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Organization of ganglioside G_{M1} in phosphatidylcholine bilayers

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Molecules of the ganglioside G_{M1} are randomly distributed in liquid-crystalline 1-palmitoyl-2-oleoyl phosphatidylcholine bilayers. This conclusion is based on a freeze-etch electron microscopic study using ferritin-conjugated cholera toxin and cholera toxin alone as ganglioside labels. The average number of G_{M1} molecules under a label is calculated by a novel method from the dependence of the fraction of bilayer area covered by the label on the mole fraction of G_{M1} in the bilayer.

Introduction

Glycosphingolipids, which are usually relatively minor components of mammalian plasma membranes, appear to be localized exclusively in the external surface of these membranes [1–4]. Gangliosides, a subclass of glycosphingolipids, bear a net negative charge conferred by one or more sialic acid residues linked to the oligosaccharide moiety of the molecule. These charged glycolipids are widely distributed in mammalian tissues. Their highest concentration occurs in the white matter of the central nervous system [5] and in particular in synaptic junctions [6]. Although gangliosides have been implicated in receptor function for a number of toxins, viruses and some peptide hormones [7], the role of the ganglioside G_{M1} as the receptor for cholera toxin is particularly well documented [8].

In this paper we examine the organization of the ganglioside G_{M1} in the bilayers of phosphatidylcholine liposomes using freeze-etch electron microscopy in conjunction with cholera toxin or cholera toxin-ferritin conjugate labeling. An analysis of the fractional coverage of the external liposome surface by the macromolecular label as a function of G_{M1} concentration in the bilayer leads to the conclusion that in this system G_{M1} is molecularly dispersed in the phosphatidylcholine bilayer. Preliminary reports of portions of this work have appeared elsewhere [9,10].

Materials and Methods

Preparation of G_{M1}

Mixed bovine brain gangliosides were extracted from upper Folch solvent (Avanti Polar Lipids, Birmingham, AL) by elution over Sephadex G-25 using deionized H_2O. The ganglioside micelles eluted at the column void volume in a preparation largely free of water-soluble peptides and salts. This procedure eliminated the tedious task of rotary evaporation of large volumes of the upper Folch solvent.

The ganglioside fraction was applied directly to DEAE-Sephadex (acetate form; 1 g ganglioside/20 g DEAE-Sephadex) and washed with excess methanol. Acidic phospholipids, sulfatides, and G_{M1} were eluted from the DEAE-Sephadex by washing with 10 mM ammonium acetate in methanol. Recovery of the majority of ganglioside was achieved by elution with 500 mM ammonium acetate in methanol. After removal of the methanol by rotary evaporation, the ganglioside fraction was

Abbreviations: POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DMPC, dimyristoylphosphatidylcholine.

dissolved in H_2O and desalted using Sephadex G-25 column chromatography. The resulting ganglioside fraction was found to be disialo- and trisialogangliosides by thin-layer chromatography ($CHCl_3$/MeOH/H_2O, 55 : 45 : 10, (0.02% $CaCl_2 \cdot 2H_2O$)). G_{M1} was prepared from the mixed gangliosides by enzymatic treatment with neuraminidase using a modification of the method of Felgner et al. [11]. The ganglioside (0.5 g) and egg phosphatidylcholine (1.0 g) were dissolved in $CHCl_3$/CH_3OH (1 : 2) and dried in a film on the side of a flask. This material was dispersed in 80 ml of a suspension containing sodium acetate (pH 5.0), 0.02% sodium azide and neuraminidase (10 units). After incubation for 24 h at 37°C, G_{M1} was isolated as described by Felgner et al. [11]. The fatty acid composition of the G_{M1} was identical to that of asialo-G_{M1}, prepared from it and reported previously [12].

Preparation of liposomes

POPC and DMPC were purchased from Avanti Biochemicals. Large multilamellar liposomes were prepared from stocks of POPC and G_{M1} dissolved in $CHCl_3$/CH_3OH (1 : 1). A total of 2.5 μmol of the dissolved phosphatidylcholine and G_{M1} were mixed together, and the solvent was evaporated under a stream of nitrogen at 22°C. The sample was kept under vacuum at least 2 h before suspension in buffered saline (150 mM NaCl/50 mM Tris-HCl (pH 7.4)/1 mM EDTA/0.02% sodium azide) by vortexing for 15 s. In other experiments the $CHCl_3$/CH_3OH solution of the lipids was sprayed onto a glass plate at room temperature using a custom-made glass spray system (Miller, I., personal communication). After the lipid-coated glass plate had been kept under vacuum for 30 min the dried lipid mixture was scraped from the plate with a razor blade and resuspended by vortexing (15 s) in Tris-buffered saline above the phase-transition temperature of the phosphatidylcholine. This second procedure proved to be a convenient way to avoid unmixing of the two lipids during solvent removal. Before use, the lipid dispersions were gently agitated in a shaker bath overnight at the desired temperature, washed once by centrifugation at 1000 × *g*, and then adjusted with Tris-buffered saline to a final concentration of 5 mM.

Preparation of ferritin-cholera toxin conjugate

The conjugation mixture contained 6 mg of cholera toxin (Sigma) and 36 mg of EM grade ferritin (Polysciences, Inc.) in 3.36 ml Tris-buffered saline (50 mM Tris/150 mM sodium chloride) containing 1 mM EDTA at pH 7.4. At room temperature, 18 μl of 8% ultrapure glutaraldehyde (Polysciences) was added dropwise while stirring vigorously. The conjugation proceeded for 1 h and the reaction was stopped by adding 0.1 M glycine, followed by reduction with 5 mM $NaBH_4$, stirred for 15 min and then dialyzed overnight against Tris-buffered saline. The conjugate was then centrifuged at 20000 × *g* for 15 min, concentrated to 3 ml with an Amicon filtration apparatus (PM-10 filter), and applied to a Bio-Gel A-5m column (1 × 167 cm) equilibrated with Tris-buffered saline. The absorbance at 440 nm of each fraction was measured to determine ferritin concentrations. Fractions containing monomeric ferritin linked to cholera toxin were pooled and concentrated 2-fold.

Preparation of labeled liposomes and freeze-etching procedures

0.02 ml of multilamellar POPC or DMPC liposomes containing varying amounts of G_{M1} were treated with a 10-fold excess of cholera toxin or cholera toxin-ferritin for 30 min. The liposomes were then washed once by adding excess buffer, centrifuging at 6000 × *g* for 5 min and resuspending the pellet in Tris-saline buffer. Small aliquots of the labeled liposomal preparations were placed on gold alloy specimen carriers and frozen in liquid Freon 22 cooled by liquid nitrogen. Specimens were freeze-fractured at −103°C and were deep-etched for 5 min at −103°C in a Balzers BAF300 freeze-etching apparatus. Specimens were then shadowed with platinum-carbon and replicas were cleaned with concentrated chromic acid/sulfuric acid solution for 2.5 min, washed with distilled water, and picked up on untreated 300-mesh copper electron microscope grids. In order to remove any residual lipid from the replica, the grids were dipped in chloroform/methanol (2 : 1, v/v). They were then examined in an Hitachi HU12A electron microscope.

Results and Discussion

The binding of cholera toxin to the surface of liposomes containing G_{M1} is essentially irreversi-

ble as judged from the label density after repeated washes by centrifugation. No agglutination of the G_{M1}-containing liposomes was observed at the saturating concentration of cholera toxin used in the experiments. The complete absence of agglutination under these conditions has been noted previously in biological membrane and phospholipid vesicle systems [13,14].

Electron microscopy

The ultrastructural appearance of freeze-etched POPC multilamellar liposomes containing varying amounts of G_{M1} and labeled with ferritin-cholera toxin is shown in Fig. 1. These specimens were quenched from 22°C, a temperature well above the main phase-transition of POPC (approx. 0°C). Control POPC liposomes without G_{M1} did not label with ferritin-cholera toxin (Fig. 1A), whereas liposomes containing increasing amounts of G_{M1} showed a corresponding increase in ferritin-cholera toxin on the outer etched surface of the liposomes (Fig. 1B and C). At concentrations of G_{M1} above 1 mol%, uniform coverage of the liposomal surfaces with the ferritin-cholera toxin marker was seen (Fig. 1C). A plot of the fractional area of liposomal surface covered by ferritin-cholera toxin as a function of the mole fraction of G_{M1} in the liquid crystalline POPC bilayer at 22°C is shown in Fig. 2.

Electron micrographs of POPC liposomes labeled with native cholera toxin are shown in Fig. 3. The native cholera toxin molecules can be resolved in the freeze-etch micrographs as individual spherical particles 86 Å in diameter. Complete coverage of the liposomal surface by the native cholera toxin was achieved at approx. 7 mol% G_{M1} (Fig. 3C), and a plot of the fractional area of liposomal surface covered by this marker as a function of the mole fraction of G_{M1} at 22°C is shown in Fig. 4.

Fig 3A and B concern the same preparation of POPC liposomes containing 1.8 mol% G_{M1}, except

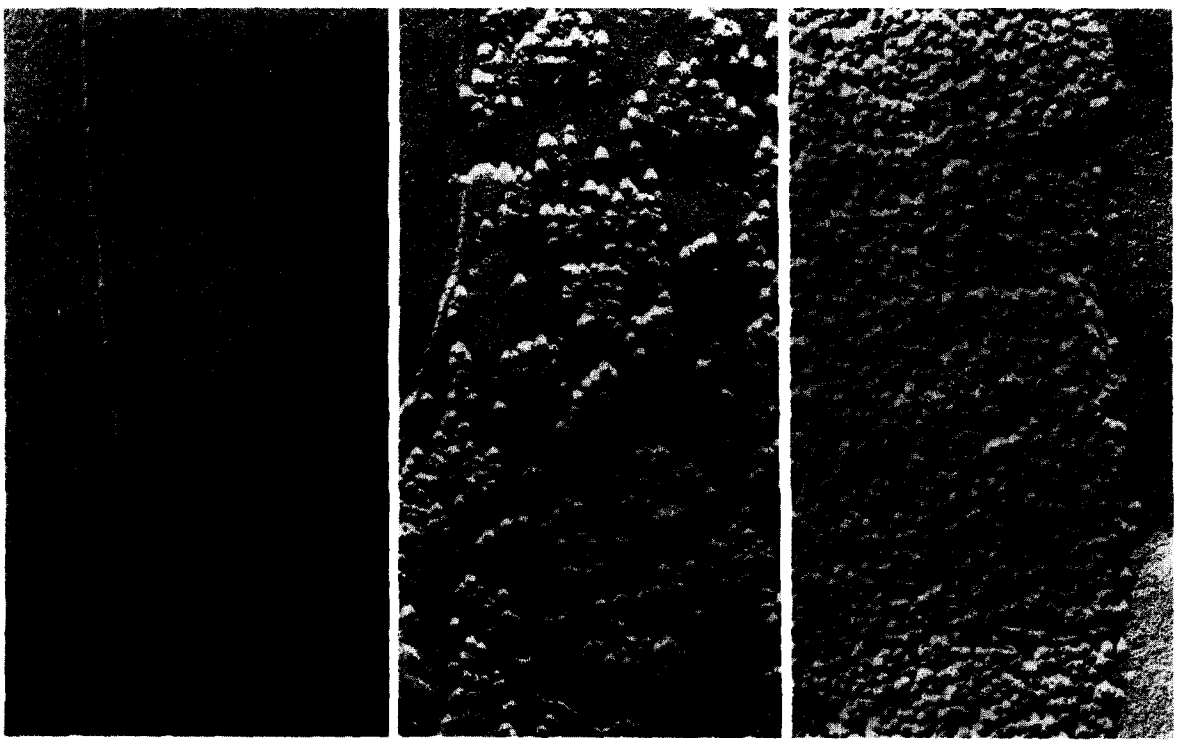

Fig. 1. Freeze-etch electron micrographs of POPC multilamellar liposomes containing varying amounts of G_{M1}, labeled with ferritin-cholera toxin, and quenched from 22°C. (A) Pure POPC liposome; (B) POPC liposome with 0.25 mol% G_{M1}; (C) POPC liposome with 2.25 mol% G_{M1}. Magnification is 100000×.

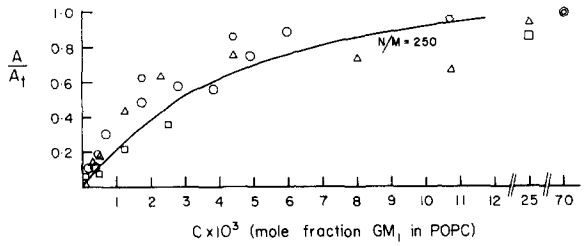

Fig. 2. The fraction of area covered by ferritin-cholera toxin labels, A/A_t is shown as a function of G_{M1} concentration in the bilayer. The matrix lipid is POPC quenched from 22°C. The solid line is calculated using Eqn. 7 with $N/M = 250$. The different symbols denote different experiments: ○, △, $A_t = 3456/\mu m^2$; ○, $A_t = 3000/\mu m^2$; □, $A_t = 3051/\mu m^2$; mixed lipids were prepared by the spray technique outlined in Materials and Methods. Mixed lipids in all other experiments were prepared by rotary evaporation from the organic solvent. Error bars are not indicated for simplicity, but are similar to those shown in Fig. 4.

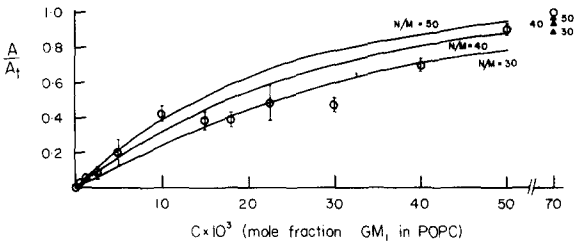

Fig. 4. The fraction of area covered by cholera toxin labels, A/A_t, is shown as a function of G_{M1}, concentration in the bilayer. Matrix lipid is POPC at 22°C. $A_t = 12764/\mu m^2$. Mixed lipids were prepared by the spray technique. Solid lines are calculated using Eqn. 7 for indicated values of N/M.

that the method of freezing and shadowing of the specimens was different. The preparation in Fig. 3A was hand-quenched in the usual manner, using liquid Freon cooled by liquid nitrogen. The preparation in Fig. 3B was frozen using the liquid helium device in Dr. John Heuser's laboratory at Washington University in St. Louis, and was rotary shadowed. Although the numerical counts per unit area of the cholera toxin molecules were remarkably similar (Fig. 3A, 4911 molecules/μm^2;

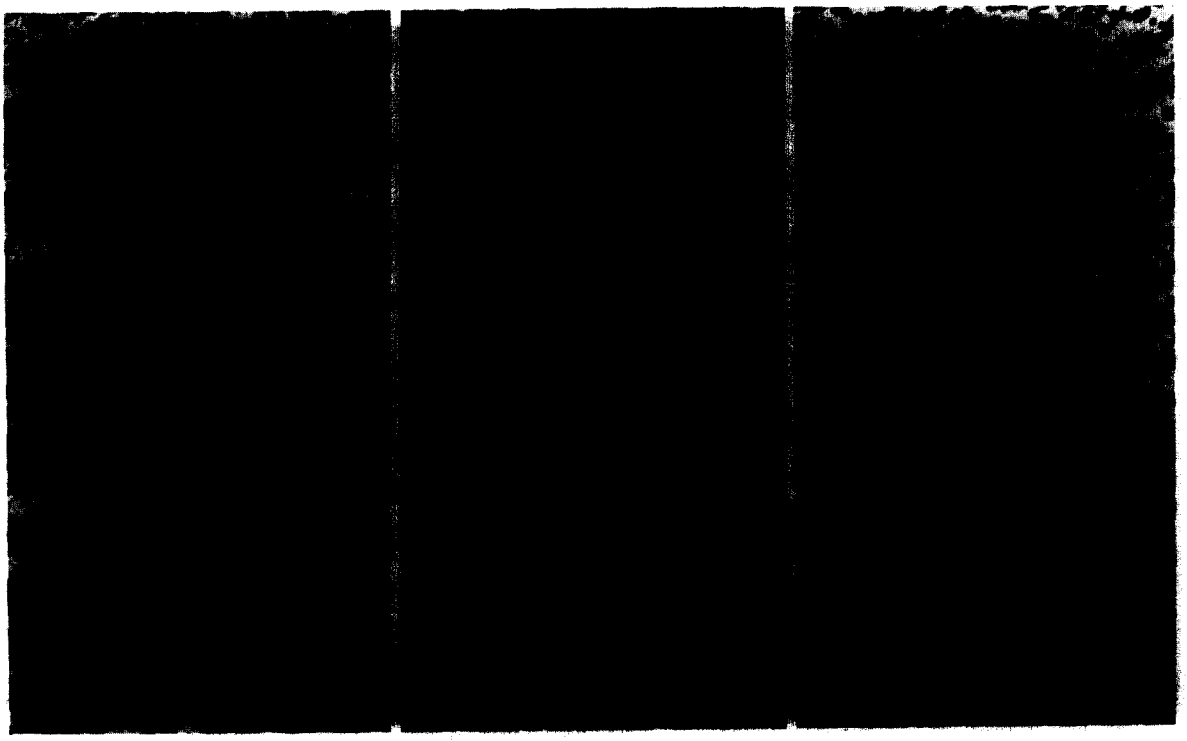

Fig. 3. Freeze-etch electron micrographs of POPC multilamellar liposomes containing varying amounts of G_{M1}, labeled with native cholera toxin, and quenched from 22°C: (A) POPC liposome with 1.8 mol% G_{M1}, quenched in liquid Freon; (B) POPC liposome with 1.8 mol% G_{M1}, quenched with a liquid-helium freezing device and rotary shadowed; (C) POPC liposome with 7 mol% G_{M1}. Magnification is approx. 300000×.

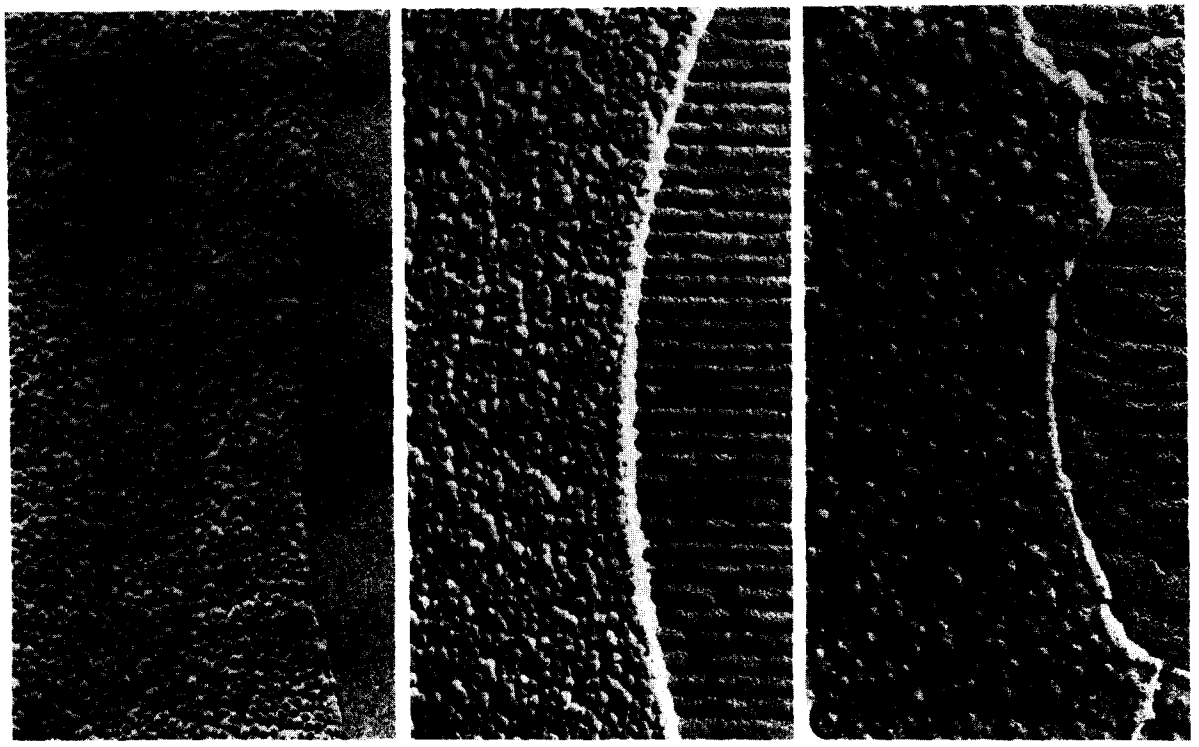

Fig. 5. Freeze-etch electron micrographs of DMPC multilamellar liposomes containing G_{M1}, labeled with ferritin-cholera toxin, and quenched from varying temperatures: (A) DMPC liposome containing 10 mol% G_{M1} quenched from 39°C; (B) DMPC liposome containing 10 mol% G_{M1} quenched from 20°C; (C) DMPC liposome containing 0.5 mol% G_{M1} quenched from 20°C. Magnification is 100000×.

Fig. 3B, 4924 molecules/μm^2), there was a difference in the pattern of labeling. The conventionally frozen preparations showed some aggregation of label, probably as a result of phase separation of the G_{M1} during the freezing process, while the liquid helium frozen specimen showed an evenly dispersed labeling pattern.

In order to compare the distribution of G_{M1} in liposomes at temperatures above and below the main phase-transition temperature of the matrix phospholipid, G_{M1} was incorporated into DMPC liposomes that were incubated and labeled with ferritin-cholera toxin at 4, 20 and 39°C. At 39°C, well above the main phase-transition of DMPC (24°C), the label was uniformly distributed over the liposomal surface but packed more densely at saturation than the POPC system at 22°C (Fig. 5A). In DMPC liposomes containing up to 10 mol% G_{M1} and incubated at 20°C, just below the main transition, the G_{M1} was evenly distributed over the liposomal surface and the labeling showed no correspondence to the prominent ridged pattern of the $P_{\beta'}$ phase (Fig 5B and C). At 4°C, below the pretransition of DMPC, uniform dispersed labeling was seen. These labeling patterns at all temperatures are markedly different from those seen in DMPC bilayers containing the neutral glycosphingolipid, asialo-G_{M1} [15].

Data analysis

The important information contained in the freeze-etch data is the average number of G_{M1} molecules under a marker. The problem of extracting this information is made difficult by the simple fact that the cross-sectional area of G_{M1} is very much smaller than the corresponding area of the ferritin-cholera toxin or cholera toxin label. In fact, each macromolecular label can cover from one to several hundred G_{M1} molecules in the liposome surface. The solution to the problem can be obtained as follows.

Consider a lattice on the surface of the lipo-

some defined by the centers of close-packed label macromolecules. In the area associated with each lattice point, there can be as many as N/M ganglioside molecules. Here N equals the cross-sectional area associated with a macromolecular label at a lattice point and M is the molecular area of a G_{M1} molecule in the bilayer surface. The N/M positions in the lattice point area in turn define a sub-lattice.

A lattice point will be occupied by a macromolecule label if from 1 to N/M ganglioside molecules are present in the area associated with the lattice point. Thus the probability, π, that a lattice point will be occupied by a label is the sum of the probabilities that from 1 to N/M ganglioside molecules are present in the sub-lattice:

$$\pi = \sum_{n=1}^{N/M} P_n \tag{1}$$

Here P_n is the probability that n G_{M1} molecules are in the area associated with each lattice point.

However,

$$\pi = 1 - P_0 \tag{2}$$

P_0, the probability of finding no G_{M1} molecule in the sub-lattice, is given by:

$$P_0 = (1-p)^{N/M} \tag{3}$$

Here p is the intrinsic probability of finding a G_{M1} molecule at any one of the N/M positions of the sub-lattice. Combining Eqns. 2 and 3, the general solution to the problem can be obtained. Thus:

$$\pi = 1 - (1-p)^{N/M} \tag{4}$$

π and p can be converted into experimental parameters by recognizing that the probability of lattice point occupancy by a label is equal to the fraction of the total area covered by macromolecular labels at a given concentration of G_{M1} in the POPC bilayer. The probability, p, of a G_{M1} molecule occurring in the lattice point area is equal to the mole fraction of G_{M1} in the bilayer. Thus,

$$\pi = A/A_t \tag{5}$$

and

$$p = C \tag{6}$$

Here A is the number of label macromolecules per unit area of bilayer at a given G_{M1} concentration, C, and A_t is the number of label macromolecules per unit area when the G_{M1} concentration is equal to or greater than that required to saturate the surface with label. C is the concentration of G_{M1} expressed as a mole fraction. Combining Eqns. 4, 5 and 6 gives:

$$A/A_t = 1 - (1-C)^{N/M} \tag{7}$$

The solid line in Fig. 2 is the best fit to the ferritin-cholera toxin data. The value of N/M obtained from this fit is 250. The best fit for the data shown in Fig. 4 obtained using cholera toxin alone as a label gives a value of $N/M = 40$. Solid lines shown in Fig. 4 correspond to values of $N/M = 50$, 40 and 30 in descending order from the top-most curve.

Let us now compare the experimentally determined values of N/M with the values for this ratio calculated from the areas associated with the close-packed macromolecular labels and the area per molecule of G_{M1}. The parameters required for this comparison are given in Table I. In the first row of the table are the molecular diameters of ferritin [16] and cholera toxin [17,18]. Assuming that these molecules are close-packed in a cubic array, the experimentally determined area associated with each is 26% larger than the circular cross-section of each molecule and if packed in hexagonal array is 18.9% larger. The calculated values of the associated areas for both cubic and hexagonal packing are given in row 2 of Table I.

TABLE I

	Ferritin	Cholera toxin
1. Molecular diameter, Å, (literature)	122	90
2. Area per molecule, Å^2 (close packed)		
cubic	$14.7 \cdot 10^3$	$8.01 \cdot 10^3$
hexagonal	$13.9 \cdot 10^3$	$7.56 \cdot 10^3$
3[a]. Area per molecule, Å^2 (from A_t)	$31.7 \cdot 10^3$	$7.85 \cdot 10^3$
4. $(N/M)_t$	264	65
5[a]. $(N/M)_{exp}$	250	40
6. $(N/M)_t/(N/M)_{exp}$	1.05	1.6

[a] Data obtained at 22°C.

The area per macromolecular label as calculated from A_t is given in row 3. Comparison of the area data in rows 2 and 3 for cholera toxin shows good agreement. This indicates that the molecules of cholera toxin at saturation are indeed close-packed on the bilayer surface. In contrast, the cross-section determined for the ferritin-cholera toxin conjugate from A_t is about twice that calculated from the known molecular diameter of ferritin. The irregular shape of this conjugate and/or intermolecular interactions between conjugates is undoubtedly the cause of this difference. However, at 39°C in DMPC bilayers (Fig. 5A), the cross-section determined from A_t is $17.6 \cdot 10^3$ Å^2. This is in much better agreement with the values in Table I, row 2, calculated from the molecular diameter. In any event, the experimentally determined cross-section of the ferritin-cholera toxin conjugate at 22°C is 4-times the value obtained for cholera toxin itself at this temperature. Using these values and the area per molecule of ganglioside, equal to 122 Å^2 as determined from monolayer studies [19], the values of $(N/M)_t$ found in row 5 can be calculated. The ratio of the value of $(N/M)_t$ to the experimentally determined value $(N/M)_{exp}$ is given in row 6. The value of the ratio for the ferritin-cholera toxin label system is almost exactly 1, whereas the value for the cholera toxin used alone as a label is 1.6. It is clear from these results that the average number of G_{M1} molecules under both types of label is essentially 1. This result leads to the conclusion that G_{M1} is molecularly dispersed in liquid crystalline POPC bilayers at room temperature in the concentration range of these studies (0 to about 5 mol% G_{M1}). The fact that each cholera toxin molecule has five binding sites for G_{M1}, all lying in a plane [18], is not reflected in the label density in these studies. This is the case because the low mole fraction of G_{M1} employed combined with the use of a very large excess of cholera toxin and the magnitude of the binding constant eliminated the subsequent recruitment of unbound G_{M1} by unoccupied G_{M1} sites on cholera toxin already bound to the surface. Under these circumstances the pattern of labeling reflects the organization of the surface at the instant of addition of label to the system.

It is interesting to examine the applicability of Eqn. 7 if microdomains rich in a molecular species which can be labeled exist in the bilayer surface. Under this condition, the probability, p, is still equal to the mole fraction of this species in the surface. This is the case, since the identification of p with C tacitly assumes that the chance of a label interacting with a particular molecular species in the bilayer is given by the fraction of the bilayer surface occupied by that species. This, to a good approximation, is given by its mole fraction. This will remain true even if this component is organized in a microdomain structure, since the probability of a label interacting with a molecule of this species is essentially independent of the surface arrangement of these molecules. However, once a molecule in a microdomain has complexed with a macromolecular label, all other similar molecules in that domain became unavailable for interaction with other labels, provided the microdomain cross-section is small compared to that of the macromolecular label. Thus, following the basic argument, the number of domains that can be accommodated in the area associated with each lattice point will be N/XM, where X is the average number of molecules which potentially can be labeled in a microdomain. As before, N and M are the cross-sectional areas of a macromolecular label and a molecule of the type which can be labeled, respectively. Thus, the experimental value of the exponent in Eqn. 7 will be $(N/XM)_{exp}$. Consequently the number, X, of the type molecules which can be labeled in a microdomain is given by:

$$X = (N/M)_t/(N/XM)_{exp} \tag{9}$$

It is clear that as the domain size increases, the experimentally determined value of the exponent in Eqn. 7 decreases. When the value of the ratio becomes less than 1, patches of label molecules will appear on the surface. It is just this set of circumstances that has been described for POPC bilayers containing the neutral glycosphingolipid, asialo-G_{M1} [15].

The concentration window which can be examined by freeze-etch electron microscopy using macromolecular labels and the data analysis outlined above extends from about 10^{-4} mole fraction of labeled molecule to 90% saturation of the bilayer surface with label. This occurs at about $1 \cdot 10^{-2}$ mole fraction for ferritin-cholera toxin

and about $5 \cdot 10^{-2}$ mole fraction for cholera toxin alone in the G_{M1}-POPC system. Obviously, nothing can be learned about molecular organization in the bilayer over the concentration range in which surface saturation of label occurs. It is apparent from Eqn. 7 that the breadth of the concentration window is controlled by the value of N/M. The window widens as N/M decreases. This occurs either as N decreases or M increases. Obviously the best circumstances obtain when $N/M = 1$.

The conclusion drawn from the electron microscope data presented in this paper is in agreement with inferences drawn from several other physical studies carried out on ganglioside-containing phospholipid bilayer systems. Differential scanning calorimetric studies suggest that, at low concentrations of gangliosides, these molecules are molecularly dispersed in dipalmitoylphosphatidylcholine bilayers [20–23]. This conclusion has also been supported by fluorescence polarization studies using diphenylhexatriene [24]. ESR investigations employing a nitroxide spin label covalently linked to the sugar moiety have also provided evidence that at low ganglioside concentrations no ganglioside clustering occurs in liquid crystalline phosphatidylcholine bilayers [25–27].

Danielle and co-workers [28], also using ESR, have, however, provided evidence that although gangliosides at low concentration in liquid-crystalline phase dipalmitoylphosphatidylcholine bilayers are randomly distributed, they appear to cluster when the matrix phospholipid is in the gel phase. A freeze-etch study of bovine brain ganglioside distribution in dielaidoylphosphatidylcholine-DPPC liposomes, labeled with native wheat germ agglutinin, indicated that although the gangliosides were distributed in both fluid and gel regions of the matrix phospholipids, the label appeared in small clusters [29]. Recently Peters and co-workers [30,31], using native *Ricinus communis* agglutinin as a label in a freeze-etch study, have reported that ganglioside molecules are localized preferentially in the troughs between the $P_{\beta'}$ phase ripples, as we had previously reported for the uncharged glycosphingolipid asialo-G_{M1} [15]. Using the freeze-etch technique, we have not observed clustering of G_{M1} in DMPC bilayers in the gel state (Fig. 5). Furthermore, we did not observe clusters or microdomains of G_{M1} in specimens that were fast-frozen by the liquid-helium method [32]; it appears that rapid freezing prevents phase separation of G_{M1} from the matrix phospholipid which may occur during the slower freezing method performed by plunging specimens into liquid Freon by hand. In our studies, we prevented unmixing of gangliosides from phospholipids during preparation of liposomes by a spraying method that facilitated rapid evaporation of the solvent. With regard to this point, it is important to understand that our conclusions are based on an analysis of the surface density of label as a function of G_{M1} concentration and not on the pattern of surface distribution of label.

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