 \times 10^{-3} M$. Moss and Vaughan[108] subsequently

demonstrated that choleragen catalyzed transfer of ADPR from NAD to arginine if the latter was added as acceptor. The guanido group of arginine was apparently the site of ADP-ribosylation. The observation was followed by a report by Trepel et al.[109] who claimed that ADP-ribosylation of choleragen or subunit A occurred under practically the same condition, where the NAD hydrolysis was observed by Moss et al.[107] In both cases the reaction occurred only in 0.2*M* K-phosphate buffer instead of Tris-HCl buffer. The latter observation may indicate formation of a complex between A subunit and NAD as an intermediate in the hydrolysis of NAD that could be precipitated with tricholoroacetic acid. A mechanism involving the ADPR transfer, either onto cholera toxin subunit A or onto an unknown protein coupled to adenylate cyclase, was suggested for the action of cholera toxin,[108,109] but the effects of the observed reactions on adenylate cyclase were not tested in these experiments. The NADase activity of choleragen and subunit A was found to be inhibited by analogues of NAD such as adenine, adenosine, AMP, ADP-ribose, nicotinamide, and NMN. It was also specifically inhibited by ganglioside GM_1.[110] More recently, the ADPR-transfer reaction catalyzed by choleragen was studied with guanidine-HCl or arginine as acceptors, and the products analyzed by NMR spectropy.[111] The results suggested that choleragen catalyzed direct displacement of the nicotinamide moiety by the guanido group into an α-configuration, in the same way other ADPR-transferases acted.

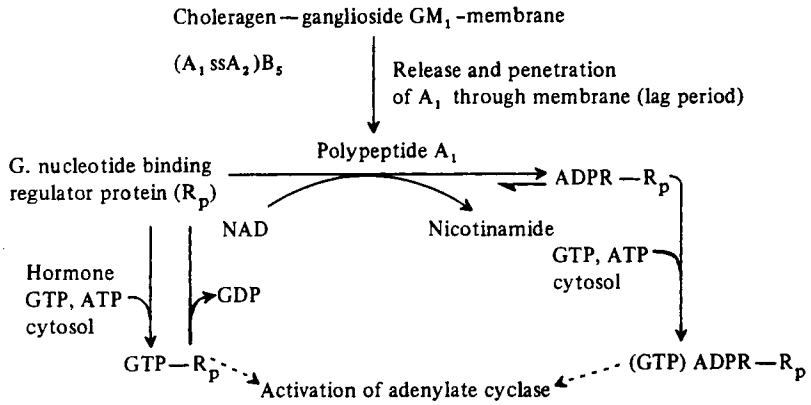
3. Involvement of GTP in the Control of Adenylate Cyclase

It has become increasingly evident in recent years that activation of adenylate cyclase with hormones involves binding of GTP at the regulatory site of the enzyme. Hydrolysis of the bound GTP to GDP causes its dissociation from the site and the return of the adenylate cyclase activity to the basal level. Substitution of GTP with the analog resistant to hydrolysis, guanosine 5′-(β,α-imino) triphosphate (Gpp[NH]p), in the system results in a persistent activation of adenylate cyclase.[112] In 1977 two groups of investigators independently obtained evidence indicating that cholera toxin in addition to GTP mimicked the effect of Gpp(NH)p on the hormone-activated adenylate cyclase, and suggested that the toxin was involved in the inhibition of GTP hydrolysis and thereby maintained adenylate cyclase in the activated state.[113,114] It was concurrently demonstrated that activation by choleragen of solubilized adenylate cyclase from brain required GTP and a cytoplasmic protein.[115] The enzyme, solubilized with Triton® X-100 and partially purified with DEAE cellulose, was found not to be stimulated by choleragen unless it was pretreated with GTP, calcium, and a protein activator. Interestingly, boiled brain supernatant was found to be more effective than GTP, calcium, and the protein activator for the choleragen action.[115] Although these studies were carried out with the "activated" choleragen, i.e., the toxin treated with SDS and dithiothreitol, the results indicated that GTP and a cytoplasmic protein, in addition to NAD, were essential for the action of polypeptide A_1.

Recently, Cassel and Pfeuffer[116] reported ADP-ribosylation of a membrane protein in pigeon erythrocytes by cholera toxin and identification of the modified protein as the GTP-binding protein. The "activated" choleragen was preincubated with cytosol and added to a suspension of partially purified membrane containing ATP and [α-^{32}P] labeled NAD. After incubation, the incorporation of ^{32}P into a protein with an M_r value of 42,000 in the membrane fraction was observed. The incorporation of ^{32}P increased with increased amount of choleragen and paralleled the rise in adenylate cyclase activity (Figure 15). The labeled protein showed an affinity to GTP-Sepharose, suggesting its identity with the GTP-binding protein of the adenylate cyclase system. Gill and Meren[117] obtained similar results independently except that several proteins other than the 42,000 M_r protein were ADP-ribosylated under their experimental con-

ditions. The latter protein represented 50 to 60% of the total toxin-specific acceptor proteins in pigeon erythrocyte membranes. It was estimated from the dose-response analyses that approximately 1500 ADPR residues per cell were incorporated when adenylate cyclase was maximally activated, and that this number was similar to that of β-adrenergic receptors per erythrocyte.[117] Another pertinent finding by Gill's group was that the reverse ADP-ribosyl transfer occurred when the modified membranes were incubated with nicotinamide and "activated" choleragen in the presence of NAD-glycohydrase, which removed newly formed NAD. The release of (^{32}P)-ADPR from the membrane paralleled the decrease in adenylate cyclase activity.

The processes of activation of membrane-bound adenylate cyclase by choleragen may now be summarized in the following scheme:



(2)

Moss and Vaughan[118] have recently isolated an ADPR-transferase from the cytosol of turkey erythrocytes. The enzyme catalyzed the transfer of ADPR from NAD to a number of arginine derivatives, lysozyme, histones, and polyarginine. It was found to stimulate rat brain adenylate cyclase in the presence of NAD, suggesting the operation of a control mechanism similar to that of activation by choleragen in animal cells.

D. Structure-Function Relationship

As discussed above, cholera toxin is a complex of one A subunit and five B subunits associated with noncovalent forces. Subunit B is involved in the attachment of the toxin onto cell surfaces through binding to the ganglioside GM_1 type structure in the membrane, and one component of subunit A, polypeptide A_1, is responsible for the activity to stimulate adenylate cyclase. The role of polypeptide A_2 is not known, but may be to serve as an anchor for A_1 to the pentameter of B in the holotoxin.

When the sequence of the first 22 amino acid residues from the NH_2-terminus of subunit B was reported,[69,72] Ledley et al.[119] noted some similarity between this structure and that of the binding subunits of several glycoprotein hormones. Using a computer "search and align" program, they found the sequence, Cys-Ala-Gln-Tyr (CAEY), in subunit B of cholera toxin to be analogous to the CAGY region in the B subunits of thyrotropin (TSH), luteinizing hormone (LH), human chorionic gonadotropin (HCG), and follicle-stimulating hormone (FSH) (Figure 16). It was suggested, on the basis of this analysis, that the sequence of CAEY or CAGY and analogous amino acids at Positions 2 to 12 residues apart might constitute a region important for the receptor binding. Further analogy in the sequence was not found in the rest of the primary structure, however.[73,74,120]

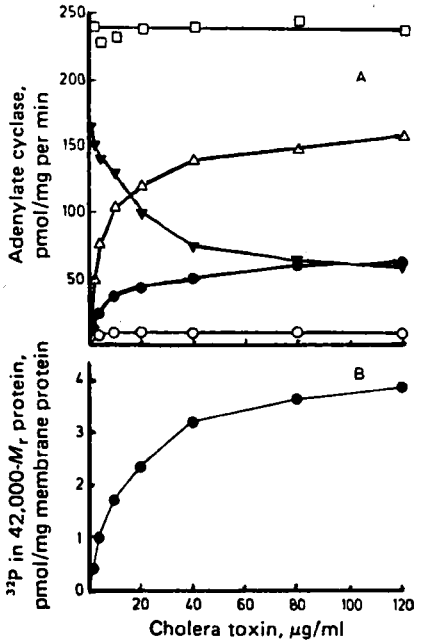


FIGURE 15. Activation of adenylate cyclase and ADP-ribosylation of the 42000 M, protein by cholera toxin. (A) Adenylate cyclase activity = 0, control; • + 0.1 m*M* GTP; Δ, + GTP + isoproterenol; ∇, + NaF. (B) Incorporation of ADPR to the 42,000 M, protein. (From Cassel, D. and Pfeuffer, T., *Proc. Natl. Acad. Sci.*, 75, 2669, 1978.)

Markel et al.[121] have recently shown that there is no immunochemical cross-reactivity between subunits of ovine luteinizing hormone and that of cholera toxin, suggesting that only a limited portion of the molecule is analogous between the hormone and the toxin and that this portion may not be the antigenic determinant.

Recent studies on the effect of chemical modification of arginine residues in subunit B on its ganglioside GM_1-binding activity have indicated that Arg-35 or the region surrounding this residue may be involved in the binding activity.[75] Arginine-73 appears to be "exposed" and may be readily reacted with cyclohexanedione without causing any change in its activity. The third arginine (Arg-67) cannot be modified unless the protein is denatured (see Figure 8). It is interesting that modification of Arg-35 also destroys the subunit's ability to precipitate with the homologous antibody.

Studies by Markel et al.[121] showed that 30% of tyrosyl residues in the B subunit could be modified by nitration without losing its immunoreactivity or its activity to compete for the toxin's binding to cell surfaces. Further nitration under denaturating conditions or succinylation of lysine residues led to inactivation of the subunit.

Little is known concerning the structure-activity relationship of polypeptide A_1. It has been established that cleavage of the disulfide bond between A_1 and A_2 polypeptides is essential for the activity of A_1 in the activation of adenylate cyclase. Modification of the SH group in A_1, however, did not cause inactivation of the protein[46] (Table 3). The results indicated that the SH-group did not play an essential role in the action of A_1 and that a change in confirmation or exposure of the region around the SH

	Position		Position
CHOLERA TOXIN (partial fragment)	1	T P Q N I T D L C A E Y H N T Q I H T L N D K I F S Y T E S L A G K R E M A I Q T F	42
TSH β CHAIN (bovine)	19	C L T I N T T V C A G Y C M T R B V B G K L F L P K Y A L S Q D V C T Y R D F M Y K	60
LH β CHAIN (bovine)	26	C I T F T T S I C A G Y C P S M K R V L P V I L P P M P E R V C T Y H E L R F A S V	67
HCG β CHAIN (human)	26	C I T V N T T I C A G Y C P T M T R V L Q G V L P A L P Z L V C N Y R D V R F E S I	67
FSH β CHAIN (human)	24	N T T(W,B, T) E T C A G Y C Y T R D L V Y K D P A K P R I Q K T C T F K E L V Y E T V	65

FIGURE 16. Analogous regions in the sequence of cholera toxin B-subunit and those of β-subunit of glycoprotein hormones. (From Ledley, F. D., Mullin, B. R., Lee, Aloj, S. M., Fishman, P. H., Hunt, L. T., Dayhoff, M. O., and Kohn, L. D., *Biochem. Biophys. Res. Commun.*, 69, 852, 1976. With permission.)

Table 3
STIMULATION OF ADENYLATE CYCLASE IN PIGEON ERYTHROCYTES MEMBRANE BY A_1 POLYPEPTIDE MODIFIED WITH IODOACETIC ACID AND *N*-ETHYLMALEIMIDE

Activator added	Adenylate cyclase activity (pmol of cAMP producted/min/mg)
None	0.88
A_1 unmodified	8.09
A_1 carboxymethylated	9.91
A_1 treated with NEM	8.46

group, as the consequence of the disulfide cleavage, was responsible for the manifestation of the activity of polypeptide A_1.

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