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Mechanism of Activation of Adenylate Cyclase by *Vibrio cholerae* Enterotoxin*

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Summary. The kinetics and properties of the activation of adenylate cyclase by cholera enterotoxin have been examined primarily in toad erythrocytes, but also in avian erythrocytes, rat fat cells and cultured melanoma cells. When cholera toxin is incubated with intact cells it stimulates adenylate cyclase activity, as measured in the subsequently isolated plasma membranes, according to a triphasic time course. This consists of a true lag period of about 30 min, followed by a stage of exponentially increasing adenylate cyclase activity which continues for 110 to 130 min, and finally a period of slow activation which may extend as long as 30 hr in cultured melanoma cells. The progressive activation of adenylate cyclase activity by cholera toxin is interrupted by cell lysis; continued incubation of the isolated membranes under nearly identical conditions does not lead to further activation of the enzyme. The delay in the action of the toxin is not grossly dependent of the number of toxin-receptor (G_{M1} ganglioside) complexes, and is still seen upon adding a second dose of toxin to partially stimulated cells. Direct measurements indicate negligible intracellular levels of biologically active radioiodinated toxin in either a soluble or a nuclear-bound form. The effects are not prevented by Actinomycin D (20 μg/ml), puromycin (30 μg/ml), cycloheximide (30 μg/ml), sodium fluoride (10 mM) or sodium azide (1 mM); KCN, however, almost completely prevents the action of cholera toxin. The action of the toxin is temperature dependent, occurring at very slow or negligible rates below certain critical temperatures, the values of which depend on the specific animal species. The transition for toad erythrocytes occurs at 15 to 17 °C, while rat adipocytes and turkey erythrocytes demonstrate a discontinuity at 26 to 30 °C. The temperature effects are evident during the lag period as well as during the exponential phase of activation. The rate of decay of the stimulated adenylate cyclase activity of cultured melanoma cells indicates a half-time of about 36 hr. The rate of exponentially increasing activity and extent of enzyme activation are related to the number of bound toxin molecules according to a Langmuir adsorption isotherm and are half-maximal when about 2000 molecules of toxin are bound per cell. It is proposed that initially cholera toxin binds ineffectively, but that it is converted to an active form during the lag phase. This process may involve lateral motion of a toxin-G_{M1} ganglioside complex within the plane of the membrane. The kinetics of adenylate

* Part of this work was reported at the 1974 meeting of the Federation of American Societies for Experimental Biology (Bennett & Cuatrecasas, 1974).

cyclase activation are consistent with the possibility that during the exponential phase a bimolecular association is proceeding between the active form of the cholera toxin and some other membrane component. The possibility is considered that the cholera toxin molecule may bind directly to adenylate cyclase. These considerations may prove useful in understanding the possible interactions of active hormone-receptor complexes with adenylate cyclase in cell membranes.

Cholera enterotoxin, a protein secreted by *Vibrio cholerae*, binds specifically and with very high affinity to cell membranes (Cuatrecasas, 1973*a–d*) and it stimulates adenylate cyclase activity in a variety of tissues (reviewed by Finkelstein, 1973; Sharp, 1973). Certain features of the activation of adenylate cyclase by cholera toxin are very different from those observed with other types of modulation of this enzyme. The toxin must be incubated with intact cells to stimulate the enzyme effectively (Kimberg *et al.*, 1971; Wolff *et al.*, 1973), although once activated the effects may persist for hours after cell lysis. A delay of 30–60 min between the addition of the toxin and the appearance of biological effects has been observed in many systems, including fluid secretion in small bowel loops (Carpenter *et al.*, 1968; Carpenter & Greenough, 1968; Pierce *et al.*, 1971*a*, *b*), lipolysis in isolated rat adipocytes (Vaughan *et al.*, 1970; Cuatrecasas, 1973*c*) and steroid secretion in cultured mouse adrenal cells (Wolff *et al.*, 1973; Donta *et al.*, 1973). A similar lag period has also been reported for stimulation of adenylate cyclase activity in intestinal mucosa (Kimberg *et al.*, 1971; Guerrant *et al.*, 1972) and melanoma cells (O'Keefe & Cuatrecasas, 1974). Once toxin stimulation of adenylate cyclase occurs, it persists with extraordinary tenacity; effects have been observed up to thirteen days after a single, brief, initial exposure in cultured mouse melanoma cells (O'Keefe & Cuatrecasas, 1974).

Despite these unusual features, significant similarities exist between the effects of cholera toxin and the action of hormones which activate adenylate cyclase. Insulin, which inhibits the lipolytic response of rat fat cells to certain hormones, is also effective in preventing or reversing the stimulation of lipolysis by cholera toxin (Vaughan *et al.*, 1970; Cuatrecasas, 1973*c*). We have recently demonstrated that cholera toxin alters the kinetic and regulatory properties of adenylate cyclase in toad erythrocytes in a manner similar to that observed with catecholamine hormones (Bennett & Cuatrecasas, 1974, 1975). These results suggest that certain aspects of cholera toxin action may resemble hormonal modulation of this enzyme, and that the mechanism of action of the toxin may help in understanding the molecular basis of hormonal regulation of adenylate cyclase.

Experimental Procedures

Cholera toxin (lot #1071), purified by the method of Finkelstein and LoSpalluto (1970), was obtained from Dr. C. E. Miller, SEATO Cholera Research Program. Choleragenoid (lot #G0673) was kindly provided by Dr. R. A. Finkelstein. Staphylococcal nuclease (6000 U/mg) was obtained from Worthington.

[α-^{32}P]ATP (20–60 Ci/mmole) was synthesized by the method of Symons (Symons, 1968; Flawia & Torres, 1972) and purified as described previously (Bennett & Cuatrecasas, 1975). Toad erythrocyte plasma membranes were prepared as described previously (Bennett & Cuatrecasas, 1975).

Adenylate cyclase assays were conducted as described elsewhere (Bennett & Cuatrecasas, 1975) using [α-^{32}P]ATP (60–100 cpm/pmole) and isolating cyclic AMP on neutral alumina columns (Ramachandran, 1971; White & Zenser, 1971). The recovery of [^{3}H]cAMP averaged 75–80%, and was determined for each sample. Assay blanks (boiled membranes) usually were less than 50 cpm per 10^6 cpm of the total radioactivity; basal (unstimulated) activities were at least 500 cpm above blank values. Except for a short, initial lag period, enzyme activity was linear (zero order kinetics) (Bennett & Cuatrecasas, 1975). Membrane protein was estimated by the method of Lowry *et al.* (1951) after heating the samples for 40 min at 100 °C with 4 N NaOH. Bovine serum albumin was used as the standard.

The iodination of cholera toxin was performed as described previously (Cuatrecasas, 1973*c*), using a modification (Cuatrecasas, 1971) of the method of Hunter and Greenwood (1962). The molecular weight and $A^{1\%}_{1\,cm}$ (280) of cholera toxin were assumed to be 90,000 and 11.4, respectively (LoSpalluto & Finkelstein, 1972; Cuatrecasas *et al.*, 1973).

Results

Time Course of Adenylate Cyclase Activation by Cholera Toxin

Preincubation of intact toad erythrocytes with cholera toxin alters the properties of adenylate cyclase of membranes (Bennett & Cuatrecasas, 1974, 1975). The basal enzyme activity is stimulated three- to fivefold, the response to catecholamines is doubled, and the stimulation of the enzyme by NaF is drastically reduced. The time course of toxin activation shows that the changes in these three parameters occur in parallel (Fig. 1). The time course is complicated, exhibiting three distinct phases: a lag period of at least 30 min, a subsequent stage of rapid change which continues for about 2 hr, and finally a period of slowly increasing activation which continues for many hours. The effects of cholera toxin do not result simply from prolonged *in vivo* activation of adenylate cyclase, since preincubation of cells with (−)-epinephrine does not elicit these changes (Bennett & Cuatrecasas, 1975). It is unlikely that the toxin acts by "stabilizing" or preventing the loss of adenylate cyclase during the preincubation period, since incubation of erythrocytes under these conditions for as long as 5 hr does not significantly alter the basal activity, hormone sensitivity, or NaF stimulation.

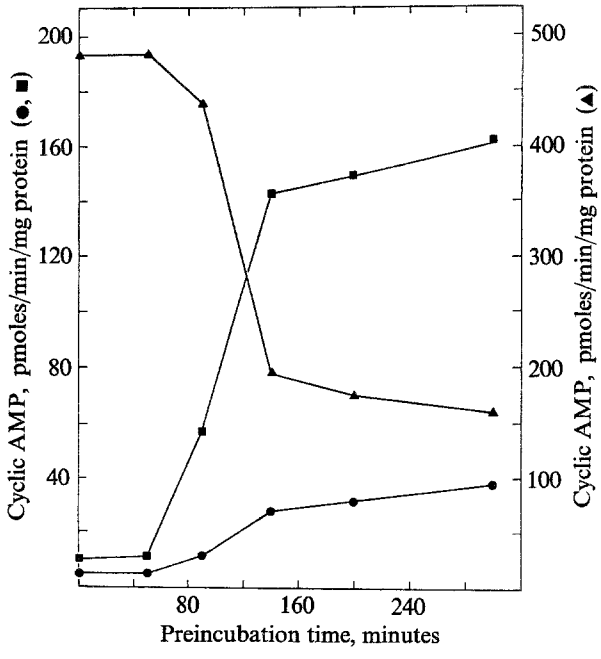

Fig. 1. Effect of increasing the time of incubation of cholera toxin with intact toad erythrocytes on the adenylate cyclase activity of plasma membranes assayed with 20 μM (−)-epinephrine (■), 20 mM sodium fluoride (▲), or with no additions (●). The cells were thoroughly washed with amphibian-Ringer's, 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 (zero incubation time) received no toxin. After 5 hr, the cells were rapidly chilled, and the plasma membrane adenylate cyclase activity determined (*see* Experimental Procedure). All values were determined in triplicate

Cholera toxin must be incubated with intact cells in order to observe any alterations in adenylate cyclase activity (Bennett & Cuatrecasas, *unpublished observation*). The progressive development of the toxin effects, depicted in Fig. 1, can be arrested at any time by breaking the cells; no further effects occur if the incubation period is continued with the broken cells or isolated membranes (Table 1). This is not explained by inactivation of adenylate cyclase, since the response to (−)-epinephrine and to NaF is relatively unaffected even after incubating the membranes for 60 min at 28 °C (Table 1).

The feasibility of interrupting the process of adenylate cyclase activation permitted a more detailed analysis of the relationship of activation to the time of incubation of the toxin with the intact cells. During the initial period of rapid change the enzyme activity increases in an exponential manner in rat fat cells as well as in toad erythrocytes (Fig. 2). A semi-log plot of adeny-

Table 1. The effect of cell lysis on the progression of the cholera toxin effects on adenylate cyclase activity of toad erythrocytes [a]

Cell treatment	Adenylate cyclase activity [b]		
	No additions	(−)-epinephrine (20 μM)	NaF (20 mM)
110 min at 28°; lysed, and incubated 60 min at 0°			
control	0.8	2.5	167.0
cholera toxin	4.8	13.6	103.9
110 min at 28°; lysed, and incubated 60 min at 28°			
control	0.6	1.1	112.3
cholera toxin	4.2	7.1	56.6
180 min at 28°; lysed, and assayed at once			
control	2.0	3.1	244.4
cholera toxin	26.6	55.5	113.1

[a] Erythrocytes were washed, suspended in amphibian-Ringer's, pH 7.5, and divided into six 30-ml portions. Cholera toxin (15 μg) was added to three of these. Some cells were incubated at 28 °C for 110 min, followed by lysis and continued incubation of the membranes for 60 min at 0 or at 28°. Other cells were incubated for 180 min at 28°, followed by lysis and immediate assay. Lysis was achieved by rapidly adding 20 ml of distilled water to 0.5 ml of packed cells, followed in 60 sec by 20 ml of amphibian Ringer's, pH 7.5, and 20 μg of staphylococcal nuclease. Plasma membrane adenylate cyclase activity was measured (*see* Experimental Procedure) in the presence of $MgCl_2$ (3.5 mM), GTP (1 mM), [α-^{32}P]ATP (1.2 mM, 63 cpm/pmole), aminophylline (5 mM), phosphoenolpyruvate (5 mM), pyruvate kinase (50 μg/ml), Tris-HCl (50 mM, pH 8.0), and 150–200 μg of membrane protein.

[b] pmoles cAMP/min/mg of protein; mean of triplicate determinations.

late cyclase activity *vs.* time of incubation permits, by interpolation, an estimate of the length of the lag phase, and clearly demonstrates the absolute nature of this delay. Rat fat cells and toad erythrocytes exhibit nearly identical lag periods of about 30 min.

Effect of Cholera Toxin Concentration and Amount of Cell-Bound Toxin on the Activation of Adenylate Cyclase

The activation of toad erythrocyte adenylate cyclase, after exposure for 4 hr at 30 °C, varies with the concentration of cholera toxin according to a simple hyperbolic relationship (Fig. 3). The process exhibits a very high apparent affinity; half-maximal effects occur at about 3×10^{-11} M toxin for

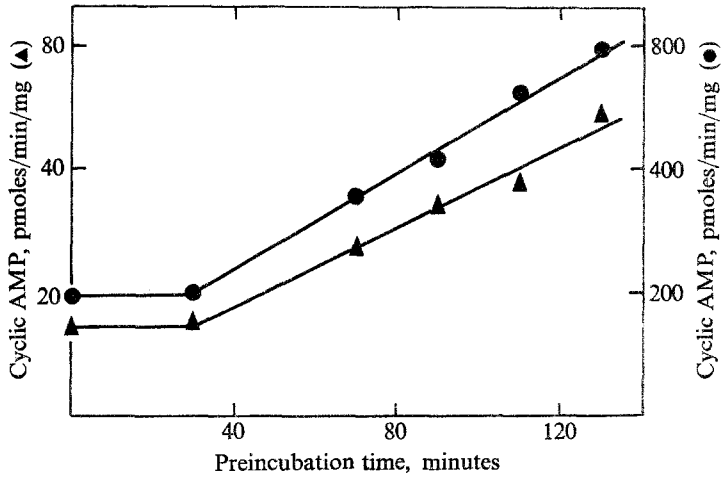

Fig. 2. Effect of increasing the time of cholera toxin preincubation with intact toad erythrocytes (▲) and rat adipocytes (●) on the plasma membrane adenylate cyclase activity. The experiment with toad erythrocytes was performed as described in Fig. 1. Adipocytes were isolated from the epididymal fat pads of eight 100–200 g rats by the method of Rodbell (1964), 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 in cold Tris-HCl (50 mM, pH 7.6) with a Brinkman Polytron (30 sec at a setting of 3.0). The suspensions were centrifuged at 40,000 $\times g$ 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 (*see* Experimental Procedure) 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. 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 plotted as the log of enzyme activity *vs.* time

activation of basal and (−)-epinephrine-stimulated activities, as well as for the inhibition of the enzyme response by NaF.

More than 90% of the ^{125}I-labeled cholera toxin in the medium binds to toad erythrocytes under the conditions (10^7 cells per ml and 10^{-11} to 10^{-9} M toxin) employed in these experiments (Cuatrecasas, 1973*a*; Bennett & Cuatrecasas, 1975). The concentration-response relationship therefore cannot be interpreted as reflecting a true affinity of the initial cholera toxin-membrane interaction. At high cell densities (greater than 10^6 cells per ml), the correct variable is not the number of molecules of toxin per liter of solution, but the number of molecules of toxin bound per erythrocyte.

The influence of the amount of toxin bound per cell on the time course of adenylate cyclase activation was determined (Fig. 4*a*, *b*). The length of

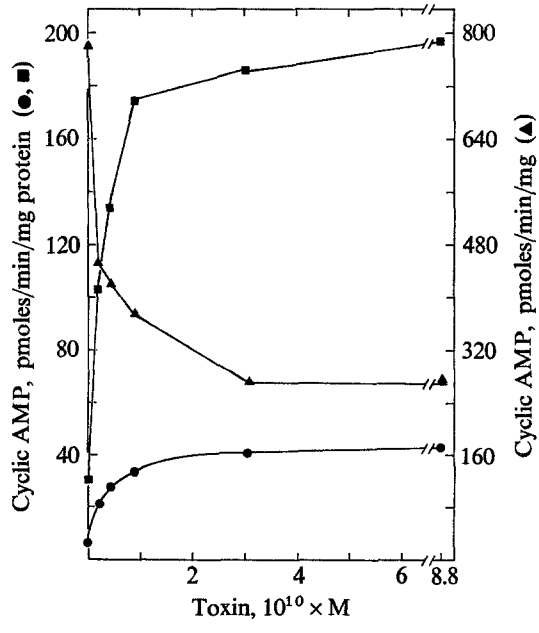

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Fig. 3. (*a*) Effect of preincubating toad erythrocytes with increasing concentrations of cholera toxin on the adenylate cyclase activity of plasma membranes assayed with 20 μM (−)-epinephrine (■), 20 mM sodium fluoride (▲) or with no additions (●). 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 (*see* Experimental Procedure). (*b*) Double-reciprocal plot of the data presented in Fig. 3*a*. The change in activity due to toxin was estimated by subtracting the control (untreated) values

the lag period does not vary, within the experimental error of 10–20 min, with increasing concentrations of cholera toxin. However, the rate of exponential change of enzyme activity clearly increases as a function of bound cholera toxin. A number which is proportional to the rate constant for the activation process can be estimated from the slope of the semi-log plot of adenylate cyclase activity *vs.* time (Fig. 4*b*). The rate constant appears to be related to the number of molecules of toxin bound per cell according to a Langmuir adsorption isotherm. Half-maximal activation is observed when about 2,200 molecules of toxin are bound per cell. This number is comparable to the apparent K_a of 1 500 molecules per cell obtained in Fig. 3 under near-equilibrium conditions.

The invariance of the length of the lag phase with cholera toxin concentration has been observed previously (Cuatrecasas, 1973*c*), and suggests

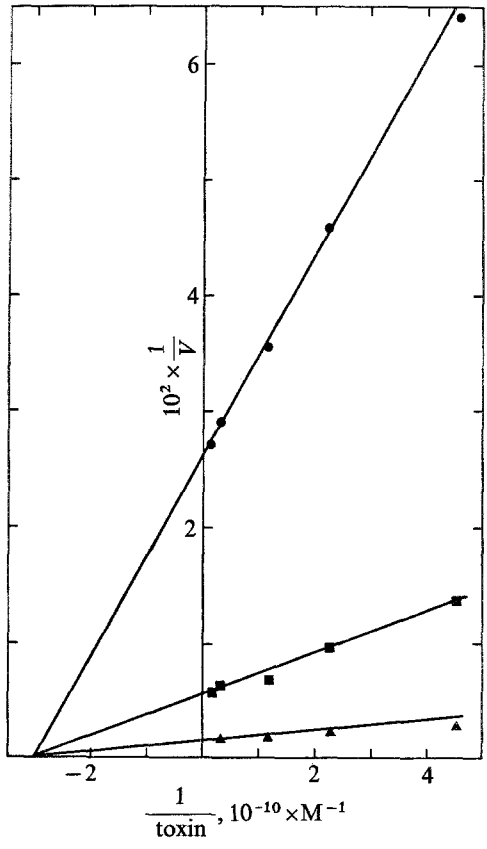

Fig. 3b

that each toxin molecule may independently experience a similar interval between contact with the cell surface and conversion to an active form. Thus, stimulation should exhibit a similar kind of latent period even if a second dose of toxin is added after a certain proportion of the cell adenylate cyclase has previously been activated by low (submaximal) concentrations of the toxin. Indeed, if high concentrations of cholera toxin are added to cells previously incubated for 147 or 240 min with submaximal concentrations of the toxin, the subsequent response to the second addition of the toxin still displays a lag period (Table 2). Virtually no effect is detected after incubating the prestimulated cells with high concentrations of the toxin for 20 min. These erythrocytes are not inherently resistant to further toxin stimulation since continued incubation with the higher toxin concentrations for longer (110 min) periods results in a small, but significant stimulation of enzyme activity (Table 2).

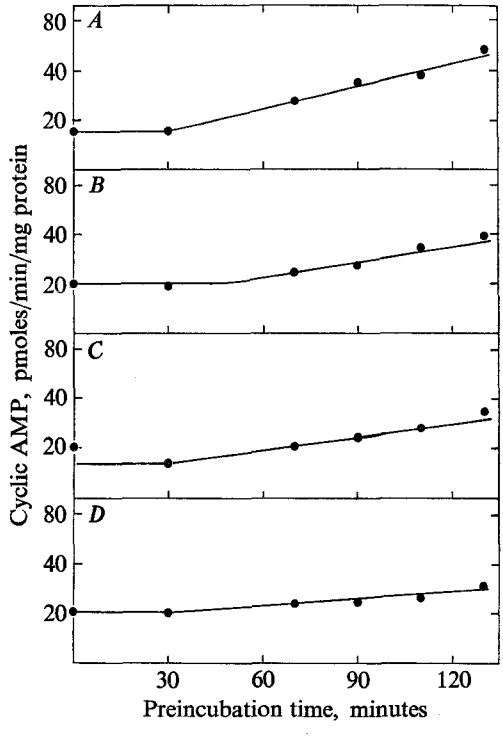

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Fig. 4. (*a*) The influence of increasing the concentration of cholera toxin on the time course of toad erythrocyte adenylate cyclase activation. Washed erythrocytes were suspended in amphibian-Ringer's and divided into twenty-four 10-ml portions, each containing 6×10^8 cells. The time course was examined as described in Fig. 1, at fixed concentrations of cholera toxin: 1.54×10^{-9} M (panel *A*), 4.4×10^{-10} M (panel *B*), 2.2×10^{-10} M (panel *C*) and 1.1×10^{-10} M (panel *D*). Adenylate cyclase activity was measured (*see* Experimental Procedure) in the presence of 20 μM (−)-epinephrine. The values were determined in triplicate, and are plotted as the log of enzyme activity *vs.* time. (*b*) Double-reciprocal plot of the slope of the semi-log plots determined from Fig. 4*a*. (*K*) *vs.* the number of molecules of cholera toxin bound per cell. The slopes (relative to panel *D*) were: panel *D*, 1.00; panel *C*, 1.7.; panel *B*, 2.05; panel *A*, 2.79, and the estimated number of molecules/cell was 1,104 (panel *D*), 2,207 (panel *C*), 4,415 (panel *B*), and 15,451 (panel *A*). Standard errors in these experiments varied between 3 and 7%

Temperature Dependence of Cholera Toxin Activation of Adenylate Cyclase

The effect of temperature on the cholera toxin-induced modification of toad erythrocyte adenylate cyclase was examined during a 4-hr incubation period (Fig. 5). Below 16 °C virtually no effect of the toxin is observed, while above this temperature the process occurs readily and in a highly temperature-dependent fashion. The increase in basal enzyme activity is

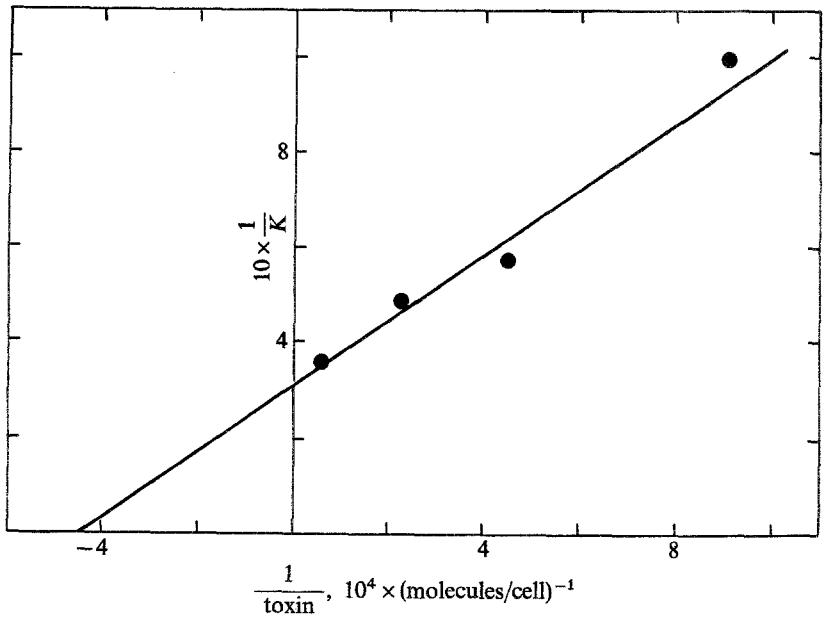

Fig. 4b

Table 2. Occurrence of a lag period in adenylate cyclase activation upon addition of cholera toxin to cells partially stimulated by the toxin [a]

Cell treatment	Adenylate cyclase activity [b]		
	No additions	(−)-epinephrine (20 μM)	NaF (20 mM)
No additions	8±1.2	19±1.2	1,287±108
No additions for 93 min, followed by low toxin (10 ng/ml) for 147 min	14±0.4	52±3	1,142±27
No additions for 93 min, followed by low toxin (10 ng/ml) for 127 min, followed by high toxin (220 ng/ml) for 20 min	14±0.4	56±2	1,220±24
Low toxin (10 ng/ml) for 240 min	18±0.5	83±6	1,020±20
Low toxin (10 ng/ml) for 130 min, followed by high toxin (220 ng/ml) for 110 min	20±1.6	104±3	905±49
No additions for 130 min, followed by high toxin (220 ng/ml) for 110 min	19±1	88±2	1,168±24

[a] Toad erythrocytes were washed, suspended in amphibian-Ringer's, pH 7.5, and divided into six 20-ml portions, each containing 9×10^8 cells. These were incubated (at 30 °C) as indicated, in the absence and presence of low or high concentrations of cholera toxin followed by a subsequent addition of a different concentration of toxin. In all cases the total time of incubation of the cells was 240 min at 30 °C. The cells were chilled, plasma membranes prepared, and adenylate cyclase activity measured as described under Experimental Procedure.

[b] pmoles cAMP/min/mg of protein; mean of triplicate determinations.

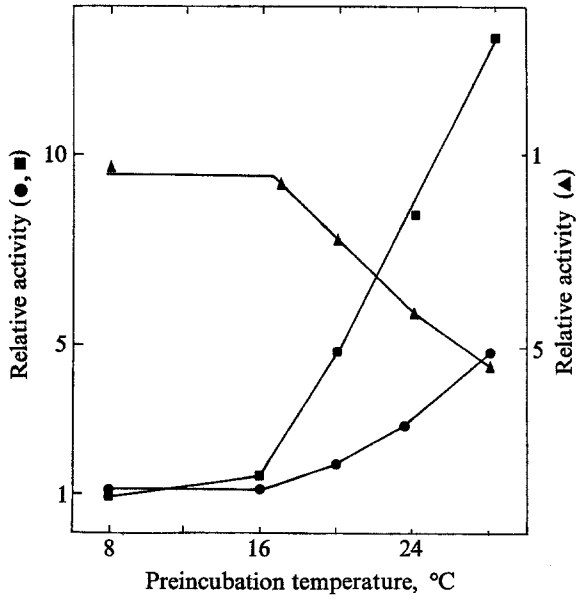

Fig. 5. Temperature dependence of the effects of cholera toxin on toad erythrocyte adenylate cyclase activity assayed either in the presence of 20 μM (−)-epinephrine (■), 20 mM sodium fluoride (▲), or with no additions (●). Washed erythrocytes were suspended in amphibian-Ringer's, pH 7.5, at 0 °C, and divided into six 30-ml portions, each containing about 6×10^8 cells. Cholera toxin (5.6 μg) was added to 5 samples, and after 20 min at 0 °C, these were placed in water baths maintained at various temperatures. One sample received no toxin and was incubated at 20 °C. After 4 hr, the cells were chilled to 0 °C and the plasma membrane adenylate cyclase activity determined. The values were determined in triplicate, and are expressed as the enzyme activity relative to a control sample which had been preincubated at 20 °C in the absence of cholera toxin

paralleled closely by the enhancement of the (−)-epinephrine response and by the inhibition of NaF stimulation.

If the dependence on temperature were confined solely to processes occurring during the lag phase, pre-exposure of the cells to toxin for 35 min at 30 °C should abolish the discontinuity at 16 °C. However, if this temperature requirement were limited to some later phase, incubation for 35 min at 30 °C after exposure of the cells to cholera toxin for 4 hr at various controlled temperatures should alter the described pattern of temperature dependence. Experiments of this type (Fig. 6) indicate the discontinuity at 16 °C remains, whether the temperature is elevated before or after incubation at controlled temperatures for 4 hr. The process occurring during the lag period thus appears to have a sharply defined temperature dependence, and, once the lag phase has passed, the rate of enzyme activation during the exponential phase is also affected by temperature.

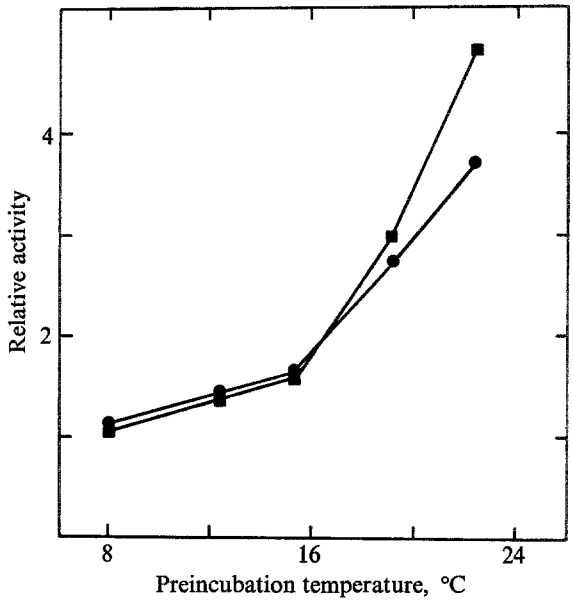

Fig. 6. Effect on adenylate cyclase activity of exposing toad erythrocytes to cholera toxin for 35 min at 30 °C before (●) and after (■) incubation of these cells with the toxin at various temperatures. Washed erythrocytes were suspended in amphibian Ringer's, pH 7.5, and divided into twelve 30-ml portions, each containing about 5×10^8 cells. The effect of preincubating with cholera toxin at various temperatures was determined as described in Fig. 5 except that one group of cells was placed in a 30 °C bath for 35 min before (●) the incubation period at the indicated temperature while the other group (■) was treated similarly after incubating at the indicated temperature. All the samples were incubated for a total time of 4 hr. The samples were assayed in the presence of 20 μM (−)-epinephrine. The values were determined in triplicate, and are expressed as the enzyme activity relative to control samples which were incubated in exactly the same way but in the absence of cholera toxin

Cells from other species also exhibit a sharp increase in the extent of adenylate cyclase activation by cholera toxin above given critical temperatures (Fig. 7). Rat adipocytes and turkey erythrocytes demonstrate an apparent temperature break between 26 and 30 °C, or about 12° higher than is observed with the toad erythrocytes. It is significant that the ambient temperature of rats and turkeys is also higher than that of amphibians.

Effect of Metabolic Inhibitors

The possible involvement of cytoplasmic events in the action of cholera toxin can be examined by the use of specific inhibitors. Very high concentrations of Actinomycin D, cycloheximide and puromycin do not prevent the

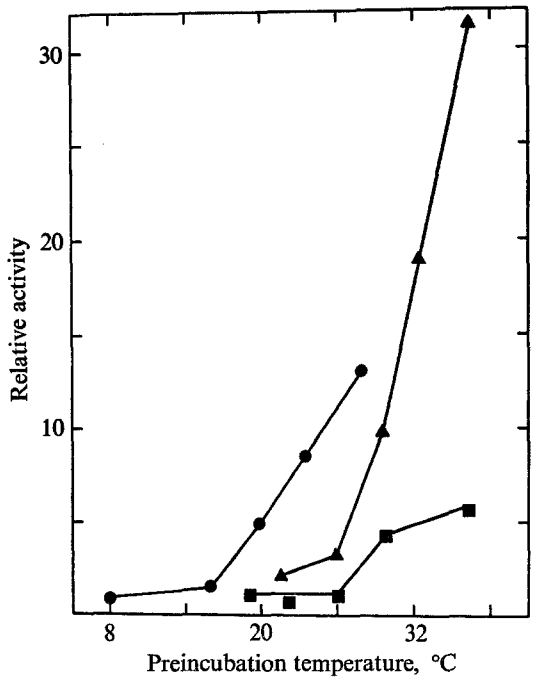

Fig. 7. Temperature dependence of the activation by cholera toxin of the adenylate cyclase activity of toad erythrocytes (●), turkey erythrocytes (■), and rat adipocytes (▲). The experiment with toad erythrocytes was performed as described in Fig. 5. Turkey erythrocytes were washed in Krebs-Ringer's bicarbonate, pH 7.4, and incubated at various temperatures with cholera toxin (0.3 μg/ml) for 4 hr. The plasma membrane adenylate cyclase activity was determined (*see* Experimental Procedures) under identical conditions as with the toad enzyme except that the concentration of ATP was 1.2 mM. Rat fat cells were prepared and incubated for 135 min at various temperatures with cholera toxin (0.2 μg/ml), as described in Fig. 2. Adenylate cyclase activity was measured at 33 °C using 0.25 mM ATP. The values were determined in triplicate, and are expressed as the enzyme activity relative to control values (cells incubated under identical conditions but in the absence of cholera toxin)

activation of toad erythrocyte adenylate cyclase by cholera toxin (Table 3). The toxin is also quite effective in the presence of 10 mM NaF and 1 mM sodium azide. However, cyanide at 1 mM inhibits the process almost completely. The effects of cholera toxin can be obtained with simple buffer systems, indicating that such effects do not depend on the presence of nutrients such as glucose or amino acids, or of macromolecules such as albumin. Moreover, toad erythrocytes which have been thoroughly washed and stored at 4 °C for 2 days still respond to the toxin when incubated in a simple Ringer's solution.

Table 3. The effect of metabolic and protein synthesis inhibitors on the stimulation of toad erythrocyte adenylate cyclase by cholera toxin [a]

Cell treatment	Adenylate cyclase activity [b]	
	No addition	(−)-epinephrine (20 μM)
No additions		
control	3.4	4.3
toxin	8.3	26.7
Actinomycin D, 20 μg/ml		
control	3.8	4.7
toxin	6.9	20.6
Cycloheximide, 30 μg/ml		
control	3.0	3.8
toxin	8.4	27.1
Puromycin, 30 μg/ml		
control	2.8	3.3
toxin	9.1	28.2
NaF, 20 mM		
control	6.4	7.7
toxin	7.0	17.0
KCN, 1 mM (pH 7.2)		
control	3.9	4.7
toxin	3.8	5.6
NaN_3, 1 mM (pH 6.9)		
control	3.1	3.8
toxin	6.6	22.1

[a] Cells were washed, suspended in amphibian-Ringer's, pH 7.5, and divided into 10-ml portions. Various inhibitors, at the concentrations indicated, were added to the samples, and these were incubated for 15 min at 30 °C. Cholera toxin (1.1 μg) was added to some of the samples, and the incubation was continued for another 130 min.
[b] pmoles cAMP/min/mg of protein; mean of triplicate determinations.

Possible Entry of [^{125}I]-Cholera Toxin into the Cytoplasm of Toad Erythrocytes

^{125}I-Labeled cholera toxin enters toad erythrocytes at a nearly negligible rate at 30 °C (Table 4). Less than 100 molecules per cell are not membrane-bound after a 90-min incubation at 30 °C under conditions where about 6,000 molecules are bound to the cell membrane. Some of the free molecules represent toxin which has dissociated into the medium during incubation and cell lysis (Table 4). The remainder is present in a soluble form in the cytoplasm. The true intracellular level of toxin must therefore be very much less than 100 molecules per cell, which is the upper limit estimated

Table 4. Possible entry of [^{125}I]cholera toxin into the cytoplasm of toad erythrocytes [a]

Incubation time (min)	% of cell-bound [^{125}I]cholera toxin		Molecules of [^{125}I]toxin per cell [b]	
	lysate [c]	medium [d]	lysate [c]	medium [d]
10	0.1		6	
20	0.3		17	
30	1.8		103	
45	0.8	0.6	46	34
60	0.6		34	
90	1.5	1.3	85	74

[a] Cells were washed and suspended in 5 ml of amphibian-Ringer's, 0.5% albumin (w/v), pH 7.5, at a concentration of 4.6×10^7 cells/ml. [^{125}I]Cholera toxin (0.22 µg, 11.8 µCi/µg) was added, and the suspension was incubated for 30 min at 0 °C. The cells were washed twice, suspended in the same buffer (10 ml), and placed in a 30° bath. The entry of bound [^{125}I]toxin into the cells was assayed at various times by filtering an aliquot of cells (0.2 ml, 0.4 µCi of bound [^{125}I]toxin) under negative pressure across an EG cellulose acetate (Millipore) membrane (0.25 µm pore size), which was then washed with ice-cold amphibian-Ringer's, 0.1% BSA (w/v), pH 7.5 (2.0 ml). The filtrate, which contained over 90% of the hemoglobin, and the filter membrane, were assayed for ^{125}I in a well-type gamma counter operating at 45% efficiency. Dissociation of bound [^{125}I]toxin into the medium was estimated independently by centrifugation of samples of the erythrocytes through an oil phase (dibutyl phthalate, dinonyl phthalate (2:1 v/v) with a Beckman microfuge (Chang & Cuatrecasas, 1974); the radioactivity in the resulting cell pellet and supernatant was thus determined separately. The values for entry (lysate) and dissociation (medium) were determined in triplicate, and are expressed as the percent of the total radioactivity, corrected for the value at zero time.

[b] 5,700 Molecules of ^{125}I-labeled cholera toxin were bound per cell initially.

[c] Uncorrected for [^{125}I]toxin which has spontaneously dissociated into the medium during incubation or cell lysis; these data thus express a maximum and clearly exaggerated figure for the possible entrance of toxin into the cell.

[d] Estimate of amount of [^{125}I]toxin which has dissociated from the cells during the incubation period.

here. It is possible that cholera toxin enters the cytoplasm and then binds avidly to the nucleus or some other particulate cell component. Although cholera toxin can bind to nuclei (Chang *et al.*, 1974), a nuclear site of cholera toxin action can readily be excluded since rat erythrocytes, which do not have nuclei, respond very well to cholera toxin (Bennett & Cuatrecasas, 1975). These findings are in agreement with previous studies on the localization of cholera toxin in fat cells (Chang *et al.*, 1974) and intestinal mucosa (Peterson *et al.*, 1972).

Duration of the Effect of Cholera Toxin

It was of interest to determine the rate of decay of the toxin-activated adenylate cyclase activity in a cultured cell system where cell division can

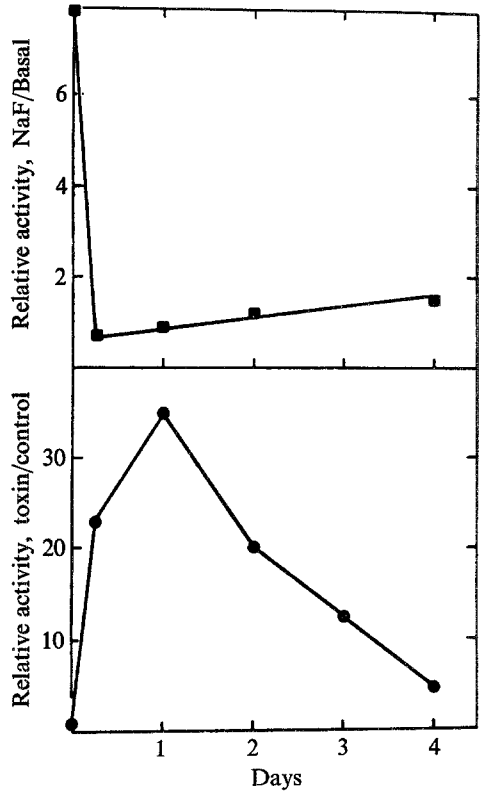

Fig. 8. Duration of the cholera toxin-induced stimulation of adenylate cyclase activity of cultured melanoma cells. Confluent monolayers (2×10^7 cells/flask), exposed to 10^{-10} M cholera toxin for 15 min on day zero only, were harvested after 6 hr and on each successive day for 4 days. Serum (10%) was added after addition of the toxin. The cell pellets were suspended in 10 ml of Tris-HCl (5 mM, pH 8.0), $CaCl_2$ (0.2 mM) $MgCl_2$ (0.2 mM) and staphylococcal nuclease (2 μg/ml) at 0 °C, homogenized for 30 sec with a Brinkman polytron (setting 3.0), centrifuged (30 min, 40,000 × *g* at 0 °C) and resuspended in Tris-HCl (50 mM, pH 8.0). Adenylate cyclase activity was determined exactly as described for toad erythrocyte membranes, except that the ATP concentration was 0.25 mM. The values were determined in triplicate and are expressed as either the activity of toxin-treated membranes relative to that of control cells incubated in parallel in the absence of toxin (●), or as the relative stimulation of toxin-treated membranes by 10 mM sodium fluoride (■)

be controlled. In cultured melanoma cells (O'Keefe & Cuatrecasas, 1974), cholera toxin induces a 30-fold activation of basal adenylate cyclase activity and concomitantly prevents the stimulation of this enzyme by NaF (Fig. 8). After a brief (15 min) exposure to cholera toxin, the stimulation of adenylate cyclase activity reaches a maximum value at about 30 hr. The activity then falls in an exponential manner with a half-life of about 36 hr. The NaF

response of the toxin-treated cells remains markedly diminished even when the stimulation of basal activity has almost disappeared, indicating that the toxin effect on adenylate cyclase is still pronounced at this time.

Discussion

Incubation of intact cells with cholera toxin has recently been demonstrated to induce profound and nearly permanent changes in the properties of adenylate cyclase through a mechanism which resembles that of hormonal stimulation (Bennett & Cuatrecasas, 1974, 1975). Furthermore, the toxin potentiates the normal hormone response of adenylate cyclase. The events which transpire between the binding of the toxin to specific membrane receptors, G_{M1} gangliosides (Cuatrecasas, 1973*b*; Holmgren *et al.*, 1973; King & van Heyningen, 1973; Hollenberg *et al.*, 1974; van Heyningen, 1974), and the activation of adenylate cyclase activity are not understood. Little has been described concerning such basic parameters as the relationship of adenylate cyclase activation to the toxin concentration, and the kinetics of this activation process have not been reported. Although the delay in the action of cholera toxin is well recognized, it is not known whether this lag is due to a slowly accelerating process or whether it reflects an absolute delay. The present studies have examined in detail the dependence of toxin action on the time of incubation, concentration of cholera toxin and temperature.

A unique aspect of the activation of adenylate cyclase is the persistence of the stimulation following cell lysis. It has been demonstrated (Bennett & Cuatrecasas, 1975) that the toxin effect remains unchanged during the period of adenylate cyclase assay using membranes isolated from stimulated cells. Continuation of the process of enzyme activation by cholera toxin can be halted by cell lysis even during the most rapid phase of activation (Table 1). This behavior differs from hormonal activation of adenylate cyclase, since effects can be obtained directly in broken cells and since the effects disappear almost immediately after dilution of the hormone (reviewed by Birnbaumer, 1973).

The adenylate cyclase activity of toad erythrocytes is modified by cholera toxin according to a triphasic time course (Fig. 1). A lag period of about 30 min is followed by an exponential increase in enzyme activity which continues for about 2 hr. A slowly increasing rate of activation then ensues, which may continue as long as 30 hr in certain cultured cells, such as melanoma cells (Fig. 8). The time course of stimulation of the basal enzyme

activity is paralleled nearly exactly by the accentuation of the catecholamine response and by the inhibition of stimulation by NaF. These three effects of cholera toxin also change in unison in dose-response curves (Fig. 3) and in temperature dependence studies (Fig. 5). Semi-log plots of adenylate cyclase activity versus time of incubation extrapolate to a starting time for the activation process of about 30 min after addition of the toxin in toad erythrocytes and rat fat cells (Fig. 2). Direct measurements of the interaction of ^{125}I-labeled cholera toxin with cell membranes (Cuatrecasas, 1973*a*) have demonstrated that above 20 °C binding occurs within 3 to 5 min. Furthermore, cells exposed to the toxin for 5 min, followed by washing, still demonstrate the characteristic delay. The data in Fig. 2 suggests that the delay represents a true lag period rather than a slowly accelerating process. Possible explanations for this phenomenon are examined in more detail below.

The degree of stimulation of enzyme activity depends on the amount of toxin added to the medium under conditions of high cell density, which promote nearly quantitative adsorption of the toxin from the medium (Figs. 3 and 4). Experiments with toad erythrocytes (Fig. 4*a*, *b*) indicate that the predominant effect of increasing the quantity of toxin is an elevation of the rate of exponential increase of enzyme activation, with no detectable effect on the length of the lag period. The rate constant for the exponential process can be estimated from the slope of the semi-log plot of activity versus time of activation, and this is related to the number of toxin-receptor complexes by a simple Langmuir adsorption isotherm (Fig. 4*b*). The half-maximal increase in the rate of exponential activation occurs with about 2,200 toxin molecules bound per cell. 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 cholera toxin, with an apparent K_a of about 1 500 toxin molecules per cell (Fig. 3).

The 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 this were the case half-maximal activation would require fewer toxin molecules with increasing length of incubation. Also, if cholera toxin acts as a "catalyst", the rate of activation should continue to increase with higher doses of cholera toxin, at least until the binding sites had been saturated. The data in Fig. 4*b* 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 the cell (about 5 ×

10^4 sites per cell)[1]. Similar considerations argue against other kinds of mechanisms based on catalytic processes initiated by interaction of the toxin with the cell. Diphtheria toxin (Gill *et al.*, 1969; Honjo *et al.*, 1971) and the bacterial colicin E_3 (Boon, 1971; Senior & Holland, 1971) provide examples of microbial products which interact with plasma membranes during an early phase and which ultimately function intracellularly by well-established catalytic processes. In these cases the concentration-response relationships obey Poisson distributions for "single hit" kinetics (Jacob *et al.*, 1952; Nomura, 1963). The data described in Fig. 3 by such analyses deviate from the predicted linear relationship (not shown). These considerations suggest that each molecule of toxin may lead to the alteration of only one adenylate cyclase enzyme. The possibility that cholera toxin itself has or develops intrinsic adenylate cyclase activity is unlikely (Bennett & Cuatrecasas, 1975), based on kinetic considerations of the activated enzyme and on the lack of activity of the toxin in human erythrocytes.

The activation of adenylate cyclase activity occurs at a very slow rate (or not at all) below certain critical temperatures which depend on the animal species studied (Figs. 5 and 7). The transition in toad erythrocytes occurs at 15–17 °C, while rat fat cells and turkey erythrocytes 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. The 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 prolonged duration of the effects of cholera toxin have been noted previously (Guerrant *et al.*, 1972; Donta *et al.*, 1973; Wolff *et al.*, 1973; O'Keefe & Cuatrecasas, 1974). The rate of decay of the toxin-stimulated adenylate cyclase activity in cultured melanoma cells has been examined in this report and elsewhere (O'Keefe & Cuatrecasas, 1974). The high degree of activation achieved falls according to a first-order relationship with a half-life of about 36 hr. This rate is at least 200-fold slower than the rate of dissociation of hormone-receptor complexes for known peptide hormones which bind to cells with very high affinity (K_D about 10^{-10} M), and suggests the existence of unusually stable cell-toxin complexes.

The data in Figs. 2 and 4 establish the absolute nature of the lag phase which is characteristically seen in the action of cholera toxin. The data are also consistent with the view (Cuatrecasas, 1973*c*) that the initial toxin-

1 The relationship of the large excess of receptors (gangliosides) to the action of the toxin is described in detail elsewhere (Bennett *et al.*, 1975).

receptor complex may be biologically ineffective[2]. High concentrations of Actinomycin D, puromycin and cycloheximide (Table 3), and drugs which disrupt microtubules (colchicine, colcemid, vincristine and vinblastine) *(unpublished observations)* or microfilaments (cytochalasin B) *(unpublished observations)* do not prevent the effects of cholera toxin. Kimberg *et al.* (1973) have also reported that cycloheximide does not significantly inhibit adenylate cyclase stimulation in the intestinal mucosa. Activation is not prevented by NaF (10 mM) or by sodium azide (1 mM) (Table 3). Erythrocytes stored for two days (4 °C) in the absence of serum and nutrients can still be affected very well by cholera toxin. The lag is not diminished with levels of cholera toxin as high as 50 μg/ml.

The possibility that the delay (lag phase) could represent the time required for the penetration of the toxin through the plasma membrane into the cytoplasm was examined by direct measurements of the entry of ^{125}I-labeled cholera toxin into erythrocytes. Very low quantities of "intracellular" toxin are observed (at most 100 molecules per cell, or about 1% of the plasma membrane-bound toxin), and nearly all of this could be accounted for by contamination occurring by simple dissociation of the membrane-bound toxin during lysis of the cells (Table 4 and Chang *et al.*, 1974). Similarly, the toxin is not bound to nuclei in significant quantities *(unpublished observations)*[3]. The small amounts of toxin which may exist in the cytoplasmic and nuclear compartments are very much less than observed K_a (1500–2200 molecules per cell) for the toxin, but could be of significance if cholera toxin has intrinsic catalytic activity or if it triggered some catalytic process in the cell. However, as discussed above, if only a few molecules of the toxin were required for complete activation single-hit kinetics should be observed.

Another possible explanation for the lag period is that the toxin must remove or inactivate some membrane component which is initially present in excess. However, once such a putative component is lost, the subsequent addition of toxin should not be accompanied by a similar delay in the expression of biological effects. This possibility was examined (Table 2) by first activating adenylate cyclase with submaximal concentrations of cholera toxin, followed by further stimulation with high toxin concentrations. The

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2 The inactive nature of the initial toxin-receptor complex is supported by studies with choleragenoid, a biologically inactive analog of the toxin which has binding properties identical to those of the toxin and which competitively inhibits the binding and action of the toxin (Cuatrecasas, 1973*d*).

3 It is noteworthy that rat erythrocytes, which are devoid of nuclei, are stimulated quite well by cholera toxin (Bennett & Cuatrecasas, 1975).

second activation still demonstrated the characteristic delay, even though adenylate cyclase had clearly been stimulated previously.

The events occurring during the lag period are thus approximately zero order with respect to the bound toxin, and they are independent of external or internal energy sources, and of RNA and protein synthesis. They are highly dependent on temperature, and do not appear to involve penetration of cholera toxin into the cytoplasm. The length of the delay appears to be independent of the amount of the initial toxin-receptor complex, which implies that concentration-dependent associations between this complex and other membrane components are not the rate-limiting events during the lag period. Many of the properties of the lag period are similar to those described for the lateral motion of membrane components in fused heterokaryons (Frye & Edidin, 1970) and in cultured muscle fibers (Edidin & Fambrough, 1973). The redistribution of membrane antigens in fused cells is also highly temperature-dependent, and occurs in the presence of sodium azide and inhibitors of protein synthesis. These processes are relatively slow, requiring about 40 min for complete mixing of membrane components at 37 °C.

It has been suggested previously (Bennett & Cuatrecases, 1975; Bennett *et al.*, 1975) that the relatively irreversible adsorption of ^{125}I-labeled cholera toxin to cell membranes (Cuatrecasas, 1973*c*) and the persistent nature of the biological effects could be explained if the toxin molecule were to be incorporated into the plasma membrane as an "integral" membrane component (Singer & Nicolson, 1972)[4]. Activation may depend on such a transformation, and the lag phase could represent the conversion of the initial, inactive[2] toxin-receptor complex to a new state such that insertion of the toxin molecule into the membrane is favored. This would proceed spontaneously above certain critical temperatures, and may involve lateral mobility of the toxin-ganglioside complex within the plane of the membrane.

The inactive nature of the initial toxin-membrane complex suggests that the biological effects of the toxin are due to interactions with other, secondary "receptor" sites. The simplest possibility is that the "receptor" is the adenylate cyclase enzyme. Thus, activation would occur by encounters between the active form of cholera toxin and adenylate cyclase. It is pertinent that the concentration-response relationships can be approximated well by a Langmuir adsorption isotherm. This type of relationship is consistent

4 It is known that with increasing time and temperature of incubation the membrane-bound toxin molecules bind progressively more strongly such that dissociation can only be achieved by dissolution of the membrane with nonionic detergents (Cuatrecasas, 1973*c*).

with the occurrence of a bimolecular interaction in the rate-limiting step of adenylate cyclase activation. Furthermore, direct evidence has recently been reported for the high affinity association of the toxin with adenylate cyclase molecules (Bennett *et al.*, 1975).

The possible interaction of these two components would depend on their diffusion properties within the two-dimensional matrix of the membrane. The frequency of this event can be estimated once the diffusion constants and average inter-molecular distances are known. Such calculations, for example, have been made for the rate of collision of rhodopsin molecules in the photoreceptor membrane (Poo & Cone, 1974).

The activation of liver membrane adenylate cyclase has recently been reported to vary in a hyperbolic manner with the amount of glucagon-receptor complex (Rodbell *et al.*, 1974). This behavior is consistent with a separate, independent step involving association between the activated glucagon-receptor complex and adenylate cyclase, as suggested recently (Cuatrecasas, 1974*a*, *b*). It has been proposed (Cuatrecasas, 1974*a*, *b*) that the general mechanism of action of hormones may involve separate bimolecular interactions between hormone-receptor complexes and functional membrane macromolecules (e.g., adenylate cyclase) within the plane of the membrane, and that important analogies may exist with the action of cholera toxin (Bennett *et al.*, 1975).

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References

Bennett, V., Cuatrecasas, P. 1974. Properties of the cholera toxin-stimulated adenylate cyclase of toad and rat erythrocyte plasma membranes and the mechanism of action of cholera toxin. *Fed. Proc.* **33**:1357 *(Abstr.)*

Bennett, V., Cuatrecasas, P. 1975. Mechanism of action of *Vibrio cholerae* enterotoxin. Effects on adenylate cyclase of toad and rat erythrocyte plasma membranes. *J. Membrane Biol. 22*:1

Bennett, V., O'Keefe, E., Cuatrecasas, P. 1975. The mechanism of action of cholera toxin and the mobile receptor theory of hormone-receptor-adenylate cyclase interactions. *Proc. Nat. Acad. Sci. 72*:33

Birnbaumer, L. 1973. Hormone-sensitive adenylyl cyclases. Useful models for studying hormone receptor function in cell-free systems. *Biochim. Biophys. Acta* **300**:129

Boon, T. 1971. Inactivation of ribosomes in vitro by colicin E_3. *Proc. Nat. Acad. Sci.* **68**:2421

Carpenter, C. C. J., Greenough, W. B. III. 1968. Response of the canine duodenum to intraluminal challenge with cholera extoxin. *J. Clin. Invest.* **47**:2600

Carpenter, C. C. J., Sack, R. B., Feeley, J. C., Steenberg, R. W. 1968. Site and characteristics of electrolyte loss and effect of intraluminal glucose in experimental canine cholera. *J. Clin. Invest.* **47**:1210

Chang, K.-J., Bennett, V., Cuatrecasas, P. 1974. Membrane receptors as general markers for plasma membrane isolation procedures – The use of ^{125}I-labelled wheat germ agglutinin, insulin and cholera toxin. *J. Biol. Chem. (In press)*

Chang, K.-J., Cuatrecasas, P. 1974. Adenosine triphosphate-dependent inhibition of insulin-stimulated glucose transport in fat cells. Possible role of membrane phosphorylation. *J. Biol. Chem.* **249**:3170

Cuatrecasas, P. 1971. Insulin-receptor interactions in adipose tissue cells: Direct measurement and properties. *Proc. Nat. Acad. Sci.* **68**:1264

Cuatrecasas, P. 1973*a*. The interaction of *Vibrio cholerae* enterotoxin with cell membranes. *Biochemistry* **12**:3547

Cuatrecasas, P. 1973*b*. Gangliosides and membrane receptors for cholera toxin. *Biochemistry* **12**:3558

Cuatrecasas, P. 1973*c*. Cholera toxin-fat cell interaction and the mechanism of activation of the lipolytic response. *Biochemistry* **12**:3567

Cuatrecasas, P. 1973*d*. *Vibrio cholerae* choleragenoid – Mechanism of inhibition of cholera toxin action. *Biochemistry* **12**:3577

Cuatrecasas, P. 1974*a*. Membrane receptors. *Annu. Rev. Biochem.* **43**:169

Cuatrecasas, P. 1974*b*. Insulin receptors, cell membranes and hormone-action. *Biochem. Pharmacol.* **23**:2353

Cuatrecasas, P., Parikh, I., Hollenberg, M. D. 1973. Affinity chromatography and structural analysis of *Vibrio cholerae* enterotoxin – Ganglioside agarose and the biological effects of ganglioside-containing soluble polymers. *Biochemistry* **12**:4253

Donta, S. T., King, M., Sloper, K. 1973. Induction of steroidogenesis in tissue culture by cholera enterotoxin. *Nature, New Biol.* **243**:246

Edidin, M., Fambrough, D. 1973. Fluidity of the surface of cultured muscle fibers. Rapid lateral diffusion of marked surface antigens. *J. Cell Biol.* **57**:27

Finkelstein, R. A. 1973. Cholera. *CRC Crit. Rev. Microbiol.* **2**:553

Finkelstein, R. A., LoSpalluto, J. J. 1970. Production of highly purified choleragen and choleragenoid. *J. Infect. Dis.* **121**:S63

Flawia, M. M., Torres, H. N. 1972. Adenylate cyclase activity in *Neurospora crassa*. I. General properties. *J. Biol. Chem.* **247**:6873

Frye, C. D., Edidin, M. 1970. The rapid intermixing of cell surface antigens after formation of mouse-human heterokargons. *J. Cell Sci.* **7**:313

Gill, D. M., Pappenheimer, A. M., Jr., Brown, R., Kurnick, J. T. 1969. Studies on the mode of action of diphtheria toxin. VII. Toxin-stimulated hydrolysis of nicotinamide adenine dinucleotide in mammalian cell extracts. *J. Exp. Med.* **129**:1

Guerrant, R. L., Chen, L. C., Sharp, G. W. G. 1972. Intestinal adenyl cyclase activity in canine cholera: Correlation with fluid accumulation. *J. Infect. Dis.* **125**:377

Hollenberg, M. D., Fishman, P. H., Bennett, V., Cuatrecasas, P. 1974. Cholera toxin and cell growth: Role of membrane gangliosides. *Proc. Nat. Acad. Sci.* **71**:4224

Holmgren, J., Lonnroth, I., Svennerholm, L. 1973. Tissue receptor for cholera exotoxin: Postulated structure from studies with G_{M1} ganglioside and related glycolipids. *Infect. Immun.* **8**:208

Honjo, T., Nishizuka, Y., Kato, I., Hayaishi, O. 1971. Adenosine diphosphate ribosylation of aminoacyl transferase II and inhibition of protein synthesis by diphtheria toxin. *J. Biol. Chem.* **246**:4251

Hunter, W. M., Greenwood, F. C. 1962. Preparation of iodine-131 labelled human growth hormone of high specific activity. *Nature* **194**:495

Jacob, F., Siminovitch, L., Wollman, E. L. 1952. Sur La Biosynthese D'Une Colicine Et Sur Son Mode D'Action. *Ann. Inst. Pasteur* **83**:295

Kimberg, D. V., Field, M., Gershon, E., Schooley, R. T., Henderson, A. 1973. Effects of cycloheximide on the response of intestinal mucosa to cholera enterotoxin. *J. Clin. Invest.* **52**:1376

Kimberg, D. V., Field, M., Johnson, J., Henderson, A., Gershon, E. 1971. Stimulation of intestinal mucosal adenyl cyclase by cholera enterotoxin and prostaglandins. *J. Clin. Invest.* **50**:1218

King, C. A., van Heyningen, W. E. 1973. Inactivation of cholera toxin by sialidase-resistant monosialoganglioside. *J. Infect. Dis.* **127**:639

LoSpalluto, J. J., Finkelstein, R. A. 1972. Chemical and physical properties of cholera exo-enterotoxin (choleragen) and its spontaneously formed toxoid (choleragenoid). *Biochim. Biophys. Acta* **275**:158

Lowry, O. H., Rosebrough, N. J., Farr, A. L., Randall, R. J. 1951. Protein measurement with the folin-phenol reagent. *J. Biol. Chem.* **193**:265

Nomura, M. 1963. Mode of action of colicines. *Symp. Quant. Biol. XXVIII*:**315**

O'Keefe, E., Cuatrecasas, P. 1974. Cholera toxin mimics melanocyte stimulating hormone in inducing differentiation in melanoma cells. *Proc. Nat. Acad. Sci.* **71**:2500

Peterson, J. W., LoSpalluto, J. J., Finkelstein, R. A. 1972. Localization of cholera toxin in vivo. *J. Infect. Dis.* **126**:617

Pierce, N. F., Carpenter, C. C. J., Elliot, H. L., Greenough, W. B. III. 1971*a*. Effects of prostaglandins, theophylline and cholera exotoxin upon transmucosal water and electrolyte movement in the canine jejunum. *Gastroenterology* **60**:20

Pierce, N. F., Greenough, W. B. III, Carpenter, C. C. J. 1971*b*. *Vibrio cholerae* enterotoxin and its mode of action. *Bacteriol. Rev.* **35**:1

Poo, M., Cone, R. A. 1974. Lateral diffusion of rhodopsin in the photoreceptor membrane. *Nature* **247**:438

Ramachandran, J. 1971. A new simple method for separation of adenosine 3′,5′-cyclic monophosphate from other nucleotides and its use in the assay of adenyl cyclase. *Analyt. Biochem.* **43**:227

Rodbell, M. 1964. Metabolism of isolated fat cells. *J. Biol. Chem.* **239**:375

Rodbell, M., Lin, M. C., Salomon, Y. 1974. Evidence for independent action of glucagon and nucleotides on the hepatic adenylate cyclase system. *J. Biol. Chem.* **249**:59

Senior, B. W., Holland, I. B. 1971. The effect of colicin E_3 upon the 30 S ribosomal subunit of *Escherichia coli*. *Proc. Nat. Acad. Sci.* **68**:959

Sharp, G. W. G. 1973. Action of cholera toxin on fluid and electrolyte movement in the small intestine. *Annu. Rev. Med.* **24**:19

Singer, S. J., Nicolson, G. L. 1972. The fluid mosaic model of the structure of cell membranes. *Science* **175**:720

Symons, R. H. 1968. Modified procedure for the synthesis of ^{32}P-labelled ribonucleoside 5′-monophosphate of high specific activity. *Biochim. Biophys. Acta* **155**:609

van Heyningen, S. 1974. Cholera toxin: Interaction of subunits with ganglioside G_{M1}. *Science* **183**:656

Vaughan, M., Pierce, N. F., Greenough, W. B. III. 1970. Stimulation of glycerol production in fat cells by cholera toxin. *Nature* **226**:658

White, A. A., Zenser, T. V. 1971. Separation of cyclic 3′,5′-nucleoside monophosphate from other nucleotides on aluminum oxide columns application to the assay of adenyl cyclase and guanyl cyclase. *Analyt. Biochem.* **41**:372

Wolff, J., Temple, R., Cook, G. H. 1973. Stimulation of steroid secretion in adrenal tumor cells by choleragen. *Proc. Nat. Acad. Sci.* **70**:2741