Glycosphingolipids: ^2H NMR Study of the Influence of Carbohydrate Headgroup Structure on Ceramide Acyl Chain Behavior in Glycolipid–Phospholipid Bilayers†

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ABSTRACT: Galactosyl- and glucosylceramide, globoside, and dihydrolactosylceramide, bearing [2,2-$^2H_2$]stearic acid, have been studied at a concentration of 10 mol % in bilayers of dimyristoylphosphatidylcholine by ^2H NMR. The quadrupolar splittings $\Delta\nu_Q$ of the C2 deuterons were measured at several temperatures in the range of 30–60 °C. Spin–lattice relaxation times T_1 of C2 deuterons were determined in the same temperature range for all lipids but globoside. T_1 values at 30 and 50 °C were unexpectedly short (6–8 ms), indicating reduced mobility of the ceramide acyl chains compared to that of the host phospholipid. At all temperatures, both $\Delta\nu_Q$ and T_1 were essentially identical for the monoglycosylated species, GalCer and GlcCer, indicating that the order and dynamics of the upper portion of the fatty acyl chain are insensitive to this small change in the headgroup structure. In the case of globoside, where the glycolipid headgroup is equivalent to that of GlcCer extended by three sugar residues, values for the quadrupolar splittings associated with the acyl chain C2-position were very close to those obtained for Gal- and GlcCer. In contrast, the $\Delta\nu_Q$ values obtained for the diglycosyl species, LacCer, were significantly different at all temperatures. This different behavior of LacCer relative to that of the other glycolipids most likely originates from an orientational change of the acyl chain at the C2-position due to the absence of a 4,5 double bond in dihydrosphingosine. T_1 values for the GlcCer and GalCer systems increased with temperature, indicating that the motions responsible for relaxation were in the short correlation time regime. T_1 for deuterons at the acyl chain C2-position of LacCer was observed to decrease with increasing temperature, indicating that the motion(s) dominating relaxation are in the long correlation time regime. Thus the mobility of the acyl chain at the 2-position is reduced in the LacCer with respect to GlcCer and GalCer.

Glycosphingolipids (GSLs),[1] the carbohydrate-bearing lipids of animal cells, have been implicated as specific recognition sites for a variety of important cellular processes, including immune events (Hakomori, 1984), cell–cell interaction (Critchly et al., 1979), and binding of viruses and proteins (Fishman & Brady, 1976). In addition, they have the potential to modulate membrane physical properties [reviewed in Curatolo (1987a)]. The functional roles of glycolipids as both recognition sites and structural elements appear to be primarily determined by the nature of the sugar headgroup. For example, certain physical properties of glycolipids, such as their high acyl chain order–disorder phase-transition temperatures, are attributed to the hydrogen-bonding network of the headgroup region rather than to acyl chain length or degree of unsaturation (Curatolo, 1987a). However, it is also thought that the antigenicity of GSLs is dependent not only on the spatial configuration of the carbohydrate chain but of the ceramide portion of the molecule as well (Hakomori, 1986). Two factors that often increase GSL antigenicity are an increase in fatty acyl chain length and the presence of an α-hydroxy group at the 2-position of the fatty acyl chain (Hakomori, 1986). These observations raise the question of whether any communication exists between the headgroup and acyl chain regions of glycolipid molecules in membranes, especially in mixed lipid systems that mimic the situation found in vivo. In other words, can changes in one region of the molecule affect the properties or behavior of the other region? Such information may be of importance in devising models of GSL function.

Previous studies of spin-labeled galactosylceramide, lactosylceramide, globoside, and the ganglioside G_{M1}, at concentrations up to 10 mol % in phospholipid bilayers, revealed essentially identical ordering and organization at C16 of the glycolipid acyl chain, in both gel and liquid-crystalline state membranes (Mehlhorn et al., 1989). The lack of observable headgroup effects in such different glycolipid species may be due to steric effects of the bulky spin label probe or, more likely, may indicate that any such effects are not observed toward the center of the membrane.

In the present study we have addressed directly by ^2H NMR the question of whether alterations to the GSL headgroup sugars are reflected in changes within the membrane at positions proximate to the interfacial region. Although such changes have been convincingly demonstrated in pure hydrated glycolipids (Maggio et al., 1981, 1985; Jarrell et al., 1987; Curatolo, 1987a,b; Thompson & Tillack, 1985; Grant, 1987), the situation is not as clear for systems in which GSL is the

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[1] Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; DPPE, L-α-dipalmitoylphosphatidylethanolamine; GalCer, galactosylceramide, Gal(β1→1)Cer; GlcCer, glucosylceramide, Glc(β1→1)Cer; LacCer, dihydrolactosylceramide, Gal(β1→4)Glc(β1→1)dihydro-Cer; globoside, GalNAc(β1→3)Gal(α1→4)Gal(β1→4)Glc(β1→1)Cer; GSL, glycosphingolipid; EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance.

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minor component, as is the case in many biological membranes. Furthermore, the acyl chains of GSLs are often significantly longer than the acyl chains of the surrounding phospholipid matrix. The systems we have selected for study were designed to approximately mimic these natural conditions. GalCer, GlcCer, LacCer, and globoside were studied at a concentration of 10 mol % in DMPC, and each GSL was synthetically modified so that its natural fatty acid was replaced with stearic acid deuteriated at the C2-position. The rationale was to examine a nonperturbing probe in close association with the sugar headgroup, yet buried at the membrane surface. The glycolipids were chosen to allow us to address both the effects of small changes (i.e., the epimerization of the 4-OH group in glucose vs galactose) and large changes (mono- vs di- vs tetrasaccharide) in headgroup structure on membrane properties. Very striking differences in behavior have been recorded for GalCer and GlcCer (Curatolo, 1987a; Thompson & Tillack, 1985; Maggio et al., 1985) and for galactosyl- and glucosylglycerolipids in mixtures with DMPC (Koynova et al., 1988). It is important to probe in detail the origin of such differential molecular properties in phospholipid membranes.

Materials and Methods

L-α-Dimyristoylphosphatidylcholine (DMPC) was obtained from Avanti Polar Lipids, Birmingham, AL. Galactosylceramide (GalCer) type II from beef brain, *N*-lignoceroyldihydrolactosylceramide (LacCer), and glucosylceramide (GlcCer) were obtained from Sigma Chemical Co., St. Louis, MO. Globoside was isolated from pig blood as described previously (Mehlhorn et al., 1988). Deuteriated solvents were from MSD Isotopes, Montreal, Canada.

[2,2-$^2H_2$]Stearic acid was synthesized from 4.3 g of methyl stearate (Aldrich, Milwaukee, WI) following the general method of Aasen et al. (1970) for inserting deuterium at the 2-position of fatty acids. The structure was confirmed by ^1H NMR. Globoside bearing [2,2-$^2H_2$]stearic acid was synthesized as described previously for the spin labeled derivative (Mehlhorn et al., 1988) with the exception that the [2,2-$^2H_2$]stearic acid was added as the acid chloride (Kopaczyk & Radin, 1965). All other glycosphingolipids bearing [2,2-$^2H_2$]stearic acid were synthesized following the procedure described previously for spin-labeled glycolipids (Sharom & Grant, 1975). Hydrolysis of the existing fatty acid was accomplished by treatment of the glycolipid (50 mg) with 1.4 g of KOH in 25 mL of methanol in a sealed glass culture tube at 95 °C for 18 h (Neuenhofer et al., 1985). Intermediate lysoglycosphingolipids were purified on a column of silicic acid (Bio-Sil A 200–400 mesh from Bio-Rad, Richmond, CA) eluted with a gradient of 20–70% $CH_3OH/CHCl_3$. The final deuterium-labeled glycolipids were purified on silicic acid columns eluted with a gradient of 0–40% CH_3OH in $CHCl_3$. Lipids were assessed for purity by thin-layer chromatography on Merck silica gel 60 plates eluted with 65:25:4 (by volume) $CHCl_3/CH_3OH/H_2O$ and developed with ninhydrin and sulfuric acid/ethanol sprays. All glycosphingolipids comigrated with standards and agreed by ^1H NMR with the nondeuteriated lipids. Lysoglycosphingolipids gave a purple stain with ninhydrin reagent, while the final product did not and was resistant to overnight exposure to aqueous KOH (5 mg/mL) at 1 atm and 20 °C.

The GlcCer, GalCer, and LacCer samples for ^2H NMR were prepared by dissolving the ^2H-labeled glycosphingolipid (5 mg) and DMPC (45 mg) in chloroform/methanol (2:1 v/v) and evaporating the mixture to dryness followed by 3 h under high vacuum. The procedure was the same for the globoside sample, except the quantities used were 6.8 mg of globoside and 31 mg of DMPC. Samples were hydrated with a 3-fold excess of deuterium-depleted water (Aldrich Chemical Co., Milwaukee, WI) in a 5-mm (o.d.) sample tube. Samples were lyophilized three times from 150 μL of deuterium-depleted water after which the hydrated samples were subjected to eight freeze–thaw cycles.

^2H NMR spectra were acquired at 30.7 MHz as described previously (Jarrell et al., 1986). Spectra were recorded by using the quadrupolar echo pulse sequence (Davis et al., 1976) with full phase cycling (Perly et al., 1985) and quadrature detection. The $\pi/2$ pulse length was 2.4 μs (5-mm solenoid coil), the pulse spacing was 60 μs, and the recycle time varied between 50 and 200 ms. Longitudinal relaxation times (T_1) were obtained with the standard inversion–recovery sequence coupled with the quadrupolar echo sequence (Perly et al., 1985). The frequency of the spectrometer was carefully set at the center of the quadrupolar powder patterns. The samples were equilibrated at a given temperature for 20–30 min prior to data acquisition; temperatures are accurate to ±0.5 °C. In general, spectra were not folded about the Larmor frequency, except for the globoside spectra and for the T_1 measurements, where they were folded to increase the signal-to-noise ratio. No spectral distortions were introduced by the folding procedure. Quadrupolar splittings were measured from the 90° oriented-sample ("dePaked") spectra, which were calculated from the powder spectra as described previously (Bloom et al., 1981).

^{31}P NMR spectra were acquired at 121.5 MHz on a Bruker MSL-300 spectrometer. The spectra were recorded by using a Hahn echo pulse sequence (Rance & Byrd, 1983) with Waltz decoupling (gated on during acquisition). The $\pi/2$ pulse length was 4.0 μs (10-mm solenoid coil), the pulse spacing was 60 μs, and the recycle time was 5.0 s.

Molecular modeling was performed on an AST Premium 386 computer by using PCMODEL version 3.0 (employing default parameters) from Serena Software, Bloomington, IN. This software utilizes an MMX force field.

Results

Figure 1 illustrates the structures of the glycosphingolipids, *N*-[2,2-$^2H_2$]stearoyl-GalCer, *N*-[2,2-$^2H_2$]stearoyl-GlcCer, *N*-[2,2-$^2H_2$]stearoyl-LacCer, and *N*-[2,2-$^2H_2$]stearoylgloboside, as arrived at by molecular modeling. It should be emphasized that the energy minimization was not performed within the context of a phospholipid bilayer, which could place constraints on the GSL, and thus the indicated orientations are not intended to corroborate the ^2H NMR data. The purpose of the figure is to accurately indicate the location of the ^2H probe at the C2-position of the fatty acyl chain, a location that should be sensitive to headgroup sugar effects on the hydrophobic region. Temperature-dependent ^2H NMR spectra of the labeled GlcCer, GalCer, and LacCer glycolipids, present at a concentration of 10 mol % in DMPC multilamellar dispersions, are shown in Figure 2. Corresponding dePaked spectra (90° orientation) (Bloom et al., 1981) appear in Figure 3. The corresponding spectra for the globoside system are presented in Figure 4. We would stress that these spectra were acquired on samples containing relatively small amounts of the deuteriated species (5 mg): a necessary condition for studying glycolipids at membrane concentrations reflective of their natural state. It is evident from the deuterium spectra that the glycolipids are in a liquid-crystalline environment. Additional verification of this is provided by the ^{31}P NMR spectra (at 30 °C) in Figure 5; these display the characteristic liquid-crystalline line shape with a $\Delta\sigma$ value of approximately 42 ppm.

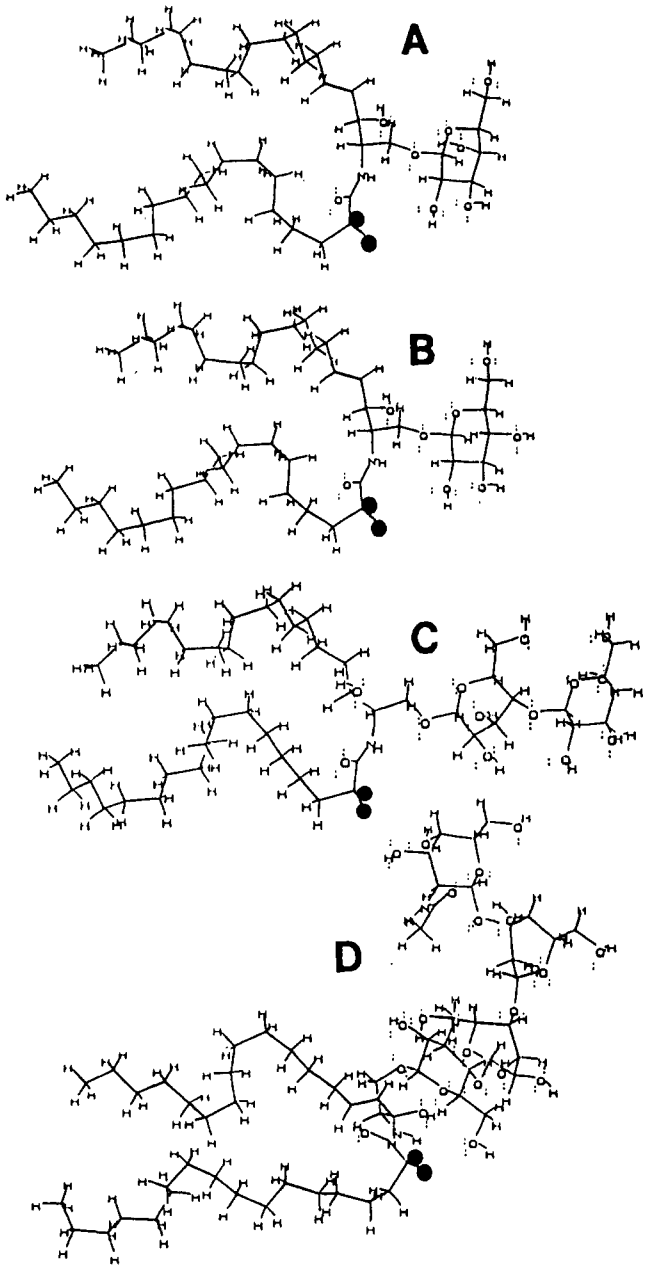

FIGURE 1: Structures of (A) galactosylceramide, (B) glucosylceramide, (C) dihydrolactosylceramide, and (D) globoside arrived at by molecular modeling. Minimum-energy structures for the sugars and ceramide backbone with an 18-carbon saturated fatty acid were calculated separately. Hydrocarbon and sugar portions were then connected, and a minimum-energy structure was calculated for the entire molecule. It should be emphasized, however, that energy minimization was not performed within the context of a phospholipid bilayer or other "neighboring" groups that might place constraints on the glycosphingolipid. Deuterium location is indicated by filled circles. The sugar linkages are Gal(β1→1)Cer, Glc(β1→1)Cer, Gal(β1→4)-Glc(β1→1)dihydro-Cer, and GalNAc(β1→3)Gal(α1→4)Gal(β1→-4)Glc(β1→1)Cer, respectively.

Two quadrupolar splittings are observed for the C2-labeled position for each of the three glycolipids. This indicates that the deuterons at this position are nonequivalent, making different angles with respect to the axis of motional averaging. Values of the quadrupolar splittings, obtained from the dePaked spectra, are listed in Table I. Initially, we direct our attention to the GalCer and GlcCer spectra. At all temperatures, the splittings for these samples are identical, indicating similar ordering and orientation of the chains at this location in the membrane. The peak height ratios of the C2 resonances of GalCer and GlcCer are also the same at comparable tem-

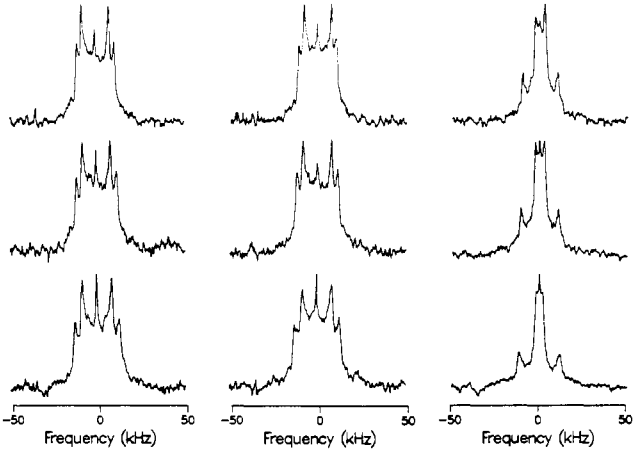

FIGURE 2: ^{2}H NMR spectra (30.7 MHz) of (left column) GlcCer, (center column) GalCer, and (right column) LacCer at (bottom row) 30 °C, (center row) 45 °C, and (top row) 60 °C, all at a concentration of 10 mol % in DMPC multilamellar dispersions. The number of acquisitions (30, 45, and 60 °C) was GlcCer (312 000, 225 960, 225 960), GalCer (312 000, 210 000, 210 000), and LacCer (396 000, 198 000, 99 000). Line broadening was 500 Hz.

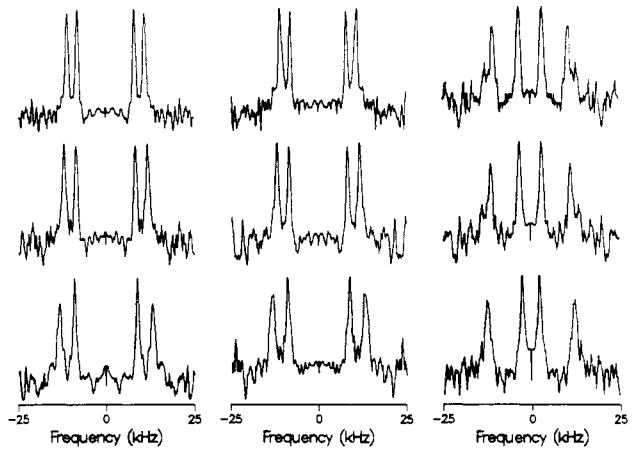

FIGURE 3: DePaked (90° orientation) spectra calculated from the corresponding powder spectra in Figure 2.

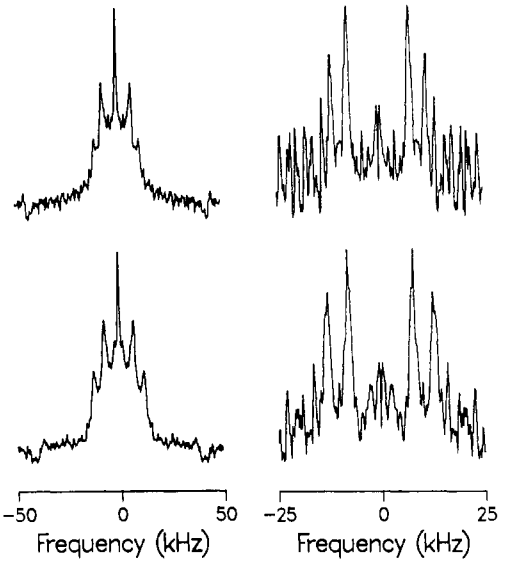

FIGURE 4: ^{2}H NMR spectra (30.7 MHz) of globoside (approximately 6.8 mg) at (bottom) 30 °C and (top) 50 °C at a concentration of 10 mol % in DMPC multilamellar dispersions (left column). The corresponding dePaked spectra are shown in the right column. The number of acquisitions was (bottom) 1.8×10^6 and (top) 1.2×10^6. Line broadening was 500 Hz.

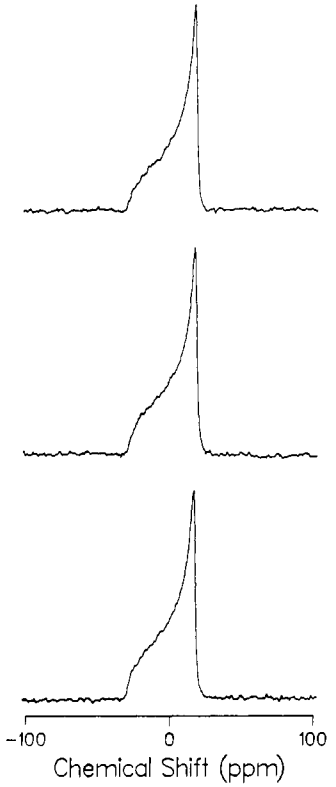

FIGURE 5: ^{31}P NMR spectra (121.5 MHz) of mixtures of (A) GlcCer, (B) GalCer, and (C) LacCer at a concentration of 10 mol % in DMPC multilamellar dispersions at 30 °C. The number of acquisitions was 128. Line broadening was 50 Hz.

Table I: Temperature Dependence of $\Delta\nu_Q$ and T_1 for *N*-[2,2-$^2H_2$]Stearoylglycosphingolipids at a Concentration of 10 mol % in DMPC Bilayers

T (°C)	GalCer	GlcCer	LacCer	globoside
		$\Delta\nu_Q$ (kHz)		
30	26.6, 18.0	26.6, 18.0	25.0, 4.6	25.4, 16.0
45	23.8, 16.8	23.8, 16.8	22.2, 6.1	
50				23.2, 15.0
60	21.9, 16.0	21.9, 16.0	20.8, 6.4	
		T_1 (ms)[a]		
30	6.1 ± 0.1	6.5 ± 0.2	7.1 ± 0.3	
50	8.1 ± 0.2	7.8 ± 0.2	5.6 ± 0.2	

[a] The errors indicated for T_1 are the standard deviations obtained from a least-squares fit of the experimental inversion–recovery data to an exponential function.

peratures. The structure of the glycolipid, globoside, is identical with that of GlcCer, but with the addition of a linear chain of three more sugar residues. Its spectra are similar to those of GlcCer and GalCer, although the outer and inner splittings are reduced by ca. 1 and 2 kHz, respectively (Table I). The ratio of outer to inner quadrupole splitting, $\Delta\nu_Q(o/i)$, is 1.59 at 30 °C, only slightly larger than the 1.48 obtained for GlcCer and GalCer. This ratio changes little with temperature, decreasing from 1.59 (30 °C) to 1.55 (50 °C) for globoside and 1.48 (30 °C) to 1.37 (60 °C) for GalCer and GlcCer as the temperature is increased. Thus the orientation and ordering of the C2-position is similar in the GlcCer, GalCer, and globoside systems, suggesting that the substantial change in headgroup size is not manifest in the average orientation of the acyl chain C2-position or in the amplitude of angular fluctuations about this direction.

The glycolipid LacCer (having a dihydrosphingosine moiety) was examined since it provides a species with a headgroup that is only one residue larger than that of GalCer and GlcCer.

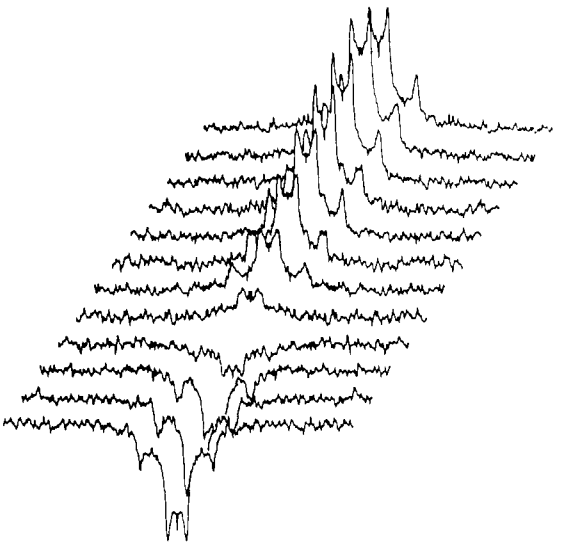

FIGURE 6: ^2H NMR spectra of LacCer (10 mol % in DMPC) at 50 °C as a function of the delay time τ in the inversion–recovery sequence. Values of τ (front to back) are 0.5, 0.75, 1.5, 2.5, 5, 7.5, 10, 12.5, 15, 20, 35, and 50 ms. The number of acquisitions was 60 000. The plot width is ±50 kHz.

In marked contrast to the results with the other lipids, the spectra of LacCer in Figure 2 reveal distinct differences in the values of the quadrupolar splittings of the C2 deuterons at all temperatures. While the outer splitting is reduced only slightly (by 1–2 kHz) with respect to that of GlcCer and GalCer at all temperatures, the inner splitting is significantly narrowed to <7 kHz. For instance, at 30 °C the ratios of the outer to inner splittings $[\Delta\nu_Q(o/i)]$ are 5.4 and 1.48 for the LacCer vs GlcCer and GalCer systems, respectively. Differences are also observed in the temperature dependence of the quadrupolar splittings: for LacCer the outer splitting decreases with increasing temperature, while the inner splitting increases (Table I). In comparison, the other glycolipids exhibit a decrease in both splittings with increasing temperature. If increasing the temperature resulted only in a decrease in molecular order, leaving the orientation of the methylene C2 segment constant, then $\Delta\nu_Q(o/i)$ should also be unchanged. However, changes in this ratio are observed (of the same magnitude in the case of GalCer and GlcCer) for the four glycolipids, indicating temperatue-dependent orientational changes. This is especially clear for the LacCer, where the changes in the two splittings are opposite.

In order to probe for headgroup-induced differences in chain dynamics, the ^2H spin–lattice relaxation times T_1 of the C2 deuterons were measured as a function of temperature. An example of a T_1 inversion–recovery experiment is shown in Figure 6 for the LacCer sample at 50 °C. T_1 values were obtained from a least-squares fit of the integrated areas of the partially relaxed spectra to an equation of the form $M(\tau_1) = M(0)[1 - A\exp(\tau_1/T_1)]$, where $M(\tau_1)$ and $M(0)$ represent the longitudinal magnetizations at times τ_1 and $\tau_1 = 0$, respectively. Three parameters were varied in the fitting routine, these being T_1, A, and $M(\infty)$; the magnetization at $\tau_1 = \infty$ (fully relaxed). In all cases, the magnetization was found to vary exponentially with time. T_1 values for three of the four glycosphingolipids obtained at 30 and 50 °C are listed in Table I; because of the small quantity of globoside, T_1 measurements were not practical. Measured values of T_1 for GlcCer and GalCer were very similar at a given temperature. Thus at 30 °C the values were 6.5 ± 0.2 and 6.1 ± 0.2 ms, respectively (Table I). These values increased to 7.8 ± 0.2 and 8.1 ± 0.2 ms, respectivley, at 50 °C. The T_1 values obtained for LacCer

are also extremely short. However, unlike the situation observed for GlcCer and GalCer, the T_1's decrease with increasing temperature, dropping from 7.1 ± 0.3 ms at 30 °C to 5.6 ± 0.2 ms at 50 °C.

Discussion

The purpose of the present study was to look for evidence of communication between the headgroup and acyl chain regions of GSL molecules. The ability to collect ^{2}H NMR spectra on small quantities of labeled glycolipid made it possible to study them in the predominantly phospholipid environment typical of their physiological state. It was intended that the use of partially synthesized ^{2}H-labeled GalCer, GlcCer, LacCer, and globoside with identical fatty acids would isolate the role of sugar headgroup changes as a determinant of glycolipid-based phenomena within the membrane. In retrospect, this proved to be not as clear in the case of LacCer, given that it had a dihydrosphingosine backbone (see further discussion below). Previous literature observations have shown both similarities and differences in glycolipid properties as a function of headgroup sugar. This has been observed in both heterogeneous and well-defined pure lipid systems (Maggio et al., 1985). Thus, both *N*-palmitoylgalactocerebroside and *N*-palmitoylglucocerebroside have T_m's of 82 °C (Skarjune & Oldfield, 1979, 1982). *N*-Stearoyldihydrogalactosylsphingosine and *N*-stearoyldihydroglucosylsphingosine have T_m's of 83.7 and 84.9 °C and transition enthalpies of 4.7 and 6.9 kcal/mol, respectively (Maggio et al., 1985). Apparently the differences in the spatial orientation of the hydroxyl group at the 4-position of the pyranose ring can lead to changes, albeit small, in the properties of membranes composed of pure glycolipids. Such observations have been extended to more complex systems in which glycolipids were minor components, for example, binary mixtures of DMPC with the glycoglycolipids 1,2-di-*O*-tetradecyl-3-*O*-β-D-galactosyl-*sn*-glycerol (14-Gal) and 1,2-di-*O*-tetradecyl-3-*O*-β-D-glucosyl-*sn*-glycerol (14-Glc) (Koynova et al., 1988). DMPC and 14-Glc formed continuous solutions above and below the phase transitions of the mixture, whereas DMPC and 14-Gal formed extended regions of solid–solid phase separation. This highlights the potential for differing behavior of galacto- and glucolipids in mixtures with phospholipids.

It is clear that, at 30 °C and above, our spectra are representative of glycolipids in a liquid-crystalline environment. This argues against phase separation of pure glycolipid domains since T_m's for GalCer, GlcCer, and LacCer are much higher. Similar results were obtained by Skarjune and Oldfield (1982), who studied *N*-palmitoyl-GlcCer at 17 mol % in bilayers of DPPC, DMPC, DPPE, and cholesterol via ^{2}H NMR. They also observed dispersion of glycolipid into the host bilayer, obtaining liquid-crystalline spectra at temperatures well below the known phase transitions of the pure GSL. It is of interest that our results are similar to those reported in the latter study despite the four-carbon discrepancy between host matrix and GSL fatty acid. One might have speculated that such a mismatch in chain length would lead to phase separation on the basis of failure of the long-chain GSL to "fit" into the host matrix.

Spectral inequivalence of deuterons at C2 of a fatty acyl chain has been observed previously for *N*-palmitoyl-GalCer (Skarjune & Oldfield, 1979), for phospholipids (Davis, 1983; Seelig & Browning, 1978), and for glyceroglycolipids (Rance et al., 1983). We found the quadrupolar splittings for GlcCer and GalCer to be identical at a given temperature, indicating similar ordering and orientation of the chains at this position. The peak height ratios of the two splittings were also the same at comparable temperatures. Thus, the orientational organization of the fatty acyl chain is the same for both glucose and galactose headgroups. The values of the quadrupolar splittings observed at 30 °C (18 and 26.6 kHz) are similar to values obtained for pure *N*-palmitoyl-GalCer (18 and 25 kHz) at 90 °C (8 °C above the gel to liquid-crystalline phase transition) (Skarjune & Oldfield, 1979). This implies a similar chain organization of GalCer in pure and mixed lipid systems. It would be of interest to see whether the acyl chain ordering of pure GlcCer is similar to the mixed lipid systems studied here, as the headgroup orientation of *N*-palmitoyl-GlcCer was found to differ in pure and mixed systems (Skarjune & Oldfield, 1982).

In light of the similarity of results obtained for GlcCer and GalCer labeled at the C2-position of the fatty acyl chain, it would be of interest to compare the headgroup orientations of the two lipids. The headgroup orientation of *N*-palmitoyl-GlcCer has been obtained by using ^{2}H NMR, but such information is not available for pure *N*-palmitoyl-GalCer. However, both lipids have been studied in their pure phases with deuterium labels at the 6-position of the sugar headgroup, and the splittings were found to differ significantly (Skarjune & Oldfield, 1979, 1982). This *may* suggest differences in the headgroup orientation of the two lipids, although motion about the C5′–C6′ bond of the headgroup cannot be discounted as the source of this difference. The situation in phospholipid membranes has not been studied.

The preceding results suggest that relatively small changes in headgroup structure do not lead to significant changes in the orientational properties of glycolipids as monitored by the acyl chain C2-position. To test the corresponding effect(s) of increasing the headgroup size, LacCer and globoside bearing [2,2-$^2H_2$]stearic acid were also examined at a concentration of 10 mol % in DMPC bilayers. GlcCer is the structural element common to the three species, with LacCer being formed from GlcCer by adding a Gal residue while globoside requires the addition of two Gal residues and a GalNAC. It should be emphasized that LacCer differs from the other lipids in that the lipid anchor has a dihydrosphingosine residue rather than the normal unsaturated sphingosine moiety. Because of the latter structural difference, it is convenient to defer discussion of the LacCer results and compare those of globoside and GlcCer since it is more straightforward to focus only on the effects of headgroup changes. The increase in headgroup size from mono- to tetrasaccharide *does* have a small effect on the ^{2}H NMR spectrum. The outer and inner quadrupolar splittings are reduced by ca. 1 and 2 kHz, respectively, and the ratio $\Delta\nu_Q$(o/i) increases slightly. Nevertheless, it is clear that the globoside spectra (Figure 4) are very similar to those of GlcCer and GalCer. Thus, it would appear that dramatic changes in headgroup structure have only a small effect on the orientation and ordering of the C2 segment and hence on the aqueous interfacial region. Whether headgroup effects are manifest in changes in acyl chain dynamics remains to be seen since sufficient sample was not available to perform T_1 measurements. The only other similar study of which we are aware, dealing with natural glyceroglycolipids, arrived at essentially the same conclusion. The C2 resonances of *Acholeplasma laidlawii* B membranes enriched in [2,2-$^2H_2$]dihydrosterculic acid were well-defined with no apparent distinction between mono- and disaccharide headgroups. Since these membranes contain substantial quantities of both monoglucosyl- and diglucosyllipids, it was concluded that both lipids had very similar conformations in the C2 region of the *sn*-2 chain (Rance et al., 1983).

In contrast to the similarity in the results obtained for the GlcCer and globoside systems, the LacCer–DMPC mixture exhibits distinct differences in the quadrupolar splittings associated with the C2-position of the glycolipid acyl chain. It is unlikely that these differences arise simply from a change in molecular ordering, since inner and outer peak splittings were affected differently rather than scaled by a constant factor (Table I). For instance, at 30 °C the ratio of the outer to inner peak splittings [$\Delta\nu_Q$(o/i)] for LacCer was 5.4 versus 1.48 for GlcCer and GalCer. This implies that the orientation of the C2 methylene group of LacCer in a phospholipid membrane is significantly different from that found in the other two glycolipids. It is also interesting that, for a given glycolipid, the ratio of outer to inner peak splittings was a function of temperature in fluid membranes. If the only effect of increasing temperature was a decrease in molecular order, leaving the orientation of the C2 methylene segment unchanged, then one would expect that $\Delta\nu_Q$(o/i) would remain constant with temperature variation. Instead, all four species display alterations in this ratio, indicating temperature-dependent orientational changes. This is particularly clear for LacCer, where the splittings changed in opposite directions.

Because of the difficulty in isolating a sufficient quantity of natural LacCer for NMR studies, we elected to modify the commercially available *N*-lignoceroyldihydrolactosylceramide. Previous studies have demonstrated that dihydro and natural LacCer have similar properties. Thus, essentially identical phase-transition temperatures of 74.4 ± 0.1 and 74.2 ± 0.1 °C have been reported for LacCer and *N*-stearoyldihydrolactosylsphingosine, respectively (Maggio et al., 1985). In contrast, changing the headgroup from a mono- to a disaccharide has a dramatic effect on the phase-transition temperature. *N*-Stearoyldihydroglucosylsphingosine and *N*-stearoyldihydrogalactosylsphingosine have similar transition temperatures of 84.9 ± 0.1 and 83.7 ± 0.1 °C, respectively, about 10 °C higher than that of the corresponding lactosyl derivative (given above). One might speculate on the basis of these studies that any effects caused by the loss of the double bond in the sphingosine residue would be much less important than effects caused by the increased size of the headgroup. However, the results with globoside indicate that changing the glycolipid headgroup from a mono- to a tetrasaccharide has only minor effects on the orientational properties of the associated lipid as monitored at the C2-position of the ceramide acyl chain. This suggests strongly that the changes observed in the LacCer system result predominantly from the loss of the olefinic residue in the sphingosine backbone.

While the residual quadrupolar splitting values give information on motional amplitudes, spin–lattice relaxation provides insight into molecular motion with rates near the Larmor frequency (ω_0). The similarity in T_1 values observed for GalCer and GlcCer indicates that the correlation times τ_c, of acyl chain motion are very similar for the two systems at both 30 and 50 °C. The increase in T_1 with temperature indicates that the motions are in the short correlation time regime ($\omega_0^2\tau_c^2 < 1$), as observed for glyceroglycolipids with glucose and mannose headgroups (Jarrell et al., 1987). Of considerable interest, however, is the *magnitude* of the relaxation times, which are much shorter than those measured at equivalent positions in phospholipids. T_1 values for the 2-position of the *sn*-2 chain of DMPC or DPPC fall in the range of 20–30 ms (Davis, 1979, 1983), which indicates a reduced mobility of the cerebroside chain compared to that of its host phospholipid. Our results are consistent with relaxation time measurements performed on *A. laidlawii* B membranes (predominantly glyceroglycolipids) enriched in [2,2-$^2H_2$]oleic acid, for which the T_1 values were on the order of 10 ms (Rance, 1980). The motional rates of glyceroglycolipid headgroups and the glycerol C3-position are also significantly slower than those of most phospholipids, with T_1's in the 4–6-ms range (Jarrell et al., 1987; Carrier et al., 1989; Auger et al., 1990). The reduced mobility of the carbohydrate headgroup thus may affect the mobility of the upper portion of the acyl chain, assuming that the rates of headgroup motion in GSLs are as slow as in glyceroglycolipids. Another possible source of the reduced chain mobility is interdigitation of the glycolipid acyl chain among the shorter acyl chains of the DMPC matrix, a phenomenon that we have suggested may occur in similar systems on the basis of both EPR (Grant et al., 1987; Mehlhorn et al., 1988) and ^2H NMR (Florio et al., 1990) observations. To the best of our knowledge, the T_1 values reported herein are among the shortest measured for the acyl chains of any lipid class.

The T_1 values obtained for LacCer at 30 and 50 °C are extremely short as observed for GlcCer and GalCer. However, unlike the latter systems, the T_1's decrease with increasing temperature, going from 7.1 ± 0.3 ms at 30 °C to 5.6 ± 0.2 ms at 50 °C. This is consistent with acyl chain motion in the long correlation time regime ($\omega_0^2\tau_c^2 > 1$) and implies that the acyl chain mobility at the 2-position is reduced in LacCer. It is of interest to note that, in a ^2H NMR study of a lactosylglyceroglycolipid, the headgroup motions were in the fast motional regime ($\omega_0^2\tau_c^2 < 1$) (Renou et al., 1989). Of the glycolipids studied to date, only gentibiose, with a disaccharide headgroup of two glucose residues in a $\beta1\rightarrow6$ linkage, was found to have headgroup motions in the slow motional regime (Carrier et al., 1989). It would be of interest to investigate whether this trend of decreasing mobility with increasing headgroup size is maintained by the larger headgroup of globoside.

The current results are of particular interest in that a lack of coupling between headgroup and acyl chains appears to be a general property of many membrane lipids. For a variety of phospholipids, with phase-transition temperatures ranging from -5 to 90 °C, the quadrupolar splittings of the C2 deuterons of both the *sn*-1 and *sn*-2 chains all fall on a relatively smooth curve when plotted as a function of reduced temperature (Seelig & Browning, 1978). Thus, variation in phospholipid headgroup structure is not observed at the C2 deuterons. A question remaining is whether there are significant differences in orientation between phospholipids and gluco- and galactolipids, since the magnitude of the C2 quadrupolar splittings is greater for the latter. A way to ascertain this is to compare the ratio of the two C2 splittings at a similar reduced temperature. If the orientation is similar but the molecular ordering differs, then the two splittings should be scaled by a constant factor. Thus, the ratio of the outer to inner splitting for DPPE at 68 °C (5 °C above T_m) is 1.43 (Seelig & Browning, 1978), which compares favorably with our results for GalCer and GlcCer at 30 °C for which the ratio is 1.48. The larger splittings observed for the glycolipids may result from increased molecular ordering. Similar results have been observed in membranes of *A. laidlawii* B (Rance et al., 1983), where phospholipid and glyceroglycolipid C2 ratios of 1.36 and 1.26, respectively, were obtained from membranes biosynthetically enriched with [2,2-$^2H_2$]oleic acid.

To summarize, the current study reveals essentially identical ordering, orientation, and dynamics at the 2-position of the fatty acyl chain of GalCer and GlcCer in a liquid-crystalline DMPC matrix. Preliminary results with globoside suggest a

similar orientation and ordering of this segment. However, in LacCer the orientation of this segment was altered, most likely resulting from the loss of the double bond at the 4,5-position of the sphingosine backbone. The rate of molecular motion of LacCer is slower than that of GlcCer and GalCer. However, the present results do not allow the origin of this effect to be attributed solely to the larger headgroup of LacCer. Corresponding motional studies on globoside and other complex glycosphingolipids would help to better delineate the influence of the carbohydrate residues on lipid motion. In all cases, the acyl chain mobility was found to be strikingly slow compared to that of phospholipids. All of the glycolipids were found to disperse in liquid-crystalline phospholipid bilayers, and no evidence of phase separation or clustering was observed. The results suggest that variations in glycosphingolipid headgroups have little effect on the orientational properties of the acyl chains, even at the 2-position.

Registry No. DMPC, 18194-24-6; gal cer, 85305-88-0; lac cer, 4682-48-8; glc cer, 85305-87-9.

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