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ORGANIZATION OF THE GLYCOSPHINGOLIPID ASIALO-G_{M1} IN PHOSPHATIDYLCHOLINE BILAYERS

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An affinity purified monovalent ferritin conjugate of *Ricinus communis* agglutinin (RCA 60) is used with freeze-etch electron microscopy to study the ultrastructural localization of the glycosphingolipid asialo-G_{M1} in multilamellar phosphatidylcholine liposomes. Dimyristoylphosphatidylcholine (DMPC) liposomes containing up to 20 mol% asialo-G_{M1} and quenched below the main transition temperature show a striking linear localization of ferritin-RCA 60 between phospholipid ridges. The glycosphingolipid localization is similar to that postulated for up to 20 mol% cholesterol in pure phosphatidylcholine bilayers by Copeland, B.R. and McConnell, H.M. (Biochim. Biophys. Acta, 599, 95–109 (1980)). Above the main phase transition temperature, asialo-G_{M1} appears to be organized into clusters, especially in palmitoyloleoylphosphatidylcholine (POPC) liposomes. This clustered distribution of glycosphingolipids seen above the phase transition temperature suggests that this type of lipid may exhibit compositional domain structure in biological membranes.

Introduction

Glycolipids are important components of biological membranes which serve as surface receptors for certain bacterial toxins [1–3], viruses [4,5] and hormones [6,7], and may play a role in cell-cell recognition and transformation [8,9]. Many blood group antigens, including the ABH, Lewis, P, Ii and Forssman antigens, are present as red cell membrane glycosphingolipids (for review, see Ref. 8). Although it has been shown that glycolipids are present on the exterior surface of membranes [10,11], there is very little information on the organization of these molecules in the plane of the membrane. Previous studies of glucocerebroside incorporated into dipalmitoylphosphatidylcholine mutlilamellar and unilamellar liposomes suggested that the glucocerebroside formed domains in the phospholipid bilayer [12,13]. Glycolipid domains could function as recognition sites of complex carbohydrate composition analogous to membrane glycoproteins.

Liposomal systems offer certain advantages for studying glycosphingolipid organization in membranes. Liposomes can be constructed from defined chemical components, and glycosphingolipids incorporated into liposomes can serve as receptors for plant lectins, which will aggregate them [14–16[. Ferritin-labelled lectins or antibodies can be used as ultrastructural markers in freeze-etch preparations to localize the glycosphingolipids in the phospholipid bilayer. This approach circumvents the problem of cross-reaction of the label with similar carbohydrate units on membrane glycoproteins or inaccessibility of glyco-

Abbreviations: DMPC, dimyristoylphosphatidylcholine; POPC, palmitoyloleoylphosphatidylcholine; RCA 60, *Ricinus communis* agglutinin 60 or ricin; RCA 120, *Ricinus communis* agglutinin 120.

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spingolipids to the label because they are covered over by protein. In addition, glycosphingolipid organization in pure phospholipid bilayers can be examined by freeze-etching under different conditions of bilayer fluidity by varying the temperature at which liposomes are labelled and quenched.

In order to study the organization of glycosphingolipids incorporated into phospholipid bilayers, we have used an affinity-purified monovalent ferritin conjugate of *Ricinus communis* agglutinin 60 (RCA 60) to determine the ultrastructural localization of asialo-G_{M1} in both multilamellar dimyristoylphosphatidylcholine (DMPC) liposomes at temperatures spanning the main phase transition temperature of DMPC and in multilamellar palmitoyloleoylphosphatidylcholine (POPC) liposomes above its main transition. We have also examined the effect of added asialo-G_{M1} on the bilayer organization of DMPC by use of differential scanning calorimetry and fluorescence polarization.

Material and Methods

Preparation of glycolipid

Asialo-G_{M1} was prepared from mixed gangliosides from bovine brain by treatment with 1 M formic acid at 80°C for one hour [17] and then purified by chromatography on Sephadex LH-20, DEAE-Sephadex A25, and Iatrobeads (6RS 8060, Iatron Laboratories, Tokyo). The purity of the asialo-G_{M1} preparation was checked by thin-layer chromatography, and the concentration was determined by a fluorescamine assay [18].

Liposome preparation

Pure DPMC and POPC were purchased from Avanti Biochemicals, Inc. Large multilamellar liposomes were prepared from stocks of DMPC and asialo-G_{M1} dissolved in chloroform. Approx. 2.5 μmol of the desired DMPC-asialo-G_{M1} mixture was placed in a round bottom flask and heated to 50°C briefly. The solvent was evaporated on a rotary evaporator at 23°C. The sample was kept under vacuum overnight. The dried lipid was heated to 30°C and suspended in phosphate-buffered saline (0.15 M NaCl containing 0.005 M phosphate buffer, pH 7.3) at the same temperature. Multilamellar liposomes were prepared by vortexing for 1 min at 30°C, then gently agitating in a shaker bath overnight at the same temperature. The liposomal suspensions were washed once by centrifugation at $1000 \times g$. Suspensions of the lipids were adjusted with phosphate buffered saline to a final concentration of 5 mM.

Multilamellar POPC liposomes containing asialo-G_{M1} were prepared in a similar manner, except that the dried lipid was suspended in phosphate buffered saline and agitated overnight at room temperature.

Preparation of RCA 60

RCA 60 and RCA 120 lectins were prepared from castor beans essentially by the method of Nicholson [19]. An affinity column was used for purification of the RCA 60 and RCA 120 lectins; it consisted of Sepharose 4B (Pharmacia) treated for 3 h with 2 M HCl at 50°C and washed with phosphate buffered saline until the pH was 7.3. The bound lectins were eluted with 0.2 M lactose, and RCA 120 and RCA 60 were separated from each other on a Sephadex G-100 column. Disc gel electrophoresis was used to check purity of the two lectins. An aliquot of RCA 60 was iodinated with ^{125}I by the method of Hunter [20] using a 10-s exposure to chloramine-T.

Preparation of a monovalent ferritin-RCA 60 conjugate

The ferritin conjugation of RCA 60 was similar to the procedure described previously [21], except that the conjugation was carried out in phosphate buffered saline with 0.2 M lactose. The conjugation reaction was stopped with 0.1 M glycine. After overnight dialysis against phosphate buffered saline, the conjugate was centrifuged 20 min at $40000 \times g$, the supernatant concentrated with an Amicon filtration apparatus, and applied to a Biogel A5m column (1×167 cm) equilibrated with 0.2 M lactose in phosphate-buffered saline. Fractions (1.5 ml) were collected and assayed for radioactivity to determine lectin concentration and for absorbance at 440 nm to determine ferritin concentration. Fractions containing approximately equimolar amounts of ferritin and lectin were combined, dialysed extensively against phosphate-buffered saline, and rechromatographed on an

acid-treated Sepharose 4B affinity column, as described in the preceding section. Unconjugated ferritin or inactivated conjugate were washed through the column with phosphate buffered saline. Bound conjugate was eluted with a continuous gradient of galactose. Fractions which did not agglutinate multilamellar liposomes containing asialo-G_{M1}, as judged by phase microscopy, but which contained radioiodinated RCA 60, were used in the labeling experiments. It was assumed that these fractions consisted of monovalent lectin-ferritin conjugates and that more tightly bound conjugates, which did agglutinate liposomes containing asialo-G_{M1}, had at least two RCA 60 molecules conjugated to a ferritin molecule.

Preparation of ferritin-labelled liposomes and freeze-etching procedures

Liposomes were allowed to equilibrate at temperatures of 4°C, room temperature, and 37°C for 24–48 h. 0.02 ml of multilamellar DMPC or POPC liposomes containing varying amounts of asialo-G_{M1} were treated with 0.2 ml of 250 μg/ml monovalent ferritin-RCA 60 conjugate at the same temperature. After a 30 min incubation, small aliquots of the incubation mixture were placed on gold alloy specimen carriers and frozen in liquid Freon 22 cooled by liquid nitrogen. This was done in a cold room for 4°C preparations and in a warm room for 37°C preparations to ensure temperature equilibrium prior to freezing in Freon. Control preparations either contained 0.2 M lactose as hapten inhibitor or consisted of liposomes lacking asialo-G_{M1}. Some room temperature preparations were washed once or twice in phosphate-buffered saline after ferritin-lectin labeling by centrifuging at $1000 \times g$ for 10 min and resuspending the pelleted labelled liposomes in phosphate-buffered saline.

Specimens were freeze-fractured at −103°C and were deep-etched for 7 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-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.

Fluorescence polarization of diphenylhexatriene

Diphenylhexatriene was used as a hydrophobic probe in scanning fluorescence polarization as in previous studies [22–24]. Multilamellar DMPC liposomes containing from 0 to 13.4 mol% asialo-G_{M1} were diluted to 0.5 mM P_i in 5 ml of buffer and 1 μl of 2 mM diphenylhexatriene in tetrahydrofuran was added at 30°C while vortexing. The sample was incubated at 30°C for 45 min in a rotary shaker. Fluorescence measurements were made with an SLM 4800S (SLM Instruments, Urbana, IL) in a T-configuration as described in a previous paper [25]. The signal intensity from a scattering control which contained no fluorescent probe was less than 1% of the sample signal intensity. Temperature was scanned at 20 deg/h. The diphenylhexatriene fluorescence polarization was analyzed using the anisotropy parameter r,

$$r = \frac{(I_{\parallel}/I_{\perp})-1}{(I_{\parallel}/I_{\perp})+2}$$

where $I_{\parallel}$ is the intensity parallel and $I_{\perp}$ is the intensity perpendicular to the incident beam.

Differential scanning calorimetry

The excess heat capacity of multilamellar liposomes containing mixtures of asialo-G_{M1} in DMPC was measured with a high sensitivity scanning calorimeter of the heat conduction type, as previously described [22,24,26]. The concentration of phospholipid was 4–5 mM.

Results

Effect of addition of glycosphingolipid on ultrastructure of DMPC liposomes

The ultrastructural appearance of freeze-fractured multilamellar liposomes composed of pure DMPC at temperatures spanning the main phase transition temperature (24°C) is shown in Fig. 1. At 23°C a prominent ridged pattern is present on both the exterior surface and on the internal fracture faces of the lamellae comprising the DMPC liposomes (Fig. 1a). The periodicity of the ridges is 120 Å, in agreement with results previously re-

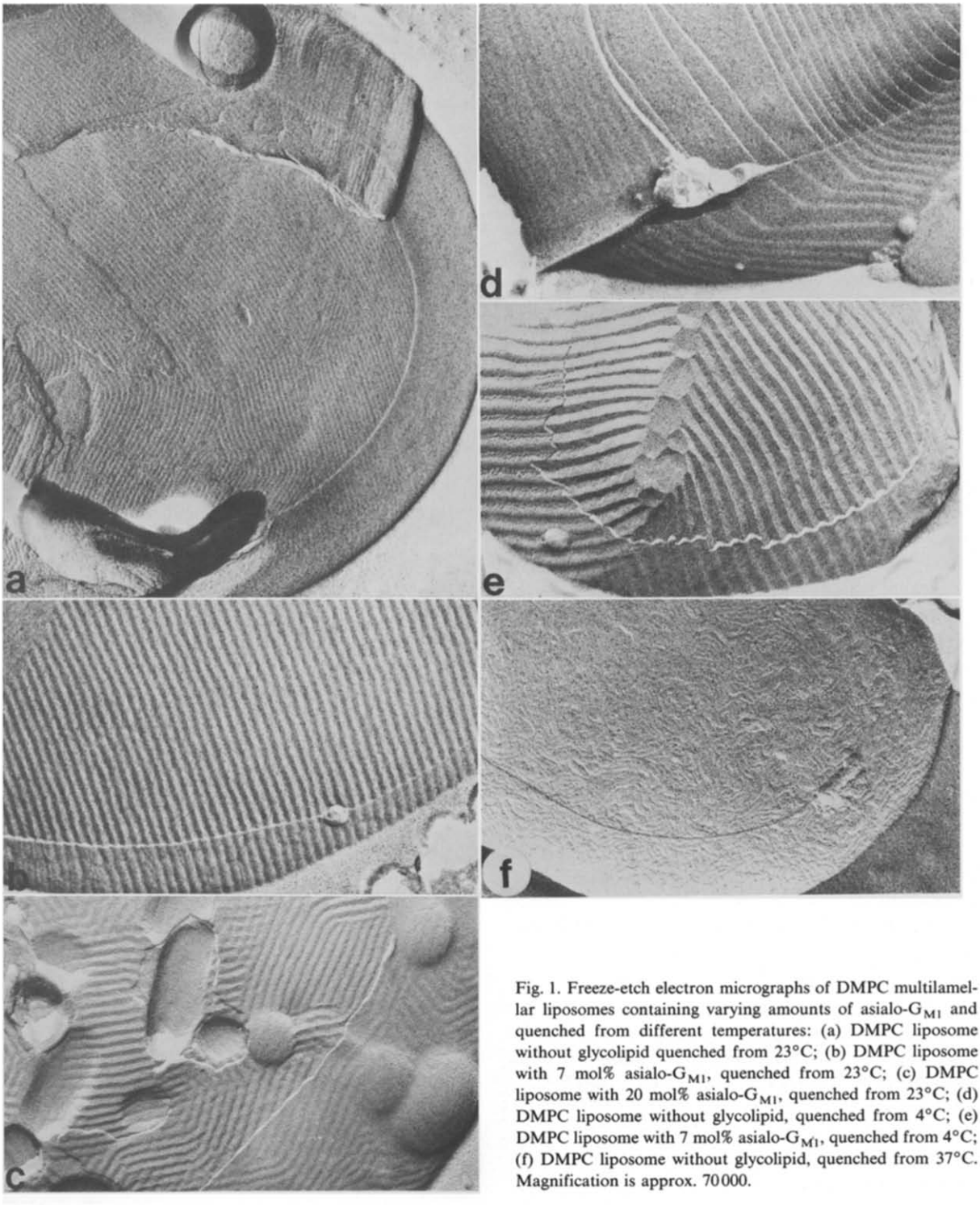


Fig. 1. Freeze-etch electron micrographs of DMPC multilamellar liposomes containing varying amounts of asialo-G_{M1} and quenched from different temperatures: (a) DMPC liposome without glycolipid quenched from 23°C; (b) DMPC liposome with 7 mol% asialo-G_{M1}, quenched from 23°C; (c) DMPC liposome with 20 mol% asialo-G_{M1}, quenched from 23°C; (d) DMPC liposome without glycolipid, quenched from 4°C; (e) DMPC liposome with 7 mol% asialo-G_{M1}, quenched from 4°C; (f) DMPC liposome without glycolipid, quenched from 37°C. Magnification is approx. 70000.

ported by Copeland and McConnell [27]. The ridge pattern is often in register through several lamellae; in other instances, merging patterns may be seen across bilayer fracture lines. At 4°C, well below the main transition temperature and pretransition temperature (14.5°C), liposomes consisting of pure DMPC show a ridged pattern which has a varying periodicity ranging from approx. 600 Å to practically smooth areas without a visible ridged pattern, as seen in Fig. 1d. At 37°C, above the main transition temperature, a 'jumbled' pattern of ridges is seen which has a periodicity of approx. 120–140 Å (Fig. 1f).

Addition of increasing amounts of the glycosphingolipid asialo-G_{M1} increases the distance between ridges in DMPC liposomes quenched from 23°C. As little as 1.3 mol% asialo-G_{M1} incorporated into DMPC liposomes increases the distance between ridges from 120 Å to 240 Å, although in these preparations both 120 Å as well as 240 Å periodicities may be seen in the same liposome (Fig. 2b). Addition of 7 to 20 mol% asialo-G_{M1} produces a ridge spacing of 240–260 Å, with elimination of the 120 Å periodicity (Figs. 1b and 1c). The amplitude of the ridges is also increased. We did not observe a linear increase in the ridge spacing with addition of increasing amounts of glycosphingolipid, such as that seen by Copeland and McConnell [27] with the addition of up to 20 mol% cholesterol to DMPC and dipalmitoylphosphatidylcholine liposomes.

Organization of glycosphingolipids in DMPC liposomes below the main transition temperature

Freeze-etch replicas of DMPC liposomes containing different amounts of asialo-G_{M1} labelled with ferritin-RCA 60 and quenched from 23°C are shown in Fig. 2. DMPC liposomes containing 1.3 mol% asialo-G_{M1} show sparse ferritin labeling on their outer surface (Fig. 2b). Addition of 7 mol% asialo-G_{M1} to DMPC liposomes results in a linear labeling pattern with monovalent ferritin-RCA 60 conjugate, as shown in Fig. 2c. Although the ferritin label is not completely continuous, ferritin molecules are distinctly seen in the bases of the ridges and are not present along the peaks. Liposomes containing 10 or 20 mol% asialo-G_{M1} have an increased amount of ferritin label on their outer surface which is in a striking linear pattern, with the ferritin occupying the bases of the 240 Å ridges (Fig. 2e). Table I shows the number of ferritin molecules per square micron counted on different surfaces of liposomes containing increasing amounts of the asialo-G_{M1}. Completely uniform labeling of the exterior surfaces was not achieved; in fact, a single washing procedure consisting of centrifugation of liposomes and resuspension in phosphate-buffered saline resulted in a decrease in labeling by as much as one-half. In spite of the fact that the liposomal preparations are not routinely subjected to washing procedures after incubation with ferritin-RCA 60, liposomes which do no contain glycosphingolipid but are treated with the ferritin-lectin probe do not show ferritin label on the outer etched surface of the multilamellar liposomes (Fig. 2a). Also, incorporation of 0.2 M lactose in the incubation mixture of ferritin-RCA 60 and asialo-G_{M1}-containing DMPC liposomes completely inhibits binding of the conjugate. Thus, there is no nonspecific binding of the

TABLE I

FERRITIN-RCA 60 BINDING SITES ON LIPOSOMES CONTAINING ASIALO-G_{M1}

Multilamellar DMPC and POPC liposomes containing varying amounts of asialo-G_{M1} were incubated at the indicated temperatures with ferritin-RCA 60 before quenching and freeze-etching. Ferritin on the surface of the liposomes was counted on electron micrographs using a Zeiss Videoplan System. Binding sites per square micron are expressed as the mean and standard deviation.

Phospholipid	Temp. (°C)	Asialo-G_{M1} (mol%)	Binding sites per μm^2
DMPC	23	1.3	213 (145)
		7.0	655 (118)
		7.0 [a]	127 (28)
		10.0	722 (54)
		13.4	720 (80)
		20.0	641 (391)
DMPC	4	1.0	141 (17)
		7.0	1087 (108)
		10.0	1735 (418)
		10.0 [a]	374 (14)
DMPC	37	7.0	130 (104)
POPC	37	7.0	313 (2)

[a] Labeled liposomes were washed 1× in phosphate-buffered saline.

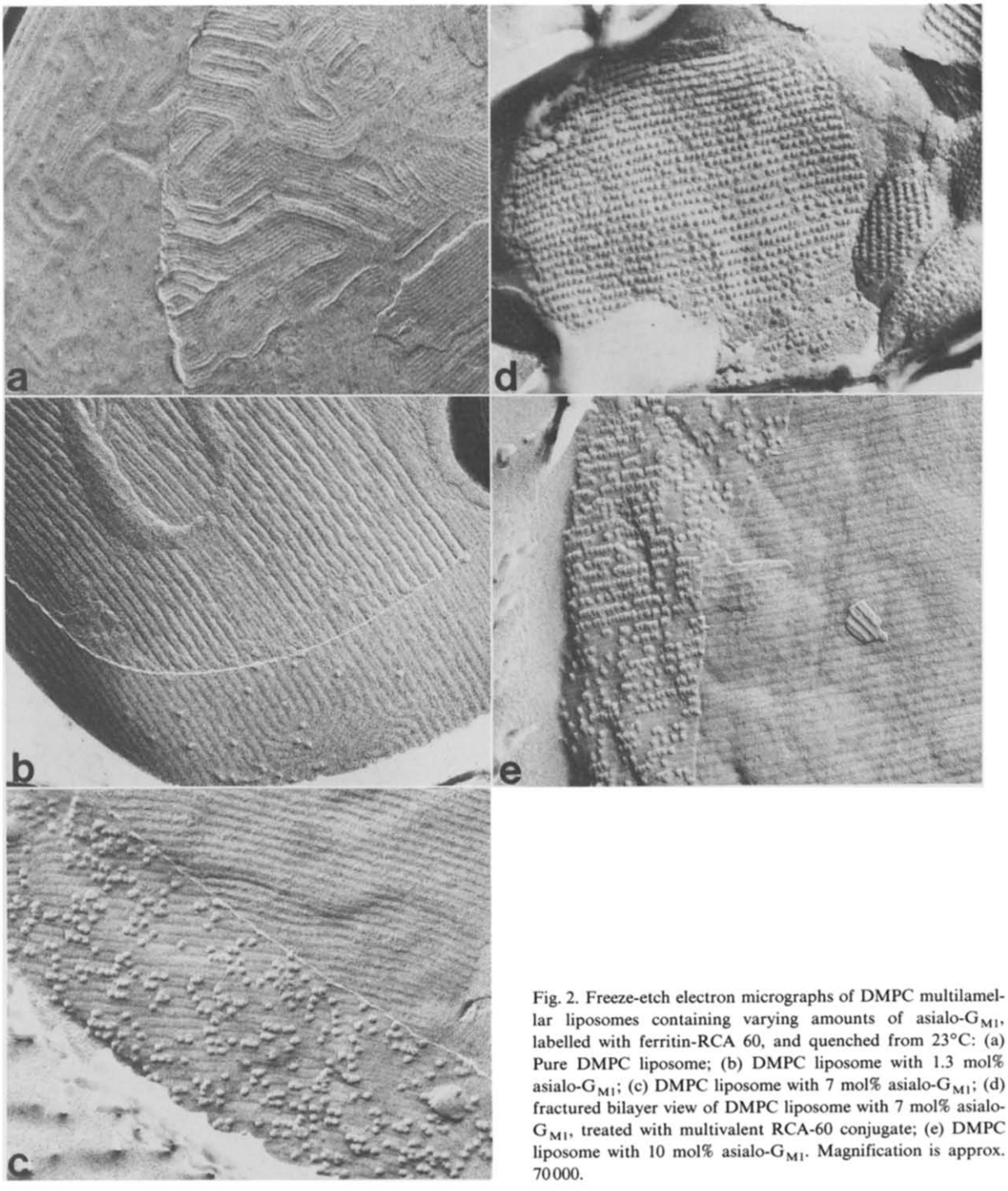
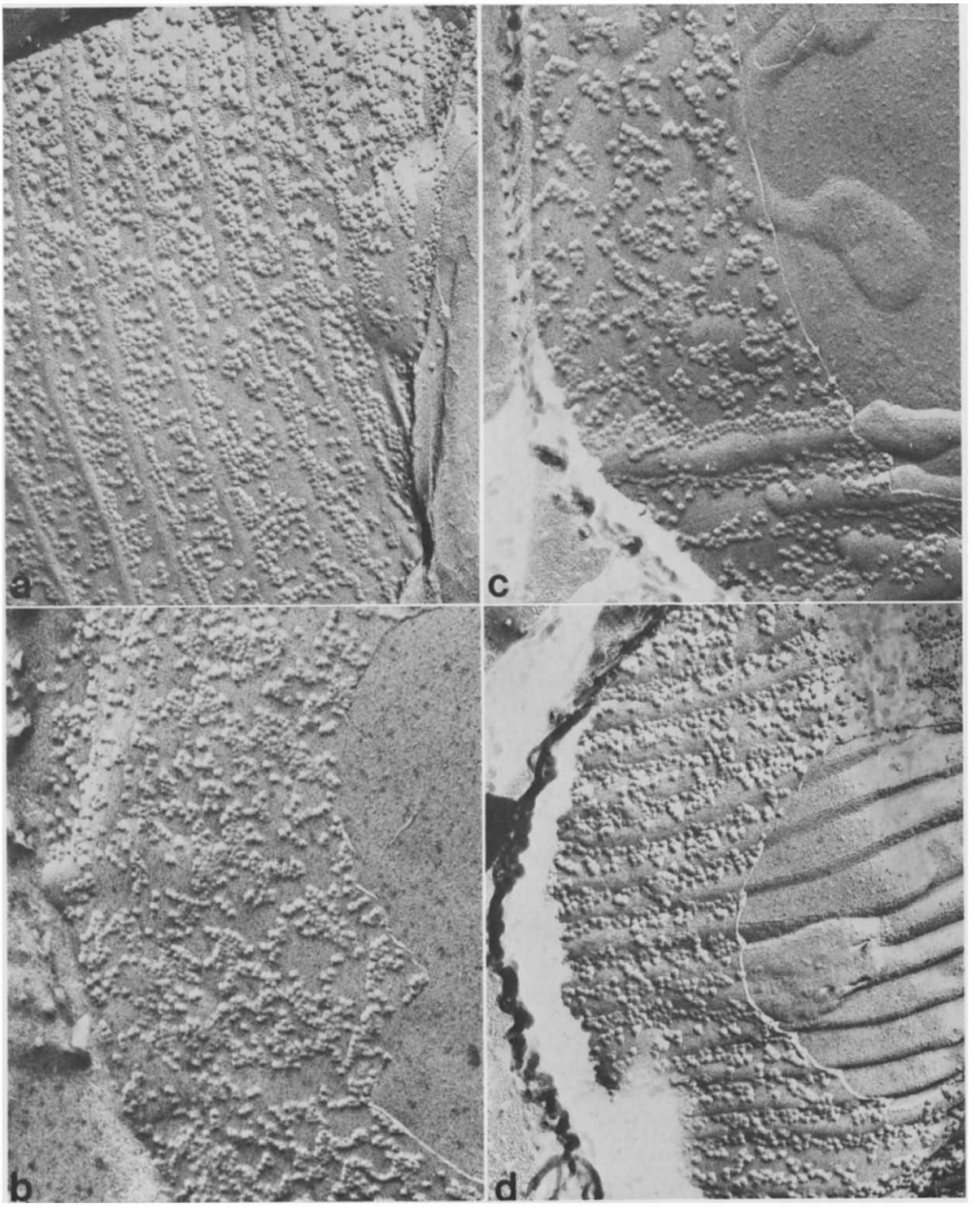


Fig. 2. Freeze-etch electron micrographs of DMPC multilamellar liposomes containing varying amounts of asialo-G_{M1}, labelled with ferritin-RCA 60, and quenched from 23°C: (a) Pure DMPC liposome; (b) DMPC liposome with 1.3 mol% asialo-G_{M1}; (c) DMPC liposome with 7 mol% asialo-G_{M1}; (d) fractured bilayer view of DMPC liposome with 7 mol% asialo-G_{M1}, treated with multivalent RCA-60 conjugate; (e) DMPC liposome with 10 mol% asialo-G_{M1}. Magnification is approx. 70000.

Fig. 3. Freeze-etch electron micrographs of DMPC multilamellar liposomes containing varying amounts of asialo-G_{M1}, labelled with ferritin-RCA 60, and quenched from 4°C: (a) DMPC liposome with 7 mol% asialo-G_{M1}, showing ridged area; (b) DMPC liposome



with 7 mol% asialo-G_{M1}, showing smooth area; (c) DMPC liposome with 7 mol% asialo-G_{M1}, showing ridged and smooth areas in same liposome; (d) DMPC liposome with 10 mol% asialo-G_{M1}. Magnification is approx. 70000.

ferritin-lectin conjugate to the outer surface of DMPC liposomes. Fig. 2d shows a freeze-fracture replica of a DMPC liposome containing 7 mol% asialo-G_{M1} which was incubated with a divalent or multivalent preparation of ferritin-RCA 60; this induced aggregation of the liposomes and trapped the ferritin label between opposing outer leaflets of adjacent liposomes. These preparations show a completely linear ferritin-labeling pattern, even though one must view the ferritin through the fractured outer leaflet of the bilayer.

As noted earlier, DMPC liposomes containing up to 20 mol% asialo-G_{M1} show both ridged and smooth areas when quenched from 4°C and examined by freeze-etch electron microscopy. When such preparations are labelled with ferritin-RCA 60 conjugate, the ferritin label is present between the ridges and in somewhat higher concentration than with liposomes quenched from 23°C (Table I; Fig. 3a and 3d). In smooth areas, the ferritin conjugate is present in a clustered arrangement on the external surface of liposomes (Fig. 3b). Fig. 3c illustrates a liposome containing both a broad ridged pattern and smooth areas, and shows the ferritin-RCA 60 conjugate to be present linearly in the areas between ridges and in a clustered arrangement in smooth areas.

Localization of glycosphingolipid in DMPC and POPC liposomes above their main transition temperatures

A freeze-etch replica of DMPC multilamellar liposomes containing 7 mol% asialo-G_{M1} labelled with ferritin-RCA 60 conjugate and quenched from 37°C is shown in Fig. 4a. There is less label present on the exterior surface of these liposomes than on preparations quenched below the main transition temperature (Table I). The ferritin-RCA 60 is present as single ferritin molecules or as groups of two or three ferritin molecules. POPC bilayers containing 7 mol% asialo-G_{M1} and quenched from room temperature showed the fer-

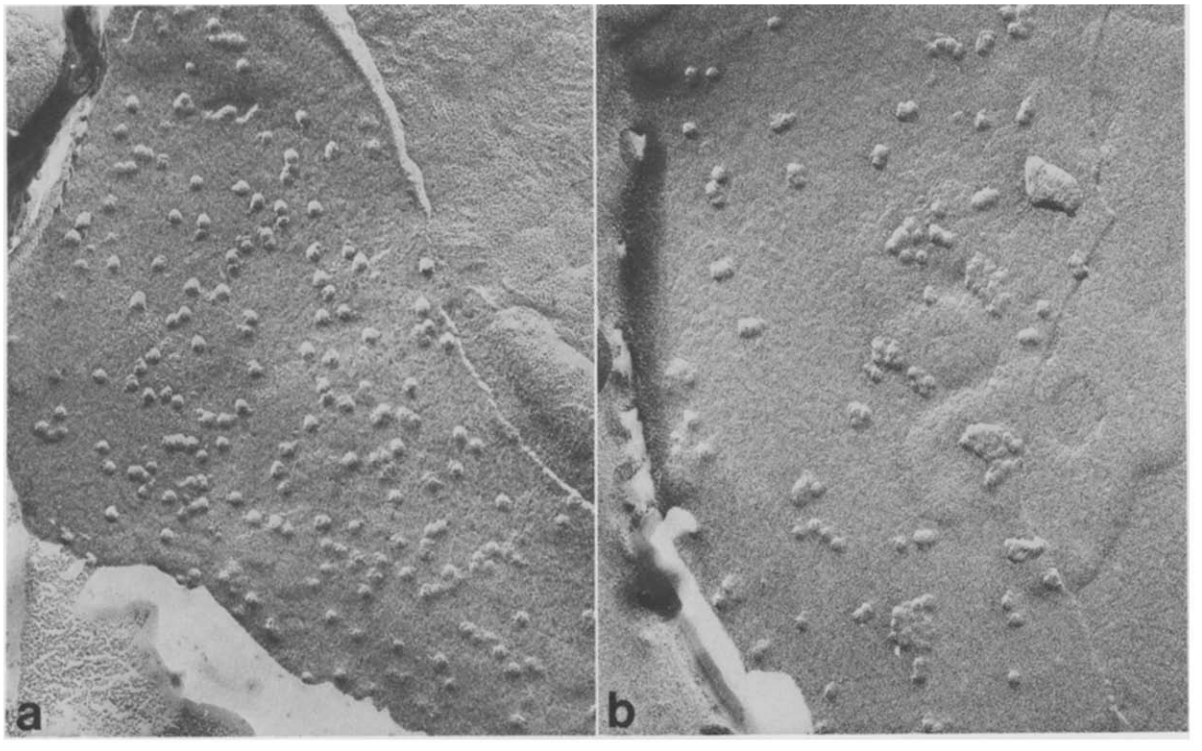


Fig. 4. Freeze-etch electron micrographs of multilamellar liposomes quenched from 37°C and labelled with ferritin-RCA 60: (a) DMPC liposome containing 7 mol% asialo-G_{M1}; (b) POPC liposome containing 7 mol% asialo-G_{M1}. Magnification is approx. 100000.

ritin label occurring in distinct clusters with fewer single ferritin molecules (Fig. 4b). Since the main transition temperature of POPC is at approx. 0°C, these samples were further above their main transition temperature when quenched from room temperature than the DMPC liposomes quenched at 37°C. POPC liposomes also showed a somewhat less distinct 'jumbled' pattern on the fractured bilayer faces than DMPC liposomes.

Fluorescence polarization of diphenylhexatriene

The diphenylhexatriene anisotropy as a function of temperature for DMPC multilamellar liposomes containing increasing amounts of asialo-G_{M1} is shown in Fig. 5A. 1.3 mol% asialo-G_{M1} resulted in decreased diphenylhexatriene anisotropy below the gel-liquid crystalline phase transition temperature and increased anisotropy above the phase transition temperature. Larger mole fractions of asialo-G_{M1} (7–13.4%) shifted the center of the transition to higher temperatures and broadened the transition.

Differential scanning calorimetry

Pure DMPC had a pretransition at 14.5°C with $\Delta H = 0.93$ kcal/mol and a main transition at 23.76°C with $\Delta H = 5.1$ kcal/mol. These data for pure DMPC are in excellent agreement with literature values, as summarized by Silvius [28]. The thermotropic behavior of DMPC multilamellar liposomes containing various amounts of asialo-G_{M1} is shown in Fig. 5B. In agreement with the fluorescence data the calorimetric scans showed a broadening of the main transition with increasing mole fractions of asialo-G_{M1}. The transition peak height decreased sharply (from 16.2 to 0.9 kcal/mol per deg) with addition of 1.3 mol% asialo-G_{M1} and further decreased (to 0.2 kcal/mol per deg) with 7 mol% asialo-G_{M1}. With 7 mol% asialo-G_{M1}, the transition broadened with the mid-point shifting up about 2°C. The addition of asialo-G_{M1} resulted in a decrease in the enthalpy of the main transition from 5.1 kcal/mol for pure DMPC multilayers to about 1.8 kcal/mol with 1.3 mol% asialo-G_{M1} and about 0.8 kcal/mol with 7 mol% asialo-G_{M1}.

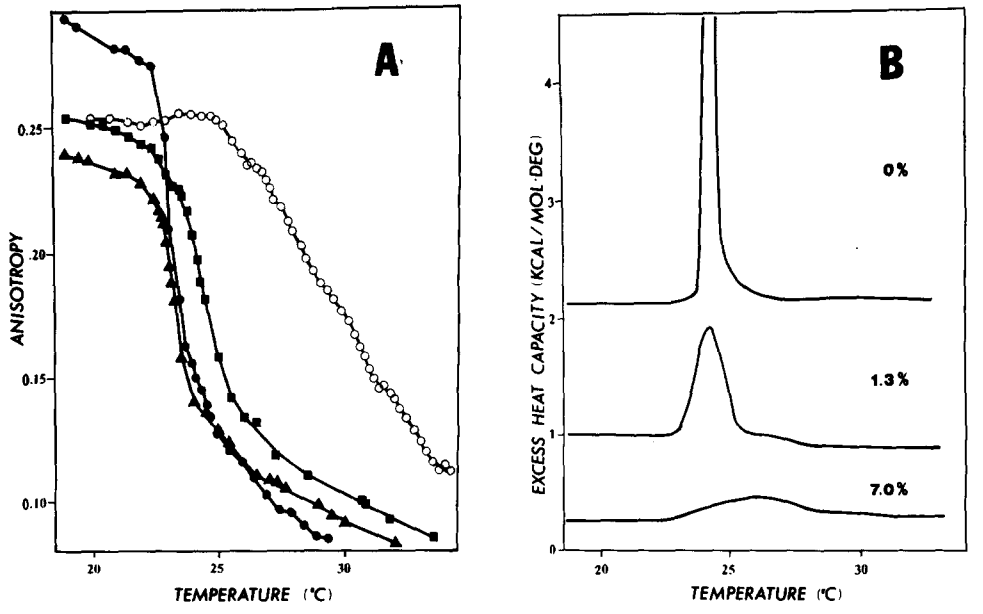


Fig. 5. (A) Anisotropy as a function of temperature. Measured using diphenylhexatriene fluorescence of DMPC multilamellar liposomes containing 0 mol% (●), 1.3 mol% (▲), 7 mol% (■) and 13.4 mol% (○) asialo-G_{M1}. (B) High sensitivity differential scanning calorimetry scans of DMPC multilamellar liposomes containing 0–7 mol% asialo-G_{M1}. For clarity, the scans have been displaced along the ordinate.

Discussion

The freeze-etching technique has proved to be very valuable for the study of the organization of the molecular components of biological membranes. With this technique, large expanses of membrane can be exposed by subliming water and buffer away from the external surface of cells to reveal features on the external surface which can be examined at high resolution and compared with views of the fractured membrane bilayer. Ferritin-labelled lectins have been used with this technique to determine the organization of erythrocyte membrane glycoproteins and to determine their relationship to the intramembranous particles [21,29,30]. Freeze-etching has also proved valuable in studying the morphologic organization of simple lipid bilayers [27,31,32]; we have used this technique to investigate the formation of 700–950 Å single-chambered liposomes from fused small sonicated vesicles [25,33]. Since very little is known about the organization of glycolipids in the external leaflets of the lipid bilayer in liposomal systems or biological membranes, we have applied labeling and freeze-etching techniques similar to those used previously in studying membrane glycoprotein organization to the localization of glycolipids in lipid bilayers.

Preparation of a monovalent labeling reagent for glycosphingolipids is necessary because of the potential problem of reorganization or clustering of glycolipids induced by a divalent or multivalent labeling reagent. In spite of the consideration that a glycosphingolipid should have only one binding site for each lectin or antibody molecule, studies of cholera toxin binding to G_{M1} ganglioside in various cellular systems have indicated that there is clustering and capping of cholera toxin binding sites [2,34]. Explanations for this include the possibility that glycolipids are bound to membrane glycoproteins which may have many receptor sites or that the cholera toxin is, in fact, labeling glycoproteins rather than glycolipids [35]. We selected RCA 60 as a labeling reagent for asialo-G_{M1} because it is believed to have only one binding site per molecule and it has a high affinity for terminal galactose in oligosaccharide units [36–38]. Glutaraldehyde conjugation of RCA 60 to ferritin does permit more than one lectin molecule to be conjugated to a ferritin molecule; however, the conjugation method that we have employed selects for conjugates containing one ferritin molecule linked to one lectin molecule. Further purification of a monovalent non-agglutinating fraction of ferritin-lectin conjugate was achieved using an acid hydrolysed Sepharose column with elution of the monovalent conjugate by a galactose gradient.

The prominent ridged pattern in DMPC phospholipid bilayers quenched at temperatures slightly below the main transition temperature has been seen by others. The finding that the ridged pattern persisted even below the pretransition temperature of DMPC (14.5°C) is similar to recent reports from McConnell's group [27] and is in contrast to earlier reports [39–44], implying that the ridged pattern was seen only at temperatures between the pretransition and main transition temperatures. Our finding of a distinctive localization of asialo-G_{M1} in DMPC bilayers has interesting parallels to the conclusions of Copeland and McConnell about cholesterol localization within DMPC and dipalmitoylphosphatidylcholine liposomes. Copeland and McConnell [27] found that in mixtures of DMPC and cholesterol, the ridge repeat distance increased as the cholesterol concentration increased from 1 to 20 mol%. They did not observe ridging at concentrations of cholesterol above 20 mol%. They proposed that ridges of a pure phosphatidylcholine phase are separated by flat strips of a 20 mol% cholesterol phase; disappearance of the ridge pattern at approximately 20 mol% cholesterol correlates with a dramatic increase in the lateral diffusion coefficient of fluorescently-labelled lipids seen at the same cholesterol concentration [45,46]. Our labeling results indicate that glycolipid insertion in the bilayer is also in the space between the ridges similar to that postulated by Copeland and McConnell for cholesterol. In contrast to cholesterol, however, addition of asialo-G_{M1} does not result in a linear increase in the distance between ridges; there is an approx. 2-fold increase in the periodicity of ridging above 1.5 mol% asialo-G_{M1}. It has been noted previously that addition of glycolipids to model membrane systems markedly increases the rigidity of phospholipid bilayers above the phase transition [47]. This is similar to the effect of cholesterol on phospholipid bilayers. The molecular basis for the

effect of cholesterol has been discussed in detail by Huang [48].

Asialo-G_{M1} and cholesterol are in some ways similar in their effect upon the thermotropic behavior of multilamellar liposomes. Anisotropy was increased above and decreased below the gel-liquid-crystalline phase transition temperature (Fig. 5A) in the presence of asialo-G_{M1}, similar to the effect of cholesterol in dipalmitoylphosphatidylcholine multilayers measured with diphenylhexatriene [49]. Both asialo-G_{M1} in DMPC multilayers and cholesterol in dipalmitoylphosphatidylcholine multilayers [26] decrease the peak height and the enthalpy of the main phase transition (Fig. 5B). Their effects differ in that a smaller mole fraction of asialo-G_{M1} is required to produce the equivalent effect of a given mole fraction of cholesterol. In addition, the main transition temperature increases as the mole fraction of asialo-G_{M1} is increased, while the addition of cholesterol is accompanied by a slight decrease in the transition temperature [26].

It is not clear why there is greater labeling of asialo-G_{M1} molecules in DMPC liposomes quenched at 4°C over that at higher temperatures. It is possible that the glycolipid molecules are more tightly bound into the phospholipid bilayer at lower temperatures, making it more difficult for the ferritin-lectin conjugate to extract glycolipids from the membrane during incubation. It is also possible that the enhanced labeling at 4°C is a result of greater affinity of the lectin-ferritin conjugate for the glycolipid receptor at this temperature. We shall attempt to resolve this question by preparing radioactively labelled glycolipid which can be incorporated into phospholipid bilayers, treated with ferritin-lectin conjugate, and the supernatant counted to determine if glycolipids are indeed extracted from the bilayer. In spite of the loss of ferritin-labelled RCA 60 experienced with repeated washing of the multilamellar liposomes, sufficient label remained on the exterior surface of the liposomes to allow conclusions to be drawn about the pattern of glycolipid organization. Nonspecific labeling was fortunately not encountered in these experiments since control liposomes which did not contain glycolipid showed no labeling with the ferritin-RCA 60 conjugate despite the lack of liposomal washing. Liposomes did not aggregate in the relatively high salt concentration of phosphate buffered saline when treated with the monovalent ferritin-RCA 60, but aggregation of liposomes with or without glycolipid was observed in low salt concentrations, presumably due to electrostatic interaction. Divalent or multivalent ferritin-lectin conjugate was useful in establishing the uniform linear labeling pattern of ferritin-RCA 60 in DMPC liposomes. By freeze-fracture we could observe deformation of the outer monolayer of lipid caused by ferritin binding to the outer liposomal surface in aggregated preparations where the fracture passed through the outermost bilayer revealing the impressions of the ferritin molecules arrayed in a linear pattern. Control experiments in which a hapten inhibitor of the RCA 60 molecule, such as galactose or lactose, completely inhibited binding of the ferritin probe to the liposomal surface insured that the labeling was specific and was not simply trapping of ferritin between opposing surfaces.

The distribution of the ferritin-RCA 60 label above the main transition temperature of DMPC suggests that asialo-G_{M1} is organized as single molecules or as small clusters on the exterior surface, in no apparent relationship to the jumbled phospholipid ridges present on the DMPC fracture faces. It is possible that the low density of labeling seen on DMPC bilayers containing asialo-G_{M1} resulted from poor affinity of the label at this temperature or from extraction of glycolipid molecules from the bilayer. POPC bilayers which contained asialo-G_{M1} suggest an even more clustered arrangement of the glycosphingolipid when quenched from room temperature, which was considerably above the main transition temperature for this phospholipid (0°C).

The clustered arrangement of the glycosphingolipid in phosphatidylcholine bilayers observed in this study both below and above the gel-liquid crystalline phase transition is in agreement with the behavior of another glycosphingolipid, glucocerebroside [12,13].

The tendency for glycolipid molecules to form organized patterns or domains in simple phospholipid bilayers suggests that these lipids may also exhibit a compositional domain structure in biological membranes. These domains may function as recognition sites of complex carbohydrate

chain composition analogous to membrane glycoproteins. Unlike membrane glycoproteins, however, glycosphingolipids may exhibit rapid lateral translation in the membrane and the concentration of glycolipids in domains and the size of the domains may be under the control of the cell and could be adjusted rapidly by such changes as local membrane curvature, phospholipid and protein composition. These compositional domains might then act as identifying surface structures to participate in cellular recognition and to alter interactions between cells.

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References

1 Cuatrecasas, P. (1973) Biochemistry 12, 3547–3558; 3558–3566
2 Revesz, T. and Greaves, M. (1975) Nature 257, 103–106
3 Critchley, D.R., Magnani, J.L. and Fishman, P.H. (1981) J. Biol. Chem. 256, 8724–8731
4 Haywood, A.M. (1974) J. Mol. Biol. 83, 427–436
5 Holmgren, J., Svennerholm, L., Elwing, H., Fredman, P. and Strannegard, O. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1947–1950
6 Mullin, B.R., Fishman, P.H., Lee, G., Ledley, F.D., Winand, R.J., Kohn, L.D. and Brady, R.O. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 842–846
7 Mullin, B.R., Aloj, S., Fishman, P.H., Lee, G., Kohn, L.D. and Brady, R.O. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1679–1683
8 Hakomori, S. (1981) Annu. Rev. Biochem. 50, 733–764
9 Magnani, J.L., Brockhaus, M., Smith, D.F., Ginsburg, V., Blaszczyk, M., Mitchell, K.F., Steplewski, Z. and Koprowski, H. (1981) Science 212, 55–56
10 Gahmberg, C.G. and Hakomori, S. (1973) J. Biol. Chem. 246, 4311–4317
11 Steck, T.L. and Dawson, G. (1974) J. Biol. Chem. 249, 2135–2142
12 Correa-Freire, M.C., Freire, E., Barenholz, Y., Biltonen, R.L. and Thompson, T.E. (1979) Biochemistry 18, 442–445
13 Correa-Freire, M.C., Barenholz, Y. and Thompson, T.E. (1982) Biochemistry 21, 1244–1248
14 Surolia, A., Bachhawat, B.K. and Podder, S.K. (1975) Nature 257, 802–804
15 Boldt, D.H., Speckart, S.F., Richards, R.L. and Alving, C.R. (1977) Biochem. Biophys. Res. Commun. 74, 208–214
16 Richards, R.L., Moss, J., Alving, C.R., Fishman, P.H. and Brady, R.O. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 1673–1676
17 Svennerholm, L., Mansson, J.E. and Li, Y. (1973) J. Biol. Chem. 248, 740–742
18 Naoi, M., Lee, Y.C. and Roseman, S. (1974) Anal. Biochem. 58, 571–577
19 Nicolson, G.L., Blaustein, J. and Etzler, M.E. (1974) Biochemistry 13, 196–204
20 Hunter, W.M. (1967) in Handbook of Experimental Immunology (Weir, D.M., ed.), pp. 608–654, Blackwell, Oxford
21 Triche, T.J., Tillack, T.W. and Kornfeld, S. (1975) Biochim. Biophys. Acta 394, 540–549
22 Suurkuusk, J., Lentz, B.R., Barenholz, Y., Biltonen, R.L. and Thompson, T.E. (1976) Biochemistry 15, 1393–1401
23 Lentz, B.R., Barenholz, Y. and Thompson, T.E. (1976) Biochemistry 15, 4521–4528, 4529–4536
24 Lentz, B.R., Freire, E. and Biltonen, R.L. (1978) Biochemistry 17, 4475–4480
25 Wong, M., Anthony, F., Tillack, T.W. and Thompson, T.E. (1982) Biochemistry, in the press
26 Estep, T.N., Mountcastle, D.B., Biltonen, R.L. and Thompson, T.E. (1978) Biochemistry 17, 1984–1989
27 Copeland, B.R. and McConnell, H.M. (1980) Biochim. Biophys. Acta 599, 95–109
28 Silvius, J.R. (1982) in Lipid-Protein Interactions (Jost, P.C. and Griffith, O.H., eds.), Vol. 2, pp. 239–281, Wiley Interscience, New York
29 Tillack, T.W., Scott, R.E. and Marchesi, V.T. (1972) J. Exp. Med. 135, 1209–1227
30 Pinto da Silva, P. and Nicolson, G.L. (1974) Biochim. Biophys. Acta 363, 311–319
31 Ververgaert, P.H.J.T., Verkleij, A.J., Verhoeven, J.J. and Elber, P.F. (1973) Biochim. Biophys. Acta 311, 651–654
32 Luna, E.J. and McConnell, H.L. (1977) Biochim. Biophys. Acta 470, 303–316
33 Schullery, S.E., Schmidt, C.F., Felgner, P., Tillack, T.W. and Thompson, T.E. (1980) Biochemistry 19, 3919–3923
34 Craig, S.W. and Cuatrecasas, P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3844–3848
35 Morita, A., Tsao, D. and Kim. Y.S. (1980) J. Biol. Chem. 255, 2549–2553
36 Olsnes, S., Saltvedt, E. and Pihl, A. (1974) J. Biol. Chem. 249, 803–810
37 Sandvig, K., Olsnes, S. and Pihl, A. (1976) J. Biol. Chem. 251, 3977–3984
38 Baenziger, J.U. and Fiete, D. (1979) J. Biol. Chem. 254, 9795–9799
39 Luna, E.J. and McConnell, H.M. (1977) Biochim. Biophys. Acta 466, 381–392
40 Luna, E.J. and McConnell, H.M. (1977) Biochim. Biophys. Acta 470, 303–316
41 Janiak, M.J., Small, D.M. and Shipley, G.G. (1976) Biochemistry 15, 4575–4580
42 Gebhardt, C., Gruler, H. and Sackmann, E. (1977) Z. Naturforsch. 32c, 581–596

43 Krbecek, R., Gebhardt, C., Gruler, H. and Sackmann, E. (1979) Biochim. Biophys. Acta 554, 1–22
44 Watts, A., Harlos, K., Maschke, W. and Marsh, D. (1978) Biochim. Biophys. Acta 510, 63–74
45 Rubenstein, J.L.R., Smith, B.A. and McConnell, H.M. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 15–18
46 Recktenwald, D.J. and McConnell, H.M. (1981) Biochemistry 20, 4505–4510
47 Sharom, F.J. and Grant, C.W.M. (1977) J. Supramol. Struct. 6, 249–258
48 Huang, C. and Mason, J.T. (1982) in Membranes and Transport: A Critical Review (Martonosi, A., ed.), Vol. 1, pp. 15–23, Plenum Publishing Co., New York
49 Lentz, B.R., Barrow, D.A. and Hoechli, M. (1980) Biochemistry 19, 1943–1954