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Spontaneous Transfer of Gangliotetraosylceramide between Phospholipid Vesicles†

Rhoderick E. Brown, István P. Sugár,‡ and Thomas E. Thompson*
Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908
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ABSTRACT: The transfer kinetics of the neutral glycosphingolipid gangliotetraosylceramide (asialo-GM_1) were investigated by monitoring tritiated asialo-GM_1 movement from donor to acceptor vesicles. Two different methods were employed to separate donor and acceptor vesicles at desired time intervals. In one method, a negative charge was imparted to dipalmitoylphosphatidylcholine donor vesicles by including 10 mol % dipalmitoylphosphatidic acid. Donors were separated from neutral dipalmitoylphosphatidylcholine acceptor vesicles by ion-exchange chromatography. In the other method, small, unilamellar donor vesicles (20-nm diameter) and large, unilamellar acceptor vesicles (70-nm diameter) were coincubated at 45 °C and then separated at desired time intervals by molecular sieve chromatography. The majority of asialo-GM_1 transfer to acceptor vesicles occurred as a slow first-order process with a half-time of about 24 days assuming that the relative concentration of asialo-GM_1 in the phospholipid matrix was identical in each half of the donor bilayer and that no glycolipid flip-flop occurred. Asialo-GM_1 net transfer was calculated relative to that of [^{14}C]cholesteryl oleate, which served as a nontransferable marker in the donor vesicles. A nearly identical transfer half-time was obtained when the phospholipid matrix was changed from dipalmitoylphosphatidylcholine to palmitoyloleoylphosphatidylcholine. Varying the acceptor vesicle concentration did not significantly alter the asialo-GM_1 transfer half-time. This result is consistent with a transfer mechanism involving diffusion of glycolipid through the aqueous phase rather than movement of glycolipid following formation of collisional complexes between donor and acceptor vesicles. When viewed within the context of other recent studies involving neutral glycosphingolipids, these findings provide additional evidence for the existence of microscopic, glycosphingolipid-enriched domains within the phospholipid bilayer.

Spontaneous, nonprotein-mediated transfer of phospholipids and phospholipid derivatives between model membranes is a well established phenomenon (Martin & McDonald, 1976, Papahadjopoulos et al., 1976; Duckwitz-Peterlein et al., 1977; Nichols & Pagano, 1981, 1982; Roseman & Thompson, 1980; McLean and Phillips, 1981; De Cuyper et al., 1983; Schroit & Madsen, 1983). Nonprotein-mediated, intervesicular transfer of cholesterol and other lipid derivatives is also well documented (Backer & Dawidowicz, 1981; McLean & Phillips, 1981, 1982; Kao et al., 1977; Doody et al., 1980). However, very little information exists on the spontaneous interbilayer transfer of glycosphingolipids. Insight into the behavior and structural organization of glycosphingolipids in membranes is important because these molecules have been implicated in cell–cell identification and recognition processes (Horowitz, 1978; Hakomori, 1981), and they can also serve as cellular attachment sites for certain bacterial toxins (Fishman, 1982) and viruses (Holmgren et al., 1980).

In previous studies from this laboratory (Correa-Freire et al., 1982) the spontaneous interbilayer transfer of the simple neutral glycosphingolipid glucosylceramide was examined and

†Supported by U.S. Public Health Service Grants GM-23573 and GM-14628.
‡Permanent address: Institute of Biophysics, Semmelweis Medical University, Budapest 1444, Hungary.

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found to be much slower ($t_{1/2}$ > 30 days) than that of phospholipids [$t_{1/2}$ < 48 h). The present study was undertaken to determine whether more complex, neutral glycosphingolipids exhibit transfer rates similar to that of glucosylceramide. The glycosphingolipid utilized in this study is asialo-GM_1 [gangliotetraosylceramide: galactosyl(β1–3)-*N*-acetylgalactosaminyl(β1–4)galactosyl(β1–4)glucosyl(β1–1)ceramide].[1] This glycosphingolipid has been localized on the surface of murine natural killer cells (Beck et al., 1982; Kasai et al., 1980; Young et al., 1980) and on the lymphoblasts of patients with acute lymphoblastic leukemia (Nakahara et al., 1980). Asialo-GM_1 also may serve as a differentiation antigen in murine cytotoxic T lymphocytes (Beck et al., 1982) and rat thymocytes (Momoi et al., 1980; Arndt et al., 1981). Therefore, asialo-GM_1 is a physiologically relevant example of the complex, neutral glycosphingolipid class.

In the present study, the kinetics of the spontaneous interbilayer transfer process have been monitored by using tritiated asialo-GM_1. The experiments described herein clearly show that the spontaneous transfer of the majority of asialo-GM_1 present in sonicated donor vesicles is very slow compared to that of phospholipids. The mechanism of asialo-GM_1 transfer is by diffusion through the aqueous buffer rather than by vesicle–vesicle collisions. In addition, the detailed kinetics of the transfer process are interpreted in terms of a compositional domain arrangement of glycolipid in the phospholipid matrix. A preliminary report on portions of this work has appeared previously (Brown & Thompson, 1983).

Materials and Methods

Preparation of Asialo-GM_1. Mixed bovine brain gangliosides were extracted from upper Folch solvent (Avanti Polar Lipids, Birmingham, AL) by elution over Sephadex G-25 using deionized H_2O. The ganglioside micelles eluted at the column void volume in a preparation largely free of water-soluble peptides and salts, thereby avoiding the tedious task of rotary evaporation of upper Folch solvent.

The ganglioside fraction was applied directly to DEAE-Sephadex (acetate form; 1 g of ganglioside/20 g of DEAE-Sephadex) and washed with excess methanol. Acidic phospholipids, sulfatides, and GM_1 were eluted from the DEAE-Sephadex by washing with 10 mM ammonium acetate in methanol. Recovery of the majority of ganglioside was achieved by elution with 500 mM ammonium acetate in methanol. After removal of the methanol by rotary evaporation, the ganglioside fraction was dissolved in H_2O and desalted by using Sephadex G-25 column chromatography. The resulting ganglioside fraction was found to be disialo- and trisialogangliosides after analyzing by thin-layer chromatography [$CHCl_3$/MeOH/H_2O, 55:45:10 (0.02% aqueous $CaCl_2$)].

Asialo-GM_1 was prepared from the purified bovine brain gangliosides by using the mild hydrolytic desialization procedure of Kasai et al. (1982). The yield of asialo-GM_1 was 50%. Gas–liquid chromatographic analysis was performed on the asialo-GM_1 fatty acyl chains as described by Correa-Freire et al. (1979). The asialo-GM_1 contained $C_{16:0}$ (0.7%), $C_{18:0}$ (91.3%), $C_{20:0}$ (6.4%), $C_{22:0}$ (0.5%), and miscellaneous components (1.1%). $C_{m:n}$ 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 Asialo-GM_1. The asialo-GM_1 was tritiated by using a modification of the methods of Suzuki & Suzuki (1972) and Radin & Evangelatos (1981). Initially, aldehyde groups were generated on the glycolipid by enzymatically oxidizing the alcohol groups at the sixth carbon of terminal galactose residues with galactose oxidase. The asialo-GM_1 (10 mg) was suspended in 5 mL of tetrahydrofuran (freshly distilled over KOH pellets). The suspension was vortexed continuously while 3.5 mL of 10 mM PIPES-Na buffer (pH 7.0) was added slowly until all the glycolipid was dissolved. Catalase (2500 units), peroxidase (1100 units), and galactose oxidase (125 units) were each suspended in 0.5 mL of 10 mM PIPES-Na buffer (pH 7.0) and added successively to the asialo-GM_1 solution. After the mixture was stirred overnight at 26 °C, an aliquot was analyzed by thin-layer chromatography [$CHCl_3$/MeOH/H_2O, 55:45:10 (0.02% $CaCl_2$)]. Only a single glycolipid spot, which migrated slightly faster than an asialo-GM_1 standard, was evident (primulin spray). After lyophilization of the reaction mixture, the enzymatically treated asialo-GM_1 was separated from the majority of the other reaction products by employing a Folch extraction. The oxidized asialo-GM_1 was stored at −20 °C in $CHCl_3$/MeOH (2:1) for as long as several months without degradation.

After generation of the aldehyde groups on asialo-GM_1, reduction back to the original carbinol was accomplished by using radioactive borohydride. The oxidized asialo-GM_1 (3–4 mg) was dissolved in 2 mL of dichloromethane/methanol (1:1). Potassium borohydride (2.5 mCi) dissolved in 0.2 mL of 0.01 N KOH was added to the glycolipid and allowed to incubate overnight at room temperature in a fume hood. Next, unlabeled potassium borohydride (10 mg) dissolved in 0.5 mL of 0.01 N KOH was incubated with the reaction mixture for 6 h at room temperature. Separation of the labeled asialo-GM_1 from free borohydride and other salts was achieved by resuspending the reaction mixture in 0.5 mL of chloroform/methanol (2:1) and eluting over a Sephadex LH-20 column (1 × 10 cm) with chloroform/methanol (2:1). Analysis of the eluted fractions (0.5 mL) by thin-layer chromatography and liquid scintillation counting revealed that the initial fractions contained asialo-GM_1 of high purity (95–97%) and high specific radioactivity (70 Ci/mol). This single step column procedure was superior to other cleanup methods such as repeated Folch washes or repeated preparative thin-layer chromatography.

Separation of Donor and Acceptor Vesicles following Incubations. Separation of donor from acceptor vesicles was accomplished by either molecular sieve or ion-exchange chromatography. In the molecular sieve method, donors were sonicated, unilamellar vesicles (20-nm diameter) prepared by the method of Barenholz et al. (1977), and acceptors were large, unilamellar vesicles (70-nm diameter) prepared by the method of Wong & Thompson (1982) or Nordlund et al. (1981). At desired time intervals, donor and acceptor vesicles were separated by elution through a Sepharose CL-2B column (40 × 0.8 cm) at 45 °C while employing an upward hydrostatic pressure of 15–20 cm. The sieve column was preequilibrated by elution with 10 μmol of either DPPC or POPC vesicles prior to the transfer experiments. Before each use, the Sepharose C1-2B column was washed with buffer at 45

[1] Abbreviations: asialo-GM_1, gangliotetraosylceramide; DPPC, dipalmitoylphosphatidylcholine; POPC, palmitoyloleoylphosphatidylcholine; DPPA, dipalmitoylphosphatidic acid; POPA, palmitoyloleoylphosphatidic acid; DMPC, dimyristoylphosphatidylcholine; SUV, sonicated unilamellar vesicle (20-nm diameter); LUV, large unilamellar vesicle (70-nm diameter); $t_{1/2}$, first-order kinetic half-time; T_m, gel-to-liquid-crystalline phase-transition temperature; PIPES, piperazine-*N*,*N*'-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; HPLC, high-pressure liquid chromatography.

°C for 6–8 h. After each use, the column was stored at room temperature. This procedure ensured high vesicle recoveries (>90%) and minimized the Sepharose C1-2B gel degradation observed when the column was left at 45 °C for periods exceeding 5–7 days. Donor vesicles had equivalent amounts of [^{3}H]asialo-GM$_1$ and [^{14}C]cholesteryl oleate (1.13×10^{-2} μCi/μmol of donor vesicle lipid) in DPPC or POPC. Incubations were carried out at total lipid concentrations from 10 to 50 mM in 20 mM PIPES-Na buffer (pH 7.0) containing 0.5 mM EDTA and 0.02% sodium azide. Vesicle mixtures were kept in glass syringes with Teflon-sealed plungers (Glenco 1-mL HPLC syringe). Use of these syringes provided an environment without air space and minimized aggregation problems associated with buffer evaporation. Styrofoam collars were placed around the necks of the syringes allowing them to be almost completely submerged in a 45 °C circulating water bath. At desired time intervals, a 0.45-mL aliquot of the vesicle mixture containing 1.0 μmol of donor vesicles and either 5-, 10-, or 20-fold excess of acceptor vesicles was loaded onto the 45 °C Sepharose CL-2B column. The column eluant was collected in 1.0-mL fractions, and each fraction (0.5 mL) was analyzed by liquid scintillation counting. Aliquots of the remaining eluant fractions were utilized in lipid phosphate determinations (Rouser et al., 1970) to monitor vesicle recovery from the column.

In the ion-exchange chromatography method, negatively charged phospholipid was included in the donor vesicles, allowing them to be separated from neutral acceptor vesicles by elution over DEAE-Sephacel minicolumns at desired time intervals. Preequilibriation of the DEAE-Sephacel minicolumns (0.7 × 2.5 cm) by elution of 1.0 μmol of DPPC fused unilamellar vesicles (Schullery et al., 1980) ensured high acceptor vesicle recoveries (90%) during the experiments. Donor vesicles had equivalent amounts of [^{3}H]asialo-GM$_1$ and [^{14}C]cholesteryl oleate (2.25×10^{-2} μCi/μmol of donor vesicle lipid) in 10 mol % DPPA and 90 mol % DPPC. Acceptor vesicles were fused unilamellar DPPC vesicles (Wong & Thompson, 1982). Vesicles mixtures were incubated at total lipid concentrations from 10 to 40 mM in the same buffer and by using the same syringes as previously described in the molecular sieve chromatographic technique. At desired time intervals, 0.1-mL aliquots of the vesicle mixture containing 0.2 μmol of donor vesicles and either 5-, 10-, or 20-fold excess of acceptor vesicles were loaded onto the DEAE-Sephacel minicolumns. These columns were prepared and run according to the procedure of McLean & Phillips (1981) except that 3.0-mL Kimax plastic syringe barrels were substituted for Pasteur pipets. After loading a sample onto a column, the acceptor vesicles were eluted with 2.0 mL of buffer, and 1.5 mL of the eluant was analyzed by liquid scintillation counting. Aliquots of the remaining eluant were utilized in lipid phosphate determinations (Rouser et al., 1970) to monitor the acceptor vesicle recovery from the minicolumns.

Kinetic Analysis. The movement of asialo-GM$_1$ $(^3H)_t$ at time t can occur by (i) spontaneous transfer $(^3H_{ST})_t$ from donor to acceptor vesicles and (ii) nonspontaneous transfer $(^3H_{NST})_t$ processes such as vesicle–vesicle fusion etc. In this study, the rate of spontaneous asialo-GM$_1$ transfer is desired. At time t the quantity of glycolipid spontaneously transferred is equal to the total transfer $(^3H_{ST})_t$ minus transfer by other processes $(^3H_{NST})_t$.

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

In order to measure the amount of nonspontaneous transfer $(^3H_{NST})_t$ at time t, [^{14}C]cholesteryl oleate was included in the donor vesicles. It was assumed that (i) [^{14}C]cholesteryl oleate can only transfer by the nonspontaneous transfer route, and (ii) the amount of [^{14}C]cholesteryl oleate movement to the acceptor fraction is proportional to the ^{3}H glycolipid amount transferring by the nonspontaneous route. Therefore, the nonspontaneous transfer process results in the movement of both [^{14}C]cholesteryl oleate and [^{3}H]asialo-GM$_1$ in proportions identical with their ratio in the system (donor plus acceptor mixture) at time zero.

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

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

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

Equation 3 gives the amount of ^{3}H glycolipid movement occurring by spontaneous transfer from donor to acceptor vesicles in experimentally observable terms. Asialo-GM$_1$ fractional transfer is computed by normalizing the $(^3H_{ST})_t$ values to the total amount of ^{3}H glycolipid present in the donor vesicles at time zero.

Kinetic data were analyzed by using the following general rate expression derived in the Appendix (eq A8):

$$\frac{X(t) - X(\infty)}{X(0) - X(\infty)} = (1 - A)\exp(-k_M t) + A\exp[-(\bar{k}_{DM} + \bar{k}_D)t] \quad (4)$$

where $X(t)$ is the fractional transfer of labeled glycolipid from the donor vesicle at time t, $X(0)$ is the total amount of labeled glycolipid present in the donor vesicle at time zero, $X(\infty)$ is the equilibrium value for the fractional transfer of labeled glycolipid from the donor vesicle at infinite time, $1 - A$ is the fraction of total glycolipid molecules which behave as a fast transferring pool, A is the fraction of total glycolipid molecules which behave as a slow transferring pool; k_M is the rate constant for departure from the fast pool from the donor vesicle, $\bar{k}_D$ is proportional to the rate constant for departure from the slow transferring pool in the donor vesicle, and $\bar{k}_{DM}$ is proportional to the rate constant for the re-formation of the fast transferring pool from the slow transferring pool in the donor vesicle.

Results

Vesicle Separation by Molecular Sieve Chromatography. Figure 1 shows typical molecular sieve elution profiles obtained at various time intervals after mixing [^{3}H]asialo-GM$_1$ containing DPPC SUV donors with DPPC LUV acceptors. Aliquots of the donor–acceptor mixture were analyzed up to 23 days after initiation of the incubation at 45 °C. The gradual shift of [^{3}H]asialo-GM$_1$ from donor vesicles to the 3-fold excess of acceptor vesicles is evident. In contrast, the movement of [^{14}C]cholesteryl oleate, a nontransferable marker, takes place in a process that is at least 5-fold slower than that of the glycolipid.

Several processes other than spontaneous interbilayer transfer could cause the movement of asialo-GM$_1$ from the donor to the acceptor vesicle fraction seen in Figure 1. A gradual breakdown of the original glycolipid to more mobile fragments would result in ^{3}H movement. However, thin-layer chromatographic analysis of the ^{3}H and ^{14}C which coeluted with the acceptor vesicles after 15 days at 45 °C indicated that the compounds were intact asialo-GM$_1$ and cholesteryl oleate, respectively (data not shown). Therefore, the observed movement of ^{3}H was not due to degradation of the labeled asialo-GM$_1$.

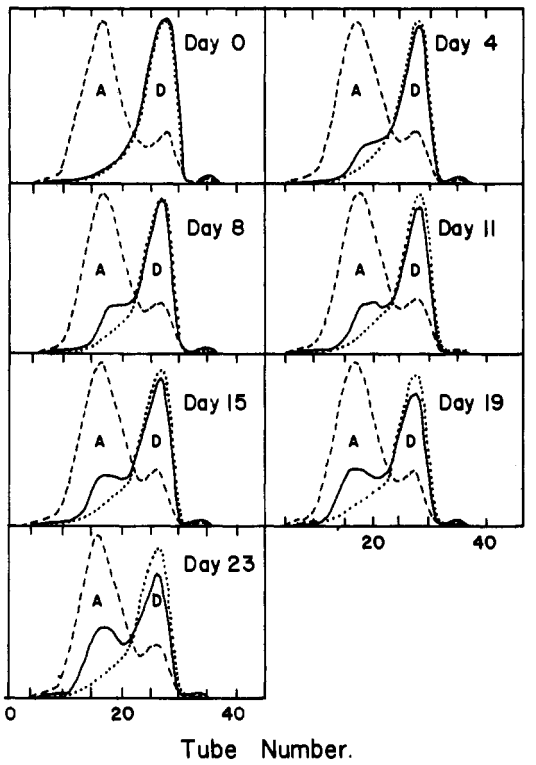

FIGURE 1: [^{3}H]Asialo-GM$_1$ intervesicular transfer at 45 °C as a function of time during use of the molecular sieve chromatographic technique. (---) Normalized lipid phosphate profile; (—) normalized [^{3}H]asialo-GM$_1$ dpm profile; (···) normalized [^{14}C]cholesteryl oleate dpm profile. The abscissas show every fifth fraction (1 mL/fraction). The acceptor-to-donor ratio was 3 where A represents the acceptor and D represents the donor elution peaks, respectively. The matrix phospholipid was DPPC (see Materials and Methods for details).

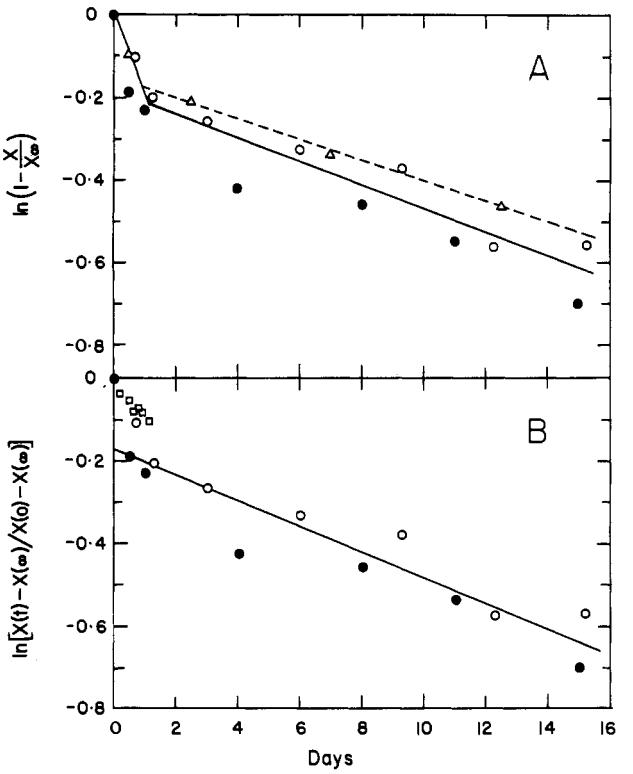

FIGURE 2: (A) First-order treatment of [^{3}H]asialo-GM$_1$ intervesicular transfer data collected by the molecular sieve chromatographic method. The form of the plot is described in detail by McLean & Phillips (1981) where X = fractional transfer of label and X_∞ = fractional transfer at infinite time. In (●), 7 mol % asialo-GM$_1$ was present in DPPC donors (donor:acceptor = 1:35). In (○), 7 mol % asialo-GM$_1$ was present in POPC donors (donor:acceptor = 1:10; see Materials and Methods for other experimental details). In (△), POPC donors were preincubated with 2.5-fold excess acceptors. After 36 h, the original acceptors were replaced with a 5-fold excess of fresh acceptors, and the incubation was resumed at 45 °C. (B) Plot of ln $[X(t) - X(\infty)/X(0) - X(\infty)]$ vs. t as described in eq A8. (●) and (○) are the same as in (A). In (□), the data were collected by ion-exchange chromatography from POPC donors containing 0.1 mol % asialo-GM$_1$ (donor:acceptor = 1:10).

Fusion of donor and acceptor vesicles could cause transfer of asialo-GM$_1$ between vesicles. However, three different experimental results argue against fusion. (i) The phosphate elution profiles of the donor and acceptor vesicles do not vary in position with respect to the void volume and each other at all selected time points (Figure 1). More importantly, the relative amount of phospholipid in the donor and acceptor fractions does not change over the experimental time course. (ii) [^{14}C]Cholesteryl oleate movement from donor to acceptor vesicles is only 5–10% after 23 days (Figure 1). In contrast, 25–30% of the total [^{3}H]asialo-GM$_1$ moves to acceptors during the same time interval (Figure 1). (iii) Control molecular sieve experiments indicated that the DPPC SUV donors can be maintained at 45 °C for at least 60 days without any fusion to LUV acceptor-size objects (R. E. Brown and T. E. Thompson, unpublished observation).

Analysis of transfer data was performed by integrating the [^{3}H]asialo-GM$_1$ and [^{14}C]cholesteryl oleate curve areas (Figure 1) corresponding to the acceptor vesicles. The acceptor vesicles were those fractions eluting prior to the crossover point of the normalized [^{3}H]asialo-GM$_1$ dpm and [^{14}C]cholesteryl oleate dpm profiles. In Figure 1, the crossover point occurs near fraction 23. Then, the amount of spontaneous asialo-GM$_1$ transfer was computed by using eq 3 as described under Materials and Methods. When the data were treated as though they were due to a reversible first-order kinetic process (McLean & Phillips, 1981), a biphasic curve resulted (Figure 2A). This result suggested the existence of two pools of asialo-GM$_1$ molecules departing from the donor vesicles at different rates. A mathematical model was developed (see Appendix) which would allow critical evaluation of this two pool model.

The general mathematical expression, which is applicable when the asialo-GM$_1$ molecules are present in the donor vesicles in two distinct pools, is provided by eq A8. Use of eq A8 results in the the curve in Figure 2B. Deconvolution of the curve in Figure 2B as described in the Appendix yields the values for the relative pool sizes and the rate constants (Table I). The faster transferring pool consists of about 17.5% of the asialo-GM$_1$ present in the donor vesicle outer monolayer. The half-time for the departure from this pool is about 8.5 h. The half-time for the departure from the slower transferring pool is about 24 days.

Transfer data were also calculated from single fractions at various positions across the acceptor vesicle profile (Figure 1). Fractions at the leading edge of the acceptor profile displayed the same biphasic kinetic behavior as later fractions (data not shown). Therefore, the biphasic kinetic behavior observed for the summed fractions (Figure 2A,B) was not due to differential transfer rates to acceptor vesicles of slightly different sizes.

Two control experiments were performed to make sure that no contaminant present in the [^{3}H]asialo-GM$_1$ could account for either of the two transferring pools. (i) The [^{3}H]asialo-GM$_1$ purity was evaluated by thin-layer chromatography using the solvent system previously described (Materials and Methods) and was found to be at least 95–97% pure (data not shown). No contaminant was present in large enough quan-

Table I: Values for the Relative Pool Sizes and the Rate Constants

experiment	donor/acceptor	A^a	$\bar{k}_D + \bar{k}_{DM}$ (day^{-1})	$t_{(D+DM)1/2}$ (day)	$1 - A$	k_M (day^{-1})	$t_{(M)1/2}$ (day)
molecular sieve							
7 mol % AGM_1 in DPPC	1/35	0.81	0.033	21	0.19	3.0	~0.2
7 mol % AGM_1 in POPC	1/10	0.84	0.028	25	0.16	1.5	~0.5
0.1 mol % AGM_1 in POPC	1/5	0.82	0.026	26	ND	ND	ND
0.1 mol % AGM_1 in DPPC	1/9	0.95	0.026	26	ND	ND	ND
ion exchange							
0.1 mol % AGM_1 in DPPC	1/5	0.84[b]	0.032[b]	21.7[b]	0.16	0.31	2.2
	1/10	0.84	0.032	21.7	0.15	0.38	1.8
	1/20	0.84	0.032	21.7	0.16	0.65	1.0
	1/10	0.84	0.032	21.7	0.16	0.41	1.7
	1/20	0.84	0.032	21.7	0.16	0.38	1.8

[a] $A \simeq N_D(0)/[N_M(0) + N_D(0)]$ as described in the Appendix. [b] Represents the values obtained by a combination of the molecular sieve data.

tities to account for either of the two observed glycolipid pools. (ii) An experiment was performed to see if the fast transferring donor pool would regenerate spontaneously. Donors containing the [^{3}H]asialo-GM_1 were incubated with a 2.5-fold excess of acceptors for 36 h at 45 °C and then eluted through a Sepharose CL-2B column to remove the acceptors. The recovered donors were mixed with fresh acceptors, and the transfer incubation was continued at 45 °C. The total time elapsed between the application of the original donor–acceptor mixture to the molecular sieve column and the restart of incubation with fresh acceptors was about 24 h. The data in Figure 2A (open triangles) show clearly that the two temporally distinct rates for glycolipid departure from the donors still remain when the kinetics of asialo-GM_1 transfer to the fresh acceptors are examined. The fast rate cannot be attributed to a relatively mobile contaminant in the system. Such a contaminant would have completely transferred to the acceptors during the first incubation, resulting in monophasic kinetics upon addition of the fresh acceptors. Thus, the faster moving glycolipid pool must be derived from the slower transferring pool of glycolipid.

Three additional conclusions can be drawn from the molecular sieve data in Table I: (i) Changing the phospholipid matrix of both the donor and acceptor vesicles from DPPC to POPC does not affect the asialo-GM_1 transfer rate. (ii) Changing the amount of asialo-GM_1 present in the donor vesicles from 0.1 to 7 mol % does not change the results dramatically. (iii) Since increasing the acceptor vesicle concentration severalfold does not significantly change the glycolipid transfer rate, asialo-GM_1 transfer is not mediated by vesicle–vesicle collisions.

Vesicle Separation by Ion-Exchange Chromatography. Since about 8 h are required to complete the vesicle separation by molecular sieve chromatography, an alternative method allowing rapid separation of donors and acceptors was employed to examine the kinetics of the faster transferring glycolipid pool. When ion-exchange chromatography was employed to remove DPPC SUV donor vesicles containing 10 mol % DPPA from DPPC LUV acceptors, slow and steady increases in the amounts of [^{3}H]asialo-GM_1 and [^{14}C]cholesteryl oleate eluting through the DEAE minicolumns were observed over the first 36 h of incubation (data not shown). However, at later time periods (>36 h), dramatic increases in the appearance of both labels in the acceptor fraction were noted. These results are in sharp contrast to the molecular sieve data which show no abrupt increase in the appearance of [^{14}C]-cholesteryl oleate in the acceptor fractions.

In order to clarify the apparent discrepancy in the data, the following control experiment was performed: SUV donor vesicles containing DPPA were incubated with LUV acceptor vesicles for approximately 90 h at 45 °C. Elution of part of the donor–acceptor mixture through a DEAE-Sephacel minicolumn indicated that over 30% of the [^{3}H]asialo-GM_1 and [^{14}C]cholesteryl oleate transferred to the acceptor fraction (data not shown). However, elution of the rest of the donor–acceptor mixture through a molecular sieve column indicated that only 12% of the [^{3}H]asialo-GM_1 and 4% of the [^{14}C]cholesteryl oleate coeluted with the LUV acceptor fractions. Furthermore, the shape of the elution profile and the recovery of vesicles indicated that donor–acceptor vesicle fusion was nearly nonexistent. This result suggested that the DPPA used to give the donor vesicles a negative charge was spontaneously transferring to acceptor vesicles. After incubations exceeding approximately 36 h, the movement of DPPA to the 10-fold excess of acceptor vesicles produced sufficient dilution of charge to allow donor vesicles to leak through the DEAE-Sephacel minicolumns.

Two other experimental results support this conclusion. (i) When POPC was substituted for DPPC as the matrix phospholipid, the rate of [^{14}C]cholesteryl oleate appearance in the acceptor fractions was accelerated. In these experiments, both donor and acceptor vesicles were 20 nm diameter, unilamellar vesicles. Molecular sieve column chromatography and quasi-elastic laser light-scattering analysis indicated that the extent of vesicle fusion was too small to account for the 20% movement of [^{14}C]cholesteryl oleate that occurred after only 12 h at 45 °C in the POPC system (data not shown). Furthermore, substitution of dicetyl phosphate or POPA for DPPA did not significantly change these results. (ii) Control experiments indicated that the half-time for the spontaneous transfer rate of [^{3}H]DPPA from DPPC 20-nm vesicles to DPPC 70-nm vesicles at 45 °C (pH 7.0) is approximately 37 h assuming that DPPA flip-flop does not occur (R. E. Brown and T. E. Thompson, unpublished observation). The fact that DPPA transfers relatively fast compared to most of the asialo-GM_1 is not surprising considering the recent work of De Cuyper et al. (1983, 1984). These investigators found that various other anionic phospholipid derivatives exhibit relatively fast transfer rates to neutral acceptor vesicles.

Due to the charge-movement complications, ion-exchange chromatography measurements of the asialo-GM_1 interbilayer transfer rate were limited to the first 36 h after mixing DPPC donor and acceptor vesicles. Correction of the data was performed as described in eq 3 (see Materials and Methods). In Figure 3, representative data are plotted as described in the Appendix (eq A9) based on a two-pool model. The large number of data points at early time (Figure 2B, open box) allows a more precise estimation of the half-time for the fast transferring glycolipid pool than the one obtained with the molecular sieve method. Values for several sets of data are compiled in Table I. They show that the average transfer half-time for the fast glycolipid pool is 42 h (1.75 days). The

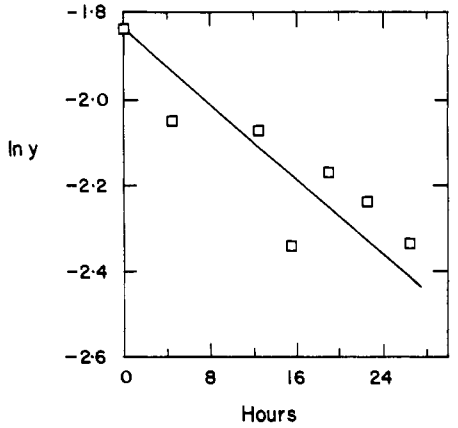

FIGURE 3: Plot of ln y vs. t as described by eq A9. Representative data were collected by ion-exchange chromatography from POPC donors containing 0.1 mol % asialo-GM$_1$ (donor:acceptor = 1:10).

results in Table I also show that variation of the acceptor vesicle concentration does not alter the transfer rate. This result suggests that spontaneous transfer is limited by the rate at which asialo-GM$_1$ molecules depart from the donor vesicles and not by the rate of vesicle–vesicle collisions (Roseman & Thompson, 1980; Doody et al., 1980; McLean & Phillips, 1981; Nichols & Pagano, 1981, 1982; Frank et al., 1983).

Discussion

Glycosphingolipids are known to be located primarily on the external surface of mammalian plasma membranes (Hakomori, 1981). They have been implicated in cell–cell identification and recognition processes (Horowitz, 1978), attachment of bacterial toxins (Fishman, 1982) and viruses (Holmgren et al., 1980) to cells, and other activities that occur at the cell surface. Spontaneous, rapid transfer of these molecules between the outer surfaces of different cells would be inconsistent with their roles as cell surface markers and receptors.

In this study, the spontaneous, nonprotein-mediated transfer rate of a complex, neutral glycosphingolipid between model membranes has been measured. Our findings indicate that interbilayer transfer for most of the asialo-GM$_1$ is an extremely slow process ($t_{1/2} \simeq 24$ days) compared to the known intervesicular transfer rates of phospholipids and cholesterol. For example, the net bilayer fluxes for nonderivatized phospholipids translate into transfer half-times of less than 48 h (Martin & MacDonald, 1976; Papahadjopoulos et al., 1976; Kremer et al., 1977; Duckwitz-Peterlein et al., 1977; McLean & Phillips, 1981; De Cuyper et al., 1983). Generally, even faster interbilayer transfer rates are found when phospholipids are labeled with either fluorescent or iodinated acyl chain derivatives (Roseman & Thompson, 1980; Nichols & Pagano, 1981, 1982; Shroit & Madsen, 1983). Other lipids such as cholesterol also are known to exhibit relatively fast interbilayer exchange rates ($t_{1/2} = 2$ h) (Backer & Dawidowicz, 1981; McLean & Phillips, 1981).

Separation of donor and acceptor vesicles by molecular sieve chromatography has allowed accurate and extended measurements of the spontaneous transfer rate of asialo-GM$_1$. Because of the stability of the donor and acceptor vesicles at 45 °C (Figure 1), kinetic measurements can be taken for at least 23 days. During this time interval, approximately 25% of the total [^{3}H]asialo-GM$_1$ in the donor vesicles spontaneously transfers to the acceptors. The majority of asialo-GM$_1$ transfers in a kinetic process whose half-time is approximately 24 days (Table I). The remainder transfers in a very rapid process.

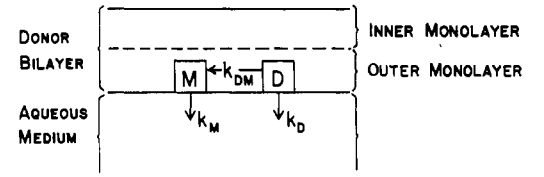

FIGURE 4: Molecular model for the arrangement of asialo-GM$_1$ in the phospholipid matrix. k_M is the rate constant for departure of asialo-GM$_1$ monomers into the aqueous medium. k_D is the rate constant for departure of asialo-GM$_1$ from domains into the aqueous medium. k_{DM} is the rate constant for the reformation of monomers from domains within the plane of the phospholipid matrix. Note: k_{DM} is not a lateral diffusion rate but rather the rate of departure from the edge of the glycolipid domain.

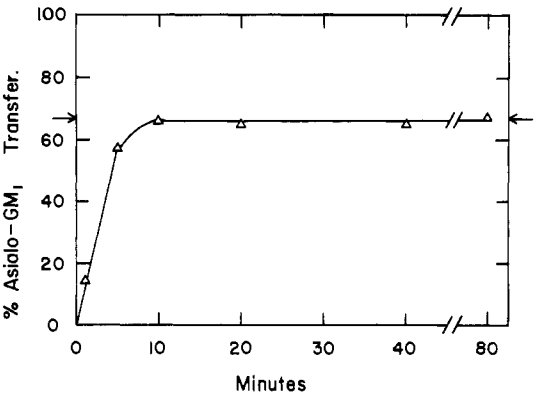

FIGURE 5: Accessibility of asialo-GM$_1$ in sonicated POPC vesicles to a soluble glycolipid transfer protein. The arrow on the ordinate indicates the amount of transfer expected if two-thirds of the asialo-GM$_1$ is initially present in the outer surface of the donor vesicle. Sonicated POPC vesicles (1.2 μmol) containing 5 mol % asialo-GM$_1$ (6×10^4 dpm of [^{3}H]asialo-GM$_1$), 10 mol % 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 as described by Wong et al. (1984)] at 37 °C in a total volume of 1.8 mL. At the indicated time, 0.3 mL of the mixture was separated on an DEAE ion-exchange minicolumn (see Materials and Methods). The data were normalized relative to the equilibrium transfer value (equals 90.9% for donor:acceptor ratio of 1:10) and to the known recovery of acceptors (88%). Control experiments indicated that only 15% of the glycolipid transfer protein activity was lost after 80 min at 37 °C in the presence of asialo-GM$_1$-containing donors.

The two different transfer rates, which are observed for asialo-GM$_1$, must reflect the molecular organization of this glycolipid in the plane of the phospholipid bilayer. The simplest identification of these two rates and their pools is as follows: Asialo-GM$_1$ molecules may coexist as both monomers and as glycolipid-enriched domains within the phospholipid matrix. The departure of the monomers from the donor vesicle outer monolayer to the aqueous medium is characterized by the rate constant k_M and is identified with the fast transferring pool. The asialo-GM$_1$ departure from the domainlike clusters to the aqueous medium is characterized by the rate constant k_D. In addition, re-formation of monomers from asialo-GM$_1$ domains within the plane of the donor bilayer is characterized by the rate constant k_{DM}. (Note, k_{DM} is not related to lateral diffusion but, rather, to the departure of monomers from the edges of glycolipid-enriched domains into the phospholipid matrix.) The processes described by k_D and k_{DM} are characteristic of the slow transferring pool of glycolipid in the donor vesicle outer monolayer. A schematic representation of this model is shown in Figure 4. Additional details are provided in the Appendix.

An alternative model to the one described in Figure 4 would associate the rate from the slow transferring glycolipid pool with the flip-flop rate of asialo-GM$_1$ from the inner to the outer

monolayer before departure from the donor surface. However, such an explanation is very unlikely for three reasons. (i) The experimental data in Figure 5 clearly show that about 67% of the asialo-GM_1 present in sonicated POPC vesicles is accessible to an externally added glycolipid transfer protein. This fractional distribution of asialo-GM_1 is also equal to the known fractional distribution of total lipid mass in the sonicated unilamellar PC vesicles (Mason & Huang, 1978). Thus, if the glycolipid flip-flop rate were the slow rate ($\bar{k}_D + \bar{k}_{DM}$) in Table I, the fast transferring pool would have to be 67% of the total glycolipid. In fact, the data show clearly that the fast pool consists of 11% of the total vesicle pool (16% of the glycolipid in the outer vesicle surface). (ii) Sharom & Grant (1977) have examined the flip-flop rate of galactosylceramide containing a spin-labeled fatty acyl chain. They found no appreciable flip-flop over a period of 5 h. (iii) The asialo-GM_1 head-group region contains four hydrophilic sugar residues, making transbilayer movement of the molecule very difficult. We therefore conclude that the slow rate cannot be the flip-flop rate but is the rate defined by the in-plane domain model.

A third model explaining the biphasic transfer kinetics associates the two pools with different molecular species of asialo-GM_1. However, two experimental observables argue against asialo-GM_1 heterogeneity being responsible. (i) Several different laboratories have reported that increasing the fatty acyl chain length of phospholipids or sphingomyelin decreases their spontaneous intervesicular transfer rates (Martin & MacDonald, 1976; Nichols & Pagano, 1982; Massey et al., 1982; DeCuyper et al., 1983; and Frank et al., 1983). The asialo-GM_1 fatty acyl chain composition reported under Materials and Methods shows that 91.3% of the asialo-GM_1 has $C_{18:0}$ and 6.4% has $C_{20:0}$ acyl chains. However, the relative sizes of the fast and slow transferring pools in Table I are 16% and 84%, respectively. (ii) In the control experiment in Figure 2A (open triangle), a faster transferring asialo-GM_1 species would be depleted from the donors during the first 36-h incubation with the acceptors. Regeneration of the faster transferring pool after addition of fresh acceptors would not be possible. Therefore, asialo-GM_1 heterogeneity cannot be the reason for the observed biphasic kinetics.

Three basic assumptions are made in order to calculate the 24-day half-time according to the in-plane domain model. (i) The [^{14}C]cholesteryl oleate present in the donor vesicles does not undergo spontaneous transfer via aqueous diffusion to acceptor vesicles. The very slight movement of [^{14}C]cholesteryl oleate to the acceptor vesicle fractions (Figure 1) is viewed as fusion or other complicating processes. Even if this assumption were wrong, the asialo-GM_1 transfer half-time would only be shortened by a few days. (ii) Approximately two-thirds of the asialo-GM_1 is available for transfer from the donor vesicles. (iii) Asialo-GM_1 transbilayer movement (flip-flop) does not occur. The validity of assumptions ii and iii are discussed above.

In this study, the asialo-GM_1 interbilayer transfer rate was found to be independent of the acceptor vesicle concentration (Table I). This result implies that the spontaneous transfer process is limited by the rate at which asialo-GM_1 molecules depart from the donor vesicles. Therefore, the mechanism of asialo-GM_1 spontaneous transfer occurs by diffusion through the aqueous medium from donor to acceptor vesicles rather than by formation of intervesicular collisional complexes. Other lipids also have been shown to transfer between bilayers by diffusion through the aqueous medium (Duckwitz-Peterlein & Moraal, 1978; Roseman & Thompson, 1980; Doody et al., 1980; McLean & Phillips, 1981; Backer & Dawidowicz, 1981; Nichols & Pagano, 1981, 1982; De Cuyper et al., 1983).

Although detailed analysis of the asialo-GM_1 "on"- and "off"-rate constants for the donor and acceptor vesicles were not performed, the experimental protocol was designed to minimize the differences in donor and acceptor off rates as well as those in their on rates [see Nichols & Pagano (1981) for detailed discussion]. For example, the matrix phospholipid comprising the donor and acceptor vesicles was always the same. Use of the same matrix phospholipid in both the donors and acceptors eliminates one complication which has been shown to produce significant differences in either the on- or off-rate constants of donor and acceptor vesicles (Nichols & Pagano, 1981). To what degree, if any, the different radii of curvature affect the on- and off-rate constants of donor and acceptor vesicles remains to be elucidated. However, it is clear that the small radius of curvature of the donor vesicles is not the reason that the overall rate of the asialo-GM_1 transfer is slow. Previous studies from this laboratory (Roseman & Thompson, 1980) have demonstrated that DMPC transfers rapidly out of donor vesicles possessing small radii of curvature.

The results of one recent study provide direct evidence for the existence of asialo-GM_1-enriched microscopic domains in liquid-crystalline bilayers (Tillack et al., 1982). Freeze–fracture electron microscopic studies were performed on multilamellar phosphatidylcholine vesicles containing asialo-GM_1. The lateral organization of this glycolipid was investigated by probing the bilayer surface with a monovalent ferritin conjugate of *Ricinus communis* agglutinin 60. At temperatures well above the gel-to-liquid-crystalline phase transition of the matrix phospholipid, the asialo-GM_1 appeared to be organized into clusters.

It is well documented that the spontaneous interbilayer transfer rate of gel-state lipids is less than their liquid-crystalline transfer rate. Duckwitz-Peterlein and co-workers (1977) showed that the spontaneous transfer rate of phospholipids from *Escherichia coli* extracts decreases dramatically below their liquid-crystalline-to-gel phase transition temperature. Furthermore, in recent studies from this laboratory (Frank et al., 1983), the spontaneous interbilayer transfer half-times of sphingomyelin between either sphingomyelin (T_m = 36 °C) or DMPC (T_m = 24 °C) donor and acceptor vesicles were approximately 300-fold longer from the gel phase (20 °C) than from the liquid-crystalline phase (50 °C). Surprisingly, the large decrease in sphingomyelin transfer rate observed when going from 50 to 20 °C persisted in a POPC donor–acceptor matrix system (T_m = −5 °C). When coupled with known X-ray diffraction and differential scanning calorimetric results (Untracht & Shipley, 1977; Barenholz et al., 1976), the temperature dependence of the sphingomyelin transfer rate in POPC suggests the presence of a sphingomylein-enriched, low temperature induced, gel-like phase that is in equilibrium with the POPC liquid-crystalline phase.

The spontaneous interbilayer transfer rate of glucosylceramide has been examined recently in this laboratory (Correa-Freire et al., 1982). Like asialo-GM_1, glucosylceramide transfers very slowly ($t_{1/2}$ > 30 days) between liquid-crystalline-phase donor and liquid-crystalline-phase acceptor vesicles at 45 °C, a temperature well below the known phase transition temperature of pure glucosylceramide (t_m = 85 °C; Barenholz et al., 1983).

The above evidence suggests that asialo-GM_1 and possibly all neutral sphingolipids exist at low concentrations as stable microscopic gel-like domains that are laterally phase separated from the majority of the liquid-crystalline phospholipid matrix. One physical property thought to be common to all neutral

sphingolipids is the ability to form hydrogen bonds with surrounding molecules (Pascher & Sundell, 1977; Barenholz & Thompson, 1980; Bunnow & Levin, 1980). Hydrogen bonding is possible due to the amide bond adjacent to the 2-carbon and the hydroxyl group adjacent to the 3-carbon of the sphingosine backbone. In glycosphingolipids, the hydrogen-bonding capability may be enhanced by the hydroxyl groups associated with the sugars in the head groups. Recent nuclear magnetic resonance experiments have supported the existence of a hydrogen-bonding pattern between neighboring cerebroside molecules (Neuringer et al., 1979; Skarjune & Oldfield, 1979). Therefore, an asialo-GM_1 hydrogen-bonding capability exists which may promote the stabilization of the microscopic, asialo-GM_1-enriched, gel-like domains that apparently occur in liquid-crystalline phospholipid bilayers.

Estimation of the activation energies associated with rate constants in the in-plane model (Figure 4) shows that the values obtained for the $\bar{k}_{DM}$ and $\bar{k}_D$ processes are reasonable compared with values reported for transfer of lipid monomers in other systems. For example, if one assumes that the activation energy for asialo-GM_1 monomer transfer is comparable with cholesterol or phospholipid transfer, then k_M is approximately 20 kcal/mol (McLean & Phillips, 1981; Massey et al., 1982). The activation energy for the $\bar{k}_{DM}$ process would be 21.4 kcal/mol if $\Delta E_D \gg \Delta E_{DM}$ or 21.8 kcal/mol if $\Delta E_{DM} = \Delta E_D$ assuming that the preexponential frequency factors for the rate constants are equal, $T = 300$ K, and $k_M/(\bar{k}_{DM} + \bar{k}_D) = 10$ (see Table I). Presumably, the slightly larger activation energy for the $\bar{k}_{DM}$ process reflects the enhanced attractive interaction provided by hydrogen bonding of the asialo-GM_1 molecules within domains. This 1.5–2.0 kcal/mol increase in activation energy is consistent with previous estimates for the hydrogen-bonding energy between certain phospholipids (Sugár & Monticelli, 1983).

In summary, the spontaneous interbilayer transfer rate of the complex, neutral glycosphingolipid, asialo-GM_1, is very slow ($t_{1/2} = 24$ days) compared with the known transfer rate of phospholipids and cholesterol. The mechanism of the asialo-GM_1 transfer process, like that of phospholipids and cholesterol, appears to be by diffusion through the aqueous medium rather than by formation of collisional complexes between vesicles. The kinetics of asialo-GM_1 transfer between model membrane systems suggest unique arrangements for this glycolipid within the plane of the phospholipid bilayer.

Acknowledgments

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Appendix

The following assumptions and statements help clarify the qualitative model shown in Figure 4 and are necessary for the quantitative description below. Assume that there are two states of asialo-GM_1 in the outer surface of donor bilayers: (i) monomeric state; (ii) domain state. The number of asialo-GM_1 molecules in the monomeric state at time t is $N_M(t)$ and in the domain state, $N_D(t)$.

Assumptions. (i) The dissociation constants, k_D and k_{DM}, are independent of the average domain size during the experiment. (ii) The interaction energy of one asialo-GM_1 molecule with another asialo-GM_1 molecule (E_{aa}) is stronger than the interaction energy between asialo-GM_1 and phosphatidylcholine molecules (E_{ap}). Consequently, $k_D \ll k_M$. (iii) The total outer surface of the acceptors is much larger than that of the donors. Therefore, the back-transfer processes are neglected in this model. (iv) Flip-flop of asialo-GM_1 is assumed to be nonexistent. (v) The shapes of glycolipid domains are elongated and irregular rather than perfectly circular because of entropic factors. Therefore, the size of the domain edge, n_E, is proportional to the domain size, n_D. Consequently, the total number of asialo-GM_1 molecules in the edges of domains is

$$N_E(t) = \sum_i n_E^i(t) = a\sum_i n_D^i(t) = aN_D(t)$$

where the summation includes every domain and a is the proportionality constant ($a < 1$). (vi) According to the experimental data in Figure 2, the fast pool becomes nearly vacant after 2 days. Therefore, $k_{DM} < k_M$. At time zero, the equilibrium distribution of monomers within the plane of the bilayer is such that the monomer concentration is highest close to the domains. Thus, at the beginning of the transfer process, the monomer concentration will decrease most rapidly near the domain in first-order fashion. Because of this rapid decrease in monomer concentration near the domains, the rate at which monomers redistribute to the domains within the plane of the bilayer will decrease drastically and will become negligible relative to the re-formation of monomers from domains after a short time.

Reaction Equations. On the basis of the above assumptions, one can obtain the following reaction equations: (i) for the number of monomers

$$dN_M(t)/dt = -k_M N_M(t) + \bar{k}_{DM} N_D(t) \quad \text{(A1)}$$

where $\bar{k}_{DM} = ak_{DM}$; (ii) for the number of asialo-GM_1 molecules in domain state

$$dN_D(t)/dt = -(\bar{k}_{DM} + \bar{k}_D)N_D \quad \text{(A2)}$$

where $\bar{k}_D = ak_D$. The solution of this system involves the first-order differential equations:

$$N_D(t) = N_D(0)\exp[-(\bar{k}_{DM} + \bar{k}_D)t] \quad \text{(A3)}$$

$$N_M(t) = \left[N_M(0) - \frac{\bar{k}_{DM}N_D(0)}{k_M - (\bar{k}_{DM} + \bar{k}_D)}\right]\exp(-k_M t) + \left[\frac{\bar{k}_{DM}N_D(0)}{k_M - (\bar{k}_{DM} + \bar{k}_D)}\right]\exp[-(\bar{k}_{DM} + \bar{k}_D)t] \quad \text{(A4)}$$

Experimentally one can determine the relative number of the asialo-GM_1 on the outer surface of the donors, i.e.

$$X(t) = [N_M(t) + N_D(t)]/[N_M(0) + N_D(0)] = [[N_M(0) - [\bar{k}_{DM}N_D(0)/[k_M - (\bar{k}_{DM} + \bar{k}_D)]]]\exp(-k_M t)]/[N_M(0) + N_D(0)] + [[N_D(0) + [\bar{k}_{DM}N_D(0)/[k_M - (\bar{k}_{DM} + \bar{k}_D)]]]\exp[-(\bar{k}_{DM} + \bar{k}_D)t]]/[N_M(0) + N_D(0)] = (1 - A)\exp(-k_M t) + A\exp[-(\bar{k}_{DM} + \bar{k}_D)t] \quad \text{(A5)}$$

where

$$A = \frac{N_D(0)}{N_M(0) + N_D(0)}\left[1 + \frac{\bar{k}_{DM}}{k_M - (\bar{k}_{DM} + \bar{k}_D)}\right] \simeq \frac{N_D(0)}{N_M(0) + N_D(0)} \quad \text{(A6)}$$

since $\bar{k}_{DM} \simeq \bar{k}_{DM} + \bar{k}_D \ll k_M$.

Determination of $[N_M(t) + N_D(t)]/[N_M(0) + N_D(0)]$ *from the Experimental Data.* (D/A) = donor lipid/acceptor lipid molar ratio; (D_o/D_i) = donor outer monolayer/donor inner

monolayer lipid molar ratio (for d = 20-nm vesicles, $D_o/D_i = 2/1$); $N_o(0)/N_i(0)$ = ratio of asialo-GM_1 molecules at the outer and inner donor surface at $t = 0$ ($D_o/D_i = N_o(0)/N_i(0) = 2/1$); (A_o/A_i) = acceptor outer/acceptor inner monolayer lipid molar ratio ($A_o/A_i = 1.087$ for large d = 100-nm unilamellar vesicles). From the experimental data, one obtains directly

$$\frac{N_M(0) + N_D(0) - [N_M(t) + N_D(t)]}{N_o(0) + N_i(0)} = \frac{N_o(0) - [N_M(t) + N_D(t)]}{N_o(0)(1 + 1/2)} = (2/3)\left[1 - \frac{N_M(t) + N_D(t)}{N_M(0) + N_D(0)}\right] \quad \text{(A7)}$$

Thus, one can simply convert the experimentally determined data into the theoretically interesting data:

$$X(t) = \frac{N_M(t) + N_D(t)}{N_M(0) + N_D(0)} = 1 - (3/2)\frac{N_M(0) + N_D(0) - [N_M(t) + N_D(t)]}{N_o(0) + N_i(0)}$$

Determination of the Value of $[N_M(\infty) + N_D(\infty)]/[N_M(0) + N_D(0)] = X(\infty)$. Assuming identical distributions of asialo-GM_1 on the outer surfaces of donor and acceptor vesicles at equilibrium

$$X(\infty) = \frac{N_M(\infty) + N_D(\infty)}{N_M(0) + N_D(0)} = \frac{D_o}{D_o + A_o} = \frac{1}{1 + (A/1.92)[(1/D)(3/2)]}$$

Therefore

$$X(\infty) = \frac{1}{1 + 0.78(A/D)}$$

where $D = D_i + D_o = (1/2)D_o + D_o = (3/2)D_o$ and $A = A_i + A_o = (1.087)^{-1}A_o + A_o = 1.92A_o$.

Fitting Procedure. It is obvious that the basic assumption of the model (i.e., the monomeric and domain pools in the donors become vacant after infinite time) is only approximately valid. Therefore, one has to correct the final result of the model (eq A5) by taking into consideration the real value of $X(\infty)$. Thus, the experimental data are normalized and

$$\frac{X(t) - X(\infty)}{X(0) - X(\infty)} = (1 - A)\exp(-k_M t) + A\exp[-(\bar{k}_{DM} + \bar{k}_D)t] \quad \text{(A8)}$$

Now one can fit the theoretical result (right side of eq A8) to the experimentally determined data (left side of eq A8).

Since the monomer pool becomes vacant after 2 days (Figure 2), the first term of the right hand side of eq A8 becomes negligible. When this condition exists ($t > 2$ days), a straight line can be fitted to the half-logarithmic plot of the experimental data (see Figure 2B). The parameters of the fitted straight line determine the rate constants of the slow process ($\bar{k}_D + \bar{k}_{DM}$) and the relative pool size (A) at time zero (see Table I). Now when these parameters are used, the fast process ($t < 2$ days) can be separated from the slow process by subtracting the slow decay from the experimental data (see eq A8):

$$y = \frac{X(t) - X(\infty)}{X(0) - X(\infty)} - A\exp[-(\bar{k}_{DM} + \bar{k}_D)t] = (1 - A)\exp(-k_M t) \quad \text{(A9)}$$

After a least-squares fitting procedure to the half-logarithmic plot of y, k_M is obtained (Table I). However, in the case of the molecular sieve experiments, the k_M values are rough estimates because only two data points are available at $t <$ 2 days. Fortunately, the ion-exchange experiments provide enough reliable data points during this early time interval. By use of the average values for $\bar{k}_D + \bar{k}_{DM}$ and A shown in Table I, the rate constant for the fast process can be obtained after application of the separation procedure (see Figure 3 and Table I).

Registry No. AGM_1, 71012-19-6; DPPC, 2644-64-6; POPC, 6753-55-5; DPPA, 19698-29-4; cholesteryl oleate, 303-43-5.

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Effects of Hydrostatic Pressure on the Molecular Structure and Endothermic Phase Transitions of Phosphatidylcholine Bilayers: A Raman Scattering Study†

P. T. T. Wong and H. H. Mantsch*
Division of Chemistry, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6
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ABSTRACT: The temperature dependences of the Raman spectra of aqueous dispersions of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) were monitored at different but constant pressures between 1 and 1210 bar. The changes observed in these Raman spectra are discussed in terms of the effects of high pressure on the phase state and molecular structure of lipid bilayers. It is demonstrated that the temperature of the endothermic gel to liquid-crystal phase transition, as well as the temperature of the pretransition, increases linearly with increasing hydrostatic pressure. The dT_m/dP values obtained from a wide range of pressures are 20.8 °C·kbar^{-1} for DPPC and 20.1 °C·kbar^{-1} for DMPC. The dT_p/dP value for DPPC is 16.2 °C·kbar^{-1}. It is also shown that the volume change that occurs at the gel to liquid-crystal transition is not constant; i.e., $d\Delta V_m/dP$ decreases by 6.2% (DPPC) or 6.3% (DMPC) per kilobar pressure. The volume change at the pretransition is also pressure dependent; the $d\Delta V_p/dP$ value of DPPC decreases by 4.7% per kilobar pressure.

Interest in the structure of biological membranes under elevated pressure has grown rapidly during the last decade (Macdonald, 1984; Wong, 1984; Yager & Peticolas, 1982; Heremans, 1982; Wann & Macdonald, 1980; Woodhouse, 1976; Trudell et al., 1974). While the experimental pressure range of interest, which also coincides with the pressure range experienced by marine organisms, is generally within 1–1000 bar (1 bar = 0.9869 atm = 101.3 kPa), studies have been performed on lipid membranes at pressures up to 32 000 bar (Wong & Mantsch, 1984).

The effect of pressure on lipid membranes is of particular interest to the study of anesthetics since the action of anesthetics can be antagonized by hydrostatic pressure (Johnson & Flagler, 1950; Lever et al., 1971; Smith et al., 1984). As anesthetics depress the transition temperature and "fluidize" the lipid bilayer, the antagonizing effect of pressure is generally considered to result from an elevation of the transition temperature and from changes induced in the dynamic structure of the lipid matrix (Trudell et al., 1975; Macdonald, 1978; Kamaya et al., 1979; Mountcastle et al., 1978).

The Raman spectroscopic technique is particularly sensitive to trans–gauche isomerizations in polymethylene chains and

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