Cogan, U., Kopelman, M., Mokady, S., & Shinitzky, M. (1976) *Eur. J. Biochem. 65*, 71-78.
Cooper, R. B., Noy, N., & Zakim, D. (1989) *J. Lipid Res. 30*, 1719-1726.
Dittmer, J. C., & Wells, M. A. (1969) *Methods Enzymol. 14*, 482-530.
Fex, G., & Johannesson, G. (1982) *Biochim. Biophys. Acta 714*, 536-542.
Fex, G., & Johannesson, G. (1988) *Biochim. Biophys. Acta 944*, 249-255.
Flewelling, R. F., & Hubbell, W. L. (1986) *Biophys. J. 49*, 531-540.
Goodman, D. S. (1984) in *The Retinoids* (Sporn, M. B., Roberts, A. B., & Goodman, D. S., Eds.) Vol. 2, pp 42-82, Academic Press, New York.
Goodman, D. S., & Raz, A. (1972) *J. Lipid Res. 13*, 338-347.
Horowitz, J., & Heller, J. (1974) *J. Biol. Chem. 249*, 4712-4719.
Kates, M. (1986) *Techniques in Lipidology*, 2nd ed., p 107, North-Holland Publishing Co., Amsterdam and London.
Klotz, I. M. (1973) *Ann. N.Y. Acad. Sci. 226*, 18.
Lai, Y. L., Wiggert, B., Liu, Y. P., & Chader, G. J. (1982) *Nature 298*, 848-849.
Noy, N., & Xu, Z.-J. (1990a) *Biochemistry* (second of three papers in this issue).
Noy, N., & Xu, Z.-J. (1990b) *Biochemistry* (first of three papers in this issue).
Ross, P. D., & Subarmanian, S. (1981) *Biochemistry 20*, 3096-3102.
Saari, J. C., Futterman, S., & Bredberg, L. (1978) *J. Biol. Chem. 253*, 6432-6436.
Sani, B. P., Titus, B. C., & Banerjee, C. K. (1978) *Biochem. J. 171*, 711-717.
Siegenthaler, G., & Saurat, J.-H. (1987) *Biochem. Biophys. Res. Commun. 143*, 418-423.
Song, C. S., Rubin, W., Rifkind, A. B., & Kappas, A. (1969) *J. Cell Biol. 41*, 124-132.
Tanford, S. (1980) *The Hydrophobic Effect, Formation of Micelles and Biological Membranes*, 2nd ed., Wiley, New York.

Spontaneous Interbilayer Transfer of Hexosylceramides between Phospholipid Bilayers†

Jeffrey D. Jones,‡ P. F. Almeida, and T. E. Thompson*
Department of Biochemistry, University of Virginia, Charlottesville, Virginia 22908
Received November 3, 1989; Revised Manuscript Received December 29, 1989

ABSTRACT: The kinetics of spontaneous transfer of various glucosyl- and galactosylceramides between 1-palmitoyl-2-oleoylphosphatidylcholine vesicles have been examined at 45 °C. Bovine brain galactosylceramides, kerasin and phrenosin, were found to transfer with biexponential kinetics. The kerasin fast pool is ~17% with a half-time of 29 h and the slow pool ~83% with a half-time of 2700 h. In contrast, semisynthetic *N*-palmitoylgalactosylceramide at the same temperature transfers with single-exponential kinetics with a half-time of 32 h. The half-time for *N*-lignoceroylgalactosylceramide under the same conditions proved to be greater than 3500 h. No concentration dependence for these half-times was found in the concentration range studied (0-10 mol %). Similar results were obtained for semisynthetic glucosylceramides. The biexponential kinetics observed for the two bovine brain ceramides, both of which are mixtures of short and long acyl chain molecules, are most probably a reflection of the strong dependence of transfer rate on acyl chain length. The very slow transfer rates of the long acyl chain hexosylceramides ensure that these molecules are lost very slowly, if at all, by spontaneous transfer from the external surface of plasma membranes; a result that is consistent with the putative biological role of glycosphingolipids.

Glycosphingolipids, a large class of lipid membrane components found almost exclusively in the external surface of the plasma membrane of eukaryotic cells (Hakomori, 1981; Barbosa & Pinto da Silva, 1983), play important rolls in cell-cell interactions, differentiation, and oncogenesis (Hakomori, 1983; Feizi, 1985). The localization of these molecules on the surface of the cell, while presumably critical for their cellular functions, would seem to make them susceptible to loss from the surface by spontaneous intermembrane transfer, a process well studied for phospholipids. Studies of the spontaneous interbilayer transfer of three glycosphingolipids between liquid-crystalline phosphatidylcholine vesicles have been reported. For Gaucher glucosylceramide the half-time of transfer at 37 and 45 °C was found to be greater than 30 days (Correa-Freire et al., 1982). In a study by Brown and co-workers (1985b) on asialo-GM_1 transfer, two kinetically distinguishable glycosphingolipid pools were observed. However, the bulk of the asialo-GM_1 transferred with a half-time of ~24 days at 45 °C. These results, which are generally consistent with the stable residence of these lipids in the external surface of plasma membranes, have been interpreted in terms of the presumptive phase structure of the glycosphingolipid/phosholipid bilayers (Thompson & Tillack, 1986; Curatolo, 1987a). In contrast to these very long half-times for transfer, the ganglioside GM_1 was observed to transfer with half-times of ~4 days (Masserini & Freire, 1986; Brown & Thompson, 1987). This observation is in agreement with other

† Supported by NIH Grant GM-23573.
‡ Present address: University of Texas Southwestern Medical Center, Department of Pharmacology, 5323 Harry Hines Blvd., Dallas, TX 75235.

0006-2960/90/0429-3892$02.50/0

evidence that suggested that this ganglioside is molecularly dispersed in phospholipid bilayers (Thompson et al., 1985).

In this paper we report the study of spontaneous interbilayer transfer betweeen phospholipid vesicles of a group of glucosyl- and galactosylceramides that differ in acyl chain length and degree of hydroxylation. A preliminary report of this work has appeared elsewhere (Jones & Thompson, 1988).

Experimental Procedures

Materials. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC)[1] and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG)[1] were purchased from Avanti Polar Lipids (Birmingham, AL). Gaucher glucosylceramide was obtained as a gift from Y. Barenholz, the Hadassah Medical School of the Hebrew University, Jerusalem. Bovine brain kerasin and phrenosin, semisynthetic galactosyl-*N*-palmitoyldihydrosphingosine (*N*-pal GalCer),[1] semisynthetic galactosyl-*N*-lignoceroyldihydrosphingosine (*N*-lig GalCer),[1] and [^{14}C]stearic acid were purchased from Sigma (St. Louis, MO). All lipids were checked for purity by TLC[1] and stored under nitrogen at 0 °C. Analysis by mass spectroscopy of the sphingoid base composition showed the bovine brain kerasin and phrenosin samples as well as the Gaucher glucosylceramide to be >95% sphingosine.

Lipid Preparation. [^{3}H]Galactosylceramides were prepared by the galactose oxidase–[^{3}H]borohydride reduction procedure described in detail by Brown et al. (1985b). The final product was purified in each case by preparative TLC on silica G plates with a solvent system of $CHCl_3$/MeOH/H_2O (85/15/1.5). [^{3}H]*N*-Palmitoyl- and [^{3}H]*N*-myristoylglucosylceramides were prepared by deacylation of Gaucher glucosylceramide and subsequent reacylation using the appropriate tritiated fatty acid. Glucosylsphingosine (lysoglucosylceramide) was prepared by hydrazinolysis of glucosylceramide as described by Suzuki et al. (1984). The purified product was obtained by elution from a silicic acid column using a polarity gradient. The product eluted at a solvent mixture of $CHCl_3$/MeOH/H_2O (50/50/5). The reacylation of lysoglucosylceramide, carried out following the procedure of Ong and Brady (1972), utilized the *N*-hydroxysuccinimide ester of the appropriate fatty acid prepared as described by Lapidot et al. (1967). The product was purified by preparative TLC as in the case of the galactosylceramides. [^{14}C]*N*-Stearoylglucosylceramide was prepared by acylation of glucosylsphingosine with [^{14}C]stearic acid.

Vesicle Preparation. Small unilamellar vesicles (SUV)[1] were prepared in Pipes[1] buffer, 10 mM KCl, 25 mM EDTA, 0.5 mM NaN_3, and 0.02% by use of probe sonication as described by Barenholz et al. (1977). The vesicles were incubated overnight at room temperature prior to experiments. Large (100 nm diameter) unilamellar vesicles (LUV)[1] were prepared by filter extrusion using a high-pressure extrusion apparatus obtained from Lipex Biomembranes Inc. (Vancouver, BC). This method has been described in detail by Hope et al. (1985). Unilamellar vesicles (70 nm) were prepared by ethanol injection as described by Nordlund et al. (1981).

Hexosylceramide Transfer Assay. Hexosylceramide transfer was monitored by separation of donor and acceptor vesicles using either molecular sieve or ion-exchange chromatography. In the sieve method, POPC donor SUV contained a given mole percent hexosylceramide, [^{3}H]hexosylceramide, and [^{14}C]cholesteryl oleate, which served as a nonexchangeable marker. POPC LUV were used as acceptors. Initially, donor and acceptor vesicles were mixed to yield lipid concentrations of 0.05 and 1.0 mM, respectively, at 45 °C. These low lipid concentrations ensured that the concentration-dependent transfer process described by Jones and Thompson (1989) was negligible in comparison to the first-order, off-rate limited process. Also, the 20-fold excess of acceptors ensured that back-transfer of hexosylceramide from acceptors to donors was negligible in the initial stages of transfer. At appropriate time intervals, a 0.5-mL aliquot of the sample mixture was placed on a Sephacryl S-1000 column. The vesicles were eluted by employing an upward hydrostatic pressure of 20–30-cm H_2O at room temperature. Fractions of 1.0 mL were collected with 0.8 mL used for scintillation counting and the remaining 0.2 mL used for phosphate determination. Transfer of [^{3}H]hexosylceramide to LUV was determined by integration of the [^{3}H]hexosylceramide and [^{14}C]cholesteryl oleate curves as described by Brown et al. (1985b). In early experiments, 70-nm POPC vesicles were used as acceptors and separation was achieved with a Sephacryl S-500 molecular sieve column.

Determination of transfer by the sieve method required at least a 6–8-h interval between data points due to the time necessary to elute and to subsequently wash the column with buffer. Thus, for time points less than 1 day, a charged vesicle assay was employed. This method permitted rapid separation of donor and acceptor vesicles. The assay was carried out essentially as described by Jones and Thompson (1989). POPC donor vesicles were prepared as described above except that 15 mol % of the negatively charged POPG was included. POPC SUV was used as acceptors. The vesicles were initially mixed to yield the concentrations given above. At appropriate time intervals, a 50-μL aliquot was placed on a minicolumn containing 0.8 mL of DEAE Sephacel that had been preequilibrated with 0.3 μmol of acceptor vesicles. The neutral acceptor vesicles were eluted with 1.2 mL of Pipes buffer.

Spontaneous transfer at time t, $[^{3}H]_{st}$, was calculated by

$$[^{3}H]_{st} = [^{3}H]_t - [^{3}H]_0[^{14}C]_t/[^{14}C]_0 \quad (1)$$

where $[^{3}H]_0/[^{14}C]_0$ is the initial ratio of the two labels in the donor vesicles. This equation corrects the observed net [^{3}H]hexosylceramide transfer, ($[^{3}H]_t$), for artifacts resulting from donor–acceptor fusion or incomplete donor–acceptor separation. The correct percent transfer is obtained by normalizing the calculated spontaneous transfer for acceptor recovery (89%). For *N*-stearoylglucosylceramide transfer, [^{14}C]hexosylceramide was used in conjunction with [^{3}H]-cholesteryl oleate.

Electron Microscopy. Negative stain electon micrographs were obtained on a Hitatchi HU12A electron microscope. Vesicles were stained with 2% uranyl formate.

Photon Correlation Spectroscopy. Quasi-elastic light scattering measurements were carried out on a Nicomp Model HN5-90 instrument equipped with a Model 170 autocorrelator.

Kinetic Analysis. Transfer data were fit to a series of exponential decay functions by the following equation

$$1 - \frac{X(t)}{X(\infty)} = \sum A_j \exp(-k_j t); \quad j = 1, 2, 3 \quad (2)$$

where $X(t)$ is the fraction of hexosylceramide transferred at time t, $X(\infty)$ is the total fraction of hexosylceramide available for transfer. A_j and k_j are the amplitudes and their associated

[1] Abbreviations: POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol; *N*-pal GalCer, galactosyl-*N*-palmitoyldihydrosphingosine; *N*-lig GalCer, galactosyl-*N*-lignoceroyldihydrosphingosine; *N*-pal GlcCer, glucosyl-*N*-palmitoylsphingosine; *N*-myr GlcCer, glucosyl-*N*-myristoylsphingosine; SUV, small unilamellar vesicle; TLC, thin-layer chromatography; EDTA, ethylenediaminetetraacetic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); DEAE, diethylaminoethyl.

Table I: Fatty Acid Composition of Bovine Brain Galactosylceramides

kerasin		phrenosin	
fatty acid	wt %	fatty acid	wt %
16:0	6.98	16:0	2.44
18:1	13.86	18:0	1.79
18:0	8.27	18:0-OH	19.0
20:1	1.90	unknown	1.41
21:1	1.83	22:0-OH	8.85
22:1	2.44	23:0-OH	9.02
22:0	3.06	24:1-OH	3.53
23:1	2.71	24:0-OH	33.22
23:0	2.92	25:1-OH	1.64
24:1	21.08	25:0-OH	9.72
24:0	17.40	26:1-OH	5.13
25:1	5.55	26:0-OH	4.26
25:0	3.76		
26:1	6.97		
26:0	1.27		

rate constants, respectively. The calculations were based on the following assumptions: (i) the rate of movement of the cholesteryl oleate nonexchangeable marker is negligible in comparison to hexosylceramide movement; (ii) the hexosylceramide is initially symmetrically disposed on the surface of the donor vesicles; (iii) the rate of transbilayer migration of the hexosylceramide is faster than the monomer desorption rate. Under assumptions ii and iii, $X(\infty)$ is set to 1.0 and the desorption rate (k_1) is determined from the apparent k_j by $(k_1)_j = (0.67)(k_j)$, where 0.67 is the fraction of hexosylceramide initially available for transfer. This fitting procedure is described by Wimley and Thompson (1990). The basis for these assumptions are discussed under Results.

Results

Bovine Brain Galactosylceramide Transfer. The fatty acid compositions of the two bovine brain galactosylceramides, kerasin and phrenosin, are given in Table I. Of significance is the relatively higher proportion of long acyl chains as well as the higher degree of unsaturation in kerasin when compared to phrenosin. As is well-known, phrenosin contains >95% hydroxylated acyl chains. Figure 1 gives ln [transfer] vs time for 5 mol % phrenosin and kerasin from POPC at 45 °C (curves A and B, respectively). In both cases, the data are best described by a biexponential decay function with approximately 20% of the GalCer moving with a relatively fast rate and the remaining fraction with a much slower rate. Amplitudes and rate constants are given in Table II for kerasin and phrenosin transfer as a function of mole percent GalCer. Comparison of the transfer rates of these two lipids shows that phrenosin exhibits a slightly larger amplitude for the fast moving fraction as well as slightly larger rate constants for both fractions. No significant differences were observed when the total concentration of GalCer in POPC was varied.

Several processes other than spontaneous GalCer transfer between vesicles could be responsible for the observed transfer kinetics. The presence of lipid breakdown products could give rise to anomalous kinetic behavior. However, extraction of

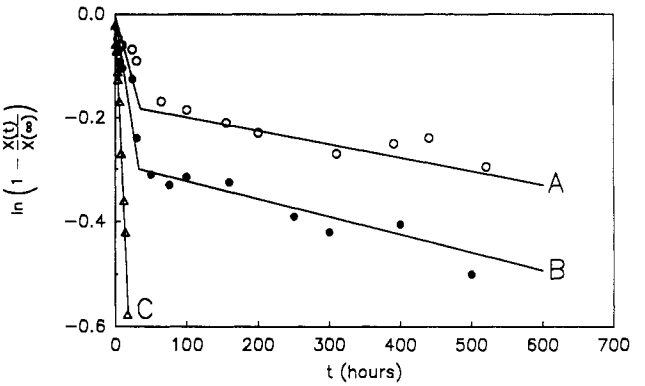

FIGURE 1: Transfer of 5 mol % [^{3}H]phrenosin (A), [^{3}H]kerasin (B), and [^{3}H]*N*-pal GalCer (C) from POPC small unilamellar vesicles at 45 °C. Data points are averages of triplicate experiments.

the lipids following each experiment and subsequent TLC analysis yielded no evidence of breakdown products. Also, GalCer transfer from structures other than SUV in the dispersions, such as micelles, could occur if the GalCer is not uniformly dispersed in POPC. However, the samples consisted entirely of small vesicles as shown by negative stain electron microscopy. Photon correlation spectroscopy yielded an average diameter of 21 nm for the vesicles. In addition, the vesicles were analyzed by molecular sieve chromatography employing Sephacryl S-500. The [^{3}H]GalCer always quantitatively coeluted with the phospholipid phosphate. Thus, it can be concluded that the GalCer was always present as components of POPC SUV. It is also possible that the GalCer did not distribute symmetrically between the two monolayers of the donor vesicle. Hence, the slow component of the transfer could be due to slow transbilayer movement of GalCer from the inner monolayer to the outer monolayer. This possibility was examined by measuring the total GalCer fraction available for rapid transfer by a specific glycolipid transfer protein (Brown et al., 1985a). It was found that two-thirds of the GalCer was transferred by the protein (data not shown). This is the fraction expected if GalCer is symmetrically disposed on the surface of SUV with a diameter of 21 nm. Transfer arising from fusion is corrected for as described in the Experimental Procedures. This value was typically less than 5%. If the movement of [^{14}C]cholesteryl oleate, the nontransferable marker, was significantly greater than this value, the results were discarded. Thus, the observed transfer kinetics must be due to spontaneous GalCer transfer between the vesicles.

Semisynthetic Galactosylceramide Transfer. Interpretation of bovine brain GalCer transfer data is complicated by the acyl chain heterogeneity found in these molecules. Thus, in order to examine specifically the effect of varying chain length of GalCer transfer, the movement of GalCer containing a single defined N-linked acyl chain was monitored. Figure 1 also shows ln [transfer] vs time for 5% *N*-pal GalCer in POPC (curve C). The data are clearly described by monoexponential kinetics. Table III gives k_1 values for *N*-pal GalCer transfer from POPC at 45 °C as a function of galactosylceramide

Table II: Parameters for Bovine Brain GalCer Transfer from POPC Vesicles at 45 °C[a]

mol %	GalCer	A_1	k_1 (h^{-1})	$t_{1/2}$ (h)	A_2	k_2 (h^{-1})	$t_{1/2}$ (h)
1	kerasin	0.19 ± 0.03	0.024	29 ± 3.2	0.81	0.00028	2500 ± 290
5	kerasin	0.16 ± 0.02	0.023	30 ± 2.9	0.84	0.00026	2700 ± 210
10	kerasin	0.17 ± 0.02	0.024	29 ± 3.1	0.83	0.00025	2800 ± 255
1	phrenosin	0.24 ± 0.04	0.030	23 ± 2.8	0.76	0.00035	2000 ± 220
5	phrenosin	0.25 ± 0.03	0.028	25 ± 3.2	0.75	0.00034	2000 ± 190
10	phrenosin	0.27 ± 0.03	0.026	27 ± 2.6	0.73	0.00038	1800 ± 210

[a] Values represent averages obtained from at least duplicate experiments consisting of at least 10 data points.

Table III: Transfer Parameters of Semisynthetic Galactosylceramides from POPC Vesicles at 45 °C[a]

mol %	GalCer	k_1 (h^{-1})	$t_{1/2}$ (h)
1	*N*-pal GalCer	0.033 ± 0.003	21 ± 2
5	*N*-pal GalCer	0.032 ± 0.002	22 ● 2
10	*N*-pal GalCer	0.029 ± 0.002	24 ● 2
5	*N*-lig GalCer	<0.0002	>3500

[a] Values represent averages obtained from at least duplicate experiments consisting of at least eight data points.

Table IV: Rate Constants for GalCer Transfer from Mixed GalCer/POPC Vesicles at 45 °C[a]

GalCer[b]	system[c]	A_1	k_1 (h^{-1})	A_2	k_2 (h^{-1})
kerasin	1% kerasin 4% *N*-pal	0.18	0.023	0.82	0.00026
kerasin	1% kerasin 4% phrenosin	0.19	0.025	0.81	0.00029
N-pal	1% *N*-pal 4% kerasin	1.0	0.032		
phrenosin	1% phrenosin 4% kerasin	0.22	0.029	0.82	0.00034

[a] Values represent averages from duplicate experiments consisting of at least 10 data points. [b] Kinetic parameters are given for the transfer of the molecular species given in this column from the defined system. [c] POPC is the third component of each system.

concentration. Analogous to the transfer of brain GalCer, no dependence on composition was observed. Also given in Table III is an approximate rate constant for the transfer of the *N*-lig GalCer. This long acyl chain molecule likewise transfers with monoexponential kinetics within the limits of observation. However, the half-time in this case is too long to measure accurately since the movement of this molecule is very small relative to experimental error over the 20-day time frame of the experiments. At time points greater than 20 days, significant movement of the nonexchangeable marker was evident. Thus, data taken after 20 days were not considered reliable. However, it is evident in comparing this molecule with *N*-pal GalCer that GalCer transfer rates depend dramatically on acyl chain length.

Galactosylceramide Transfer from Mixed Systems. In view of this dependence of transfer rate on chain length, it seems likely that the biphasic transfer kinetics of the bovine brain galactosylceramides could arise from acyl chain heterogeneity. In order to examine this possibility, vesicles were prepared containing GalCer with defined chain heterogeneity and the movement of a specific [^{3}H]GalCer was monitored. Results are given in Table IV. The half-time of *N*-pal GalCer transfer from POPC in the presence of 4 mol % kerasin agrees with that obtained for the POPC/*N*-pal GalCer system. Conversely, the kinetics of kerasin transfer are likewise unaffected by inclusion of 4 mol % *N*-pal GalCer. In addition, the rates of kerasin and phrenosin transfer in the ternary system with POPC are identical with those observed for each GalCer in a binary POPC/GalCer system. Thus, it is quite evident that the transfer rates of these molecules exhibit no significant dependence on the total GalCer content of the vesicles in the concentration range examined.

Glucosylceramide Transfer. In order to define more systematically the effect of acyl chain length of hexosylceramide transfer rates, as well as to examine possible headgroup effects, the transfer of glucosylceramide containing N-linked palmitic, myristic, and stearic acids was examined. Table V gives the half-times of transfer of these molecules between POPC vesicles. The transfer half-time for *N*-stearoyl-GlcCer is an order of magnitude longer than that for the *N*-palmitoyl derivative, which in turn is 2.5 times longer than that observed for *N*-myristoyl-GlcCer. In addition, the transferable fraction for the fast moving *N*-myristoyl-GlcCer was observed to be significantly higher than the expected two-thirds. Thus, transbilayer movement is evidently faster than desorption for this molecule. Since desorption rates are more sensitive to acyl chain length than flip-flop rates (Homan & Pownall, 1988), it is reasonable to assume that transbilayer movement rates are faster than desorption rates for all hexosylceramides studied. It proved technically impossible to carry experiments for the other hexosylceramides over the many days necessary to determine directly the size of the transferable fraction. As described under Experimental Procedures, the results presented in this paper were obtained under the assumption of fast transbilayer movement. An analysis assuming very slow transbilayer movement increased the rate constants by no more than 10% in any system.

Table V: Semisynthetic Glucosylceramide Transfer Rates from POPC Vesicles at 45 °C[a]

GlcCer	k_1 (h^{-1})	$t_{1/2}$ (h)
N-myristoyl-GlcCer	0.079 ± 0.004	8.8 ± 0.4
N-palmitoyl-GlcCer	0.031 ± 0.004	22 ± 2.8
N-stearoyl-GlcCer	0.0034 ± 0.0005	204 ± 31

[a] Values represent averages obtained from duplicate experiments consisting of at least four data points.

Discussion

The results presented in the preceding section clearly show that the spontaneous interbilayer transfer rates of hexosylceramides decrease markedly with increasing acyl chain length. The rates, however, neither depend strongly on the state of hydroxylation of the acyl chain nor appreciably differ for galactosyl- and glucosylceramides with the same acyl chain. Somewhat surprisingly, at the relatively low concentrations of galactosylceramides studied, the transfer rate of a specific GalCer is independent of the total concentration of that galactosylceramide or other species of galactosylceramides in the POPC bilayer. The very slow transfer rates of the long acyl chain hexosylceramides ensure that these molecules are lost very slowly, if at all, by spontaneous transfer from the external surface of plasma membranes; a result that is consistent with the putative biological role of glycosphingolipids.

The biexponential kinetics observed for the transfer of kerasin and phrenosin most likely reflect the presence of both long and short acyl chain species in these bovine brain ceramides. This can be understood by noting that the rate of transfer of *N*-pal GalCer averaged over the data given in Table III (0.031 h^{-1}) is approximately equal to the rates of transfer of the fast pool of phrenosin (0.028 h^{-1}) and of kerasin (0.024 h^{-1}) as given in Table II. Furthermore, if the amplitude of the relatively fast moving pool is arbitrarily identified with the fraction containing C-16 and C-18 acyl chains (Table I), quite good agreement is obtained for phrenosin and only a slight discrepancy is observed for kerasin. As similar concordance is seen between the rough transfer rate obtained for *N*-lignoceryl-GalCer (<0.0002 h^{-1}) given in Table III and slow-moving fractions of kerasin (0.00026 h^{-1}) and phrenosin (0.00036 h^{-1}) shown in Table II. The biexponential kinetics are, however, only a limiting approximation to the true transfer behavior. The actual off-rate kinetics are defined by a sum of exponentials with each species of GalCer desorbing with its own rate constant. The fit to biexponential kinetics simply represents the best approximation that can be obtained within the precision limits of the experiments. This rationalization is clearly in accord with the observation that the transfer rate of a specific galactosylceramide is independent of the con-

centration of total galactosylceramides in the concentration range examined (0–10 mol %).

An exponential decrease of transfer rates with increasing acyl chain length is well established for phospholipid transfer (Nichols & Pagano, 1981, 1982; Ferrell et al., 1985; McLean & Phillips, 1984; Massey et al., 1982). This dependence is thought to reflect the increase in activation energy with increasing acyl length associated with the formation of the activated complex for monomer transfer [see Nichols (1985) for a detailed discussion]. It seems reasonable that the energy for transition-state formation for hexosylceramide monomer desorption from phospholipid bilayers should depend on acyl chain length in a similar manner.

Understanding hexosylceramide transfer from POPC vesicles is complicated, however, by the propensity of hexosylceramide/phosphatidylcholine systems to exhibit coexistence of a hexosylceramide-rich gel phase in equilibrium with a phosphatidylcholine-rich liquid-crystalline phase over a wide range of temperature and composition. In contrast, the vast majority of two-component phospholipid systems in which spontaneous interbilayer transfer has been studied are one-phase, liquid crystalline. Thus, an alternative but less likely explanation for the biexponential transfer kinetics observed with the beef brain ceramides in POPC may be sought in the possible coexistence of liquid-crystalline and gel phases in the systems. It is well established that the transfer rates from gel-phase lipids are at least several orders of magnitude less than the rates from liquid-crystalline phases at the same temperature (Thompson & Tillack, 1985). Thus, if both phases coexist in the bilayer and the interbilayer transfer rate between phases is of the same order of magnitude as the desorption rate from the gel phase, then biexponential kinetics will be observed. This explanation, which is supported by other evidence, has been used to rationalize the biexponential transfer kinetics of asialo-GM_1 in POPC at 45 °C. The asialo-GM_1 used in this study, however, had greater than 90% stearoyl acyl chains (Brown et al., 1985).

The nonideality of mixing in hexosylceramide/phospholipid bilayers has been examined in a number of studies [for reviews, see Thompson and Tillack (1985) and Curatolo (1987b)]. The basis for this nonideality must rest at least in part in the extensive hydrogen-bonding capacity of the hexose headgroup and the sphingosine base. Both intra- and intermolecular hydrogen bonding are believed to be responsible for the fact that the thermotropic behavior of hexosylceramides in aqueous dispersions is essentially independent of acyl chain content. For example, both C-16 and C-24/C-26:1 GalCer exhibit main gel–liquid-crystalline phase transitions at 83 °C with transition enthalpies of 13.9 and 16.6 kcal/mol, respectively (Curatolo & Jungalawa, 1985). The nonideality of mixing of hexosylceramides in phospholipid bilayers must also reflect mismatches in acyl chain length between the ceramide and phospholipid components as well as the intramolecular methylene chain mismatches in ceramides with long acyl chains. A mismatch between the methylene chain of the N-linked acyl group and the methylene chain of the sphingosine base provides the capacity for molecular interdigitation across the bilayer in gel phases that could enhance the stability of hexosylceramide-enriched gel phases (Bunow, 1979). Acyl chain mismatch may not, however, be of major importance. Gardham and Silvius (1989) found that the mixing behavior of the long-chain *N*-lignoceroyl-GalCer in DPPC multibilayers is quite similar to the behavior of *N*-palmitoyl-GalCer earlier studied by Ruocco et al. (1983). This result, in conjunction with phase diagrams reported for phosphatidylcholine/hexosylceramide mixtures (Correa-Freire et al., 1979; Barenholz et al., 1983; Maggio et al., 1985; Curatolo, 1986; Bunow & Levin, 1988; Johnston & Chapman, 1988; Rintoul et al., 1988) suggests that the solubility of hexosylceramides in a DPPC-enriched liquid-crystalline phase is roughly independent of acyl chain length. Thus it appears that headgroup and sphingosine base interactions, not acyl chain length, are primarily responsible for inducing phase separation of hexosylceramides in hexosylceramide/DPPC systems.

On the basis of the information outlined above, it is clear that the spontaneous transfer kinetics of the hexosylceramides in POPC vesicles discussed in this paper allow little to be concluded about bilayer phase structure in these vesicles. The biexponential kinetics observed for phrenosin and kerasin transfer are well explained by the presence of both long and short acyl chain species in these natural products combined with the observed dependence of the transfer rate on acyl chain length. The marked decrease in transfer rate with increasing acyl chain length is readily explained by the increase in energy required to form the monomeric transition state as the chain length is increased. In the same sense, it cannot be concluded that GalCer and GlcCer are molecularly dispersed in POPC vesicles under these conditions, although the half-times observed for *N*-pal GalCer and *N*-pal GlcCer are quite similar to that observed for POPC transfer in liquid-crystalline vesicles at the same temperature. The results do not rule out the possibility that these short acyl chain hexosylceramides are present in phase-separated gel domains. For this to be the case, however, the intrabilayer reequilibration rate between gel and liquid-crystalline domains must then be fast relative to the rate of transfer from the putative gel domains.

The transbilayer movement of *N*-myr GlcCer is faster than the desorption rate. Homan and Pownall (1988) examined rates of transbilayer movement of a series of phospholipids in POPC small unilamellar vesicles. It was shown that these rates depend primarily on headgroup structural features such as size and relative polarity. The monoglycosyl headgroups of hexosylceramides are less polar than those of phospholipids, due to the absence of charges. Thus, it is not surprising that these molecules show faster transbilayer movement than those typically observed for phospholipids. Rates of transbilayer movement could not be evaluated for the other hexosylceramides examined. However, the study by Homan and Pownall (1988) showed that transbilayer movement rates are much less sensitive to acyl chain length than are desorption rates. For example, when the acyl chain at position 1 of 2-9-(1-pyrenyl)nonanoylphosphatidylcholine was increased from C-8 to C-12, the transbilayer movement rate decreased by a factor of approximately 4 while the desorption rate decreased by a factor of approximately 50. Thus, although one would expect hexosylceramide transbilayer movement rates to decrease with increasing chain length, it is reasonable to assume that transbilayer movement is fast relative to desorption for all hexosylceramides examined in this study.

Acknowledgments

We thank Margaretta Allietta and Philip Rock for carrying out the electron microscopy, Mike Kinter for the mass spectroscopy, and Matt Junker for the preparation of glucosylsphingosine.

References

Barbosa, M. L. E., & Pinto da Silva, P. (1983) *Cell 33*, 959–966.

Barenholz, Y., Gibbes, D., Litman, B. J., Goll, J., & Thompson, T. E. (1977) *Biochemistry 16*, 2806–2810.

Barenholz, Y., Freire, E., Thompson, T. E., Correa-Freire, M. C., Bach, D., & Miller, I. R. (1983) *Biochemistry 22*, 3497–3501.

Brown, R. E., & Thompson, T. E. (1987) *Biochemistry 26*, 5454–5460.

Brown, R. E., Stephenson, F. A., Markello, T., Barenholz, Y., & Thompson, T. E. (1985a) *Chem. Phys. Lipids 38*, 79–93.

Brown, R. E., Sugar, I. P., & Thompson, T. E. (1985b) *Biochemistry 24*, 4082–4091.

Bunow, M. R. (1979) *Biochim. Biophys. Acta 574*, 542–546.

Bunow, M. R., & Levin, I. W. (1988) *Biochim. Biophys. Acta 939*, 577–586.

Correa-Friere, M. C., Freire, E., Barenholz, Y., Biltonen, R. L., & Thompson, T. E. (1979) *Biochemistry 18*, 442–445.

Correa-Freire, M. C., Barenholz, Y., & Thompson, T. E. (1982) *Biochemistry 21*, 1244–1248.

Curatolo, W. (1986) *Biochim. Biophys. Acta 861*, 373–376.

Curatolo, W. (1987a) *Biochim. Biophys. Acta 906*, 111–136.

Curatolo, W. (1987b) *Biochim. Biophys. Acta 906*, 137–160.

Curatolo, W., & Jungalawa F. B. (1985) *Biochemistry 24*, 6608–6613.

Feizi, T. (1985) *Nature (London) 314*, 53–57.

Ferrell, J. E., Jr., Lee, K.-J., & Huestis, W. H. (1985) *Biochemistry 24*, 2857–2864.

Gardam, M., & Silvius, J. R. (1989) *Biochim. Biophys. Acta 980*, 319–325.

Hakomori, S. (1981) *Annu. Rev. Biochem. 50*, 733–764.

Hakomori, S. (1983) *Handbook of Lipid Research: Sphingolipid Biochemistry* (Hanahan, D. J., Ed.) Vol. 3, pp 327–380, Plenum Press, New York.

Homan, R., & Pownall, H. C. (1988) *Biochim. Biophys. Acta 938*, 155–166.

Hope, M. J., Bally, M. B., Webb, G., & Cullis, P. R. (1985) *Biochim. Biophys. Acta 812*, 55–65.

Johnston, D. S., & Chapman, D. (1988) *Biochim. Biophys. Acta 939*, 603–614.

Jones, J. D., & Thompson, T. E. (1988) *Biophys. J. 53*, A1779.

Jones, J. D., & Thompson, T. E. (1989) *Biochemistry 28*, 129–134.

Lapidot, Y., Rappoport, S., & Wolman, Y. (1967) *J. Lipid Res. 13*, 819–822.

Maggio, B., Ariga, T., Sturtevant, J. M., & Yu, R. K. (1985) *Biochim. Biophys. Acta 818*, 1–12.

Masserini, M., & Freire, E. (1987) *Biochemistry 26*, 237–242.

Massey, J. B., Gotto, A. M., Jr., & Pownall H. J. (1982) *Biochemistry 21*, 3630–3636.

McLean, L. R., & Phillips, M. C. (1984) *Biochemistry 23*, 4624–4630.

Nichols, J. W. (1985) *Biochemistry 24*, 6390–6398.

Nichols, J. W., & Pagano, R. E. (1981) *Biochemistry 20*, 2783–2789.

Nichols, J. W., & Pagano, R. E. (1982) *Biochemistry 21*, 1720–1726.

Nordlund, J. R., Schmidt, C. F., Dicken, S. N., & Thompson, T. E (1981) *Biochemistry 20*, 3227–3241.

Ong, D. E., & Brady, R. N. (1972) *J. Lipid Res. 13*, 819–822.

Rintoul, D. A., Welti, R., & Song, W. (1988) *Biophys. J. 53*, 126a.

Ruocco, M. J., Shipley G. G., & Oldfield, E. (1983) *Biophys. J. 43*, 91–101.

Suzuki, Y., Hirabayashi, Y., & Matsumoto, M. (1984) *J. Biochem. 95*, 1219–1222.

Thompson, T. E., & Tillack, T. W. (1985) *Annu. Rev. Biophys. Biophys. Chem. 14*, 361–386.

Thompson, T. E., Allietta, M., Brown, R. E., Johnson, M. L., & Tillack, T. W. (1985) *Biochim. Biophys. Acta 817*, 229–237.

Wimley, W. C., & Thompson, T. E. (1990) *Biochemistry 29*, 1296–1303.