Asymmetric Incorporation of Trisialoganglioside into Dipalmitoylphosphatidylcholine Vesicles†

P. L. Felgner, E. Freire, Y. Barenholz, and T. E. Thompson*

ABSTRACT: Results are presented which demonstrate that purified trisialoganglioside spontaneously incorporates into preformed phospholipid vesicles. Determinations of the extent of incorporation were made by separating large unilamellar dipalmitoylphosphatidylcholine vesicles containing incorporated ganglioside from micellar ganglioside on a Sepharose-2B column. Incorporation occurs without appreciably altering the vesicular character of the phospholipid bilayer as judged by the maintenance of an outside/inside ratio, determined by ^{31}P NMR, comparable to that of the original vesicles. All of the incorporated ganglioside is accessible to neuraminidase, indicating that incorporation occurs only on the outer face of the bilayer. The thermotropic behavior of these asymmetric dipalmitoylphosphatidylcholine–trisialoganglioside vesicles, examined by high sensitivity scanning calorimetry, strongly suggests that the incorporated ganglioside is intercalated into the outer monolayer of the vesicle bilayer. Calorimetric studies indicate that the ganglioside stabilizes these vesicular structures by inhibiting the fusion of small vesicles that occurs below the phase-tranisition temperature. These structures are a representative model system, which like the mammalian plasma membrane contain an asymmetric distribution of glycosphingolipid in the outer surface.

Unlike phospholipids which form bilayer systems, gangliosides form micellar solutions in aqueous media. Micellar weights are usually $(1–5) \times 10^5$, and critical micelle concentrations are 10^{-5} M or less (Barenholz et al., 1980; Formisano et al., 1979; Corti et al., 1980). The type of aggregate obtained when phospholipids and gangliosides are codispersed depends upon the relative composition of these two components (Hill & Lester, 1972; Cestaro et al., 1980). When the ganglioside content is greater than about 50 mol %, cosonicated dispersions are almost entirely micellar. When the mole percent of ganglioside is 10% or less, cosonication produces a dispersion of vesicles (Barenholz et al., 1980). These cosonicated vesicular systems generally have both lipid components distributed equally in the two surfaces of the bilayer. Similar results are obtained with other means of codispersion (Bunow & Bunow, 1979).

In this paper, we report the formation of unilamellar phosphatidylcholine vesicles containing trisialoganglioside incorporated into the outer surface only of the vesicle bilayer. Since plasma membrane ganglioside in many mammalian cells appears to be localized almost exclusively in the outer surface of this membrane (Steck & Dawson, 1974; Dawson, 1978; Hansson et al., 1977; Stoffel, 1975), this compositionally asymmetric bilayer system is the direct analogue of the plasma membrane bilayer of the mammalian cell. It is formed under conditions which permit spontaneous ganglioside transfer from ganglioside micelles to phosphatidylcholine bilayers. This paper describes the formation of asymmetric bilayers by utilizing both large and small unilamellar vesicles formed from 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine. A preliminary report of this work has appeared elsewhere (Felgner et al., 1980).

Materials and Methods

The vesicle preparations used in this paper are of two types, small unilamellar vesicles (SUV)[1] and fused unilamellar vesicles (FV). SUV where prepared by sonication followed by fractionation by utilizing differential centrifugation (Barenholz et al., 1977). FV were prepared essentially as described by Schullery and co-workers (Schullery et al., 1980). Dipalmitoylphosphatidylcholine (DPPC) obtained from Avanti Biochemicals (Birmingham, AL) was dried from a chloroform solution and placed under vacuum for at least 24 h. The dried lipid was suspended in 50 mM KCl containing 0.02% sodium azide to give a concentration in the range of 60–100 mM. The sonication time was about 30 min for volumes of 2–4 mL by using a Heat Systems W-350 sonifier. The sonicated vesicles were kept above 45 °C and centrifuged at 40 000 rpm for 20 min in a Beckman Ti50 rotor preheated to 55 °C. The supernatant was carefully pipetted away from the pelleted titanium and residual multilamellar vesicles. This supernatant was then incubated at room temperature for 5 days. Over this period, a marked increase in turbidity occurred, and the solution gradually became milky white. After the incubation, the vesicles were applied to a Sepharase CL-2B column and eluted with 50 mM KCl containing 0.02% sodium azide. The large vesicle fraction (FV), accounting for approximately 60% of the lipid, was pooled. Void volume material was discarded as was the small vesicle fraction. Electron microscopy indicated that the FV preparation contained vesicles 700 ± 100 Å in diameter as shown in Figure 1.

Gangliosides were purified by adapting several published procedures in order to process conveniently gram quantities of ganglioside (Nagai & Iwamori, 1980; Momoi et al., 1976; Ando & Yu, 1977; Fredman et al., 1980). The upper Folch extract (10 L) from 3 kg of bovine brain obtained commercially from Avanti Biochemicals (Birmingham, AL) was adsorbed to 50 g of DEAE-Sephadex A-25 (acetate form) by stirring at room temperature for 1 h. The Sephadex was collected in a large Büchner funnel and washed with chloroform–methanol–H_2O (30:60:8) and then with 20 mM ammonium acetate in methanol. This material was packed into a column and eluted with 0.2 M ammonium acetate in

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[1] Abbreviations used: SUV, small unilamellar vesicles; FV, fused unilamellar vesicles (FUV in figures); DPPC, dipalmitoylphosphatidylcholine; PE, egg phosphatidylethanolamine.

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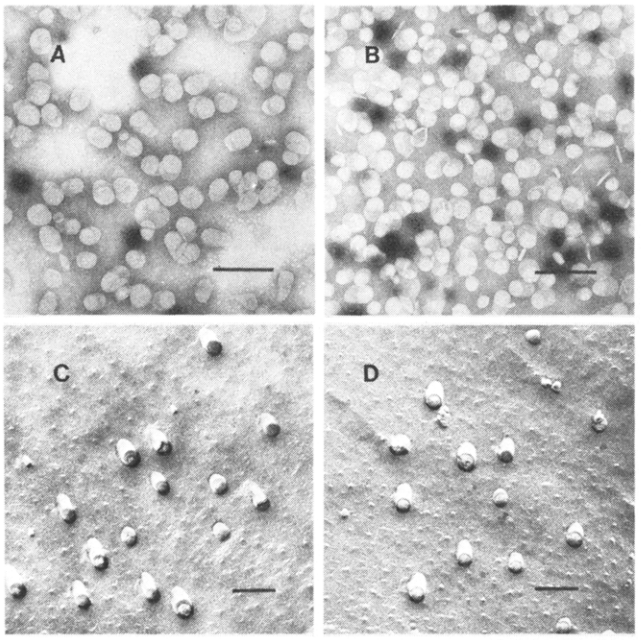

FIGURE 1: Electron microscopy by negative staining and freeze–etch of FV with and without ganglioside. FV at a concentration of 7 μmol/mL were incubated with (B, D) and without (A, C) 8 mol % trisialoganglioside for 18 h at 46 °C and then brought to room temperature. Samples were prepared for negative staining in 1% ammonium molybdate (A, B) or were frozen and etched (C, D). The bars in each figure are 0.1 μm.

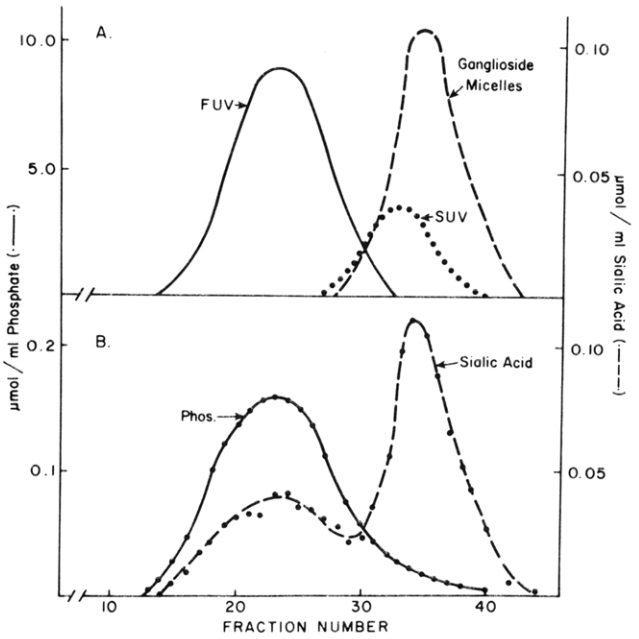

FIGURE 2: Separation of dipalmitoylphosphatidylcholine in FV from ganglioside micelles by Sepharose column chromatography. (A) To a Sepharose CL-2B column (30 × 1.6 cm; void volume 21.9 mL; included volume 60.8 mL; fraction size 1.45 mL; in 0.05 M KCl–0.02% sodium azide) were applied aliquots of FV, SUV, and several different preparations of trisialoganglioside micelles. Typical profiles of each are presented. (B) To a 2-mL aliquot of FV (4 mM) prepared as described under Materials and Methods was added 33 mol % trisialoganglioside from an aqueous stock solution (7 mM ganglioside). The sample was incubated for 1 h at 46 °C and brought to room temperature, and 0.8 mL of the sample was passed over the Sepharose column. Fractions were assayed for phosphate according to Bartlett (1959) and for sialic acid by the recorsinol method according to Cassidy et al. (1966). The recovery of phospholipid phosphorus was 90%.

methanol. The eluate was dried in a flash evaporator. The dried material was dissolved in 50 mL of water and desalted by Sephadex G-25 column chromatography, and the aqueous crude ganglioside was lyophilized to yield a faintly yellow powder. A 400-mg sample of the crude ganglioside was then applied to a DEAE-Sepharose column (2.5 × 25 cm; acetate form in methanol) and eluted with a linear gradient of ammonium acetate (0–0.5 M) in methanol. Peak III, which elutes between 0.2 and 0.3 M salt, contains trisialoganglioside. This material was dried, dissolved in water, desalted by Sephadex G-25 column chromatography, and lyophilized. A 200-mg aliquot of dried trisialoganglioside was applied to an Iotrobeads column (2.5 × 40 cm) and eluted with a linear gradient (2 L) from 50% to 80% methanol in chloroform. This material gave a single spot by thin-layer chromatography (65:35:8 chloroform–methanol–2.5 M ammonia) and matched authentic standards of GT1b obtained from Supelco (State Collage, PA). This material could contain trace amounts of other trisialogangliosides which have escaped our detection (Nagai & Iwamori, 1980). Hence, we designate this fraction trisialoganglioside rather than GT1b.

Calorimetry was carried out as described by Suurkuusk et al. (1976) with vesicles or multilamellar liposomes at a concentration from 10 to 20 mM in 50 mM KCl containing 0.02% sodium azide.

Results and Discussion

Separation of Ganglioside Micelles from Phospholipid Vesicle Bound Ganglioside. In order to determine the amount of vesicle-associated ganglioside in micelle–vesicle mixtures, it was necessary to find a means of separating vesicles from micelles. Attempts to separate small sonicated vesicles from micelles on a Sepharose CL-2B column failed. Despite the large difference in the molecular weight of the vesicles and micelles (2 000 000 and 250 000, respectively), the elution profiles of the two species essentially coincide as shown in Figure 2A. This is probably due to the nonspherical shape of ganglioside micelles reported by Corti and co-workers (Corti et al., 1980) or to their large negative charge. An effort was therefore made to prepare larger unilamellar vesicles that could be resolved from the micelles on a Sepharose CL-2B column. One such preparation is the dispersion of large unilamellar vesicles (FV) which form spontaneously from small sonicated dipalmitoylphosphatidylcholine vesicles after incubation below the phase transition for 5 days (Schullery et al., 1980). These unilamellar vesicles are about 700 Å in diameter and can be readily resolved from ganglioside micelles on a Sepharose CL-2B column as shown in Figure 2A.

When ganglioside micelles and FV are incubated together above the phase transition for 1 h prior to molecular sieve chromatography, the profile in Figure 2B is obtained. In this experiment, the mole percent of total ganglioside is 33. The coelution of ganglioside with phospholipid phosphorus indicates a tight association between ganglioside and vesicles. The mole percent of ganglioside in the peak fraction of the phosphate profile is 8.08 ± 1.0. Upon rechromatography of the fractions in this peak, after room temperature incubation for 2 days, the phospholipid and ganglioside coeluted (experiment not shown). Subsequent experiments with longer incubation times have indicated that the maximum amount of ganglioside that can be incorporated is approximately 10–12%. Details of these experiments will be published at a later date.

Maintenance of Vesicle Integrity after Ganglioside Addition. If the fluorescent dye 6-carboxyfluorescein is trapped inside phospholipid vesicles at concentrations greater than 25 mM and the extravesicular 6-carboxyfluorescein then removed by Sephadex G-25 column chromatography, most of the intrinsic fluorescence is quenched (Blumenthal et al., 1977). An

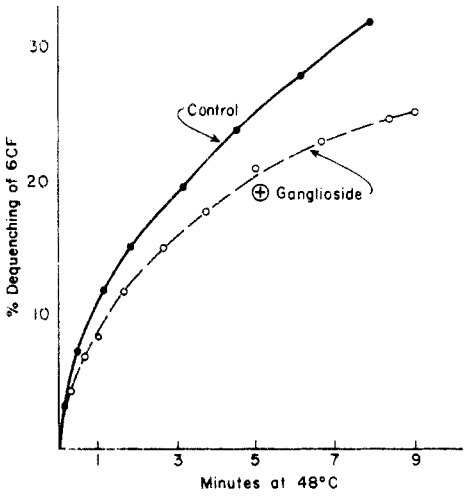

FIGURE 3: Effect of ganglioside on 6-carboxyfluorescein leakiness from FV. FV (4 mM) were incubated with 6-carboxyfluorescein (25 mM, pH 7.5) for 24 h at 46 °C. Independent experiments indicated that this procedure allowed entry of the 6-carboxyfluorescein into the vesicles, and equilibration of the inner compartment with outer compartment was complete. At the end of the incubation, the vesicles were passed through a short Sephadex G-25 column at room temperature, equilibrated with an isosmotic solution of KCl, to remove untrapped 6-carboxyfluorescein. The 6-carboxyfluorescein-containing FV were injected into cuvettes (final concentration 0.015 mM dipalmitoylphosphatidylcholine) containing isosmotic KCl (48 °C) ± trisialoganglioside (0.056 mM), and the fluorescence was monitored with time. Kinetic studies show that substantial amounts of ganglioside are incorporated into the vesicle during the 9-min duration of the experiment (unpublished observation).

increase in the fluorescence of such a suspension indicates leakage of the fluorophore from the vesicles. The data in Figure 3 show that the addition of ganglioside to the outer surface of the phospholipid vesicles does not increase the slow intrinsic rate of release of 6-carboxyfluorescein, indicating that the addition of ganglioside does not cause vesicle rupture or loss of bilayer integrity as a permeability barrier. In fact, ganglioside incorporation actually decreases the rate of fluorophore release.

Additional data support this conclusion. The electron micrographs of negatively stained and freeze-fractured FV before and after the addition of ganglioside shown in Figure 1 reveal no changes in the size or shape of the vesicles. The molecular sieve data presented in Figure 2B show no shift in the FV elution profile after these vesicles have been incubated for 2 days with excess ganglioside above the phospholipid phase-transition temperature. In addition, no change in the ratio of external to internal surface ^{31}P as determined by NMR with Mn^{2+} as a broadening reagent was detectable after ganglioside incorporation (data not shown).

Asymmetry of Addition of Ganglioside to FV. Since the enzyme neuraminidase from *C. perfringens* does not permeate the bilayer of phospholipid vesicles, the sialic acid accessibility to this enzyme can be used to determine the fraction of sialic acid in the outer face of the vesicle. Figure 4B shows that in cosonicated systems 35% of the ganglioside is inaccessible to neuraminidase. The diameter of these vesicles is close to 200 Å. Thus, about 35% of the total bilayer area is on the internal surface of the vesicle wall. This result then indicates that the ganglioside is evenly distributed in both surfaces of the bilayer as expected in this cosonicated system. In contrast to this distribution, Figure 4A indicates that the ganglioside incorporated into FV by spontaneous transfer from micelles is all accessible to neuraminidase. Since micellar ganglioside was completely removed by molecular sieve chromatography prior to neuraminidase treatment, this result indicates that the

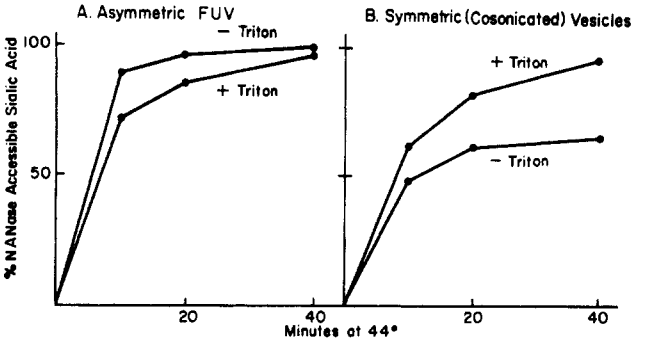

FIGURE 4: Neuraminidase accessibility in asymmetric FV and cosonicated vesicles. For panel A, the trisialoganglioside-containing vesicles from a Sepharose column similar to that described in Figure 1 were pooled (0.48 μmol/mL dipalmitoylphosphatidylcholine, 0.145 μmol/mL NANA). Sialic acid released by neuraminidase in the presence and absence of 0.1% Triton was monitored as a function of time at 44 °C, pH 5.6 (Cassidy et al., 1966). For panel B, the vesicles were prepared by comixing dipalmitoylphosphatidylcholine (25 μmol) and trisialoganglioside (3.4 μmol of sialic acid) in chloroform and methanol and cosonicating the dried lipid in 2 mL of 50 mM KCl–0.02% sodium azide. Sialic acid release was monitored as in panel A. The end product of enzymatic hydrolysis under these conditions with or without Triton is monosialoganglioside (Ando & Yu, 1977).

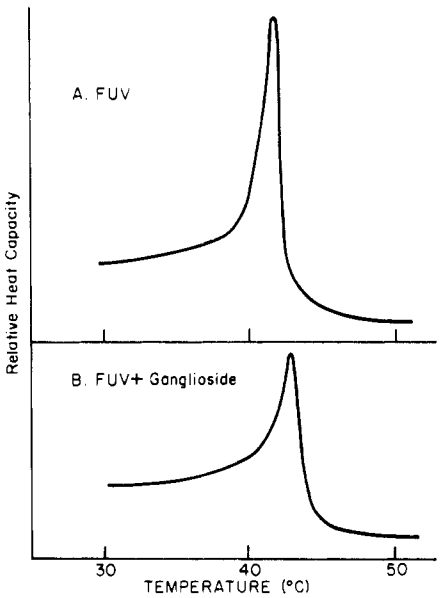

FIGURE 5: Differential scanning calorimetry heating curve of FV with and without ganglioside. FV (4.6 μmol/mL) were incubated for 18 h at 46 °C with (B) or without (A) trisialoganglioside (8 mol %). Each sample was scanned from 20 to 60 °C at a rate of 15 °C/h.

ganglioside associated with the FV is on the outer surface of the vesicle.

Thermotropic Behavior of FV-Containing Ganglioside. The gel–liquid crystalline phase transition of dipalmitoylphosphatidylcholine in FV shown in Figure 5A is similar to that displayed by multilamellar liposomes with a T_m of 41.6 °C and an enthalpy of 8.1 kcal/mol of lipid. The transition is, however, somewhat broadened with a half-width of about 1.0 °C compared to less than 0.5 °C for multilamellar liposomes (Suurkuusk et al., 1976). The asymmetric incorporation of 8 mol % ganglioside leads to an increase of 1.5 °C in the T_m and a reduction in the enthalpy of the transition to 7.0 kcal/mol as shown in Figure 5B. These data suggest that the added ganglioside on the external surface of the vesicle is intercalated among the phospholipid molecules of the vesicle bilayer.

Inhibition by Ganglioside of Vesicle Fusion. Coincident with the fusion of SUV to form FV, there is a time-dependent

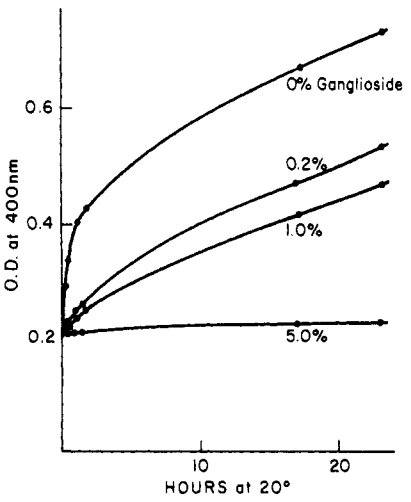

FIGURE 6: Ganglioside inhibition of SUV fusion. Dipalmitoylphosphatidylcholine and SUV (10 mM in 50 mM KCl, 0.02% sodium azide) were prepared by sonication at 50 °C. The vesicles were centrifuged (20 min, 40K) while still hot, trisialoganglioside micelles were added at the indicated mol %, and the vesicles were brought to room temperature. The turbidity changes was monitored as a function of time at 400 nm. Sepharose CL-2B column chromatography of the vesicles containing 5 mol % ganglioside after 2 days at room temperature confirmed that they did not fuse.

increase in turbidity (Schullery et al., 1980). This time dependence in turbidity measured at 400 nm can be seen in the upper curve in Figure 6. The data in this figure also indicate that the addition of increasing amounts of micellar ganglioside to SUV prior to incubation below T_m markedly reduces the rate of the turbidity increase. This result shows that ganglioside associates with the small unilamellar vesicles and in so doing slows the spontaneous fusion process leading to FV formation. This action may be the result of the negative surface charge on the vesicles imparted by incorporated ganglioside which prevents vesicle–vesicle association, a necessary prerequisite to fusion. Changes in the surface hydration resulting from added sugar moieties and the asymmetric addition of mass could also inhibit fusion. Preliminary experiments with cosonicated mixtures of neutral glycosphingolipids and DPPC show, however, that the fusion rates of SUV are little affected by the incorporation of the neutral glycosphingolipid (M. Freire, unpublished experiments).

Asymmetric Association of Ganglioside to SUV. Since the separation of SUV trisialoganglioside micelles by Sepharose column chromatography was not complete, an alternative separation procedure was developed. On the basis of the difference in density between phospholipid vesicles (about 1.0 g/cm^3) and ganglioside micelles (1.3–1.4 g/cm^3), separation can be achieved by equilibrium density centrifugation on a gradient containing D_2O. Deuterium oxide was used instead of sucrose or dextran in order to eliminate osmotic effects. By this procedure, it was determined that ganglioside associates with small vesicles. The neuraminidase accessibility assay indicates that ganglioside-containing SUV, formed by spontaneous transfer, have the ganglioside asymmetrically distributed with all incorporated ganglioside accessible to the neuraminidase. This result is identical with that obtained for FV as discussed above. These experiments also suggest that SUV cannot incorporate by transfer as much ganglioside as FV under similar conditions (data not shown). We are currently examining this result in more detail.

Integrity of SUV after Ganglioside Addition. An alternative procedure to the one used in Figure 3 for assessing vesicle integrity was used with SUV containing 10% egg phosphatidylethanolamine (PE). In this procedure, the amino group reactive reagent trinitrobenzenesulfonate was used to determine the concentration of phosphatidylethanolamine on the outer leaflet of the SUV (Litman, 1973). The data (not presented) show that the accessibility of phosphatidylethanolamine for reaction with trinitrobenzenesulfonate remains approximately constant even after incubation of the vesicles for 40 h at 48 °C in the presence of excess ganglioside. This result indicates that the vesicles remain intact. If the vesicles had been ruptured by the addition of ganglioside or mixed micelles had formed, the accessibility of phosphatidylethanolamine to this reagent would have increased (Cestaro et al., 1980). Under the conditions employed, all the PE is accessible after disruption of the vesicles by the addition of Triton X-100.

Thermotropic Behavior of SUV-Containing Ganglioside. Differential scanning calorimetry of small unilamellar vesicles of dipalmitoylphosphatidylcholine formed by sonication shows two thermal transitions, one at 36.8 °C and the other at 41.6 °C. These two transitions are associated with SUV and FV, respectively. During incubation below the phase-transition temperature, the enthalpy of the higher temperature transition increases at the expense of the enthalpy of the lower temperature transition. The rate of this process reflects the fusion of SUV to form FV (Suurkuusk et al., 1976; Schullery et al., 1980). Addition of excess ganglioside to dispersions of SUV prevents this fusion process as reflected in a time-independent heat capacity vs. temperature plot (data not shown). This result is consistent with the inhibition of turbidity shown in Figure 6. The addition of ganglioside also causes an increase in the phase-transiton temperature of SUV about 1.2 °C with little change in the enthalpy (6.3 kcal mol^{-1}). This evidence together with the thermotropic behavior obtained with FV-containing ganglioside strongly suggests that added ganglioside is intercalated into the phospholipids in the outer bilayer surface.

The asymmetric incorporation of gangliosides into phospholipid bilayers by transfer from ganglioside micelles has several important implications for the study of biological membranes. First, since plasma membrane ganglioside in many mammalian cells appears to be localized almost exclusively in the external surface of this membrane (Steck & Dawson, 1974; Dawson, 1978; Hansson et al., 1977; Stoffel, 1975), the compositionally asymmetric bilayers described in this report are the direct analogue of the plasma membrane bilayer of mammalian cells. These lipids have been known for some years to act as antigenic determinants (Horowtiz, 1978). Recent evidence suggests that certain of these lipids may serve as specific receptors (Fishman & Brady, 1976; Helting et al., 1977; Moss & Vaughan, 1979). There is also a growing body of evidence that ganglioside compositional alterations are associated with transformation in many cell types in cultures as well as in neoplastic cell lines (Oseroff et al., 1973; Brady & Fishman, 1974; Hakomori, 1975; Steiner et al., 1978). In addition, these lipids as well as other glycosphingolipids have been postulated by Roseman (1974) to play an important role in the cell–cell adhesion. The compositionally asymmetric system described in this report provides a plasma membrane model which may be used to examine the putative roles of gangliosides in cellular functions.

Second, the addition of mass to one face of the bilayer by ganglioside transfer from the micelle is similar to the general process thought to occur during membrane biogenesis in which mass is added biosynthetically to one face only of the growing membrane (Coleman & Bell, 1978). The asymmetric addition of mass creates a mass imbalance between the two bilayer faces. Three consequences may be the result of this mass

imbalance. (1) It is possible that the amount of lipid added to the outer face is limited by the mass imbalance. (2) The mass imbalance may drive a transbilayer migration of lipid which releaves the imbalance. (3) In large radius unilamellar vesicles, the mass imbalance may cause local changes in bilayer curvature which result in evagination and the production of smaller radius of curvature vesicles. The compositionally asymmetric bilayer described in this paper affords a simple system with which to examine the consequences of mass imbalance as they occur in membrane biogenesis.

Third, intact cells as well as cell membrane preparations have been shown in several instances to absorb gangliosides added to the ambient medium. Associated with this absorption is acquisition of certain ganglioside-mediated cell functions (Callies et al., 1977; Spiegel et al., 1979; Fishman et al., 1976, 1977; Cuatrecasas, 1973; Hakomori, 1975). It seems quite probable that the process operative in these instances is the same process of ganglioside transfer from micelle to bilayer described in this paper. Further study of the parameters governing this process in simple bilayer systems will provide a basis for continuing attempts to modify the external surface of cell membranes by ganglioside addition.

Acknowledgments

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