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Interaction of Cholera Toxin with Ganglioside G_{M1} Receptors in Supported Lipid Monolayers†

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ABSTRACT: Lipid monolayers formed at the air-water interface containing the ganglioside G_{M1} in egg yolk phosphatidylcholine have been transferred according to the Langmuir-Blodgett technique to (a) glass cover slips coated with octadecyl- or hexadecyltrichlorosilane and (b) carbon-coated electron microscope grids. Monolayer transfer has been demonstrated with fluorescence microscopy, by the transfer of a fluorescent phospholipid analogue, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine or Lucifer yellow labeled G_{M1} (LY-G_{M1}), incorporated into the lipid monolayer. Incubation of supported monolayers with solutions of fluorescein-labeled cholera toxin (FITC cholera toxin) resulted in specific binding of the toxin to monolayers containing G_{M1}, as revealed by fluorescence microscopy. Lateral diffusion coefficients were measured for both the receptor (LY-G_{M1}) [$(3.9 \pm 2.1) \times 10^{-8}$ cm^2/s] and the receptor-ligand complex (G_{M1}-FITC cholera toxin) [$(8.9 \pm 3.2) \times 10^{-9}$ cm^2/s] according to the technique of fluorescence recovery after photobleaching. In separate studies, G_{M1}-containing monolayers transferred to electron microscope grids were incubated with solutions containing unlabeled cholera toxin, followed by negative staining with uranyl acetate. Electron microscopy revealed patches of stained cholera toxin molecules (diameter ~70 Å) in crystalline, two-dimensional hexagonal arrays. Optical diffraction and image reconstruction showed the arrangement of the cholera toxin molecules in a planar hexagonal cell, a = 81 Å. These initial reconstructions give structural information to a resolution of ~30 Å and indicate a doughnut-shaped molecule with a central aqueous channel.

Cholera toxin is produced by the Gram-negative bacterium *Vibrio cholerae*. The toxin molecule (M_r ~84 000) is considered to be an assembly of five identical B subunits (protomer M_r ~11 600) and a single A subunit (M_r ~27 000). Cell surface binding is associated with the B subunits, and the cell surface receptor is the ganglioside G_{M1}. The biological effect is mediated by penetration of the A subunit [a disulfide-linked A1:A2 (M_r ~22 000:~5400) heterodimer] through the host membrane. Following reduction of the disulfide bond, the A1

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peptide catalyzes the NAD^+-requiring[1] ADP-ribosylation and activation of adenylate cyclase. The increased levels of cAMP perturb ion transport processes, and in intestinal cells, for example, acute fluid loss and diarrhea can result. For reviews of cholera toxin chemistry, biological activity, and pathology, see Holmgren (1981), van Heyningen (1983) and Fishman (1982).

In a variety of studies, the cell surface receptor for cholera toxin has been shown conclusively to be the sialic acid containing glycosphingolipid ganglioside G_{M1} (Hollenberg et al., 1974; van Heyningen et al., 1971; King & van Heyningen, 1973; Cuatrecasas, 1973). The gangliosides reside exclusively in the extracellular monolayer of cell membranes (Fishman & Brady, 1976), and the cholera toxin binding function has been shown to be associated with the oligosaccharide moiety of G_{M1} (Fishman et al., 1978). At present, few details of the structure of cholera toxin or the cholera toxin–G_{M1} complex are known.

Our own interests have focused on the properties and interactions of glycosphingolipids, including gangliosides, in model membrane systems (Ruocco et al., 1981; Curatolo et al., 1977). Two recent observations have led us to consider a new approach to the study of cholera toxin–G_{M1} assemblies. First, studies by McConnell and colleagues have demonstrated that lipid monolayers formed at the air–water interface can be transferred to solid supports (glass, quartz, etc.) coated with an alkylating agent (von Tscharner & McConnell, 1981) and their properties (e.g., lateral diffusion) monitored (Smith et al., 1979). By incorporation of lipid haptens in the transferred monolayer, innovative studies of antigen–antibody complexes (Hafeman et al., 1981) and cell-associated recognition processes (Watts et al., 1984) have been possible. Second, Uzgiris and Kornberg (1983) have used a similar rationale, in this case using carbon-coated electron microscope grids as the support, to form two-dimensional crystals of a lipid hapten–monoclonal antibody complex. The structure of the negatively stained complex was studied by electron microscopy and image reconstruction methods, resulting in a 60-Å resolution description of the hapten-bound antibody. Subsequent binding of the C1q component of complement allowed a low-resolution structural study of this three-component system, lipid hapten–monoclonal antibody–complement (Uzgiris & Kornberg, 1983).

The key to these studies was the incorporation of a receptor into a lipid monolayer. It occurred to us that since ganglioside G_{M1} is stable in lipid monolayers (Maggio et al., 1978), the interaction of cholera toxin with G_{M1}-containing lipid monolayers transferred onto either glass supports or electron microscope grids could be studied. Thus, in this paper we report (1) the successful transfer of G_{M1}-containing phosphatidylcholine monolayers to solid supports, (2) the specific binding of cholera toxin to supported monolayers containing G_{M1}, (3) fluorescence photobleaching studies providing lateral diffusion coefficients of the cholera toxin–G_{M1} complexes, and (4) the formation of two-dimensional crystals of the cholera toxin–G_{M1} complex as visualized by electron microscopy/image reconstruction methods.

Materials and Methods

Isolation of Ganglioside G_{M1}. Bovine brain gangliosides were extracted according to the procedure of Svennerholm and Fredman (1980) while the separation of the gangliosides into mono-, di-, and trisialogangliosides (G_{M1}, G_{D1a}, and G_{T1b}) was accomplished by following the procedure of Myers et al. (1984). Briefly, freshly obtained bovine brain (150 g) was homogenized in a high-speed blender for 2 min at 4 °C with 450 mL of water. The aqueous mixture was poured into 1620 mL of methanol with constant stirring at room temperature, and 810 mL of chloroform was then added. Stirring was continued for 0.5 h, and the mixture was centrifuged at 17000*g* for 10 min. The supernatant was filtered through Celite 545, and the brown residue was reextracted by homogenizing with 300 mL of water and pouring the aqueous mixture into 1200 mL of chloroform/methanol (1:2 v/v), followed by centrifugation and filtration. The extracts were combined in a 6-L conical flask, and 780 mL of water was added to produce a final chloroform:methanol:water ratio of 1.0:2.0:1.4 (v/v). The mixture was inverted several times without shaking and left overnight. The upper phase was siphoned off and set aside while 450 mL of methanol was added to the lower phase with vigorous shaking. Then 300 mL of 0.01 M KCl was slowly added, and the solvents were carefully mixed. After overnight separation of the two phases, the upper phase was again siphoned off, combined with the previous upper phase, and evaporated to dryness at 30 °C in the presence of the antifoaming agent isobutyl alcohol. The residue was dissolved in 150 mL of chloroform/methanol/water (60:30:4.5 v/v), allowed to stand at room temperature overnight, and then centrifuged. The supernatant was decanted and evaporated to dryness at 30 °C. The residue was dissolved in 60 mL of water and dialyzed against water for 48 h, followed by at least two changes of distilled water over the next 48 h. The mixture of pure gangliosides was then lyophilized for ~48 h to give ~1200 mg of an off-white, fluffy residue.

The separation of the mixed gangliosides was achieved by column chromatography on a solvent gradient of chloroform/methanol/water. Briefly, 220 g of Iatrobeads (8060, Iatron Laboratories, Tokyo, Japan) was packed into a 420 mm × 60 mm column by using a solvent system of chloroform/methanol/water (65:24:4 v/v). Approximately 2400 mg of mixed ganglioside was dissolved in the minimum amount of the above solvent mixture and placed on the column. The elution was done on a stepwise gradient of chloroform/methanol/water (65:25:4, 60:30:5, and 60:35:8 v/v), with continuous monitoring of 10-mL fractions of eluant by thin-layer chromatography (TLC) on silica gel using ganglioside standards from Supelco (Bellefonte, PA). Monosialoganglioside (G_{M1}) and disialoganglioside (G_{D1a}) were obtained with the solvent system chloroform/methanol/water (60:30:5 v/v) while trisialoganglioside G_{T1b} was obtained with the solvent system chloroform/methanol/water (60:35:8 v/v). The yield of the pure ganglioside from 2400 mg of mixed ganglioside was ~100 mg of G_{M1}, ~250 mg of G_{D1a}, and ~100 mg of G_{T1b}.

Fluorescent Labeling of Ganglioside G_{M1} and Cholera Toxin. The labeling of G_{M1} with Lucifer yellow CH (Molecular Probes, Eugene, OR) has been previously described (Spiegel, 1985). Briefly, a 100 mM acetate buffer (pH 5.5) containing 150 mM NaCl and 2.0 mM $NaIO_4$ was reacted with G_{M1} (1 mg/mL) at 0 °C for 30 min. The oxidation was stopped by adding 0.5 mL of 50% glycerol, and the solution was dialyzed extensively against distilled water and lyophilized. After the oxidation, 70% of the starting ganglioside was recovered and dissolved in 5.0 mL of phosphate-buffered saline. Lucifer yellow CH was added until 5 mM, and the solution was incubated at 0 °C for 20 h. To this solution was added

[1] Abbreviations: NBD-PE, *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine; LY, Lucifer yellow; FITC, fluorescein isothiocyanate; FRAP, fluorescence recovery after photobleaching; NAD^+, oxidized nicotinamide adenine dinucleotide; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; EYPC, egg yolk phosphatidylcholine.

$NaCNBH_3$ until 10 mM, and the reduction was allowed to proceed for 15 min at 25 °C, followed by extensive dialysis and lyophilization. The entire labeling process was monitored by TLC on silica gel G uniplates (Analtech, Newark, DE) using a chloroform/methanol/2.5 N ammonium hydroxide (60:40:9 v/v) solvent system and visualized by H_2SO_4 charring. The Lucifer yellow CH labeled ganglioside (LY-G_{M1}) could also be detected under UV light prior to charring.

Cholera toxin was purchased from Calbiochem/Behring Diagnostics (La Jolla, CA); it was shown to be pure by gel filtration on Sephadex G-150 and by SDS–polyacrylamide gel electrophoresis and was used without further purification. The toxin was supplied as a lyophilized powder and was dissolved in buffer (50 mM Tris-HCl, 200 mM NaCl, 3 mM NaN_3, and 1 mM EDTA, pH 7.5) to give 1 mg of protein/mL of solution. A stock solution of 1.0 mg/mL fluorescein isothiocyanate (FITC) in 0.1 M $NaHCO_3$ (pH 8.2) was prepared. To a 1.0-mL solution of cholera toxin was added 0.1 mL of 0.1 M $NaHCO_3$ buffer and, with constant stirring, 0.1 mL of FITC solution. The mixture was allowed to react at room temperature for 1 h. The unbound FITC was then separated from the FITC cholera toxin conjugate by passing the mixture over a Sephadex G-150 column. Protein was monitored at 280 nm and fluorescein at 470 nm. The conjugate elutes in a 50 mM Tris-HCl buffer system as a single peak in the initial column fractions. These fractions were pooled and dialyzed against cold distilled water and lyophilized. Although in separate experiments we have demonstrated FITC labeling of cholera toxin B subunits (data not shown), we cannot say at this stage whether labeling occurs exclusively on the B subunits.

Preparation of Solid Supports. Two types of hydrophobic solid supports have been used. For fluorescence experiments, alkylated glass cover slips were prepared (Thompson et al., 1984). The cover slips were first boiled for 30 min in Linbro 7X detergent (Flow Labs, Inglewood, CA)/water (1:5 v/v), rinsed extensively with deionized water, dried at 130 °C for 1 h, and placed into a plasma cleaner (Harrick, PDC-3XG) containing a residual of argon gas. After plasma cleaning for 5 min the cover slips were individually alkylated by immediately immersing them in an 80% hexadecane, 12% carbon tetrachloride, 8% chloroform, and 0.1% octadecyltrichlorosilane or hexadecyltrichlorosilane solution for 15–30 s. Upon slow removal they should rapidly shed the solution. After being coated, the cover slips were washed with chloroform, allowed to dry in air, and stored until use. For electron microscopy experiments, carbon-coated silver grids were used. Silver grids were used to avoid corrosion problems associated with prolonged incubations in the salt solutions. The grids were prepared by covering one surface with a 0.5% Formvar film and then coating that surface with a thin layer of carbon by use of an evaporator (Varian, Mountain View, CA). The carbon surface remains hydrophobic enough for monolayer transfer for only 12–24 h, so the grids must be used immediately.

Monolayer Preparation and Transfer. Figure 1 illustrates each step in the process of transferring lipid monolayers from the air–water interface to a solid support. Monolayers were prepared at the air–water interface by applying a lipid-containing solution dropwise inside a confining boundary. The boundary was provided by tying a piece of surgical thread into a circle (~12.5-cm diameter) and coating it with bees wax (benzene/wax solution) so that it floats. The lipid solution contained egg yolk phosphatidylcholine (EYPC) (Lipid Products, Nutfield, England) as a matrix lipid and the ganglioside G_{M1} as a receptor, and in some experiments the

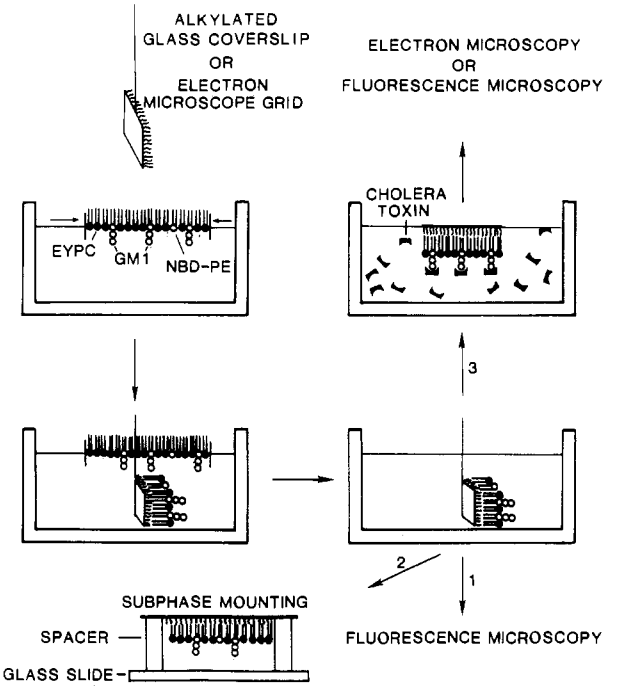


FIGURE 1: Schematic diagram outlining the technique of monolayer transfer from the air–water interface to a hydrophobic solid support (glass cover slip or electron microscope grid). The solid support is either (1) removed from the subphase and viewed directly by fluorescence microscopy, (2) mounted while still in the subphase to a glass slide with a double-faced adhesive, or (3) removed from the subphase and transferred to a solution of either fluorescent-labeled or unlabeled cholera toxin.

fluorescent probe *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)phosphatidylethanolamine (Avanti Polar Lipids, Birmingham, AL) was also incorporated. The lipids were applied to the surface in a chloroform/methanol (2:1 v/v) solvent, which was allowed to evaporate for 30–60 min. After evaporation, the lipids were compressed into a monolayer by applying a "piston" oil (EYPC) to the region outside of the thread. The piston oil has a spreading pressure (~40 dyn/cm) and forces the lipid molecules within the thread to compress into a constant pressure monolayer [see Gaines (1966) and Green et al. (1973)]. Either a carbon-coated electron microscope grid or an alkylated glass cover slip was attached to a holder by a piece of double-sided tape, the holder being attached to a motorized Langmuir–Blodgett mechanical dipping system. Several different dipping systems have been used, and the important factor for monolayer transfer seems to be maintaining a constant dipping rate of 1 mm/min or less. After the solid support was passed through the monolayer, the monolayer lipids were transferred with their alkyl chains interacting with the hydrophobic support and their polar head groups facing outward.

The transferred monolayer was kept in the subphase after dipping and the surface swept clean of the remaining monolayer and the piston oil by using a vacuum and a stream of nitrogen gas. At this point the transferred monolayers may be (1) removed from the subphase into the air and visualized in a dry state by fluorescence microscopy or (2) transferred, while still in the subphase, to a glass slide. The cover slip is attached to the glass slide by placing it on top of two strips of double-faced adhesive tape such that the polar groups of the monolayer are facing down toward the glass slide (see Figure 1). The adhesive tape provides sufficient elevation such that the monolayer head groups are not in contact with the

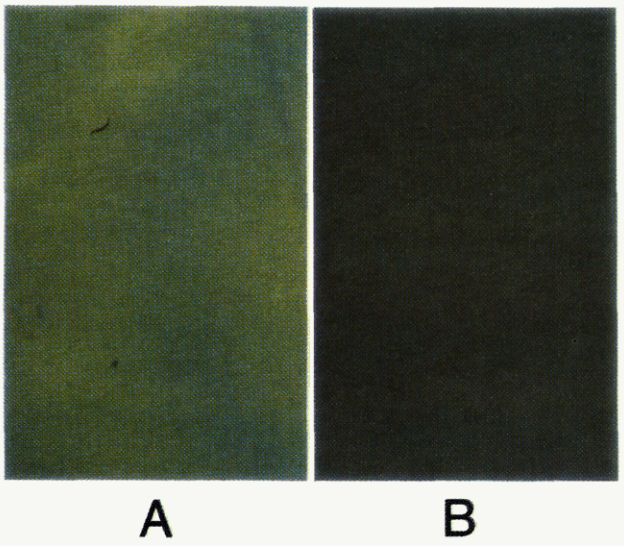


FIGURE 2: Fluorescence photomicrograph of a transferred monolayer containing 5.0 mol % G_{M1} in EYPC plus 3.0 mol % NBD-PE (A) and a control where the fluorescent probe NBD-PE was omitted (B).

glass slide, and upon removal of the slide from the subphase, the surface tension retains the water between the cover slip and the glass slide, assuring constant hydration of the monolayer. Fluorescent-labeled cholera toxin solutions may be injected into this space and then washed after the desired incubation time. In some experiments it was necessary to retransfer the cover slip to a clean glass slide after incubation with the fluorescent-labeled cholera toxin due to nonspecific binding of the toxin to the glass slide. An alternative method (3), involves removing the deposited monolayers from the subphase and incubating with a solution of either fluorescent-labeled or unlabeled cholera toxin. All three methods were used successfully.

Fluorescence Microscopy. Fluorescence microscopy was performed on a Zeiss (Oberkochen, FRG) photomicroscope equipped with a fluorescence illumination and detector system. The illumination was provided by a 100-W mercury lamp, and the detection of fluorescence was through an exciter–barrier filter and reflector combination objective for FITC fluorescence (Zeiss, Oberkochen, FRG). The fluorescence was photographed with a Nikon camera (FX-35A) and semiquantitated with a transmitted light photomicrographic attachment (Nikon, Microflex UFX).

Fluorescence Recovery after Photobleaching (FRAP). The experimental system and method of data analysis have been described in detail elsewhere (Smith & McConnell, 1978; Hafeman et al., 1984). An argon ion laser (Spectra Physics, Model 164-05, Mountain View, CA) provided bleaching through a Ronchi ruling system and low-intensity observational radiation. The fluorescence was observed through a Zeiss (Oberkochen, FRG) Photomicroscope III and measured with a photomultiplier tube (RCA 34103A). The entire system was interfaced to an Archives microcomputer that stored and analyzed the data. The data were analyzed by a least-squares fit to a single exponential, and the efficiency of fluorescence bleaching and the percent fluorescence recovery in the bleached areas were determined. The time constant for recovery of fluorescence was recorded. When necessary, statistical methods involving multiexponential fits of the data were employed. The experiments were performed by using 50–400-ms bleach times, 100–450-ms chop periods, a 40× dry objective, and 50 and 133 line/in. Ronchi rulings. The temperature of the microscope stage was held constant (±0.1 °C) with a heating/cooling bath. All samples were maintained in a fully

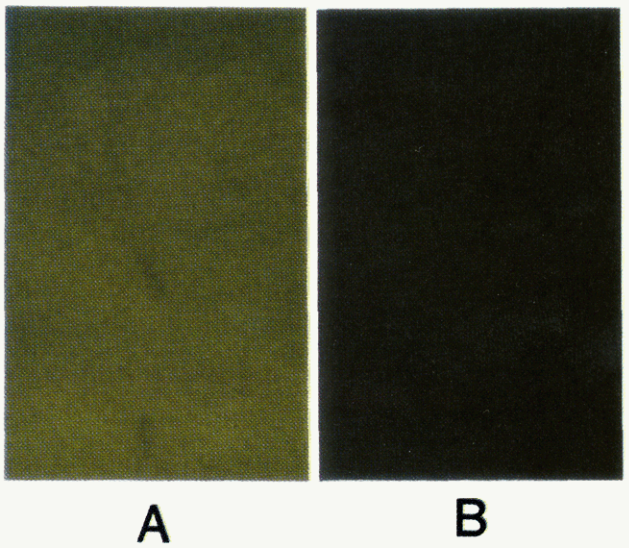


FIGURE 3: Fluorescence photomicrograph of a transferred monolayer containing 5.0 mol % G_{M1} in EYPC incubated with a 0.1 mg/mL FITC-labeled cholera toxin (A) and a control monolayer containing EYPC only, incubated with a 0.1 mg/mL FITC-labeled cholera toxin (B). Both monolayers were incubated with the FITC-labeled cholera toxin for 1 h, followed by washing with distilled water.

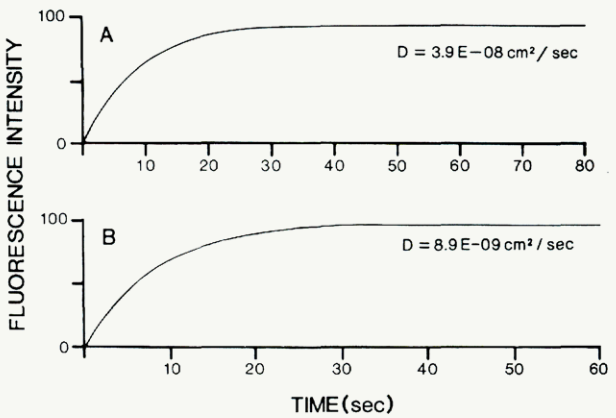
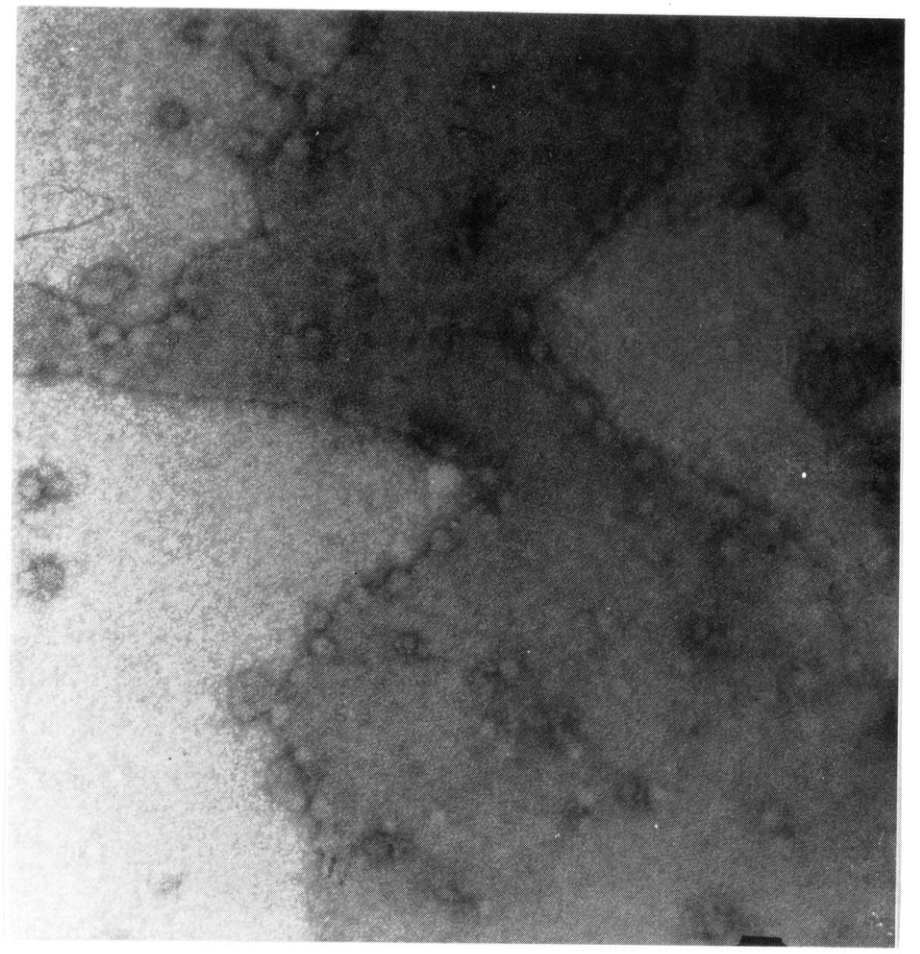


FIGURE 4: Fluorescence recovery after photobleaching (FRAP) recovery curves for a transferred monolayer containing 5.0 mol % Lucifer yellow CH labeled G_{M1}, in EYPC (A), and a transferred monolayer containing 1.0 mol % G_{M1}, in EYPC plus FITC-labeled cholera toxin (B). Both monolayers were maintained hydrated by using the subphase mounting technique (see Materials and Methods), and the diffusion coefficients represent the mean values of several measurements. The plotted curves are the single exponential computer fits to the original data.

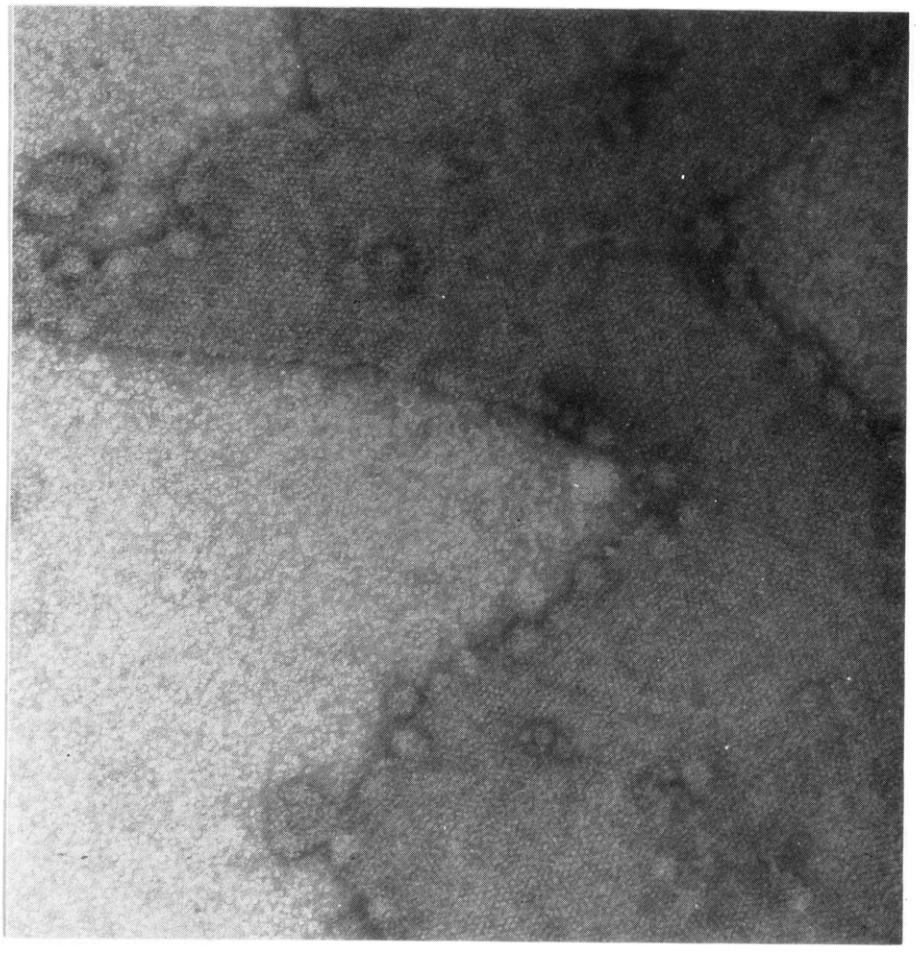
hydrated state for the duration of the experiment (see previous section), and no significant fluorescence recovery occurred when the sample was bleached in the absence of a Ronchi ruling.

Electron Microscopy and Image Reconstruction. The transfer of a monolayer to carbon-coated silver electron microscope grids has been described above. After monolayer transfer, the grids were removed from the subphase and immediately floated on the surface of a drop of 0.1 mg/mL cholera toxin in a 50 mM Tris-HCl, 200 mM NaCl, 3 mM NaN_3, and 1 mM EDTA, pH 7.5, buffer. The grids were floated such that the head groups of the lipids were exposed to the cholera toxin. Incubation times ranged from 30 min to 75 h. After incubation of supported monolayers with the cholera toxin, the grids were removed and washed 3 times by immersing them in distilled water and gently agitating. The grids were stained with 1% uranyl acetate for 30 s, blotted dry, and stored in a grid holder until use. Electron microscopy was performed on both Hitachi HU 11C and Phillips EM420

A



B



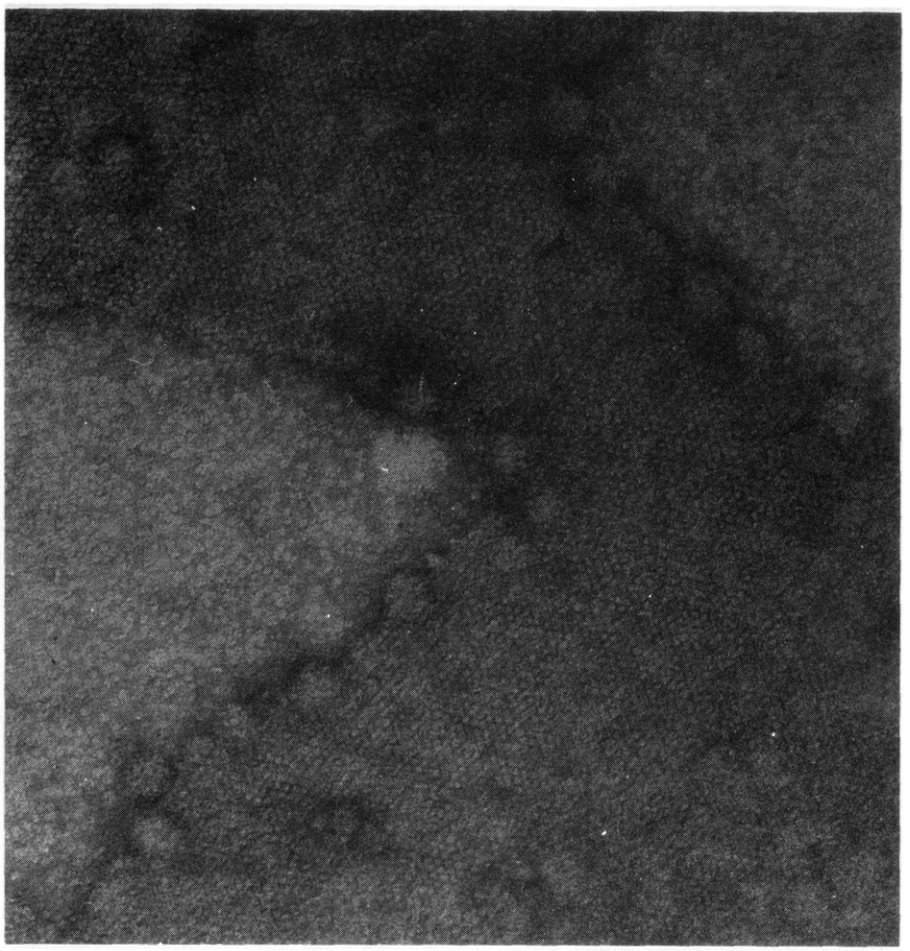


FIGURE 5: Electron micrographs of negatively stained cholera toxin–G_{M1} ordered domains at different magnifications: (A) 84240×, (B) 128570×, and (C) 181805×.

microscopes under minimal dose procedures. Optical diffraction was carried out with a surveying optical diffractometer (Salmon & De Rosier, 1981) on regions of the electron micrograph negatives corresponding to the most crystalline domains. For image reconstruction, the most ordered regions were digitized with an Optronics P1000 Photoscan microdensitometer and displayed on a Grinnel graphics screen (512 × 512 pixels). The displayed images were Fourier-transformed and analyzed with a VAX 11/780 minicomputer using standard Fourier imaging programs.

RESULTS

Transfer of Monolayers to Solid Supports. The ability to transfer a monolayer containing the receptor for cholera toxin (the ganglioside G_{M1}) to solid supports is critical in the evolution of our experiments. Since G_{M1} contains a negatively charged sialic acid in its head group, it could act as a detergent at high enough concentrations. Figure 2A is a fluorescence micrograph of an EYPC monolayer containing 5 mol % G_{M1} and 3 mol % of the fluorescent probe NBD-PE transferred to an alkylated glass slide. Figure 2B is a fluorescence micrograph of an identical monolayer but with the fluorescent NBD-PE probe omitted. The green fluorescence in Figure 2A is characteristic of NBD-PE, and its presence is indicative of a successful transfer of the monolayer. In addition, successful transfer of monolayers has been demonstrated with LY-G_{M1} (data not shown). Experiments monitoring the loss of surface lipid from the air–water interface maintained at constant pressure confirm that the Langmuir–Blodgett procedure quantitatively transfers a single monolayer to the solid support [see, for example, Weis et al. (1982)]. These transfer experiments have been repeated with carbon-coated silver electron microscope (EM) grids acting as the solid support. Again, transfer of fluorescent NBD-PE to the grid is observed (data not shown).

Interaction of Cholera Toxin with G_{M1}-Containing Supported Monolayers. Although Figure 2 demonstrates that it is possible to transfer monolayers in the presence of G_{M1}, it does not determine whether the G_{M1} has indeed been incorporated into the monolayer or if it is in the proper orientation to bind cholera toxin. To establish the presence and binding activity of G_{M1} in our system, an assay utilizing a fluorescein isothiocyanate (FITC) conjugated cholera toxin was developed (see Materials and Methods). The conjugate elutes from the Sephadex G-150 column at a position corresponding to an 85000-Da species, the molecular weight of AB_5 cholera toxin (data not shown). The absorption of the later fractions corresponds to unbound FITC (λ = 470 nm). Fractions corresponding to FITC-labeled cholera toxin were pooled and used without further purification.

The binding assay used to detect the presence of the cholera toxin receptor in supported monolayers involved incubating the transferred monolayers with solutions of FITC cholera toxin (see Figure 1, method 3) for various times, washing, and assaying for binding with fluorescence microscopy. The lipid monolayers contained between 1.0 and 5.0 mol % G_{M1} in EYPC, transferred to alkylated glass cover slips. The fluorescence micrograph in Figure 3A is of a monolayer containing 5.0 mol % G_{M1} in EYPC incubated with a 0.1 mg/mL solution of FITC cholera toxin for 1 h. An identical monolayer not containing the ganglioside G_{M1} does not bind FITC cholera toxin (Figure 3B). The specific fluorescence

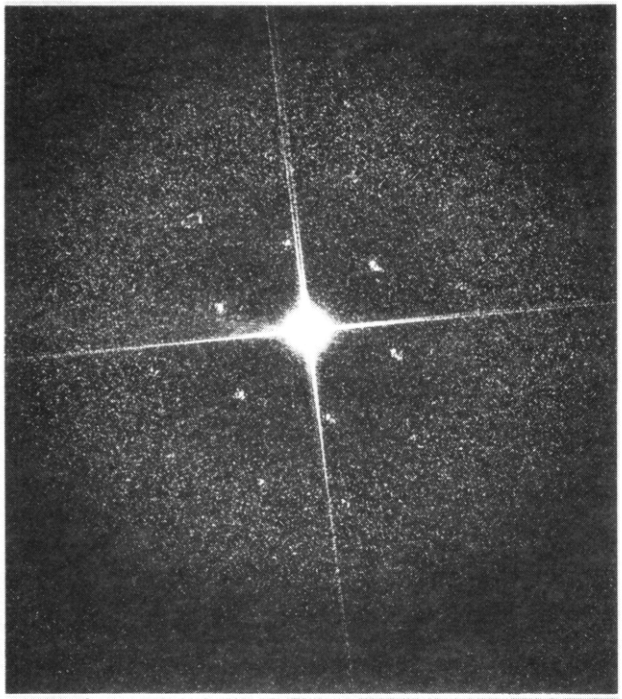


FIGURE 6: Optical diffraction pattern of an ordered crystalline array of ganglioside G_{M1} bound cholera toxin.

binding found only when G_{M1} is present in the monolayer supports the idea of the incorporation and proper external orientation of the G_{M1} within the supported monolayers, thus allowing for the binding of the labeled cholera toxin. The absence of fluorescence in monolayers without G_{M1} (Figure 3B) further demonstrates the specificity of the interaction and the lack of significant background binding of the toxin to the EYPC matrix lipid.

Fluorescence Recovery after Photobleaching (FRAP). The lateral diffusion coefficient (D) of lipids in fluid membranes is fast with values of $D \sim 10^{-7}$–10^{-8} cm^2/s (Wu et al., 1977; Rubenstein et al., 1979). In contrast, lipids in solid bilayers have D values several orders of magnitude slower; $D < 10^{-10}$ cm^2/s (Rubenstein et al., 1979). By incorporation of the fluorescent-labeled ganglioside LY-G_{M1} into supported lipid monolayers containing EYPC, the lateral diffusion coefficient of the receptor has been determined by FRAP. A representative plot of relative fluorescence intensity vs. time for a 5 mol % LY-G_{M1}/EYPC monolayer at 26 °C is shown in Figure 4A. From these recovery curves the lateral diffusion coefficient of G_{M1} was determined to be $D = (3.9 \pm 2.1) \times 10^{-8}$ cm^2/s. This diffusion coefficient agrees with those of other fluid lipid bilayer systems and shows that the supported monolayer has fluid characteristics.

When FITC-labeled cholera toxin is incubated with monolayers containing unlabeled G_{M1}, a specific interaction is observed (see Figure 3). The diffusion coefficient for this G_{M1}–cholera toxin receptor–ligand complex has been determined by FRAP, and a typical fluorescence recovery curve is shown in Figure 4B. The monolayer contained 1.0 mol % G_{M1} in EYPC and was incubated with a 0.25 mg/mL solution of labeled cholera toxin for 20 min at 26 °C. The lateral diffusion coefficient was determined to be $(8.9 \pm 3.2) \times 10^{-9}$ cm^2/s. This is approximately 4 times slower than the receptor alone, but when the errors are considered, the difference is probably not significant.

For EYPC monolayers containing 3.0 mol % NBD-PE, the diffusion coefficient of the probe at 26 °C was $(5.6 \pm 0.1) \times 10^{-8}$ cm^2/s. As the concentration of G_{M1} in the EYPC monolayer was increased, the diffusion coefficient of the NBD-PE probe progressively decreased, reaching a value of $(1.7 \pm 0.4) \times 10^{-8}$ cm^2/s at 20.0 mol % G_{M1} in EYPC (data not shown).

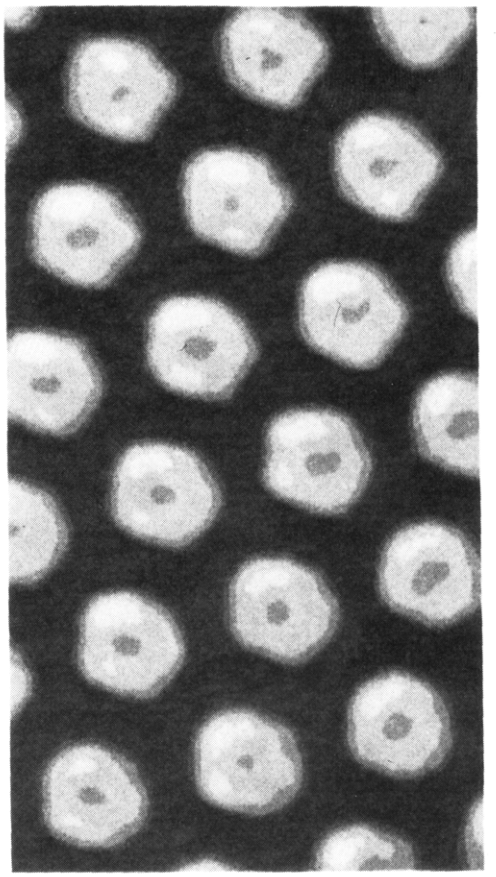


FIGURE 7: Image reconstruction of a crystalline domain of ganglioside G_{M1} bound cholera toxin at ~30-Å resolution. The computer-generated diffraction pattern was filtered (see text) prior to the back Fourier transform.

Crystallization of the Cholera Toxin–G_{M1} Complex. The lipid monolayers containing G_{M1} in EYPC were transferred to hydrophobic electron microscope grids (carbon coated), incubated on solutions of cholera toxin, washed, and stained with 1.0% uranyl acetate. Conditions such as temperature, time of incubation, and concentration of both G_{M1} and cholera toxin were all varied in an attempt to optimize the formation of ordered arrays. Figure 5 shows electron micrographs of a 5.0 mol % G_{M1} in EYPC monolayer incubated with a 0.1 mg/mL cholera toxin solution for 70 h at room temperature (25 °C). Figure 5A is a low-magnification view, which shows crystalline domains of the negatively stained cholera toxin molecules as circular (diameter ~70 Å), stain-excluding particles packed in a regular lattice. In other regions, although stained particles are present, no lattice formation has occurred. Parts B and C of Figure 5 show higher magnification views of the same crystalline domain. At higher magnification the hexagonal packing of the G_{M1}–cholera toxin complex becomes apparent (see arrowed regions) and the circular particles show evidence of staining in the center. We note the interesting macrostructures formed at the interface between the ordered and unordered regions. In a control experiment where G_{M1} was omitted from the monolayers, no stained particles were observed in the electron micrographs (data not shown).

Optical diffraction was carried out on the most ordered crystalline domains, and a typical diffraction pattern is shown in Figure 6. Reflections corresponding to a hexagonal lattice are observed and extend out to the third or fourth order re-

flections. This lattice corresponds to a hexagonal unit cell in real space, $a = 81$ Å. The image on the electron micrograph was digitized and displayed on a graphics device. By boxing off different regions of the digitized image, the optimal crystalline domains could be identified and the Fourier transform calculated. These transforms (corresponding to the optical diffraction patterns described above) were high and low frequency filtered to remove noise, and a hexagonal mask corresponding to the unit cell parameters was applied to the transform before the second (back) transform was calculated. An image-reconstructed view, at ~30-Å resolution, of the cholera toxin–G_{M1} complex is shown in Figure 7. At this resolution the cholera toxin molecule can be seen to pack into a hexagonal lattice with each individual cholera toxin molecule (diameter ~70 Å) surrounded by six neighbors. The cholera toxin molecule appears to be doughnut shaped with a hole in the center (diameter ~20 Å), and there is a suggestion that the molecule exhibits pseudo-fivefold symmetry.

Discussion

Supported lipid monolayers represent an excellent system for studying the structure and dynamics of membrane receptor–ligand interactions. The ability to transfer the monolayers from the air–water interface to solid supports and to assess the interaction between the receptor and ligand is easily monitored by using fluorescent lipids and fluorescence microscopy. In systems where the receptor is a lipid (e.g., ganglioside), the formation of monolayers containing these receptors is easily accomplished due to the miscibility of the receptor in neutral lipid (e.g., PC) monolayers. However, if the receptor is a cytoplasmic or membrane protein, then incorporation into a monolayer system obviously poses problems. Methods of circumventing these problems have been developed; these include derivatizing fatty acids to the protein (Thompson et al., 1984; Huang, 1985) for better insertion into the lipid hydrocarbon environments or incorporating the proteins into unilamellar liposomes and then fusing these to a solid support such that supported planar bilayer membranes are formed (Watts et al., 1984). After utilization of these procedures, the lateral mobility of supported cytoplasmic proteins has been determined (Huang, 1985), as well as the two-dimensional crystallization of a fatty acid derivatized enzyme–effector complex (H. O. Ribi and R. D. Kornberg, personal communication).

The parameters investigated in this study of the ganglioside G_{M1}–cholera toxin system were lateral diffusion and two-dimensional crystallization. With the fluorescence recovery after photobleaching technique, diffusion coefficients of 3.9×10^{-8} cm^2/s and 8.9×10^{-9} cm^2/s were determined for the G_{M1} and the G_{M1}–cholera toxin complex, respectively, in supported EYPC monolayers. The diffusion coefficient of G_{M1} measured here in supported lipid monolayers is in close agreement with measurements made on human fibroblasts with incorporated rhodamine- and fluorescein-labeled G_{M1} ($\sim 10^{-8}$ cm^2/s) (Spiegel et al., 1984) and eosin-labeled G_{M1} incorporated into liposomal bilayers (4.7×10^{-9} cm^2/s) (Goins et al., 1986). A diffusion coefficient of 10^{-8} cm^2/s is typical of fluid membrane conditions and is similar to values obtained for phospholipids in bilayers. Thus, it appears that, at concentrations of 5.0 mol % G_{M1} in EYPC, the G_{M1} molecules are freely diffusing and have not patched into G_{M1}-containing networks. However, in studies utilizing the fluorescent probe NBD-PE to monitor the surface properties, reduced lateral motion was observed at concentrations of between 1.0 and 20.0 mol % G_{M1}; however, the lateral diffusion coefficient of NBD-PE was reduced by a factor of only 3–4 (data not shown). For monolayers containing higher mole percent G_{M1}, a double exponential gave a better fit to the recovery curve and corresponded to two populations with lateral diffusion coefficients of $\sim 10^{-8}$ cm^2/s and $\sim 10^{-9}$ cm^2/s, respectively. The slower diffusing species might represent regions in which G_{M1} molecules have patched (Thompson et al., 1985).

When cholera toxin is bound to G_{M1}, the lateral diffusion coefficient of the complex is very similar to the unbound G_{M1} receptor, consistent with the theory of planar diffusion developed by Saffman and Delbrück (1975). With their model, it can be shown that the lateral diffusion of a molecule within a lipid membrane of the hydrodynamic model is essentially independent of the molecular weight of the diffusing species.

Clearly, the ability of the G_{M1}–cholera toxin complex to diffuse rapidly in the plane of the supported monolayer permits intermolecular collision processes to occur, these presumably leading to nucleation of two-dimensional crystals. Following short incubation times, stained electron micrographs show small crystalline domains containing small numbers (100–1000) of cholera toxin molecules (data not shown); longer incubation times allow crystal growth to occur and very large two-dimensional crystals form (see Figure 5, for example). While the quality of the crystals provides for only a low-resolution description of the G_{M1}–cholera toxin complex, already several interesting features are apparent. The G_{M1}-bound cholera toxin molecules pack in a hexagonal lattice parallel to the monolayer surface, dimension $a = 81$ Å, clearly visible in the original electron micrograph (see Figure 5) and confirmed by the optical transform (Figure 6). At present, the resolution provided by the two-dimensional crystals is ~30 Å and thus detailed interpretation of the reconstructed image is not justified. However, two structural features are worth pointing out. First, both the original electron micrograph (Figure 6) and the filtered reconstructed image (Figure 7) show evidence of stain penetration at the center of the 70 Å diameter particle, indicating that the cholera toxin molecule has a doughnut shape with a solvent-accessible channel at its center. Second, and most interesting, individual cholera toxin molecules in the reconstructions (Figure 7) provide initial evidence for the subunit organization of cholera toxin bound to a membrane surface with some indication of a pentameric arrangement of subunits. Although it would be tempting to associate this picture with the organization of the five B subunits of each cholera toxin, caution should be exercised at this stage of the analysis. In this respect we make note of the issue of packing objects with perfect fivefold symmetry in hexagonal lattices. We should also point out that other two-dimensional rectangular crystals of G_{M1}-bound cholera toxin have been observed, as well as crystals of G_{M1}–cholera toxin B subunits (R. A. Reed, J. Mattai, and G. G. Shipley, unpublished observations).

Currently, we are trying to grow better two-dimensional crystals of cholera toxin with the objective of providing a higher resolution description of the subunit organization of receptor-bound cholera toxin. These studies will clearly benefit from a tilt series electron microscopy study. In the long term, three-dimensional crystals (Sigler et al., 1977) will be required to provide details of the subunit structure and intersubunit interactions.

Added in Proof

Rectangular and hexagonal two-dimensional crystals of G_{M1}-bound cholera toxin β subunits have recently been reported (Ludwig et al., 1986). At 17-Å resolution, a pentameric doughnut-shaped organization of the β subunits is again indicated.

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