Glycosphingolipid Backbone Conformation and Behavior in Cholesterol-Containing Phospholipid Bilayers†

Katherine S. Hamilton,‡ Harold C. Jarrell,*,§ Kathleen M. Brière,§,∥ and Chris W. M. Grant*,‡

Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1, and Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

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ABSTRACT: ^2H NMR spectroscopy was used to consider correspondence between existing single-crystal X-ray data for glycosphingolipids and their ceramide backbone conformation in fluid phospholipid membranes. A monoglycosylated sphingolipid, glucosylceramide (GlcCer), which represents the core structure of many important glycosphingolipids, was derived by partial synthesis through replacement of all native fatty acids with the 18-carbon species, stearic acid, deuterated at C_2. *N*-[2,2-$^2H_2$]stearoyl-GlcCer was used to probe glycosphingolipid orientation and motion at low concentration in "fluid" phospholipid bilayers composed of dimyristoylphosphatidylcholine (DMPC), with and without physiological amounts of cholesterol. Spectral analysis, aided by stereoselective monodeuteration of the GlcCer fatty acid at C_2, demonstrated that glycosphingolipid average acyl chain backbone conformation in fluid phospholipid membranes, with or without cholesterol, is likely closely related to that predicted from single crystal X-ray studies [Pascher, I. (1976) *Biochim. Biophys. Acta 455*, 433–451; Pascher, I., & Sundell, S. (1977) *Chem. Phys. Lipids 20*, 175–191]. To test the generality of this observation, specific comparisons were made involving galactosylceramide (GalCer) and globoside. GalCer provided a glycolipid differing only in monosaccharide stereochemistry (galactose vs glucose). Globoside permitted isolation of the effect of headgroup size, since it is derived from GlcCer via extension of the carbohydrate portion by the oligosaccharide, GalNAcβ1 → 3Galα1 → 4Gal attached in β1 → 4 linkage to the Glc residue. Spectra obtained for *N*-[2,2-$^2H_2$]stearoyl-GalCer in the range 30 °C to 45 °C were within experimental error of those seen for *N*-[2,2-$^2H_2$]stearoyl-GlcCer uniformly dispersed in fluid membranes, indicating that orientational properties at the level of the fatty acid are extremely similar for these two species. Spectra obtained from *N*-[2,2-$^2H_2$]stearoylgloboside consistently showed quadrupole splittings that were up to 10% smaller than those seen for the monoglycosyl lipids in a given membrane, likely reflecting somewhat greater orientational disorder. The presence of 33 mol % cholesterol accentuated spectral differences between glycolipids having long vs short carbohydrate headgroups. An important characteristic feature of glycolipids in eukaryotes is their common possession of very long fatty acids—for which X-ray data, and NMR data for C_2, do not exist. GalCer with deuterium-labeled 24-carbon ([2,2-$^2H_2$]lignoceroyl) fatty acid was synthesized to address this issue. At 10 mol % in DMPC/cholesterol matrices, its spectra proved to be remarkably similar to those for the 18-carbon analogue described above; suggesting that in cell membranes the same conclusions apply regarding orientation at C_2.

Glycosphingolipids (GSLs),[1] the carbohydrate-bearing lipid components of animal cells, serve as structural elements and recognition sites at the plasma membrane outer surface. Considerable insight has been gained regarding the molecular characteristics of GSLs through study of the pure crystalline species and their aqueous dispersions (Curatolo, 1987a,b). It has been proposed however, that interactions such as hydrogen bonding which are considered important in stable spatial arrangements typical of single crystals, or which require highly uniform packing as in single-component bilayers, may not be as influential in the complex and fluid environment of cell membranes. Hence, there is impetus to extend studies of pure glycolipids to systems that mimic certain key aspects of biological membranes. Typical of such systems are phospholipid bilayers bearing relatively small quantities of glycolipid. It is clear in such systems that surrounding membrane lipids both affect and are affected by the presence of the glycolipid and that the interactions control GSL function in a manner modulated by GSL structure and dynamics (Maggio et al., 1985a; Thompson & Tillack, 1985; Curatolo, 1987a,b; Grant, 1987). We report here the employment of wideline ^2H NMR to appraise the backbone orientational arrangement of glycolipids in fluid bilayers of DMPC and the possible effects of a physiological concentration of cholesterol.

In considering whether key features suggested by X-ray studies carry over to glycolipids dispersed at low concentrations in membranes rich in phospholipid and cholesterol, a group of species labeled with deuterium at the C_2 (α) position of the single GSL fatty acid was studied. ^2H NMR spectroscopy of probes at C_2 may be expected to be sensitive to backbone structure near the membrane surface: the C–D bond orientation and motional properties are major determinants of wideline spectra (Seelig, 1977; Davis, 1983; Smith, 1984). The monoglycosyl lipid, GlcCer, was chosen as an example of a simple neutral glycolipid since it is the key structural unit of a very wide range of important GSLs. Globoside, whose biosynthesis involves extension of the GlcCer carbohydrate

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* To whom correspondence should be addressed.

‡ University of Western Ontario.

§ National Research Council of Canada.

∥ Present address: Institute for Biodiagnostics, 435 Ellice Ave., Winnipeg, Manitoba, Canada R3B 1Y6.

[1] Abbreviations: GSL, glycosphingolipid; DMPC, L-α-dimyristoylphosphatidylcholine; GlcCer, Glcβ1 → 1 ceramide; GalCer, Galβ1 → 1 ceramide; globoside, GalNAcβ1 → 3 Galα1 → 4Galβ1 → 4Glcβ1 → 1 ceramide.

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portion by the trisaccharide GalNAcβ1 → 3Galα1 → 4Gal in β1 → 4 linkage, provided a suitable complex glycolipid for comparison. Galactosylceramide (GalCer) was included as a monoglycosyl species which differs from GlcCer only in carbohydrate stereochemistry and which has been the subject of a variety of key studies relating to GSL receptor function. In each case the fatty acid attached to the sphingosine backbone was the common 18-carbon saturated species, stearic acid. DMPC—a phosphatidylcholine with saturated 14-carbon fatty acids—was employed as host bilayer matrix since, in cell membranes, GSL fatty acids tend to be longer than those of surrounding phospholipids.

An unusual feature of GSLs is that a high proportion of their natural fatty acids are commonly very long: up to 24–26 carbon atoms. It has been demonstrated that this can importantly modulate receptor and structural roles of GSLs (Alving et al., 1970; Hakomori, 1986; Crook et al., 1986; Curatolo & Neuringer, 1986). X-ray studies of glycolipids with long-chain fatty acids have not been reported, nor have long-chain fatty acids specifically deuterated at C_2 that would permit their wideline $^2H_2$ NMR spectral analysis. Hence, a GalCer derivative with 24-carbon fatty acid, *N*-[2,2-$^2H_2$]-lignoceroyl-GalCer, was synthesized for comparison with the shorter chain analogues described above.

MATERIALS AND METHODS

L-α-Dimyristoylphosphatidylcholine (DMPC) was purchased from Avanti Polar Lipids, Birmingham, AL. Glucosylceramide (GlcCer), galactosylceramide (GalCer), cholesterol, and glucopsychosine were obtained from Sigma Chemical Co., St. Louis, MO. Globoside was isolated from porcine blood as described previously (Mehlhorn et al., 1988a,b). [2(*S*)-$^2H_1$]Palmitic acid was a generous gift from Dr. A. P. Tulloch of the Plant Biotechnology Institute, National Research Council of Canada. Deuterated solvents were from MSD Isotopes, Montreal, Canada.

[2,2-$^2H_2$]Stearic acid and [2,2-$^2H_2$]lignoceric acid were synthesized from methyl stearate and methyl lignocerate, respectively (Aldrich, Milwaukee, WI), using the method of Aasen et al. (1970) for inserting deuterium at the C_2 position of fatty acids. Structures were confirmed by ^1H NMR. Lysoglycosphingolipid intermediates were synthesized using the method of Neuenhofer et al. (1985). The syntheses of *N*-[2,2-$^2H_2$]stearoyl- and *N*-[2,2-$^2H_2$]lignoceroyl-GalCer, *N*-[2,2-$^2H_2$]stearoylgloboside, and *N*-[2(*S*)-$^2H_1$]palmitoyl-GlcCer were performed by reacting the lyso intermediates with the acid chloride derivative of the appropriate fatty acid (Kopaczyk & Radin, 1965). *N*-[2,2-$^2H_2$]Stearoyl-GlcCer was prepared as described previously (Sharom & Grant, 1975; Florio et al., 1990). Chromatography techniques for characterization and purification of lipids were as described in previous work (Fenske et al., 1991). Deuterated GSLs comigrated with nondeuterated standards, and their ^1H NMR were consistent with those of their nondeuterated counterparts.

Sample preparation techniques and details of NMR methodology have been outlined previously (Fenske et al., 1991), with the exception that some samples in the present study were run using a 10-mm solenoid coil with 4.5-μs $\pi/2$ pulse lengths. Samples for ^2H NMR consisted of 6–20 mg (8–17 μmol) of dideuterated GSL or 23 mg (33 μmol) of the monodeuterated GlcCer, which represented 10 mol % of the total phospholipid in each case. X-ray coordinates from the Cambridge Data Bank were manipulated using software by Biosym Technologies (San Diego, CA) on a Silicon Graphics platform.

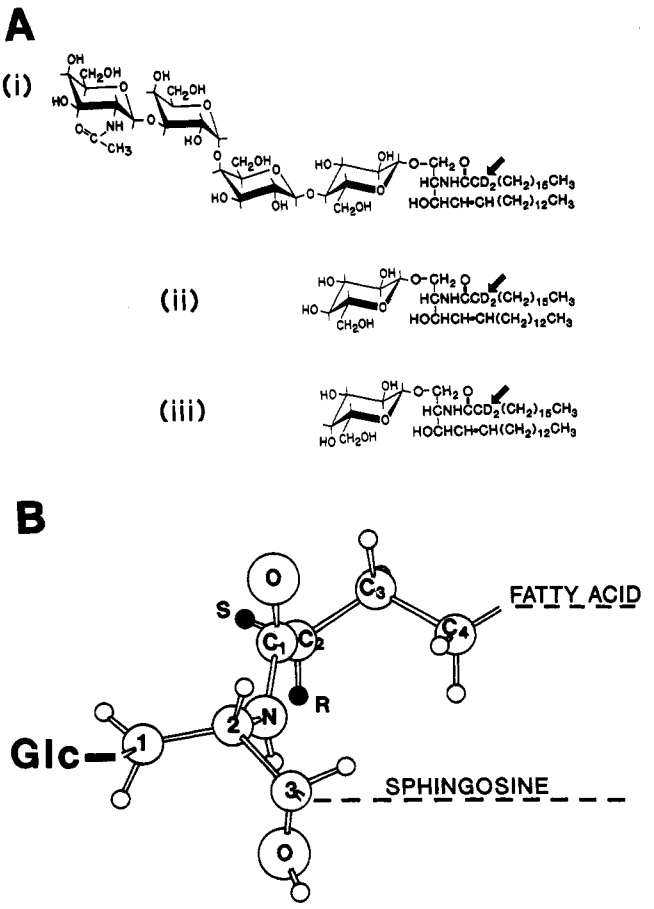

FIGURE 1: (A) Chemical structures of the major deuterium-labeled glycolipids used in this study: (i) *N*-[2,2-$^2H_2$]stearoylgloboside, (ii) *N*-[2,2-$^2H_2$]stearoyl-GlcCer, and (iii) *N*-[2,2-$^2H_2$]stearoyl-GalCer (arrows point to the deuterated sites). (B) Stereoscopic partial structure of GlcCer depicting single crystal stereochemistry and spatial arrangement of atoms in the region deuterated. The glucose headgroup is indicated (Glc) attached to the first of three carbon atoms in the sphingosine backbone (indicated as 1, 2, 3). The first four carbon atoms of the fatty acid (C_1–C_4) are marked. Deuterons are shown as filled circles attached to C_2 of the fatty acid, and their *pro-R* and *pro-S* stereochemical designations are given. Heavy dotted lines indicate extension of the fatty acid and sphingosine chains parallel to the long molecular axis.

RESULTS

Figure 1A presents chemical structures for the three glycolipid families studied in this work. In each case deuterium nuclei were located at C_2 (adjacent to the fatty acid carbonyl function). Note that GlcCer is a structural subunit of globoside and that GlcCer and GalCer differ only in functional group orientation within the single sugar residue. Figure 1B is a stereoscopic representation of the ceramide backbone near the point of fatty acid attachment, as redrawn from the X-ray crystallographic studies of Pascher (1976) and Pascher and Sundell (1977) which dealt with *N*-(α-OH-stearoyl)dihydrogalactosylceramide and a group of related compounds. The deuterated region displays the important feature that carbon 2 (indicated as 2 in Figure 1B) and N-H of sphingosine, along with C=O and C_2 of the attached fatty acid, can form a relatively rigid planar group of 6 atoms that is oriented perpendicularly toward the sphingosine chain. A significant tendency to this orientation of the plane of the fatty acyl linkage in membranes would involve the fatty acyl chain bending sharply at C_2 such that only the portion below this point is parallel to the molecular long axis—a feature for which X-ray evidence has been found in structures relevant to both the GSL fatty acid (Pascher, 1976; Pascher & Sundell,

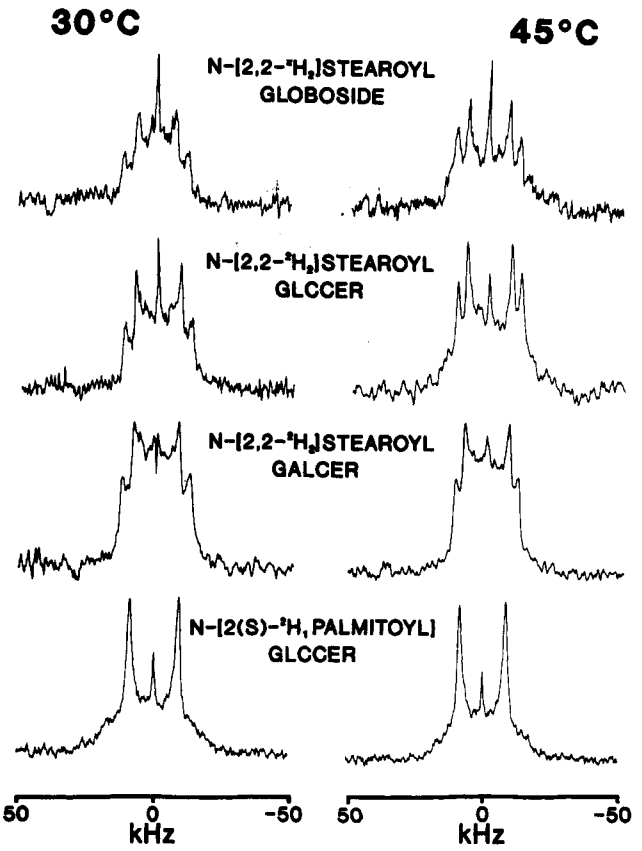

FIGURE 2: ^2H NMR (30.7-MHz) powder spectra for multilamellar vesicles of GlcCer, GalCer, and globoside dispersed at 10 mol % in DMPC. Number of acquisitions for each sample at 30 °C and 45 °C: 1 800 000, 400 000 (*N*-[2,2-$^2H_2$]stearoylgloboside); 312 000, 225 960 (*N*-[2,2-$^2H_2$]stearoyl-GlcCer); 312 000, 210 000 (*N*-[2,2-$^2H_2$]stearoyl-GalCer); 240 000, 200 000 (*N*-[2(*S*)-$^2H_1$]palmitoyl-GlcCer).

1977; Nyholm et al., 1990) and the *sn*-2 chain of phospholipids (Hitchcock et al., 1974; Pearson & Pascher, 1979).

Figure 2 shows ^2H NMR powder spectra of *N*-[2,2-$^2H_2$]-stearoyl-GlcCer, *N*-[2,2-$^2H_2$]stearoyl-GalCer, and *N*-[2,2-$^2H_2$]stearoylgloboside in DMPC at 30 °C and 45 °C. Their appearance is consistent with rapid axially symmetric motion (Seelig, 1977; Davis, 1983; Smith, 1984). The observation of spectra compatible with glycolipids in a liquid crystalline phase, at temperatures below their main transition temperature [84 °C, 82–85 °C, and 40 °C for GlcCer, GalCer, and globoside, respectively (Ruocco et al., 1983; Maggio et al., 1985b; Curatolo & Jungalwala, 1985)], indicates that the GSL molecules involved are dispersed throughout the (fluid) host matrix (Skarjune & Oldfield, 1982; Florio et al., 1990; Fenske et al., 1991). In all spectra, each of the two deuterons at the α-carbon (C_2) gives rise to a distinct pair of peaks (i.e., a distinct "Pake doublet"). This demonstrates that the C–^2H bonds at C_2 are magnetically inequivalent. In the case of similarly deuterated dipalmitoylphosphatidylcholine in pure, fluid bilayers, such inequivalence has been demonstrated to result from the carbon–deuterium bonds at these locations having different orientations relative to the lipid "motional director": the *pro-R* deuteron was shown to give rise to the Pake doublet with the larger splitting (Engel & Cowburn, 1981). Inspection of Figure 1B suggests that, if GSL features related to those indicated persist in the fluid DMPC membranes studied here, then orientational inequivalence of the C_2 carbon–deuterium bonds should indeed exist, leading to differences in the associated ^2H quadrupole spectral splittings for the two deuterons.

Table I: Quadrupole Splittings,[a] $\Delta\nu_Q$, for Deuterated Glucosylceramide (GlcCer), Galactosylceramide (GalCer), and Globoside, at 10 mol % in Lipid Bilayers of DMPC, in the Presence and Absence of 33 mol % Cholesterol[b]

deuterated glycolipid	$\Delta\nu_Q$ (±1 kHz)			
	without cholesterol		33 mol % cholesterol	
	30 °C	45 °C	30 °C	45 °C
[α-$^2H_2$]globoside	25.4, 16.0	23.9, 15.5	28.2, 11.0	27.1, 12.3
[α-$^2H_2$]GalCer	26.6, 18.0	23.8, 16.8	30.4, 15.8	29.6, 16.4
[α-$^2H_2$]GlcCer	26.6, 18.0	23.8, 16.8	31.6, 15.3	29.8, 15.9
[α-$^2H_1$]GlcCer	19.4	17.8	19.8	19.2

[a] Splittings were measured from powder spectra and have an estimated uncertainty of up to ±1 kHz. [b] In each case the single 18-carbon fatty acid was substituted at C_2 with one (α-$^2H_1$) or two (α-$^2H_2$) deuterium nuclei.

Assuming that the C(NH)(C=O)$C_2{}^2H_2$ fragment of the ceramide moiety can be held in a favored average molecular geometry in fluid bilayer membranes, which seems reasonable on the basis of earlier work (Skarjune & Oldfield, 1979; Fenske et al., 1991), then it is possible to consider the spatial orientation of the GSL C_2–^2H bonds using the relationship describing deuterium quadrupole splitting, $\Delta\nu_Q$:

$$\Delta\nu_Q = {}^3/_8(e^2Qq/h)(S_{mol})(3\cos^2\theta_i - 1) \qquad (1)$$

where e^2Qq/h is the nuclear quadrupole coupling constant (170 kHz for an aliphatic C–^2H (Seelig, 1977)), S_{mol} is the molecular order parameter (assuming axially symmetric order) describing orientational fluctuations of the lipid molecule relative to the bilayer normal, and θ_i is the average orientation of each C–^2H bond relative to the bilayer normal. In the case of fluid-phase phospholipids (Seelig, 1977) and glyceroglycolipids (Jarrell et al., 1987), S_{mol} for the lipid backbone is in the range 0.6–0.7 (Seelig, 1977; Jarrell et al., 1987; Mayer et al., 1990). If the corresponding molecular fluctuations are similar to those of phospholipids and glyceroglycolipids, the geometrical parameters θ_i may be estimated from $\Delta\nu_Q$ values using eq 1.

The values of $\Delta\nu_Q$ measured for GlcCer in DMPC at 30 °C are 18.0 and 26.6 kHz (Table I), with an uncertainty of up to ±1 kHz. Using eq 1 and S_{mol} of 0.6–0.7, θ_i values of 46 ± 1° (or 64 ± 1° if $\Delta\nu_Q$ is <0) and 42 ± 1° (or 70 ± 2° if $\Delta\nu_Q$ is <0), respectively, are calculated. Consideration as to how these values relate to GSL molecular geometry in fluid phospholipid membranes is aided by examination of Figure 1B and by the fact that the signs associated with the calculations for the two deuterons at C_2 must be opposite (Seelig, 1977; Engel & Cowburn, 1981). The pairing of 46° and 42° or 64° and 70° is not compatible. The X-ray crystallographic data upon which the stereoscopic structure in Figure 1B are based indicate that the substituent at the *pro-S* position of the fatty acid C_2 should make the smaller angle with the molecular long axis, while the substituent at the *pro-R* position makes the larger angle.

Ambiguity as to the assignment of the two spectral splittings to *pro-S* vs *pro-R* C_2 deuterons was eliminated using palmitic acid monodeuterated at C_2 with *S* stereochemistry to synthesize the corresponding *N*-[2(*S*)-$^2H_1$]palmitoylGlcCer. ^2H NMR spectra of this lipid system (Figure 2) clearly demonstrate that, as in the case of phospholipids (Engel & Cowburn, 1981), each pair of peaks in α-dideuterated GlcCer is associated with only one of the C_2 deuterons (rather than with one of two possible conformations in slow exchange). Comparison of the spectra and tabulated quadrupole splittings for the monodeuterated and dideuterated GlcCer shows that the

smaller $\Delta\nu_Q$ values are assignable to the *pro-S* deuteron, while the larger ones, by default, must correspond to deuterium in the *pro-R* configuration.

Increasing the temperature of the GlcCer/DMPC sample from 30 °C to 45 °C reduced the outer and inner quadrupole splittings by 10% and 7%, respectively, to 23.8 and 16.8 kHz. The similarity of these temperature-induced changes in the outer and inner splittings suggests that the major effect of the temperature elevation was an increase in the amplitude of GSL whole body motions (i.e., wobble), corresponding to a decrease in S_{mol}.

Inspection of Figure 2 and Table I reveals that the ^{2}H NMR spectra of *N*-[2,2-$^2H_2$]stearoyl-GalCer dispersed in fluid bilayers of DMPC are indistinguishable from those already discussed for GlcCer. Small changes in orientation of the C_2 methylene moiety can lead to substantial alterations in the residual quadrupole splittings. For example, a change of +5° or −5° in the *pro-S* deuteron orientation would lead to $\Delta\nu_Q$ values of 27 and 7 kHz, respectively, as calculated from eq 1 using 0.6 for S_{mol}. For the *pro-R* deuteron the corresponding values would be 18 and 30 kHz. Thus, there appears to be very little orientational effect of a change in the single carbohydrate residue from Glc to Gal.

The measured quadrupole splittings, $\Delta\nu_Q$, for globoside at 30 °C were 25.4 and 16.0 kHz—somewhat reduced from the GlcCer values (26.6 and 18.0 kHz) (Table I). Nevertheless, as described above, this change falls within a range which would be expected with fairly minor conformational or orientational ordering effects. Thus, the molecular impact of extending the GlcCer carbohydrate chain by 3 residues is relatively minor at the acyl chain C_2 position, amounting to only a few percent change in conformation or molecular ordering. As was the case with GlcCer (and GalCer), raising the temperature from 30 °C to 45 °C caused a slight uniform decrease in the quadrupole splittings (Table I): by 6% and 3% for outer and inner splittings, respectively. At 45 °C spectral differences between GlcCer and globoside are within experimental error.

Figure 3 shows ^{2}H spectra typical of the same deuterated GSLs in DMPC bilayers containing 33 mol % cholesterol. The measured $\Delta\nu_Q$ values are listed with those for the cholesterol-free membranes in Table I. An adequate description of the spectra would be that the addition of cholesterol to the fluid phospholipid membrane has led to an increase in the outer splitting and a decrease in the inner splitting for GlcCer, GalCer, and globoside. However an important alternative explanation would be that what has actually occurred is a switch in the peak assignments with the addition of cholesterol, via some effect on GSL conformation. This possibility was ruled out by use of the monodeuterated derivative, *N*-[2(S)-$^2H_1$]palmitoyl-GlcCer. From comparison of the spectra for the stereospecifically deuterated (*S*) isomer in Figures 2 and 3, it appears that the inner splitting in spectra of the dideuterated analogues remains associated with the *pro-S* deuteron. It will be noted that the splittings for the stereoselectively deuterated GlcCer are not identical to those for the dideuterated GlcCer. This is presumably because the chain is 18 carbons in the latter case and 16 carbons in the former. Chain mismatch with the (14-carbon) host matrix is thus greater in the case of the (18-carbon) dideuterated species. It may be that the presence of cholesterol enhances the consequences of this mismatch by ordering and extending the acyl chains. Given an unambiguous spectral assignment and the measured spectral splittings (31.6 and 15.3 kHz) for GlcCer in the presence of cholesterol, θ_i were calculated using

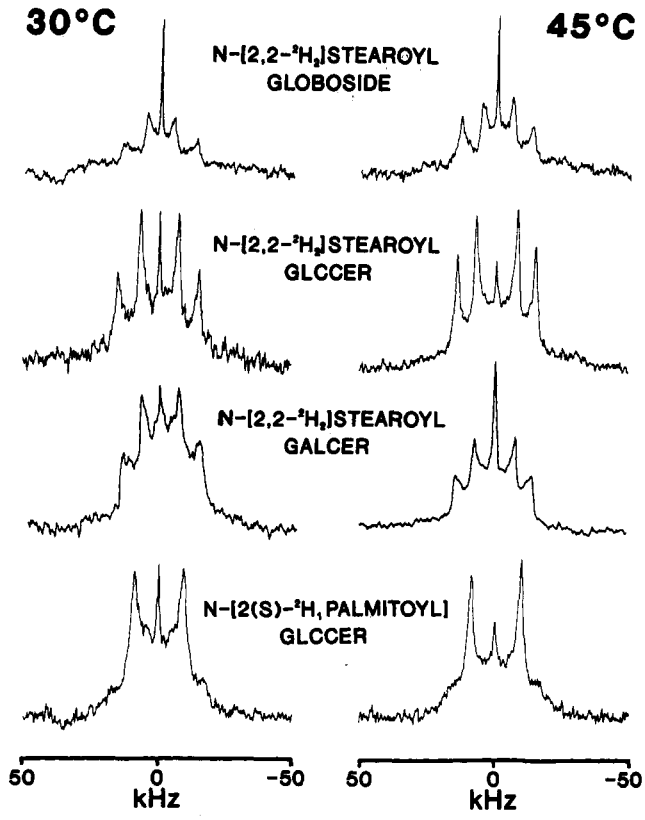

FIGURE 3: ^{2}H NMR (30.7-MHz) powder spectra for multilamellar vesicles of GlcCer, GalCer, and globoside dispersed at 10 mol % in DMPC containing 33 mol % cholesterol. Number of acquisitions for each sample at 30 °C and 45 °C: 640 000, 560 000 (*N*-[2,2-$^2H_2$]-stearoyl-globoside); 518 160, 495 000 (*N*-[2,2-$^2H_2$]stearoyl-GlcCer); 400 000, 100 000 (*N*-[2,2-$^2H_2$]stearoyl-GalCer); 280 000, 200 000 (*N*-[2(*S*)-$^2H_1$]palmitoyl-GlcCer).

eq 1 as already described [but substituting S_{mol} = 0.8–1 (Seelig, 1977; Jarrell et al., 1987; Mayer et al., 1990)], to give angles of 49 ± 1° and 67 ± 2°, respectively, relative to the molecular long axis. A minor reorientation of C_2 in Figure 1B, associated with chain extension, would readily account for the cholesterol-induced spectral changes seen here.

Raising the temperature of the *N*-[2,2-$^2H_2$]stearoyl-GlcCer sample from 30 °C to 45 °C led to a 6% decrease in the outer spectral splitting, from 31.6 to 29.8 kHz, while the inner splitting increased 4% from 15.3 to 15.9 kHz (Table I). Once again, GalCer gave spectra indistinguishable within experimental error from those recorded for GlcCer under the same conditions, and the same conclusions regarding its conformation must apply. A similar pattern was seen for the complex glycolipid: the outer splitting decreased by 4% from 28.2 to 27.1 kHz, while the inner splitting increased 11% from 11.0 to 12.3 kHz. These numerical differences from GlcCer (and GalCer) must be viewed with caution, since the experimental error in a given spectrum is ±0.5–1 kHz. However, several observations may be made. First, it is clear that the changes induced by temperature elevation are small, considering the potential sensitivity of the relationship between spectral splitting and conformation at C_2 already mentioned. Second, the magnitude and pattern of temperature-induced spectral change is the same for GlcCer and globoside. Third, since a drop in molecular order, which is expected to arise from increased sample temperature, should decrease both spectral splittings by the same fraction; it would appear that some (small) conformational change has occurred with sample warming for both GlcCer and globoside when cholesterol is present in the membrane. Fourth, as was seen in the absence

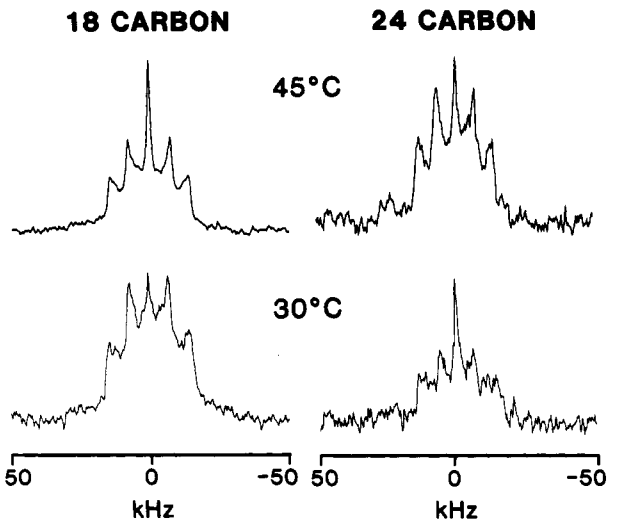

FIGURE 4: ^2H NMR (30.7-MHz) powder spectra for multilamellar vesicles of "long"- and "short"-chain GalCer at 10 mol % in DMPC containing 33 mol % cholesterol. Number of acquisitions for each sample at 30 °C and 45 °C: 400 000, 100 000 (*N*-[2,2-$^2H_2$]stearoyl-GalCer); 400 000, 175 000 (*N*-[2,2-$^2H_2$]lignoceroyl-GalCer). Spectral splittings for *N*-[2,2-$^2H_2$]lignoceroyl-GalCer at 30 °C and 45 °C were determined to be 13.8, 28.8 kHz and 15.4, 28.0 kHz (±1 kHz), respectively.

of cholesterol, the quadrupole splittings for globoside were always smaller than those observed for GlcCer for a given set of conditions.

Addition of cholesterol to the DMPC host matrix accentuated the spectral differences among the structurally different GSLs employed in this study. Thus, adding cholesterol increased the GlcCer outer splitting by 19–25% and decreased the inner splitting 5–15%, while for globoside the effect was to increase the outer splitting by 11–13% and to decrease the inner splitting by 21–31%. In the absence of cholesterol the difference in outer spectral splittings between GlcCer and globoside ranged between 0 and 4%, while with cholesterol added it was 9–11%. In the absence of cholesterol the difference in inner spectral splittings between GlcCer and globoside ranged between 8 and 11% while with cholesterol it was 23–26%. This behavior extended to the palmitic acid (16-carbon) monodeuterated derivative of GlcCer compared to its stearic acid (18-carbon) dideuterated analogue; their splittings were within 1 kHz in the absence of cholesterol, but this increased to 4 kHz in the presence of cholesterol.

All previous literature measurements of phospholipid and glycolipid bond orientation at fatty acid C_2 have involved species of homogeneous length in the range 14–18 carbon atoms. However, systems comprised of GSLs with very long fatty acids, in phospholipid host matrices with shorter fatty acids, have special relevance as a common natural situation. The receptor implications of such combinations have been particularly considered with regard to GalCer and sulfate substituted GalCer in phosphatidylcholine/cholesterol bilayers (Alving & Richards, 1977; Alving et al., 1980; Crook et al., 1986). To measure the effect, the 24-carbon fatty acid derivative, *N*-[2,2-$^2H_2$]lignoceroyl GalCer, was derived by partial synthesis and included in cholesterol-containing bilayers of DMPC analogous to those employed in these earlier studies of immunoglobulin binding. Figure 4 shows spectra of this system for direct comparison with the data for shorter chain GSLs. Analysis of these spectra clearly indicates the similarity of this long-chain GSL to the 18-carbon analogue in DMPC/cholesterol membranes at 30 and 45 °C. The measured quadrupole splittings for the 24-carbon analogue are 13.8 and 28.8 kHz (±1 kHz) at 30 °C and 15.4 and 28.0 kHz (±1 kHz) at 45 °C. Although slightly smaller, these values are not significantly different from those for the shorter chain (GalCer) analogue in the same membrane at the same temperature.

DISCUSSION

An important feature to emerge from X-ray studies of single crystals of sphingosine-related compounds and *N*-(α-OH-stearoyl)dihydrogalactosylceramide is the possible existence of a characteristic fatty acid conformation near the polar headgroup; it appears that this chain may bend sharply at C_2 away from the plane of the fatty acyl linkage (and the plane of the bilayer) to run parallel to the molecular long axis (Pascher, 1976; Pascher & Sundell, 1977). The same crystal structure attribute has been found in the *sn*-2 chain of phospholipids (Hitchcock et al., 1974; Pearson & Pascher, 1979). Carry over of this X-ray derived feature into fluid membranes has fundamental implications for glycolipids in cells, since it is associated with a key arrangement of the planar amide structure in the backbone, imposing motional and conformational restrictions on the molecule. Seelig and Browning (1978) concluded from ^2H NMR studies of deuterated phospholipids in fluid membranes that C_2 of the *sn*-2 fatty acid chain appeared to have an orientation consistent with the crystal structure for DMPC; and evidence exists that the (single) GSL fatty acid displays conformational behavior in fluid membranes similar to that of the *sn*-2 fatty acid of phospholipids (Sharom & Grant, 1977; Skarjune & Oldfield, 1979; Florio et al., 1990; Fenske et al., 1991). In addition, ^2H NMR spectra of pure hydrated GalCer have been shown to be compatible with the existence of a sharp bend in the fatty acid chain away from the membrane surface at C_2 (Skarjune & Oldfield, 1979).

In the present work, in each case, spectra of GSLs deuterated at C_2 and dispersed in fluid phospholipid-rich membranes were found to consist of a pair of Pake doublets. Stereospecifically monodeuterated GlcCer provided for the first time unambiguous assignment of these spectral splittings. The inner splitting (inner Pake doublet) proved to be associated with the *pro-S* deuteron at C_2. This finding is the same result obtained for the only other lipid which has been so assigned, pure dipalmitoylphosphatidylcholine in liquid crystal form (Engel & Cowburn, 1981). These authors addressed the fact that the observation of two separate Pake doublets for phospholipids deuterated at C_2 of the *sn*-2 chain could be explained by the existence of two long-lived rotational conformers or by (magnetic) nonequivalence of the *pro-R* and *pro-S* deuterons. Their observation that the *pro-R* form gave rise to a single Pake doublet with the larger splitting demonstrated that nonequivalence was the case: the C–H (C–D) bonds at C_2 appear to have different average orientations relative to the motional director axis. They concluded that their ^2H NMR spectra for phospholipids were consistent with X-ray data giving a *pro-R* angle of about 90° and a *pro-S* angle of about 34° to the molecular long axis. Thus, inequivalence of deuterons at the fatty acyl C_2 position of GSLs, in addition to the *sn*-2 fatty acid C_2 position of glycerolipids, now appears to be a general feature in lipid bilayer systems. Individual deuterated methylene groups at other acyl chain locations on GSLs and on glycerolipids give rise to single Pake doublets in fluid bilayers (Skarjune & Oldfield, 1979; Seelig, 1977; Seelig & Browning, 1978; Davis, 1983; Smith, 1984; Fenske et al., 1991). Compatibility between X-ray conformation at C_2 and that present in fluid

membranes is likely a result of the rigid nature of the fatty acyl linkage (Pascher, 1976) combined with the sharp bend at C_2 of the fatty acid chain, which makes conformational reorientation difficult for this methylene group. However, libration about this orientation in fluid membranes is expected to make the observed conformation an average one. The results found in the present work are consistent with previous findings that there is considerable similarity between the membrane behavior of GSLs and phospholipids at greater membrane depths (Skarjune & Oldfield, 1979; Tillack & Thompson, 1985; Grant, 1987; Florio et al., 1990), since orientation at the level of the amide link and C_2 of the fatty acid would be an important determinant of the behavior of the rest of the acyl chain.

Spectral assignments in these experiments were proven using a selectively deuterated GlcCer. Hence, it is conceivable that the GalCer and globoside conformations are in fact very different (e.g., with reversed spectral peak assignment), and only fortuitously gave similar spectra. This is unlikely, however, for several reasons. First, the relationship between carbon–deuterium bond orientation and spectral splitting is very sensitive to small changes at C_2. Second, the qualitative and quantitative similarities among spectra for the different glycolipids were preserved with the addition of cholesterol, including the response to temperature alteration. This is particularly true in the case of GalCer, which gave results in all cases within experimental error of measured values for GlcCer. In spite of measurable spectral differences seen between the monoglycosylated GlcCer and the tetraglycosylated globoside, particularly in membranes containing physiological amounts of cholesterol, the very considerable resemblance of their spectra must be seen as evidence of basic similarity. The essentially identical nature of the GlcCer and GalCer conformations is remarkable in light of the fact that the headgroups involved have been observed to have very different H-bonding properties (Koynova et al., 1990).

An important factor to consider is that, in the absence of cholesterol, phospholipid bilayers exhibit striking degrees of cooperativity in their response to temperature variation and also very high degrees of "fluidity" or "rigidity" relative to cell membranes (Yeagle, 1985; Vist & Davis, 1990; Sankaram & Thompson, 1990). Oldfield et al. (1978) studied the effect of cholesterol addition in pure DMPC by ^2H NMR. Interestingly, in the latter study cholesterol increased both outer and inner spectral splittings for the phospholipid, while the effect on the glycolipids seen in our work was to increase the value of the larger splitting and to decrease that of the smaller. This need not reflect noteworthy intrinsic differences between phospholipids and glycolipids, since the NMR technique is very sensitive to small changes in orientation. The presence of 33 mol % cholesterol had no effect on the quantitative equality of spectral parameters for GlcCer and GalCer. It did affect the spectral differences which were detected between GlcCer and globoside, and it did induce a dependence of conformation on temperature, raising the interesting possibility that cholesterol may accentuate functional effects of headgroup structural differences among glycosphingolipids. The outer spectral splitting, associated with the *pro-R* stereoisomeric deuteron, increased, while the inner splitting decreased for both: but quantitatively, the effect was greater for the *pro-R* deuteron in the case of GlcCer and for the *pro-S* deuteron of globoside. The exaggerated difference between GlcCer with 18- and 16-carbon fatty acid chains in the presence of cholesterol may arise from the greatly increased orientational order of the (14-carbon) host matrix and GSL acyl chains, which become rigidly extended in membranes with high cholesterol content.

The observation that spectral quadrupole splittings differed between GlcCer and globoside implies that the C_2 deuterons have different orientations for the two GSLs and/or that they have different degrees of orientational order while the same average orientation is maintained. The fact that the much larger globoside headgroup gave consistently smaller quadrupole splittings relative to those of GlcCer and that outer and inner splittings changed proportionately for a given alteration of conditions strongly suggests that the primary effect is reduced molecular order for the GSL with the larger headgroup. This is reminiscent of observations by Maggio et al. on pure hydrated GSLs, that increasing the size of the GSL carbohydrate portion led to steric interference and resultant less orderly packing (1985, 1988). It is certainly reasonable that a similar phenomenon could operate among GSL and phospholipid headgroups in membranes. In the host matrix containing cholesterol, the spectral effect of the larger oligosaccharide chain was again consistent with the globoside backbone undergoing axial motions of somewhat greater amplitude.

A common feature of GSLs, which differentiates them from other membrane lipids, is possession of very long fatty acids. The significance of this feature has been examined in detail for GalCer by previous workers in critical experiments that relate to membrane recognition (Alving et al., 1980; Kannagi et al., 1982; Crook et al., 1986). Its effect on GSL backbone conformation in membranes has not been previously addressed by spectroscopic or X-ray techniques. Given the extreme sensitivity of this technique to C–D bond orientation, the observation that spectra for GalCer with 24-carbon fatty acid follow very closely the same pattern at C_2 as do those for shorter chain species is relevant to mechanistic explanations of long-chain effects on GSL receptor function. The latter have considered extent of headgroup protrusion from the membrane, headgroup orientation, glycolipid lateral arrangement, and glycolipid dynamics (Alving et al., 1980; Kannagi et al., 1982; Crook et al., 1986; Mehlhorn et al., 1988a).

CONCLUSIONS

Magnetic inequivalence of deuterium nuclei at C_2, reported for fatty acids at the *sn*-2 position of phospholipids in a wide range of membranes (Davis, 1983; Smith, 1984), is clearly also a feature of GSL fatty acids. This is true for GSLs having up to 24 carbon atoms in common (shorter chain) phospholipid-rich fluid membranes, with and without cholesterol. Use of a stereoselectively deuterated glycolipid and the highly sensitive relationship between C–D bond orientation and ^2H NMR spectral characteristics made it possible to demonstrate close correspondence in such membranes between GlcCer and phospholipids. Average spatial orientation determined for C_2 deuterons in GlcCer in fluid phospholipid-rich membranes proved to be compatible with conclusions from X-ray crystallography data. Spectral findings, and thus presumably backbone orientation in the membranes studied, were identical within experimental error for a GSL, GalCer, with very different H-bonding characteristics. The addition of a linear neutral trisaccharide in $\beta 1 \rightarrow 4$ linkage to GlcCer did not significantly alter the basic observation, although the degree of whole body orientational order at the membrane surface appeared to be decreased by some 10%. In cholesterol-containing membranes the spectra suggested that a small conformational change near the *N*-acyl carbonyl region occurs—presumably one consistent with stiffening and ex-

tension of the fatty acid chain (Oldfield et al., 1978)—but that once again critical features of the X-ray derived conformation are retained.

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REFERENCES

Aasen, A. J., Lauer, W. M., & Holman, R. T. (1970) *Lipids 5*, 869–877.
Alving, C. R., Urban, K. A., & Richards, R. L. (1980) *Biochim. Biophys. Acta 600*, 117–125.
Crook, S. J., Boggs, J. M., Vistnes, A. I., & Koshy, K. M. (1986) *Biochemistry 25*, 7488–7494.
Curatolo, W. (1987a) *Biochim. Biophys. Acta 906*, 111–136.
Curatolo, W. (1987b) *Biochim. Biophys. Acta 906*, 137–160.
Curatolo, W., & Jungalwala, F. B. (1985) *Biochemistry 24*, 6608–6613.
Curatolo, W. & Neuringer, L. J. (1986) *J. Biol. Chem. 261*, 17177–17182.
Davis, J. H. (1983) *Biochim. Biophys. Acta 737*, 117–171.
Engel, A. K., & Cowburn, D. (1981) *FEBS Lett. 126*, 169–171.
Fenske, D. B., Hamilton, K., Jarrell, H. C., Florio, E., Barber, K. R., & Grant, C. W. M. (1991) *Biochemistry 30*, 4503–4509.
Florio, E., Jarrell, H. C., Fenske, D. B., Barber, K. R., & Grant, C. W. M. (1990) *Biochim. Biophys. Acta 1025*, 157–163.
Grant, C. W. M. (1987) in *Gangliosides and Modulation of Neuronal Functions* (Rahman, H., Ed.) pp 119–138, NATO ASI Series Cell Biology, Springer-Verlag, Berlin.
Hakomori, S.-I. (1986) *Chem. Phys. Lipids 42*, 209–233.
Hitchcock, P. B., Mason, R., Thomas, K. M., & Shipley, G. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3036–3040.
Jarrell, H. C., Jovall, P. A., Giziewicz, J. B., Turner, L. A., & Smith, I. C. P. (1987) *Biochemistry 26*, 1805–1811.
Kannagi, R., Nudelman, E., & Hakomori, S.-I. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 3470–3474.
Kopaczyk, K. C., & Radin, N. S. (1965) *J. Lipid Res. 6*, 140–145.
Koynova, R. D., Kuttenreich, H. L., Tenchov, B. G., & Hinz, H.-J. (1988) *Biochemistry 27*, 4612–4619.
Mayer, C., Grobner, G., Muller, K., Weisz, K., & Kothe, G. (1990) *Chem. Phys. Lett. 165*, 155–161.
Lee, C. W. B., Waugh, J. S., & Griffin, R. G. (1986) *Biochemistry 25*, 3737–3742.
Maggio, B., Ariga, T., Sturtevant, J. M., & Yu, R. K. (1985b) *Biochemistry 24*, 1084–1092.
Maggio, B., Ariga, T., Sturtevant, J. M., & Yu, R. K. (1985a) *Biochim. Biophys. Acta 818*, 1–12.
Maggio, B., Albert, J., & Yu, R. K. (1988) *Biochim. Biophys. Acta 945*, 145–160.
Mehlhorn, I. E., Barber, K. R., & Grant, C. W. M. (1988a) *Biochim. Biophys. Acta 943*, 389–404.
Mehlhorn, I. E., Florio, E., Barber, K. R., Lordo, C., & Grant, C. W. M. (1988b) *Biochim. Biophys. Acta 939*, 151–159.
Neuenhofer, S., Schwarzmann, G., Egge, H., & Sandhoff, K. (1985) *Biochemistry 24*, 525–532.
Nyholm, P.-G., Pascher, I., & Sundell, S. (1990) *Chem. Phys. Lipids 52*, 1–10.
Oldfield, E., Meadows, M., Rice, D., & Jacobs, R. (1978) *Biochemistry 17*, 2727–2740.
Pascher, I. (1976) *Biochim. Biophys. Acta 455*, 433–451.
Pascher, I., & Sundell, S. (1977) *Chem. Phys. Lipids 20*, 175–191.
Pearson, R. H., & Pascher, I. (1979) *Nature 281*, 499–501.
Ruocco, M. J., Shipley, G. G., & Oldfield, E. (1983) *Biophys. J. 43*, 91–101.
Sankaram, M. B., & Thompson, T. E. (1990) *Biochemistry 29*, 10676–10684.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.
Seelig, J., & Browning, J. L. (1978) *FEBS Lett. 92*, 41–44.
Sharom, F. J., & Grant, C. W. M. (1977) *J. Supramol. Struct. 6*, 249–258.
Sharom, F. J., & Grant, C. W. M. (1975) *Biochem. Biophys. Res. Commun. 67*, 1501–1506.
Skarjune, R., & Oldfield, E. (1979) *Biochim. Biophys. Acta 556*, 208–218.
Skarjune, R., & Oldfield, E. (1982) *Biochemistry 21*, 3154–3160.
Smith, I. C. P. (1984) *Biomembranes 12*, 133–168.
Thompson, T. E., & Tillack, T. W. (1985) *Annu. Rev. Biophys. Biophys. Chem. 14*, 361–386.
Vist, M. R., & Davis, J. H. (1990) *Biochemistry 29*, 451–464.
Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 267–287.