Cholera Toxin–Fat Cell Interaction and the Mechanism of Activation of the Lipolytic Response†

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ABSTRACT: The possible mechanisms by which the interaction of cholera toxin with isolated fat cells leads to an enhancement in the rate of lipolysis were examined, with special emphasis on the biochemical basis of the marked lag phase which exists before the onset of the lipolytic response is evident. Cells exposed to cholera toxin can be washed and replaced with fresh medium after 8 min or after 60 min of incubation at 37° without altering the characteristic 1-hr lag phase or the subsequent course of the lipolytic response. During the lag phase lipolytic metabolites or products are not secreted and accumulated in the incubation medium. Fat cells obtained from young rats (50–100 g) are much more sensitive to cholera toxin than cells obtained from large (>200 g) animals. Increasing the concentration of cholera toxin in the medium to very high values does not appreciably shorten the lag phase of the lipolytic response. In the presence of cholera toxin the lipolytic effects of epinephrine and of glucagon are not appreciably altered during the period of the lag phase. During the course of incubation the toxin does not appear to be secreted into the medium in an immediately active form. Incubation of fat cells at 37° in the absence of toxin does not alter the subsequent course of toxin-induced lipolysis. The lag phase is very dependent on the temperature of incubation. Prolonged incubation of the cell–toxin complex at 4 or 24° does not modify the length of the lag phase upon subsequent incubation at 37°. The length of the lag phase is the same whether the cells are incubated at 37 or 48°. The lipolytic response of fat cells to cholera toxin can be effectively inhibited by insulin or by alloxan, compounds which are capable of inhibiting the activity of adenylate cyclase. The inhibition by insulin is equally effective whether it is added at the start of the incubation with toxin or whether it is added after the lag period has transpired. Alloxan, however, becomes progressively less effective when it is added after increasing periods of incubation of the toxin–cell complex. Various inhibitors of prostaglandin biosynthesis or action (indomethacin, sodium salicylate, diphloretin phosphate) and inhibitors of RNA and protein synthesis (actinomycin D, cycloheximide, puromycin) do not affect the lipolytic activity of cholera toxin. With increasing length and temperature of incubation of the cell–toxin complex the rate and extent to which the complex can spontaneously dissociate are progressively decreased. However, even with very prolonged incubation the toxin does not appear to form stable covalent bonds with membrane macromolecules. The length of the lipolytic lag phase cannot be decreased by exposing the cells for a brief period to cholera toxin in the presence of low concentrations of detergent (to enhance permeability). Despite the ability of tetanus toxin to bind to gangliosides, this bacterial toxin has no lipolytic or antilipolytic activities in fat cells, even when ganglioside-treated fat cells are used. Tetanus toxin does not compete with ^{125}I-labeled cholera toxin for binding to membranes. It is suggested that cholera toxin initially forms an inactive toxin–ganglioside receptor complex on the cell membrane, and that this complex is transformed into a biologically active complex by a special transition which involves a major, spontaneous relocation of the complex within the two-dimensional structure of the membrane.

The fulminant gastrointestinal losses of water and electrolytes which occur in clinical *Vibrio cholerae* infection appear to be caused by a specific enterotoxin whose action is mediated by stimulation of adenylate cyclase activity in the epithelial cells of the small intestine with a consequent increase in the intracellular concentrations of cyclic 3′,5′-adenosine monophosphate[1] (reviewed by Pierce *et al.*, 1971a,b; Field, 1971). Although in clinical infection the enterotoxin is not absorbed from the intestine well enough to reach the general circulation in sufficient quantities to cause systemic manifestations, the toxin is capable of stimulating adenylate cyclase activity in all tissues which have so far been examined. In such tissues it can stimulate metabolic responses which are mediated by cAMP, such as glycogenolysis in liver and platelets (Zieve *et al.*, 1970) and lipolysis in isolated fat cells (Greenough *et al.*, 1970; Vaughn *et al.*, 1970; van Heyningen *et al.*, 1971).

One of the most peculiar characteristics of the action of cholera toxin is the consistent lag period or delay which exists between the time of intraluminal addition of the toxin and the time of onset of fluid output by small bowel loops (Carpenter *et al.*, 1968; Pierce *et al.*, 1971a,b; Sharp and Hynie, 1971; Carpenter and Greenough, 1968). Similar lag periods have been observed in the effect of cholera toxin on the stimulation of adenylate cyclase activity in intestinal mucosal cells (Kimberg *et al.*, 1971), isolated fat cells[2] and frog erythrocytes,[2] on the stimulation of short circuit current across the isolated rabbit ileum (Field *et al.*, 1968), and on the lipolytic response of isolated fat cells (Vaughn *et al.*, 1970).

Recent studies which measured directly the binding of ^{125}I-labeled cholera toxin to isolated fat cells and to liver and intestinal cell membranes suggest that the basic toxin–membrane interaction is very similar in all these tissues (Cuatre-

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[1] Abbreviation used is: cAMP, cyclic 3′,5′-adenosine monophosphate.

[2] G. V. Bennett and P. Cuatrecasas, manuscript in preparation.

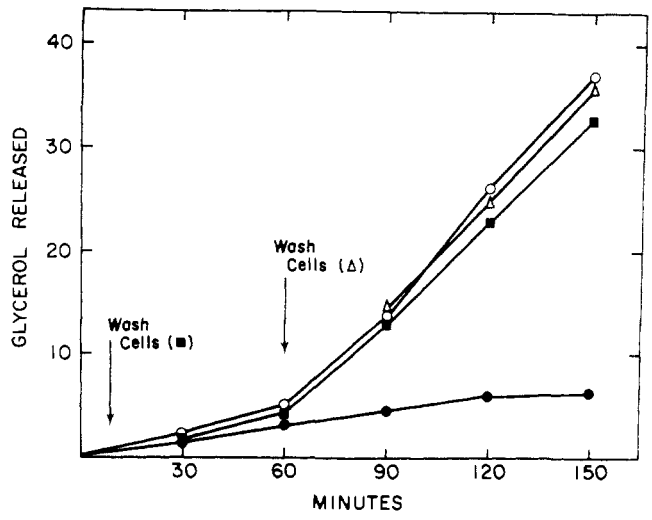

FIGURE 1: Effect of washing fat cells after exposure to cholera toxin on the lipolytic response of the cells. The cells were incubated at 37° in Krebs–Ringer–bicarbonate buffer, 3% albumin (w/v), in the absence (●) and presence of 2 μg/ml of cholera toxin. After 9 (■) and 60 (Δ) min some of the toxin-treated cells were washed extensively with the same buffer and the incubation was continued at 37° in the absence of added toxin to the medium. In some cell suspensions (○) the toxin in the medium was not removed. Lipolysis is described as the micromoles of glycerol released per millimole of triglyceride.

casas, 1973b,c). Evidence has been presented which indicates that the normal receptors for cholera toxin may be special membrane-localized gangliosides (Cuatrecasas, 1973c). The biological effects of cholera toxin on fat cells have been shown to depend in a critical way on the number of toxin molecules which are bound to the cells, probably through interaction with gangliosides. It has also been demonstrated directly that the toxin–cell complex forms extremely rapidly and that the complex, although reversible under certain conditions, is very stable (Cuatrecasas, 1973c). These considerations might thus account for the persistence of the biological effect (many hours) which is observed after a tissue is exposed to cholera toxin for only a few minutes (Pierce *et al.*, 1971a,b). The binding studies, however, do not explain the peculiar delay in the onset of the biological action of cholera toxin since the quantitative aspects of the cell- and liver membrane–toxin interaction remain essentially unchanged during incubation periods (37°) as long as 3 hr (Cuatrecasas, 1973c). It appears that activation of biological effects may be dependent on relatively slow membrane or cytoplasmic events which must follow formation of the initial toxin–cell complex. It is apparent that the nature of such changes cannot easily be elucidated further by simple quantitative measurements of equilibrium binding between the toxin and cell.

The present report describes studies designed to explore in more detail the nature of the latency phase and the manner by which the toxin–receptor complex, once formed, might produce biological effects. It is suggested that the active form of the toxin–receptor complex is not formed initially but requires an important reorientation or reorganization within the structure of the membrane.

Experimental Procedure

Materials. Cholera toxin (lot 1071), purified by the method of Finkelstein and LoSpalluto (1970) and obtained from Dr. R. S. Northrup, SEATO Cholera Research Program, was prepared under contract for the National Institute of Allergy and Infectious Diseases by Dr. R. A. Finkelstein, The University of Texas Southwestern Medical School, Dallas, Tex. Tetanus toxin (lot T-1), with an activity of one mouse MLD unit per 0.6 μg, was a generous gift from Dr. C. Hardegree, Food and Drug Administration. L-Norepinephrine, alloxan, sodium salicylate, bovine brain gangliosides (fraction II), actinomycin D, and cycloheximide were purchased from Sigma. Indomethacin was obtained from Merck Sharp and Dohme. Puromycin was a gift from Dr. D. Nathans. Purified disialoganglioside and trisialoganglioside were purchased from Supelco, Inc., Bellefonte, Pa. GD_{1a} ganglioside was a gift from Dr. R. Brady, National Institutes of Health. Bovine crystalline zinc-insulin (24 units/mg) and glucagon were purchased from Eli Lilly. Samples of diphloretin phosphate were from Nelson Laboratories, Irvine, Calif. Bovine albumin fraction V was from Armour Pharmaceuticals.

Procedures. Isolated fat cells were prepared from Sprague-Dawley rats by the method of Rodbell (1966). Unless indicated otherwise the rats used weighed 90–120 g. Lipolysis was studied (Cuatrecasas, 1969) by determining the concentration of glycerol in the medium by the method of Ryley (1955). Liver membranes were prepared by homogenization and differential centrifugation in 0.25 M sucrose (Cuatrecasas, 1972a,b; Illiano and Cuatrecasas, 1972). Membrane protein was determined by the method of Lowry *et al.* (1951) after heating at 100° for 30 min in 1 M NaOH; bovine albumin was used as the standard.

The procedures used to prepare ^{125}I-labeled cholera toxin and to measure the specific binding of the toxin to fat cells have been described (Cuatrecasas, 1973b). Unless otherwise stated the dissociation of the fat cell- or membrane-^{125}I-labeled toxin complex was studied by initiating the dissociative process by the addition of 15–30 μg/ml of native cholera toxin as described in the legends.

Results

General Lipolytic Properties of Cholera Toxin. The characteristic period of about 1 hr (Vaughn *et al.*, 1970; Cuatrecasas, 1973c) which must transpire between the addition of cholera toxin and the initiation of significant lipolysis in fat cells is demonstrated in Figure 1. In agreement with earlier reports (van Heyningen *et al.*, 1971), removal of the toxin from the medium by washing the cells after an exposure period of 8 min (37°) results subsequently in nearly a maximal lipolytic response of the cells; 3 hr after this washing procedure the rate of lipolysis is still nearly unaltered (Figure 1). No significant changes in lipolysis occur if the cells are washed after exposure to cholera toxin for 60 min at 37° (Figure 1).

The dependence of the lipolytic response of fat cells on the concentration of cholera toxin is highly dependent on the size and age of the rats from which the cells originate (Figure 2). Fat cells obtained from very young animals (50 g) respond very well to concentrations of toxin under 5 ng/ml; in these cells a maximal lipolytic response can be obtained with about 20 ng/ml of cholera toxin. Relatively small lipolytic responses are obtained with concentrations of cholera toxin under 20 ng/ml in fat cells of rats weighing more than 200 g, and in these cells the maximal effects require more than 150 ng of toxin/ml. Studies of the binding of ^{125}I-labeled cholera toxin to fat cells obtained from rats of differing weight indicate that the number of binding sites per unit surface area is greater in the smaller cells compared to the large cells. These results are in agreement with the observations that the lipo-

TABLE I: Inhibition by Insulin of Cholera Toxin Induced Lipolysis in Fat Cells.[a]

Additions	Glycerol Released[b]
None	5.8 ± 0.8
Insulin	4.2 ± 0.4
L-Epinephrine	31.8 ± 2.2
+ insulin	10.2 ± 1.0
Cholera toxin	28.8 ± 1.5
+ insulin	6.7 ± 0.8

[a] Fat cells were incubated at 37° for 2 hr in Krebs–Ringer–bicarbonate buffer containing 3% albumin and, where indicated, insulin (20 microunits/ml), L-epinephrine (0.5 μg/ml), and cholera toxin (1.3 μg/ml). [b] Micromoles of glycerol released per millimole of triglyceride in 2 hr.

lytic response of a fat cell to cholera toxin is related to the number of toxin molecules bound to that cell, and that in a given cell population similar biological responses can be achieved by increasing either the number of receptors per cell or the concentration of the toxin in the medium (Cuatrecasas, 1973c). It is not yet known whether the decreased toxin sensitivity or decreased toxin-binding capacity with increasing age reflects changes in the ganglioside composition of the cell membrane.

It is also pertinent that with certain batches of serum albumin the lipolytic responses of fat cells are very small and require large concentrations of cholera toxin, regardless of the size of the rats from which the cells are obtained. This effect of the albumin can be totally abolished by dialyzing the albumin, at 20% (v/v), for 2 days at 4° against Krebs–Ringer–bicarbonate buffer. Some commercially available fatty acid poor albumin preparations also strongly suppress toxin-induced lipolysis and also require dialysis before use. The fatty acid poor albumin preparations do not appear to offer special advantages in this type of study of lipolysis.

Effects of Insulin and of Alloxan on Cholera Toxin Induced Lipolysis. The lipolytic effect of cholera toxin on isolated fat cells is inhibited by low concentrations of insulin (Table I). The inhibitory effect of insulin is equally evident whether insulin is added to the cells before the cholera toxin or whether it is added 15, 30, 60, or 90 min after the cells have been incubated at 37° with cholera toxin (Table II). The lipolytic effect of cholera toxin is thus inhibited by insulin in a manner very similar to the inhibition by insulin of L-epinephrine-induced lipolysis (Table I). The major difference in the effects of these two lipolytic agents is the presence of the dramatic lag phase with cholera toxin but not with L-norepinephrine (Cuatrecasas, 1973c). It appears that once the lipolytic effect of cholera toxin is initiated (following the lag phase) the mechanism by which this lipolytic response is sustained may not differ fundamentally from that which mediates the response to L-epinephrine.

Alloxan, a compound which inhibits selectively and reversibly the basal and hormone-stimulated activities of adenylate cyclase in various tissues (Cohen and Bitensky, 1969), is also capable of inhibiting the lipolytic response of fat cells to cholera toxin (Table III). Concentrations of alloxan in the range of 0.2–5 mM are effective, and the concentration required to achieve half-maximal inhibition of lipolysis is about 0.8 mM. The concentration required to produce 50% inhibition of adenylate cyclase activity in isolated preparations of various tissues is about 10^{-4} M (Cohen and Bitensky, 1969). Unlike the effects observed with insulin (Table II), the inhibition of lipolysis by alloxan is progressively less effective when the drug is added to cells which have been incubated with cholera toxin for increasing lengths of time (Table IV). It is therefore apparent that insulin is a more effective agent than alloxan for blocking and reversing the lipolytic effects of cholera toxin. Alloxan (5 mM) and insulin (5 μg/ml) do not modify the binding of ^{125}I-labeled cholera toxin to membranes even in

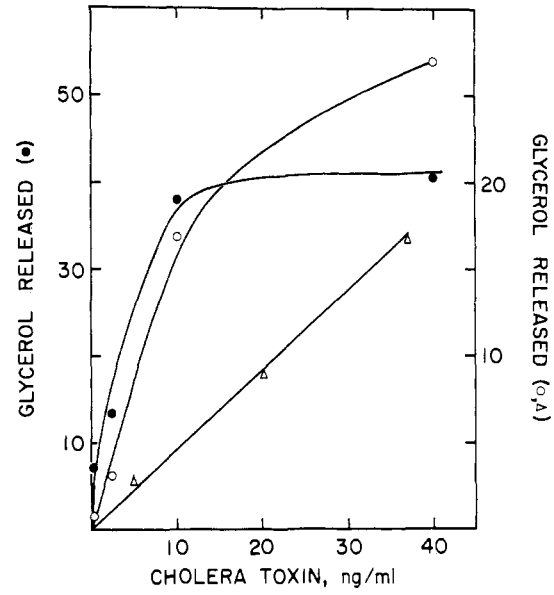

FIGURE 2: Effect of rat size on the lipolytic response of the isolated fat cells to cholera toxin. Isolated fat cells from the epididymal fat pads of rats weighing 50 (●), 100 (○), or 200 g (△) were incubated at 37° for 160 min in Krebs–Ringer–bicarbonate buffer containing 3% (w/v) albumin and the indicated concentration of cholera toxin. Lipolysis is given as micromoles of glycerol released per millimole of cell triglyceride.

TABLE II: Effect of Length of Incubation on the Protection by Insulin of Cholera Toxin Induced Lipolysis in Fat Cells.[a]

Conditions of Incubation	Glycerol Produced[b] 30 min	60 min	90 min	150 min	Protection by Insulin (%)
No additions	3.1	3.7	3.8	5.0	
Cholera toxin	3.5	6.0	7.3	34.3	
Cholera toxin + insulin					
after 0 min				10.6	80
15 min				10.5	80
30 min				9.5	85
60 min				11.7	85
90 min				13.8	82

[a] Fat cells were incubated for various times at 37° in Krebs–Ringer–bicarbonate buffer containing 3% (w/v) albumin and 10 ng/ml of cholera toxin. Insulin (2 ng/ml) was added at various times after the beginning of the incubation, and the incubations were continued for a total of 150 min. The effects of insulin in the absence of cholera toxin are very minimal (Table I). [b] Micromoles per millimole of cell triglyceride.

TABLE III: Inhibition of Cholera Toxin Stimulated Lipolysis in Fat Cells by Alloxan.[a]

Additions		Glycerol Released[b]
None		4.7 ± 0.5
Cholera toxin, 0.8 μg/ml		22.3 ± 1.8
Alloxan, 4 mM		3.3 ± 0.2
Cholera toxin, 0.8 μg/ml + alloxan,	40 μM	23.4 ± 1.6
	0.16 mM	20.1 ± 1.4
	0.80 mM	12.6 ± 0.9
	4 mM	5.3 ± 0.5

[a] Isolated fat cells were incubated at 37° for 2 hr in Krebs–Ringer–bicarbonate buffer containing 3% (w/v) albumin and, where indicated, cholera toxin and alloxan. [b] Micromoles of glycerol per millimole of triglyceride.

concentrations much greater than those which are effective in inhibiting toxin-induced lipolysis.

Effect of Cholera Toxin on Epinephrine- and Glucagon-Induced Lipolysis. A possible reason which can be suggested to explain the lag phase in the lipolytic response of fat cells to cholera toxin is that this protein initially produces an effect on the cell which is opposed to the effect which predominates after a prolonged period of incubation. Such an effect might be effective in inhibiting any type of lipolytic stimulus which is presented to the cells during the latency phase of the responses. Such a mechanism can be excluded on the basis of experiments which demonstrate that the lipolytic responses of fat cells to L-epinephrine or to glucagon are unaltered by cholera toxin during the period of time in which the toxin is itself not lipolytic (Table V).

Effect of Toxin Concentration on the Lag Phase of the Lipolytic Response. The lag phase of the lipolytic response of fat cells to cholera toxin is not a reflection of a lack of saturation of the binding sites for this protein. Increasing the concentration of cholera toxin to extremely high values does not appreciably modify the relatively weak lipolytic response which is observed during the first hour of incubation (Figure 3).

TABLE IV: Effect of Length of Incubation on the Protection by Alloxan of Cholera Toxin Induced Lipolysis in Fat Cells.[a]

Conditions of Incubation	Glycerol Produced[b] 30 min	60 min	90 min	160 min	Protection by Alloxan (%)
No additions	3.7	4.0	4.0	5.6	
Cholera toxin	4.0	4.0	11.4	40.1	
Cholera toxin + alloxan					
after 0 min				17.9	63
15 min				22.7	50
30 min				27.4	37
60 min				29.6	30
90 min				37.3	25

[a] Fat cells were incubated for various times at 37° in Krebs–Ringer–bicarbonate buffer containing 3% (w/v) albumin and 10 ng/ml of cholera toxin. Alloxan (1 mM) was added at various times after the beginning of the incubation, and the incubations were continued for a total of 160 min. [b] Micromoles per millimole of cell triglyceride.

TABLE V: Effect of Cholera Toxin on the Lipolytic Response of Fat Cells to Epinephrine and Glucagon.[a]

Additions	Glycerol Released[b] 20 min	40 min	60 min
None	2.2 ± 0.3	3.9 ± 0.4	7.2 ± 0.7
Cholera toxin	1.9 ± 0.2	4.3 ± 0.3	8.8 ± 0.5
L-Epinephrine	20.4 ± 1.6	38.7 ± 3.1	55.8 ± 7.0
Glucagon	10.7 ± 0.8	19.5 ± 1.4	28.6 ± 3.1
Cholera toxin +			
L-epinephrine	21.6 ± 1.9	39.4 ± 2.9	60.1 ± 5.2
+ glucagon	9.8 ± 1.1	20.0 ± 0.9	30.4 ± 2.8

[a] Fat cells were incubated at 37° for various time periods in Krebs–Ringer–bicarbonate buffer containing 3% (w/v) albumin and, where indicated, cholera toxin (1 μg/ml), L-epinephrine (2 μg/ml), and glucagon (0.5 μg/ml). [b] Micromoles of glycerol released per millimole of triglyceride.

Production of Lipolytic Substances and Alteration of the Toxin in the Medium. The possibility must be entertained that during the first hour of incubation the cell is generating, as a result of the action of cholera toxin, lipolytic substances which are released into the medium and which when present in sufficient quantity can initiate and maintain the lipolytic response. This thesis is very unlikely since replacement of the medium after incubating the cells with cholera toxin for 1 hr does not alter the subsequent, rapid lipolytic response (Figure 1).

Experiments were designed to examine on fresh cells the lipolytic effects of the toxin-containing medium which had been previously incubated for 60 min at 37° with isolated fat cells. These experiments demonstrated that the medium of fat cells incubated with cholera toxin does not contain substances capable of eliciting immediate lipolytic responses on fat cells.

Effect of Preincubating Fat Cells at 37° without Toxin. Experiments were performed to test the possibility that the characteristic lag phase of cholera toxin induced lipolysis was an inherent property of the fat cells which was independent of the presence of cholera toxin. In principle it is possible, for example, that during the preparation and isolation of the fat cells some selective damage was incurred by the cells, and that spontaneous repair of synthesis might occur during the 1-hr lag phase which would permit the cells to respond immediately to the toxin. This possibility was excluded by demonstrating that fat cells preincubated for 90 min at 37 and 4° in the absence of cholera toxin respond similarly with the typical 1-hr lag period when subsequently incubated with cholera toxin.

Effect of Temperature on the Lag Phase of Toxin-Induced Lipolysis. The data already presented indicate that within minutes cholera toxin binds to fat cells in sufficient quantity to elicit a lipolytic response after incubation of the cell–toxin complex for 1 hr at 37°, even in the absence of the toxin in the medium. Experiments were performed to examine the effect of temperature of the cell–toxin incubation on the length of this lag period. Exposure of fat cells to cholera toxin for periods varying between 30 and 90 min at 4° does not shorten the length of the lag phase observed when these cells are subsequently incubated at 37°. Similarly, incubation of the cell–toxin complex at 24° for 30 and 60 min has no appreciable

TABLE VI: Effect of Inhibitors of Prostaglandin Synthesis and Action on the Lipolytic Response of Fat Cells to Cholera Toxin.[a]

Addition	Glycerol Released[b] 100 min	160 min
No additions	6.0 ± 0.3	10.8 ± 0.8
Cholera toxin	15.0 ± 1.0	34.0 ± 2.0
+ indomethacin,		
20 μg/ml	18.0 ± 0.4	36.0 ± 1.1
100 μg/ml	18.2 ± 0.9	37.0 ± 1.4
+ sodium salicylate,		
50 μg/ml	15.5 ± 0.8	33.5 ± 1.2
200 μg/ml	13.0 ± 0.6	32.5 ± 0.9
+ diphloretin phosphate,		
10 μg/ml	15.3 ± 0.6	34.8 ± 1.4
100 μg/ml	14.4 ± 0.9	31.5 ± 1.0

[a] Isolated fat cells were preincubated for 10 min at 24° in Krebs–Ringer–bicarbonate buffer containing 1% (w/v) albumin and the indicated prostaglandin inhibitor. Cholera toxin (80 ng/ml) was added and the cells were incubated at 37° for 100 and 160 min before determining the content of glycerol in the medium. [b] Micromoles of glycerol released per millimole of triglyceride.

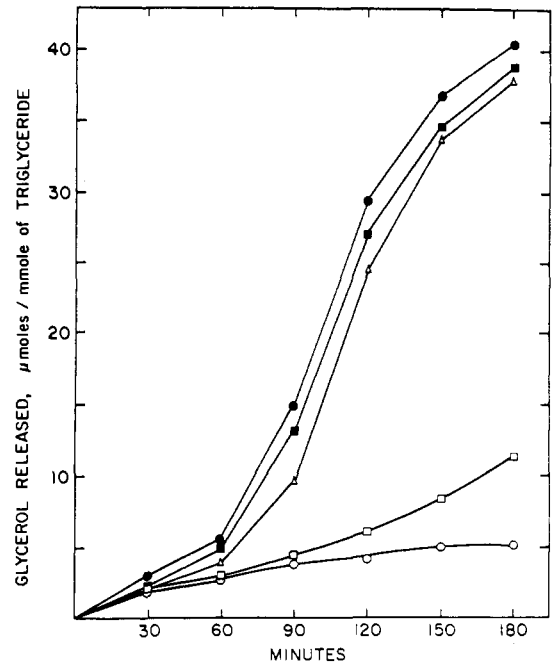

FIGURE 3: Effect of concentration of cholera toxin on the lipolytic response of isolated fat cells. Fat cells obtained from 190-g rats were incubated at 37° in Krebs–Ringer–bicarbonate buffer containing 3% (w/v) albumin and no toxin (○), or toxin at concentrations of 10 ng/ml (□), 60 ng/ml (△), 3 μg/ml (■), and 100 μg/ml (●).

effect on the lag period of the lipolytic process observed at 37°. Preincubation at 48° for 30 min results in a fall in the magnitude of the subsequent lipolytic response at 37°, but the length of the lag phase is not measurably shorter than that observed when the preincubation is performed at 37°. The results suggest that the processes occurring in the lag period, which are essential for the initiation of toxin-induced lipolysis, are dependent on exposure of the cell–toxin complex to a temperature higher than 24°. Furthermore, the absence of an additional effect by increasing the temperature from 37 to 48° suggests that a relatively sharp transition temperature may exist for the specific, unknown events of the lag period.

Effect of Inhibitors of Prostaglandin Synthesis. The effects of cholera toxin on isolated rabbit ileal mucosal segments are nearly identical with those which are produced by the exogenous administration of prostaglandins PGA and PGE_2 (Pierce *et al.*, 1971a,b; Greenough *et al.*, 1969). It has been suggested that these compounds may be natural mediators of cholera toxin (Bennett, 1971), and Katz and Fink (1972) have recently reported that aspirin, which inhibits the biosynthesis of prostaglandins in various tissues (Collier, 1971; Vane, 1971; Smith and Willis, 1971; Ferreira *et al.*, 1971), can block cholera toxin induced intestinal secretion in the cat. The hypothesis that the toxin-induced lipolysis of fat cells may be mediated by endogenous prostaglandins, and that the lag period may be related to processes involved in the biosynthesis of prostaglandins, was tested by examining the effects of compounds which interfere with the action of endogenous prostaglandins (Table VI). The inhibitors of prostaglandin biosynthesis, indomethacin and sodium salicylate (Collier, 1971; Vane, 1971; Smith and Willis, 1971; Ferreira *et al.*, 1971), as well as the antagonist diphloretin phosphate (Eakins, 1971), did not attenuate the lipolytic response of fat cells to cholera toxin even at concentrations (100–200 μg/ml) very much higher than are required to exert effects in most tissues, including fat cells (Illiano and Cuatrecasas, 1971). Furthermore, these compounds did not change the length or nature of the lag period (Table VI). These results suggest that prostaglandins are not involved in the basic lipolytic mechanism of cholera toxin. This is in agreement with the conclusions of Kimberg *et al.* (1971) who, based on the observation that cholera toxin could further increase the intracellular concentration of cAMP in intestinal mucosa maximally stimulated by prostaglandins, felt that effects of cholera toxin and of prostaglandins were mediated by different processes.

Effects of Inhibitors of RNA and Protein Synthesis. The possibility was considered that the lipolytic effects of cholera toxin on fat cells might be dependent on processes which require the synthesis of RNA or protein, and that the occurrence of such processes might be the basis for the lag period of cholera toxin action. Moritz *et al.* (1972) have recently reported that pretreatment of loops of rabbit jejunum with cycloheximide abolished the secretory response of this tissue to cholera toxin. These possibilities were examined by testing the effects of actinomycin D, cycloheximide, and puromycin on toxin-induced lipolysis in fat cells (Table VII). Even very high concentrations of these inhibitors were without effect on either the length of the lag phase or on the magnitude of the lipolytic response. Thus, there is no evidence that the lipolytic property of cholera toxin in fat cells is mediated by steps requiring RNA or protein synthesis.

Effect of Time and Temperature of Incubation on the Rate of Dissociation of the Cholera Toxin–Fat Cell Complex. It has already been demonstrated (Cuatrecasas, 1973b,c) that ^{125}I-labeled cholera toxin binds in a few minutes to membranes or to fat cells, and that the quantity of cholera toxin bound is not significantly changed upon a prolonged period of incubation at 24 or 37°. It has also been shown (Cuatrecasas, 1973c) that despite the very high affinity of the cell–toxin interaction the complex is largely reversible if this property is examined soon after (*e.g.*, 15 min, 24°) an apparent equilibrium of binding is achieved. Such data, however, do not indicate whether the

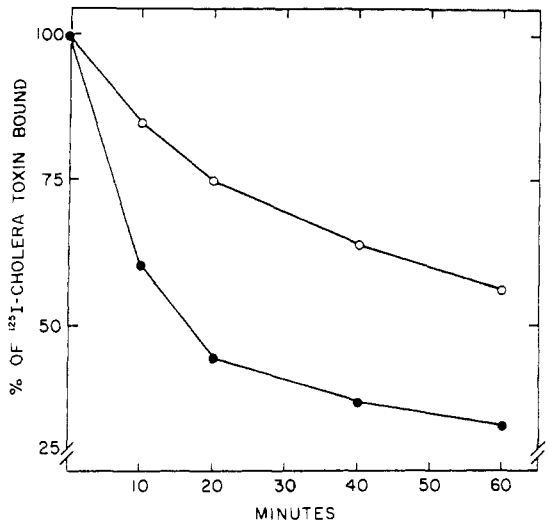

FIGURE 4: Effect of incubating liver membranes with ^{125}I-labeled cholera toxin at 24 (●) and 37° (○) on the subsequent rate of spontaneous dissociation of the toxin–membrane complex at 24°. Liver membranes (140 μg of protein/ml) were incubated in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and ^{125}I-labeled cholera toxin (5 ng, 20 μCi/μg) for 50 min at 24 (●) or 37° (○). The dissociation reaction was begun (zero time) by adding native cholera toxin (15 μg/ml) to all the samples; the specific binding of ^{125}I-labeled toxin to the membranes was determined by filtration procedures (Cuatrecasas, 1973b) after incubating the samples for various periods at 24°.

nature of the bonding interaction is changing during the subsequent period of incubation at 37°. Studies were therefore performed to determine if the reversibility of the binding reaction, or the rate of dissociation of the complex, were dependent on the length and temperature of incubation of the complex.

The rate of spontaneous dissociation (at 24°) of the liver membrane–toxin complex is clearly decreased if the membranes are preincubated for 50 min with cholera toxin at 37° compared to 24° (Figure 4). Perhaps even more dramatic changes occur in the *extent* of dissociation of the membrane- or cell–toxin complex with increasing length or temperature of incubation of the complex (Figure 5). After incubating liver membranes with ^{125}I-labeled cholera toxin for 3 min at 24°, virtually all of the radioactivity is demonstrably dissociable.

TABLE VII: Effect of Actinomycin D, Puromycin, and Cycloheximide on the Lipolytic Response of Fat Cells to Cholera Toxin.[a]

Addition	Glycerol Released[b]		
	60 min	90 min	150 min
None	5.0 ± 0.5	7.0 ± 0.4	11.8 ± 0.8
Cholera toxin	6.0 ± 0.6	18.0 ± 1.0	29.0 ± 1.5
+ actinomycin D	6.6 ± 0.8	19.0 ± 1.4	27.0 ± 1.7
+ cycloheximide	6.7 ± 0.4	21.0 ± 0.9	30.5 ± 1.5
+ puromycin	6.3 ± 0.7	22.2 ± 1.8	30.0 ± 0.8

[a] Isolated fat cells were incubated at 37° in Krebs–Ringer–bicarbonate buffer, 3% (w/v) albumin, containing cholera toxin (0.5 μg/ml), actinomycin D (45 μg/ml), cycloheximide (30 μg/ml), and puromycin (45 μg/ml) as indicated. [b] Micromoles of glycerol released per millimole of triglyceride.

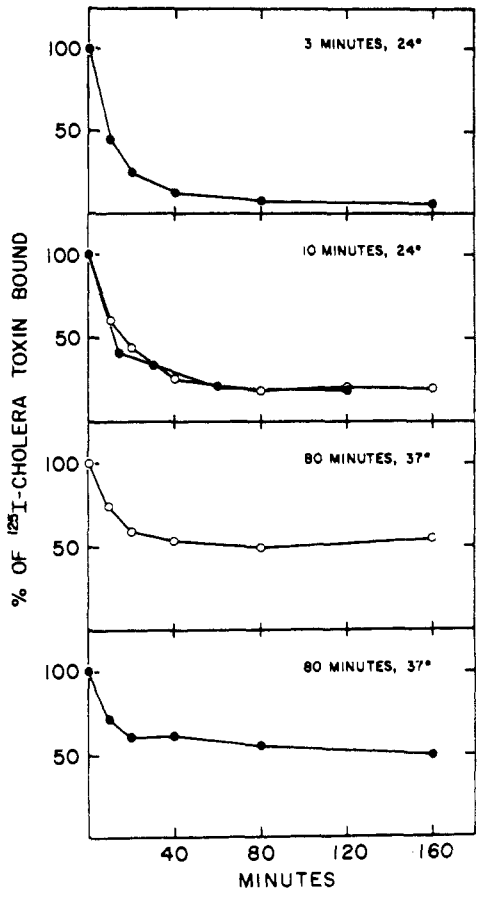

FIGURE 5: Effect of time and temperature of incubation of isolated fat cells (○) and liver membranes (●) with ^{125}I-labeled cholera toxin on the subsequent rate and extent of dissociation of the complex at 37°. Fat cells (about 5 × 10^6 cells) or liver membranes (210 μg of protein/ml) were incubated for various time periods at 24 or 37° in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% albumin and ^{125}I-labeled cholera toxin (10 ng, 12 μCi/μg). The dissociation reaction was begun by adding native cholera toxin (20 μg/ml) to all the samples and immediately incubating at 37°. At the indicated time intervals the specific binding of ^{125}I-labeled cholera toxin was determined by filtration procedures.

After a 10-min period of incubation at 24°, however, about 20% of the total membrane-bound radioactivity does not dissociate readily; the pattern of dissociation in this case is nearly identical for the fat cell- and liver membrane–toxin complexes (Figure 5). If fat cells or liver membranes are incubated for 80 min at 37° with ^{125}I-labeled cholera toxin an initial dissociative reaction is observed but about 50% of the toxin does not dissociate readily; under these conditions the dissociation pattern is again nearly the same for fat cells as for isolated liver membranes. It is clear from these studies that the rate, pattern, and extent of dissociation of the cholera toxin–cell complex are altered with increasing length and temperature of incubation.

Elution of Membrane-Bound Toxin. Serious attempts have been made to elute and isolate the radioactive cholera toxin from the cell membranes after varying periods of incubation of the toxin–cell complex. It was hoped that such studies might help to determine if with increasing length of incubation the toxin is chemically altered or is covalently bound to other macromolecules, and if the toxin eluted after prolonged exposure to fat cells is lipolytic without a lag phase.

Most (about 70%) of the radioactive cholera toxin which is bound to fat cells after an incubation period of 60 min at 37°

is recovered in the particulate fraction (40,000*g*, 20 min) of homogenates of thoroughly washed cells. Although the total amount of radioactive material present in such preparations is quite small, a more serious problem has been the inability to find conditions which are effective in eluting the toxin from the membranes which do not irreversibly denature the protein and which do not cause total dissolution of the membrane. The ^{125}I-labeled cholera toxin, for example, can be eluted from the cell membranes with 0.1 M acetic acid, 6 M urea, 5 M guanidine-HCl, or 1–2% sodium dodecyl sulfate. It is known, however, that after exposing low concentrations of native ^{125}I-labeled cholera toxin to these solvents the ability to bind to membranes or to elicit a biological response in fat cells is irreversibly lost regardless of whether the denaturant is removed by dialysis or whether its concentration is decreased by dilution (Cuatrecasas, 1973b).[3]

Various detergents have also been used in an attempt to dissociate the ^{125}I-labeled toxin–membrane complex. Triton X-100 and sodium deoxycholate, which at certain concentrations are effective in causing nearly complete dissolution of membrane proteins, are virtually without effect on the binding of ^{125}I-labeled cholera toxin to liver membranes (Table VIII). The use of such detergents to dissociate the membrane–toxin complex and to test the ability of the eluted toxin to bind again to fresh membranes is therefore of very limited value since it is not known that the toxin–receptor complex (in solution) is actually dissociated. Although sodium dodecyl sulfate appears to interfere with the binding of ^{125}I-labeled toxin, its effects on cholera toxin are apparently irreversible.

For the reasons just described it has not been possible to determine whether the membrane-bound ^{125}I-labeled toxin can, after elution, bind to fresh cells or membranes with the same properties as the native ^{125}I-labeled toxin, whether the eluted toxin is biologically active, or whether a lipolytic response could be elicited by such material without a lag phase. It has been determined that the ^{125}I-labeled cholera toxin which is eluted with 5 M guanidine-HCl from fat cell or liver membranes after a 90-min period of incubation of the complex at 37° migrates as a single radioactive band on sodium dodecyl sulfate disc gel electrophoresis; the behavior (mol wt ~10,000) of this material is identical with that of unused ^{125}I-labeled cholera toxin.[3] It thus appears very likely that cholera toxin does not form stable covalent linkages with membrane components, even after prolonged periods of incubation.

Effect of Toxin Binding in the Presence of Triton X-100 on the Lag Phase of the Lipolytic Response. A possible explanation for the lag phase in the lipolytic response to cholera toxin is that after the toxin binds to the membrane the toxin or toxin–ganglioside complex must undergo a major relocation within the structure of the membrane, possibly a penetration into its inner phase, or possibly a complete transmembrane transfer. The ability of cholera toxin to tolerate exposure to the nonionic detergent, Triton X-100, suggested a possible means of testing this if conditions could be found for exposing intact cells to this detergent at concentrations which might increase general membrane permeability but which would not adversely affect the lipolytic integrity of the cell. It would be important if under such conditions the lipolytic effect of cholera toxin were to be evident without a lag period.

Exposure of fat cells for 8 min at 24° to 0.5 and 0.1% Triton X-100 in Krebs–Ringer–bicarbonate buffer, 3% (w/v) albumin, followed by dilution of the cells and thorough washing results in a total loss of responsiveness of the cells to cholera toxin and to epinephrine and glucagon. The cells are severely damaged by such treatment. A similar treatment with 0.02% Triton X-100 only decreases by 30–40% the lipolytic response of the cells to cholera toxin. The response to the toxin is unaffected by exposing the cells for this brief period to 0.005% Triton X-100. Experiments were therefore performed in which fat cells were incubated for 8 min at 24° with 4 μg/ml of cholera toxin in the presence of 0.001, 0.005, and 0.02% Triton X-100. After thorough washing, the cells were incubated for various times at 37° in Krebs–Ringer–bicarbonate buffer, 3% (w/v) albumin, in the absence and presence of cholera toxin (0.15 μg/ml), to determine the rates of lipolysis of the cells. All the cell samples demonstrated equivalent lipolytic responses, the nature or length of lag phase was unchanged in the cells preincubated with the detergent and toxin, and the second addition of cholera toxin (after washing the cells) did not augment the lipolytic response in any of the samples. Thus, although cholera toxin was bound to the cells to a maximal degree in the presence of detergent concentra-

TABLE VIII: Effect of Exposure of Cholera Toxin to Detergents on the Ability of the Toxin to Bind to Liver Membranes.[a]

Conditions of Preincubation	Sp Binding of ^{125}I-Labeled Cholera Toxin (%)
No detergent	100
Triton X-100, 0.4%	96
2.0%	94
Sodium desoxycholate, 0.4%	100
2.0%	90
Sodium dodecyl sulfate, 0.4%	38
1.0%	30
2.0%	33

[a] ^{125}I-Labeled cholera toxin (7.6×10^8 cpm/ml; 10 μCi/μg) was preincubated at 24° for 60 min in Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and the indicated detergent. The samples were then diluted eightfold with the same buffer, and 20-μl aliquots were added to 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% albumin (w/v) and liver membranes (0.6 mg of protein/ml). Specific binding was determined after incubating the samples for 15 min at 24°. Aliquots (20 μl) of the detergent-treated ^{125}I-labeled toxin were also added to samples containing no liver membranes to determine if the presence of the detergent caused adsorption of the toxin to the Millipore filter. Triton X-100 and sodium deoxycholate caused no such adsorption, and the binding in the absence of liver membranes was less than 0.1% of the radioactivity in the medium. However, in the three sodium dodecyl sulfate samples given in the table the binding of ^{125}I-labeled toxin in the absence of membranes was 10, 22, and 26%, respectively, of the total radioactivity in the medium. The values given in the table have been corrected for this nonspecific binding. The binding of ^{125}I-labeled toxin was not appreciably altered in separate experiments in which the concentrations of Triton X-100 and deoxycholate in the final membrane-incubation medium were tenfold higher than those described here.

[3] P. Cuatrecasas, I. Parikh, and M. D. Hollenberg, manuscript in preparation.

tions which should have at least temporarily rendered the cells "leaky," the biological response and the tardiness of its appearance were not altered.

Studies with Tetanus Toxin. In view of the very potent binding of disialogangliosides and trisialogangliosides to tetanus toxin (van Heyningen and Miller, 1961; van Heyningen and Mellanby, 1968; van Heyningen, 1959), it was of interest to determine if some of the properties described in the present series of studies on cholera toxin would also be applicable to tetanus toxin. No lipolytic activity could be demonstrated by incubating fat cells at 37° for 150 min in Krebs–Ringer–bicarbonate buffer containing 3% (w/v) albumin and tetanus toxin in the concentration range of 16 mg/ml to 80 μg/ml.

The possibility was considered that perhaps the lack of lipolytic activity of tetanus toxin could result from the relative lack of the specific receptor gangliosides for this toxin on the fat cell membranes. Because of the demonstration that exogenous gangliosides can be spontaneously incorporated into intact fat cell membrane, and that such ganglioside-treated fat cells exhibit large lipolytic responses to concentrations of cholera toxin that in normal cells are ineffective (Cuatrecasas, 1973c), studies were performed to determine if preincubation of fat cells with various types of gangliosides could endow on these cells biological responsiveness to tetanus toxin. Fat cells were accordingly preincubated at 24° for 60 min with crude brain gangliosides (60 μg, 0.12 mg), and 0.5 mg/ml), ganglioside GD_{1a} (60 μg/ml), trisialoganglioside (2.5 μg/ml), or disialoganglio side (2.5 μg/ml). After thoroughly washing these cells by previously described procedures (Cuatrecasas, 1973c), the lipolytic responses to tetanus toxin (2–200 μg/ml) were examined. No significant stimulation of glycerol production could be demonstrated under all the conditions tested. Moreover, tetanus toxin (50 μg/ml) did not inhibit the lipolytic response of fat cells to L-epinephrine (1 μg/ml), and it did not modify the lag period or magnitude of the cholera toxin induced lipolysis in normal or ganglioside-treated fat cells.

Furthermore, preincubation of liver membranes for 5 min with concentrations of tetanus toxin between 8 μg and 0.4 mg/ml did not reduce the binding of ^{125}I-labeled cholera toxin (20 ng/ml) to these membranes. Thus, the specificity of binding of tetanus toxin does not significantly overlap with that of cholera toxin. Unfortunately, it has not yet been possible to examine directly the binding of iodinated tetanus toxin to membranes because of the unavailability of sufficiently pure toxin.

Discussion

The present studies have attempted to examine the possible mechanisms by which cholera toxin activates a lipolytic response in isolated fat cells, which is presumably a reflection of the ability of this toxin to stimulate the activity of adenylate cyclase in the cell membranes of a variety of tissues (Field, 1971; Sharp and Hynie, 1971; Kimberg *et al.*, 1971; Guerrant *et al.*, 1972; Schafer *et al.*, 1970; Chen *et al.*, 1971; Parkinson *et al.*, 1972; Evans *et al.*, 1972; Baker *et al.*, 1971).[2] Attempts in many laboratories to reproducibly demonstrate direct stimulatory effects of cholera toxin on isolated cell membranes or on other broken-cell preparations have so far failed. The effects of the toxin on the activity of this enzyme in subcellular fractions are demonstrable only if the intact tissue is incubated with the toxin at 37° for at least 1 hr before the tissue is homogenized. The inability to demonstrate direct effects of the toxin on subcellular preparations may be related to the occurrence, in all tissues so far examined (Sharp and Hynie, 1971; Vaughn *et al.*, 1970; Pierce *et al.*, 1971a,b; Carpenter *et al.*, 1968; Carpenter and Greenough, 1968; Kimberg *et al.*, Field *et al.*, 1968), of a characteristic lag period before the onset of the biological effect can be detected. Since the activity of adenylate cyclase in broken-cell preparations is relatively labile at higher temperatures, and cannot withstand incubation at 37° for more than 30 min, it has been most difficult to test directly the *in vivo* observations in subcellular fractions. Furthermore, since the present studies point to the requirement for temperatures greater than 24°, even very prolonged periods of incubation at 4°, where the enzyme activity is more stable, will probably not be adequate to demonstrate enzyme activation in isolated membranes. Since the activity of the toxin in a simple subcellular system has not yet been clearly demonstrated, and since the *in vivo* effects demonstrate an unusual lag phase, the possibility must be considered that the biochemical basis for the *in vivo* activity of the toxin may be mediated by more complicated events which require the metabolic integrity of the intact cell. In attempting to study this possibility, special attention must be given to the biological property of cholera toxin which is perhaps its most unique feature—the latency in its onset of action.

On the basis of the present as well as previous studies a number of possible explanations for the delay in onset or basis of action of cholera toxin can reasonably be excluded. It is quite clear that the continued presence of the toxin in the medium is not a necessary feature of cholera toxin action. Furthermore, the cell-bound toxin molecule is not slowly altered into an immediately active species which is released into the medium, from where it can exert its biological effects independently of the original cell-bound native molecules. The biological activity of the toxin appears to be exerted by the continual presence on the cell of those molecules which initially bind very rapidly and tightly to the cell membrane. Since the removal of the incubation medium after the lag period has passed does not alter the subsequent lipolytic response (Figure 1), it is apparent that the biological response is not generated by the production of lipolytic metabolites into the medium. This is supported by the finding that isolated, washed membrane preparations obtained from toxin-treated tissues exhibit elevated activities of adenylate cyclase (Sharp and Hynie, 1971; Kimberg *et al.*, 1971; Guerrant *et al.*, 1972; Chen *et al.*, 1972, 1971; Evans *et al.*, 1972; Parkinson *et al.*, 1972).[2] These observations exclude the possibility that the slow accumulation of metabolites in the medium or in the cell cytoplasm is responsible for either the lag phase or the actual stimulatory activity of the toxin.

Cholera toxin does not initially act by generally suppressing the lipolytic capacity of the cells since during the lag period the lipolytic responses of the cells to epinephrine and to glucagon are not modified (Table V). Thus, the possibility of a paradoxical response to a unique toxin-receptor interaction, characterized initially by an inhibition of adenylate cyclase activity, is unlikely. Furthermore, the possibility that simultaneous initial interactions with heterogeneous stimulatory and inhibitory receptors might initially produce a predominantly suppressive effect is similarly unlikely.

The lag phase of cholera toxin action is not apparently explained by a lack of saturation of cell binding sites or by the existence of simple diffusion barriers in the transmembrane transport of the toxin (Figure 3). Furthermore, incubation of fat cells in the absence of cholera toxin does not alter the nature of the lag phase which is observed upon subsequent addition of toxin, indicating that the lag phase is not simply a

period of time required for the synthesis or repair of components which may have been damaged during preparation of the cells.

If, as described above, the lag period does not reflect the time which is required to accumulate important metabolites, it might still represent the time period required for the synthesis of a critical component of the cell membrane. It is unlikely that the biosynthesis of gangliosides is a limiting factor since the binding of toxin to these components on the cell surface is immediate, since concentrations of toxin far below saturation result in detectable lipolytic responses with the characteristic lag period, and since the incorporation of biologically significant exogenous gangliosides can greatly enhance the lipolytic response of cholera toxin without altering the nature of the lag period. Also, it is unlikely that stimulation of prostaglandin synthesis is involved in the lag phase because of the unusually long period of time involved and because inhibitors of prostaglandin biosynthesis do not alter the lag phase or the actual response of the toxin (Table VI). These results also suggest that stimulation of adenylate cyclase activity by cholera toxin, when it does occur, is not mediated by prostaglandins. Kimberg *et al.* (1971) have come to similar conclusions by a very different experimental approach.

An important possibility which must be considered is that cholera toxin exerts its effects by increasing the synthesis of a membrane protein which is required for the subsequent enzymatic response to cholera toxin, or that it stimulates the synthesis of the enzyme adenylate cyclase itself. The former possibility, although feasible, is awkward since it requires that a selective process be established such that the binding of toxin to specific receptors stimulates intracellular biosynthetic steps of proteins which would be incorporated into the membrane, thus leading either to an increase in the basal enzymatic activity of the enzyme or to a transformation of the original toxin–receptor interaction into one that is effective in modifying the activity of the enzyme. The possibility that cholera toxin stimulates the synthesis of adenylate cyclase may be more plausible. The inability to modify the effect of cholera toxin by very high concentrations of inhibitors of RNA and protein synthesis (Table VII) makes both of these possibilities much less likely. Furthermore, if the total amount of adenylate cyclase protein in the membrane is increased as a result of the action of cholera toxin, it would be expected that the activity in the presence of NaF would also be greater in the treated compared to the control tissues. Sharp and Hynie (1971) have found that the NaF activity of the mucosa of rabbit intestine is actually somewhat decreased after treating with cholera toxin. Kimberg *et al.* (1971) studying guinea pig intestinal mucosa and Chen *et al.* (1972) studying human tissue also found no increase in the NaF-stimulated adenylate cyclase activity after exposure to cholera toxin, arguing strongly against a mechanism based on an increase in the synthesis of adenylate cyclase. Recent studies on the effects of the toxin on frog erythrocyte adenylate cyclase indicate that the kinetic and regulatory properties of the enzyme, rather than the total quantity of enzyme, are altered.[2]

A most unusual feature of the biological effects of cholera toxin is the specific pattern of the lag phase. Virtually no activity at all is detected during the latency phase, the delay is of very long duration, the onset of activation occurs very abruptly, and once activation of lipolysis begins its rate increases very sharply and it persists over a long period of time. The lag phase is clearly not due to a lack of sufficiently sensitive detection techniques. Since the toxin binds completely within a few minutes, it can be expected that if a simple chain of metabolic events leading to a critical end product were to be immediately initiated, at very early times a given steady-state amount of this material would have been formed and its effects should be detectable. Such considerations alone argue against a number of potential explanations of the action of cholera toxin, including those which suggest that the biosynthesis of important proteins (including adenylate cyclase) must intervene before activation of lipolysis becomes evident.

The ease with which the stimulation of lipolysis by cholera toxin is inhibited by insulin (Tables I and II) and by alloxan (Tables II and III) suggests that the mechanism of toxin activation of adenylate cyclase, once it occurs, may generally be very similar to that commonly observed with a variety of hormones such as epinephrine, glucagon, and adrenocorticotropic hormone. It is pertinent that insulin, which can inhibit adenylate cyclase activity in isolated membrane preparations (Illiano and Cuatrecasas, 1972; Hepp, 1971; Flawiá and Torres, 1973a,b; DeAsúa *et al.*, 1973), is quite effective in reversing the lipolytic effect of cholera toxin even if it is added to the cells after the lag phase has passed (Table II). It may thus be that the actual stimulation of enzyme activity occurs by relatively direct effects of the toxin–ganglioside complex on adenylate cyclase by processes analogous to those which occur in many other well-recognized hormonal systems. The major unusual feature of cholera toxin action might then be the lag phase, which would in effect represent a transition from an inactive toxin–receptor complex to an active one.

The hypothesis is advanced that the initial toxin–ganglioside complex at the cell surface is biologically inactive, and that subsequent changes occur spontaneously by special membrane rearrangements which convert the complex into an active form. The active form of the complex would modulate the activity of adenylate cyclase (and possibly other membrane processes) by mechanisms presently unknown but perhaps similar to those of well-known hormonal systems. The transition from an inactive to an active receptor complex would, by virtue of the length of time, temperature dependence, and very abrupt nature of the lag phase, involve a very slow process for which a substantial "barrier" exists. It is very pertinent that choleragenoid, a protein derived from cholera toxin, appears to bind to exactly the same receptors as the toxin and its binding properties are nearly identical, yet this protein is biologically inactive, and it is thus a potent competitive antagonist of the toxin (Cuatrecasas, 1973d). Choleragenoid presumably forms the same initially inactive receptor complex and lacks the properties required for the subsequent transition to an active state. It is suggested that the transition may primarily represent a structural reorganization of the complex within the membrane. The conversion of an inactive to an active complex would thus not represent a change in the chemical nature of the toxin–receptor interaction or complex, but would rather represent a change in the nature of the interaction of the complex itself with other membrane structures. Considerable evidence exists which indicates that various kinds of molecules in biological membranes are relatively free to move, diffuse, or migrate laterally within the two-dimensional plane of the membrane (Singer and Nicolson, 1972). Examples are known in which cell surface proteins (*e.g.*, immunoglobulins) spontaneously rearrange, form patches, aggregate, and grossly migrate over large areas of the membrane upon special kinds of external perturbations (Singer and Nicolson, 1972). It is reasonable to suggest that the interaction of cholera toxin with membrane-bound gangliosides would substantially change or perturb the natural position or behavior of those membrane gangliosides and lead spontaneously to their relocation in

new, more thermodynamically favored locations. The complex in its newly acquired membrane location might effectively perturb new vicinal structures, such as adenylate cyclase. The length of the lag period may be in part explained by the fact that the total number of toxin molecules bound per cell is very small (10^4 or less). The effect of temperature may be related to special phase transitions of the membrane lipids. In addition, the existence of a true lag phase followed by an accelerating rate of modification of adenylate cyclase may suggest that the toxin may be initially affecting a process which is not rate limiting in the overall sequence of events leading to activation of the enzyme. This could occur, for example, if the activated toxin complex were to somehow neutralize or inactivate membrane components (inhibitory to adenylate cylase) which are normally present in excess quantity.

In this general scheme the structural transition of the complex within the membrane could result from major morphologic reorganizations of large regions of the cell membrane. The transition might also represent a relocation of the complex from an external to an internal position on the cell membrane, which is a change which would be difficult to initiate and therefore may be sufficiently slow to explain the qualitative nature of the lag phase; once this transitional barrier was overcome, however, the inner localization of the complex could be very stable. The outer-to-inner membrane transition could occur either by a flip-flop mechanism or by more conventional means such as membrane invaginations and reorganizations.

The striking changes in the extent to which the cholera toxin–membrane complex can dissociate with increasing time and temperature of incubation (Figures 4 and 5), the apparent absence of covalent linkages between the toxin and membrane components even after prolonged incubation, and the lack of alternative, reasonable explanations for rationalizing the available data indicate that the hypotheses described above must be examined carefully. Studies are under way to experimentally test these possibilities by freeze etching and other morphological tools applicable to the study of cell membranes.[4]

It is of interest that the action of diptheria toxin on cells is also characterized by a substantial, true lag phase (Gill *et al.*, 1973). It is known that this toxin very slowly traverses the cell membrane, gaining access into the cytoplasm where it inactivates elongation factor 2 and thus inhibits protein synthesis. The length of the lag phase is presumably explained by the relatively slow rate of entrance into the cell while the true nature of the lag phase is probably explained by the fact that elongation factor 2 normally exists in excess of that required for protein synthesis.

The inability of tetanus toxin, a protein known to interact very strongly with di- and trisialogangliosides (van Heyningen, 1959; van Heyningen and Miller, 1961; van Heyningen and Mellanby, 1968), to compete with cholera toxin for binding or to elicit lipolytic or antilipolytic responses even in ganglioside-treated cells suggests that the activity of cholera toxin is dependent on highly specific interactions of this toxin with monosialogangliosides of the cell membrane. Thus, the biological activity of cholera toxin probably cannot be mimicked simply by perturbing membrane gangliosides.

The ability of insulin and alloxan to reverse the lipolytic effects of cholera toxin suggests that these compounds may be of therapeutic value in clinical cholera infection. In this respect insulin may be especially useful in view of its potency, its effectiveness during all phases of the lipolytic response, and the relative lack of systemic manifestations upon its administration by the oral route. From the tenacious and protracted nature of the cell–toxin interaction described here it can be anticipated that effective reversal of the toxic effects of cholera toxin will require interruption of the ongoing biological effects of the toxin in addition to elimination of the free toxin in the medium.

[4] In collaboration with Dr. Vincent T. Marchesi.

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Vibrio cholerae Choleragenoid. Mechanism of Inhibition of Cholera Toxin Action†

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ABSTRACT: Choleragenoid, a biologically inactive protein which is derived from the exotoxin of *Vibrio cholerae*, blocks the binding of ^{125}I-labeled cholera toxin to liver membranes as effectively as the parent toxin. The binding of ^{125}I-labeled cholera toxin to membranes which have been incubated with choleragenoid and subsequently washed is suppressed to the same extent as when the membranes are incubated with cholera toxin. The rate of dissociation of the cholera toxin–membrane complex is the same whether it is measured in the presence of cholera toxin or of choleragenoid. Choleragenoid does not elicit a lipolytic response in isolated fat cells. When the cells are preincubated for 15 min at 24° with choleragenoid, there is potent inhibition of cholera toxin induced lipolysis. The concentration of choleragenoid required to inhibit by 50% the lipolytic response of 0.2 μg/ml of cholera toxin is about 40 ng/ml (7×10^{-10} M). Choleragenoid exhibits virtually no inhibitory effect if the fat cells are incubated (15 min, 24°) first with cholera toxin. The ability of choleragenoid to block the lipolytic activity of cholera toxin is explicable in terms of its ability to block the binding of the toxin to the membrane receptor by its own occupancy of that receptor. Cholera toxin and choleragenoid are equally active in blocking the binding of ^{125}I-labeled choleragenoid to liver membranes. Preincubation of ^{125}I-labeled choleragenoid with gangliosides blocks the ability of the iodoprotein to bind to membranes. Incubation of membranes with gangliosides (followed by washing of the membranes) greatly increases the binding sites on the membrane for choleragenoid. The present studies indicate that cholera toxin and choleragenoid interact with the same receptor (probably gangliosides) on the cell surface, and that the properties of this interaction are very similar if not identical for both proteins. Choleragenoid is thus a structural analog of cholera toxin which appears to bind to the receptor with unaltered affinity but which produces a biologically unproductive complex with the receptor. It is suggested that the choleragenoid– and cholera toxin–receptor complexes as initially formed are both very similar and inactive, and that the toxin– but not the choleragenoid–receptor complex is capable of undergoing a subsequent rearrangement within the structure of the membrane which converts it into a biologically active form.

In the process of purification of an active exotoxin from *Vibrio cholerae*, Finkelstein and LoSpalluto (1969, 1970) have purified another protein which is immunologically identical with the toxin but which is lacking in biological activity in intestinal (Finkelstein and LoSpalluto, 1969, 1970) and fat cell (Vaughan *et al.*, 1970) preparations. This protein, which has been called choleragenoid (Finkelstein and LoSpalluto,

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