Effect of Glycosphingolipid Fatty Acid Chain Length on Behavior in Unsaturated Phosphatidylcholine Bilayers: A ^2H NMR Study†

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ABSTRACT: Deuterium-labeled glycosphingolipids, *N*-lignoceroyl[d_{47}]galactosylceramide (24:0 fatty acid form) and *N*-stearoyl[d_{35}]galactosylceramide (18:0 fatty acid form) were prepared by partial synthesis. These probe-labeled species, differing only in the lengths of their fatty acids, were compared via ^2H NMR with regard to arrangement and behavior in bilayers of the monounsaturated phospholipid 1-stearoyl-2-oleoylphosphatidylcholine (SOPC). Results were used to consider the physical significance of the great range of common acyl chain lengths that is a frequent feature of cell membrane glycosphingolipids. *N*-lignoceroyl[d_{47}]- and *N*-stearoyl[d_{35}]galactosylceramide were incorporated at concentrations ranging from 5 to 50 mol % into unsonicated phospholipid liposomes, and their spectra were analyzed in the range +73 to −14 °C. For the 18:0 fatty acid derivative, first spectral moments, M_1, were calculated and plotted as a function of temperature for each sample composition. Spectral inspection for regions of phase coexistence, in conjunction with consideration of M_1 curves, permitted derivation of phase diagram boundaries which were then refined using spectral subtraction techniques. The phase diagram for galactosylceramide with short fatty acid in SOPC was compared to the corresponding phase diagram for its long-chain analogue, derived previously in the same fashion [Morrow, M. R., Singh, D., Lu, D., & Grant, C. W. M. (1992) *Biochim. Biophys. Acta 1106*, 85–93]. The binary phase diagrams referred to above, which reflect the behavior of short- and long-chain glycolipids in a common phospholipid host matrix, displayed important similarities and differences. In fluid membranes, the behavior appeared to be remarkably alike, as reflected in superimposable fluidus curves over the concentration range studied. Similarity was also seen in gel phase membranes at low glycolipid concentration (<20 mol %), although the long-chain species demonstrated greater capacity to stabilize the gel phase membrane against the gel/fluid transition. At higher mole fractions (>20 mol %), the long-chain glycolipid displayed a much greater tendency to gel/fluid phase separation and also to gel phase immiscibility which was not seen for the short-chain analogue. Both glycolipids were miscible in the liquid-crystal phase. A tendency for glycosphingolipid to order phosphatidylcholine membranes was evident in all samples. Partial synthesis of *N*-lignoceroyl[d_7]-galactosylceramide, selectively deuterated on carbon atoms near the methyl terminus, permitted analysis of the novel ^2H NMR spectrum of its perdeuterated analogue and refinement of its phase diagram. The availability of an essentially complete spectral analysis also permitted derivation and comparison of order parameter profiles for short- and long-chain glycolipid fatty acids in the fluid 18-carbon fatty acid host matrix. The gradient of order parameter with respect to distance from the sugar headgroup for the 24-carbon glycolipid was comparable to that for the 18-carbon analogue to the depth of C_{14}, beyond which point its slope became sharply reduced.

Glycosphingolipids (GSLs),[1] the carbohydrate-bearing lipids of higher animal cells, are known to be involved as recognition sites and structural elements of the plasma membrane. It is considered that GSL physical arrangement within the membrane, as determined by their molecular features, may be an important modulator of their phenotypic expression as recognition sites, in addition to being a prime determinant of their structural influence [for reviews, see Hakomori (1981), Thompson and Tillack (1985), Curatolo (1987), and Grant (1987)]. The (membrane-inserted) ceramide portion of the GSL has been repeatedly identified as a major contributor to such events in cells and model membranes (Alving et al., 1980; Yoshino et al., 1982; Kannagi et al., 1982; Crook et al., 1986). Since the sphingosine portion of the ceramide backbone is often fairly homogeneous, a key variable feature that may determine receptor function is the fatty acid, which commonly ranges from 18 to 24 and 26 carbon atoms in sharp contrast to the 16- and 18-carbon species that typify membrane phospholipids. In pursuing the significance of this phenomenon, Carl Alving and co-workers were able to quantitate the effect of glycolipid fatty acid chain length on antibody binding to galactosylceramide (GC) in phosphatidylcholine liposomes and suggested that stronger immune interaction with longer chain species was due to relatively greater carbohydrate headgroup protrusion from the membrane, resulting from mismatch of the long fatty acid chain with the host matrix (Alving & Richards, 1977; Alving et al., 1980). This has come to be accepted as a fundamental example of receptor crypticity. Very similar experiments have been carried out more recently using sulfated GC, with apparently related observations (Crook et al., 1986). It has been suggested that an alternative contributor to fatty acid

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[1] Abbreviations: GSL, glycosphingolipid; SOPC, 1-stearoyl-2-oleoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; 18:0 GC, *N*-stearoylgalactosylceramide; 24:0 GC, *N*-lignoceroylgalactosylceramide.

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length effects is their modulation of glycolipid lateral distribution in membranes, determined by fatty acid chain length mistmatch (Utsumi et al., 1984). In addition, it seems likely that dynamic processes involving surface receptors for macromolecules contribute to their receptor function (Brulet & McConnell, 1977; Balakrishnan et al., 1982; Petrossian & Owicki, 1984).

There have now been a number of studies of the phase behavior of GSLs possessing single pure fatty acids in phospholipid membranes, making it possible to specifically consider the effects of glycolipid fatty acid nature on their membrane behavior. A phase diagram derived for GC with 16-carbon saturated fatty acid (16:0 GC) in a phospholipid host matrix with the same fatty acids, (dipalmitoylphosphatidylcholine (DPPC)) is characterized by solid-phase immiscibility and a considerable temperature range of fluid/gel phase coexistence at GSL fractions greater than 0.2 (Ruocco et al., 1983). The corresponding DSC-derived phase diagram for 24:0 GC in DPPC displays features of solid-phase immiscibility over the entire composition range (Gardam & Silvius, 1989). Gardam and Silvius concluded from their calorimetry experiments with 24:0 GC and sulfated 24:0 GC in DPPC that 24:0 GSLs are not markedly more prone to segregate laterally in phosphatidylcholine-rich bilayers than are their shorter chain analogues reported in the literature. Moreover, in a study of four families of GSLs with spin-labeled fatty acids incorporated at less than 10 mol % in several host matrices, we were able to measure no differential phase separation for 18- vs 24-carbon species (Mehlhorn et al., 1989). However, recent monolayer studies of GC indicate that unsaturated fatty acids increase GSL miscibility with DPPC (Ali et al., 1989).

GC has been of particular interest to immunologists and physical biochemists because of its nature as a fundamental GSL. Properties of pure 24:0 GC and 16:0 GC in fully hydrated form are known from DSC and X-ray diffraction work (Ruocco et al., 1983; Curatolo & Jungalwala, 1985; Reed & Shipley, 1987; Gardam & Silvius, 1989). Both 24:0 GC and 16:0 GC display fluid/gel main transitions in the range 82–85 °C and exhibit metastable polymorphism at lower temperatures. In a previous paper, we explored the applicability of ^{2}H NMR to analysis of the phase behavior of GC having 24-carbon (24:0) fatty acid in phospholipid bilayer systems (Morrow et al., 1992). The host matrix chosen was SOPC, a monounsaturated phosphatidylcholine having 18-carbon fatty acids and a fluid/gel transition temperature of 6 °C (Davis & Keough, 1985). Pairing of a GSL having longer chain with a typical membrane phospholipid represents a model for common natural scenarios. The results were used to infer a partial temperature–composition phase diagram. For GSL concentrations less than 20 mol %, the two lipids were found to be miscible in both the liquid-crystal and ordered phases, except in a small temeprature range over which fluid and gel phases coexist. Above the three-phase line, liquid crystal was found to coexist with a GSL-rich ordered phase over a wide range of temperatures.

In the present work, we have extended the NMR approach, employing 18:0 GC and 24:0 GC deuterated in the fatty acid chain, to examine the extent to which GSL fatty acid chain length determines phase behavior in an SOPC matrix and to better characterize the factors underlying lipid organization in such systems. The partial phase diagram obtained for the 18:0 GC in SOPC was compared to that for the corresponding system with the longer chain (24:0) GC. Location of the nonperturbing spectroscopic probe on the glycolipid itself permitted us to examine, with high sensitivity, samples in which GSLs were minor membrane components—a biologically important scenario. Since the assignment of ^{2}H NMR wide-line spectra for perdeuterated long-chain fatty acids in shorter chain host matrices has not been reported previously, several questions that arose regarding interpretation of the 24:0 GC/SOPC system were addressed by examining mixtures prepared with 24:0 GC specifically deuterated at the fatty acid methyl group and adjacent two methylenes. The availability of line assignments for spectra corresponding to perdeuterated 24:0 GC in SOPC made it possible to compare the order parameter profiles for the 24:0 and 18:0 GC fatty acids in the GSL/SOPC system.

MATERIALS AND METHODS

Partial synthesis of *N*-lignoceroyl[d_{47}]GC (24:0[d_{47}]GC) and sources of 1-stearoyl-2-oleoylphosphatidylcholine (SOPC), beef brain galactosyl ceramide (GC), and lignoceric[d_{47}] acid were as described previously (Morrow et al., 1992). Synthesis, characterization, and purification of *N*-stearoyl[d_{35}]GC (18:0[d_{35}]GC) were via routes similar to those involved with the long-chain analogue: 18 mg (56 μmol) of perdeuterated stearic acid (Aldrich) was converted to the acid chloride and subsequently combined with 27 mg (61 μmol) of lyso GC (Kopakczyk & Radin, 1965). Yield was 60% [26 mg (34 μmol)] of deuterated GSL. Deuterated 24:0 GC's comigrated in the TLC system described above with the faster-running spot of native beef brain GC (the "non-hydroxy" fraction), while the 18:0 derivative ran just beneath it. The specifically deuterated 24-carbon derivative, *N*-lignoceroyl[d_7]GC (24:0[d_7]GC), was prepared by coupling lignoceric acid deuterated on the terminal methyl and the two adjacent methylenes using the procedure employed for the perdeuterated analogues.

Lignoceric[ω-d_7] *Acid.* 1,20-Eicosanedioic acid dimethyl ester (TCI, Tokyo) was refluxed with $Ba(OH)_2$ in dry methanol, followed by extraction with the addition of HCl/ether (Durham et al., 1967a). After removal of the ether, the resulting monomethyl ester derivative was refluxed with $SOCl_2$ to produce the corresponding acid chloride. The organocadmium product of 1-bromobutane[d_9](MSD Isotopes) was linked with the above acid chloride (Hubbel & McConnell, 1971) to produce a 24-carbon fatty acid monomethyl ester with ketone group at C_{20}. The ketone function was reduced via reaction with hydrazine monohydrate (Aldrich)/potassium hydroxide (Durham et al., 1967b). The final product was purified by silicic acid column chromatography eluting with $CHCl_3$. Reactions were followed by thin-layer chromatography (Merck Silica Gel 60), eluting with 20:30:1 hexane/ether/formic acid. Identification was confirmed with mass spectrometry and ^{1}H NMR.

Fully hydrated lipid bilayer structures containing measured amounts of deuterated GC in SOPC were prepared as described elsewhere (Morrow et al., 1992). Samples with the highest deuterium concentrations contained between 15 and 17 mg of labeled lipid. Samples (10 and 5 mol %) contained 5–8 mg because of the limited sample tube volume. ^{2}H NMR spectra for phase diagram generation were collected from 73 to −14 °C, generally at 3 °C intervals. For the lowest four concentrations, the interval was reduced to 1 °C in the region of two-phase coexistence. Precautions taken to minimize the presence of metastable GC phases (Ruocco et al., 1983; Gardam & Silvius, 1989; Reed & Shipley, 1987, 1989) were described in earlier work, as were technical details of the ^{2}H NMR spectroscopy involved (Morrow et al., 1992). For the sample having selectively deuterated fatty acid chains, oversampling was by a factor of 4 so that the effective digitizer dwell times were further doubled. ^{2}H NMR difference

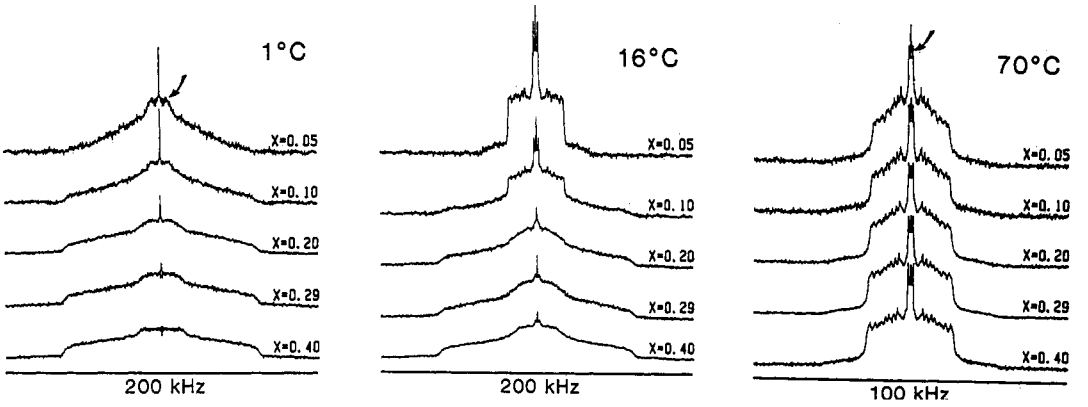

FIGURE 1: Concentration dependence of ^{2}H NMR spectra for *N*-stearoyl[d_{35}]galactosylceramide (18:0[d_{35}]GC) in SOPC at the temperatures 1, 16, and 70 °C. Spectra are arranged in vertical groups with glycolipid mole fraction and temperature indicated. Curved arrows indicate features associated with the fatty acid terminal deuteromethyl group for the 1 °C (gel phase) and 70 °C (fluid phase) 0.05 mole fraction samples.

spectroscopy (Huschilt et al., 1985; Vist & Davis, 1990; Morrow et al., 1991) was used to determine tie line endpoints in selected two-phase regions of the phase diagram for the GC/SOPC mixtures. The dependence of orientational order on position along the GSL fatty acid chain for selected samples was examined using smoothed order parameter profiles (Sternin et al., 1988; Lafleur et al., 1989) obtained from "de-Paked" spectra (Bloom et al., 1981).

RESULTS

The initial stage of the present study was to derive the phase diagram for galactosylceramide with relatively short saturated fatty acid (18:0[d_{35}]GC) in SOPC, employing ^{2}H NMR techniques applied previously to 24:0[d_{47}]GC in the same phospholipid matrix (Morrow et al., 1992). Line shape and splittings of the deuterated glycolipid spectra were used to identify spectral characteristics corresponding to single-phase and two-phase coexistence regions of the binary phase diagram. The preliminary phase diagram derived in this way was then refined by ^{2}H NMR difference spectroscopy.

Figure 1 displays representative spectra of perdeuterated 18:0[d_{35}]GC in SOPC for a series of glycolipid mole fractions at three temperatures. Each spectrum can be identified as corresponding to gel phase, liquid-crystal phase, or a superposition of spectral components for these phases. In the 1 °C group, all of the samples are in the gel phase and display broad spectral features well known for acyl-chain-deuterated phospholipids (Seelig, 1977; Davis, 1983; Smith, 1984) and also reported for GSLs (Skarjune & Oldfield, 1979; Florio et al., 1990; Morrow et al., 1992) below their main transition temperatures. At 16 °C, the spectrum for the 10 mol % GSL sample demonstrates a component consisting of sharp powder (Pake) doublets characteristic of the liquid-crystal phase and indicating rapid axially symmetric motion of the glycolipid labeled chain. At incrementally higher temperatures, this fluid phase feature became evident in the spectra of samples having progressively higher glycolipid content and accounted for a larger proportion of each sample (data not shown). At 70 °C, all samples display only liquid-crystal spectra. There was no evidence in any of the spectra for the coexistence of two distinct ordered phases such as was found for 24:0 GC in SOPC (Morrow et al., 1992). Spectra in Figure 1 appear to demonstrate that GSL chain order, as reflected in the magnitude of quadrupole splitting, increases with GSL concentration. This is particularly evident for the methyl deuteron splittings (curved arrows in 1 and 70 °C series).

Figure 2 shows the temperature dependence of the first spectral moment, M_1, for five 18:0 GC concentrations. This

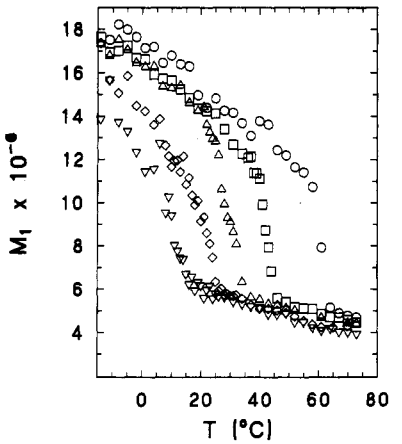

FIGURE 2: Temperature dependence of the first spectral moment, M_1, for *N*-stearoyl[d_{35}]galactosylceramide in SOPC at mole fractions x = 0.53 (○), x = 0.35 (□), x = 0.24 (△), x = 0.10 (◇), and x = 0.05 (▽).

parameter is proportional to the weighted average of the spectral splittings (Davis, 1983) and generally increases with chain order. In this form of data analysis, it is evident that orientational ordering in gel and liquid-crystal phases is greater for samples with higher glycolipid concentration. The temperature range over which M_1 rises sharply provides a rough estimate of the extent of two-phase coexistence for each sample. Along with inspection of the spectra for each sample, this provided a first approximation to the phase diagram. The boundaries of two-phase coexistence were refined and extended using spectral subtraction to extract spectral components corresponding to endpoint concentrations at a given temperature (Huschilt et al., 1985; Vist & Davis, 1990; Morrow et al., 1991, 1992). This approach assumes that spectra in the two-phase region are superpositions of spectra corresponding to the coexisting endpoint compositions with proportions determined by the lever rule. An example with particular relevance to the comparison made in the present work is shown in Figure 3. It depicts spectral subtractions used in defining phase diagram features showing a subtraction at 31 °C: A and B being the observed spectra. The spectrum in Figure 3C is A – (0.35)B giving an endpoint at x_f = 0.16, and Figure 3D is B – (0.20)A, giving an endpoint at x_g = 0.52. The solidus endpoint value is half that previously determined for the long-chain glycolipid under similar conditions (vida infra) and imposes a nonzero slope on the solidus.

Figure 4 shows the resultant phase diagram for 18:0 GC in SOPC, together with the corresponding phase diagram for 24:0 GC [data points are taken from Morrow et al. (1992)]

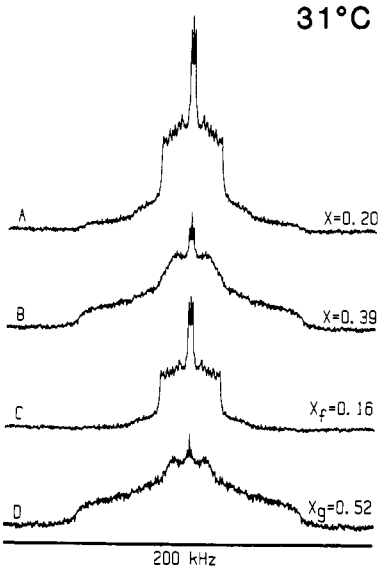

FIGURE 3: Example of spectral subtraction testing the possibility of a horizontal solidus at low temperature for 18:0 GC in SOPC. Data are shown for 31 °C. Spectra A and B are observed spectra. Spectrum C is A – (0.35)B, giving an endpoint at x_f = 0.16 (fluidus point); spectrum D is B – (0.20)A, giving an endpoint at x_g = 0.52 (solidus point).

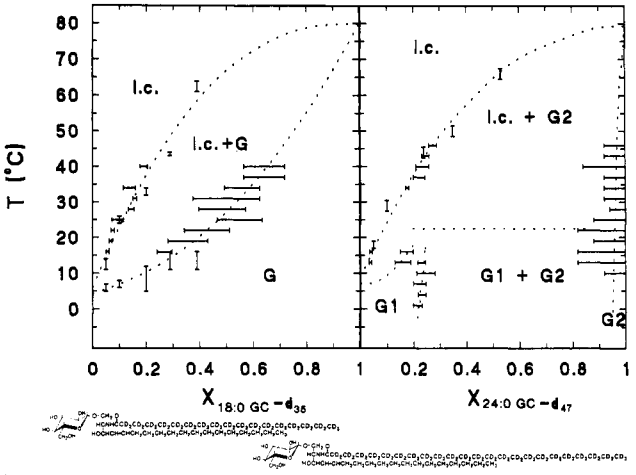

FIGURE 4: Proposed phase diagrams for (A) *N*-stearoylgalactosylceramide (18:0 GC) and (B) *N*-ligoceroylgalactosylceramide (24:0 GC) in SOPC. The structures of the glycolipids involved are illustrated as inserts below their corresponding phase diagrams. Vertical error bars represent estimated range of uncertainty in boundary crossings determined by inspection of spectra and consideration of M_1. Horizontal bars indicate the range of experimental values obtained using spectral subtraction techniques with various spectral combinations. The structures of the glycolipids are indicated as inserts to their corresponding plots. Primary data points used in construction panel B are from Morrow et al. (1992).

in the same SOPC matrix. Dashed lines are projected probable phase diagram boundaries based on the experimental data. Comparison of Figure 4 panels A and B indicates that the glycolipid which has the same chain length as the SOPC host matrix displays greater miscibility than does the glycolipid which has a very long fatty acid. Above 20 mol % glycolipid there is a marked difference between the behaviors of the two glycolipids in the region of the solidus: ordered phase miscibility exists for 18:0 GC in SOPC, while ordered phase immiscibility is seen for 24:0 GC. However, striking similarity is evident in the points determining the fluidus curves, which are superimposable within experimental error. Moreover, at glycolipid concentrations of less than 20 mol %, the solidus behavior differs only quantitatively, being higher for 24:0 GC.

In order to better understand the factors involved in determining the phase diagram characteristics, spectral features of 24:0 GC and 18:0 GC were examined in detail. Figures 5 panels A and B display liquid-crystal phase spectra for 10 mol % 24:0[d_{47}]GC and 18:0[d_{35}]GC, respectively, in SOPC. Figure 5 panels C and D show spectra for the same deuterated GSLs in SOPC in the two-phase coexistence and gel phase regions of the phase diagrams. While the spectra in Figure 5A,B are characteristic of axially symmetric chain motion, as expected for a perdeuterated acyl chain in a liquid-crystalline phase (Seelig, 1977; Davis, 1983; Smith, 1984; Florio et al., 1990), the patterns of intensity for the two samples are very different. In particular, there is relatively more intensity in the central region of the fluid phase spectra for the sample containing the longer chain GC (Figure 5B). The shape of the 24:0 GC perdeuterated chain spectrum suggests that the orientational order parameter profile for this long-chain glycosphingolipid in a shorter chain host matrix is unusual toward the methyl terminus. The splittings for the most ordered deuterons (those toward the polar headgroup) are similar for the sample containing 24:0 GC and that containing 18:0 GC.

Given the differences in the phase diagrams for the two glycolipids, there are potential difficulties in comparing the spectra shown in Figure 5C,D at a given temperature. At 10 and 22 °C, the spectra for 10 mol % 18:0 GC in SOPC (Figure 5D) are probably superpositions of liquid-crystal and gel phase spectra. For 24:0 GC in SOPC at 10 °C and corresponding concentration (Figure 5C), other possibilities must be considered in light of the complexity of the phase diagram in this region. However, on the basis of consideration of the spectra of a selectively deuterated analogue under similar conditions (see below), it was possible to determine that the spectrum for 10 mol % 24:0 GC at 10 °C was more consistent with coexistence of a phospholipid-enriched liquid-crystal phase and a GSL-enriched ordered phase than with coexistence of a GSL-rich ordered phase (G_2) and another ordered phase (G_1) having lower GSL concentration. This confirms related aspects of the 24:0 GC/SOPC phase diagram reported previously (Morrow et al., 1992).

For 10 mol % 24:0 GC and 10 mol % 18:0 GC at 4 and –2 °C (Figure 5C,D), the spectra are probably single-component gel (ordered) phase spectra. The gel phase spectra for the sample containing the shorter chain glycolipid (Figure 5D) are similar to gel phase spectra from single-component bilayers of perdeuterated diacylphosphatidylcholines such as DPPC and DSPC. In particular, there is a prominent feature with a width of about 13 kHz (curved arrow) which corresponds to the methyl group in the ordered bilayer. The prominent edges at about ±64 kHz (i.e., splitting 128 kHz) in the corresponding spectra of the long-chain glycolipid suggest a nearly rigid lattice (Davis, 1983).

On the basis of order parameter profiles that are well known for phospholipids (Seelig, 1977; Davis, 1983; Smith, 1984), and to a lesser extent for glycosphingolipids (Sharom & Grant, 1977; Skarjune & Oldfield, 1979; Florio et al., 1990), one would normally assign the spectral peaks to corresponding CD_2 groups by considering the central splitting (Pake doublet) of each spectrum as arising from the terminal CD_3 and by working outward, assigning the splittings in order of increasing magnitude to the next carbon in the acyl chain until the largest splittings have been assigned to CD_2 groups near the COOH terminus. However, such studies have all been carried out in membranes in which the fatty acid in question closely matched the average chain length of the host membrane. In order to confirm that the segment of the 24-carbon chain giving rise

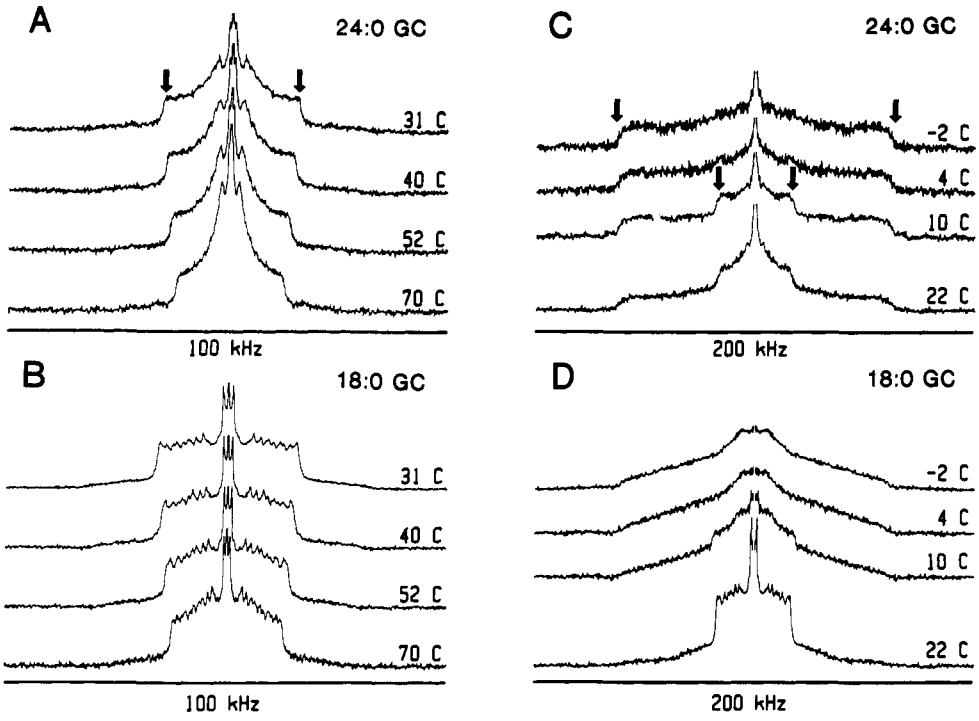

FIGURE 5: Comparison of spectral features between 24:0[d_{47}]- and 18:0[d_{35}]GC at low concentration in phospholipid bilayer membranes. (A and B) Liquid-crystal phase spectra for 10 mol % 24:0[d_{47}]GC and 18:0[d_{35}]GC, respectively, in SOPC. (C and D) Spectra for the same deuterated GSLs in SOPC in the two-phase coexistence and gel phase regions of their phase diagrams. For 10 mol % 18:0 GC in SOPC, the spectra at 22 and 10° C are probably superpositions of liquid-crystal and gel phase spectra. For both samples, the spectra at 4 and −2 °C are probably pure ordered phase spectra. Paired arrows indicate prominent spectral features of width ~34 and ~128 kHz described in the text.

to the smallest splittings did indeed correspond to the methyl terminus, 24:0[d_7]GC was prepared with deuterium labels substituted for protons only on the methyl group and the two methylene carbons immediately above it (C_{23} and C_{22}). Figure 6A shows spectra for 8 mol % 24:0[d_7]GC in SOPC for a range of membrane conditions. Figure 6B shows a comparison of the selective label spectra to corresponding spectra obtained with the long-chain perdeuterated species (i.e., 24:0[d_{47}]GC). In all cases, the spectra of the terminal methyl group and its two nearest methylenes appear to account for the spectral components with the smallest quadrupolar splittings in the perdeuterated chain spectra, confirming that orientational order likely decreases monotonically toward the methyl end of the chain despite the considerable mismatch in the 24:0 GC and SOPC chain lengths.

The spectra of Figure 6B also permit one to address an important point regarding the phase behavior of the 24:0 GC/SOPC system in a manner which was not possible using only the perdeuterated chain glycolipid. In the spectrum representing GC with perdeuterated (d_{47}) fatty acid in SOPC at 10 °C, there is a prominent feature with edges near ±17 kHz (arrows). This is similar both to the plateau splitting in the liquid-crystalline phase of 24:0[d_{47}]GC in SOPC (e.g., Figure 5A at 31 °C) and to the width of the methyl feature in the glycolipid-rich gel phase of the same mixture (Morrow et al., 1992). Other evidence was used in earlier work (Morrow et al., 1992) to place the 10 mol % 24:0[d_{47}]GC sample, at 10 °C, in the region of the phase diagram corresponding to an ordered phase poor in glycolipid. The spectra in Figure 6B confirm the earlier interpretation of the phase diagram and resolve the apparent difficulty with the assignment of the feature with edges at ±17 kHz. First, note that, in known liquid-crystalline phase spectra of the 24:0[d_7]GC (e.g., 40 or 70 °C in Figure 6B), the splittings for the methylene deuterons near the methyl terminus remain much smaller than the largest liquid-crystal splittings (which correspond to the plateau deuterons) in the perdeuterated (d_{47}) GC spectra. At 10 °C, in the specifically labeled lipid spectrum, deuterons on the two carbons nearest the methyl group give rise to a feature with a width of about 34 kHz. This feature, although clearly not from a liquid sample, is somewhat narrower than would be expected for methylene deuterons in an ordered phase of uniform chain length and is presumably a consequence of the low orientational order at the methyl end of the 24-carbon chain in the predominantly SOPC bilayer. It is significant though that in the 10 °C specific label (24:0[d_7]GC) spectrum there is no evidence of the small splittings which would be associated with a coexisting liquid-crystal phase. In consequence, the feature in the 10 °C perdeuterated lipid spectrum having a width of about 34 kHz cannot be due to coexisting liquid-crystalline domains and must arise either from the methyl group in a 24:0 GC-rich highly ordered (G_2) phase or from methylene deuterons near the methyl end of the chain in the less ordered (G_1) phase. Since it displays the largest splitting in the spectrum of the sample with selectively located labels, the 34-kHz wide feature must correspond to the methylene groups near the methyl terminus in a gel phase. This is not consistent with the presence of substantial amounts of 24:0 GC-rich (G_2) phase. Thus, by exclusion, it is clear that under these conditions of temperature and composition the sample is in the 24:0 GC-poor ordered (G_1) phase.

Having assigned the innermost doublets for 24:0[d_{47}]GC in the SOPC matrix using the selectively deuterated analogue, it is possible to construct order parameter profiles for a lipid with very long fatty acid in a host membrane having shorter fatty acids. Smoothed order parameter profiles were obtained using the method of Sternin et al. (1988) and Lafleur et al. (1989). A typical result is illustrated in Figure 7 along with the corresponding profile for the 18:0 derivative. Examination of the order parameter profiles for 18:0 GC and 24:0 GC at 10 mol % in SOPC provides some insight into the basis of the similarities and differences noted above regarding their

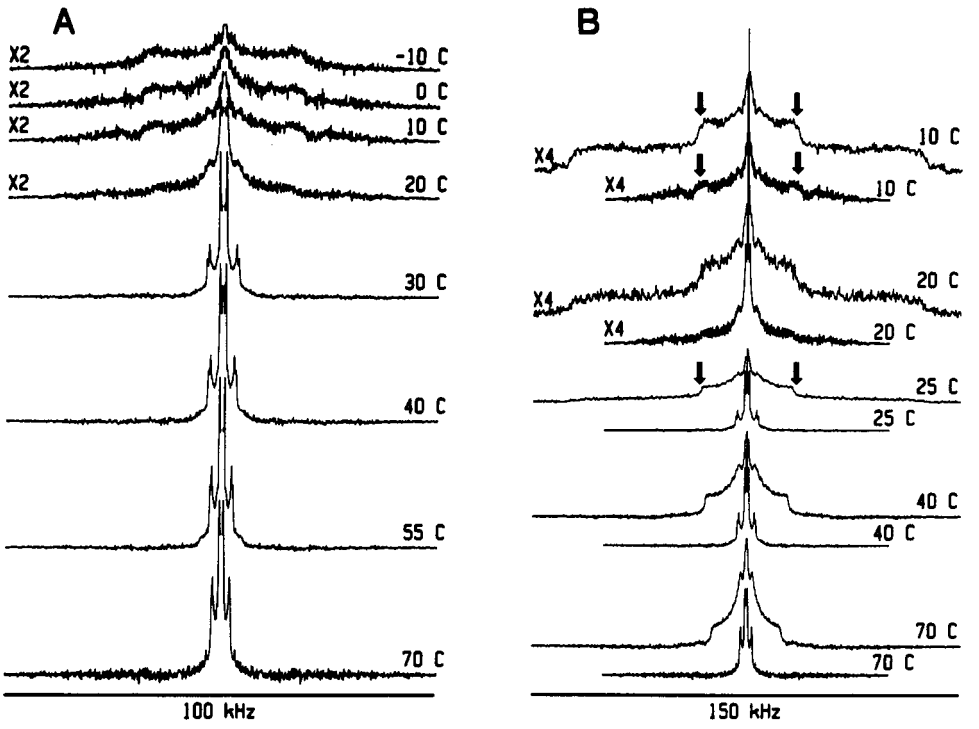

FIGURE 6: Comparison of samples containing *N*-lignoceroyl[d_7]GC (24:0[d_7]GC) (i.e., specifically labeled on the fatty acid terminal methyl group and the two adjacent methylene groups), with corresponding samples in which the long-chain fatty acid was perdeuterated (i.e., 24:0[d_{47}]GC), at low mol % in SOPC. This comparison makes possible determination of the contribution to the perdeuterated sample spectra, from deuterons toward the methyl terminus of the acyl chain. (A) Spectra for 24:0[d_7]GC in SOPC matrices having a wide range of fluidity properties between 10 and 70 °C. (B) Direct comparison of selected spectra for the perdeuterated and specifically labeled 24:0 GC in SOPC. The spectra of the methyl and last two methylenes appear to account for the narrowest components of the perdeuterated chain spectra. The prominent feature with edgnes near +17 kHz (splitting 34 kHz) in the 24:0 GC/SOPC spectra at 10 °C is indicated by arrows in panel B. This value is similar to the plateau splitting in the liquid-crystalline phase of 24:0[d_{47}]GC in SOPC (panel B at 25 °C) in the same mixture. Spectra shown for 24:0[d_7]GC contained 8 mg of deuterated glycolipid. GSL concentration was 10 mol %.

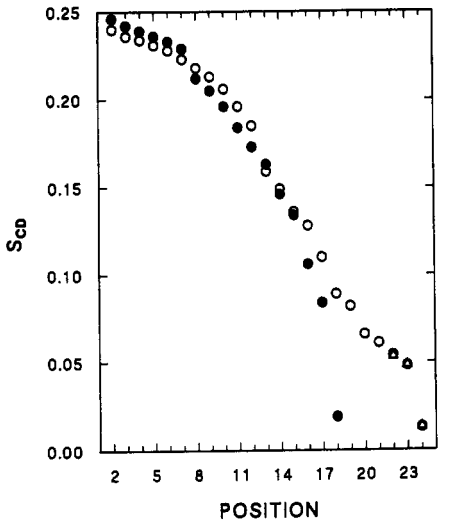

FIGURE 7: Order parameter profiles for *N*-stearoyl[d_{35}]GC (●), *N*-lignoceroyl[d_{47}]GC (○), and *N*-lignoceroyl[d_7]GC (△), all dispersed in SOPC at 10 mol %. Profiles for the 18:0 fatty acid are based on previous spectral assignments for phospholipids and glycolipids (Seelig, 1977; Skarjune & Oldfield, 1979; Davis, 1983; Smith, 1984; Florio et al., 1990). Profiles for the 24:0 analogous are based upon experiments represented by Figures 5 and 6. Smoothed order parameter profiles include C_2 for simplicity.

behavior in SOPC bilayers. It will be seen that the profiles for the upper portions of the chains (to a membrane depth of C_{14}) are very similar in these fluid membranes, in spite of the six-carbon chain length difference, while below C_{14} the order parameter profiles of the long-chain species diverge strikingly from the shape normally associated with perdeuterated phospholipid and glycolipid fatty acid chains in the (length-homogeneous) systems studied previously (Seelig, 1977; Davis, 1983; Smith, 1984; Florio et al., 1990), while that associated with the 18:0[d_{35}]GC species is typical.

DISCUSSION

A significant concept that has developed in studies of GSL function is that the length of the single glycolipid fatty acid may modulate their membrane receptor properties and intermolecular interactions. The question was approached in this work by examining GC with perdeuterated fatty acid, utilizing the two common extremes of 18- and 24-carbon species. The phospholipid chosen as host matrix, SOPC, has 18-carbon fatty acids. This in itself might be considered a basis for behavior differences between the 18:0 GC and 24:0 GC studied; for instance, one might propose relative failure to "fit" within the host matrix in the case of the long-chain species. Certainly the role of (phospholipid) fatty acid chain length in modulating membrane properties and component arrangement within phospholipid-based membranes has been widely considered (Lee, 1977b). It is well known that binary mixtures of phospholipids with fatty acids differing significantly in length tend to phase separate and that this effect is proportional to the difference in length. The situation is less thoroughly documented for binary mixtures of phospholipids in which one member has mixed chain length fatty acids (i.e., in which the *sn*-1 and *sn*-2 fatty acids are mismatched); however, phospholipid phase separation induced by intramolecular fatty acid mismatch has been documented (Mason, 1988; Gardam & Silvius, 1989). The interpretation in the case of binary phospholipid mixtures is somewhat more complicated than with glycolipid/phospholipid mixtures, since phospholipid main transition temperatures are very sensitive to chain length. As a rule one cannot alter phospholipid fatty acid chain length without altering the fluid/gel transition

temperature. 18:0 GC and 24:0 GC, on the other hand, undergo fluid/gel transitions in the same narrow temperature range [reflecting the general observation that GSL main transitions are determined primarily by carbohydrate structure (Maggio et al., 1985)]. Hence this is not a confounding factor in our study, and the results seen can be attributed to direct molecular consequences of the chain length difference rather than to secondary effects such as differences in transition temperature. The only striking difference in 18:0 GC vs 24:0 GC phase behavior in this internally controlled experiment with sensitive probes on the GSL itself was in the solidus curves. In the case of 24:0 GC in SOPC, there appear to be two types of ordered phase spectra, reflecting solid-phase immiscibility, at intermediate GSL concentrations. One of these, which we have designated G_1, is relatively enriched in SOPC. The other ordered phase, designated G_2, appears to be nearly pure 24:0 GC. For 18:0 GC in SOPC, only one ordered phase spectrum was identified over the concentration range studied.

GSLs have relatively high phase transition temperatures, in the range 45–85 °C (Maggio et al., 1985). This may be seen as a reflection of weaker overall attractive forces among phospholipids compared to glycolipids, perhaps related to greater GSL potential for hydrogen bonding (Boggs, 1987; Curatolo, 1987). The "typical" cell membrane phospholipid has a cis monounsaturated fatty acid at the *sn*-2 position, with the result that the phase transition temperature of such a lipid is generally 40–80 °C lower than that of a GSL (Lee, 1977a; Davis & Keough, 1985). SOPC exhibits a main transition at 6 °C (Davis & Keough, 1985), while both 18:0 GC and 24:0 GC undergo gel/fluid phase transitions between 82 and 85 °C (Ruocco et al., 1983; Curatolo & Jungalwala, 1985; Reed & Shipley, 1987; Gardam & Silvius, 1989). It was apparent in our phase diagrams that the degree of orientational order was greater in membranes with higher glycolipid content. This is a clear example of the phenomenon reflected in literature observations that glycolipids can increase order in phospholipid membranes (Sharom et al., 1976; Tinker et al., 1976; Sharom & Grant, 1977; Tkaçzuk & Thornton, 1979; Bertoli et al., 1981; Uchida et al., 1981). In the present work, we determined that the phenomenon occurs in both gel and liquid-crytal phases. It is interesting that the longer chain fatty acid GSL stabilized the gel phase phospholipid host matrix more at low GSL concentrations than did the shorter chain analogue. Spectra for the long-chain GC differed from those commonly associated with (shorter) chain-perdeuterated pure liquid-crystal spectra [i.e., those seen for a phosphatidylcholine (Seelig, 1977; Davis, 1983; Smith, 1984) or GSL (Florio et al., 1990) in a membrane of relatively homogeneous chain length], in that there was greater concentration of intensity toward the center of the spectrum. This type of spectrum has, however, been reported recently for small concentrations of DSPC with perdeuterated fatty acids in nondeuterated DMPC (Morrow et al., 1991), which is also a case of a longer labeled chain incorporated into a bilayer whose thickness is determined primarily by a shorter chain lipid. In the present article, we were able to demonstrate unequivocally that the source of this unusual feature is the methyl terminal end of the fatty acid chain.

There were important similarities and differences between short- and long-chain GSL behavior. The similarities were particularly strong at low GSL concentrations in the membrane and in very fluid membranes. These are presumably at the core of several recent reports in the literature that there is surprising correspondence in phase behavior between long- and short-chain GSLs. Thus, in four families of glycolipids with spin-labeled fatty acids, we saw no markedly greater tendency for the long-chain species to phase separate when dispersed at less than 10 mol % in phosphatidylcholine membranes (Mehlhorn et al., 1989). In a recent DSC study it was reported that 24:0 GC shows no evidence of being significantly more prone to segregate laterally in DPPC bilayers than is GC with a shorter fatty acid, based upon comparison with literature information (Gardam & Silvius, 1989). An analysis of GC with unsaturated fatty acid did, however, conclude that they may influence associative behavior in membranes (Ali et al., 1990), as may hydroxylation of the GSL fatty acid (Curatolo & Jungalwala, 1985; Crook et al., 1986; Johnston & Chapman, 1988).

The order parameter profiles measured here for long-chain glycolipids shed light on the source of the similarities and differences in the behavior of 18:0 and 24:0 GC. The quantitative similarity seen in the order parameter profiles along most of the chain (in the upper region of the membrane) indicates that the basic fit of the GC molecule within the fluid host matrix is the same regardless of chain length. This would account for the similarity in behavior of the molecule as a whole. In contrast, the methyl-terminal 10 carbons described a divergent curve of order parameters, not previously recorded in the literature (which deals with membranes of homogeneous chain length). Thus it is clear that this portion has importantly different motional properties that make its behavior discontinuous with the rest of the molecule. One would expect this effect to be most pronounced in gel phase membranes, i.e., in situations in which spatial crowding is at a maximum. This maybe reflected in the relative similarity of the 18:0 and 24:0 GC phase diagrams in fluid regions, where the "extra" length can be accommodated, and their dissimilarities in gel phase regions.

Glucosylceramide and sphingomyelin have been observed to be selectively distributed in the plasma membrane of a cultured intestinal epithelial cell line, and one possibly proposed was that this relative enrichment could be driven by self-aggregation of GSLs into a precursor (micro)domain in cholesterol-poor organelles prior to plasma membrane insertion (Simons & van Meer, 1988). Our results would suggest that there is not a significant driving force for GC separation in highly fluid phospholipid membranes based solely on fatty acid chain length, but in ordered membranes (e.g., membranes with very high glycolipid content at physiological temperatures) such a phenomenon could take place.

Phase diagrams have been derived previously by calorimetry for natural gangliosides from beef brain in the SOPC matrix studied here (Bunow & Bunow, 1979). Interestingly, the fatty acid composition of these complex, acidic GSLs is almost totally 18:0 when derived from a beef brain source, yet their phase behavior in SOPC resembled that found here for 24:0 GC rather than 18:0 GC. The main transition of gangliosides is much closer to that of SOPC than is the GC transition (Maggio et al., 1985b). Presumably the greater tendency of gangliosides to phase separate might be based on headgroup effects. However, Bach et al. (1982) recorded a calorimetric study of native GM_1 from the same source that showed characteristics of complete miscibility in egg phosphatidylcholine. In POPC, GC with natural fatty acid mixture (from a beef brain source) showed solid-phase immiscibility and striking phase separation of coexisting fluid (phospholipid enriched) and rigid (glycolipid enriched) domains over a wide range of temperature and composition, extending to membranes with very low glycolipid content (Curatolo, 1986). GC from this source has a high proportion of long chain fatty acids, and α-OH fatty acids. The phase diagram determined

by us for 24:0 GC in SOPC is reminiscent of that found by Shipley and co-workers for GC with 16:0 fatty acid in DPPC: the latter showed a horizontal solidus above GSL mole fraction 0.2, solid-phase miscibility below this GSL concentration, and peritectic behavior at GSL mole fractions in the range 0.2–0.3 (Ruocco et al., 1983). A phase diagram for GC with longer chain (24:0) fatty acid in the same (DPPC) matrix did not show solid-phase miscibility at low GSL concentrations (Gardam & Silvius, 1989). This apparent difference in longer chain GC is in the same direction seen in the present work.

CONCLUSIONS

In this internally controlled study of 18:0 and 24:0 GC in SOPC bilayers, there was a significant behavioral difference based on glycolipid fatty acid chain length. There was also important behavioral similarity. Differences were in the direction of increased tendency to phase separation for the longer chain species, particularly in ordered membranes. This tendency to long-chain glycolipid self-association was reduced in membranes containing smaller quantities of glycolipid, which is the most common situation in plasma membranes of cells. The similarity of behavior was striking in highly fluid membranes. The source of differences between 24:0 GC and 18:0 GC seemed to be somewhat localized to the methyl-terminal 10 carbon atoms of the fatty acid, in that the orientation and order were similar to a membrane depth of 14 carbons, diverging markedly beyond that point. The order parameter profile established here for a glycolipid with long-chain fatty acid in a membrane matrix rich in phosphatidylcholine having shorter fatty acids (a common natural scenario) differs strikingly from familiar literature profiles for length-homogeneous membranes.

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