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STRUCTURE AND FUNCTION OF CHOLERA TOXIN AND HORMONE RECEPTORS

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The enterotoxin from Vibrio cholerae is a protein of 100,000 mol wt which stimulates adenylate cyclase activity ubiquitously. The binding of biologically active ^{125}I-labeled choleragen to cell membranes is of extraordinary affinity and specificity. The binding may be restricted to membrane-bound ganglioside G_{M1}. This ganglioside can be inserted into membranes from exogenous sources, and the increased toxin binding in such cells can be reflected by an increased sensitivity to the biological effects of the toxin. Features of the toxin-activated adenylate cyclase, including conversion of the enzyme to a GTP-sensitive state, and the increased sensitivity of activation by hormones, suggest analogies between the basic mechanism of action of choleragen and the events following binding of hormones to their receptors. The action of the toxin is probably not mediated through intermediary cytoplasmic events, suggesting that its effects are entirely due to processes involving the plasma membrane. The kinetics of activation of adenylate cyclase in erythrocytes from various species as well as in rat adipocytes suggest a direct interaction between toxin and the cyclase enzyme which is difficult to reconcile with catalytic mechanisms of adenylate cyclase activation. Direct evidence for this can be obtained from the comigration of toxin radioactivity with adenylate cyclase activity when toxin-activated membranes are dissolved in detergents and chromatographed on gel filtration columns. Agarose derivatives containing the "active" subunit of the toxin can specifically adsorb adenylate cyclase activity, and specific antibodies against the choleragen can be used for selective immunoprecipitation of adenylate cyclase activity from detergent-solubilized preparations of activated membranes. It is proposed that toxin action involves the initial formation of an inactive toxin-ganglioside complex which subsequently migrates and is somehow transformed into an active species which involves relocation within the two-dimensional structure of the membrane with direct perturbation of adenylate cyclase molecules (virtually irreversibly). These studies suggest new insights into the normal mechanisms by which hormone receptors modify membrane functions.

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INTRODUCTION

Cholera enterotoxin (choleragen), an oligomeric protein of about 100,000 mol wt secreted by Vibrio cholerae, stimulates ubiquitously adenylate cyclase activity in mammalian tissues (1, 2), as well as avian (3, 4) and amphibian (4–6) erythrocytes. The initial event in the action of choleragen involves a high affinity binding to cell surface receptor sites (4, 7–13) which are believed to be G_{M1} monosialogangliosides (see Fig. 1) (4, 7–12, 14–20). G_{M1} competes very effectively for the binding of ^{125}I-labeled cholera toxin to liver membranes (7, 8) and it blocks the biological effects of the toxin (8, 14–20).

Although high concentrations of certain complex sugars and glycoproteins will also block binding (Table I), choleragen exhibits a remarkable specificity for glycosphingolipids containing sialic acid (gangliosides), particularly G_{M1} (Tables II, III). No binding competition is observed with sulfatides, sphingomyelin, psychosine, or crude brain cerebrosides (Table II). The simple glycosphingolipids, such as glucose-ceramide, galactose-ceramide, and galactose-galactose-ceramide, are also relatively inert, although

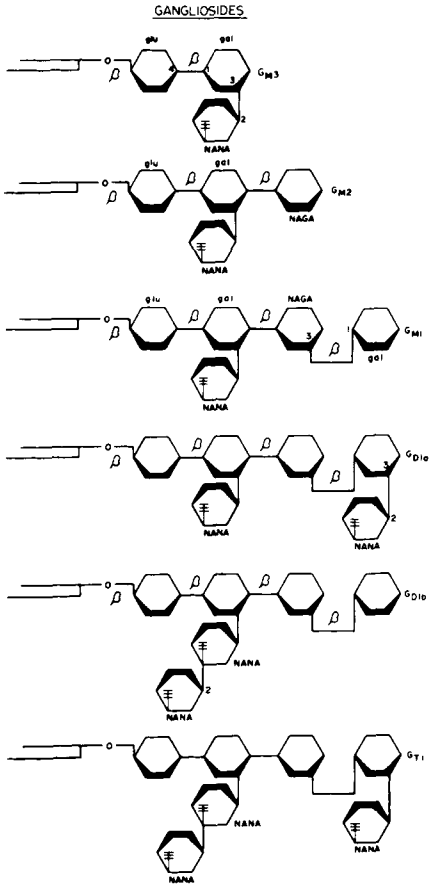

Fig. 1. Structures of some commonly occurring gangliosides. The nomenclature is that of Svennerholm (74).

TABLE I. Effect of Special Glycopeptides and Oligosaccharides on the Specific Binding of Cholera Toxin to Liver Membranes[1]

Oligosaccharide	% ^{125}I-labeled cholera toxin bound
Fetuin glycopeptide I, 85 μM	85
Fetuin glycopeptide II, 85 μM	98
Thyroglobulin glycopeptide I, 85 μM	86
Thyroglobulin glycopeptide II, 85 μM	84
γG-Glycopeptide, 0.9 μM	100
γM-Glycopeptide, 0.1 μM	96
Lacto-N-tetraose (Gal $\xrightarrow{\beta 1,3}$ GlcNAc $\xrightarrow{\beta 1,3}$ Gal ⟶ GlcNAc),[2] 1 mM	80
Lacto-N-neotetraose (Gal $\xrightarrow{\beta 1,4}$ GlcNAc $\xrightarrow{\beta 1,4}$ Gal $\xrightarrow{\beta 1,4}$ GlcNAc),[2] 1 mM	92

[1] ^{125}I-labeled cholera toxin (50 ng/ml) was preincubated at 24°C for 60 min in Krebs-Ringer-bicarbonate buffer containing 0.1% (w/v) albumin and the compound indicated in the Table. Samples of these solutions were then incubated at 24°C for 15 min with Krebs-Ringer-bicarbonate containing 0.1% (w/v) albumin and 40 μg/ml of liver membrane protein. Specific toxin binding was determined by filtration procedures as described in the text.

[2] Gal, galactose; GlcNAc, N-acetylglucosamine.
From Cuatrecasas (7).

more complex derivatives formed by sequential addition of terminal galactose and N-acetylgalactosamine residues result in an increased potency in blocking binding of ^{125}I-choleragen (Table II). The addition of sialic acid in 3, 2 linkage with the galactose of galactose-glucose-ceramide to form G_{M3} monosialoganglioside results in at least a 20-fold increase in inhibitory potency (Table III). Sequential additions of N-acetylgalactosamine to G_{M3} to form G_{M2}, and of galactose to G_{M2} to form G_{M1} result in an increase of 100-fold and 50-fold, respectively, in the apparent affinity for choleragen (Table III). Further addition of N-acetylneuraminic acid to G_{M1} reduces the inhibitory ability by 40- to 100-fold. The approximate concentrations of gangliosides required for half-maximal inhibition of [^{125}I] choleragen binding are: G_{M1} (0.02 μg/ml), G_{D1a} (0.7 μg/ml), G_{M2} (1 μg/ml), G_{T1} (1.8 μg/ml, and G_{M3} (50 μg/ml) (7).

Exogenous G_{M1} can incorporate spontaneously into cell membranes, resulting in an increased capacity for binding of ^{125}I-labeled choleragen (up to 10-fold), as well as an enhanced sensitivity to the biological effects of the toxin (8, 21). The fact that reconstituted G_{M1} can function as a biologically effective membrane receptor strongly supports the notion that this glycolipid is the natural cell surface binding site for choleragen. Further evidence is provided by a study of transformed mouse cell lines from the same parent strain which exhibit loss of specific enzymes involved in the de novo biosynthesis of membrane gangliosides (10). The binding of [^{125}I] choleragen and the sensitivity to stimulation of adenylate cyclase activity and inhibition of DNA snythesis vary in parallel with the ganglioside composition of these cells. The SV40-transformed cell line which lacks chemically detectable G_{M2}, G_{M1}, and G_{D1a}, as well as UDP-N-acetylgalactosamine: G_{M3} N-acetylgalactosaminyltransferase activity, is the least sensitive to the biological effects and binds the least amount of choleragen (Fig. 2).

TABLE II. Effect of Various Glycosphingolipids and Other Glycolipids on the Specific Binding of ^{125}I-Labeled Cholera Toxin to Liver Membranes[1]

Compound[2]	Concentration in preincubated mixture (μg/ml)	% Binding of ^{125}I-labeled cholera toxin
None		100
Gal 1 → Cer	100	100
	5	100
Glc 1 → Cer	100	100
	5	100
Gal 1 $\xrightarrow{\beta}$ 4Glc 1 → Cer	100	100
	5	100
Gal 1 $\xrightarrow{\beta}$ 4Gal 1 $\xrightarrow{\beta}$ 4Glc 1 → Cer	100	80
GalNAcl 1 $\xrightarrow{\beta}$ 3 Cal 1 $\xrightarrow{\alpha}$ 4Gal 1 $\xrightarrow{\beta}$ 4 Glc 1 $\xrightarrow{\beta}$ Cer	50	35
	20	44
	5	68
HSO_3 → 3Gal 1 → Cer (sulfatide)	150	100
Gangliosides (crude, bovine brain)	50	0
	10	0
	1	2
	0.25	5
	0.50	73
Psychosine (Gal 1 → spingosine)	100	100
Cerebrosides	500	100
Sphingomyelin	100	100

[1] ^{125}I-Labeled cholera toxin (0.1 μg/ml) was preincubated at 24°C for 60 min in 0.2 ml of Krebs-Ringer-bicarbonate buffer containing 0.1% (w/v) albumin and the compound indicated in the Table. Samples (50 μl) were then added to incubation mixtures consisting of 0.2 ml of Krebs-Ringer-bicarbonate buffer, 0.1% (w/v) albumin, and 50 μg of liver membrane protein. After 20 min at 24°C the specific binding of the toxin to the membranes was determined by filtration on cellulose-acetate filters. In the absence of liver membranes none of these compounds caused adsorption of the toxin to the filters. The final concentration of the various glycolipids in the membrane incubation mixture is five times lower than that indicated in the Table

[2] Cer, ceramide; Gal, galactose; Glc, glucose; GalNAc, N-acetylgalactosamine. From Cuatrecasas (7).

Agarose derivatives of gangliosides have been prepared which are capable of adsorbing choleragen nearly quantitatively at concentrations as low as 10^{-11} M (22). Moreover, soluble ganglioside polymers prepared by coupling the terminal sialic acid residues to the amino groups of branched copolymers of lysine and alanine also prevent the binding of ^{125}I-labeled choleragen to liver membranes and abolish the lipolytic activity of choleragen on fat cells (22). These macromolecular derivatives of gangliosides apparently do not incorporate into membranes as do the free glycolipids, and thus may provide useful therapeutic tools for the management of clinical cholera (22).

Choleragen is composed of two major subunits, one of about 60,000 mol wt ("binding" subunit) which is responsible for the binding interaction with cell membranes, and another of approximately 36,000 mol wt ("active" subunit) which confers biological activity to the molecule (20, 22–25). Choleragenoid, a 60,000 mol wt protein derived from choleragen (1), which contains only the binding subunit (20, 22–24), competitively

TABLE III. Effect of Specific Gangliosides on the Binding of ^{125}I-Labeled Cholera Toxin to Liver Membranes[1]

Ganglioside[2]	Concentration in preincubation mixture (μg/ml)	% Binding of ^{125}I-labeled cholera toxin
None		100
G_{M3} (Cer → 1Glc4 $\xrightarrow{\beta}$ 1Gal3 $\xrightarrow{\beta}$ 2NANA)	50	71
	25	95
	6	100
G_{M3} (Cer → 1Glc4 $\xrightarrow{\beta}$ 1Gal3 $\xrightarrow{\beta}$ 2N-glycolyl-NA)	50	50
	20	60
	5	82
G_{M2} (Cer → 1Glc4 $\xrightarrow{\beta}$ 1Gal4 $\xrightarrow{\beta}$ 1GalNAc) 3 ↑α 2NANA	5	16
	1.6	28
	0.5	65
	0.2	84
G_{M1} (Cer → 1Glc4 $\xrightarrow{\beta}$ 1Gal4 $\xrightarrow{\beta}$ 1GalNAc3 $\xrightarrow{\beta}$ 1 Gal)[3] 3 ↑α 2NANA	0.35	0
	70 ng/ml	18
	20 ng/ml	45
	6 ng/ml	74
	1 ng/ml	90
G_{D1a} (Cer → 1Glc4 $\xrightarrow{\beta}$ 1Gal4 $\xrightarrow{\beta}$ 1GalNAc3 $\xrightarrow{\beta}$ 1Gal3) 3 ↑α 2NANA ↑α 2NANA	5	20
	1.6	34
	0.5	43
	0.2	70
G_{T1} (Cer → 1Glc4 $\xrightarrow{\beta}$ 1Gal4 $\xrightarrow{\beta}$ 1GalNAc3 $\xrightarrow{\beta}$ 1Gal3) 3 ↑α 2NANA ↑α 2NANA 8^{α}→ 2NANA	10	43
	2.5	31
	0.8	85
	0.2	90

[1] These experiments were performed as described in Table II.

[2] Nomenclature according to Svennerholm (74); Cer, ceramide; Glc, glucose; Gal, galactose; GalNAc, N-acetylgalactosamine; NANA, N-acetylneuraminic acid.

[3] After digestion of G_{M1} (0.1 mg/ml) with neuraminidase (20 μg/ml) for 3 hr at 37°C in 0.1 M sodium acetate (pH 6.2)-2 mM $CaCl_2$, the binding of toxin in the presence of 1 ng/ml of the ganglioside was 80%. Under similar conditions digestion of G_{D1a} and G_{T1} resulted in a very large enhancement of inhibitory activity. From Cuatrecasas (7).

inhibits the action of choleragen (4, 9, 18, 26) and has been shown to bind to the same membrane sites as and with an affinity identical to that of choleragen (9). No biological effects have yet been attributed unequivocally to pure choleragenoid.

When choleragen or choleragenoid are treated with mild acid (27) or heated in sodium dodecyl sulfate (22, 23, 28) the 60,000 mol wt subunit of both proteins is converted to small components with a molecular weight of 8,000–10,000. These peptides

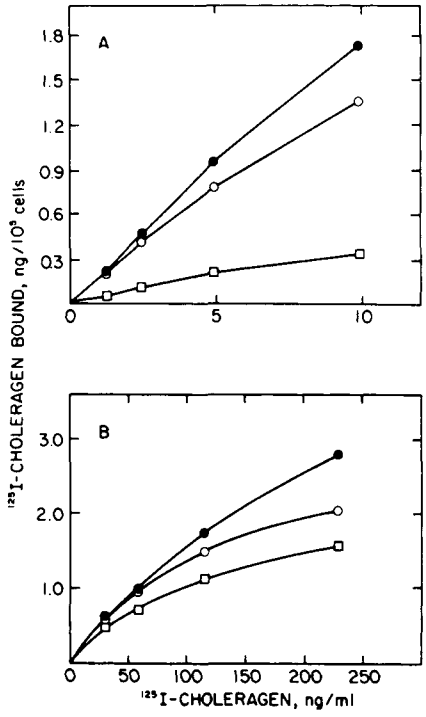

Fig. 2. Binding of cholera toxin to transformed AL/N cells. •, TAL/N, P = 46; ○, TAL/N, P = 269, □, SVS AL/N. (A) Binding to 8 × 10^4 cells in 0.4 ml of buffer. (B) Binding to 3.2 × 10^5 cells in 0.2 ml of buffer. From Hollenberg et al. (10).

may be closely related or identical, which suggests the possibility that each 10,000 mol wt subunit may be capable of binding one molecule of G_{M1} ganglioside, and that the 60,000 mol wt component is thus multivalent. Direct measurements of the stoichiometry of ganglioside binding to choleragen are not available at this time. However, the observation that cholera toxin-treated cells bind specifically to G_{M1} covalently attached to agarose beads (S. Craig and P. Cuatrecasas, in preparation) (Fig. 3a, b) demonstrates that the toxin is at least bivalent.

Choleragen and choleragenoid are potentially useful as probes for detection and quantitation of a special class of cell surface glycolipids which most probably are G_{M1} monosialogangliosides. These membrane molecules are of particular interest since the complement of cell surface gangliosides can be altered dramatically following virus- or carcinogen-induced transformation (29–32) or extensive culturing of spontaneously transformed cell lines (33, 34). Choleragen and choleragenoid can be radioiodinated to specific activities as high as 10^7 cpm per picomole, and therefore provide a sensitivity greatly exceeding (by 10^3-10^4) that of the chemical methods now available. The extremely high affinity of the binding of these proteins to their membrane sites (K_D less than 10^{-10} M) and the fact that they do not penetrate cells in detectable amounts (5) also permit their use as plasma membrane markers during membrane isolation procedures (35).

Choleragen is not toxic to cells. Despite the profound stimulation of adenylate cyclase activity, cultured cells exposed to choleragen remain viable (10, 36, 37), and the

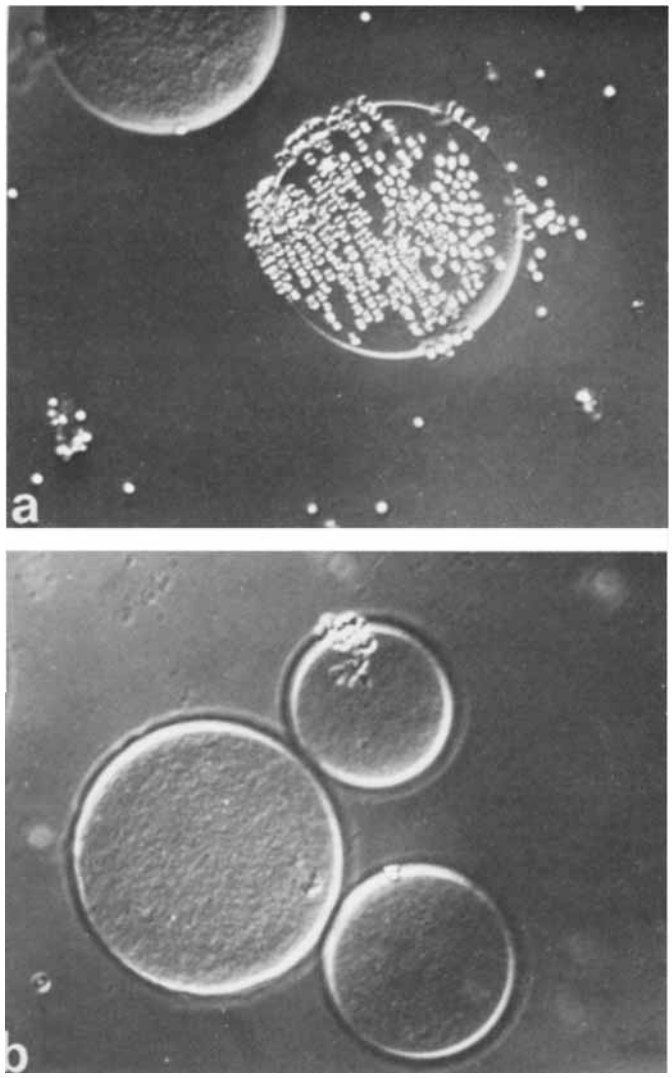

Fig. 3. Specific binding of toxin-treated rat lymphocytes to ganglioside-Sepharose beads. (a) Toxin-treated lymphocytes incubated with ganglioside-Sepharose; (b) untreated lymphocytes incubated with ganglioside-Sepharose.

enzyme activity eventually returns to normal (5, 36). Choleragenoid offers a potential advantage over choleragen as a membrane marker in that it is apparently metabolically inert.

The activation of adenylate cyclase by cholera toxin exhibits some unusual properties that make this protein unique among other known modulators of cyclase activity. Only a brief exposure to the toxin is required for full expression of the biological effects, and these cannot be reversed by extensive washing of the cells (4, 15, 36, 38) or by addition of specific antibody (39) or other inhibitors of cholera toxin binding to cell membranes (38, 40). Despite the rapidity of the initial toxin-cell interaction, a lag period of 20–30 min must transpire before any effects are observed (1, 2, 5, 18, 36, 38, 40–42). Once

activation of adenylate cyclase has occurred, the stimulated state of the enzyme is maintained during cell lysis and membrane isolation procedures (1, 2), and persists even after solubilization of the membranes with nonionic detergents (43, 44). The effects of cholera toxin exhibit a remarkable duration in living cells, and are observed for days after a single brief exposure (5, 36, 41, 45, 46).

Properties of Choleragen-Stimulated Adenylate Cyclase

The characteristics of the cholera toxin-activated adenylate cyclase of toad (4, 6) and turkey (3) erythrocytes, rat fat cells (47, 48), and rat liver (43) have been examined recently. The stimulated enzyme exhibits marked changes in the regulatory properties which resemble specifically the alterations attending hormonal activation (4, 6, 47). Toxin stimulation converts the enzyme of toad red cells to a purine nucleotide-sensitive state such that 5′GTP, 5′ITP, and with much less affinity, 5′ATP stimulate the activity three- to five-fold (Fig. 4) (4, 6). Toxin treatment of rat fat cells elicits qualitatively similar alterations in the membrane adenylate cyclase, although the effects of 5′GTP are more complicated in this system (47). Purine nucleotides preferentially stimulate adenylate cyclase activity assayed in the presence of catecholamines in these cells (4, 47) as well as in the case of a variety of other tissues and hormones (49-56). In contrast, NaF stimulation occurs independently of and is relatively unaffected by these nucleotides (Fig. 4).

In addition to mimicking the effects of hormones, choleragen also increases the sensitivity of adenylate cyclose to stimulation by these agents. The maximal response of

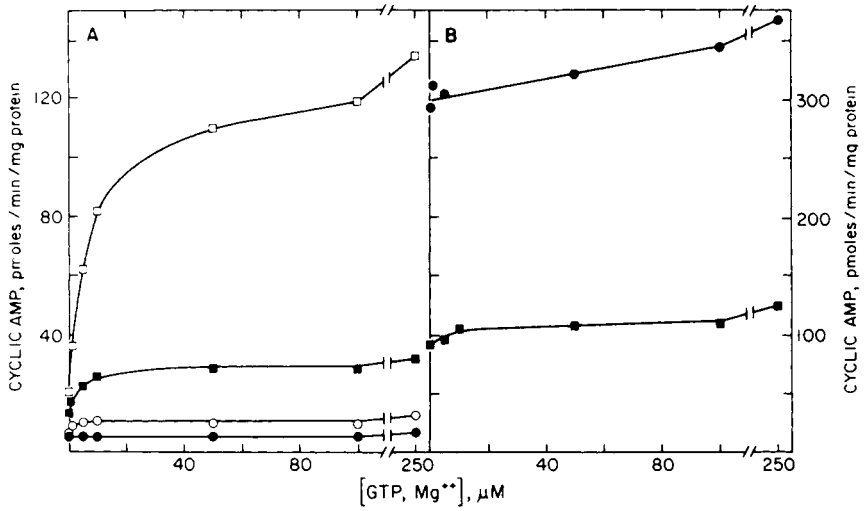

Fig. 4. Effect of increasing the concentration of 5′GTP, Mg^{++} on the adenylate cyclase activity of control (●, ○) and cholera toxin-treated (■, □) toad erythrocyte plasma membranes assayed in the presence (○, □) and absence (●, ■) of 20μM (−) epinephrine (Fig. 4a), and in the presence of 10 mM sodium fluoride (Fig. 4b). Erythrocytes (about 2×10^9 cells) were incubated in 30 ml of amphibian Ringer's in the presence and absence of cholera toxin (0.25 μg/ml) for 4 hr at 30°C. The plasma membrane adenylate cyclase activity was determined (15 min, 30°C) in the presence of $MgCl_2$ (6.2 mM), [α-^{32}P] ATP (0.5 mM, 96 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0), 200–210 μg of membrane protein and increasing concentrations of GTP-Mg^{++}. The values are expressed as the mean of duplicate determinations. From Bennett and Cuatrecasas (4).

toad erythrocyte adenylate cyclose to catecholamines is increased (Fig. 4) (4, 6), while the apparent affinity of these hormones is increased in the toxin-treated enzyme of rat erythrocytes (Fig. 5) (4, 6). An increased sensitivity of adenylate cyclase activity to catecholamines has been reported for turkey erythrocytes (3) and rat fat cells (47, 48). Choleragen also increases the apparent affinity of rat fat cell adenylate cyclase for activation by glucagon, ACTH, and vasoactive intestinal peptide (47), and the affinity of the enzyme of rat liver for glucagon (43).

The stimulation by choleragen appears to differ significantly from the response elicited by NaF, and in toad erythrocytes these agents activate adenylate cyclase in a mutually exclusive fashion (4, 6). NaF prevents the usual activation of the toxin enzyme by GTP (Fig. 4) and catecholamines (4, 6) and, conversely, toxin depresses the stimulation by NaF (Fig. 4). Similar inhibitory effects of toxin on activation by fluoride ion have been observed in turkey erythrocytes (3).

Choleragen most probably does not interact directly with hormone receptors. Direct measurements have shown no influence of high concentrations of glucagon or isoproterenol on the apparent affinity or maximal extent of binding of [^{125}I] choleragen to fat cell membranes (47). Moreover, after binding the toxin exhibits an absolute, temperature-dependent lag period (see below) during which the properties of adenylate cyclase with or without hormones are unaltered (4–6). Simple occupation of the binding site for choleragen is not sufficient to affect the cyclase; choleragenoid, an analog missing the "active" subunit which is a potent competitive antagonist (9, 18, 26), binds with

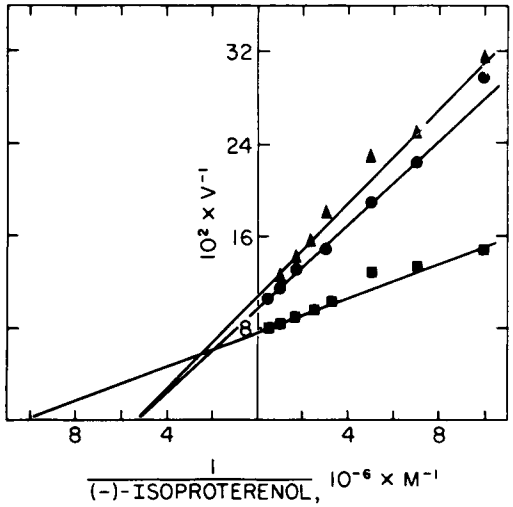

Fig. 5. Effect of increasing the concentration of (−) isoproterenol on the adenylate cyclase activity of control (●), choleragenoid-treated (▲), and cholera toxin-treated (■) rat erythrocyte plasma membranes. Rat erythrocytes were washed in Krebs–Ringer-bicarbonate, pH 7.4, and then incubated in the same buffer for 3 hr at 37°C with either toxin (0.28 μg/ml) or choleragenoid (0.28 μg/ml), or with no additions. The plasma membrane adenylate cyclase activity was determined (12 min, 30°C) in the presence of $MgCl_2$ (6.5 mM), [α-^{32}P]-ATP (0.27 mM, 200 cpm/pmole), GTP (0.16 mM), phosphoenolpyruvate (5.4 mM), pyruvate kinase (55 μg/ml), aminophylline (5 mM), Tris-HCl (50 mM, pH 8.0), 100 μg of membrane protein, and increasing concentrations of (−) isoproterenol. The data are expressed as a double reciprocal plot of isoproterenol-stimulated cyclase activity (stimulated-basal) vs. concentration of isoproterenol. From Bennett and Cuatrecasas (4).

identical affinity to the same ganglioside receptors (9), but has no effect on adenylate cyclase or its sensitivity to hormones (4, 6, 47) (Fig. 5).

Features of the activation of adenylate cyclase by choleragen, including conversion of the enzyme to a GTP-sensitive state and the alteration in the apparent affinities for activation by hormones, suggest analogies between the basic mechanism of action of choleragen and the events following binding of hormones to their receptors. Cholera toxin may thus provide an experimentally accessible model for the interaction of hormone receptors with adenylate cyclase. The possibility is discussed below that the action of choleragen involves mobility of the initial toxin-ganglioside complex in the plane of the membrane with the ultimate formation of an active complex between toxin and the cyclase. The implications for the mechanism of hormone-receptor-cyclase interactions are then considered within the context of a recent proposal that hormone receptors are free to diffuse within the plane of the membrane, and that, when complexed with hormone, they encounter and modify membrane enzymes by analogy with protein-protein associations in solutions.

Properties of the Process of Activation of Adenylate Cyclase by Choleragen

The kinetics and properties of the process of activation of adenylate cyclase by choleragen have recently been described in detail (5). When choleragen is incubated with intact cells, it stimulates adenylate cyclase in the subsequently isolated plasma membranes according to a triphasic time course. This consists of a true lag period of approximately 30 min, followed by a stage of exponentially increasing activity which continues for 110–130 min, and finally a period of slow activation (Fig. 6). The influence of choleragen on basal activity, catecholamine sensitivity, and the activation by fluoride occur in parallel (Fig. 6). The progressive development of the toxin effects can be arrested at any time by lysis of the cells (5).

A semilog plot of adenylate cyclase activity vs. time of incubation during the initial period of exponential change permits, by interpolation, an estimate of the length of the lag phase (Fig. 7). The fact that the semilog plots do not extrapolate through 0 time indicates that the delay is absolute in nature rather than a period of slowly accelerating activation. Thus, the initial toxin-membrane complex is incapable of activating adenylate cyclase as has been proposed previously (5, 38). It is of interest that such different cells as rat adipocytes and toad erythrocytes exhibit nearly identical delays of about 30 min (Fig. 7).

The events occurring during the lag period are not grossly dependent on the number of toxin-receptor complexes, and they are independent of external or internal energy sources and of RNA and protein biosynthesis (5, 38, 47).[1] They are highly dependent on temperature, and do not appear to involve penetration of cholera toxin into the cytoplasm (5). Direct measurements of the interaction of ^{125}I-labeled choleragen with cell membranes (7) have demonstrated that above 20°C almost complete binding occurs within 3–5 min. Furthermore, cells exposed to the toxin for 5 min, followed by washing, still demonstrate the characteristic delay (38).

It is unlikely that the latent period represents the time required for choleragen to remove or inactivate some cell component which is initially present in excess and is therefore rate limiting. Such a hypothesis would predict that once the putative molecule was lost, subsequently added toxin should experience no delay in activation of cyclase.

[1] It is significant in this regard that activation of adenylate cylcase by choleragen occurs with extensively washed, purified plasma membrane preparations from rat fat cells (Sahyoun, N., and Cuatrecasas, P., Proc. Natl. Acad. Sci. U.S.A., in press [1975].

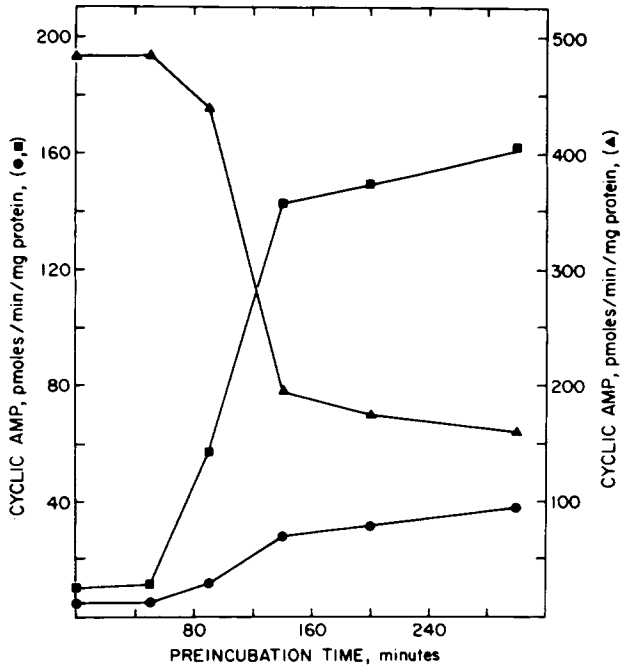

Fig. 6. Effect of increasing the time of incubation of cholera toxin with intact toad erythrocytes on the adenylate cyclase activity of plasma membranes assayed with 20 μM (−) epinephrine (■), 20 mM sodium fluoride (▲), or no additions (●). The cells were thoroughly washed with amphibian Ringer's, pH 7.5, suspended in this buffer, and divided into six 30-ml portions, each containing about 2×10^8 cells. The cell samples were incubated in a 30°C water bath and cholera toxin (5.6×10^{-11} moles) was added at various times. One sample (0 incubation time) received no toxin. After 5 hr, the cells were rapidly chilled, and the plasma membrane adenylate cyclase activity (15 min, 30°C) determined, as described (5). All values were determined in triplicate. From Bennett and Cuatrecasas (5).

It has been shown, however, that the lag is still present after adding a second dose of toxin to partially stimulated cells (5). These results suggest that each toxin-ganglioside complex must independently undergo some slow process before activation of adenylate cyclase can proceed. The possibility is considered below that during the lag phase the hydrophobic[2] (20) 36,000 mol wt subunit of toxin inserts into the membrane bilayer and thus assumes an active form.

The activation of adenylate cyclase activity occurs at a very slow rate below certain critical temperatures which depend on the animal species studied (Fig. 8). The transition in toad erythrocytes occurs at 15–17°C, while rat fat cells and turkey eythrocytes exhibit a discontinuity at 26°–30°C. The temperature effect is observed not only for the lag period, but also for the later, exponential phase of activation. These data suggest that membrane phospholipids may be involved in the action of cholera toxin, and that some degree of lipid "fluidity" may be required throughout the process of enzyme activation.

The degree of stimulation of enzyme activity depends on the number of membrane-bound toxin molecules. During the initial phase of activation, the predominant effect of

[2] Unpublished data.

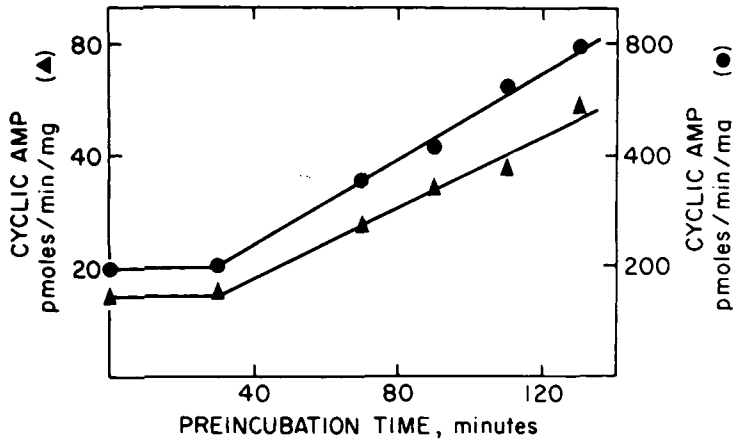

Fig. 7. Semilog plot of the time course of adenylate cyclase activation by cholera toxin in rat adipocytes (●) and toad erythrocytes (▲). The experiment with toad erythrocytes was performed as described in Fig. 6. Isolated epididymal fat cells of eight 100–200-g rats were suspended in oxygenated Krebs-Ringer's bicarbonate, 2% BSA (w/v), pH 7.4, and divided into six 15-ml portions. These were incubated at 37°C under an atmosphere of 95%, O_2, 5% CO_2. Cholera toxin (5.6 μg) was added at various times. After 130 min, the cells were chilled and homogenized (Brinkman Polytron, setting 3.0/30 sec) in cold Tris-HCl (50 mM, pH 7.6). The suspensions were centrifuged at 40,000 × g for 30 min at 0°C, and the membrane pellets were resuspended in Tris-HCl (50 mM, pH 8.0). Adenylate cyclase activity was determined under identical conditions as with the toad enzyme except that the concentration of ATP was 0.25 mM and the assay was conducted at 33°C (5). The enzyme activity of toad and rat membranes was determined in the presence of 20 μM (−) epinephrine. The values were determined in triplicate and are expressed as a semilog plot of enzyme activity vs. time. From Bennett and Cuatrecasas (5).

increasing the amount of cell-bound toxin is an elevation in the rate of exponential increase of enzyme activity. The rate constant for the exponential process can be estimated from the slope of the semilog plot of activity vs. time of incubation (Fig. 7), and this value is related to the number of toxin-receptor complexes by Michaelis-Menten saturation kinetics (Fig. 9). The half-maximal increase in the rate of exponential activation with toad erythrocytes occurs with about 2,200 toxin molecules bound per cell (Fig. 9). The extent of enzyme activation at near equilibrium conditions (4 hr at 30°C) also depends in a hyperbolic manner on the amount of bound choleragen, with an apparent K_a of about 1,500 toxin molecules per cell (Fig. 10).

The quantitative similarity of the dependence of the rate constant and extent of enzyme activation on the amount of cell-bound toxin suggests that catalytic processes are not involved in the sense that one toxin molecule does not lead progressively to stimulation of many enzyme molecules. If such were the case, half-maximal activation would require fewer toxin molecules with increasing length of incubation. Furthermore, if cholera toxin acts as a "catalyst," the rate of activation should continue to increase with higher doses of choleragen, at least until the membrane binding sites have been saturated. The data in Fig. 9 demonstrate, however, that the rate of activation is saturable with respect to bound toxin, and the half-maximal value (2,200 molecules per cell) is quite small compared to the total binding capacity of these cells (about 50,000 sites per erythrocyte [4]). Similar considerations argue against other mechanisms based on catalytic processes initiated by interaction of toxin with the cell.

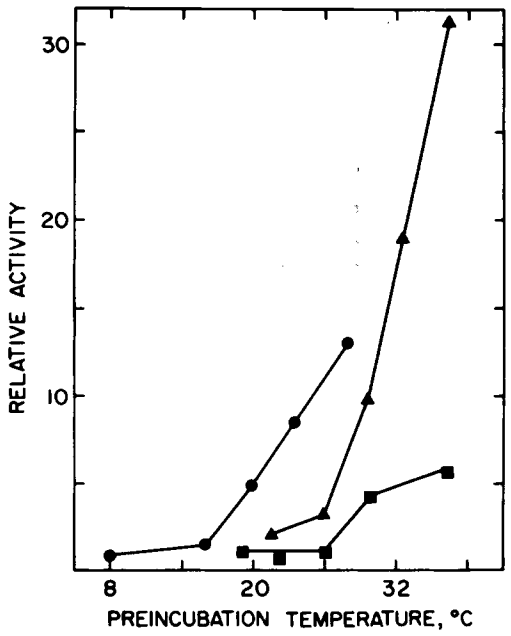

Fig. 8. Temperature dependence of the activation by cholera toxin of the adenylate cyclase activity of toad erythrocytes (•), turkey erythrocytes (■), and rat adipocytes (▲). Toad and turkey erythrocytes were washed in amphibian Ringer's and Krebs-Ringer bicarbonate, respectively, and incubated at various temperatures with cholera toxin (0.3 μg/ml) for 4 hr. The plasma membrane adenylate cyclase activity was determined as described (5). Rat fat cells were prepared and incubated for 135 min at various temperatures with cholera toxin (0.2 μg/ml), as described in Fig. 7. Adenylate cyclase activity was determined in triplicate, and the values are expressed as the enzyme activity relative to control values (cells incubated under identical conditions but in the absence of cholera toxin). From Bennett and Cuatrecasas (5).

Diphtheria toxin (57, 58) and the bacterial colicin E_3 (59, 60) provide examples of microbial products which initially interact with cell membranes, and ultimately function intracellularly by well-established enzymatic mechanisms. In these cases the concentration-response relationships obey Poisson distributions for "single hit" kinetics (61, 62). The data described in Fig. 10 clearly deviate from the predicated linear relationship (not shown).

These considerations suggest that each molecule of toxin may lead to the alteration of only one adenylate cyclase enzyme and imply that at some point these molecules interact directly. The inactive nature of the initial toxin-ganglioside complex implies that the biological effects of toxin are due to interactions with other, secondary "receptor" sites. The simplest possibility, in view of the lack of evidence for a catalytic mechanism, is that the "receptor" is the adenylate cyclase molecule itself. Thus activation would occur by a relatively stable association between the active form of choleragen and adenylate cyclase. It is pertinent that the concentration-response relationships for the toxin-membrane complex can be approximated by a Langmuir adsorption isotherm. This type of relationship is consistent with the occurrence of a bimolecular interaction between bound choleragen and another molecule in the rate-limiting step of adenylate cyclase activation. The absolute nature of the latency phase between binding of choleragen and the onset of activation

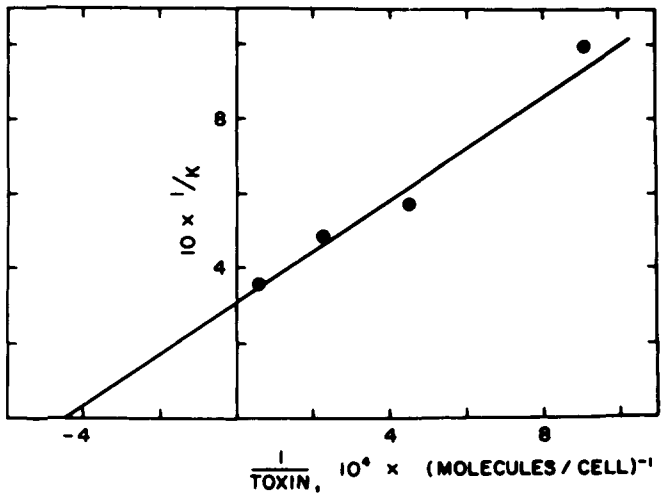

Fig. 9. Double reciprocal plot of the slope (K) of semilog plots of time courses of toad erythrocyte adenylate cyclase activation vs. the number of molecules of cholera toxin bound per cell. Washed erythrocytes were suspended in amphibian Ringer's and divided into 24 10-ml portions, each containing 6×10^8 cells. The time course was examined as described in Fig. 7, at fixed concentrations of cholera toxin: 15,400 molecules/ cell, 4,440 molecules/cell, 2,200 molecules/cell, and 1,100 molecules/cell. Adenylate cyclase activity was measured in the presence of 20 μM (−) epinephrine (5). The values were determined in triplicate. From Bennett and Cuatrecasas (5).

of cyclase indicates that some transformation process must be occurring during this time which requires at least 20–30 min for even the most advanced choleragen molecules. Thus, the proposed association of choleragen with cyclase would not begin until after the lag period.

Studies with Detergent-Solubilized Adenylate Cyclase

Evidence has been reported recently suggesting that following the initial binding interaction, choleragen, or some portion of the molecule forms a detergent-stable complex directly with adenylate cyclase (43). Activation of adenylate cyclase by choleragen is retained following conversion of the membranes with Lubrol PX to a nonsedimentable form (30 min at 250,000 X g) (43, 44), and after gel filtration on agarose columns in the presence of detergent (43). Furthermore, a small peak of [^{125}I] choleragen corresponding to about 1,000 molecules per cell is associated with adenylate cyclase activity on gel filtration provided the enzyme has been activated by labeled choleragen (Fig. 11). The direct association of at least a portion of the small radioactive peak with adenylate cyclase is supported by the finding that antisera directed against choleragen will specifically immunoprecipitate solubilized choleragen-stimulated adenylate cyclase activity (43). It is pertinent that agarose derivatives containing the "active" subunit can specifically adsorb adenylate cyclase activity (43). The fact that about 90% of the membrane-bound choleragen is not associated with adenylate cyclase on gel filtration (Fig. 11) is not surprising; the apparent K_m for stimulation of adenylate cyclase in toad erythrocytes (Fig. 10) and cultured melanocytes (34) and fibroblasts (10) occurs with 1,500–3,000 molecules of toxin bound per cell, whereas specific binding is half-maximal at about 20,000 or more molecules per cell. Studies using choleragenoid to "decrease"

the number of toxin-binding sites suggest that these excess sites represent equivalent "spare" receptors (43).

It is of interest to consider the mechanism by which active membrane-bound toxin may encounter and complex with adenylate cyclase. The fact that equivalent receptors are present in excess over adenylate cyclase molecules implies that a specific mechanism exists for translocating the toxin to adenylate cyclase following formation of the initial toxin-membrane complex. Otherwise, virtually all of the ganglioside receptors would have to exist in direct contiguity with adenylate cyclase. Since some cells such as cultured human fibroblasts may bind as many as 10^6 toxin molecules per cell, this situation would pose serious geometrical problems. Furthermore, direct visualization of the initial binding of fluorescent choleragen to fibroblasts indicates a random, nonclustered distribution of toxin molecules (S. Craig and P. Cuatrecasas, in preparation). Relocation of the bound toxin would most likely occur by lateral diffusion of the toxin-ganglioside complex within the plane of the membrane, since rotation through the membrane bilayer to the inner membrane surface may be energetically unfeasible for larger molecules (63). It is unlikely that toxin enters the cytoplasm in a soluble form; direct measurements of the intracellular level of ^{125}I-labeled choleragen in toad erythrocytes indicate that considerably less than 1% of bound toxin is present in cell lysates after a 90-min incubation at 30°C (5). It is also improbable that pinocytosis of bound choleragen is an obligatory step, since anucleate rat erythrocytes, which are incapable of pinocytosis (64), respond well to choleragen (4, 6) (Fig. 5).

Studies with Fluorescent Derivatives of Choleragen

Studies with fluorescein-labeled choleragen provide direct evidence for the possibility of redistribution of choleragen-receptor complexes on cell surfaces (reference 65, and S. Craig and P. Cuatrecasas, manuscript in preparation). Viable lymphocytes which are membrane stained with fluorescent cholera toxin at 0°C and then fixed exhibit a smooth ring fluorescence (Fig. 12a) and/or random micropatches when viewed under the fluorescence microscope. If the cells stained at 0°C are subsequently incubated for 20 min at 37°C, the smooth ring fluorescence redistributes into macropatches, polar caps with residual surface fluorescence, or small tight caps without detectable residual surface fluorescence (Fig. 12b, c). The multivalency of cholera toxin (see above) appears to be essential to its ability to redistribute membrane toxin receptors, since agents which act to reduce the effective valence of membrane-bound cholera toxin, e.g., anti-cholera toxin antibody, inhibit patching and redistribution very effectively. These observations suggest that cross-linking of toxin receptors may be a necessary step in the process of redistribution of membrane-bound choleragen.

Binding and redistribution of fluorescent cholera toxin can be observed using concentrations of fluorescent cholera toxin as low as 10^{-10} M, which is well within the physiological range for cholera toxin activation of adenylate cyclase in rat mesenteric lymph node lymphocytes. Thus the events described probably reflect the properties of the biologically relevant toxin receptors (G_{M1} ganglioside).

Proposed Mechanism of Action of Cholera Toxin

The proposed sequence of events in the action of choleragen involves first formation of an inert toxin-ganglioside complex which is converted to an active state by a time- and temperature-dependent process which requires a minimum of 20–30 min. The active form of membrane-bound choleragen diffuses laterally in the two-dimensional plane of

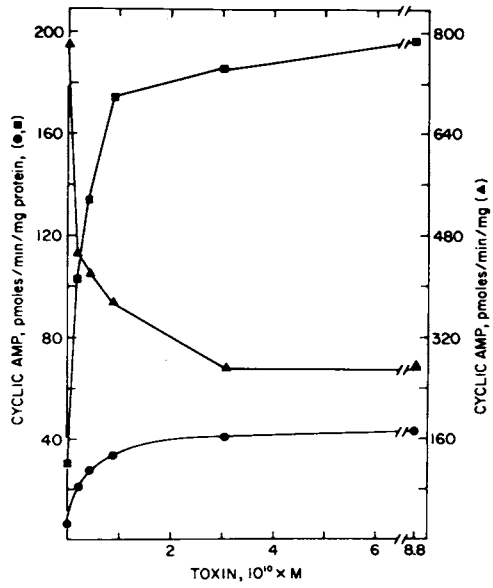

Fig. 10a. Effect of preincubating toad erythrocytes with increasing concentrations of cholera toxin on the adenylate cyclase activity of plasma membranes assayed with 20 μM (–) epinephrine (■), 20 mM sodium fluoride (▲), or with no additions (●). Cells were washed with amphibian Ringer's, pH 7.5, suspended in this buffer, and divided into six 45-ml portions, each containing 6×10^8 cells. Various concentrations of cholera toxin were added, and the cell suspensions were incubated for 4 hr at 30°C before preparation of the plasma membranes for assays of adenylate cyclase activity (5). Nearly all of the choleragen is membrane bound under the conditions employed in this experiment ($>10^7$ cells/ml, $10^{-11}-10^{-9}$ M choleragen) (4, 7). The amount of cell-bound toxin can be estimated by dividing the total number of toxin molecules by the number of erythrocytes: 10^{-10} M toxin = 2.7×10^{12} molecules/45 ml = 4,500 molecules/cell.

the plasma membrane and thus encounters and complexes with adenylate cyclase, resulting in activation of the enzyme. The possible interaction of these two molecules would depend on their diffusion properties within the matrix of the membrane. The collision frequency can be estimated once the diffusion constants and average intermolecular distances are known. Such calculations, for example, have been made for the rate of collision of rhodopsin molecules in the photoreceptor membrane (66). These steps may be viewed schematically:

1. Choleragen + membrane $\rightleftharpoons$ choleragen$_{I}$-membrane (inactive)
2. Choleragen$_{I}$-membrane $\rightharpoonup\!\!\rightharpoonup$ choleragen$_{II}$-membrane (active),
3. Choleragen$_{II}$-membrane + adenylate cyclase-membrane $\rightleftharpoons$ choleragen-adenylate cyclase-membrane.

This model is consistent with the kinetics and concentration-response relationships for the action of choleragen, and would explain the acute dependence on temperature of the activation process. The possibility of lateral motion of membrane-bound choleragen is suggested by the presence of huge numbers of functionally equivalent "spare" receptors, and has been demonstrated directly with fluorescent derivatives of choleragen (reference 65; S. Craig and P. Cuatrecasas, manuscript in preparation). Diffusion of choleragen within the plane of the membrane could explain the apparent paradox in the

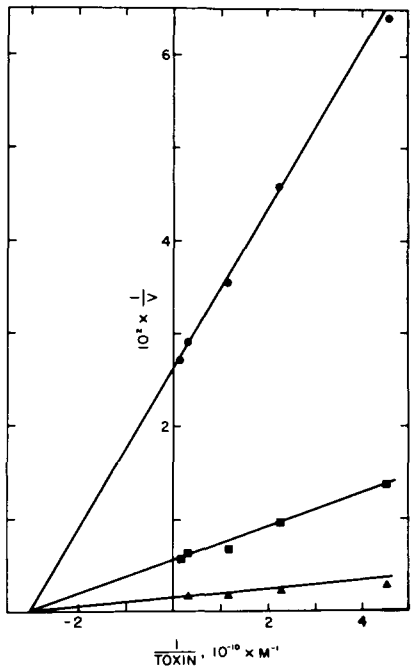

Fig. 10b. Double reciprocal plot of the data presented in Fig. 10a. The change in activity due to toxin was estimated by subtracting the control (untreated) values. Half-maximal activation occurs at approximately 3×10^{-11} M choleragen, which corresponds to about 1,500 molecules of choleragen bound per erythrocyte. From Bennett and Cuatrecasas (5).

intestine where toxin binding occurs initially on the brush borders (67), while adenylate cyclase activity is localized on the lateral and basal surfaces of the mucosal cells (68). Bound toxin may simply move in the plane of the membrane directly to the adenylate cyclase without having to penetrate these cells and traverse the cytoplasm. Estimates of the diffusion constants for membrane proteins (66, 69–71) suggest values in the range of 10^{-9}–10^{-10} cm^2/sec, which should be sufficient to explain the time course of activation of adenylate cyclase which continues over a period of 2–3 hr.

The basis for the absolute nature of the lag period is not understood at this time. This process is approximately zero order with respect to bound toxin, and is still observed after adding a second dose of toxin to partially stimulated cells (5). This behavior suggests that each toxin molecule may independently experience a similar interval between contact with the cell surface and conversion to an active form. On the basis of the extreme persistence of the toxin's biological effects (5, 36, 45, 46), and the irreversible binding of ^{125}I-labeled choleragen to cell membranes (38) it has been proposed (4, 5, 43) that during the lag phase, choleragen, or some portion of the molecule, may become incorporated into the phospholipid bilayer as an "integral" membrane protein (63) and that this is the biologically active form of the molecule. It is pertinent that the subunit (36,000 mol wt) which confers biological activity to choleragen (20, 22–25), has strongly hydrophobic properties[3] (20). According to this view, the lag period would represent the time required

[3] Unpublished data.

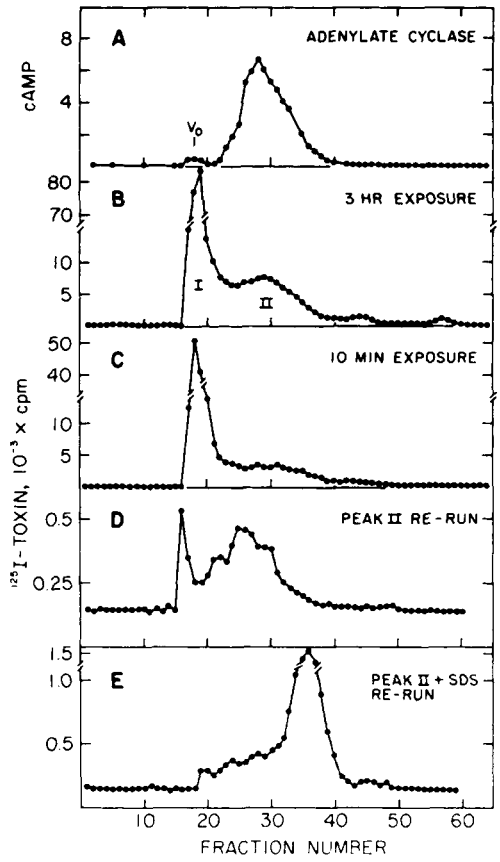

Fig. 11. Sepharose 6B chromatography of Lubrol PX-solubilized adenylate cyclase from fat cells incubated with ^{125}I-labeled cholera toxin. Cells from 25 rats (200 g) were incubated at 37°C for 3 hr. ^{125}I-Labeled cholera toxin (2.4 μg, 9.4 μCi/μg) was added either at the beginning of the incubation or for the final 10 min. Samples (0.75 ml) of the 250,000 × g membrane detergent extracts were applied to Sepharose 6B columns (1 cm × 57 cm; flow rate 10 ml/hr; 0.6 ml fractions) and cyclase activity (panel A) was determined. Panels A and B refer to cells exposed to toxin for 3 hr while panel C is for the 10-min exposure to ^{125}I-labeled toxin. The peak of cyclase in panel A (fractions 28–30) was rechromatographed with (panel E) or without (panel D) heating the sample in 1% sodium dodecyl sulfate (60 min at 40°C). From Bennett et al. (43).

for conversion of the initial, inactive toxin-ganglioside complex to a new state such that insertion of the toxin molecule into the membrane is favored. This process would occur spontaneously above certain critical temperatures, and may involve lateral mobility of the toxin-ganglioside complex within the plane of the membrane.

Analogies Between the Action of Choleragen and the Mode of Activation of Adenylate Cyclase by Hormones

The postulated lateral movement of membrane-bound choleragen and subsequent interaction with adenylate cyclase is similar to general features of the mobile receptor model recently proposed for the modulation of adenylate cyclase by hormones (43, 72, 73) which emphasizes the fluidity of cell membranes. It was suggested that hormone

receptors, which in their free state are considered to exist uncomplexed with the enzyme, are free to diffuse laterally along the plane of the membrane as has been observed with other "integral" membrane proteins (63). Modulation of adenylate cyclase activity would occur as the result of brief, direct encounters between the enzyme and hormone receptors within the membrane matrix. The dynamic interaction between cyclase and receptors would be analogous to the associations between molecules in three-dimensional aqueous solution, and could be described in terms of rates of association and dissociation. Productive collisions would presumably occur only if the receptors were complexed with the appropriate hormone. The following equilibria should exist:

1. $H + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} H \cdot R$

2. $H \cdot R + AC \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} H \cdot R \cdot AC$

3. $H \cdot R \cdot AC \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} H + R \cdot AC$

4. $H \cdot R \cdot AC \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} H \cdot R + AC$

5. $R + AC \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} R \cdot AC,$

where H refers to hormone, R to hormone receptor, and AC to adenylate cyclase. Choleragen may be visualized as functioning as an "active" hormone receptor which can stimulate adenylate cyclase in the absence of complexed hormone and which dissociates very slowly.

Several unique predictions follow from this scheme which cannot be encompassed within concepts of a static receptor-adenylate cyclase complex (43, 72, 73). For example, hormones may dissociate from free receptors at different rates than from the cyclase-receptor complex. It should therefore be possible to modify the binding properties of hormones by affecting the proportion of receptors complexed with adenylate cyclase without directly perturbing the hormone binding site of the receptor. Since enzyme activation involves two separate and sequential steps, $H + R \rightleftharpoons H \cdot R$ and $H \cdot R + AC \rightleftharpoons H \cdot R \cdot AC$, the apparent K_a for hormones and the extent of activation may not depend in a simple way on the binding properties of the hormone.

These considerations could explain the unusual findings that choleragen increases the extent of catecholamine stimulation in toad erythrocyte (4–6) (Fig. 4), the extent and affinity in turkey erythrocytes (3), and the apparent affinity for cyclase stimulation by a variety of hormones in mammalian cells (4, 6, 43, 47, 56) (Fig. 5). This phenomenon may reflect the stabilization of hormone-receptor-cyclase complexes by choleragen (3, 4, 47). The average residence time of each hormone-receptor complex would be lengthened resulting in an increased efficiency of activation, and perhaps an altered rate of dissociation of membrane-bound hormones. It is of interest in this regard that choleragen has recently been demonstrated to decrease the rate of spontaneous dissociation of specifically bound ^{125}I-labeled glucagon from liver membranes (43). A novel aspect of the mobile receptor theory and the proposed mechanism of action of choleragen is the concept of functionally

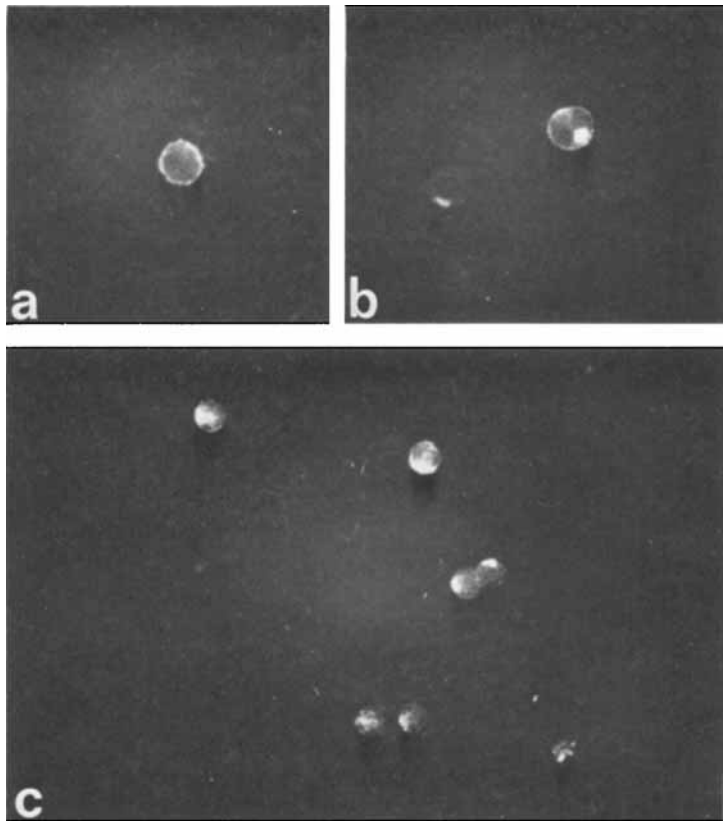

Fig. 12. Patterns of surface fluorescence on rat lymphocytes incubated in FITC-cholera toxin. (a) Lymphocytes were incubated at 0°C for 30 min; the fluorescence is randomly distributed on the surface of the cell. (b and c) Lymphocytes, stained with FITC-cholera toxin at 0°C for 30 min, were then incubated at 37°C for 20–30 min. Various patterns of FITC-cholera toxin redistribution on the membrane are observed.

important dynamic collisions and associations between membrane proteins. This has been postulated previously on the basis of estimates of the collision frequency between rhodopsin molecules in rod outer segment membranes (66).

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