Interaction of *Vibrio cholerae* Enterotoxin with Cell Membranes†

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ABSTRACT: The interaction of biologically active ^{125}I-cholera toxin of very high specific activity (about 1000 Ci/mmol) with isolated fat cells and liver membranes of the rat has been studied. There is nearly quantitative binding of the ^{125}I-labeled toxin in the medium (at 10^{-10} M) to liver cell membranes, and very low concentrations of native toxin compete effectively for binding. Binding to liver membranes or fat cells occurs very rapidly, being complete in about 5 min (at 24°) with concentrations of the toxin as low as 7×10^{-11} M. In both liver membranes and fat cells the binding of toxin is a saturable process over the concentration range under 1 μg of toxin/ml. A single fat cell can bind a maximum of about 2×10^4 molecules of cholera toxin, and liver membranes bind a maximum of about 0.8 μg of toxin/mg of membrane protein. The dissociation constants for formation of the initial toxin–fat cell and toxin–liver membrane complexes are estimated at about 4.6×10^{-10} and 1.1×10^{-9} M, respectively. Relatively high concentrations of some simple galactoside sugars produce moderate inhibition of toxin binding to membranes. Certain glycoproteins, notably fetuin and thyroglobulin, can bind cholera toxin and inhibit the binding of the toxin to membranes. However, proteolytic digests of these glycoproteins and the isolated, purified glycopeptides do not significantly inhibit toxin binding to membranes. Various glycosphingolipids are very potent inhibitors of cholera toxin binding to membranes. The most potent of these is the monosialoganglioside, GM_1, which is effective in final concentrations as low as 1 ng/ml. Very little if any effect on cholera toxin binding is observed by digesting liver membranes with a variety of proteolytic enzymes, and digestion with neuraminidase increases the binding of toxin to membranes. Extraction of glycolipids from liver membranes results in virtually total loss of toxin binding, and the binding activity can be recovered in the ganglioside fraction of these extracts. The possibility is considered that membrane gangliosides constitute the natural biological receptors for cholera toxin.

The acute diarrhea of clinical cholera results from the action of a specific exotoxin of *Vibrio cholerae* on the epithelial cells of the small intestine (Finkelstein and LoSpalluto, 1969; LoSpalluto and Finkelstein, 1972; Carpenter, 1971; Pierce *et al.*, 1971; Burrows, 1968; Finkelstein, 1969). The enterotoxin has recently been purified and chemically characterized (Finkelstein and LoSpalluto, 1969, 1970; LoSpalluto and Finkelstein, 1972; Richardson and Noftle, 1970). It is a protein with a mol wt of 84,000, it is probably composed of six subunits, it has five–six intrachain disulfide bridges but no free sulfhydryl groups, and it lacks significant quantities of lipid or hexose (LoSpalluto and Finkelstein, 1972).

Much evidence has recently accumulated which indicates that the effects of cholera toxin on the flow of salts and fluid into the lumen of the intestine are mediated by cyclic 3′,5′-adenosine monophosphate[1] (Field, 1971; Pierce *et al.*, 1971; Carpenter, 1971). Cholera toxin increases the intracellular content of cAMP in intestinal cells by enhancing the activity of adenylate cyclase in these cells (Sharp and Hynie, 1971; Kimberg *et al.*, 1971; Guerrant *et al.*, 1972; Schafer *et al.*, 1970; Chen *et al.*, 1971, 1972; Parkinson *et al.*, 1972; Evans *et al.*, 1972). The toxin is not ordinarily absorbed from the intestine into the general circulation in sufficient quantities to produce systemic manifestations. However, concentrations of the toxin as low as those which are effective in the intestine are capable of stimulating adenylate cyclase activity and of initiating biochemical responses characteristically mediated by cAMP, in a variety of tissures such as isolated fat cells (Greenough *et al.*, 1970; Vaughn *et al.*, 1970; van Heyningen *et al.*, 1971; Evans *et al.*, 1972), platelets (Zieve *et al.*, 1970), liver (Baker *et al.*, 1971; Gorman and Bitensky, 1972), and lymphocytes, fibroblasts in tissue culture (Hollenberg and Cuatrecasas 1973),[2] and frog erythrocytes.[2] These studies suggest that cholera toxin can selectively interact with ubiquitous components of all cells, and that the fundamental mechanism by which this toxin–cell interaction modifies the activity of membrane-bound adenylate cyclase may be very similar in all cells.

The activity of cholera toxin has been studied primarily *in vivo* using the ligated ileal loop of rabbits and dogs (Finkelstein *et al.*, 1964; De, 1969; Sack and Carpenter, 1969). Introduction of cholera toxin into canine jejunal loops for just a few minutes followed by removal of the free toxin in the lumen by thorough washing results in increased net secretion of fluids and electrolytes which persist for many hours (Pierce *et al.*, 1971). This very rapid fixation of toxin, the persistent nature of the effect after such a brief exposure, and the ability of the toxin to selectively activate an enzyme in the cytoplasmic membrane suggest very specific binding to receptor structures located in the cell membrane. The introduction of simpler assay systems, such as those which utilize isolated fat cells and liver, offers many convenient advantages in the study of the nature of the interaction of cholera toxin with cells and membranes. The present studies describe procedures which permit direct measurement and study of toxin–cell membrane interactions. Some properties of this interac-

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

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

tion are presented, and the possible role in this interaction of various structural components of the cell membrane is investigated. Subsequent reports will deal in detail with the complex manner by which the toxin–membrane interaction may modify adenylate cyclase activity (Cuatrecasas, 1973b–d).[2]

Experimental Procedure

Materials. Cholera toxin (lot 1071), purified by the method of Finkelstein and LoSpalluto (1970), 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. Lacto-*N*-tetraose and lacto-*N*-neotetraose were gifts from Dr. V. Ginsburg, National Institutes of Health. Dr. S. Kornfeld, Washington University School of Medicine, St. Louis, Mo., kindly provided samples of human γG-immunoglobulin glycopeptide (McM, in Kornfeld *et al.*, 1971a,b) and human γM-immunoglobulin glycopeptide (C_B-type III, in Hickman *et al.*, 1972). Glycopeptides I and II from fetuin (Spiro, 1962a,b) and glycopeptides I and II from thyroglobulin (Spiro and Spiro, 1965) were a generous gift of Dr. R. G. Spiro, Harvard University Medical School. Purified human red blood cell glycoprotein was a gift from Dr. V. Marchesi, National Institutes of Health. The following purified glycolipids were obtained from Dr. H. R. Sloan, National Institutes of Health; Gal1→Cer, Glc-1→Cer, Gal1→4Glc1→Cer, G_{M1} ganglioside, sulfatide (galactosyl 3-sulfate ceramide), and psychosine (galactosylsphingosine). Dr. S. Hakamori, University of Washington, kindly provided samples of *N*-acetylhematoside, *N*-glycolylhematoside, globoside, G_{M1} ganglioside, and G_{D1} ganglioside. Dr. R. Brady, National Institutes of Health, generously provided G_{M2} ganglioside, G_{M1} ganglioside, and G_{D1a} ganglioside. Purified *Escherichia coli* β-galactosidase was a gift from Dr. E. Steers, National Institutes of Health.

The following compounds were obtained from Sigma: D-arabinose, D-melibiose, D-mannosamine, D-glucosamine, mucic acid, methyl α-D-galactoside, D-fucose, L-fucose, *N*-acetylneuraminic acid, *N*-acetylneuramin lactose, chitin (poly-(*N*-acetylglucosamine)), hyaluronic acid, *N*-acetyl-D-mannosamine, *N*-acetylglucosamine, D-gluconic acid, D-glucuronic acid, D-xylose, D-galactosamine, collagen (calf skin), starch, mucin (bovine submaxillary gland), heparin, stachyose, thiodigalactoside (D-galactopyranoside β-thiogalactopyranoside), bovine brain gangliosides (type II), isopropyl β-D-thiogalactopyranoside, cerebrosides (bovine brain), psychosine (DL-sphingosyl β-D-galactoside), methyl β-D-thiogalactoside, and polymixin B-sulfate. The following were purchased from Pfanstiehl Laboratories, Waukegan, Ill: raffinose, D-galactose, D-glucose, xylan, esculin, L-rhamnose, glucoheptose, 3-*O*-β-D-galactopyranosyl-D-arabinose, D-fructose, sucrose, lactose, maltose, D-mannoheptulose, galacturonic acid, D-mannose, L-arabinose, gulonic acid, glucuronic acid lactose. The following were purchased from Nutritional Biochemicals, Cleveland, Ohio: lima bean trypsin inhibitor, β-lipoprotein (bovine Cohn fractions IV-4 and III-0), human β-globulin (Cohn fraction III), thyroglobulin, phospholipase C (*Clostridium perfringens*). Galactose oxidase, trypsin, lysozyme, and neuraminidase from *C. perfringens* were purchased from Worthington Biochemical Corporation, Freehold, N. J. The following were obtained from Miles Laboratories, Kankakee, Ill.: horse glycoprotein (Cohn fraction VI), human α-globulins (Cohn fraction IV), human β-globulins (Cohn fraction III), rabbit glycoprotein (Cohn fraction VI), human glycoprotein (Cohn fraction VI), thyrotropin (bovine), thyroglobulin (porcine). *N*-Acetyl-D-galactosamine, *p*-aminophenyl β-D-thiogalactoside, Pronase, and hyaluronidase were purchased from Calbiochem. Methyl β-D-galactopyranoside was obtained from Fox Chemical Co., Los Angeles, Calif. Disialoganglioside (G_{D1a}) and trisialoganglioside (G_{T1}) were purchased from Supelco, Bellefonte, Pa.

Preparation of Iodinated (^{125}I-Labeled) Cholera Toxin. Before iodination, cholera toxin (0.3 ml of 5 mg/ml of 50 mM Tris-HCl–1 mM EDTA–3 mM NaN_3–0.2 M NaCl, pH 7.5) was chromatographed on a 12-ml G-75 Sephadex column equilibrated with 0.25 M sodium phosphate buffer, pH 7.4. Omission of this chromatographic step results in very poor iodination of cholera toxin, possibly because of interference by the solutes in the buffer. The $A^{1\%}_{1\,cm}$ (280 nm) for the toxin was assumed to be 11.42 (LoSpalluto and Finkelstein, 1972). Iodination was performed by a modification (Cuatrecasas, 1971a) of the procedures described by Hunter and Greenwood (1962). Twenty microliters of Chloramine-T (2.5 mg/ml) was added to 0.15 ml of 0.25 M sodium phosphate buffer (pH 7.4) containing 50 μg of cholera toxin and 1.5–2 MCi of carrier-free $Na^{125}I$ freshly prepared by Union Carbide. After 30 sec (24°) 20 μl of sodium metabisulfite (5 mg/ml) was added and after 10 sec 0.2 ml of 0.1 M sodium phosphate buffer (pH 7.5), containing 0.1% (w/v) albumin, was added and the sample was applied on a 12-ml column of Sephadex G-50 equilibrated with 0.1 M sodium phosphate buffer (pH 7.4) containing 0.1% (w/v) albumin. The radioactive material in the void volume of the column was pooled. To determine the specific activity of the iodotoxin an aliquot of the reaction mixture (before Sephadex chromatography) was diluted with 0.1 M sodium phosphate buffer (pH 7.4) containing 0.5% (w/v) albumin to determine the per cent of total radioactivity which had been incorporated into protein; this value, determined according to the precipitability of radioactivity by 7% trichloroacetic acid, was generally near 50%. The specific activity of the iodinated toxin varied between 5 and 18 μCi/μg, depending on the particular lot and concentration of $Na^{125}I$ used. If a mol wt of 84,000 is assumed (LoSpalluto and Finkelstein, 1972) for the toxin, this represents a specific activity of about 1000 Ci/μmol, or less than 1 mol of ^{125}I/mol of protein. About 90% of the iodotoxin was precipitated by 7% trichloroacetic acid in 0.1 M sodium phosphate buffer (pH 7.4)–0.5% (w/v) albumin. Nearly 80% of the radioactive toxin could be specifically bound to liver membranes (to be described shortly). About 80% of the iodotoxin adsorbs to selective cholera toxin affinity columns prepared with ganglioside–agarose;[3] the portion which does not adsorb to such columns does not bind at all to liver membranes or to fat cells[3] and is presumed to be a contaminating protein or inactivated toxin. About 80–90% of the freshly prepared radioactive toxin is present as a single, discrete band on sodium dodecyl sulfate disc gel electrophoresis.[3]

The lipolytic activity of the freshly prepared iodotoxin, measured over a broad range of toxin concentrations on isolated fat cells (Greenough *et al.*, 1970; Vaughn *et al.*, 1970), was indistinguishable from the native toxin. The ^{125}I-labeled cholera toxin was stored at −20° in 0.1 M sodium phosphate buffer (pH 7.4)–0.1% albumin. After 1 week of such storage progressive loss of activity was detectable. This probably represents inactivation of a certain proportion of toxin mole-

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

cules since the loss of activity is manifest by a fall in the proportion of the total radioactivity present in the incubation medium which can be specifically bound to liver membranes or cells. Furthermore, it can be demonstrated that in such samples the same proportion of radioactivity which does not bind to liver membranes also does not adsorb to selective affinity columns. The binding behavior of those molecules which still bind to membranes and which adsorb to affinity columns is not appreciably altered. Thus, even aged iodotoxin preparations can be used for certain purposes. After storage at −20° for 3 weeks about 50% of the radioactivity does not bind to liver membranes.

Assay for Binding of ^{125}I-Labeled Toxin to Membranes and Cells. The assay procedures used to measure the specific binding of ^{125}I-labeled cholera toxin to fat cells and membrane preparations were similar to those described earlier for measuring the binding of iodinated derivatives of insulin (Cuatrecasas, 1971a,b) and plant lectins (Cuatrecasas, 1973e). Isolated fat cells (10^4–10^5/ml) or liver membranes (10–500 μg of protein/ml) were incubated for 15–30 min at 24° in 0.2 ml of Krebs–Ringer–bicarbonate buffer (pH 7.4) containing 0.1% (w/v) albumin and the iodinated toxin. Three milliliters of the same buffer (ice cold) are added to the tubes, the contents are poured on 25-mm cellulose acetate Millipore filters (EGWP for membranes, EAWP for cells) which are positioned with vacuum in a multiple sample filtration manifold, and the filters are washed under vacuum with 10 ml of ice-cold Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin. The steps of dilution, filtration, and washing consume 10–15 sec. The filters are counted in a well-type γ counter using disposable plastic tubes.

In the absence of cells or membranes less than 0.2–0.4% of the freshly prepared ^{125}I-labeled toxin is adsorbed to the cellulose acetate Millipore filters. Mixed cellulose filters (HAWP) could not be used since 30–40% of the cholera toxin applied adsorbs to them. All of the binding data presented except that in Figure 1 are described in terms of "specific" binding, as described in studies of the binding of ^{125}I-labeled insulin (Cuatrecasas, 1971a,b) or plant lectins (Cuatrecasas, 1973e) to cells. Specific binding refers to the amount of ^{125}I-labeled protein bound to the cells or membranes which can be specifically "displaced" by adding 5 μg/ml of native cholera toxin to the cells or membranes before addition of the iodoprotein. Generally the contribution of nonspecific binding to the total quantity of radioactive uptake is very small since virtually all of the bound radioactivity can be selectively blocked by prior addition of the native toxin.

Procedures. Isolated fat cells were prepared from Sprague-Dawley rats (90–120 g) by the method of Rodbell (1966). Liver membranes (microsomal fraction) were prepared by homogenization and differential centrifugation in 0.25 M sucrose (Cuatrecasas, 1972a,b; Illiano and Cuatrecasas, 1972). 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. Liver membrane glycoprotein was prepared by the procedure of Marchesi and Andrews (1971) using lithium diiodosalicylate.

Glycolipids were extracted from liver membranes by procedures known to completely extract gangliosides from tissues (Suzuki, 1965; Puro, 1970; Spence, 1969). Membranes (18 mg of protein/ml), suspended in 50 mM Tris-HCl buffer (pH 7.4), were lyophilized and extracted by stirring at 40° for 2 hr with 20 vol (of the original membrane volume) of chloroform–methanol (2:1, v/v). The residue obtained by centrifugation was extracted for 2 hr at 40° with chloroform–methanol (1:2, v/v). The residue was suspended in distilled water, homogenized (Polytron, Brinkmann), and diluted appropriately with Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin. The organic phases were combined, evaporated to dryness, and dissolved in 10% (v/v) aqueous methanol. The water-soluble gangliosides from the above organic extracts, which were evaporated and lyophilized, were extracted (Saito and Hakomori, 1971) by the addition of 3 ml of chloroform–methanol (2:1, v/v) and 0.5 ml of 0.1 M KCl. The lower phase was extracted three times with 1 ml of chloroform–methanol–0.1 M KCl (1:10:10, v/v). The pooled aqueous upper phases were lyophilized and redissolved in distilled water.

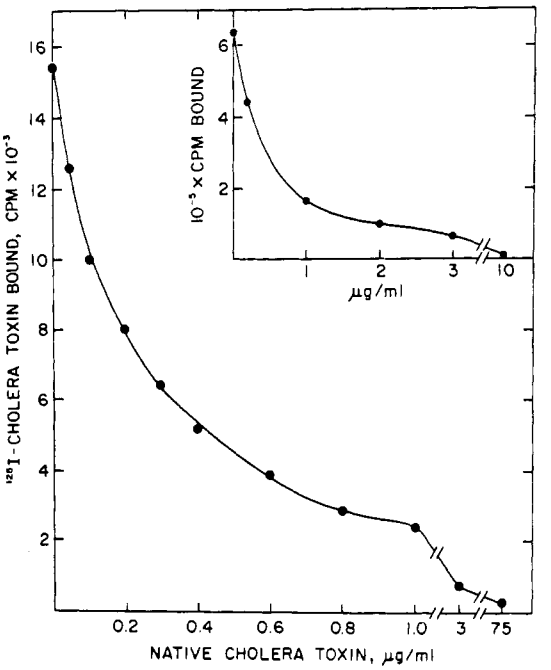

FIGURE 1: Effect of native cholera toxin on the binding of ^{125}I-labeled cholera toxin to liver membranes. The indicated concentration of native toxin was added to 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and 50 μg of membrane protein. After incubating for 5 min at 24° ^{125}I-labeled cholera toxin (15 ng/ml, 9 μCi/μg) was added and the samples were incubated for 15 min at 24°. Binding to the membranes was determined by filtration over cellulose-acetate Millipore filters as described in the text. The inset describes a similar experiment in which the concentration of ^{125}I-labeled cholera toxin in the incubation medium was increased by more than 20-fold (0.4 μg/ml); the concentration of protein in this experiment was 0.75 mg/ml.

Results

General Properties of the Binding of Cholera Toxin. The iodotoxin binds very effectively to liver cell membranes (Figure 1). Even at concentrations below 10^{-10} M a substantial proportion (more than 50%) of the total toxin present in the medium is bound to the membranes. This indicates the existence of either very numerous binding sites on the membrane or of few binding sites of very high affinity. This binding is displaced sharply by very low concentrations of native cholera toxin (Figure 1). It is notable that the binding is nearly totally displaced by concentrations of native toxin which are less than 5 μg/ml. This indicates that the nonspecific binding of iodotoxin is a very minor component of the total binding. As expected from the observation that under these conditions little free iodotoxin remains in the medium, the concentration of native toxin required to cause 50% displacement of binding

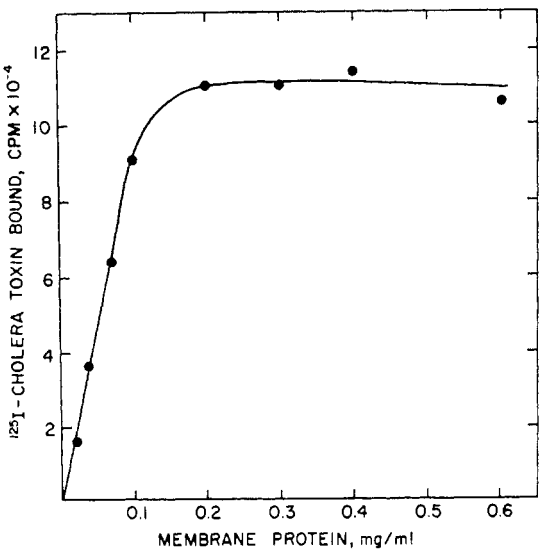

FIGURE 2: Effect of liver membrane concentration on the binding of ^{125}I-labeled cholera toxin. The indicated concentration of membrane protein was incubated for 15 min at 24° in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and 1.4×10^5 cpm (9 μCi/μg) of ^{125}I-labeled cholera toxin. Specific binding was determined as described in the text.

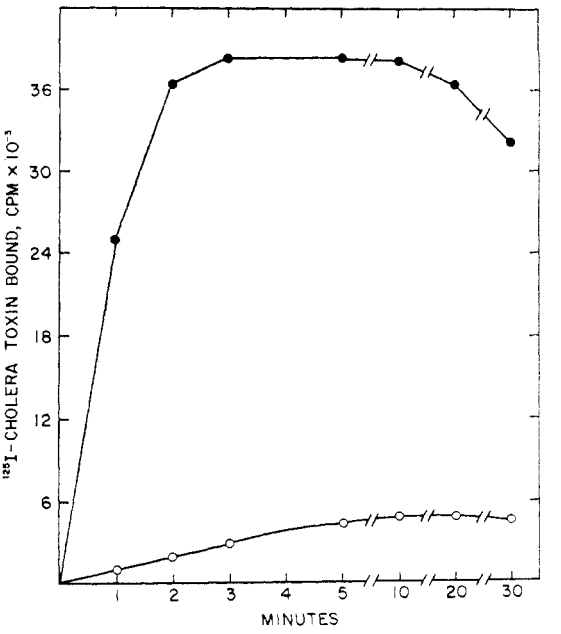

FIGURE 3: Time course of binding of ^{125}I-labeled cholera toxin to liver membranes at 24°. Liver membranes (40 μg of protein) were incubated at 24° in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and 1.3×10^4 cpm (○) or 8.2×10^4 cpm (●) of ^{125}I-labeled cholera toxin (sp act. 10 μCi/μg). Specific binding was determined by filtration as described in the text.

will depend quite critically on the concentrations of membrane protein and of ^{125}I-labeled toxin which are used. Therefore, without additional data such simple displacement curves cannot be used to accurately calculate a dissociation constant for the toxin–membrane complex. It is nevertheless apparent that toxin binds avidly to membranes, and that it is possible to design experimental conditions which in displacement studies permit the detection of concentrations of native toxin as low as 10^{-10} M. Such procedures may be generally useful in the design of convenient assays for cholera toxin.

The binding of ^{125}I-labeled toxin is linearly related to the concentration of liver membrane provided the concentration of toxin is not limiting. Nearly 80% of the ^{125}I-labeled toxin can be specifically bound to liver membranes when sufficiently high concentrations of membrane are used (Figure 2). This probably represents nearly quantitative binding of cholera toxin since study of the supernatant radioactivity shows that the material which is not bound under these conditions does not bind at all when added to fresh membranes. Furthermore, affinity chromatography on ganglioside–agarose columns[3] demonstrates that in such preparations about 20% of the radioactivity does not adsorb to the column and, when tested after such chromatography, does not bind at all to liver membranes. This proportion (about 20%) of radioactivity thus represents contaminating protein or denatured toxin. If this represents denatured toxin it is unlikely to have resulted from the iodination procedure since affinity chromatography of the native toxin reveals that about 20% of the total protein applied to the column does not adsorb and is devoid of biological activity.[3]

The binding of ^{125}I-labeled cholera toxin to liver membranes occurs very rapidly (Figure 3). Binding equilibrium at 24° is achieved within 10 min with a concentration of cholera toxin as low as 7×10^{-11} M. With a concentration of toxin near 4×10^{-10} M, binding is complete in 3 min. The rapid nature of the binding process is consistent with the great avidity of membranes for toxin which is described above. A slight fall in the binding of toxin to membranes is frequently observed when the period of incubation is prolonged for periods longer than 20 min (Figure 3).

Cholera toxin also binds very rapidly to isolated fat cells (Figure 4). Binding is complete within a few minutes with a toxin concentration of 7×10^{-10} M. No discernible difference occurs in the rate or extent of binding of toxin to fat cells at 24° compared to 37°. This is of some importance since it is known that exposure of fat cells to cholera toxin for just a few minutes followed by washing of the cells to remove the free toxin is sufficient to elicit a lipolytic response in these cells, but that regardless of the concentration of toxin used a 60-min period of incubation at 37° is required before a lipolytic response is apparent (Greenough *et al.*, 1970; Vaughn *et al.*, 1970; Cuatrecasas, 1973b,c). The relation of this lag period to the phenomena of binding is the subject of separate publications (Cuatrecasas, 1973b,c).[2] The binding of ^{125}I-labeled cholera toxin to fat cells is nearly completely displaced by native toxin in a manner very similar to that described for liver membranes (Figure 1).

The rate and extent of dissociation of the complex formed between cholera toxin and liver membranes or fat cells are very complicated processes which are dependent on the time and temperature to which the complex is exposed before the kinetics of the dissociation are studied (Cuatrecasas, 1973c).

The binding of cholera toxin to liver membranes (Figure 5) and to isolated fat cells (Figure 6) is a saturable process with respect to toxin when this is examined with concentrations of the toxin less than about 1 μg/ml. In both tissues an additional binding process is discerned when much higher concentrations of the toxin are used. Since the biological effects of cholera toxin are ordinarily at their maximum with concentrations of toxin under 1 μg/ml, it is very likely that the saturable process readily detected with low concentrations of the toxin reflects the biologically significant interaction. With liver membranes (Figure 5) the concentration of toxin required to achieve 50% saturation is about 130 ng/ml (1.5×10^{-9} M), and the mem-

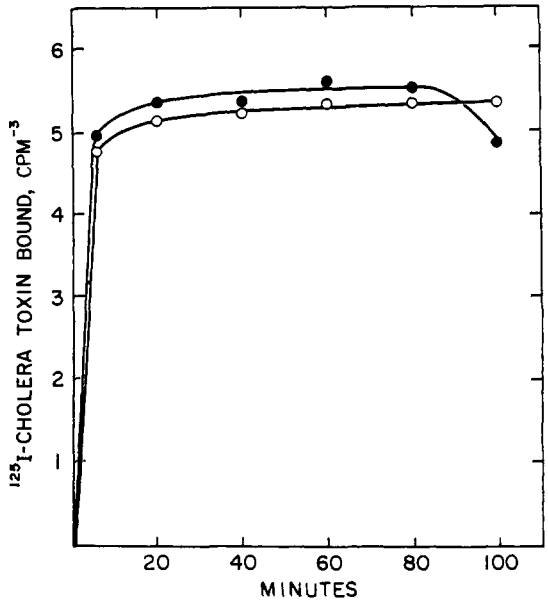

FIGURE 4: Time course of binding of cholera toxin to isolated fat cells at 24° (○) and at 37° (●). Isolated fat cells (about 4.3 × 10^5 cells) were incubated in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 4% (w/v) albumin and ^{125}I-labeled cholera toxin (1.3 × 10^6 cpm, 12 ng).

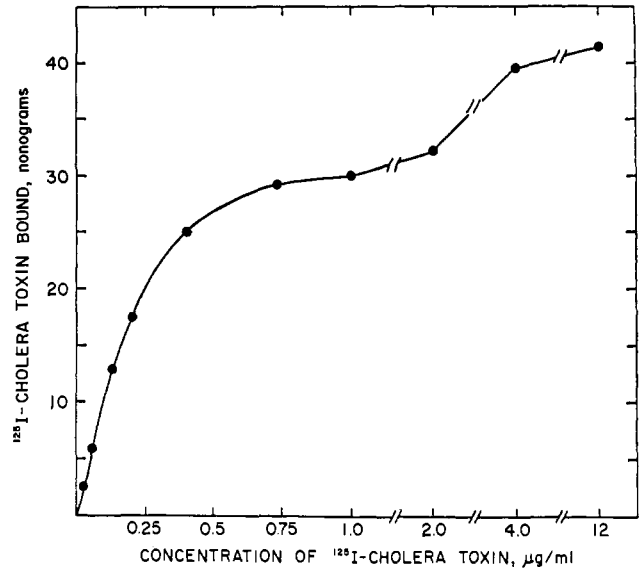

FIGURE 5: Effect of increasing concentrations of cholera toxin on the specific binding to liver membranes. Samples containing 0.2 ml of Krebs–Ringer–bicarbonate buffer, 0.1% albumin, 36 μg of membrane protein, and the indicated concentration of ^{125}I-labeled cholera toxin were incubated at 24° for 30 min. Specific binding was determined by filtration over cellulose acetate filters as described in the text. The samples used to correct for nonspecific binding (see Experimental Procedures) contained 220 μg of native toxin/ml.

branes can bind a maximum of about 0.83 μg of toxin/mg of membrane protein.

The saturable binding process observed in isolated fat cells (Figure 6) demonstrates that the toxin concentration required for half-maximal saturation is about 30 ng/ml (about 3 × 10^{-10} M). Since the binding of toxin to fat cells, unlike that observed with liver membranes, does not ordinarily result in the removal of very appreciable quantities of toxin from the medium, this half-maximal concentration is probably a more reasonable approximation of the dissociation constant of toxin–cell complex formation. It is quite pertinent that when the biological activity of cholera toxin is studied in isolated fat cells obtained from rats of the same weight (90–120 g) as are used in the binding studies, the dependence of lipolysis on toxin concentration is very similar to that described by the saturable portion of the curve of Figure 6 and the concentration required for the half-maximal effect is near 30 ng/ml (Cuatrecasas, 1973b).

From the data presented in Figures 5 and 6 it is possible to calculate for the saturable binding process the concentration of the toxin–membrane or toxin–cell complex, the total receptor, the free toxin, and the free receptor for various concentrations of cholera toxin added to the medium. These data can in turn be used directly to estimate dissociation constants (K_d) for complex formation using the simple expression, K_d = [T][R]/[T–R], where [T] and [R] refer to the concentrations of free toxin and free receptor, respectively, and [T–R] refers to the concentration of the toxin–receptor complex. The dissociation constants thus calculated are 1.1 ± 0.1 × 10^{-9} M (liver membranes) and 4.6 ± 0.3 × 10^{-10} M (fat cells). These kinetic parameters must be considered only as approximations of the early, initial phase of receptor interaction. It is known that with progressive incubation the rate of dissociation of the complex falls progressively (Cuatrecasas, 1973c); thus, the overall dissociation constants probably decrease very significantly with increasing length of incubation.

From the data described in Figure 6 it can be calculated that 3.2 ng of toxin can be bound per 10^6 fat cells, and that one fat cell can bind a maximum of about 22,000 molecules of cholera toxin. This represents a relatively small number of binding sites. For a fat cell having a diameter of 30 μm, the surface density for binding sites is about 8/μm². This density is nearly the same as calculated for insulin receptors in these cells (Cuatrecasas, 1971a) but it is about 10^4 times less dense than the fat cell binding sites for the plant lectins, wheat germ agglutinin, and concanavalin A (Cuatrecasas, 1973e).

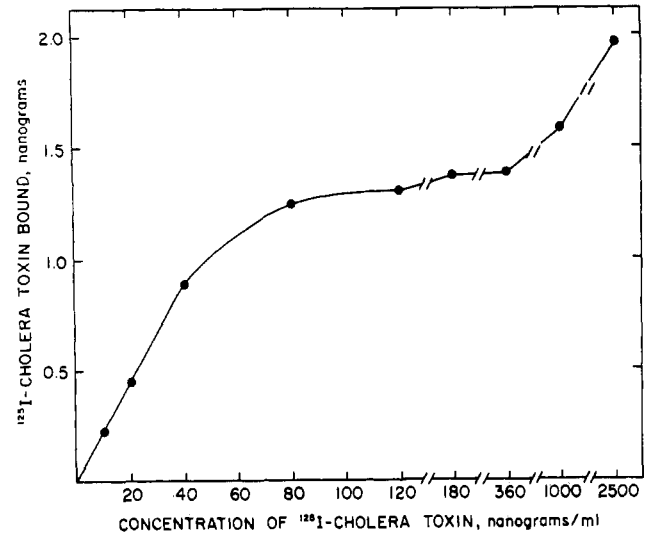

FIGURE 6: Effect of increasing the concentration of cholera toxin on the binding to isolated fat cells. Isolated fat cells (about 4 × 10^5 cells) obtained from 110-g rats were incubated for 30 min at 24° in 0.2 ml of Krebs-Ringer-bicarbonate buffer containing 0.1% (w/v) albumin and the indicated concentration of ^{125}I-labeled cholera toxin (1.2 μCi/μg). Specific binding was determined by filtration over EHWP Millipore filters as described in the text. The samples used to correct for nonspecific binding (see Experimental Procedures) contained 100 μg of native toxin/ml.

TABLE I: Simple Sugars Which Alter the Binding of Cholera Toxin to Liver Cell Membranes.[a]

Sugar	Concn (M)	Specific Binding of ^{125}I-Labeled Cholera Toxin (cpm)
None		29,400 ± 500
p-Aminophenyl β-D-thiogalactopyranoside	0.2	16,700 ± 300
Methyl β-D-galactopyranoside	0.1	20,100 ± 300
Methyl α-D-galactopyranoside	0.1	21,700 ± 200
Methyl β-D-thiogalactopyranoside	0.1	23,400 ± 700
Iospropyl β-D-thiogalactopyranoside	0.1	22,100 ± 400
D-Galactose β-D-thiogalactopyranoside	0.1	15,300 ± 300
Stachyose[b] (α-D-Gal-α-D-Gal-α-D-Glu-β-D-Fru)	30 mM	14,300 + 500
Raffinose[b] (α-D-Gal-α-D-Glu-β-D-Fru)	50 mM	27,000 ± 400

[a] ^{125}I-Labeled cholera toxin (0.1 μg/ml) was preincubated with the sugar indicated below before testing its binding to liver membranes as described in Table I. The concentration of sugar in the final membrane incubation mixture is five times lower than that indicated in the table. None of these sugars caused the toxin to bind to the Millipore filters in the absence of liver membranes. [b] Gal, galactose; Glu, glucose; Fru, fructose.

^{125}I-Labeled cholera toxin can be demonstrated to bind similarly strongly to red blood cells and to membranes which are prepared from scrapings of rat small intestinal mucosal cells by procedures similar to those used to prepare liver membranes. For example, human blood cells can specifically bind about 8 pg of cholera toxin/10^6 cells when the concentration is 2.7×10^{-10} M. The binding of toxin to red blood cells is apparently strong although these cells appear to have much fewer binding sites than fat cells. Although the binding of toxin to intestinal cell preparations has not been studied in detail, the general features of rapid binding and extensive clearance of the toxin from the medium resemble closely the patterns described for liver cell membranes.

Binding of Denatured Cholera Toxin. Boiling cholera toxin for 10 min, a procedure which abolishes the biological activity of the toxin (Sharp and Hynie, 1971), destroys the ability of the protein to bind to liver membranes. No specific binding of iodotoxin to membranes can be detected after incubating ^{125}I-labeled toxin (0.1 μg/ml) at 24° for 10 min in solutions of 0.1% (w/v) albumin containing (a) 4.5 M guanidine-HCl, (b) 5 M urea, (c) 1% (w/v) sodium dodecyl sulfate, (d) 0.1 N HCl, (e) 0.2 M acetic acid, or (f) 0.1 M NaOH. Such solutions were cooled to 4°, diluted tenfold with Krebs–Ringer–bicarbonate buffer–0.1% albumin, and neutralized before assaying for binding to membranes. The capacity to bind to membranes was not regained by dialyzing these solutions at 4° for 24 hr against Krebs–Ringer–bicarbonate or 0.05 M Tris-HCl buffer, pH 7.5. Such denatured preparations of ^{125}I-labeled toxin also do not bind to isolated fat cells. Urea (6 M) and solutions having a pH below 3.5 are known to cause dissociation of cholera toxin to components having a mol wt of ~15,000 (LoSpalluto and Finkelstein, 1972). Although the apparent dissociation of cholera toxin into subunits is reversible with concentrations of protein above 0.5 mg/ml (LoSpalluto and Finkelstein, 1972),[9] no significant reconstitution occurs with the low concentrations of cholera toxin used in the present studies. The present studies indicate that the subunits of cholera toxin are not capable of interacting significantly with cell membranes.

Effects of Sugars on the Binding of Toxin. A large number of simple sugars and saccharide compounds were screened for their ability to inhibit the binding of ^{125}I-labeled cholera toxin to liver membranes. The following sugars had no effect on binding when the ^{125}I-labeled toxin (50 ng/ml) was preincubated with 50–200 mM sugar for 60 min at 24° before addition (fivefold dilution) to liver membranes: D-glucose, D-mannose, D-arabinose, L-arabinose, D-fucose, L-fucose, D-fructose, D-xylose, L-rhamose, lactose, cellobiose, sucrose, maltose, melibiose, 3-*O*-β-D-galactopyranosyl-D-arabinose, glucoheptose, mannoheptulose, galacturonic acid, glucuronic acid, gulonic acid, glucuronic acid lactone, hyaluronic acid, D-glucosamine, D-galactosamine, D-mannosamine, *N*-acetyl-D-glucosamine, *N*-acetyl-D-galactosamine, *N*-acetyl-D-mannosamine, methyl α-D-glucopyranoside, methyl β-D-glucopyranoside, methyl α-D-mannopyranoside, *O*-nitrophenyl D-galactopyranoside, *N*-acetylneuraminic acid, *N*-acetylneuramin lactose, esculin, xylan, glycogan, starch, heparin, chitin, and polymixin B-sulfate. D-Galactose was the only monosaccharide which exhibited some capacity to inhibit toxin binding, although a minimal concentration of 0.2 M was required and the effects (20% inhibition) were very small. The following combinations of sugars (each at 0.1 M) were also tested and found to be ineffective in inhibiting the binding to toxin to membranes: D-galactose plus *N*-acetyl-D-glucosamine plus *N*-acetylneuraminic acid; D-galactose plus D-fucose plus *N*-acetyl-D-glucosamine plus D-mannose; D-mannose plus *N*-acetylneuraminic acid.

A number of α- and β-substituted galactosides demonstrated modest but definite competition for binding (Table I). Relatively high concentrations of these sugars were required, however, and none was effective in displacing the toxin–membrane complex once it had formed. These results nevertheless suggested that the specificity of toxin binding to cell membranes might be directed at least in part toward a saccharide component of the cell membrane.

Effect of Glycoproteins and Glycopeptides on the Binding of Toxin. The possibility, suggested above, that cell surface saccharides may be at least in part responsible for the specificity of toxin binding was examined by studying the effect on the binding process of a number of soluble glycoproteins (Table II). Several glycoproteins are clearly capable of binding cholera toxin and of inhibiting the binding of the toxin to liver membranes. Fetuin is one of the most effective glycoproteins, and it is one of the few with which it is possible to study carefully the effect of varying its concentration because it does not itself adsorb to cellulose acetate filters. Fetuin at a concentration of 50 μg/ml inhibited binding by 37%, at 150 μg/ml it inhibited binding by 58%, and at 0.5 mg/ml it virtually abolished binding.

A highly purified, major human red blood cell membrane glycoprotein (Marchesi and Andrews, 1971) modestly inhibited the binding of toxin to liver membranes although this occurred only with very high concentrations of the protein (Table II). A preparation of liver membrane glycoprotein prepared by the procedure of Marchesi and Andrews (1971) was quite effective in inhibiting toxin binding, but much of this activity probably resulted from contaminating glycolipids since more than 80% of the activity was lost after extraction

TABLE II: Binding of Cholera Toxin to Various Glycoproteins, Lipoproteins, and Serum.[a]

Glycoprotein	Binding of ^{125}I-Labeled Cholera Toxin (cpm)	
	With Liver Membranes	Without Liver Membranes
None	19,300 ± 300	900 ± 100
Fetuin, 0.5 mg/ml	1,800 ± 200	1,100 ± 200
150 μg/ml	8,600 ± 400	800 ± 100
50 μg/ml	12,700 ± 300	900 ± 100
Human RBC glycoprotein, 0.5 mg/ml	14,100 ± 300	1,400 ± 200
100 μg/ml	18,700 ± 700	1,100 ± 200
Rat liver membrane glycoprotein,[b] 80 μg/ml	6,500 ± 400	900 ± 100
20 μg/ml	14,600 ± 600	1,000 ± 200
Thyroglobulin, 15 mg/ml	26,400 ± 400	27,400 ± 1,000
Horse serum glycoprotein, 20 mg/ml	25,100 ± 400	26,100 ● 800
2 mg/ml	18,700 ± 300	6,200 ± 300
Thyrotropin, 25 mg/ml	24,600 ± 1,100	23,900 ± 900
Human α-globulin, 25 mg/ml	10,300 ± 300	11,400 ± 300
5 mg/ml	7,100 ± 200	1,200 ± 200
Bovine β-lipoprotein (IV-4), 50 mg/ml	24,500 ± 900	20,700 ± 1,100
0.5 mg/ml	18,100 ± 600	1,400 ± 200
Bovine β-lipoprotein (III-0), 50 ng/ml	23,100 ± 700	21,300 ± 700
0.5 ng/ml	17,900 ± 900	1,800 ● 300
Rabbit serum glycoprotein, 25 mg/ml	14,200 ± 500	2,200 ± 200
Human β-globulin, 30 mg/ml	15,900 ± 600	7,700 ± 500
0.5 mg/ml	18,700 ± 800	1,200 ± 200
Human serum glycoprotein, 25 mg/ml	20,500 ± 700	1,000 ± 100
Bovine γ-globulin, 25 mg/ml	19,500 ± 300	900 ± 200
Ovomucoid, 30 mg/ml	17,200 ± 500	1,000 ± 0
Lima bean trypsin inhibitor, 30 mg/ml	19,100 ± 900	1,100 ± 100
Egg albumin	19,200 ± 300	1,000 ± 100
Serum, undiluted	1,200 ± 200	1,600 ± 600
1:10 dilution	12,600 ± 500	1,200 ± 300

[a] ^{125}I-Labeled cholera toxin (3.2×10^4 cpm, 15 ng/ml) was preincubated at 24° for 60 min in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% albumin and the protein indicated in the table below. Samples (50 μl) of each incubation mixture were then added to 0.2 ml of the same buffer without other additions or containing 50 μg of liver membrane protein. After incubating at 24° for 30 min the incubation mixtures were filtered over EGWP Millipore filters as described in the text. Since some proteins adsorb strongly to the filters in the absence of liver membranes it was possible to measure directly the binding of ^{125}I-labeled cholera toxin to these proteins. The data in the presence and absence of liver membranes are therefore presented. A number of proteins thought not to be glycoproteins had no effect when tested at 0.5 mg/ml: pancreatic ribonuclease, lysozyme, serum albumin, concanavalin A, wheat germ agglutinin, and hemoglobin. [b] At least 80% of the inhibiting activity is lost after extracting this material with chloroform–methanol (2:1, v/v) as described under Experimental Procedures.

with chloroform–methanol (2:1, v/v). The binding of toxin to certain glycoproteins like thyroglobulin, thyrotropin, and bovine β-lipoprotein can be demonstrated in the absence of liver membranes since a substantial portion of these glycoproteins adsorb to cellulose acetate filters (Table II). However, the binding in these instances must be very weak because very high concentrations of the glycoprotein are required. After exhaustive digestion of thyroglobulin with trypsin or Pronase (Spiro, 1962a) the peptide mixture does not cause binding of ^{125}I-labeled toxin to the filters in the absence of liver membranes. This peptide mixture, however, is capable of inhibiting toxin binding at concentrations equivalent to 1–5 mg/ml of native thyroglobulin. No effect could be demonstrated with bovine γ-globulin, human serum glycoprotein, ovomucoid, and lima bean trypsin inhibitor. Human serum was very effective in inhibiting the binding of cholera toxin to liver membranes (Table II).

Digestion of fetuin (4 mg/ml) for 4 hr at 37° with *C. perfringens* neuraminidase (50 μg/ml) in 0.1 M sodium acetate buffer (pH 6.5)–2 mM $CaCl_2$ resulted in a 30% enhancement of inhibition of toxin binding to membranes when the fetuin was tested at a concentration of 0.1 mg/ml. The principal glycopeptides of fetuin, thyroglobulin, and thyrotropin were prepared by digesting these glycoproteins with papain followed by extensive dialysis (Spiro, 1962a). The crude glycopeptide mixtures produced virtually no inhibition of binding of toxin to liver membranes when tested at a concentration equivalent to 1 mg/ml of the original glycoprotein. These results suggest that the portions of the native glycoproteins which can bind the toxin are contained in minor, small, and dialyzable glycopeptides, or that the polypeptide backbone of the intact glycoprotein contributes very significantly to the toxin interaction.

The toxin-inhibitory activity of a number of important purified glycopeptides was examined (Table III). The principal glycopeptides isolated from fetuin (Spiro, 1962a,b) were very weak inhibitors, in accord with the observation that proteolytic digestion of this glycoprotein diminishes its ability to bind cholera toxin. Similarly, the major glycopeptides isolated from thyroglobulin (Spiro and Spiro, 1965), γG-immunoglobulin (Kornfeld *et al.*, 1971), and γM-immuno-

TABLE III: Effect of Special Glycopeptides and Oligosaccharides on the Specific Binding of Cholera Toxin to Liver Membranes.[a]

Oligosaccharide	% ^{125}I-Labeled Cholera Toxin Bound
Fetuin glycopeptide I, 85 μM	85
Fetuin glycopeptide II, 85 μM	98
Thyroglobulin glycopeptide I, 85 μM	86
Thyroglobulin glycopeptide II, 85 μM	84
γG-Glycopeptide, 0.9 μM	100
γM-Glycopeptide, 0.1 μM	96
Lacto-*N*-tetraose (Gal$\xrightarrow{\beta 1,3}$GlcNAc$\xrightarrow{\beta 1,3}$Gal$\xrightarrow{\beta 1,4}$GlcNAc),[b] 1 mM	80
Lacto-*N*-neotetraose (Gal$\xrightarrow{\beta 1,4}$GlcNAc$\xrightarrow{\beta 1,3}$Gal$\xrightarrow{\beta 1,4}$GlcNAc),[b] 1 mM	92

[a] ^{125}I-Labeled cholera toxin (50 ng/ml) was preincubated at 24° for 60 min in Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and the compound indicated in the table. Samples of these solutions were then incubated at 24° for 15 min with Krebs–Ringer–bicarbonate containing 0.1% (w/v) albumin and 40 μg/ml of liver membrane protein. Specific toxin binding was determined by filtration procedures as described in the text. [b] Gal, galactose; GlyNAc, *N*-acetylglucosamine.

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

Compound[b]	Concn in in Pre-incubation Mixture (μg/ml)	% Binding of ^{125}I-Labeled Cholera Toxin
None		100
Gal1→Cer	100	100
	5	100
Glc1→Cer	100	100
	5	100
Gal1$\xrightarrow{\beta}$4Glc1→Cer	100	100
	5	100
Gal1$\xrightarrow{\beta}$4Gal1$\xrightarrow{\beta}$4Glc1→Cer	100	80
GalNAc1$\xrightarrow{\beta}$3Gal1$\xrightarrow{\alpha}$4Gal1$\xrightarrow{\beta}$4Glc1$\xrightarrow{\beta}$Cer	50	35
	20	44
	5	68
HSO_3→3Gal1→Cer (sulfatide)	150	100
Gangliosides (crude, bovine brain)	50	0
	10	0
	1	2
	0.25	5
	0.05	73
Psychosine (Gal1→sphingosine)	100	100
Cerebrosides	500	100
Sphingomyelin	100	100

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

globulin (Hickman *et al.*, 1972) demonstrated very weak inhibition of toxin binding to liver membranes. Lacto-*N*-neotetraose was ineffective, while lacto-*N*-tetraose demonstrated some inhibiting activity at relatively high concentrations. Thus, the separated major oligosaccharide structures of those glycoproteins which can bind cholera toxin appear to interact only very weakly if at all with the toxin.

Effect of Glycolipids on the Binding of Toxin. Although the studies described above with glycoproteins and glycopeptides were consistent with the view that carbohydrate moieties may play some role in the specificity of binding of cholera toxin, it was not possible to demonstrate directly that isolated or protein-bound oligosaccharide sequences of even complex structure alone possessed sufficient specificity or affinity to account for the very tight binding of cholera toxin to cell membranes. Glycolipids were therefore studied to examine if the determinants for toxin binding might reside in such molecules.

The most simple glycosphingolipids, such as galactose→ceramide, glucose→ceramide, and galactose→galactose→ceramide, do not appear to inhibit the binding of cholera toxin to membranes (Table IV). However, compounds of slightly greater complexity result in definite inhibition. The sequential addition of terminal galactose and *N*-acetylgalactosamine residues to galactose→galactose→ceramide and galactose→galactose→glucose→ceramide, respectively, results in sharp increases in inhibitory potency. Even greater complexity of structure is apparently associated with much greater potency, as suggested by the studies performed with a crude mixture of brain gangliosides (Table IV). No inhibition was detected with sulfatide, sphingomyelin, psychosine, and crude mixtures of bovine brain cerebrosides.

Studies performed with a series of purified gangliosides (Table V) reveal that the sequential terminal additions of *N*-acetylgalactosamine to ganglioside G_{M3} to form G_{M2} and of galactose to G_{M2} to form G_{M1} are accompanied by very large increases in toxin-inhibitory potency. The substitution of one or two additional residues of *N*-acetylneuraminic acid to ganglioside G_{M1} (di- and trisialogangliosides) results in a substantial fall in inhibitory capacity. It is difficult to determine whether the presence of the *N*-acetylneuraminic acid moiety in G_{M1} is an essential determinant; digestion of this ganglioside with neuraminidase slightly enhances the inhibitory potency of this ganglioside (Table V) but it is known that the sialic acid group of G_{M1} is resistant to hydrolysis by neuraminidase (Kolodny *et al.*, 1970). Marked improvement in potency can be demonstrated by similarly digesting ganglioside G_{D1a} or G_{T1} with neuraminidase, conditions known to convert these gangliosides to G_{M1} (Kolodny *et al.*, 1970).

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

Ganglioside[b]	Concn in Preincubation Mixture (μg/ml)	% Binding of ^{125}I-Labeled Cholera Toxin
None		100
G_{M3} (Cer→1Glc4$\overset{\beta}{\rightarrow}$1Gal3$\overset{\beta}{\rightarrow}$2NANA)	50	71
	25	95
	6	100
G_{M3} (Cer→1Glc4$\overset{\beta}{\rightarrow}$1Gal3$\overset{\beta}{\rightarrow}$2*N*-glycolyl-NA)	50	50
	20	60
	5	82
G_{M2} (Cer→1Glc4$\overset{\beta}{\rightarrow}$1Gal4$\overset{\beta}{\rightarrow}$1GalNAc)	5	16
3	1.6	28
↑α	0.5	65
2NANA	0.2	84
G_{M1} (Cer→1Glc4$\overset{\beta}{\rightarrow}$1Gal4$\overset{\beta}{\rightarrow}$1GalNAc3$\overset{\beta}{\rightarrow}$1Gal)[c]	0.35	0
3	70 ng/ml	18
↑α	20 ng/ml	45
2NANA	6 ng/ml	74
	1 ng/ml	90
G_{D1a} (Cer→1Glc4$\overset{\beta}{\rightarrow}$1Gal4$\overset{\beta}{\rightarrow}$1GalNAc3$\overset{\beta}{\rightarrow}$1Gal3	5	20
3 ↑α		
↑α 2NANA	1.6	34
2NANA	0.5	43
	0.2	70
G_{T1} (Cer→1Glc4$\overset{\beta}{\rightarrow}$1Gal4$\overset{\beta}{\rightarrow}$1GalNAc3$\overset{\beta}{\rightarrow}$1Gal3	10	43
3 ↑α	2.5	31
↑α 2NANA	0.8	85
2NANA8$\overset{\alpha}{\rightarrow}$2NANA	0.2	90

[a] These experiments were performed as described in Table IV. [b] Nomenclature according to Svennerholm (1964); Cer, ceramide; Glc, glucose; Gal, galactose; GalNAc, *N*-acetylgalactosamine; NANA, *N*-acetylneuraminic acid. [c] After digesting G_{M1} (0.1 mg/ml) with neuraminidase (20 μg/ml) for 3 hr at 37° in 0.1 M sodium acetate (pH 6.5)–2 mM $CaCl_2$, the binding of toxin in the presence of 1 ng/ml of the ganglioside was 80%. Under similar conditions digestion of G_{D1a} and G_{T1} resulted in a very large enhancement of inhibitory activity.

The relative order of inhibitory potency of the glycosphingolipids studied, with the approximate concentration required to obtain half-maximal inhibition, is: G_{M1} (20 ng/ml), G_{D1a} (0.7 μg/ml), G_{M2} (1 μg/ml), G_{T1} (1.8 μg/ml), GL4 (17 μg/ml), G_{M3} (50 μg/ml), and G_{M3} monosialohematoside (130 μg/ml). Since in these experiments the concentrations given represent those utilized in the mixture which was used to preincubate the ganglioside with the ^{125}I-labeled toxin before it was added to the membrane, the actual concentrations in the final mixture are five times lower. Direct inhibition studies in which all the components are added simultaneously and toxin binding is measured at equilibrium were not possible because of the propensity of gangliosides to be spontaneously incorporated into membranes (Cuatrecasas, 1973b). Under these conditions the kinetics of the binding interaction are very complex and preclude accurate calculations of ganglioside–toxin dissociation constants. Nevertheless, it is clear that cholera toxin can bind to various gangliosides, and the apparent affinity of certain gangliosides, especially G_{M1}, is very great since the data described above suggest that the dissociation constant for this ganglioside is not less than 25 ng/ml (about 10^{-8} M).

Effect of Enzymic Digestions of Membranes. Digestion of liver cell membranes with very high concentrations of trypsin, Pronase, lysozyme, phospholipase C, hyaluronidase, and galactose oxidase had little or no effect on the ability of these membranes to bind cholera toxin (Table VI). It is of interest that digestion of the membranes with neuraminidase or with β-galactosidase resulted in a slight but significant enhancement in the ability of these membranes to bind toxin. The effects of neuraminidase may result from the conversion of more complex gangliosides to G_{M1} (Kolodny *et al.*, 1970).

Effect of Extracting the Membrane Glycolipids. The binding of cholera toxin to liver membranes is nearly abolished by treating these membranes with chloroform–methanol under conditions known to be very effective in extracting tissue gangliosides (Table VII). Furthermore, the water-soluble material present in the chloroform–methanol extract is very effective in inhibiting the binding of toxin to native membranes. Fractionation of the organic extract of the membranes by procedures which selectively separate gangliosides (see Experimental Procedures) yields a ganglioside fraction which inhibits toxin binding to membranes. These results are consistent with the view that gangliosides in membranes are primarily or solely responsible for the binding of cholera toxin to these membranes.

TABLE VI: Effect of Digesting Liver Membranes with Various Enzymes on the Specific Binding of Cholera Toxin.[a]

Enzyme	Sp Binding of ^{125}I-Labeled Cholera Toxin (cpm)
None	93,400 ± 300
Pronase	85,200 ± 800
Trypsin	90,300 ± 900
Neuraminidase	115,600 ± 800
Lysozyme	94,500 ± 500
β-Galactosidase	106,400 ± 700
Phospholipase C	93,600 ± 800
Hyaluronidase	92,400 ± 900
Galactose oxidase	94,000 ± 1000

[a] Liver membranes (0.8 mg/ml) were digested at 37° for 40 min in 0.2 ml of Krebs–Ringer–bicarbonate buffer containing 0.1% (w/v) albumin and Pronase (1.0 mg/ml), trypsin (1.0 mg/ml), neuraminidase (0.1 unit) from *C. perfringens*, lysozyme (0.5 mg/ml), phospholipase C (0.2 mg/ml) from *C. perfringens*, hyaluronidase (0.5 mg/ml), or galactose oxidase (0.5 mg/ml). Soybean trypsin inhibitor (2.0 mg/ml) was added to the samples containing trypsin. ^{125}I-Labeled cholera toxin (1.4×10^5 cpm) was added to the samples and the specific binding was determined after incubating at 24° for 40 min. Digesting the membranes with Pronase or trypsin for 90 min at 37° did not further decrease toxin binding.

TABLE VII: Effect of Extracting Gangliosides of Liver Membranes on the Specific Binding of Cholera Toxin.[a]

Membranes	Sp Binding of ^{125}I-Labeled Cholera Toxin (cpm)	
	3.9×10^4 cpm Toxin	1.8×10^5 cpm Toxin
Control	16,100 ± 700	72,800 ± 2100
Extracted	800 ± 100	4,200 ± 300
Control + combined organic extract	6,200 ± 300	32,500 ± 1400
Control + extracted gangliosides	10,800 ± 500	41,100 ± 1800

[a] Two-milliliter samples of liver membranes containing 36 mg of protein in 0.1 M Tris-HCl buffer, pH 7.5, were lyophilized and either resuspended in 2 ml of distilled water (control membranes) or extracted with chloroform–methanol, as described in the text, and resuspended in 2 ml of distilled water (extracted membranes). The resuspended membranes were homogenized (Polytron), diluted 50-fold with Krebs–Ringer–bicarbonate buffer containing 0.1% albumin, and assayed for specific binding of ^{125}I-labeled cholera toxin as described in the text; 0.2 ml of the membrane suspension (90 μg of protein/ml) was incubated at 24° for 20 min with 3.9×10^4 cpm or 1.8×10^5 cpm of ^{125}I-labeled cholera toxin. The organic extracts were combined, evaporated to dryness, and suspended in 6 ml of 10% (v/v) aqueous methanol; these were then used in 20-fold dilution to determine possible inhibition of binding of ^{125}I-labeled toxin to the control liver membranes. Gangliosides, separated from the combined organic extract by the procedures described in the text, were similarly suspended and tested for inhibition of toxin binding.

Discussion

The use of ^{125}I-labeled cholera toxin derivatives of very high specific activity permits direct measurements of the interaction of cells and membrane preparations with the toxin over the range of toxin concentration which effectively produces biological effects in cells. By the procedures described here it is possible to examine in relatively simple systems the mechanism of action of cholera toxin. Furthermore, the binding assay utilizing liver membranes may serve as a simple, convenient, and sensitive assay for cholera toxin.

Cholera toxin binds to isolated fat cells in a saturable manner with respect to toxin over the concentration range (1–100 ng/ml) of the toxin which is effective in activating lipolysis in fat cells (Cuatrecasas, 1973c; Greenough *et al.*, 1970; Vaughn *et al.*, 1970). The binding of toxin to these cells is very fast and is of very high affinity, which is consistent with the ability of the toxin to stimulate lipolysis after it is exposed to fat cells for just a few minutes and is followed by washing of the cells (van Heyningen *et al.*, 1971; Cuatrecasas, 1973c). This is in part explained by the fact that the rate of dissociation of the cell–toxin complex is relatively slow (Cuatrecasas, 1973b). Of greater importance, however, is the observation that the rate and extent of dissociation of the toxin–membrane complex decrease progressively with increasing time and temperature of incubation of the complex (Cuatrecasas, 1973c).

Cholera toxin clearly has very important specificity properties which are directed to carbohydrate moieties present on cell surfaces. Its binding specificity thus resembles other proteins which bind to saccharides of cell surfaces, such as concanavalin A (Sharon and Lis, 1972), lentil (Kornfeld *et al.*, 1971a,b), and red kidney bean (Kornfeld and Kornfeld, 1970) phytohemagglutinins. The specificity of the latter proteins, however, appears to reside entirely in the carbohydrate determinants of membrane glycoproteins, and such determinants are generally retained intact in simple sugars or isolated oligosaccharides. The studies on the effect of glycoproteins and glycopeptides on the binding of toxin to membranes indicate that the oligosaccharide sequences of such structures may alone not possess sufficient information to promote very tight binding of the toxin. It is at present not clear why the ability of certain glycoproteins to bind cholera toxin is so drastically reduced or abolished upon digestion of the glycoproteins with proteolytic enzymes.

The effects of specific gangliosides point dramatically to the critical contribution of carbohydrates to the specificity of binding. The precise nature or linkage form of the sugars in the oligosaccharide portion of the ganglioside appears to contribute in an important manner to the binding properties, although the binding of toxin is clearly not restricted to a unique ganglioside species. Unfortunately, it is not yet known what role the ceramide portion of the ganglioside plays in the very tight binding which is observed between cholera toxin and the complex gangliosides.

The very high apparent affinity and the specificity of binding of certain gangliosides to cholera toxin suggest that these glycolipids may be the natural cell membrane "receptors" for cholera toxin. The loss of toxin binding activity from membranes by glycolipid extraction procedures and the recovery of the binding activity in the ganglioside fraction of these organic solvent extracts are consistent with this view. Perhaps more definitive proof for the identity of gangliosides with the receptors for cholera toxin is the observations that exogenous gangliosides can be incorporated into intact cells

and membranes, that the binding of cholera toxin to such ganglioside-treated preparations can be greatly enhanced, and that the sensitivity of cells with respect to their biological response to cholera toxin can be greatly enhanced by pretreating these cells with gangliosides (Cuatrecasas, 1973b).

It has recently been demonstrated that incubation of cholera toxin with brain gangliosides blocks the biological effects of cholera toxin on isolated fat cells (van Heyningen *et al.*, 1971; Cuatrecasas, 1973b) and on the small intestine (van Heyningen *et al.*, 1971). By preincubating cholera toxin with increasing concentrations of G_{M1} ganglioside it is possible to demonstrate that the loss of the biological effect of the toxin parallels the loss of binding of the ^{125}I-labeled toxin to the fat cells (Cuatrecasas, 1973b). This supports further the contention that the binding activity being studied in this report is a reasonable measurement of the initial cell–toxin interaction which eventually leads to biologically significant events. The specific interrelationships between the biological activity and the binding of cholera toxin to cells and membranes are considered in detail elsewhere (Cuatrecasas, 1973b–d).

It is of some interest that other bacterial toxins which have exquisitively potent biological effects on certain mammalian tissues can also be inactivated by incubation with gangliosides. Tetanus toxin binds di- and trisialogangliosides very strongly (van Heyningen, 1959; van Heyningen and Miller, 1961; van Heyningen and Mellanby, 1968), and the activity of botulinus toxin can be blocked with trisialogangliosides (Simpson and Rapport, 1971a,b).

The availability of biological substances which can bind cholera toxin very tightly provides the basis for the development of selective adsorbents which can be used to purify the toxin by affinity chromatography (Cuatrecasas and Anfinsen, 1971; Cuatrecasas, 1972c). Ganglioside– and fetuin–agarose derivatives have been prepared which very effectively absorb cholera toxin and which can be used for purposes of purification.[3] Such adsorbents also have potential therapeutic utility.

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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).

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Gangliosides and Membrane Receptors for Cholera Toxin†

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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-

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