18, 1341.
Simpson, L. L., and Rapport, M. M. (1971b), *J. Neurochem. 18*, 1751.
Spence, M. W. (1969), *Can. J. Biochem. 47*, 735.
Spiro, R. G. (1962a), *J. Biol. Chem. 237*, 382.
Spiro, R. G. (1962b), *J. Biol. Chem. 237*, 646.
Spiro, R. G., and Spiro, M. J. (1965), *J. Biol. Chem. 240*, 997.
Suzuki, K. J. (1965), *J. Neurochem. 12*, 629.
Svennerholm, L. (1964), *J. Lipid Res. 5*, 145.
van Heyningen, W. E. (1959), *J. Gen. Microbiol. 20*, 310.
van Heyningen, W. E., Carpenter, W. B., Pierce, N. F., and Greenough, W. B. III (1971), *J. Infect. Dis. 124*, 415.
van Heyningen, W. E., and Mellanby, J. (1968), *J. Gen. Microbiol. 52*, 447.
van Heyningen, W. E., and Miller, P. A. (1961), *J. Gen. Microbiol. 24*, 107.
Vaughn, M., Pierce, N. F., and Greenough, W. B. III (1970), *Nature (London) 226*, 658.
Zieve, P. D., Pierce, N. F., and Greenough, W. B. (1970), *Clin. Res. 18*, 690.

Gangliosides and Membrane Receptors for Cholera Toxin†

Pedro Cuatrecasas‡

ABSTRACT: Brain gangliosides and the glycoprotein fetuin inhibit the lipolytic response of fat cells to cholera toxin but not to epinephrine. The ability of various concentrations of ganglioside G_{M1} to inhibit the binding of ^{125}I-labeled cholera toxin to fat cells or liver membranes parallels the inhibition of toxin-induced lipolysis by G_{M1}. During prolonged periods of incubation the ganglioside inhibition of toxin binding is reversed and binding can then exceed that observed in the absence of gangliosides. When isolated fat cells or liver membranes are preincubated with gangliosides and then washed, there is a very large increase in the binding of cholera toxin to these tissues. This increased binding is due primarily to an increase in the total number of binding sites for the toxin. The rates of association of toxin with normal and with ganglioside-treated tissues are identical, but the rate of dissociation of the complex is slightly lower in the ganglioside-treated membranes. The presumed ability of gangliosides to become spontaneously incorporated into membranes is a rapid process which is temperature dependent. Treatment of erythrocytes with gangliosides also results in an increase in toxin binding to these cells; this new property of the erythrocytes is retained for at least 24 hr at 24°, indicating that the ganglioside–membrane complex is very stable. Ganglioside-treated fat cells demonstrate a greatly increased sensitivity toward the lipolytic effects of cholera toxin but not to L-norepinephrine. The lipolytic effect of concentrations of the toxin which in normal cells have minimal effects is increased tenfold by treating cells with gangliosides. This effect of gangliosides is to increase the sensitivity but not the maximal lipolytic response to cholera toxin. The biological response is proportional to the number of toxin molecules specifically bound to the cells, and this number can be increased either by raising the toxin concentration in the medium or by increasing the number of receptors per cell by treatment with gangliosides. Addition of free gangliosides to the medium does not effectively reverse the already formed toxin–membrane complex, in part because of the ability of gangliosides to be incorporated rapidly into membranes. The evidence indicates that gangliosides can be spontaneously incorporated into membranes in a manner which creates new and stable binding sites for cholera toxin. These sites are kinetically similar to the normal toxin receptors, and their interaction with toxin leads to identical biological effects. Gangliosides thus appear to be the normal membrane receptors for cholera toxin. These receptors can be experimentally manipulated to study the nature of the toxin–receptor interaction and its relationship to activation of the biological response.

Methods have recently been described (Cuatrecasas, 1973b) for measuring directly the interaction between intact fat cells or membranes from liver and intestinal cells and a purified exotoxin from *Vibrio cholerae*. This enterotoxin, which is responsible for the gastrointestinal manifestations of clinical cholera (Finkelstein and LoSpalluto, 1969; LoSpalluto and Finkelstein, 1972; Carpenter, 1971; Pierce *et al.*, 1971; Burrows, 1968; Finkelstein, 1969), is thought to act by increasing the intracellular concentration of cyclic 3′,5′-adenosine monophosphate[1] (Sharp and Hynie, 1971; Kimberg *et al.*, 1971; Guerrant *et al.*, 1972; Schafer *et al.*, 1970; Chen *et al.*, 1971, 1972; Evans *et al.*, 1972; Parkinson *et al.*, 1972). Cholera toxin has been found to stimulate adenylate cyclase activity in all tissues examined to date (Pierce *et al.*, 1971; Field, 1971). The general properties of the toxin–membrane interaction do not appear to depend in an important way on the specific tissue which is utilized for study (Cuatrecasas, 1973b). Binding of ^{125}I-labeled cholera toxin to membranes occurs very rapidly and is quite extensive even at concentra-

† From the Department of Medicine and the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205. *Received January 23, 1973.* Supported by grants from the National Science Foundation (GB-34300), the American Cancer Society (BC-63), the National Institute of Arthritis and Metabolic Diseases (AM14956), and The Kroc Foundation. Part of this work was reported at the 65th Annual Meeting of the American Society for Clinical Investigation (Cuatrecasas, 1973a).

‡ Recipient of U. S. Public Health Service Research Career Development Award AM31464.

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

tions of the toxin lower than 10^{-10} M. The toxin can bind to certain glycoproteins, such as fetuin or thyroglobulin, but not to proteolytic digests or purified glycopeptides from these proteins. Prior incubation of the toxin with very low concentrations of certain gangliosides, especially ganglioside G_{M1}, blocks the ability of the toxin to subsequently bind to membranes, and extraction of membrane gangliosides destroys the ability of the membranes to bind cholera toxin (Cuatrecasas, 1973b). These studies suggested that membrane gangliosides may be the natural cellular receptors for this toxin.

The present studies explore in more detail the interrelationships between the cholera toxin–ganglioside interaction, the binding of the toxin to membranes, the ganglioside content of the membrane, and the biological activity of the toxin. It is demonstrated that prior incubation of toxin with gangliosides inhibits in parallel the ability of the toxin to bind to cells and to activate a biological response in these cells. Furthermore, it is demonstrated that isolated fat cells and membranes into which exogenous gangliosides have been spontaneously incorporated exhibit greatly enhanced binding of cholera toxin and a much greater sensitivity to the biological effects of the toxin. These studies strongly suggest that gangliosides are the membrane receptors which are responsible for binding the toxin as well as for the subsequent biological actions of the toxin.

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. Fetuin, L-norepinephrine, and bovine brain ganglioside G_{M1} preparations were kindly provided by Dr. H. Sloan and by Dr. R. Brady, National Institutes of Health.

Procedures. ^{125}I-Labeled cholera toxin (5–20 μCi/μg) was prepared as previously described (Cuatrecasas, 1973b). The procedures used to detect the binding of ^{125}I-labeled cholera toxin to cells and membranes have been described (Cuatrecasas, 1973b). The molecular weight of cholera toxin was assumed to be 84,000 and the $E^{1\%}_{1\,cm}$ (280 nm) 11.41 (LoSpalluto and Finkelstein, 1972). Isolated fat cells were prepared from Sprague-Dawley rats (90–120 g) by the method of Rodbell (1966). Lipolysis was studied 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.

Results

Inhibition by Gangliosides of Toxin Binding and Lipolysis. The lipolytic response of isolated fat cells to cholera toxin, unlike the response of these cells to L-norepinephrine, is (Greenough *et al.*, 1970; Vaughn *et al.*, 1970) characterized by a lag period of about 60 min when the cells are incubated at 37° (Figure 1). Prior incubation of the toxin with brain gangliosides, as described earlier (van Heyningen *et al.*, 1971), profoundly decreases the lipolytic response to the toxin. In contrast, gangliosides have little or no effect on the lipolysis

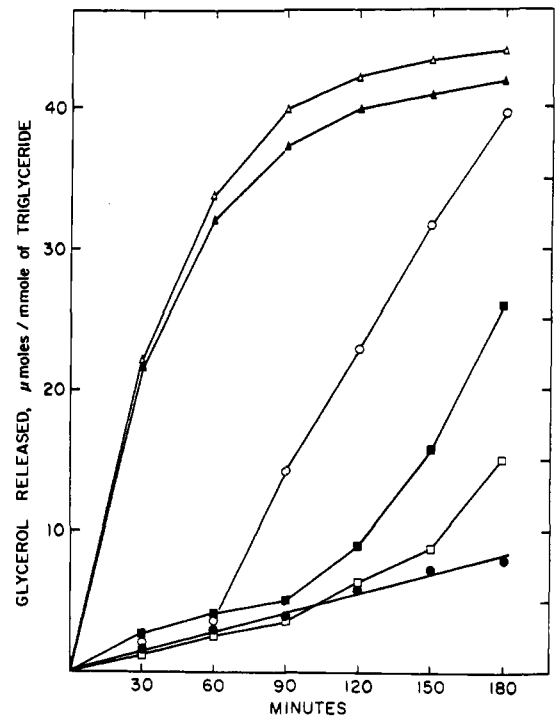

FIGURE 1: Effect of bovine brain gangliosides and fetuin on cholera toxin and on L-epinephrine stimulated lipolysis in isolated fat cells. Fat cells were incubated at 37° in Krebs–Ringer–bicarbonate buffer containing 3% (w/v) albumin, in the absence of additional compounds (●) or with 3 μg/ml of cholera toxin (○), 3 μg/ml of cholera toxin plus 0.1 mg/ml of gangliosides (□), 3 μg/ml of cholera toxin plus 0.4 mg/ml of fetuin (■), 3 μg/ml of L-epinephrine (△), and 3 μg/ml of L-epinephrine plus 0.1 mg/ml of gangliosides (▲). The cholera toxin was preincubated with gangliosides at 24° for 20 min before addition to the cells. Fetuin was added to the cells 5 min (24°) before the addition of cholera toxin. At the concentrations used here fetuin and gangliosides in the absence of cholera toxin did not significantly alter lipolysis.

induced with L-norepinephrine. Fetuin is also able to decrease the lipolytic response of fat cells to cholera toxin, in accord with the observation (Cuatrecasas, 1973b) that this glycoprotein prevents the binding of ^{125}I-labeled toxin to cells and membranes.

The inhibition of toxin action by gangliosides and fetuin is most pronounced during the first 2 hr of the lipolytic response (Figure 1). With increasing periods of incubation there is an apparent reversal of inhibition which is more marked for the fetuin samples than it is for the ganglioside-treated samples. This is consistent with a time-dependent, progressive dissociation of the fetuin- and ganglioside–toxin complexes with concomitant formation of cell–toxin complexes. This point will be examined in more detail later in this report. With concentrations of ganglioside higher than those used in the experiments described in Figure 1 it is possible to demonstrate that no reversal of inhibition occurs during a 200-min period of incubation at 37°. Similarly, if the very potent inhibitory ganglioside, G_{M1} (Cuatrecasas, 1973b), is used it is very difficult to demonstrate reversal of the inhibitory effect during the course of the incubation.

Purified ganglioside G_{M1} is very effective in blocking the binding of ^{125}I-labeled cholera toxin to liver membranes (Figure 2). Under the conditions of the experiment described in Figure 2, in which the concentration of toxin is 10^{-9} M, the final concentration of G_{M1} required to achieve half-maximal inhibition of binding is about 7×10^{-9} M (11 ng/ml). The concentrations of ganglioside G_{M1} required to block the

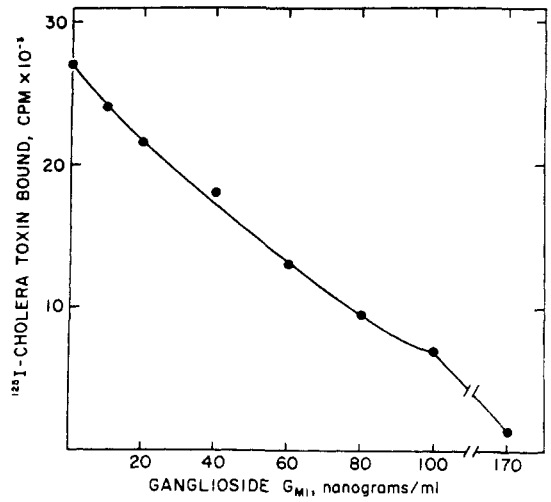

FIGURE 2: Effect of incubating cholera toxin with G_{M1} ganglioside on the specific binding of the toxin to liver membranes. ^{125}I-Labeled cholera toxin (100 ng, 3.5×10^5 cpm) was incubated in 0.2 ml of Krebs–Ringer–bicarbonate buffer, 0.1% albumin, for 60 min at 24° with G_{M1} at the indicated concentration. A portion (50 μl) of this incubation was then added to 0.2 ml of the same buffer containing 40 μg of membrane protein. The actual concentration of the ganglioside in the membrane-containing sample was therefore five times lower than that indicated in the figure. After incubating for 15 min at 24° the specific binding to membranes was determined (Cuatrecasas, 1973b) as described in the text.

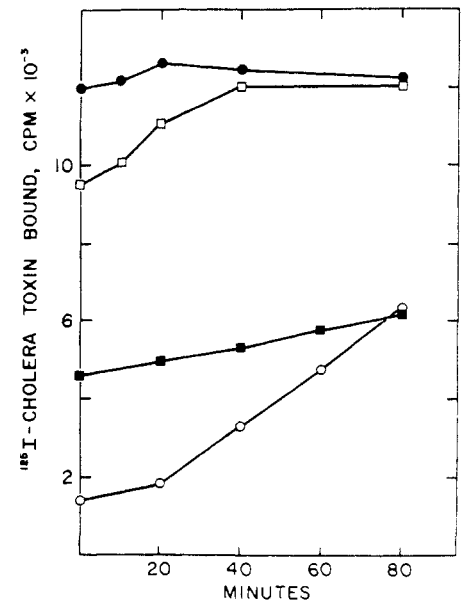

FIGURE 3: Effect of bovine brain gangliosides, fetuin, and galactose on the time course of binding of cholera toxin to liver membranes at 37°. Liver membranes (50 μg of protein) were incubated in 0.2 ml of Krebs–Ringer–bicarbonate buffer, 0.2% (w/v) albumin, for 5 min at 24° without additions (●) or with 0.5 mg/ml of gangliosides (○), 1 mg/ml of fetuin (■), and 10 mg/ml of D-galactose (□). ^{125}I-Labeled cholera toxin (5 ng, 2.2×10^4 cpm) was added to all the samples and specific binding was determined (Cuatrecasas, 1973b) after incubating for 3 min at 24° (zero time). The samples were then incubated at 37° for varying time periods.

binding of ^{125}I-labeled toxin to intact fat cells are very similar to those described in Figure 2 for liver membranes, although even slightly lower concentrations of the ganglioside are effective in this tissue.

Ganglioside G_{M1} is as effective in blocking the lipolytic effect of the toxin (Table I) as it is in inhibiting its binding to cell membranes. Despite the differences in the nature of the conditions utilized in the membrane-binding and lipolytic studies, the concentration range of G_{M1} required to observe inhibition is quite similar in both types of studies. Under the conditions described in Table I, a concentration of G_{M1} of 10^{-8} M completely inhibits the lipolytic response of 2×10^{-9} M cholera toxin. The slightly greater inhibitory effectiveness of G_{M1} in fat cells compared to liver membranes is probably a reflection of the much greater content of toxin-binding sites in the liver membranes compared to the fat cells (Cuatrecasas, 1973b). Because of the inherent complexities of a system in which, as will be described, the nature of the interactions is changing with time, it is not possible with the data presented here to calculate meaningful dissociation constants for the interactions measured or to apply kinetic interpretations which are based on equilibrium assumptions.

TABLE I: Inhibition of Cholera Toxin Induced Lipolysis in Isolated Fat Cells by Ganglioside G_{M1}.[a]

Addition	Glycerol Released[b] 30 min	60 min	120 min
None	20 ± 4	37 ± 6	74 ± 7
Cholera toxin, 0.26 μg/ml	22 ± 4	90 ± 7	340 ± 18
Ganglioside G_{M1}, 50 ng/ml	21 ± 3	44 ± 7	82 ± 8
Cholera toxin, 0.26 μg/ml			
+ ganglioside G_{M1}, 50 ng/ml	20 ± 4	42 ± 3	77 ± 9
+ ganglioside G_{M1}, 12.5 ng/ml	22 ± 5	40 ± 5	75 ± 8
+ ganglioside G_{M1}, 2.5 ng/ml	19 ± 4	78 ± 7	260 ± 10
+ ganglioside G_{M1}, 0.5 ng/ml	23 ± 4	95 ± 5	349 ± 15

[a] Cholera toxin (13 μg/ml) was preincubated for 60 min at 24° with varying concentrations of ganglioside G_{M1} in Krebs–Ringer–bicarbonate buffer containing 0.1% albumin. Samples of these incubations were then added to fat cell suspensions, which were incubated for various periods of time at 37° in Krebs–Ringer–bicarbonate buffer containing 3% (w/v) albumin. The concentration described for each addition is that present in the final cell incubation medium. [b] Micromoles of glycerol released per millimole of triglyceride.

Incorporation of Gangliosides into Membranes and the Binding of Toxin. In previous experiments the binding of ^{125}I-labeled cholera toxin to cells or membranes has been examined after a 15- to 20-min period of incubation since it has been demonstrated that in the absence of compounds which can compete with the cells or membranes for the binding of toxin an apparent equilibrium is generally achieved within a few minutes (Cuatrecasas, 1973b). However, it was important to examine the changes in binding which occur during a prolonged period of incubation at 37° in the presence of compounds which inhibit binding, especially in view of the spontaneous reversal of the inhibition of lipolysis which is observed (Figure 1) in fat cells incubated with fetuin or ganglioside. As anticipated earlier, the depression in toxin binding observed when fetuin or ganglioside is present in the medium, or that which can be caused by extremely high concentrations of D-galactose, is spontaneously reversed during prolonged incubation at 37° (Figure 3). The reversal with D-galactose is rapid and complete, as expected from the very weak affinity of this sugar for cholera toxin. It was peculiar, however, that under certain conditions the rate of recovery from ganglioside inhibition was much faster than the rate of recovery from fetuin

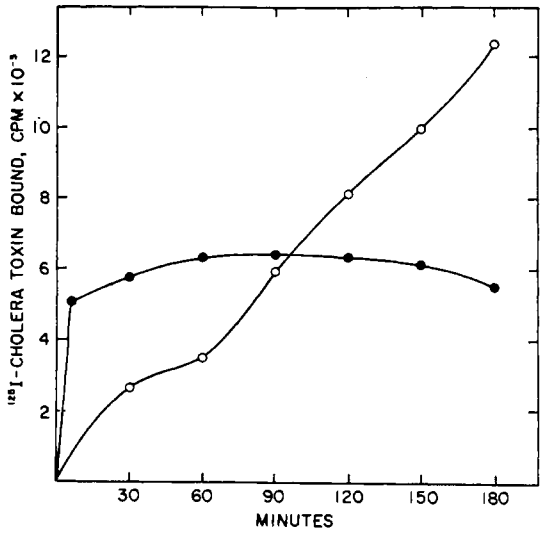

FIGURE 4: Effect of gangliosides on the time course of binding of cholera toxin to isolated fat cells. Fat cells (about 4 × 10^5 cells) were incubated for varying times at 37° in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 3% (w/v) albumin, ^{125}I-labeled cholera toxin (1.3 × 10^5 cpm, 12 ng), and in some cases (○) 5 μg/ml of bovine brain gangliosides. Gangliosides were added to the cells 5 min (24°) before addition of ^{125}I-labeled cholera toxin.

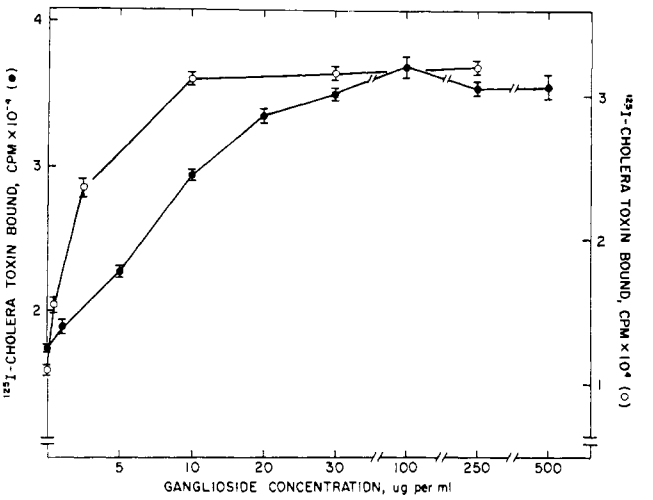

FIGURE 5: Effect of preincubating isolated fat cells (○) and liver membranes (●) with increasing concentrations of bovine brain gangliosides on the specific binding of cholera toxin. Fat cells (2 × 10^6 cells/ml) and liver membranes (1 mg of protein/ml) were preincubated at 24° for 60 min in Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and the indicated concentration of gangliosides. The cells were washed four times with 8 vol of the same buffer (without gangliosides). The membranes were diluted tenfold, centrifuged at 35,000*g* for 20 min, and resuspended in the same buffer. Specific binding of ^{125}I-labeled cholera toxin (4.5 × 10^4 cpm) was determined as described in the text after incubating the membranes (30 μg of protein) and fat cells (2.5 × 10^5 cells) at 24° for 15 min in 0.2 ml of Krebs–Ringer–bicarbonate buffer, 0.1% (w/v) albumin.

inhibition, despite the fact that the binding of toxin to ganglioside is much stronger than that to fetuin, as evidenced in this experiment (Figure 3) by the more profound initial depression of binding in the presence of ganglioside.

This unexpectedly rapid reversal of toxin binding in the presence of ganglioside can also be observed in isolated fat cells (Figure 4). It is apparent here that under certain conditions prolonging the incubation for periods longer than 90 min can result in the binding of much greater quantities of toxin to the cells which are incubated in the presence of gangliosides compared to those incubated in the absence of gangliosides.

This paradoxical reversal and the enhancement of toxin binding with prolonged incubation in the presence of gangliosides are dependent on the presence of a large quantity of membranes or cells relative to the concentration of ^{125}I-labeled toxin and on the presence of relatively low concentrations of gangliosides.

The results of the above experiments clearly indicated that the gangliosides in the medium were not acting simply by binding the toxin and thus restricting its availability to the cells. To explain the enhancement in binding the gangliosides must in addition be acting by modifying the toxin in the medium, by altering the properties of the cell membrane in a way that facilitates toxin binding, or by promoting the insertion of preformed, intact toxin–ganglioside complexes into the membrane. It was possible to readily distinguish between these possibilities by performing experiments in which the binding of ^{125}I-labeled toxin to fat cells and membranes was studied after these tissues were incubated with gangliosides and then washed to remove the gangliosides from the medium before measuring toxin binding. It is clear that following such a simple preincubation of fat cells or liver membranes with gangliosides there is a very substantial increase in the quantity of toxin which is bound to these tissues (Figure 5). The binding of ^{125}I-labeled toxin to ganglioside-treated and washed cells or membranes is complete within minutes and, as with the untreated tissues, the steady-state binding of ^{125}I-labeled toxin does not change appreciably during a prolonged period of incubation at 37°. Furthermore, the ability of native cholera toxin to displace the increased ^{125}I-labeled toxin binding of the ganglioside-treated tissues is very similar to that observed with untreated tissues (Cuatrecasas, 1973b); more than 95% of the radioactivity can be displaced by preincubating the tissues for 3 min with native toxin (5 μg/ml) before addition of the ^{125}I-labeled toxin.

In the experiment described in Figure 5 the nearly threefold increase in the binding of toxin to liver membranes which is observed with the highest ganglioside concentration used does not reflect saturation of the potential toxin binding sites on the membrane since nearly all of the radioactivity (about 90%) present in the medium which can potentially bind has complexed to the membranes. Addition of fresh membranes does not result in additional binding of any of the residual radioactivity in the medium. The binding of toxin to fat cells in this experiment, however, is not similarly limited by the amount of toxin in the medium, suggesting that the ganglioside molecules which can be properly incorporated into the membrane are finite in number. It is also of interest that the membrane incorporation of gangliosides in a manner detectable by an enhancement in toxin binding occurs with lower ganglioside concentrations in fat cells compared with liver membranes (Figure 5).

Although the apparent incorporation of gangliosides into cells and membranes is dependent on the time and temperature used in the incubation, the maximal effects occur very rapidly (Table II). Even at 4° the process is complete in about 30 min.

Nature of the Increased Binding of Toxin to Ganglioside-Treated Tissues. The simplest explanation for the above observations is that exogenous gangliosides can be spontaneously incorporated into the membrane in a way which effectively retains their capacity to bind the toxin in the medium. However, the data so far presented do not clearly describe whether the increase in the binding of toxin to ganglioside-

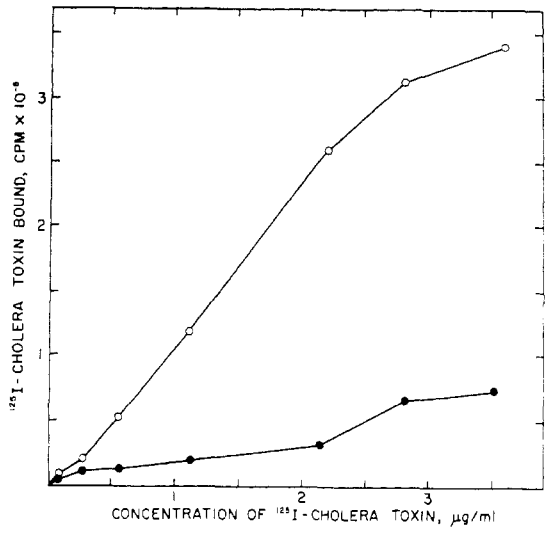

FIGURE 6: Effect of increasing the concentration of cholera toxin on the binding of the toxin to ganglioside-treated liver membranes. Liver membrane suspensions (3 mg of protein/ml) were incubated for 50 min at 24° in Krebs–Ringer–bicarbonate buffer, 0.1% (w/v) albumin, in the absence (●) and presence (○) of 0.1 mg/ml of bovine brain gangliosides. The suspensions were then diluted tenfold with the same buffer (without gangliosides) and centrifuged for 20 min at 30,000*g*. The pellets were resuspended in the same buffer, and 0.2-ml fractions containing 14 μg of membrane protein were incubated for 15 min at 24° with varying concentrations of ^{125}I-labeled cholera toxin (2 μCi/μg).

TABLE II: Effect of Preincubating Isolated Fat Cells and Liver Membranes with Gangliosides on the Enhancement of Binding of Cholera Toxin.[a]

Conditions of Preincubation	Specific Binding of ^{125}I-Labeled Cholera Toxin (cpm)	
	Fat Cells	Liver Membranes
No gangliosides, 4°, 20 min	5,700 ± 200	17,000 ± 400
24°, 60 min	5,800 ± 200	17,200 ± 600
37°, 60 min	5,200 ± 100	16,800 ± 500
Gangliosides, 4°, 10 min	9,100 ± 200	21,000 ± 900
4°, 30 min	14,200 ± 600	29,400 ± 700
4°, 60 min	15,400 ± 200	28,800 ± 500
24°, 10 min	21,600 ± 500	35,600 ± 600
24°, 60 min	20,800 ± 400	36,400 ± 900
37°, 60 min	22,400 ± 700	38,300 ± 200

[a] Liver membranes (3 mg of protein/ml) and isolated fat cells (5×10^5 cells), suspended in Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin, were preincubated for varying periods at different temperatures with and without 200 μg/ml of bovine brain gangliosides. The membrane suspensions were then diluted tenfold and centrifuged at 36,000*g* for 20 min, and the pellets were resuspended in Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin. The fat cells were washed four times with 5 vol of the same buffer. The specific binding of ^{125}I-labeled cholera toxin (30 ng/ml, 10 μCi/μmole) to 0.2 ml of liver membranes (0.20 mg of protein/ml) and to 0.2 ml of fat cells (about 1×10^6 cells/ml) was determined as described in the text.

treated tissues results from an increase in the total number of sites available for the toxin or from an increase in the affinity for the toxin of the already existing sites. From experiments in which the binding of toxin is examined as a function of the concentration of the toxin it is readily apparent that in ganglioside-treated membranes there is a very substantial increase in the total number of binding sites for cholera toxin (Figure 6). As described earlier (Cuatrecasas, 1973b), a saturable binding process in the range of about 1 μg of toxin/ml is seen in untreated liver membranes. In the treated membranes this plateau of binding disappears. The differences in toxin binding between the untreated and treated membranes are therefore much more striking if the concentration of toxin used to detect binding is greater than 0.4 μg/ml. Under the experimental conditions described in Figure 6, where the concentration of membrane protein is very low so that at virtually no concentration of toxin tested is the amount of toxin in the medium limiting, the treated membranes bind seven–eight times more toxin than the native membranes at concentrations of ^{125}I-labeled toxin greater than 1 μg/ml.

Despite the obvious existence of a large increase in the total number of binding sites for toxin in ganglioside-treated tissues, it was of interest to determine if such tissues also exhibit at least some gross changes in the apparent affinity of the toxin–membrane complexes. Because of the inherent difficulties in calculating accurate dissociation constants in such a complex system by measurements which depend on the achievement of equilibrium states, the approximate relative affinities were experimentally assessed by measurements of the rates of formation and of dissociation of the toxin–membrane complexes in ganglioside-treated and untreated membranes.

As described earlier, the rate of binding of cholera toxin to membranes is very fast in the native and in the ganglioside-treated membrane. Even when the concentration of the membrane or of the toxin was reduced appreciably in order to permit comparative measurements of the rapidity of binding, no discernible differences were observed between the native and treated membranes. Small differences, however, could be detected in the rates of spontaneous dissociation of the membrane–toxin complexes (Figure 7). The rate of dissociation of the toxin from the ganglioside-treated membranes is somewhat slower than that observed with the untreated membranes. These data indicate that the affinity of cholera toxin for membranes treated with exogenous gangliosides is at least as great, and probably somewhat greater, than the affinity of the toxin for native membranes. Although the very large increase in the binding of toxin to ganglioside-treated tissues appears to result primarily from an increase in the total number of toxin-binding sites, a somewhat enhanced affinity of the complex may also be a contributing factor.

Heightening the Biological Response to Cholera Toxin by Treating Cells with Gangliosides. It is pertinent that the sensitivity of the lipolytic response of isolated fat cells to cholera toxin can be markedly enhanced by prior treatment of the cells with crude brain gangliosides and with ganglioside G_{M1} (Figure 8). By simply incubating the cells at 24° for 30 min with 3 μg/ml of ganglioside G_{M1} or with 0.1 mg/ml of crude brain gangliosides, followed by thorough washing of the cells to remove the excess ganglioside in the medium, very large increases can be achieved in the lipolytic responses to submaximal concentrations of cholera toxin. In these experiments (Figure 8) a concentration (20 ng/ml) of cholera toxin which is virtually without effect in the native cells is able to maximally stimulate lipolysis in the G_{M1}-treated cells; at 150 min, for example, the lipolytic response to this toxin concentration is

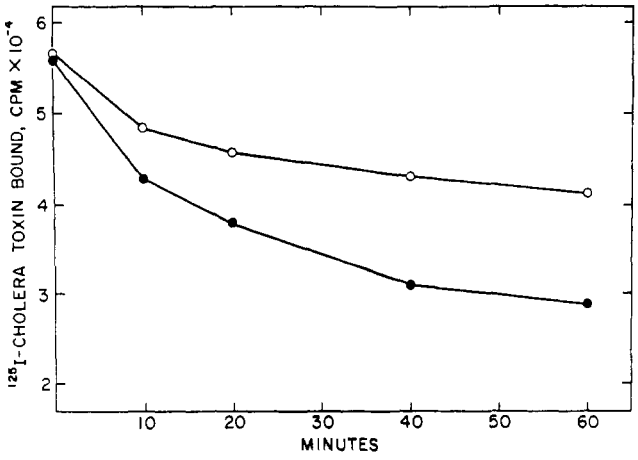

FIGURE 7: Rate of dissociation of cholera toxin–liver membrane complex using normal liver membranes (●) and membranes preincubated with gangliosides and washed (○). Liver membranes (6.4 mg of protein/ml) were incubated at 24° for 50 min in Krebs–Ringer–bicarbonate buffer, 0.1% (w/v) albumin in the absence (●) and presence (○) of bovine brain gangliosides (0.1 mg/ml). The samples were diluted tenfold with the same buffer and centrifuged (20 min, 30,000*g*). The pellets were resuspended in the same buffer and 0.2-ml samples (containing 0.32 mg of protein) were incubated at 24° for 20 min with ^{125}I-labeled cholera toxin (9×10^4 cpm, 20 μCi/μg). Native cholera toxin was added to the samples and the binding was determined after incubating at 24° for various time periods. In these experiments the binding at zero time in both membrane preparations was the same because the concentration of ^{125}I-labeled toxin in the medium was limiting; the amount of membrane protein was sufficiently high to bind essentially all of the toxin in the medium in both cases.

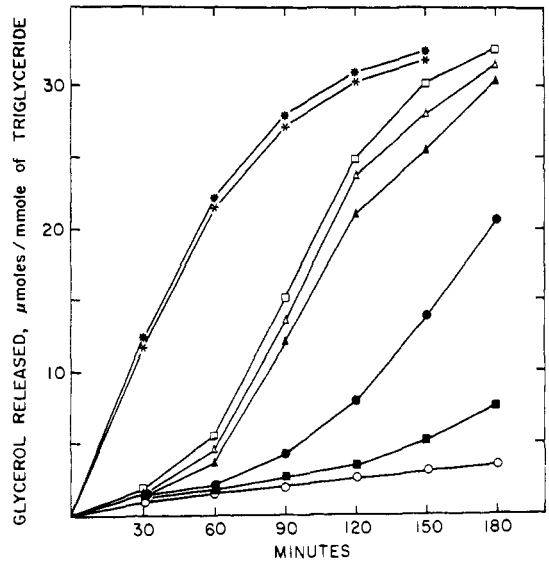

FIGURE 8: Effect of preincubating fat cells with bovine brain gangliosides and with ganglioside G_{M1}, followed by washing of the cells, on cholera toxin and epinephrine induced lipolysis. Isolated fat cells from rats weighing 190 g were incubated for 30 min at 24° in Krebs–Ringer–bicarbonate buffer, 0.1% (w/v) albumin, in the absence of other compounds (○, ■, ●, ×), in the presence of 0.1 mg/ml of brain gangliosides (△, ▲, +), or in the presence of 3 μg/ml of ganglioside G_{M1} (□). The cell suspensions were washed four times with the same buffer. The cells were then incubated at 37° for varying time periods in Krebs–Ringer–bicarbonate buffer, 3% (w/v) albumin, containing no other additions (○) or containing 20 ng/ml (■, ▲) or 1 μg/ml (●, △, □) of cholera toxin, or 2 μg/ml of L-epinephrine (+, X). In the absence of lipolytic agents the cells pretreated with gangliosides or G_{M1} produced the same amount of glycerol as the control cells (○). The response of the untreated cells to very high concentrations (50 μg/ml) of toxin is equal to the response described by □. The effect of preincubating cells with 0.5 mg/ml of gangliosides was not significantly greater than that of 0.1 mg/ml (△); 40 μg/ml of gangliosides produces nearly maximal effects. The sensitivity of fat cells to cholera toxin is dependent on the size of the rats from which the cells are obtained; with cells from smaller (100 g) animals nearly maximal lipolytic responses can be achieved with about 50–100 ng of toxin/ml (Cuatrecasas, 1973c).

more than ten times greater in the treated than in the native cells. At all time points, the response of G_{M1}-treated cells to 20 ng/ml of cholera toxin is greater than the response of the untreated cells to 1 μg/ml of toxin.

This dramatic influence of exogenous gangliosides on the lipolytic response of fat cells to cholera toxin is not observed in the lipolytic response of the cells to L-norepinephrine (Figure 8). Even with very low, submaximal concentrations of L-norepinephrine no effect is observed by preincubating the cells with gangliosides.

It is of interest that despite the greatly enhanced sensitivity of ganglioside-treated cells to cholera toxin, the characteristic lag period (Figure 1) which is observed in the lipolytic response of normal cells to the toxin is not appreciably altered (Figure 8). As indicated in the legend to Figure 8, even higher concentrations of G_{M1} or of crude brain gangliosides than were used in that experiment do not result in greater lipolytic responses or in the abolition of the latency phase. Thus, the lipolytic response of the ganglioside-treated cells resembles very closely the normal response of untreated cells to cholera toxin. The only apparent difference appears to be that the treated cells can respond to much lower concentrations of the toxin compared to the untreated cells. These observations, which are consistent with the data which demonstrate much greater binding of toxin to ganglioside-treated cells, suggest that within limits the lipolytic response of a fat cell to toxin is dependent in an important way on the number of toxin molecules bound to receptors on that cell. Furthermore, the toxin molecules which are bound to experimentally introduced binding sites (receptors) may be biologically equivalent to the natural membrane sites for cholera toxin.

Enhancement of Toxin Binding in Erythrocytes by Exogenous Gangliosides. Prior treatment of intact human erythrocytes with gangliosides results in an increase in toxin binding which is similar to that described for fat cells and liver membranes (Table III). Thus, in analogy with the ability of virtually all cell membranes to bind cholera toxin with very high affinity, it may be that all such membranes may also be manipulable with respect to the insertion of exogenous gangliosides and the consequent acquisition of new toxin-binding sites. The experiments with erythrocytes also indicate that the results described earlier using liver membranes and fat cells are not artifacts resulting from damage to the membranes during their isolation. The simplicity and ready availability of the erythrocyte suggest that this may be a useful tissue in which to study in detail the mechanisms involved in the spontaneous insertion of gangliosides into membranes as well as the nature of the interaction of these molecules with cholera toxin. For example, it is possible to demonstrate with erythrocytes that the enhanced binding capacity for toxin which follows treatment of these cells with gangliosides is a very stable new property of the cells. Erythrocytes treated with gangliosides (and washed) can be kept for 24 hr at 24° without losing their new capacity to bind more toxin than the controls (Table III). It thus appears that gangliosides, once incor-

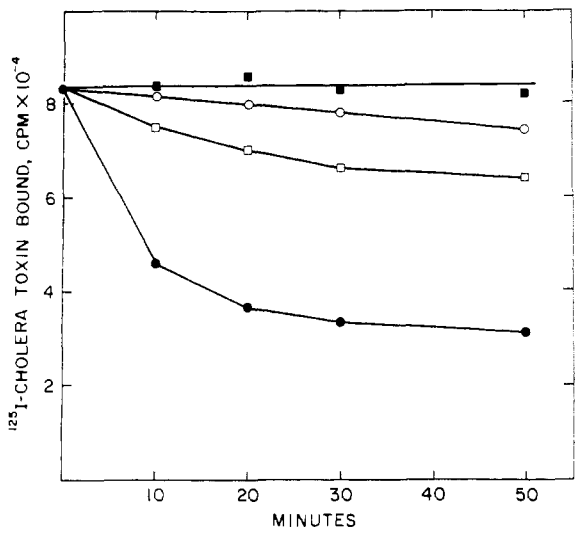

FIGURE 9: Rate of spontaneous dissociation at 37° of the liver membrane–cholera toxin complex (●) and the effect of bovine brain gangliosides (○) and fetuin (□) on reversing the complex. Liver membranes (80 μg of protein) were incubated at 24° for 20 min in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and 2×10^5 cpm of ^{125}I-labeled cholera toxin (10 μCi/μg). Native cholera toxin (50 μg) was added to some samples (●) and the incubation was continued at 37° for various periods to determine spontaneous dissociation (●). Other samples were similarly incubated at 37° with no additions (■), or after adding 5 mg/ml of bovine brain gangliosides (○) or 5 mg/ml of fetuin (□).

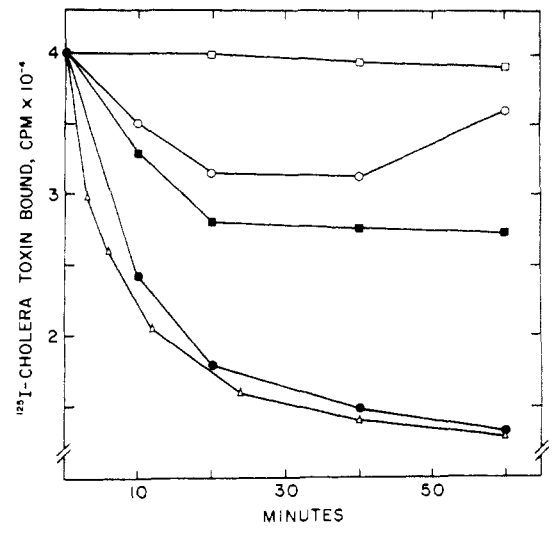

FIGURE 10: Effect of gangliosides on the rate of dissociation of the liver membrane–cholera toxin complex. Liver membranes (60 μg of protein) were incubated at 24° for 15 min in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and ^{125}I-labeled cholera toxin (9×10^4 cpm, 10 μCi/μg). The following were then (zero time) added: 3 μg of native cholera toxin (●, △), 3 μg of native cholera toxin followed in 3 min by 50 μg of gangliosides (■), and 50 μg of gangliosides (○). Samples containing no additions were also included (□). The suspensions were incubated at 24° (■, □, ○, ●) or at 37° (△) for various intervals before determining the binding of the toxin to the membranes.

TABLE III: Enhancement of Binding of Cholera Toxin by Treating Erythrocytes with Gangliosides.[a]

Cells	Concn of ^{125}I-Labeled Cholera Toxin (Sp Cpm Bound)[b]		
	1.5×10^4 cpm (5×10^{-11} M)	6.0×10^4 cpm (2×10^{-10} M)	2.4×10^5 cpm (8×10^{-10} M)
Fresh cells			
Untreated	4500	11,500	24,000
Treated with gangliosides	5400	19,000	66,500
Cells after 24 hr			
Untreated		16,000	24,400
Treated with gangliosides		51,000	77,000

[a] Human erythrocytes (1.5×10^9 cells/ml) were incubated at 24° for 60 min in Krebs–Ringer–bicarbonate buffer, 0.1% (w/v) albumin, in the absence or presence of 0.125 mg/ml of crude bovine brain gangliosides. The cells were washed five times by centrifugation using five times the original volume of buffer. The cells (7.5×10^8 cells/ml) were incubated at 24° for 20 min in 0.2 ml of Krebs–Ringer–bicarbonate buffer, 0.1% (w/v) albumin, containing the indicated concentration of ^{125}I-labeled cholera toxin (sp act., 20 μCi/μg). To examine the stability of the ganglioside–cell complex the cells were left standing at 24° for 24 hr, at which time they were washed, resuspended in the same buffer, and reexamined for binding of ^{125}I-labeled cholera toxin. In all cases the ability of native toxin (20 μg/ml) to block more than 90% of the binding of iodotoxin to these cells was confirmed. [b] Counts per minute per 0.2 ml of incubation mixture.

porated into the membrane, can be retained in a very stable complex.[2]

Dissociation of the Toxin–Membrane Complex and the Effect of Gangliosides in the Medium. The demonstration that the membrane–toxin complex can normally dissociate spontaneously (Figure 7) suggested the possibility that addition of gangliosides to the medium might be capable of reversing the toxin–membrane interaction by binding tightly the toxin molecules which would normally spontaneously dissociate from the complex. The ability of gangliosides themselves to be incorporated into the membrane with a resultant enhancement of binding suggested that such a reversal of toxin binding might be difficult to achieve. Nevertheless, because of the possibility that very large concentrations of gangliosides in the medium might compete effectively, and because of the potential importance of such a reversibility on the therapeutic approaches to clinical cholera, experiments were performed to test this possibility.

It was not possible to demonstrate that even very high concentrations of gangliosides could very effectively reverse the binding of cholera toxin to membranes (Figure 9). The reversal in binding was in fact more pronounced with fetuin than with ganglioside, suggesting again that the simple ability of gangliosides in the medium to bind toxin very tightly is not alone a sufficient property to cause reversal of binding. The complicated nature of the effect of gangliosides and its ineffectiveness in reversing toxin binding are also illustrated in experiments which utilize lower concentrations of gangliosides (Figure 10). Gangliosides cause an initial fall in binding, but this is much smaller than is observed with native toxin, and with increasing time of incubation the enhancement in

[2] It has recently been demonstrated that cholera toxin modifies adenylate cyclase activity in frog erythrocytes in a manner similar to that seen in mammalian tissues: G. V. Bennett and P. Cuatrecasas, manuscript in preparation.

binding described earlier in this report is observed. Furthermore, the addition of native toxin and ganglioside together demonstrates a slower rate of dissociation than is observed with the native toxin alone. This clearly demonstrates that the interaction of free gangliosides with the membrane is a concomitant process which occurs to some extent during the early phases of the incubation.

Discussion

The most compelling reasons for believing that membrane gangliosides are the normal or natural receptors which bind cholera toxin and which mediate its biological effects come from the studies performed with tissues which have been briefly pretreated with gangliosides. Such tissues demonstrate markedly enhanced binding of cholera toxin and a much greater sensitivity to the biological effects of the toxin.

The very tight and selective binding which occurs between cholera toxin and gangliosides in solution, and the similarly tight binding which occurs to ganglioside-treated cells and membranes, suggest that free, exogenous gangliosides can be incorporated spontaneously into membrane structures under very mild conditions. Using radioactively labeled ganglioside G_{M1} it has recently been possible to verify the suggestion that exogenous gangliosides can be readily incorporated into a variety of membranes by simple incubation procedures.[3] Such artificially incorporated gangliosides must be anchored in the membrane in a way that permits retention of very strong affinity for both the membrane and the toxin. As described for erythrocytes (Table III), the membrane–ganglioside complex appears to be stable for at least 24 hr at 24°. The relatively large size and the highly amphiphilic nature of gangliosides such as G_{M1} may well permit the formation of stable membrane complexes which are still capable of tightly and selectively binding proteins (*e.g.*, cholera toxin) present in the aqueous medium. Since the highly specific toxin-binding determinants of the gangliosides appear to reside primarily or exclusively in the carbohydrate end of the ganglioside molecule (Cuatrecasas, 1973b), it is possible to speculate that the highly apolar *N*-acylsphingosine portion of the molecule may be primarily involved in the interaction with the hydrophobic matrix of the membrane while the hydrophilic, carbohydrate domain of the molecule is exposed to the medium. The head groups of gangliosides, being about 30 Å long and as large as the hydrocarbon tails of the molecule (Hill and Lester, 1972), should thus be readily assessible to interaction with cholera toxin. The ability of gangliosides to form micellar lamellar complexes and to interact with other lipids (Hill and Lester, 1972; Gammack, 1963; Ohki and Hono, 1970; Parsegian, 1966) may be very pertinent to the mechanisms by which these molecules form stable complexes with biological membranes. The importance and nature of the interactions between amphiphiles, proteins, and biological membranes have been reviewed recently (Tanford, 1972).

The properties of the toxin–membrane interaction are very similar in the native and ganglioside-treated membranes. The only major difference between these membrane preparations appears to be in the total number of binding sites available for the toxin. This suggests that the binding of cholera toxin to native cells and membranes, in analogy with the binding to ganglioside-treated membranes, results from an interaction between the toxin and membrane-bound gangliosides.

[3] G. V. Bennett and P. Cuatrecasas, unpublished data.

The inability to significantly reverse the cell-bound toxin by addition of exogenous gangliosides appears to be related, at least in part, to the incorporation of gangliosides into the membrane. In experiments designed to examine this (Figures 7, 9, and 10) it appears that the interactions of (a) free ganglioside with free toxin, (b) free ganglioside with membrane-dissociating toxin, (c) membrane with toxin dissociating from the membrane and from the ganglioside complex, (d) ganglioside with membrane, and (e) toxin with membrane-bound ganglioside are all occurring simultaneously. The principal conclusion, however, is that since it is not possible to experimentally demonstrate dissociation of membrane-bound toxin by addition of gangliosides it is unlikely that free gangliosides can be useful as agents for reversing the biological effects of the toxin.

These observations are supported by the inability to reverse or very significantly attenuate the lipolytic response of fat cells to cholera toxin by adding gangliosides even as soon as 5–10 min after exposure of the cells to the toxin (van Heyningen *et al.*, 1971; LoSpalluto and Finkelstein, 1972; Pierce *et al.*, 1971). The explanation for this and for the poor reversibility by gangliosides of toxin binding is even more complicated than suggested above since it has been demonstrated that the spontaneous rate and extent of dissociation of the toxin–membrane complex decrease with increasing length of incubation, resulting with time in an increasingly more stable complex (Cuatrecasas, 1973c). Furthermore, the possibility must be considered that the affinity of membrane-bound gangliosides for cholera toxin may be even greater than that of free gangliosides.

One of the most significant findings of the present studies is the demonstration that incubation of fat cells with gangliosides under conditions which greatly increase the number of binding sites for cholera toxin in these cells also results in a large increase in the sensitivity of these cells toward the biological effects of the toxin. In these studies the lipolytic response of normal or of ganglioside-treated fat cells appears to be critically dependent on the quantity of toxin which is bound within the range of concentration which is below that which elicits the maximal response. Ganglioside treatment of cells does not modify the basal or the maximal lipolytic response to the toxin. Instead it permits the achievement of equal biological responses with much lower concentrations of cholera toxin. Since at equal concentrations of cholera toxin the ganglioside-treated cells bind considerably more toxin that the untreated cells, it follows that the biological response is linked closely to the total number of toxin–receptor interactions, and that the number of such interactions can be increased in an approximately equivalent manner by either increasing the number of receptors per cell or by increasing the concentration of the toxin in the medium.

These studies, coupled with those which demonstrate that incubation of the toxin with brain gangliosides or with ganglioside G_{M1} inhibits in a closely parallel manner both the binding and the biological effects of the toxin, strongly support the contention (Cuatrecasas, 1973b) that the toxin–cell interactions being measured by the present techniques are a reasonable measure of the biologically significant receptors for cholera toxin. The close coupling of toxin binding and biological activity demonstrated in these studies is in itself important in understanding the mechanisms by which receptor interactions can lead to biological activation. Perhaps more important, however, is the fact that the binding sites created by the incorporation of exogenous gangliosides are almost certainly sites which can interact with cholera toxin to initiate

biological responses very similar to or identical with those which occur in the untreated cells.

It thus appears that the addition of exogenous gangliosides to cells may be equivalent to the artificial reconstruction of a biologically significant membrane–receptor system. Since the primary action of cholera toxin appears to be the stimulation of a membrane-bound adenylate cyclase activity (Field, 1971; Pierce *et al.*, 1971; Carpenter, 1971; Parkinson *et al.*, 1972; Evans *et al.*, 1972; Sharp and Hynie, 1971; Kimberg *et al.*, 1971; Gorman and Bitensky, 1972),[3] the system under study may be a useful means of studying the mechanisms by which membrane receptors in general can modulate the activity of this important enzyme.

Acknowledgment

The helpful discussions with Dr. Vincent T. Marchesi and the technical assistance of Ms. Lily Wu are gratefully acknowledged.

Added in Proof

Since submission of this manuscript two reports have appeared describing effects of gangliosides on the activity of cholera toxin: Pierce (1973) and Holmgren *et al.* (1973).

References

Burrows, W. (1968), *Annu. Rev. Microbiol. 22*, 245.
Carpenter, C. C. J. (1971), *Amer. J. Med. 50*, 1.
Chen, L. C., Rohde, J. E., and Sharp, G. W. G. (1971), *Lancet 1*, 939.
Chen, L. C., Rohde, J. E., and Sharp, G. W. G. (1972), *J. Clin. Invest. 51*, 731.
Cuatrecasas, P. (1972a), *Proc. Nat. Acad. Sci. U. S. 69*, 318.
Cuatrecasas, P. (1972b), *Proc. Nat. Acad. Sci. U. S. 69*, 1277.
Cuatrecasas, P. (1973a), *J. Clin. Invest. 52*, 22a.
Cuatrecasas, P. (1973b), *Biochemistry 12*, 3547.
Cuatrecasas, P. (1973c), *Biochemistry 12*, 3567.
Evans, D. J., Jr., Chen, L. C., Curlin, G. T., and Evans, D. G. (1972), *Nature (London), New Biol. 236*, 137.
Field, M. (1971), *N. Engl. J. Med. 284*, 1137.
Finkelstein, R. A. (1969), *Tex. Rep. Biol. Med. 27*, 181.
Finkelstein, R. A., and LoSpalluto, J. J. (1969), *J. Exp. Med. 130*, 185.
Finkelstein, R. A., and LoSpalluto, J. J. (1970), *J. Infect. Dis. 121*, 563.
Gammack, D. B. (1963), *Biochem. J. 88*, 373.
Gorman, R. E., and Bitensky, M. W. (1972), *Nature (London) 235*, 439.
Greenough, W. H. III, Pierce, N. F., and Vaughn, M. (1970), *J. Infect. Dis. 121*, 5111.
Guerrant, R. L., Chen, L. C., and Sharp, G. W. G. (1972), *J. Infect. Dis. 125*, 377.
Hill, M. W., and Lester, R. (1972), *Biochim. Biophys. Acta 282*, 18.
Holmgren, J., Lonnroth, I., and Svennerholm, L. (1973), *Scand. J. Infect. Dis. 5*, 77.
Illiano, G., and Cuatrecasas, P. (1972), *Science 175*, 906.
Kimberg, D. V., Field, M., Johnson, J., Henderson, A., and Gershon, E. (1971), *J. Clin. Invest. 50*, 1218.
LoSpalluto, J. J., and Finkelstein, R. A. (1972), *Biochim. Biophys. Acta 257*, 158.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Ohki, S., and Hono, O. (1970), *J. Colloid Interface Sci. 32*, 270.
Parkinson, D. K., Ebel, H., DiBona, D. R., and Sharp, G. W. G. (1972), *J. Clin. Invest. 51*, 2292.
Parsegian, A. (1966), *Trans. Faraday Soc. 62*, 848.
Pierce, N. F. (1973), *J. Exp. Med. 137*, 1009.
Pierce, N. F., Greenough, W. B. III, and Carpenter, C. C. (1971), *J. Bacteriol. Rev. 35*, 1.
Rodbell, M. (1966), *J. Biol. Chem. 241*, 140.
Ryley, J. R. (1955), *Biochem. J. 59*, 353.
Schafer, D. E., Lust, W. D., Sircar, B., and Goldberg, N. D. (1970), *Proc. Nat. Acad. Sci. U. S. 67*, 851.
Sharp, G. W. G., and Hynie, S. (1971), *Nature (London) 229*, 266.
Tanford, C. (1972), *J. Mol. Biol. 67*, 59.
van Heyningen, W., Carpenter, W. B., Pierce, N. F., and Greenough, W. B. III, (1971), *J. Infect. Dis. 124*, 415.
Vaughn, M., Pierce, N. F., and Greenough, W. B. III (1970), *Nature (London) 226*, 658.