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Mechanism of Action of Choleragen

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Choleragen exerts its effect on cells through activation of adenylate cyclase. Choleragen initially interacts with cells through binding of the B subunit of the toxin to the ganglioside G_{M1} on the cell surface. Subsequent events are less clear. Patching or capping of toxin on the cell surface may be an obligatory step in choleragen action. Studies in cell-free systems have demonstrated that activation of adenylate cyclase by choleragen requires NAD. In addition to NAD, requirements have been observed for ATP, GTP, and calcium-dependent regulatory protein. GTP also is required for the expression of choleragen-activated adenylate cyclase. In preparations from turkey erythrocytes, choleragen appears to inhibit an isoproterenol-stimulated GTPase. It has been postulated that by decreasing the activity of a specific GTPase, choleragen would stabilize a GTP-adenylate cyclase complex and maintain the cyclase in an activated state. Although the holotoxin is most effective in intact cells, with the A subunit having 1/20th of its activity and the B subunit (choleragenoid) being inactive, in cell-free systems the A subunit, specifically the A_1 fragment, is required for adenylate cyclase activation. The B protomer is inactive. Choleragen, the A subunit, or A_1 fragment under suitable conditions hydrolyzes NAD to ADP-ribose and nicotinamide (NAD glycohydrolase activity) and catalyzes the transfer of the ADP-ribose moiety of NAD to the guandino group of arginine (ADP-ribosyltransferase activity). The NAD glycohydrolase activity is similar to that exhibited by other NAD-dependent bacterial toxins (diphtheria toxin, Pseudomonas exotoxin A), which act by catalyzing the ADP-ribosylation of a specific acceptor protein. If the ADP-ribosylation of arginine is a model for the reaction catalyzed by choleragen in vivo, then arginine is presumably an analog of the amino acid which is ADP-ribosylated in the acceptor protein. It is postulated that choleragen exerts its effects on cells through the NAD-dependent ADP-ribosylation of an arginine or similar amino acid in either the cyclase itself or a regulatory protein of the cyclase system.

Key words: **choleragen, adenylate cyclase, Escherichia coli enterotoxin, diphtheria toxin, Pseudomonas exotoxin A, NAD glycohydrolase, ADP-ribosyltransferase, ganglioside G_{M1}**

Abbreviations: G_{M1} – galactosyl-N-acetylgalactosaminyl-[N-acetylneuraminyl]-galactosylglucosylceramide; App(NH)p – adenylyl imidodiphosphate; App(CH)p – adenylyl diphosphonate; Ap(CH)pp – adenylyl (α,β-methylene) diphosphonate; Gpp(NH)p – guanylyl imidodiphosphate.

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INTRODUCTION

Although cyclic AMP appears to play a pivotal role in cellular metabolism in animal tissues, the factors controlling its synthesis by adenylate cyclase (EC 4.6.1.1.), degradation by the cyclic nucleotide phosphodiesterase(s), and interaction with other proteins such as the cyclic nucleotide-dependent protein kinases are poorly understood. It is believed that several hormones, neurotransmitters, pharmacologic agents, and bacterial toxins exert their effects on cells through the activation of adenylate cyclase. The biochemical basis of activation by none of these agents has been defined. It seems probable, however, that choleragen, an enterotoxin from Vibrio cholerae, activates adenylate cyclase by a mechanism that differs in several ways from those utilized by hormones and other agents (for reviews on the historical, clinical, and biochemical aspects of choleragen, see Refs. 1–22).

Choleragen is an oligomeric protein of approximately 72,000–86,000 daltons, containing two dissimilar subunits, A and B [23–30] ; the holotoxin oligomer is composed of one A (27,000–29,000 daltons) and 4–6 B (11,600 daltons) subunits [25, 28–41]. The A subunit consists of two peptide chains, A_1 (20,000–25,000 daltons) and A_2 (2,500–9,700 daltons), linked through a single disulfide bond [25–27, 29–34]. The complete amino acid sequence of the B subunit and parts of the A_1 and A_2 peptides have recently been determined [30, 32, 36–38, 41, 42]. It has been noted that choleragen shares certain homologies of sequence with the glycopepetide hormones [43–45].

The initial event in the activation of adenylate cyclase by choleragen in intact cells almost certainly involves binding of the toxin to the cell-surface receptor, the monosialoganglioside G_{M1} [14, 26–29, 35, 46–91]. The specific interaction of the toxin with the ganglioside is mediated through the B subunit of the toxin [14, 27, 29, 35, 39, 50, 51, 53, 61, 65, 66, 85, 87, 90, 92–99]. Subsequent events leading to the time-dependent activation of adenylate cyclase are less clear. The delay in activation [53, 65, 66, 99–106] may represent the time required for lateral motion of the toxin on the cell surface [68, 83, 100, 107, 108], penetration of the active A_1 peptide into or through the plasma membrane [31, 65, 99] and, very likely, covalent modification of a cellular protein in an NAD-dependent ADP-ribosylation reaction catalyzed by the A_1 peptide [85, 109–112]. Although effects of cycloheximide have been reported [113,114], activation of adenylate cyclase by choleragen appears to be independent of protein synthesis [115]. Once activated, adenylate cyclase activity in cells may remain elevated for days [116], and adenylate cyclase activated by choleragen in intact cells [97, 117, 118] or in homogenates [119] is stable to solubilization.

In the following sections, we have attempted to summarize evidence for the current views of choleragen action. These studies, albeit far from complete, may provide some clues to the mechanisms involved in the control of adenylate cyclase activity and the regulation of cyclic AMP concentrations.

II. REQUIREMENTS FOR ACTIVATION OF ADENYLATE CYCLASE BY CHOLERAGEN

Studies of activation of adenylate cyclase by choleragen in cell-free systems have defined some of the cofactor requirements (II.2, II.3) and identified the enzymatic and binding functions, respectively, of the A and B subunits of choleragen (II.1, III.2). The factors believed to be essential for activation of adenylate cyclase by choleragen in broken cells and their possible functions are shown in Table I. It has also been established that, although the putative receptor, ganglioside G_{M1}, may be absolutely required for choleragen activation of cyclase in an intact cell, it can be bypassed in cell-free systems (II.1).

TABLE I. Requirements for Activation of Adenylate Cyclase by Choleragen in Cell-Free Systems

Component	Proposed function	References
A_1 peptide of choleragen	Catalyzes transfer of ADP-ribose from NAD to arginine	65, 130, 131
NAD	Substrate for choleragen-catalyzed ADP-ribosylation	77, 79, 99, 109, 110, 119, 130, 131, 135–138
GTP	? (Required for expression of activity of enzyme activated in intact or in broken cells)	136, 137
Calcium-dependent regulatory protein	? (Required with solubilized brain enzyme for expression of activity)	139
ATP	?	110, 131, 138
Cytosolic factors	? (ATP, GTP, and/or calcium-dependent regulatory protein may be among these)	109, 110, 119

II.1. Role of Ganglioside G_{M1} in Intact Cells and in Cell-Free Systems

Ganglioside G_{M1} is believed to serve as the receptor for choleragen on the cell surface [14, 26–29, 35, 46–91]. It was initially observed that a mixture of gangliosides inhibited the action of the toxin on the gut [46] and subsequently confirmed that ganglioside mixtures will block choleragen action in several systems [56, 66, 69]. The ganglioside responsible for interaction with choleragen appears to be G_{M1}. G_{M1} can precipitate toxin from solution [51, 59, 60, 81], can interfere with choleragen action [48, 50–53, 55, 60–63, 65, 71, 81, 82, 89], can block the binding of ^{125}I-choleragen [47, 76, 88] and when immobilized on Agarose beads [49] or as a ganglioside-cerebroside complex [27], can bind choleragen. In several instances, it has been shown that G_{M1} is much more effective than other gangliosides which differ only in the structure of the oligosaccharide moiety [47, 51, 52, 62, 83]. Indeed, although it has a lower affinity for choleragen than does the ganglioside, the oligosaccharide of G_{M1} will bind to the toxin and inhibit its precipitation by G_{M1} [59, 60, 81, 90].

Treatment of cells with neuraminadase, which converts trisialo- and disialogangliosides to G_{M1}, produced increases in G_{M1} content [83], choleragen responsiveness [57, 66] and choleragen binding [52, 54, 80, 83]. It was further found that prior incorporation of G_{M1} increased the capacity of cells or membrane preparations to bind choleragen [48, 67, 70, 76, 80, 83] and enhanced the responsiveness of cells to the toxin [48, 65, 67, 68, 74, 75, 89]. In several types of cells and tissues, there seems to be a correlation between G_{M1} content and choleragen binding [58, 64, 67], and it has been observed that modifications of the toxin molecule that decrease its ability to bind G_{M1} decrease its activity toward cells [73].

In an attempt to relate quantitatively G_{M1} content and choleragen action, chemically transformed fibroblasts grown in defined medium in the absence of serum, a source of ganglioside [120, 121], were studied. These cells contained no detectable G_{M1} and did not accumulate cAMP in response to choleragen [72, 77, 91]. Following incubation of the cells with G_{M1}, but not with other gangliosides, an increase in binding of and responsiveness to

choleragen was observed [72, 77; unpublished data]. All data were consonant with the conclusion that G_{M1} is the choleragen receptor in these cells, although its role in fat cells has recently been questioned [74]. It should be noted that a large fraction of G_{M1} in cells, whether of endogenous or exogenous origin, may not be involved in the action of choleragen [75, 99].

Interactions of choleragen with gangliosides have also been investigated using liposomal model membranes. ^{125}I-choleragen was bound to liposomes containing G_{M1} [78] but not in large quantities to those containing other gangliosides. The specific interaction of choleragen or its B protomer with G_{M1} liposomes was associated with alterations in permeability of the model membranes [78, 86, 122] and under some conditions with precipitation of these structures [123].

Changes in the fluorescent and circular dichroic properties of choleragen in solution [79, 85] are induced by G_{M1} but not by other gangliosides. These may result from the interaction of G_{M1} with lysyl residues in the toxin [124]. Identical effects are induced by the oligosaccharide derived from G_{M1} but not by those from other gangliosides [90]. Indirect evidence that choleragen interacted with the oligosaccharide moiety was provided by the observation that binding of the toxin to G_{M1} in cells and membranes prevented enzymatic oxidation of galactose in the ganglioside [79, 84]. Using equilibrium dialysis and gel filtration multivalent, high-affinity binding of G_{M1} oligosaccharide by choleragen and choleragenoid has been directly studied [87, 90]. Although the multivalent nature of the interaction was not unexpected, given the number of B subunits in each choleragen molecule [30, 31], the tendency of gangliosides to self-associate in solution made it difficult to interpret results of similar studies with G_{M1} itself [87]. Indeed, the molar ratio of G_{M1} to choleragen apparently required to inactivate toxin has been reported to range from 1:1 to 3:1 [51, 59, 60, 82].

The changes in physical properties of the toxin induced by G_{M1} ganglioside or oligosaccharide appear to be related entirely to interactions with the B subunit of the toxin [51, 61, 85, 87, 90, 92, 98]. Choleragenoid (see review in Ref. 1) is an inactive form of choleragen [39, 92, 97, 125–128] which consists solely of B subunits identical to those present in choleragen [27, 30, 32–34, 39]. It blocks choleragen binding to cells and membranes [92, 95] and choleragen action on intact cells [39, 50, 53, 65, 66, 92–94, 96, 97, 99] but has no effect on lysed cells [65]. Choleragenoid interacts with G_{M1} or its oligosaccharide [51, 61, 87, 92, 98] and undergoes alterations in its fluorescent and circular dichroic spectra [85, 90]; these observations are consistent with the view that it is the B subunit which is responsible for interaction of choleragen with the cell-surface receptor.

Although G_{M1} seems to be absolutely required for effects of the toxin on intact cells, choleragen activation of adenylate cyclase in a cell-free system can proceed in the apparent absence of ganglioside, as was shown with mouse fibroblasts [77]. Nor does G_{M1} inhibit choleragen action in lysates as it does in intact cells [65]. The requirement for G_{M1} for adenylate cyclase activation by choleragen in intact cells is paralleled by a requirement for the entire choleragen complex, consisting of both the A (or activating) and B (or binding) subunits [35]. The activity of the A subunit or A_1 peptide alone in intact cells is less than 1/20th that of the holotoxin [35, 65, 118, 129–131]. In a cell-free system, in which the ganglioside receptor can be bypassed, the B subunit is not necessary [65, 98, 129], and there is no lag before activation is observed [131]. The A subunit alone is active, and its effects are not blocked by ganglioside [35, 131, 132]. In several types of cell-free preparations, membranes and detergent-solubilized or solubilized and partially purified adenylate cyclase, activation has been obtained with either the A subunit or the A_1 pep-

tide [65, 129–131], or a membrane-generated choleragen breakdown product believed to be related to A [133–135]. Modification of the single sulfhydryl in A_1 did not affect the activity of the peptide [130]. When intact holotoxin is used for activation of adenylate cyclase in a cell-free system, it must first be incubated with a sulfhydryl reducing agent, presumably to release A_1 peptide [110, 130], whereas when A_1 is used, dithiothreitol is not required [130]. The necessity for release of A_1 has, however, been questioned in a recent report of studies with cross-linked choleragen [89], and further information will be required to resolve this point.

II.2. Identification of NAD as a Necessary Cofactor

Observations reported by Gill [109, 110] and confirmed in other laboratories [77, 79, 119, 130, 131, 133–139] led to the conclusion that NAD is required for choleragen activation of adenylate cyclase. NAD could be partially replaced by NMN, NADP, or deamino NAD [109]. Thionicotinamide adenine dinucleotide was slightly better than NAD; FAD, α-NAD and ADP-ribose were ineffective with pigeon erythrocyte systems. In the rat liver system [138], NADH, NADP, and NADPH were inactive. In systems that were not dependent on exogenous NAD and that presumably contained endogenous nucleotide, the addition of an NAD glycohydrolase revealed an NAD requirement for choleragen activation of adenylate cyclase [109, 110, 135]. An NAD effect has not been demonstrated in all systems [129]. In these cases, endogenous NAD may be sufficient to support activation, although where investigated it has been found that maximal activation of adenylate cyclase requires NAD in millimolar concentrations [109, 110, 135, 138, 139].

In addition to NAD, ATP and cytosolic factor(s) are required to obtain activation of several adenylate cyclases [65, 109, 110, 119, 131, 135–139]. Stimulation of activation by ATP has also been documented with the hepatic, sarcoma, and brain cyclases [131, 135, 138, 139]. Since App(NH)p, App(CH)p, and Ap(CH)pp can replace ATP in the pigeon erythrocyte system [20], utilization of the compound in a phosphorylation reaction is unlikely. As discussed below (II.3), GTP is also required for expression of the adenylate cyclase activated by choleragen in intact cells in cell-free systems [96, 127, 136–139]. The relative roles of GTP and ATP have not been defined. Those systems in which requirements for NAD, ATP and/or cytosolic factors have been demonstrated are listed in Table II.

II.3. Effects of GTP and Calcium-Dependent Regulatory Protein

Recognition of the importance of GTP for expression of hormone-activated adenylate cyclase initially stemmed from the work of Rodbell and his collaborators [140–145]. Cuatrecasas and co-workers demonstrated that following treatment of intact cells with choleragen full expression in vitro of the activated cyclase required GTP [7, 96, 127]. In some cases, expression of choleragen-activated enzyme required a preliminary incubation with GTP before initiation of the assay [139]. When choleragen was used to activate adenylate cyclase in a cell-free system, GTP was required in addition to ATP and NAD [136, 139, 146]. The soluble cyclase system from brain needed a preliminary incubation with GTP in order to achieve maximal activity [139]. In this case, however, full expression of the cyclase after toxin activation was not observed unless the calcium-dependent regulatory protein was also added [139]. This protein, which was first identified by its ability to activate cyclic nucleotide phosphodiesterase [147, 148], has been purified from many tissue and organ systems [149–156]. It is a low-molecular-weight protein [148–151, 153, 157], relatively stable to heating [148, 154, 158–161], which requires calcium for activity

TABLE II. Requirements for Choleragen Activation of Adenylate Cyclase

Tissue	Preparation	Cofactor requirements	References
Pigeon erythrocytes	Lysates	NAD, supernatant factors, ATP	65, 109, 110
Pigeon erythrocytes	Membranes	NAD	130
Rat liver	Homogenate	NAD	119
Rat liver	Plasma membranes	NAD, ATP	138
Rat liver	Solubilized (Lubrol PX)	NAD, ATP	131
Rat brain	Solubilized, DEAE-cellulose purified	NAD, ATP, GTP, calcium-dependent regulatory protein	139
Rat adrenal	Particulate fraction	GTP	146
Bovine adrenal	Membranes	GTP	146
Bovine thyroid	Membranes	NAD	79
Mouse lymphoma (S49)	Plasma membranes	NAD, GTP	136
Mouse neuroblastoma	Particulate fraction	NAD, GTP	137
Mouse fibroblasts (transformed)	Homogenate	NAD	77
Mouse ascites cells (sarcoma 180)	Plasma membrane Fragments	NAD	133–135

[151, 154, 158, 159, 162–165] and undergoes a conformational change after binding calcium [154–157, 166, 167]. It is similar to other calcium-binding proteins such as troponin C [152, 153, 157, 165, 166]. Cheung and co-workers [168–170] and Brostrom et al [161, 171] independently found that this protein was required for demonstration of fluoride and Gpp(NH)p effects on brain and fibroblast adenylate cyclases, respectively. With the solubilized brain adenylate cyclase system, little activity was observed in the absence of the calcium-dependent regulatory protein whether or not choleragen was present [139]. Although calcium-dependent regulatory protein did not increase percentage activation by toxin, it markedly enhanced the activity of both toxin-treated and basal adenylate cyclases. In addition to its activation of phosphodiesterase and adenylate cyclase, it has also been shown to enhance ATPase activity [172, 173] (for review see Ref 174).

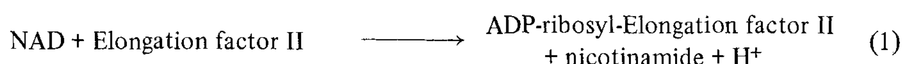
Proteins that bind GTP and may influence the activity of the hepatic and myocardial cyclases [175, 176] and the pigeon and turkey erythrocyte enzymes [177–180] have been described. The role of these GTP-binding proteins in the expression of the choleragen-activated cyclase has not been determined. Several investigators have postulated that activation of adenylate cyclase by choleragen results from stabilization of an adenylate cyclase–GTP complex [136, 137]. A model for choleragen action based on a postulated role for GTP in the expression of adenylate cyclase activity has been offered by Cassel and Selinger [181]. They found that activity of the turkey erythrocyte adenylate cyclase was stimulated by choleragen and isoproterenol. A GTPase in these preparations was stimulated by isoproterenol [181–184]; the isoproterenol-stimulated GTPase was in turn inhibited by choleragen [181]. They proposed that the inhibition of GTPase activity by choleragen would account for the activation of cyclase. It was postulated that there exists a specific GTPase associated with the cyclase and that choleragen, by inhibiting this GTPase, decreases GTP breakdown, thus permitting the cyclase to remain in an active state [181, 182].

III. ENZYMATIC ACTIVITY OF CHOLERAGEN

The relative impurity of available adenylate cyclase preparations makes it difficult to resolve components of the system and to define mechanisms of activation. Recent studies on the enzymatic activities of choleragen, as they are performed in the absence of cellular materials, circumvent this problem and have provided evidence to support the hypothesis that activation of adenylate cyclase by the toxin involves the ADP-ribosylation of a cellular protein (III.2).

III.1. Mechanism of Action of Other NAD-Dependent Bacterial Toxins

NAD is required for the action of several bacterial toxins; of these, diphtheria and Pseudomonas toxins have been the best-studied [185–213]. Both toxins interfere with protein synthesis in susceptible intact cells and in cell-free systems [185, 186, 192, 196, 200–207]. Inhibition of protein synthesis is believed to result from the toxin-catalyzed, NAD-dependent ADP-ribosylation of a single amino acid in elongation factor II [188, 189, 191, 192, 194, 195, 199, 200, 213] (Reaction 1).

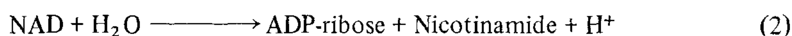


$$\text{NAD} + \text{Elongation factor II} \longrightarrow \text{ADP-ribosyl-Elongation factor II} + \text{nicotinamide} + H^+ \quad (1)$$

This reaction requires a ternary complex between the toxin, elongation factor II, and NAD and can be demonstrated in the absence of other cellular components.

Although the native toxins are inactive in cell-free systems, limited tryptic digestion and reduction of diphtheria toxin or incubation of Pseudomonas toxin with urea and dithiothreitol reveals their ability to utilize NAD as a substrate in the ADP-ribosylation of elongation factor II [208–212]. Release of the enzymatically active fragment of diphtheria toxin [208–211] or mild denaturation of Pseudomonas exotoxin A [212], while enhancing the ability of the toxin to ADP-ribosylate elongation factor II, diminishes its ability to interact with and inhibit protein synthesis in intact cells. Only a relatively small portion of the diphtheria toxin [193, 210] or exotoxin A [198] molecule is responsible for enzymatic activity. Indeed, species of only ~ 24,000–25,000 daltons, which can catalyze the ADP-ribosylation of elongation factor II, have been isolated from tryptic digests of diphtheria toxin or from Pseudomonas cultures [193, 198, 208, 210]. The remaining portions of the proteins are thought to contain binding determinants which permit interaction of the toxin with cells and/or penetration of the active component [209].

In the absence of elongation factor II, the active peptide of diphtheria toxin (Fragment A) and the modified Pseudomonas toxin will catalyze the hydrolysis of NAD to ADP-ribose and nicotinamide [193, 198] (Reaction 2).



$$\text{NAD} + H_2O \longrightarrow \text{ADP-ribose} + \text{Nicotinamide} + H^+ \quad (2)$$

This reaction proceeds at a rate much less than that of the ADP-ribosylation reaction and is thought not to be relevant to the biologic effects of the toxins [213]. The existence of this reaction does indicate that these toxins are capable of activating the ribosyl-nicotinamide bond of NAD in the absence of a ternary complex with the acceptor-protein.

A comparison of some properties of diphtheria toxin and Pseudomonas exotoxin A with those of choleragen is given in Table III (III.2).

TABLE III. Comparison of Enzymatic and Physical Properties of Choleragen, Diphtheria Toxin, and Pseudomonas Exotoxin A

	Choleragen	Diphtheria toxin	Exotoxin A	References
Molecular weight of native toxin	~ 84,000	~ 63,000	~ 66,000–71,500	23–30, 198, 200, 208–212
Subunits	A,B	One chain	One chain	
Molecular weight of active subunit or fragment	~ 23,500	~ 24,000	~ 26,000–27,000	25–27, 29–34, 193, 198, 208–212
NAD glycohydrolase activity	Present	Present	Present	111, 193, 198
ADP-ribosyltransferase activity with arginine as an acceptor	Yes	Not determined	Not determined	112
Protein acceptor	Not determined	Elongation factor II	Elongation factor II	188, 189, 191, 192, 194–199

III.2. NAD Glycohydrolase and ADP-Ribosyltransferase Activities of Choleragen

It was proposed initially that activation of adenylate cyclase by choleragen proceeded by interaction of the toxin with the cyclase [7, 97, 100, 129]. In view of the mechanistic similarities between choleragen and several bacterial toxins utilizing NAD, however, it appeared that activation of adenylate cyclase by choleragen might proceed through the ADP-ribosylation of an intracellular protein [85, 109, 111, 112]. This protein has not, unfortunately, been identified, and a reaction analogous to Reaction 1 has not been demonstrated. Choleragen does, however, catalyze Reaction 2, the hydrolysis of NAD to ADP-ribose and nicotinamide [85, 111] (Table IV, Reaction IA). This NAD glycohydrolase activity resides in the A_1 fragment of the toxin (unpublished data). It is independent of the B or binding subunit and the ganglioside receptor, and it requires no cellular components for demonstration [111]. The reaction is greatly enhanced by increasing salt concentrations [111]. Dithiothreitol, which presumably promotes cleavage of the disulfide bond linking the A_1 and A_2 fragments, is required when either choleragen or A subunit, but not when the purified alkylated A_1 peptide, is the source of enzyme [111; unpublished data]. The delay observed when the reaction is initiated with A subunit or holotoxin, ie, when the first exposure to thiol occurs in the assay, is not observed when the reaction is initiated with reduced and alkylated A_1. High salt concentrations are, however, still required [111; unpublished data].

Inclusion of arginine in the assay mixture greatly accelerates the production of [carbonyl-^{14}C]nicotinamide from [carbonyl-^{14}C]NAD catalyzed by choleragen [112]. Other basic amino acids, lacking a guanidino group, such as lysine and histidine, are ineffective. L- and D-arginine are both effective, although D-arginine is slightly better in accelerating [carbonyl-^{14}C]nicotinamide formation. Citrulline, in which the guanidino group of arginine is replaced by a ureido moiety, is totally inactive, suggesting that the activity of arginine is related to the guanidino group. Guanidine itself is, in fact, active

TABLE IV. NAD Glycohydrolase and ADP-Ribosyltransferase Activities of Choleragen

	References
I. Reactions catalyzed by choleragen	
A. NAD ⟶ ADP-ribose + nicotinamide + H^+	111
B. NAD + L-arginine ⟶ ADP-ribose-L-arginine + nicotinamide + H^+	112
II. Activation of adenylate cyclase by choleragen	
Hypothesis:	
NAD + acceptor protein ⟶ ADP-ribose-acceptor protein + nicotinamide + H^+	109, 111, 112

TABLE V. Effect of Guanidino Compounds and Amino Acids in ADP-Ribosyltransferase Reaction Catalyzed by Choleragen ([112] and unpublished data)

(Release of [^{14}C]nicotinamide from [^{14}C]NAD)	
Increased	No effect
L-Arginine	L-Lysine methyl ester
D-Arginine	L-Histidine methyl ester
L-Arginine methyl ester	L-Histidyl-L-lysine
Guanidine	L-Ornithine
Agmatine	L-Citrulline

(Table V). The role of arginine as an acceptor for the ADP-ribosyl moiety of NAD was established by isolating the reaction products formed during incubation of [adenine-U-^{14}C]NAD and L-[^{3}H]arginine with choleragen. In addition to nicotinamide, a compound was found on thin-layer chromatograms which had a mobility intermediate between that of ADP-ribose and NAD. This product contained adenine and arginine in a 1:1 ratio consistent with its tentative identification as ADP-ribosyl arginine [112].

The fact that choleragen exhibits both NAD glycohydrolase [111] (Reaction IA, Table IV) and ADP-ribosyltransferase activities [112] (Reaction IB, Table IV) does not, of course, necessarily mean that these reactions are involved in its mechanism of action. It does, however, establish that choleragen has an NAD site. Because NAD is required for choleragen activation of adenylate cyclase and because the NAD glycohydrolase activity of choleragen is similar to that of both diphtheria toxin and Pseudomonas exotoxin A, it is reasonable to propose that adenylate cyclase activation results from the choleragen-catalyzed ADP-ribosylation of an appropriate acceptor protein. Such a reaction would be analogous to Reaction (1) catalyzed by diphtheria and Pseudomonas toxins (see Table III). Although the natural protein acceptor has not been identified, if it is assumed that the ADP-ribosyltransferase activity of choleragen is a model for the biologically relevant reaction catalyzed by the toxin, it seems probable that arginine is an analog of the acceptor amino acid in that protein. Thus, it is suggested that activation of adenylate cyclase by choleragen may result from the ADP-ribosylation of an arginine or similar residue on a protein which is either the cyclase itself or is a regulatory component of the cyclase

system* (Table IV, Reaction II). It should be noted that arginine methyl ester, in which the carboxyl moiety is blocked, should more accurately mimic the acceptor protein, and it is, in fact, a better substrate for the toxin [112].

IV. MECHANISTIC SIMILARITIES BETWEEN CHOLERAGEN AND ESCHERICHIA COLI ENTEROTOXIN

Certain strains of Escherichia coli elaborate two enterotoxins that may be involved in the pathogenesis of "traveler's diarrhea" [217–222]. One of these, a high-molecular-weight [223–225], heat-labile toxin [62, 218, 224, 226–228], resembles choleragen in its mechanism of action [62, 224, 228–233] (Table VI) and probably exerts its effects by increasing intracellular cyclic AMP [62, 233–235; for review, see 5, 12, 20, 236, 237]. The E coli enterotoxin has not been purified to homogeneity; it appears, however, to exist in both high-molecular-weight (2×10^6) and low-molecular-weight (20,000) species [223–225, 238–241]. While thus differing from choleragen, the E coli enterotoxin does have immunologic cross-reactivity [62, 224, 227, 235, 238, 240–245], probably with the B or ganglioside-binding subunit of choleragen [241, 243]. There is some evidence that the E coli enterotoxin will interact with gangliosides [62, 63, 71, 235, 246]. Although enterotoxin seems to have a lower affinity than that of choleragen for G_{M1} in some systems [50, 53],† its action can be inhibited by the ganglioside [62].

TABLE VI. Comparison of Choleragen and Heat-Labile E coli Enterotoxin

Characteristics	Choleragen	Enterotoxin	References
Genetic determinant	Bacterial chromosome	Plasmid	236, 250
Molecular weight	84,000	20,000 to several million	23–30, 223–225, 238–241
Subunit structure	Two dissimilar subunits	Not determined	23–30
Cell surface receptor	Ganglioside G_{M1}	May be a ganglioside (but affinity lower than that of choleragen)	14, 26–29, 35, 46–91, 235, 246
Activation of adenylate cyclase	NAD-dependent	NAD-dependent	77, 79, 109, 110, 119, 130, 131, 133–139, 241
NAD glycohydrolase and ADP-ribosyltransferase activities	Present	Present	112, 248, 249

*It has recently been reported that choleragen will catalyze ADP-ribosylation of its A subunit and some glycopeptide hormones [214, 215]. It has also been reported that the A_1 peptide of choleragen is a substrate for protein kinase [216]. The relevance of these reactions in the cellular action of the toxin remains to be determined.

†The action of E coli enterotoxin, however, is not blocked by choleragenoid [50, 53].

The E coli enterotoxin activates adenylate cyclase in intact cells [218, 228, 231, 233] and in a cell-free system [224, 247]. There is no delay in the action of enterotoxin in cell-free systems [217]. As shown by Gill for choleragen [110], activation of cyclase in the pigeon erythrocyte system is dependent on NAD, ATP, and supernatant factor(s) [241]. The highly purified enterotoxin isolated by Richardson exhibits both NAD glycohydrolase and ADP-ribosyltransferase activities similar to those of choleragen [248, 249]. The conditions for demonstration of enzymatic activity and the affinities for substrates (NAD and arginine) differ considerably from those of choleragen, however, suggesting that the catalytic units of the two toxins are structurally distinct proteins. The fact that both of these toxins which activate adenylate cyclase catalyze the transfer of ADP-ribose from NAD to arginine lends further support to the hypothesis that activation results from ADP-ribosylation of the cyclase itself or of a protein that regulates its activity.

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