Kinetics of Transfer of Gangliosides from Their Micelles to Dipalmitoylphosphatidylcholine Vesicles†

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ABSTRACT: Two aspects of the kinetics of transfer of ganglioside from micelles to dipalmitoylphosphatidylcholine vesicles have been examined: (i) The first aspect is the rate of transfer of ganglioside from micelles at very low ganglioside/phospholipid ratios. Under these conditions the rate of incorporation into vesicles is independent of the vesicle concentration, indicating that transfer occurs by diffusion of ganglioside molecules through the aqueous phase and not by collision of micelles and vesicles. The initial transfer of monosialoganglioside is slower ($t_{1/2}$ = 2 h) than that of trisialoganglioside ($t_{1/2}$ = 0.5 h). The rate of transfer decreases during the transfer process. This decrease in rate depends on the character of the micelles and not on the ganglioside content of acceptor vesicles. The initial rate of transfer decreases sharply with decreasing temperature. (ii) The second aspect is the rate of transfer of ganglioside from micelles to phospholipid vesicles at high ganglioside/phospholipid ratios. In the presence of excess ganglioside, the level of incorporation into vesicles saturates when the ganglioside content of the vesicles reaches 12–15 mol %. This saturation level is not markedly dependent on the number of sialic acid residues in the ganglioside.

Gangliosides are a minor lipid component of the plasma membrane of mammalian cells. They are located almost exclusively in the outer surface of these membranes (Fishman & Brady, 1976; Hakomori, 1981; Steck & Dawson, 1974; Dawson, 1978; Hansson et al., 1977; Stoffel, 1975). In neurons gangliosides account for 10 mol % of the total lipid in the plasma membrane. Since this class of lipid is located in the outer surface of this structure, the concentration there is about 20 mol % (Hakomori, 1981; Suzuki, 1972; Eichberg et al., 1969; Ledeen, 1978; Breckenridge et al., 1972; DeVries & Zmachinski, 1980). Recently, however, it has been suggested that gangliosides may be present in the cytosol of nerve endings (Tettamanti et al., 1980).

Sialic acid in gangliosides and glycoproteins contributes substantially to the net negative charge on cell surfaces (Ambrose, 1966; Burry & Wood, 1979). Although in peripheral tissues most of this sialic acid is covalently linked to protein, in brain the sialic acid is found primarily as a component of gangliosides (Brunngraber, 1979; Ledeen, 1978). Relatively little is known about the functional roles of gangliosides on cell surfaces. However, this class of molecules appears to be involved in cell–cell interactions (Rauvala, 1981; Kleinman et al., 1979) and nerve regeneration (Gorio et al., 1980) and serves as receptor sites for peptides hormones (Mullin et al., 1976; Bremer & Hakomori, 1982), bacterial toxins (Fishman & Brady, 1976; Lee et al., 1979; Rodgers, & Snyder, 1981), and viruses (Holmgren et al., 1980). Hakomori (1975) has reported that there is a correlation between the ganglioside composition and malignancy in certain cell lines.

In a previous publication (Felgner et al., 1981a) we reported that purified gangliosides transfer from their micelles to large unilamellar vesicles formed from dipalmitoylphosphatidylcholine (Schullery et al., 1980). The ganglioside is stably incorporated, and its acyl chains are intercalated into the hydrocarbon region of the phospholipid bilayer. This incorporation is asymmetric with the sugar residues entirely in the outer surface of the vesicle. There is no measurable transbilayer migration of ganglioside. These structures may be considered to be a representative model system, which like the mammalian plasma membrane contains an asymmetric distribution of glycosphingolipid on the outer surface. In this paper we examine some of the details of the kinetics of transfer of gangliosides from their micelles. A preliminary report has been presented elsewhere (Felgner et al., 1981b).

Materials and Methods

Materials. Dipalmitoyl- and 1-palmitoyl-2-oleylphosphatidylcholines were obtained from Avanti Biochemicals (Birmingham, AL).

Ganglioside GT1b was prepared as previously described (Felgner et al., 1981a). GM1 was made by exhaustive hydrolysis of GT1b with neuraminidase by the following procedure to ensure identity of the acyl chain of both gangliosides studied. A total of 100 mg of GT1b and 10 mg of egg phosphatidylcholine were dissolved in 30:60 chloroform–methanol and dried in a film on the side of a flask. The egg phosphatidylcholine was used to promote hydrolysis of the ganglioside by the neuraminidase (Cestaro et al., 1980). This material was then dispersed in 50 mL of aqueous buffer containing 20 mM sodium acetate (pH 5.6) and 0.02% sodium azide, and 2 units of neuraminidase (*C. perfringens*, type VI; Sigma Chemical Co.) was added. This solution was incubated for 24 h at 37 °C and then desalted by passage over a Sephadex G-25 column (4 cm × 20 cm in H_2O). DEAE in the acetate form (from 2.5 g of dry DEAE A-25) was added to the desalted solution and stirred at room temperature for 15 min to adsorb the ganglioside. The DEAE was packed into a small column and washed with 30:60:8 chloroform–methanol–H_2O and then with pure methanol. Adsorbed GM1 was eluted with 75 mM ammonium acetate in methanol, dried, dissolved in H_2O, desalted by Sephadex column chromatography, and lyophilized. This ganglioside was then loaded on an Iatrobead (6RS-8060) column (Iatron Labs, Inc.) in 20%

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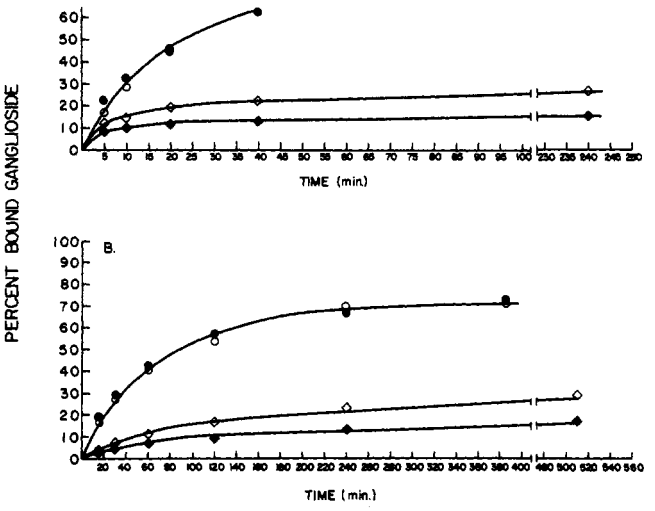

FIGURE 1: Rate of incorporation of micellar ganglioside into DPPC unilamellar vesicles at 45 °C. Aqueous phase was 50 mM KCl. (A) Ganglioside GT1b to DPPC mole ratio: 0.004 (●), 0.017 (○), 0.41 (◇), and 0.77 (◆). (B) Ganglioside GM1 to DPPC mole ratio: 0.017 (●), 0.034 (○), 0.31 (◇), and 0.63 (◆).

methanol in chloroform and eluted with a step gradient up to 70% methanol.

Phospholipid Vesicle Preparation. Large dipalmitoylphosphatidylcholine (DPPC)[1] unilamellar vesicles were prepared by the method of Schullery et al. (1980). Large unilamellar vesicles of 1-palmitoyl-2-oleylphosphatidylcholine (POPC)[1] were formed following a minor modification of the ethanol injection method described by Nordlund et al. (1981). Lyophilized POPC was dissolved in absolute ethanol to give a solution containing 30 μmol of phospholipid/mL. This solution was injected slowly (~3 mL/h) into rapidly stirring 50 mM KCl. The resulting vesicle dispersion was concentrated on an Amicon filter and then passed over an agarose 4B molecular sieve column to size the vesicles and remove the residual ethanol. This preparation was carried out at 21 °C.

Separation of Ganglioside Micelles from Phosholipid Vesicles. Ganglioside micelles, prepared as described by Felgner et al. (1981a), were separated from large fused dipalmitoylphosphatidylcholine vesicles on a Sepharose CL-4B column (Felgner et al., 1981a). The column was prepared by pouring a thick slurry of Sepharose CL-4B in 50 mM KCl into a disposable 6-mL plastic syringe fitted over the bottom end with a fine mesh Teflon cloth (50 μm pore size). The filled syringe was then inserted into a 15-mL conical glass centrifuge tube and centrifuged in a swinging bucket, International clinical centrifuge at full speed until most of the excess water was removed, but not to complete dryness (about 2 min). A 0.2-mL aliquot of a ganglioside–phospholipid vesicle mixture was placed on the small column, and the tube was centrifuged as before. Vesicles containing associated ganglioside collected in the bottom of the tube, while ganglioside micelles remained trapped in the gel. About 8% of the vesicle phosphorus was recovered. In the absence of large phospholipid vesicles, no ganglioside passed through the column under these conditions. The extent of incorporation of ganglioside into DPPC vesicles was determined by comparing the [sialic acid]/[phosphate] ratio in the original incubation mixture with the ratio obtained in the fraction that passed through the column.

Phosphorus was assayed according to Bartlett (1959) and sialic acid by the thiobarbituric acid method after hydrolysis at 80 °C for 1 h in 0.1 N H_2SO_4 (Warren, 1959).

[1] Abbreviations: DPPC, dipalmitoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleylphosphatidylcholine; cmc, critical micelle concentration.

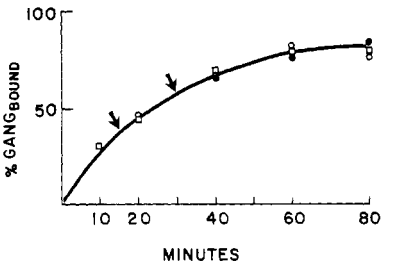

FIGURE 2: Effect of the addition of fresh DPPC vesicles after partial transfer of GT1b. To a preparation of phospholipid vesicles (19 μmol/mL), incubated at 45 °C, was added ganglioside GT1b from an aqueous stock solution to give a final concentration of 0.15 μmol/mL. The time course of disappearance of micelles was followed as in Figure 1 (□). After 15 min (○) or at 30 min (●), as denoted by the arrows, phospholipid vesicles without incorporated ganglioside were added to two separate aliquots of the original reaction mixture, and the disappearance of micelles was followed. The molar amount of vesicles added in the second addition was equivalent to the amount of original phospholipid vesicles. All solutions were 50 mM in KCl.

Results

Transfer Kinetics as a Function of Mole Ratio of Ganglioside to Phospholipid. Representative data in Figure 1 show the time dependence of the incorporation of GT1b (A) and GM1 (B) into large unilamellar vesicles of DPPC from micelles of these gangliosides at 45 °C. Incorporation is plotted as the percent of total system ganglioside present in the acceptor phosphatidylcholine vesicles as a function of time. Each curve in the two panels (A, B) was obtained at a specific mole ratio of ganglioside to phospholipid. The uppermost curve in each panel is the limiting time course of incorporation at low mole ratios of ganglioside to phospholipid. For GT1b the upper curve is the time course for mole ratios less than 0.016, and for GM1 less than 0.034. At and below these ratios, all ganglioside is eventually incorporated into the phospholipid vesicles. The composition of the resulting vesicles containing both ganglioside and phospholipid is thus the same as the composition of the initial mixture of ganglioside micelles and phospholipid vesicles.

At mole ratios of ganglioside to phospholipid higher than these limiting values, the percents of total ganglioside incorporated eventually reach essentially time-independent values which decrease with increasing values of the ganglioside to phospholipid ratio. The compositions of the resulting vesicles appear to reach a maximum, time-invariant value of 12–15 mol % ganglioside. This point will be discussed further in connection with Figure 4. Let us now consider in more detail the time course of ganglioside transfer at low values of the mole ratio of ganglioside to phospholipid.

Transfer Kinetics at Low Mole Ratios of Ganglioside to Phospholipid. It is apparent from the upper curves in Figure 1 that the rate of ganglioside transfer is independent of the mole ratio of ganglioside to phospholipid. This fact is shown in a different manner by the data in Figure 2. In this experiment DPPC vesicles were incubated at 45 °C. The percent incorporation at successive times is indicated by the open squares. To aliquots of this system were added sufficient additional vesicles at 15 or at 30 min to reduce to half the ganglioside to phospholipid mole ratio. The time course of ganglioside incorporation in these two systems is indicated by the open and closed circles, respectively. The times of addition are indicated by the arrows. It is clear that the time course is not influenced by the additions of DPPC vesicles.

Although the rate of ganglioside incorporation into DPPC vesicles at low values of the ganglioside to phospholipid ratio

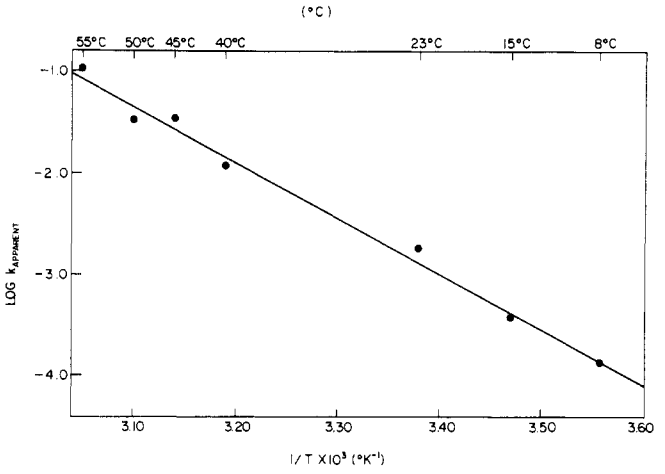

FIGURE 3: Temperature dependence of the apparent first-order rate constant for transfer of GT1b from micelles to phospholipid vesicles at values of the ganglioside to DPPc mole ratio less than 0.02. The log k apparent is plotted vs. the reciprocal of the absolute temperature. Experiments at 45 and 50 °C utilized DPPC vesicles. POPC vesicles were used for the rest of the experiments. Aqueous phase was 50 mM KCl.

is independent of concentration of vesicles, a plot of the data in Figure 1 as the log percent transfer vs. time is nonlinear. The rate of transfer markedly decreases with time. For GM1, the initial half-time for transfer is 2 h and for GT1b is 0.5 h.

Temperature Dependence of the Initial Rate of Ganglioside Transfer at Low Ganglioside to Phospholipid Mole Ratios. Figure 3 is an Arrhenius plot of the initial rate constant determined from the data in Figure 1 at low values of the ganglioside to phospholipid mole ratio and data obtained at other temperatures. Acceptor vesicles made from DPPC were used at 45 and 50 °C, and vesicles made from POPC were used over the entire temperature range. The rate constant decreases markedly with decreasing temperature with an apparent activation energy of 22.8 kcal mol^{-1}.

Transfer Kinetics at High Mole Ratios of Ganglioside to Phospholipid. It is apparent in Figure 1 that at mole ratios of ganglioside to phospholipid higher than the limiting values shown in the uppermost curves in both panels, the percentage of total ganglioside bound to vesicles reaches values which are essentially time independent. These limiting values decrease with increasing values of the total system ganglioside to phospholipid mole ratio. At long times these systems are comprised of mixtures of ganglioside micelles and vesicles containing limiting amounts of incorporated ganglioside. Figure 4 shows the increase in the ganglioside content of the phospholipid acceptor vesicles with time. For both GM1 and GT1b transfer there is an initial rapid phase followed by a much slower phase. In contrast to the situation obtained at low ganglioside to phospholipid mole ratios discussed above, the slower phase evident in these curves does not depend on the character of the donor micelle. This is shown in the data presented in Figure 5 which was obtained in the following experiment. Ganglioside GT1b was incubated with DPPC acceptor vesicles at a total system mole ratio of ganglioside to phospholipid equal to 0.18. After 18 h at 46 °C the transfer time course is in the slow phase as shown in Figure 4. At this point the system was divided into three aliquots. To one was added sufficient ganglioside-free DPPC acceptor vesicles to bring the total system mole ratio to 0.046 (●). To a second aliquot was added sufficient ganglioside to give a total system mole ratio of 0.32 (□). The third aliquot served as a control (○). The time course of incorporation of the three systems was then followed for an additional 7 h. The data in Figure

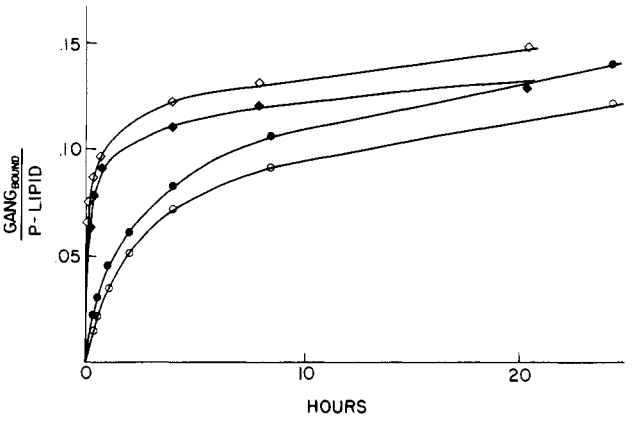

FIGURE 4: Apparent maximum limit of incorporation of ganglioside into DPPC vesicles at larger values of the ganglioside to phospholipid mole ratio. For GM1 the original phospholipid phosphate concentration was 0.5 μmol/mL, and the ganglioside concentration was either 0.16 (○) or 0.31 μmol/mL (●). For GT1b the original phospholipid concentration was 0.21 μmol/mL and the ganglioside concentration either 0.86 (◇) or 0.16 μmol/mL (◆). Incubations were carried out at 46 °C in 50 mM KCl.

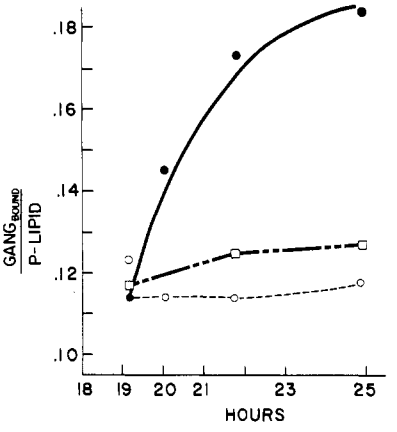

FIGURE 5: Effect of the addition of fresh DPPC vesicles or GT1b micelles to DPPC vesicles containing upper limit concentrations of GT1b. Initial phospholipid concentration was 0.8 μmol/mL, and GT1b concentration was 0.14 μmol/mL ([gang]/[phos] was 0.18). The sample was incubated at 46 °C for 18 h and divided into three aliquots. To one aliquot were added additional phospholipid vesicles to give a final concentration of 1.4 μmol/mL phospholipid and a ganglioside/phospholipid mole ratio of 0.046 (●). To a second aliquot were added additional ganglioside micelles to give a final concentration of 0.32 μmol/mL and a ganglioside/phospholipid ratio of 0.36 (□). The third aliquot was used as a control (○). All systems 50 mM in KCl.

5 show that the ganglioside micelles remaining after 18 h of transfer were still capable of acting as ganglioside donors exhibiting rapid phase kinetics. They also show that the addition of more ganglioside micelle leads to the incorporation of only a small additional amount of ganglioside into the acceptor vesicles.

Discussion

The spontaneous transfer of membrane lipids between bilayers, biological membranes, and lipoproteins has been examined in a number of laboratories (Roseman & Thompson, 1980; Doody et al., 1980; Nichols & Pagano, 1981a,b; McLean & Philips, 1981; Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Papahadjopoulos et al., 1976; Kao et al., 1977; Massey et al., 1980). Although in principal this transfer can occur either upon collision of lipid-containing structures or by transfer of molecules through the aqueous

phase, the available experimental evidence strongly suggests that transfer takes place by the latter mechanism in all systems. Transfer through the aqueous phase has generally been established in these studies by the observation that the kinetics of transfer are independent of donor and acceptor system concentrations (Roseman & Thompson, 1980). It is thus not surprising that ganglioside transfer from micelle to phospholipid bilayer is concentration independent by this criterion. The data in Figure 2 show not only that the time course of ganglioside transfer is independent of acceptor vesicle concentration but also that it is not dependent on the ganglioside content of acceptor vesicles. This must be the case since addition of acceptor vesicles containing no ganglioside to the vesicle population containing about 50% of the total system ganglioside does not perturb the time course shown in Figure 2. This inference is substantiated by the observation that the time course of ganglioside incorporation at low ganglioside to phospholipid ratios does not depend on the type of phospholipid (data not shown). It is thus clear that the decrease in transfer rate with time must be due to a change in the nature of the ganglioside micelle as the concentration of donor micelles decreases due to transfer of ganglioside to acceptor vesicles. These results strongly support the view that at low ganglioside to phospholipid system ratios, the transfer of ganglioside is limited by the rate at which molecules leave the ganglioside micelle.

This interpretation is consistent with the observation that the transfer rate is substantially larger for GT1b than for GM1. Electrostatic considerations suggest that the three negative charges per molecule of GT1b would lead to a larger off-rate than would the one negative charge carried by GM1. It is also consistent with the observation that Ca^{2+}, which is known to bind strongly to ganglioside micelles, decreases the rate of ganglioside transfer from micelles (P. L. Felgner and T. E. Thompson, unpublished observations).

There is only limited information in the literature about the structure and physical properties of ganglioside micelles. Values of the critical micelle concentration (cmc)[1] in aqueous media have been determined by various methods. Most striking is the lack of agreement among these values which range from 10^{-10} to 10^{-4} M (Gammack, 1963; Howard & Bruton, 1964; Yohe & Rosenberg, 1972; Yohe et al., 1976; Formisano et al., 1979). A recent quasi-elastic light-scattering study by Corti and co-workers (1980) places the cmc at less than 10^{-6} M for both GM1 and GD1a. This study reports a micelle weight of (4–5) $\times$ 10^5 in reasonable agreement with earlier values reported by Yohe and co-workers (1976) but larger than the value of (1–3.5) $\times$ 10^5 reported by Formisano et al. (1979). Most studies show that there is considerable heterogeneity in micellar size and indicate that both micellar size and heterogeneity depend on the system concentration of ganglioside (Corti et al., 1980). These observations are consistent with the suggestion that the character of the donor ganglioside micelle changes as the concentration of micelles decreases due to transfer of ganglioside to acceptor vesicles.

It is also possible that the character of the donor micelle changes because of the transfer of phospholipid into the ganglioside micelle from the acceptor vesicles. On the basis of the data of Roseman & Thompson (1980), the rate of transfer of phosphatidylcholine to the ganglioside micelles should be less than one-tenth the rate of ganglioside transfer to vesicles. We have not been able to demonstrate experimentally the presence of dipalmitoylphosphatidylcholine in residual ganglioside micelles, but we have observed the transfer of dimyristoylphosphatidylcholine into ganglioside micelles from vesicles composed of this phospholipid (P. L. Felgner, Y. Barenholz, and T. E. Thompson, unpublished observation).

Gel filtration experiments carried out by Formisano and co-workers (1979) in an attempt to establish the cmc of mixed gangliosides showed that the dissociation rate of ganglioside from micelles was very slow at 24 °C. A similar observation was recorded by Harris & Thornton (1978) in an NMR study of aqueous ganglioside systems at 4 °C. The long lifetimes of ganglioside micelles after dilution observed by these workers are in marked contrast to the relatively rapid transfer of ganglioside to phospholipid vesicles at 46 °C observed in the present work and in previous studies (Felgner et al., 1981a). However, the data presented in Figure 3 show a very strong decrease in the transfer rate with decreasing temperature. The small transfer rates at 24 and 4 °C are consistent with the observations of Formisano et al. (1979) and Harris & Thornton (1978).

The origin of this large temperature dependence of the transfer rate must lie in alterations in micellar structure which occur with temperature. Differential scanning calorimetric measurements on aqueous dispersions of GM1 micelles show no evidence of a phase transition in the temperature range 12–83 °C (Sillerud et al., 1979). Similar results were obtained for GM1 and GD1a micellar systems at a concentration of 15 mg/mL by Hinz et al. (1981), who did, however, note a gradual increase in heat capacity over the temperature range from 10 to 60 °C. These authors also observed a marked increase in the half-width of the ^1H NMR signals from the methylene groups with decreasing temperature in GM1 and GD1a dispersions over the same temperature range. The results indicate that structural changes in the ganglioside micelle occur in the temperature range from 10 to 60 °C which result in a marked increase in molecular motion. This increase in molecular motion might be expected to result in the large increase in the ganglioside off-rate inferred from the data in Figure 3.

At high total system ganglioside to phosphatidylcholine ratios, the initial rapid net rate of transfer decreases markedly when about 10–12 mol % ganglioside has been incorporated into the phosphatidylcholine vesicle. Although not zero, the rates of incorporation become very small at about 12–15% ganglioside. The data in Figure 5 indicate that this limitation of ganglioside incorporation is dependent on the characteristics of the acceptor vesicle and not on the nature of the donor micelle. We have shown previously that the transferred ganglioside is present entirely in the outer monolayer of the phosphatidylcholine acceptor vesicle (Felgner et al., 1981a). Thus at 12–15 mol % ganglioside, the outer vesicle surface actually has a concentration of 24–30 mol % ganglioside. Since no transbilayer migration of ganglioside occurs, a sizable mass imbalance must exist between the two monolayers of the vesicle bilayer. It is possible that three consequences, or a combination of three consequences, result from the mass imbalance. (i) The amount of ganglioside added to the outer vesicle face is limited by the mass imbalance. (ii) The mass imbalance drives a transbilayer migration of phosphatidylcholine which tends to relieve the imbalance. (iii) In large radius unilamellar vesicles of the type used in this study, the mass imbalance causes local changes in bilayer curvature which may result in evaginations leading ultimately to the formation of smaller radius of curvature vesicles. Under the conditions we have employed we have not found evidence that the mass imbalance leads to the formation of smaller vesicles.

The very small rate of ganglioside incorporation observed above 12–15 mol % may be the result of a transbilayer mi-

gration of phosphatidylcholine which relieves the mass imbalance. Experiments are currently under way to examine this possibility. A more general interest in mass imbalance transbilayer migration of lipids derives from a consideration of mechanisms which may operate to distribute lipids in a bilayer during membrane biogenesis. Since the site of lipid biosynthesis is always asymmetrically located with respect to a given membrane in the cell, it is possible that mass imbalance driven transbilayer lipid migration is an important process in vivo.

The transfer of ganglioside from micelles to phosphatidylcholine vesicles which we have studied must be the basic process involved in the addition of gangliosides from the aqueous phase to the plasma membrane of cells in culture as described by several workers [for a summary, see Hakomori (1981)]. The results of our study suggest that the rate of incorporation of ganglioside may be independent of the cell type and dependent only upon the molecular species of ganglioside. Since the transfer is dependent upon the type of ganglioside molecule, transfer from a mixture of gangliosides may not result in the incorporation of that mixture into the cell membrane after a specific time interval.

Registry No. DPPC, 2644-64-6; POPC, 6753-55-5; ganglioside GT1b, 59247-13-1; ganglioside GM1, 37758-47-7.

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