Spontaneous Transfer of Ganglioside GM_1 between Phospholipid Vesicles†

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ABSTRACT: The transfer kinetics of the negatively charged glycosphingolipid II^3-*N*-acetylneuraminosylgangliotetraosylceramide (GM_1) were investigated by monitoring tritiated GM_1 movement between donor and acceptor vesicles. After appropriate incubation times at 45 °C, donor and acceptor vesicles were separated by molecular sieve chromatography. Donors were small unilamellar vesicles produced by sonication, whereas acceptors were large unilamellar vesicles produced by either fusion or ethanol injection. Initial GM_1 transfer to acceptors followed first-order kinetics with a half-time of about 40 h assuming that GM_1 is present in equal mole fractions in the exterior and interior surfaces of the donor vesicle bilayer and that no glycolipid flip-flop occurs. GM_1 net transfer was calculated relative to that of [^{14}C]cholesteryl oleate, which served as a nontransferable marker in the donor vesicles. Factors affecting the GM_1 interbilayer transfer rate included phospholipid matrix composition, initial GM_1 concentration in donor vesicles, and the GM_1 distribution in donor vesicles with respect to total lipid symmetry. The findings provide evidence that GM_1 is molecularly dispersed at low concentrations within liquid-crystalline phospholipid bilayers.

Glycosphingolipids are membrane lipid components that appear to be localized almost exclusively in the outer leaflet of eukaryotic plasma membranes (Hakomori, 1981; Barbosa & Pinto da Silva, 1983). These glycosylated ceramides have been implicated in a variety of processes involving cellular interactions, differentiation, and oncogenesis (Hakomori, 1983; Feizi, 1985). Glycosphingolipids containing sialic acid, commonly known as gangliosides, are of particular interest because of their relative abundance in selected mammalian tissues (Nagai & Iwamori, 1984). Certain gangliosides are thought to modulate receptor function (Bremer & Hakomori, 1984) while others are highly antigenic (Hakomori & Young, 1983; Rapport & Huang, 1984). Gangliosides also serve as cellular attachment sites for certain viruses and bacterial toxins (Markwell et al., 1984; Suzuki et al., 1985; Keusch et al., 1986). The role of II^3-*N*-acetylneuraminosylgangliotetraosylceramide (GM_1)[1] as the receptor for cholera toxin is particularly well documented (Fishman, 1982).

Because of the wide variety of membrane-related events in which gangliosides participate, it is important to gain insight into how these glycolipids are transferred between various cellular compartments and delivered to the membrane surface. Axonal transport of gangliosides from the neuron cell body to the neuron endings has been studied in several animal systems (Forman & Ledeen, 1972; Ledeen et al., 1976; Landa et al., 1979; Yates et al., 1984). Other insights into intracellular ganglioside transport have been provided by experiments on neuroblastoma and glial cells in culture (Miller-Podraza & Fishman, 1982, 1983, 1984). The actual mechanism(s) of glycolipid movement in cells remain(s) poorly elucidated although both vesicular and protein-associated glycolipid transport have been suggested (Burkart et al., 1982; Sonnino et al., 1979; Tettamanti, 1984).

In an effort to identify and characterize basic physical properties which govern intermembrane transfer of glycosphingolipids and their organization in the surface of membranes, we have previously studied the intermembrane movement of certain neutral glycolipids (Correa-Freire et al., 1982; Brown et al., 1985b). In this paper, we continue that effort by examining the spontaneous transfer of ganglioside GM_1 between phospholipid vesicles. The resulting kinetic analysis provides information about the rate at which ganglioside GM_1 moves between pure phospholipid membranes and leads to the conclusion that most of this glycolipid is molecularly dispersed within the phosphatidylcholine matrix. Preliminary reports on portions of this work have appeared previously (Brown et al., 1986).

Materials and Methods

Preparation of Ganglioside GM_1. Mixed bovine brain gangliosides were isolated from upper Folch extract (Avanti Polar Lipids, Birmingham, AL) and processed on Sephadex G-25 and DEAE-Sephadex columns as described previously (Brown et al., 1985b). The resulting ganglioside fraction was comprised of disialo- and trisialogangliosides. GM_1 was prepared by treating the isolated ganglioside mixture with neuraminidase using a modified method of Felgner et al. (1983). 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 comprised of sodium acetate (pH 5.0), 0.02% sodium azide, and neuraminidase (10 units). After incubation for 24 h at 37 °C, GM_1 was isolated as described by Felgner et al. (1983).

Gas–Liquid Chromatography. GM_1 fatty acyl chain analysis was performed by using the gas–liquid chromatographic system described by Hresko et al. (1985). Fatty acids were released from GM_1 by heating in 1 N HCl in aqueous methanol (CH_3OH/H_2O, 82:18 v/v) for 18 h at 70 °C (Naoi et al., 1974) and methylated with diazomethane (Kates, 1972).

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[1] Abbreviations: GM_1, II^3-*N*-acetylneuraminosylgangliotetraosylceramide; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; DPPA, dipalmitoylphosphatidic acid; DPPC, dipalmitoylphosphatidylcholine; SUV, sonicated unilamellar vesicle(s) (25-nm diameter); LUV, large unilamellar vesicle(s) (70-nm diameter); $t_{1/2}$, first-order kinetic half-time; EDTA, ethylenediaminetetraacetic acid.

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Fatty acid methyl esters (dissolved in hexane) were eluted by using a temperature program (160–220 °C; 3 °C/min and detected at 250 °C. The GM_1 contained $C_{16:0}$ (0.4%), $C_{18:0}$ (91.6%), $C_{20:0}$ (7.4%), $C_{22:0}$ (0.2%), and miscellaneous components (0.4%). $C_{m:n}$ designates the fatty acyl chain where m is the number of carbon atoms and n is the number of double bonds. The values are reported as weight percent.

Preparation of Tritiated GM_1. Ganglioside GM_1 was tritiated by using the procedure described by Brown et al. (1985b). Briefly, alcohol groups at the sixth carbon of GM_1-terminal galactose residues were enzymatically oxidized by using galactose oxidase. The resulting aldehyde groups were reduced back to their original carbinol configurations using tritiated borohydride. Labeled GM_1 was separated from free potassium borohydride and other salts by elution through a Sephadex LH-20 column. Over 98% of the [^{3}H]GM_1 comigrated with authentic GM_1 standard when evaluated by analytical thin-layer chromatography in $CHCl_3/CH_3OH/H_2O$ (55:45:10) containing 0.02% $CaCl_2$.

Donor and Acceptor Vesicle Preparation and Incubation Conditions. Donors were sonicated unilamellar vesicles (25-nm diameter) (SUV) prepared by the method of Barenholz et al. (1977) except that the centrifugation time was reduced to 35–40 min due to the increased density of vesicles containing 7 mol % GM_1. Acceptors were large unilamellar vesicles (LUV) prepared by the method of Wong et al. (1982) or Nordlund et al. (1981). Details of donor and acceptor vesicle preparation and incubation during transfer experiments are discussed by Brown et al. (1985b). In order to avoid the possibility of vesicle fusion, at least a 5-fold molar excess of EDTA relative to ganglioside GM_1 was maintained in the buffer.

Donor and Acceptor Vesicle Separation following Incubations. At desired time intervals, donor and acceptor vesicles were separated by elution through a Sephacryl S-500 column (35 × 0.8 cm) at 45 °C while employing an upward hydrostatic pressure of 30–35 cm. The sieve column was prepared, used, and stored as described previously (Brown et al., 1985b) except that Sephacryl S-500 was used instead of Sepharose C1-2B. Sephacryl S-500 possesses structural properties allowing faster resolution of donor and acceptor vesicles (2–3 h) compared to Sepharose C1-2B (6–8 h). Other advantages of using Sephacryl to fractionate phospholipid vesicles have been discussed previously (Reers et al., 1984).

Kinetic Analysis. Ganglioside GM_1 movement at time t, $(^3H)_t$ can occur by (i) spontaneous transfer, $(^3H_{ST})$, between donor and acceptor vesicles and (ii) nonspontaneous transfer, $(^3H_{NST})$, processes such as vesicle–vesicle fusion. In this study, the quantity of interest is the ganglioside GM_1 spontaneous transfer. At time t, the GM_1 transferred spontaneously is equal to the total observable transfer $(^3H)_t$ minus the nonspontaneous transfer contributions $(^3H_{NST})_t$:

$$(^3H_{ST})_t = (^3H)_t - (^3H_{NST})_t \quad (1)$$

In order to estimate the nonspontaneous transfer at various time intervals, [^{14}C]cholesteryl oleate was included in the donor vesicles. If one assumes that [^{14}C]cholesteryl oleate only transfers to acceptors by the nonspontaneous route (e.g., fusion), then any [^{14}C]cholesteryl oleate eluting in the acceptor fractions will be accompanied by a proportional amount of [^{3}H]GM_1 also moving by the nonspontaneous route. The relative proportions of each label in the acceptor fractions will be identical with their original ratio in the donor–acceptor mixture at time zero:

$$(^3H_{NST})_t/(^{14}C)_t = (^3H)_0/(^{14}C)_0 \quad (2)$$

Equation 2 expresses the glycolipid nonspontaneous transfer, $(^3H_{NST})_t$, in experimentally observable terms. Solving eq 2 for $(^3H_{NST})_t$ and substituting back into eq 1 yield

$$(^3H_{ST})_t = (^3H)_t - [(^3H)_0(^{14}C)_t/(^{14}C)_0] \quad (3)$$

Equation 3 gives the ^{3}H-glycolipid movement occurring by spontaneous transfer from donor to acceptor vesicles in experimentally observable terms.

Transfer data were analyzed by integrating [^{3}H]GM_1 and [^{14}C]cholesteryl oleate curve areas (Figure 1) corresponding to the donor and acceptor vesicle fractions. Acceptors were those fractions eluting prior to the crossover point of the normalized [^{3}H]GM_1 dpm and [^{14}C]cholesteryl oleate dpm profiles. In Figure 1, the crossover point occurs near fraction 29. The amount of spontaneous GM_1 transfer is computed as departure from donor fractions relative to time zero data. The data are treated as departure from donors rather than as arrival to acceptors because the highest [^{3}H]GM_1 dpm levels at early time points are in the donor fractions. Thus, the precision of the early time point data is maximized. However, control calculations using the [^{3}H]GM_1 arriving at the acceptors are in agreement with the departure from donor calculations.

When the data are treated as a reversible first-order kinetic process, the following rate expression is used:

$$\frac{X(t) - X(\infty)}{X(0) - X(\infty)} = \exp(-kt) \quad (4)$$

where $X(t)$ is the glycolipid fractional transfer from donor vesicles at time t, $X(0)$ is the total amount of glycolipid present in the donors at time zero, $X(\infty)$ is the calculated equilibrium value for glycolipid fractional transfer from the donors at infinite time, and k is the rate constant for departure from the donor vesicles.

Kinetic rate parameters were determined by applying an iterative nonlinear least-squares analysis (Johnson & Frasier, 1985). The best fit was determined after evaluating the data using one- or two-exponential functions with or without a constant equilibrium value on a Control Data Corp. Cyber 730 computer.

Results

Figure 1 shows the elution profiles obtained at various time intervals after mixing DPPC SUV donors containing 7 mol % [^{3}H]GM_1 with 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) LUV acceptors. [^{3}H]GM_1 movement from the slower eluting donor vesicles to the faster eluting acceptor vesicles is evident. The elution position of donor vesicles is clearly delineated by [^{14}C]cholesteryl oleate which serves as a nontransferable marker during the 45 °C incubations.

Careful control experiments were performed to ensure that the observed tritium movement was due to intervesicular GM_1 transfer. In the first control experiment, the possibility of ganglioside degradation to mobile, tritiated fragments was examined. Tritium which eluted with acceptor vesicles was anaylzed by thin-layer chromatography. The tritiated material comigrated with authentic GM_1 standard (data not shown). By similar analysis, no degradation of [^{14}C]cholesteryl oleate was found. Therefore, tritium movement in Figure 1 does reflect GM_1 movement in the system.

Another control experiment was performed to make certain that [^{3}H]GM_1 movement was due to intervesicular transfer rather than donor–acceptor fusion. In this experiment, both donors and acceptors were small sonicated POPC vesicles. The donors contained 7 mol % [^{3}H]GM_1 and a trace of [^{14}C]-

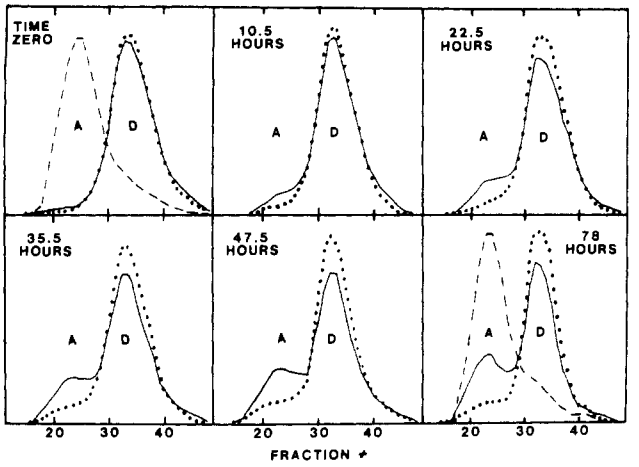

FIGURE 1: [^{3}H]GM_1 intervesicular transfer at 45 °C as a function of time. (---) Normalized lipid phosphate profiles of donor–acceptor mixtures; (—) normalized [^{3}H]GM_1 dpm profile; (···) normalized [^{14}C]cholesteryl oleate dpm profile. The matrix phospholipid is DPPC, and the acceptor to donor ratio is 10.1. D represents donor vesicle elution, and A represents acceptor vesicle elution. Each eluted fraction contains 1.0 mL. (See Materials and Methods for details.)

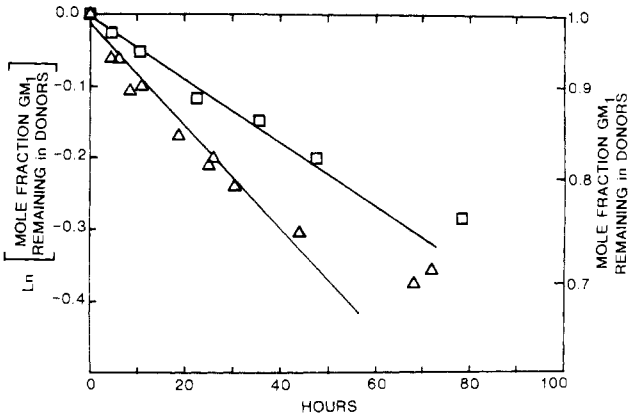

FIGURE 2: Natural logarithm of the mole fraction of GM_1 remaining in donors as a function of time. The starting GM_1 concentration in the donor vesicles is 7 mol %. (△) represents GM_1 departure from POPC SUV donors at 45 °C when the acceptor to donor ratio is 12.4; (□) represents GM_1 departure from DPPC SUV donors at 45 °C when the acceptor to donor ratio is 10.1. The mathematical description of the left-hand ordinate is ln $\{[X(t) - X(\infty)]/[X(0) - X(\infty)]\}$. A complete explanation is provided by eq 4 under Materials and Methods.

cholesteryl oleate. After incubation of donor and acceptor vesicle mixtures for various time intervals extending from 0 to 15 days, molecular sieve chromatographic analysis was performed to assess the extent of fusion. Almost no fusion could be detected by either of the radioactive labels (data not shown). Additional information indicating negligible amounts of vesicle fusion is illustrated by the results in Figure 1. First, the total lipid phosphate elution profiles of the donor and accedptor vesicles show no change over the experimental time course. Second, [^{14}C]cholesteryl oleate movement from donor to acceptor vesicles is quite low (5–7%). In contrast, over 30% of the total [^{3}H]GM_1 moves to acceptors during the same time interval (Figure 1). If fusion were responsible for label movement, one would expect both labels to move at the same rate to the acceptor fractions. This clearly is not the case.

Having established that [^{3}H]GM_1 movement is due to spontaneous intervesicular transfer of GM_1, we performed kinetic analysis of the data (see Materials and Methods). The results in Figure 2 show that initial GM_1 departure from donor vesicles follows single-exponential decay kinetics. The single-exponential decay occurs regardless of whether the donor

Table I: Values for GM_1 and Asialo-GM_1 Rate Constants[a]

glycolipid	matrix	k (h^{-1})[b]	$t_{1/2}$ (h)[b]
GM_1	POPC	0.0173	40.2
GM_1	DPPC	0.0125	55.4
asialo-GM_1 (slow pool)[c]	POPC	0.0012	594.0
asialo-GM_1 (fast pool)[c]	POPC	ND[d]	ND
asialo-GM_1 (slow pool)[c]	DPPC	0.0014	504.0
asialo-GM_1 (fast pool)[c]	DPPC	0.0154	45.0

[a] The data were calculated by assuming that 67% of the glycolipid is in the donor vesicle outer monolayer available for transfer and that glycolipid flip-flop does not occur. The rate constants for asialo-GM_1 were obtained from Brown et al. (1985b). [b] Temperature 45 °C. [c] The slow pool comprised 84% of the asialo-GM_1 whereas the fast pool comprised 16% of the asialo-GM_1 in the donor vesicle outer monolayer. The more accurate fast pool rate constants were determined by the ion-exchange method. [d] Not determined.

Table II: GM_1 Rate Constant Variation as a Function of Starting Concentration in Donor Vesicles

GM_1 starting concn in donors (mol %)	k (h^{-1})[a]	$t_{1/2}$ (h)[a]
15.0	0.0391	17.7
7.0	0.0173	40.2
4.0	0.0189	38.1
1.0	0.0179	38.7
0.1	0.0094	74.0

[a] Temperature 45 °C.

phospholipid matrix is POPC or dipalmitoylphosphatidylcholine (DPPC). When analyzed by an iterative nonlinear least-squares fitting routine (Johnson & Frasier, 1985), the best fit occurred when a single-exponential function with one equilibrium value was applied to the GM_1 transfer data. The resulting GM_1 departure rate constants for POPC donors are listed in Table I. Slightly faster rates of GM_1 transfer are observed when DPPC is the donor matrix rather than POPC. However, quite different rates are observed if one compares GM_1 transfer kinetics with those of its desialylated derivative. In previous studies, we examined the intervesicular transfer of asialo-GM_1 (Brown et al., 1985b). The majority of asialo-GM_1 (~84%) present in the donor vesicle outer monolayer transferred to acceptor vesicles very slowly ($t_{1/2} > 500$ h). The remaining 16% or so transferred much faster to acceptors ($t_{1/2} = 45$ h). The transfer rate of this minor asialo-GM_1 pool is very similar to that observed for GM_1 (Table I).

The calculation producing the rate constants in Table I requires that the total glycolipid pool size in the donors be known. The following experiment was carried out to determine the GM_1 transbilayer distribution in donor vesicles. Sonicated unilamellar POPC vesicles containing 7 mol % GM_1 were mixed with a 10-fold excess of POPC SUV acceptors in the presence of glycolipid transfer protein (Wong et al., 1984; Brown et al., 1985a). Figure 3 shows the amount of GM_1 transferred to acceptors over a 2-h incubation at 37 °C. It is clear that approximately two-thirds of the GM_1 moves rapidly to the acceptors.

For the purpose of calculating the GM_1 kinetic rate constant, the ganglioside pool size available for spontaneous transfer is approximately 67% of the total starting amount in the donor vesicles. The contribution of the 33% remaining within the donor inner monolayer is not considered because transbilayer redistribution of inner monolayer GM_1 is assumed to be negligible due to the high polarity of the charged glycolipid head group. The transbilayer redistribution rate of a spin-labeled GM_1 derivative has been examined in sonicated unilamellar egg phosphatidylcholine vesicles at 23 °C (Sharom

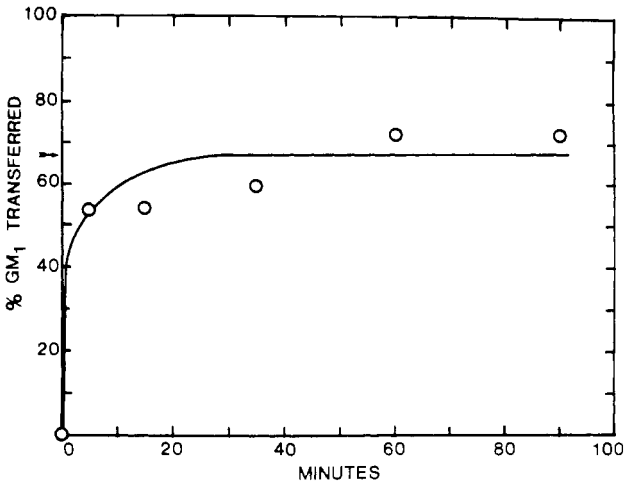

FIGURE 3: Accessibility of GM_1 in sonicated POPC vesicles to a soluble glycolipid transfer protein. The arrow on the ordinate indicates the transfer expected if two-thirds of the GM_1 is initially present in the vesicle outer surface. Sonicated POPC vesicles (1.2 μmol) containing 5 mol % GM_1 (6×10^4 dpm of [^{3}H]GM_1), 10 mol % dipalmitoylphosphatidic acid (DPPA), and a trace of [^{14}C]cholesteryl oleate (6×10^4 dpm) were incubated with sonicated POPC acceptors (12.0 μmol) and glycolipid transfer protein [60 μg; isolated by method of Brown et al. (1985)a] at 37 °C in a total volume of 1.8 mL. At the indicated time, 0.3 mL of the mixture was separated on a DEAE ion-exchange minicolumn. The data are normalized relative to the equilibrium transfer value (90.9% for a donor to acceptor ratio of 1:10) and to the known recovery of acceptors (87%). Control experiments indicated that less than 10% of the glycolipid transfer protein activity was lost after 90 min at 37 °C in the presence of GM_1-containing donors.

& Grant, 1978). These authors reported no evidence of ganglioside flip-flop in fluid lipid bilayers.

Variation of GM_1 Initial Concentration in Donors. Table II shows the kinetic rate constants obtained when the starting GM_1 concentration in POPC donor vesicles is varied between 0.1 and 15 mol %. Over the entire concentration range, a general trend of increasing transfer rate with increasing GM_1 concentration is observed. However, very little change in the transfer rate is noted between 1 and 7 mol %.

Departure of Asymmetrically Incorporated GM_1 from Donor Vesicles. The following experiment was performed to see if transfer of GM_1 that is asymmetrically oriented in the donor is different from GM_1 that is distributed in each of the bilayer surfaces. The results in Figure 3 show that, in donor vesicles produced by cosonicating GM_1 and POPC, approximately 65–70% of the GM_1 is present in the vesicle outer monolayer. This is very close to the expected values for phosphatidylcholine mass distribution in vesicles of this size. The asymmetrical donors were produced by making POPC SUV and subsequently incubating with GM_1 micelles to yield a final glycolipid concentration of 7 mol % in the outer surface of the POPC SUV bilayer. In previous studies in this laboratory, Felgner et al. (1983) examined the structures obtained when GM_1 micelles are incubated with DPPC unilamellar vesicles. When present in amounts yielding final GM_1 concentrations below 12–15 mol % in the vesicles, all of the GM_1 rapidly transfers to the vesicles, and the micelles disappear. GM_1 monomers depart from their micelles and transfer through the aqueous phase into the phospholipid vesicles. The fact that the ganglioside is localized exclusively in the outer monolayer of the phospholipid vesicles has been shown by using bilayer-impermeant probes such as neuraminidase (Felgner et al., 1981).

When GM_1 was incorporated asymmetrically into sonicated unilamellar vesicles to a concentration of 7 mol % in the vesicle outer surface, this GM_1 transferred to acceptor vesicles in a single-exponential process with a rate constant of 0.0347 h^{-1} ($t_{1/2}$ = 20.0 h) at 45 °C. In contrast, the GM_1 transfer rate constant for donor vesicles produced by cosonicating 7 mol % GM_1 with POPC was 0.0173 h^{-1} ($t_{1/2}$ = 40.2 h). Therefore, GM_1 that is asymmetrically oriented in the donors transfers significantly faster than GM_1 that is mass distributed in the donors.

Discussion

Gangliosides are believed to be synthesized by a membrane-associated multi-glycosyltransferase system located in the Golgi apparatus (Roseman, 1970; Tettamanti, 1984; Yusuf et al., 1985). Following synthesis, the gangliosides are delivered to the plasma membrane and become spatially oriented in the outer surface so that their polar glycosyl groups are projecting toward the external cellular environment (Hakomori, 1981; Barbosa & Pinto da Silva, 1983). The spatial orientation of gangliosides in the plasma membrane outer surface is of prime importance in their cellular functions. Gangliosides have been implicated in cellular adhesion and recognition (Hakomori, 1983), modulation of receptor function (Bremer & Hakomori, 1984), and cellular antigenicity and oncogenesis (Hakomori & Young, 1983; Feizi, 1985). Gangliosides also serve as cellular attachment sites for certain viruses and bacterial toxins (Fishman, 1982; Markwell et al., 1984; Suzuki et al., 1985).

After spending their metabolic lifetime at the plasma membrane outer surface, gangliosides are transported to lysosomes where enzymatic degradation occurs (Gatt, 1970; Tettamanti, 1984). The mechanism of ganglioside transport to the plasma membrane and ultimately to lysosomes remains to be elucidated. Whether ganglioside transport occurs via vesicles (Pagano, 1983; Tettamanti, 1984) or lipoprotein complexes (Conzelmann et al., 1982; Sonnino et al., 1979; Brown et al., 1985a) or free molecules or a combination of these mechanisms is still a controversial issue.

The present study was undertaken to identify and characterize the kinetic parameters governing spontaneous transfer of ganglioside molecules between phospholipid bilayers. To date, very little information is available about GM_1 intervesicular transfer (Brown et al., 1986; Masserini & Freire, 1986) although some data have been reported for GM_1 transfer between phospholipid vesicles and high-density lipoproteins (Kwok et al., 1981; Shen et al., 1981).

In the two-component lipid system employed here, the GM_1 spontaneous transfer rate provides clues about the interactions of GM_1 molecules with other GM_1 molecules and with phospholipid molecules in the vesicle surface. The ease with which GM_1 desorbs from the vesicle bilayer is indicative of the immediate lipid environment surrounding the GM_1 molecules. One can then make predictions about the lateral arrangements of GM_1 in the bilayer surface that are consistent with the kinetic data.

The experiments reported here indicate that ganglioside GM_1 does spontaneously transfer at 45 °C from small sonicated phosphatidylcholine vesicles to large unilamellar phosphatidylcholine vesicles. The GM_1 transfer half-time is about 40 h from a POPC matrix and 55 h from a DPPC matrix when the starting concentration of GM_1 is 7 mol % in the donor vesicles. As the GM_1 starting concentration in the donors is increased from 0.1 to 15 mol %, the GM_1 transfer rate gradually increases although very little change in GM_1 transfer rate was noted between 1.0 and 7.0 mol % GM_1. The range of GM_1 starting concentration was selected so as to ensure a bilayer configuration for the donors. Several investigators have

demonstrated that if ganglioside GM_1 concentrations exceed 25–30 mol % in phosphatidylcholine vesicles, then mixed micelles of GM_1 and phospholipid coexist with vesicles in the system (Sillerud et al., 1979; Barenholz et al., 1980). The presence of such GM_1–phospholipid mixed micelles would be expected to increase the observed transfer rate since ganglioside transfer from their pure micelles into phospholipid vesicle acceptors is known to occur on a much faster time scale than the rates reported for GM_1 interbilayer transfer in Tables I and II (Felgner et al., 1981; 1983). However, this clearly is not the case in the present studies because 15 mol % GM_1 is never exceeded. More likely, the correlation between increasing transfer rate and increasing GM_1 donor vesicle concentration simply reflects the increase in negative surface charge density in the donor vesicle due to GM_1. Alternatively, varying the GM_1 concentration may alter the donor vesicle radius of curvature and produce changes in the GM_1 transfer rate. Masserini et al., (1985) reported that increasing the GM_1 concentration in sonicated egg phosphatidylcholine vesicles decreases the vesicle radius. The fact that vesicle size influences lipid interbilayer transfer rates has been predicted in theoretical calculations (Jahnig, 1984) and, recently, has been reported for cholesterol and other lipid derivatives (Fugler et al., 1985; Wolkowicz et al., 1984).

Other parameters also influence ganglioside interbilayer transfer. The experiments described here indicate that ganglioside GM_1 departure from sonicated phosphatidylcholine donor vesicles is affected by the GM_1 distribution and arrangement in the vesicles. When GM_1 is incorporated by addition to the outer monolayer of preformed sonicated vesicles, the interbilayer transfer rate to large unilamellar acceptors is about 2-fold faster than when the GM_1 is cosonicated with the phosphatidylcholine to form donors. The donor vesicles resulting from incubation with GM_1 micelles are structurally unique. Felgner et al. (1981) have shown that all of the ganglioside becomes incorporated into the vesicle outer monolayer. Thus, the vesicles are asymmetric with respect to ganglioside distribution. Also, the GM_1 added to preformed vesicles is extra lipid mass integrated into only one of the monolayers. The integration of this GM_1 into the vesicle outer monolayer might produce an intermolecular packing stress. If such a packing stress occurs, relief could be achieved by increasing the initial desorption rate of GM_1 and/or the matrix phospholipid upon mixing with acceptor vesicles. On the other hand, one might argue that the intermolecular stress is short-lived and rapidly relieved by a totally different mechanism, such as transbilayer migration (flip-flop) of phosphatidylcholine to the donor vesicle inner monolayer. However, when similarly stressed vesicles are monitored using NMR shift reagents, no phosphatidylcholine flip-flop is measurable (P. L.-G. Chong and T. E. Thompson, unpublished observation). If it is true, however, that phospholipid flip-flop does occur, then an alternative interpretation would be needed to explain the increased GM_1 desorption rate to acceptor vesicles. One possible alternative is the following: During formation of the asymmetrically arranged GM_1 donor vesicles, the GM_1 molecules departing from their micelles may preferentially integrate into the smallest vesicles of the SUV preparation. In contrast, GM_1 that is incorporated into SUV by colyophilization and cosonication becomes uniformly distributed throughout the SUV population. The faster GM_1 transfer rate observed for the asymmetrically arranged GM_1 would simply reflect the more favorable desorption from the smaller donors. In fact, although difficult to measure in sonicated vesicle preparations, preliminary experiments support the contention that GM_1 molecules departing from their micelles do selectively integrate into the smallest vesicles of a given population (R. E. Brown and T. E. Thompson, unpublished observation). Obviously, more work is required to solve this problem.

In any case, the transfer half-time for 7 mol % ganglioside GM_1 is nearly an order of magnitude shorter than that determined for the neutral glycosphingolipid glucosylceramide (Correa-Freire et al., 1982). Similarly, GM_1 transfers much faster to acceptor vesicles than does the majority ($\sim$84%) of its desialylated derivative, asialo-GM_1 (Brown et al., 1985b). Interestingly, the balance of asialo-GM_1 ($\sim$16%) transfers to acceptors at the same rate as GM_1. The GM_1 transfer rate is also very similar to the interbilayer exchange rate reported for POPC at 37 °C (McLean & Phillips, 1981). The interbilayer transfer rates of other fluid-phase phospholipids and sphingomyelins are also known to occur as fast or faster than POPC (Martin & MacDonald, 1976; Duckwitz-Peterlein et al., 1977; Frank et al., 1983).

The simplest interpretation of the GM_1 transfer rate data is the following: The majority of GM_1 is molecularly dispersed in POPC and DPPC bilayers. This arrangement of GM_1 is in sharp contrast to that of the neutral glycosphingolipids gangliotetraosylceramide and glucosylceramide, which have been shown to exist predominantly as gel-like clusters in phosphatidylcholine vesicles near physiolgical temperatures (Rintoul, 1986; Thompson & Tillack, 1985; Brown et al., 1985b; Tillack et al., 1982; Correa-Freire et al., 1979). Several corroborating pieces of evidence indicate that a molecularly dispersed arrangement of GM_1 in the bilayer best explains the transfer data. Recently, we have reported freeze–etch electron microscopic studies in which native cholera toxin or a cholera toxin–ferritin conjugate was used to label GM_1 which had been incorporated into POPC liposomes. An analysis of the results indicates that GM_1 is molecularly dispersed at 37 °C when present in the concentration interval 0–5 mol % (Thompson et al., 1985). Differential scanning calorimetric studies, electron spin resonance experiments, and electrical conductance measurements also suggest that ganglioside molecules are molecularly dispersed in phosphatidylcholine bilayers when present at low concentration [Maggio et al., 1985; for a review, see Thompson and Tillack (1985)]. A similar conclusion was reported by investigators using fluorescence depolarization techniques to study ganglioside behavior in phosphatidylcholine bilayers (Uchida et al., 1981; Rintoul et al., 1986).

The differences and similarities in the spontaneous transfer rates of ganglioside GM_1 and asialo-GM_1 are particularly striking because these molecules are virtually identical except for the presence or absence of *N*-acetylneuraminic acid. Thus, *N*-acetylneuraminic acid can be thought of as a fine-tuning element that modulates the transfer process by shifting the relative pool sizes of glycolipid departing from the bilayer surface at fast ($t_{1/2} \cong 40$ h) and slow rates ($t_{1/2} \cong 500$ h). The fast and slow transfer rates, in turn, provide indirect information about the glycolipid lateral arrangement within the phospholipid bilayer. The modulatory role of *N*-acetylneuraminic acid in such processes may have important physiological implications. For example, certain mammalian sialidases are thought to be localized within the plasma membrane (Schengrund et al., 1972; Zeigler & Bach, 1985). Stimuli that trigger enzymatic removal of sialic acid from glycolipids situated in the plasma membrane would be expected to promote glycolipid lateral rearrangement within the cell surface. Such rearrangements of glycolipids would provide the cell with a molecular mechanism for rapid surface modification during

cell interaction–recognition events. Sialylation and desialylation are already known to be critical processes which dramatically affect the clearance of erythrocytes and lymphocytes from circulation (Schauer, 1985). Thus, the functional importance of sialylation and desialylation is recognized although the resulting molecular changes occurring at the plasma membrane surface remain to be elucidated.

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Dependence on Phospholipid Composition of the Fraction of Cholesterol Undergoing Spontaneous Exchange between Small Unilamellar Vesicles†

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ABSTRACT: Spontaneous cholesterol exchange between small unilamellar vesicles comprised of different phospholipids and their binary mixtures has been studied in order to understand the factors involved in the establishment and maintenance of intracellular cholesterol distributions. Exchange was performed from neutral donor vesicles containing different cholesterol concentrations, traces of [^{3}H]cholesterol, and [^{14}C]cholesteryl oleate as a nonexchangeable marker. The acceptor vesicles, in 10-fold excess, had the same composition, but 15 mol % phosphatidylglycerol was included to permit chromatographic separation. Data were best fitted by a single exponential and a base value. In donor vesicles containing only one phospholipid, the kinetic rate constants agreed with data reported previously; however, the base values were larger than the expected equilibrium value of 9.09%. The size of this nonexchangeable pool and the exchange rate were found to depend on the type of phospholipid. In binary phospholipid donor systems, well above the transition temperatures of the lipid components, the exchange parameters were preferentially closer to those of one component according to the order POPC > DMPC > DPPC > bovine brain SPM.

It is well-known that the distribution of cholesterol among the various subcellular membranes of any specific type of cell is not uniform. For example, in the rat hepatocyte the plasma membrane contains 28.0 wt % cholesterol while in the endoplasmic reticulum, the site of cholesterol biosynthesis, the concentration is only 6 wt %. In the inner mitochondrial membrane the concentration is less than 1% (Thompson & Huang, 1986).

The process of spontaneous cholesterol exchange between phospholipid vesicle bilayers has been studied in an attempt to understand some of the factors controlling the establishment and maintenance of this cholesterol distribution. Several studies in small unilamellar phosphatidylcholine vesicles have shown that cholesterol moves between vesicles by transfer through the aqueous phase (Backer & Dawidowicz, 1981; McLean & Phillips, 1981, 1982). The data also suggest that the rate-limiting step is the desorption of cholesterol from the donor membrane. The same results have been obtained in cholesterol-transfer studies between biological membranes (Lange et al., 1979, 1983). The ultimate cholesterol concentration in the various membranes could be a result of a partitioning equilibrium, which depends on membrane composition. In fact, Wattenberg and Silbert (1983) showed that the differences in cholesterol concentration between various membrane fractions were retained when any two fractions were incubated together.

In a recent study we reexamined the kinetics of the spontaneous exchange of [^{3}H]cholesterol between small unilamellar vesicles of 1-palmitoyl-2-oleoylphosphatidylcholine and found two kinetically defined cholesterol pools (Bar et al., 1986). About 80% of the total cholesterol exchanges with first-order kinetics ($t_{1/2}$ = 90 min), in good agreement with previous studies, while the remaining 20% was found to be nonexchangeable in the time frame of the experiments (8 h). This nonexchangeable pool of cholesterol together with differences in the rates of exchange may contribute to the unequal distribution of cholesterol between membranes in the same cell. We now report studies on the kinetics of [^{3}H]cholesterol exchange between small unilamellar vesicles comprised of different phospholipids and binary mixtures of these phospholipids.

Materials and Methods

Materials. Cholesterol was obtained from Nu-Chek-Prep, Inc. (Elisian, MN). Bovine brain sphingomyelin, 1,2-diacyl-*sn*-glycero-3-phosphocholines, and phosphatidylglycerols were purchased from Avanti Polar Lipids, Inc. (Birmingham,

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