Glycosphingolipid Headgroup Orientation in Fluid Phospholipid/ Cholesterol Membranes: Similarity for a Range of Glycolipid Fatty Acids

Michael R. Morrow,* Dev M. Singh,‡ and Chris W. M. Grant‡
*Department of Physics, Memorial University of Newfoundland, St. John's, Newfoundland, Canada A1B 3X7; and ‡Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1

ABSTRACT Galactosyl ceramide (GalCer) was labeled for nuclear magnetic resonance (NMR) spectroscopy by replacement of a hydrogen atom at C_6 of the galactose residue with deuterium. Wideline ^2H NMR of [d_1]GalCer permitted consideration of a mechanism traditionally entertained for cell surface recognition site modulation: that the nature of the fatty acid attached to the sphingosine backbone of glycosphingolipids (GSLs) importantly influences carbohydrate headgroup orientation. Comparison was made among various glycolipid fatty acids by altering hydroxylation, saturation, and chain length. Studies were carried out in unsonicated bilayer membranes mimicking several important characteristics of cell plasma membranes: fluidity, low GSL content, predominant [*sn*-2]monounsaturated phosphatidylcholine (PC) (1-palmitoyl-2-oleoyl PC), and the presence of cholesterol. Spectroscopy was performed on samples over a range of temperatures, which included the physiological. ^2H NMR spectra of [d_1]GalCer having 18-carbon saturated fatty acid (stearic acid), *cis*-9-unsaturated fatty acid (oleic acid), D- and L-stereoisomers of α-OH stearic acid, or 24-carbon saturated fatty acid (lignoceric acid), were importantly similar. This argues that for GSLs dispersed as minor components in fluid membranes, variation of the glycolipid fatty acid does not provide as much potential for direct conformational modulation of the carbohydrate portion as has sometimes been assumed. However, there was some evidence of motional differences among the species studied. The ^2H NMR spectra that were obtained proved to be more complex than was anticipated. Their features could be approximated by assuming a combination of axially symmetric and axially asymmetric glycolipid motions. Presuming the appropriateness of such an analysis, at a magnetic field of 3.54 T (23.215 MHz), the experimental spectra suggested predominantly asymmetric motional contributions. At the higher field of 11.7 T (76.7 MHz, equivalent to a proton frequency of 500 MHz), spectra indicated dominance by axially symmetric rotational modes. There was also evidence of some bilayer orientation in the stronger magnetic field. The unusual observation of spectral differences between the two magnetic field strengths may involve a diamagnetic response to high field on the part of some liposome physical characteristics.

INTRODUCTION

GSLs, the carbohydrate-bearing lipids of higher animal cells, have been implicated as specific recognition sites, as elements that contribute to membrane physical properties, and as modulators of signal transduction pathways at the membrane level (Thompson and Tillack, 1985; Curatolo, 1987a,b; Hakomori, 1989). Numerous physical studies have focused on the relationship between GSL structure and behavior, inasmuch as these seem to be associated with GSL function (reviewed in Hakomori, 1981; Thompson and Tillack, 1985; grant, 1985; Curatolo, 1987a,b). In the case of recognition events, it has been observed that the extent of glycolipid carbohydrate headgroup participation can be importantly modulated by the nature of their (single) fatty acid. It has often been pointed out that this key aspect of the phenomenon of receptor "crypticity" (Hakomori, 1981) could arise from fatty acid modification of GSL carbohydrate spatial arrangement (Alving et al., 1980; Stromberg et al., 1991; Stewart and Boggs, 1993; Kiarash et al., 1994). Implicit in the majority of such studies has been the concept that fatty acid-based differences can be manifest in systems without membrane proteins. However, relatively few direct structural measurements have been carried out on glycolipid carbohydrate headgroups in membranes reflective of the complex, fluid lipid mixtures found in cells.

In the present work we set out to study GSL fatty acid effects on carbohydrate orientation in membranes containing cholesterol and small amounts of glycolipid. GalCer was chosen for this investigation, because it has formed the basis of numerous important analyses of GSL characteristics and behavior, including their x-ray crystal structure (Nyholm et al., 1990) and the original systematic studies of receptor crypticity (Alving et al., 1980; Utsumi et al., 1984; see also Stewart and Boggs, 1993). Its behavior as a minor component in fluid membranes seems similar to that of other GSLs (Mehlhorn et al., 1988, 1989; Fenske et al., 1991, Hamilton et al., 1993). GalCer has clearly been demonstrated to be importantly influenced by its single fatty

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Address reprint requests to Dr. Christopher Grant, Department of Biochemistry, University of Western Ontario, London, Ontario N6A 5C1, Canada. Tel.: 519-661-3065; Fax: 519-661-3175; E-mail: cgrant@voyager.biochem.uwo.ca.

Abbreviations used in this article: GSL, glycosphingolipid; GalCer, galactosyl ceramide; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-3-*sn*-PC; 18:0 GalCer, N-stearoyl galactosyl ceramide; 18:1 GalCer, N-oleoyl GalCer; D-α-OH 18:0 GalCer, N-(D-α-hydroxy stearoyl) GalCer; L-α-OH 18:0 GalCer, N-(L-α-hydroxy stearoyl) GalCer; 24:0 GalCer, N-lignoceroyl GalCer.

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acid, both in its bulk physical properties (Curatolo and Jungalwala, 1985; Reed and Shipley, 1989) and in its role as a recognition site for macromolecules (Alving et al., 1980; Stewart and Boggs, 1993). Samples of this glycolipid, deuterated in the exocyclic hydroxymethyl group of the carbohydrate residue ([d_1]GalCer), were prepared having different ceramide compositions based on fatty acid alterations, which are often implicated as modulators of its membrane function. These were extensively examined in unsonicated liposomes of POPC containing cholesterol.

MATERIALS AND METHODS

POPC was obtained from Avanti Polar Lipids (Birmingham, AL) and was used without additional purification. GalCer from a beef brain source was also from Avanti Polar Lipids. Cholesterol was from Sigma (St. Louis, MO). Methods for fatty acid replacement and deuteration of GalCer have been described elsewhere (Singh et al., 1992a).

Preparation of fully hydrated lipid bilayer membranes for these experiments was as described in previous work (Morrow et al., 1992). In all cases, initial sample hydration was with phosphate-buffered deuterium-depleted water, pH 7.4, and subsequent hydration was with deuterium-depleted water. The total lipid used per sample was typically 100–200 mg, of which 10–14 mg (11.6–16.2 μmol) was deuterated, and the total final volume was 500–700 μl. Typically samples were incubated at 75°C to assure diffusional equilibrium within the bilayer before recording spectra. In general, the spectral features described were not significantly affected by sample history. In particular, spectra were unaffected by prolonged heating above 80°C with vigorous vortexing versus rapid warming to only 55°C, and they could be reproduced in fresh samples.

^{2}H NMR spectra were collected by using the quadrupole echo pulse sequence (Davis et al., 1976) at 23.215 MHz on a home-built instrument (Simatos et al., 1990) and at 76.7 MHz on a Varian Unity 500 spectrometer (Varian, Palo Alto, CA) equipped with a Doty 5-mm solenoid probe. At the lower field, the $\pi/2$ pulse length was between 2.3 μs and 2.7 μs, and pulse separation in the echo sequence was typically 60 μs. Signal-to-noise ratio of the free induction decay was improved by oversampling by a factor of 4, as described by Prosser et al. (1991). Effective digitizer dwell time was thus 4 μs. For each carbohydrate-deuterated sample, up to 400,000 transients were collected per spectrum, with a repetition time of 0.35 s. For spectra of chain-perdeuterated samples, between 8,000 and 60,000 transients were collected with a dwell time of 2 μs and oversampling by a factor of 2 so that the effective dwell time was 4 μs. Pulse separation was 40 μs. At the higher field, $\pi/2$ pulse length was between 5.5 μs and 5.7 μs. Pulse separation in the echo sequence was typically 20–30 μs. Repetition time was typically 80 ms, although values as high as 1000 ms were tried, with no significant effect on results. Powder patterns were simulated by using a program developed by Griffin and co-workers (Wittebort et al., 1987).

RESULTS

Fig. 1 illustrates glycolipid structures and carbohydrate deuteron locations referred to in the present work. Note that the deuteron at C_6 of the galactose residue in [d_1]GalCer (i.e., on the exocyclic hydroxymethyl group) can have *pro*-R or *pro*-S stereochemistry and that these two sites are spectrally inequivalent. The C_5-C_6 bond, about which restricted internal rotation might occur, is indicated (*solid arrow*).

Fig. 2 displays typical ^{2}H NMR powder spectra obtained at a magnetic field of 11.7 T for GalCer having deuteration at C_6 of the sugar residue ([d_1]GalCer). In each case glycolipids were dispersed in POPC/cholesterol membranes (mole ratio 7:90:30). The results are identifiable in terms of the two-doublet (four-line) ^{2}H NMR powder spectra originally reported by Skarjune and Oldfield (1979) for fluid bilayers of pure [d_1]GalCer having 16-carbon saturated fatty acid. We have consistently observed such two-doublet spectra for [d_1]GalCer dispersed as a minor component in fluid POPC bilayers (Singh et al., 1992a), and similar results exist for the galactose residue of lactosyl diglyceride (Renou et al., 1989). The interpretation of these past experiments has been that suggested by Skarjune and Oldfield (1979): one doublet arises from each of the (equal) populations of the two possible stereoisomeric deuteration sites at C_6 of the galactose residue. Such spectra are considered to reflect predominantly axially symmetric glycolipid whole-body rotation about an axis perpendicular to the bilayer, i.e., the glycolipid long axis. The "90° edge" quadrupole splittings represented in two-doublet

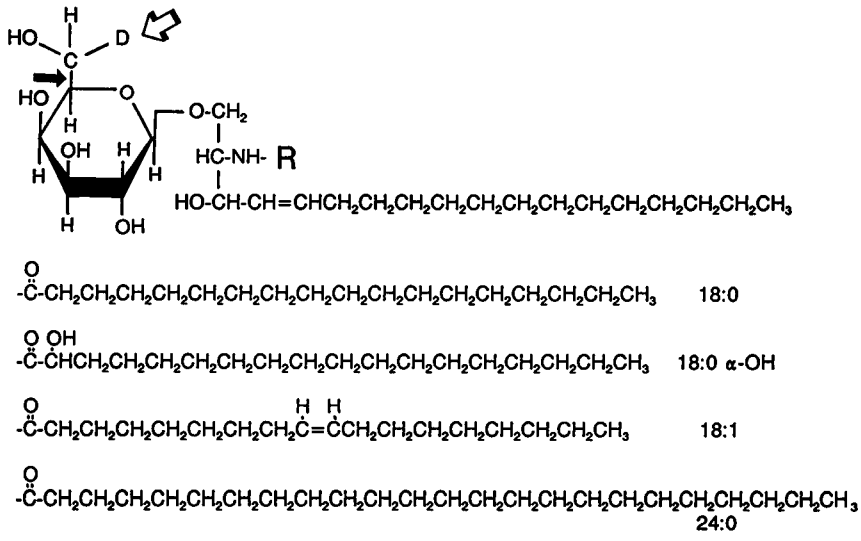

FIGURE 1 Chemical structures for the headgroup-deuterated GalCers ([d_1]GalCer) studied in the present work. The deuterated exocyclic hydroxymethyl group (C_6) of the carbohydrate residue is indicated (*open arrow*), as is the C_5-C_6 bond about which this group might undergo restricted rotation. R refers to the fatty acid attached in amide linkage: shown from top to bottom, the 18-carbon species (stearic, α-OH-stearic, and oleic acids, respectively) and the 24-carbon saturated lignoceric acid.

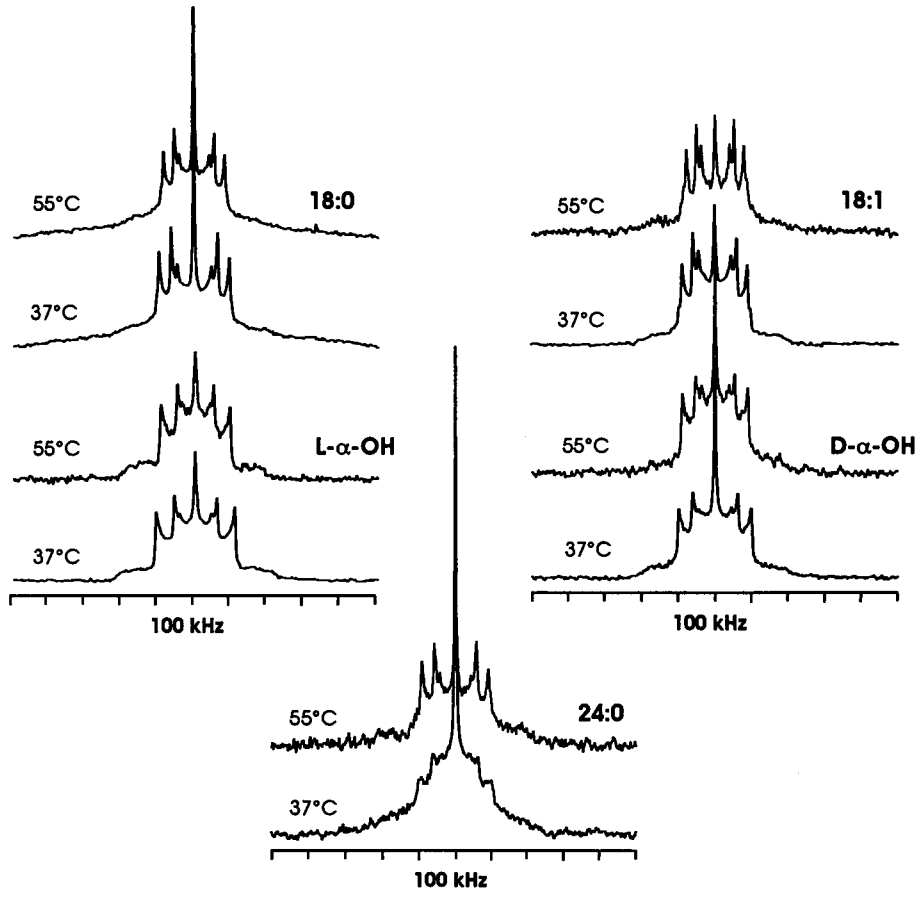

FIGURE 2 Typical ^2H NMR powder spectra collected at high field (11.7 T, 76.7 MHz) for GalCer deuterated at C_6 of the sugar residue ([d_1]GalCer). Glycolipids dispersed at 7 mol % in POPC/cholesterol bilayers. 18:0 [d_1]GalCer (18:0); 18:1 [d_1]GalCer (18:1); L-α-OH 18:0[d_1] GalCer (L-α); D-α-OH 18:0[d_1]GalCer (D-α); 24:0 [d_1]GalCer (24:0). Spectra were collected while cooling, after initial incubation at high temperature, and have been normalized. Each spectrum represents 100,000 accumulated transients.

spectra are well known to be sensitive reflections of C-D bond orientation and spatial order (Seelig, 1977; Davis, 1983; Smith, 1984). These quadrupole splittings were measured for both doublets and are listed in Table 1 for comparison among the different fatty acid derivatives.

An inner doublet of low intensity is also present in each spectrum of Fig. 2. As shown below, this may reflect a small probability of C-D bond orientation along the third tetrahedral direction about the C_5-C_6 axis, and thus slow but nonnegligible asymmetric rotation around this axis superimposed upon the axially symmetric rotation expected for the molecule as a whole.

Fig. 3 shows typical spectra obtained at a magnetic field of 3.54 T for [d_1]GalCer, with a range of modifications to the 18-carbon fatty acid chain. A striking feature is the presence of a prominent nondoublet component, with a base of between 25 kHz and 35 kHz width and intensity peaked toward the center. As indicated above, this was not the anticipated spectral shape. Fig. 4 comprehensively presents such data for a wide range of temperatures. In addition to the nondoublet feature, some of the spectra in Figs. 3 and 4 display attributes reminiscent of the two-doublet spectra already described (*arrows* in Fig. 4). This was most obvious for the species with short-chain saturated fatty acids. Where the two- doublet-like feature appeared, its relative contribution was not a sensitive function of temperature. It should be noted that the features seen in Figs. 2–4 could be observed both in fresh samples and in samples that had been through repeated cycles of spectroscopy. Thus, observed differences in the spectra at the two field strengths were not a consequence of sample degradation.

As was the case for experimental results obtained at higher field (Fig. 2), the aspect of spectra in Figs. 3 and 4 most relevant to the question being addressed is their similarity at a given temperature for all fatty acids tested. However, in considering the unexpected features of spectra obtained in the present work, and their possible implications for GSL fatty acid effects on behavior, we examined species deuterated in the fatty acid itself and attempted simulation of spectra for species deuterated in the carbohydrate portion, as described below.

TABLE 1 Quadrupole splittings for the two-doublet spectra obtained at high field

GalCer fatty acid	$\Delta\nu_Q$ (±0.5 kHz)	
	37°C	55°C
18:0	13.2, 19.8	11.4, 17.2
18:1	12.2, 18.0	10.6, 16.0
L-α-OH	12.2, 22.0	10.3, 19.4
D-α-OH	12.6, 20.2	11.0, 18.2
24:0	13.0, 20.7	12.0, 18.8

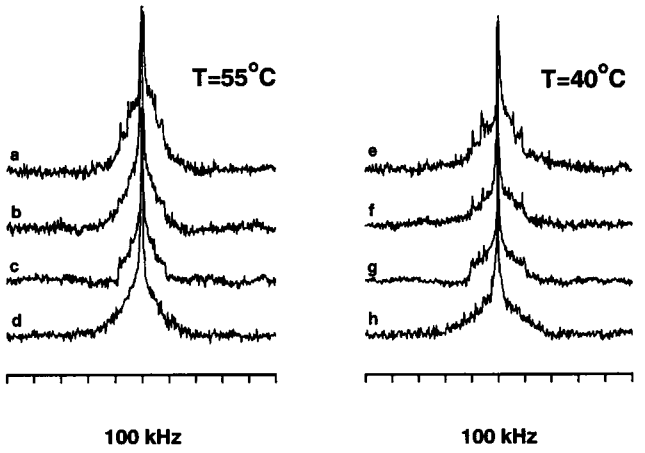

FIGURE 3 Selected normalized ^{2}H NMR powder spectra collected at 3.54 T (23.215 MHz) for GalCer deuterated at C_6 of the carbohydrate residue ([d_1]GalCer). Glycolipid dispersed at 7 mol % in unsonicated POPC/cholesterol (3:1 mol ratio) bilayers; examples are shown for two temperatures. 18:0 [d_1]GalCer (*a, e*); D-α-OH 18:0 [d_1]GalCer (*b, f*); L-α-OH 18:0 [d_1]GalCer (*c, g*); 18:1 [d_1]GalCer (*d, h*). Each spectrum represents between 300,000 and 400,000 accumulated transients.

Fig. 5 displays spectra, obtained at the lower magnetic field, for 18:0 GalCer (per)deuterated in the fatty acid chain rather than in the carbohydrate residue. These spectra are characteristic of symmetric rotation and typify examples well known for perdeuterated glycerolipids (Seelig, 1977; Davis, 1983; Smith, 1984) and GSLs (Skarjune and Oldfield, 1979; Singh et al., 1992b; Morrow et al., 1993). They are included so that a number of considerations can be addressed. First, there is the question of whether the motion responsible for the predominantly nondoublet carbohydrate headgroup spectrum seen at low field is reflected in departures from the usual axially symmetric spectra displayed by chain deuterons in the liquid crystalline phase. A related question is whether the coexisting populations apparent in the low field headgroup spectra can be distinguished by their chain-deuteron spectra. A further question, prompted by the weak intensity of the carbohydrate deuteron spectra, is whether there might be an additional ordered phase for which the spectrum of the headgroup deuteron is not apparent because of short transverse relaxation times (e.g., a gel phase characterized by motions slow on the NMR timescale of 10^{-5} s) (Oldfield et al., 1978; Meier et al., 1986; Renou et al., 1989; Hamilton et al., 1994). The perdeuterated-chain spectra in Fig. 5 suggest that the glycolipids exist as a homogeneous population in a single fluid phase. The same homogeneous behavior throughout the temperature range represented by Fig. 5 was seen for all the other GalCer species with perdeuterated fatty acids (data not shown).

Our goal in carrying out spectral simulations for GalCer deuterated in the sugar residue was not, primarily, to identify the specific motions involved, but to gain some understanding of the magnitude and nature of changes in headgroup behavior that might lead to the spectral effects observed. In particular, we wished to examine the extent to which spectra might be altered by reasonable variations in the rates of motion among accessible carbon-deuterium bond orientations. In approaching these simulations, we noted that the peaked nondoublet components seen in Figs. 3 and 4 are suggestive of spectra seen for lipids undergoing axially asymmetric motion (Huang et al., 1980; Meier et al., 1986; Auger et al., 1990). Axially asymmetric motion includes all two-fold "flips" and threefold (or higher) flip rotation among unequally populated sites. The observation that the width of the nondoublet component is considerably less than 120 kHz would indicate that elements of the electric field gradient tensor, which are subject to averaging by asymmetric reorientation, also reflect additional averaging by other motions that reduce their magnitude. In addition, the prominence of the central region, compared with the case for fast axially asymmetric reorientation, may indicate that some aspect of the headgroup reorientation is occurring at an intermediate rate on the ^{2}H-NMR timescale. The axial symmetry of the spectra in Fig. 5 (deuterons in the acyl chain) suggests that those features of the hydroxymethyl group motion that are responsible for the spectral asymmetry in Figs. 3 and 4 may be localized within the headgroup portion of the glycolipid.

On one occasion we observed that a prominent axially asymmetric component with distinct edges appeared in the spectrum of one sample after storage at room temperature (Fig. 6). The occurrence of this spectrum may have been related to partial drying, inasmuch as addition of water followed by mixing with a glass rod led to reappearance of spectra such as those in Figs. 3 and 4. As described below, this observation may provide additional insight into the spectra obtained more generally in our experiments.

Spectral simulations were carried out using a program developed by Griffin and co-workers (Wittebort et al., 1987). Motions specifically taken into account were the simplest ones capable of accounting for our observations: diffusion of the whole glycolipid about its long axis and restricted rotation about the bond (C_5-C_6) attaching the deuterated hydroxymethyl group to the sugar ring (Fig. 1). A particular consideration in choosing motions for the simulation is the fact that an unsplit spectrum implies that one of the diagonal elements of the underlying average electric field gradient tensor is zero. This in turn generally indicates that the principal axis of the electric field gradient tensor and the axis about which the asymmetric reorientation takes place are separated by close to the magic angle (Griffin, 1981). The orientation of the hydroxymethyl group is such that twofold exchange around the C_5-C_6 axis and asymmetric reorientation about the molecular long axis are both capable of giving rise to axially asymmetric electric field gradient tensors with one near-zero diagonal element. This does not necessarily rule out more complex motions and should not be interpreted as uniquely determining the properties of molecular motions underlying the observed spectra. In particular, no attempt has been made to incorporate a wobbling motion of the molecular long axis.

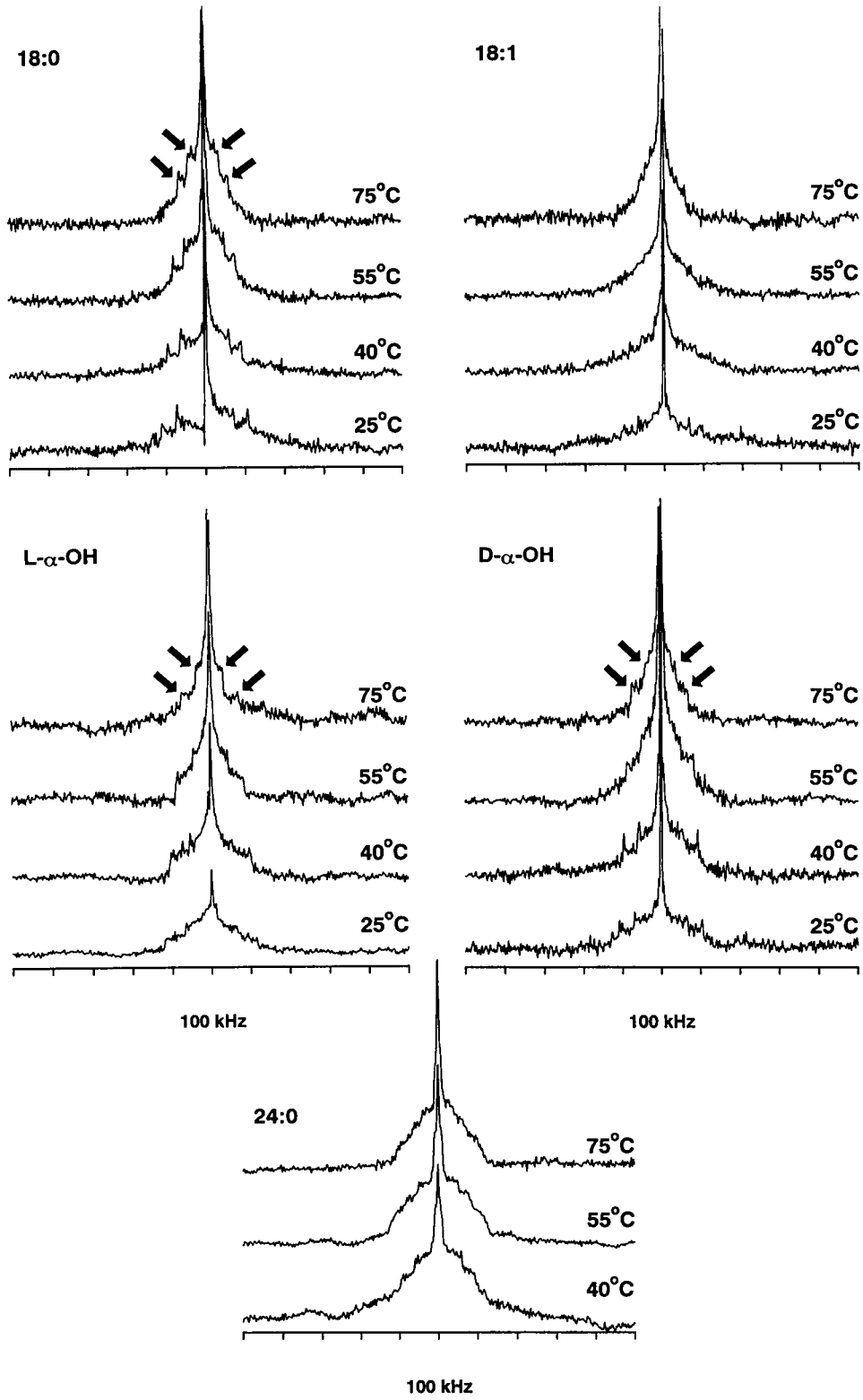

FIGURE 4 Arrayed normalized ^2H NMR powder spectra collected at 3.54 T (23.215 MHz) for GalCer deuterated at C_6 of the sugar residue ([d_1]GalCer). Glycolipid dispersed at 7 mol % in unsonicated POPC/cholesterol (3:1 mol ratio) bilayers (the same samples for which spectra in Figs. 2 and 3 were recorded). 18:0 [d_1]GalCer (18:0); 18:1 [d_1]GalCer (18:1); L-α-OH 18:0 [d_1]GalCer (L-α); D-α-OH 18:0 [d_1]GalCer (D-α); 24:0 [d_1]GalCer (24:0). Spectra were collected while cooling, after initial incubation at high temperature. Each spectrum represents between 300,000 and 400,000 accumulated transients. Arrows indicate two-doublet-like features referred to in the text.

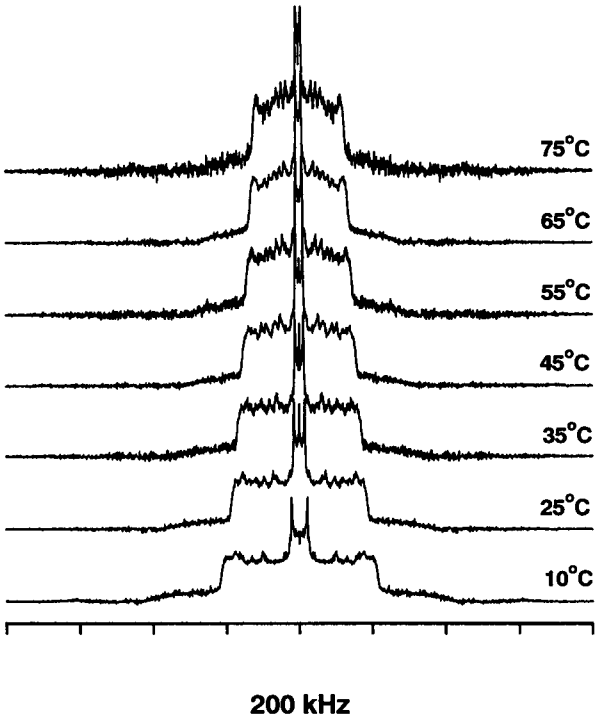

FIGURE 5 Arrayed ^{2}H NMR powder spectra (at 3.54 T) for 18:0 GalCer with perdeuterated fatty acid chain. N[d_{35}]-stearoyl GalCer was dispersed in unsonicated POPC/cholesterol multilamellar vesicles of the same composition and under the same conditions as those represented by Figs. 2–4.

In fluid membranes, rotational diffusion of amphiphilic lipids is generally axially symmetric. In contrast, rotation about C_5-C_6 is thought to be among three major conformers of unequal probabilities (Renou et al., 1989): such motion clearly is intrinsically axially asymmetric. On the basis of molecular modeling of energy-minimized x-ray crystallographic structures, we took the average angular separation between the C_6-H, C_6-D, and C_6-OH bonds to be 108.6° (i.e., it seems likely that the environment around C_6 is slightly distorted from tetrahedral). For purposes of spectral simulation, this deuterated exocyclic hydroxymethyl group was oriented relative to the axis of symmetric rotation such that the spherical coordinates for the bond directions giving rise to the two major doublets were (θ_1,f_1) = (60.3°, 0°) and (θ_2,f_2) = (128.8°, 90.68°), where f = 0 is defined by the projection of the first bond direction on the plane perpendicular to the C_5-C_6 "rotation" axis. In such an analysis, the minor doublet arises from a C-D bond having (θ_3,f_3) = (122.5°, 265.9°). Choice of simulation parameters was guided by the appearance of the 18:0 GalCer spectra at 55°C in Figs. 2, 4, and 6. Key simulated spectra are presented in Fig. 7.

Although not strictly necessary, it was natural to associate the axis of axially symmetric motion with the long molecular axis. The simulated spectrum in Fig. 7 *a*, which closely approximates key features of the high field spectra recorded in Fig. 2, was arrived at by assuming that glycolipid "whole body" rotational diffusion is fast and axially symmetric (see caption to Fig. 7), although rotation among the three non-

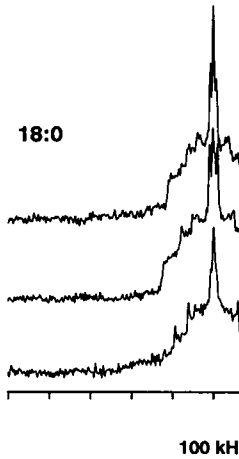

FIGURE 6 ^{2}H NMR spectra for a sample of 18:0 [d_1]GalCer corresponding to those in Figs. 3 and 4 after some apparent (inadvertent) dehydration during storage at room temperature. Features returned to those represented in Figs. 3 and 4 after addition of water and gentle stirring.

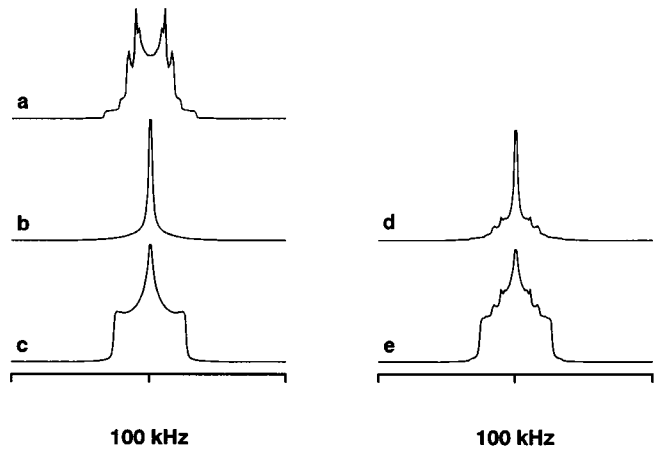

FIGURE 7 Simulated spectra calculated for GalCer deuterated at C_6 of the sugar residue ([d_1]GalCer) assuming various contributions from axially symmetric and axially asymmetric rotational modes involving the glycolipid. Threefold jumps were permitted about one axis presumed to be coincident with the glycolipid long axis, and twofold exchange was considered between sites around the C_5-C_6 bond axis attaching the deuterated hydroxymethyl group. Details are described in the text. (*a*) Axially symmetric threefold jumps about the GalCer long axis with a rate $k_r = 5 \times 10^7$ s^{-1}. Rotation about C_5-C_6 was considered to be slow on the NMR timescale. Population of the site associated with the inner doublet was 10%. (*b*) Axially symmetric threefold jumps about the glycolipid long axis with a rate $k_r = 6 \times 10^7$ s^{-1}, along with twofold exchange about the C_5-C_6 bond axis with a rate $k_f = 2 \times 10^4$ s^{-1}. (*c*) Axially asymmetric jumps about the glycolipid long axis (threefold exchange with probabilities of 0.453, 0.334, and 0.213) at a rate $k_r = 6 \times 10^7$ s^{-1}, along with twofold exchange about the C_5-C_6 bond direction with a rate $k_f = 10^6$ s^{-1}. (*d*) Superposition of simulation (*a*) and (*b*) with the latter weighted by a factor of 5. (*e*) Superposition of simulation (*a*) and (*c*) with the latter weighted by a factor of 5. The weighting used in superpositions (*d*) and (*e*) reflect differences in intensity of the simulated spectra attributable to transverse relaxation effects that are not apparent in (*a*), (*b*), and (*c*), which have been scaled to approximate the scaling used for the experimental spectra.

equivalent conformers about C_5-C_6 is very slow. The concept of highly restricted rotation about the latter bond was suggested by Skarjune and Oldfield (1979) in the first ^2H NMR study of membrane surface carbohydrate. A recent reference to this exocyclic hydroxymethyl group as part of a careful investigation of galactosyl diglyceride bilayers favored interpretation in terms of rapid rotation about C_5-C_6, although it did not discount the possibility of slow rotation (Renou et al., 1989). It will be seen in our analysis that the two major doublets of roughly equivalent intensity correspond to the two major C-D bond orientations about C_5-C_6. The minor (inner) doublet is then associated with the small population of the third C-D bond orientation about C_5-C_6. The population of the conformer giving rise to the minor doublet was taken to be 10% for purposes of simulation. Axially symmetric motion was simulated by 120° jumps of the entire molecule, with a rate of $k_r = 5 \times 10^7\ s^{-1}$.

The simulation shown in Fig. 7 *b* was obtained by allowing twofold exchange between the (θ_1,f_1) and (θ_2,f_2) bond directions around the C_5-C_6 axis to occur simultaneously with axially symmetric 120° jumps around the molecular long axis. For simplicity, the C-D orientation giving rise to the minor doublet in the previous simulation was left unpopulated. The rate for axially symmetric rotation about the glycolipid long axis was effectively identical to that used for the simulation in Fig. 7 *a*. Fig. 7 *d* shows the result of superimposing the simulations displayed in 7 *a* and 7 *b*, weighting the latter by a factor of 5. It will be seen that this is a good approximation to some of the spectra obtained at low magnetic field. From the fact that the addition of two spectra approximated the experimental ones, it would seem that the headgroup environment may not be homogeneous.

Another option that was considered by spectral simulation was the effect of permitting rotation of the entire glycolipid within the membrane to become significantly asymmetric. This was achieved by allowing the three orientations around the glycolipid long axis to be populated with unequal probabilities. A typical result is shown in Fig. 7 *c*. The probabilities were chosen to yield a spectrum with a base matching the width of the 55°C spectrum of Fig. 6. The rate for exchange around this axis was the same as in Fig. 7 *b*. The rate for twofold exchange between the (θ_1,f_1) and (θ_2,f_2) bond directions around the C_5-C_6 bond axis was adjusted to $k_f = 10^6\ s^{-1}$ in order to flatten the shoulders in the simulated spectrum.

Fig. 7 *e* shows the superposition of 7 *a* and 7 *c* with the latter being weighted by a factor of 5. This superposition is a good approximation to the features seen in the 55°C spectrum of Fig. 6, except for the narrow central doublet. It is interesting to note that the simulated intensity of the nondoublet component, relative to the doublet component, is lower in Fig. 7 *d* than in 7 *e*. This is consistent with the experimentally observed relationship between the intensities for the two types of nondoublet spectral component (18:0 $[d_1]$GalCer spectra in Figs. 4 and 6, respectively).

DISCUSSION

The primary aim of the present work was to consider the mechanism whereby glycolipid fatty acid variation may modulate GSL receptor function in fluid membranes whose compressibility is limited (Needham et al., 1988) by the presence of cholesterol. To this end, wideline NMR spectra were compared for nonperturbing deuterium nuclear probes within the carbohydrate headgroup of a GSL as a function of GSL fatty acid. For molecules undergoing rapid axially symmetric rotation in membranes, spectral splittings are related in a fairly uniquely determined fashion to C-D bond orientation and to the degree of time-dependent angular excursion about this direction (Seelig, 1977; Davis, 1983; Smith, 1984). Thus the key result can be found in the similarity of the quadrupolar splittings listed in Table 1: carbohydrate headgroup orientation and order are importantly similar among the glycolipids tested. Variations of only ± 1 kHz in quadrupolar splitting were found (Table 1), which would be readily explained by orientational differences of no more than a few degrees. In addition, the fundamental sameness of the axially asymmetric spectral features at a given field strength must be seen as an argument for similar behavior among the species studied.

Nevertheless, there were measurable spectral differences among the various fatty acid derivatives. The L-α-OH derivatives showed the greatest dissimilarity in quadrupolar splitting. Although the L-α-OH isomer is not a natural fatty acid in typical eukaryotic systems, it provides some indication of an upper limit for the effect of a fatty acid-OH group. At low field, spectra for the 18:1 and 24:0 $[d_1]$GalCer species displayed a triangular component with little evidence of the two-doublet feature that was present in the other fatty acid derivatives. This is likely not related to phase behavior because, of the five species studied, 18:1 and 24:0 GalCer have the lowest and highest phase transition temperatures, respectively (Curatolo and Jungalwala, 1985; Reed and Shipley, 1989). At high field, the 37°C spectrum for 24:0 $[d_1]$GalCer was noticeably more triangular than the others. We have also observed the latter result for more complex headgroup-deuterated GSLs with long-chain fatty acids (C. Grant, unpublished data), and there is evidence of it in published $[d_1]$GalCer spectra in membranes without cholesterol (Singh et al., 1992a). The latter observations suggest that glycolipid fatty acid variation can measurably alter GSL motional characteristics in complex membranes in which the GSL is a minor component.

The literature on fatty acid effects surrounding GSL receptor function has been covered in several recent papers (Stewart and Boggs, 1993; Kiarash et al., 1994; Hamilton et al., 1994; Singh et al., 1995) and will not be dealt with in detail here. Certainly, important evidence exists that GSL recognition function can be altered by fatty acid variation, and this phenomenon has been recorded both in natural membranes and in simple lipid bilayers. A common and reasonable interpretation of such observations has been that fatty acid features can

modulate orientation or arrangement of the carbohydrate recognition site at the membrane surface. Hence it is noteworthy that a range of GalCer species, each deuterated at C_6 of the sugar residue but characterized by different fatty acids, displays virtually indistinguishable spectral splittings when dispersed as minor components in highly fluid bilayer membranes of POPC (Singh et al., 1992a) and in matrices containing cholesterol. A possible explanation could be that the recognition site differences among the glycolipids having different fatty acids are only manifest when bound to a macromolecule, e.g., that they reflect differences in relative ease of displacement of bound water or ease of conformational distortion. It is interesting that our results suggest motional changes in the 24:0 fatty acid species, since the placement of a very long-chain fatty acid on a GSL has traditionally been associated with altered receptor function (Alving et al., 1980; Stewart and Boggs, 1993; Kiarash et al., 1994). A related consideration is that GSLs display greatly slowed axial diffusion under at least some of the conditions employed in important past crypticity measurements, as recently noted (Hamilton et al., 1994).

POPC (phase transition temperature −3°C) (Davis and Keough, 1985) provided a monounsaturated PC similar to species common in natural membranes. A partial binary phase diagram exists for POPC mixtures with cholesterol (Thewalt and Bloom, 1992). On the basis of these facts, and assuming that our GSL concentration was low enough to represent a minor perturbation on the POPC/cholesterol host matrix, we anticipated that the ternary mixture would be homogeneous at the relatively high temperatures considered. Consistent with such an interpretation, the basic spectral features seen for a given glycolipid were present throughout the temperature range of our experiments. Nevertheless, there have been important indications that there may be phase coexistence in membranes containing high concentrations of cholesterol and sphingolipids, and also preferential associations (Demel et al., 1977; Johnston and Chapman, 1988; Silvius, 1992).

In the present experiments the deuterated hydroxymethyl group of $[d_1]$GalCer gave rise to spectral features that were readily understandable if one assumed motional contributions from axially asymmetric modes. To our knowledge, this is the first report of axially asymmetric lipid ^{2}H NMR spectra in fluid membranes, although similar narrow asymmetric spectra have been recorded for mobile side chains of polypeptides (reviewed in Opella, 1986). The spectral simulations presented suggest that at least two types of motion contribute distinctly to the experimental spectra and that observed differences might be accounted for by the restriction or partial restriction of one or the other of these modes. For example, the only significant change in parameters required to go from the simulation of Fig. 7 *b* to that of 7 *a* was the elimination of twofold exchange between sites around the C_5-C_6 bond. Thus the spectral lineshape differences that were seen could have their bases in motional sensitivity to modest changes. It is noteworthy that it was possible to simulate the major features of the high and low field spectra without having to alter underlying headgroup geometry.

The question arises as to why magnetic field strength should affect spectral symmetry, as seemed to be the case in the present work. In a number of the spectra obtained at high magnetic field, there was enhancement of intensity in the doublet edges associated with molecules rotating about axes oriented perpendicular to the magnetic field. This suggests partial field-induced sample orientation. Magnetic field-induced lipid bilayer orientation has been described in detail by others (Seelig et al., 1985; Speyer et al., 1987; Reinl et al., 1992; Qui et al., 1993) and is dependent on field strength. The work of Speyer et al. (1987) is particularly relevant to the present experiments. At ~9.4 T, they noted strong orientation of deuterated sphingomyelin dispersed at 60 mol % in fluid bilayers of dimyristoyl PC. The authors reported that this was not seen with deuterated PC alone. They noted further that field-induced orientation was maximal in fluid liposomes and concluded that it may arise from a combination of liposome reorientation and deformation in the field. Sphingomyelin shares the same ceramide backbone found in GalCer (with phosphocholine replacing the sugar residue). Reinl et al. (1992) commented on an enhanced magnetic orientation of phospholipid vesicle samples in the presence of cholesterol and discussed the possibility that this might reflect a reduction in bilayer-bending stiffness. Qui et al. (1993) also noted cholesterol effects, although it reduced orientation in their case. In our experiments there may have been effects such as alteration in hydroxymethyl group motion induced by modified bilayer separation, strain, or hydration resulting from partial magnetic orientation. It is interesting to consider the possibility that changes in the observed spectra, and thus the corresponding motion of the hydroxymethyl group, might reflect changes in the nature of hydrogen bonding attributable to progressive equilibration of the headgroup hydration state. Following the logic of Speyer et al. (1987), both interbilayer stacking distance and membrane curvature would likely be disturbed, at least locally, by the vesicle strain that must accompany partial orientation in a magnetic field. Hence cycling the sample into a large magnetic field might provide a mechanism to disturb the interbilayer environment on a microscopic level and to promote equilibration of the bilayer headgroup region.

CONCLUSIONS

GalCer with deuterium probe in the carbohydrate headgroup was studied by wideline ^{2}H NMR in membranes containing cholesterol for indications of a mechanism whereby glycolipid fatty acid nature might control its function as a recognition site. Carbohydrate orientation at the surface of the fluid bilayer membranes that were involved seemed not to be sensitive to the fatty acid variations tested. The spectra obtained indicated the presence of asymmetric GSL rotational modes, and there was evidence of motional differences based on fatty acid structure.

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REFERENCES

Alving, C. R., K. A. Urban, and R. L. Richards. 1980. Influence of temperature on complement-dependent immune damage to liposomes. *Biochim. Biophys. Acta.* 600:117–125.

Auger, M., M.-R. Van Calsteren, I. C. P. Smith, and H. Jarrell. 1990. Glycerolipids: common features of molecular motion in bilayers. *Biochemistry.* 29:5815–5821.

Curatolo, W. 1987a. The physical properties of glycolipids. *Biochim. Biophys. Acta.* 906:111–136.

Curatolo, W. 1987b. Glycolipid function. *Biochim. Biophys. Acta.* 906: 137–160.

Curatolo, W., and F. B. Jungalwala. 1985. Phase behavior of galactocerebrosides from bovine brain. *Biochemistry.* 24:6608–6613.

Davis, J. H. 1983. The description of membrane lipid conformation, order and dynamics by ^{2}H-NMR. *Biochim. Biophys. Acta.* 737:117–171.

Davis, J. H., K. R. Jeffrey, M. Bloom, M. I. Valic, and T. P. Higgs. 1976. Quadrupolar echo ^{2}H MR spectroscopy in ordered hydrocarbon chains. *Chem. Phys. Lett.* 44:390–394.

Davis, P. J., and K. M. W. Keough. 1985. Chain arrangements in the gel state and the transition temperatures of phosphatidylcholines. *Biophys. J.* 48:915–918.

Demel, R. A., J. W. C. M. Jansen, P. W. M. Van Dijck, and L. L. M. Van Deenen. 1977. The preferential interaction of cholesterol with different classes of phospholipids. *Biochim. Biophys. Acta.* 465:1–10.

Fenske, D. B., K. Hamilton, H. C. Jarrell, E. Florio, K. R. Barber, and C. W. M. Grant. 1991. Glycosphingolipids: ^{2}H NMR study of the influence of carbohydrate headgroup structure on ceramide acyl chain behavior in glycolipid-phospholipid bilayers. *Biochemistry.* 30:4503–4509.

Grant, C. W. M. 1985. Fundamentals of physico-chemistry of glycolipids in membranes. *In* Gangliosides and Modulation of Neuronal Function. H. Rahmann, editor. Springer-Verlag, Berlin. 119–138.

Griffin, R. G. 1981. Solid state nuclear magnetic resonance of lipid bilayers. *Methods Enzymol.* 72:108–174.

Hakomori, S.-I. 1981. Glycosphingolipids in cellular interaction, differentiation, and oncogenesis. *Annu. Rev. Biochem.* 50:733–764.

Hakomori, S.-I. 1989. Aberrant glycosylation in tumors and tumor-associated carbohydrate antigens. *Adv. Cancer Res.* 52:257–331.

Hamilton, K. S., K. Briere, H. C. Jarrell, and C. W. M. Grant. 1994. Acyl chain length effects related to GSL crypticity in phospholipid membranes: probed by ^{2}H NMR. *Biochim. Biophys. Acta.* 1190:367–375.

Hamilton, K. S., H. C. Jarrell, K. M. Briere, and C. W. M. Grant. 1993. Glycosphingolipid backbone conformation and behavior in cholesterol-containing phospholipid bilayers. *Biochemistry.* 32:4022–4028.

Huang, T. H., R. P. Skarjune, R. J. Wittebort, R. G. Griffin, and E. Oldfield. 1980. Restricted rotational isomerization in polymethylene chains. *J. Am. Chem. Soc.* 102:7379–7381.

Johnston, D. S., and D. Chapman. 1988. A calorimetric study of the thermotropic behaviour of mixtures of brain cerebrosides with other brain lipids. *Biochim. Biophys. Acta.* 939:603–614.

Kiarash, A., B. Boyd, and C. Lingwood. 1994. Glycosphingolipid receptor function is modified by fatty acid content. *J. Biol. Chem.* 269: 11138–11146.

Mehlhorn, I. E., K. R. Barber, E. Florio, and C. W. M. Grant. 1989. A comparison of physical behaviour amongst four GSL families. *Biochim. Biophys. Acta* 986:281–289.

Mehlhorn, I. E., E. Florio, K. R. Barber, C. Lordo, and C. W. M. Grant. 1988. Evidence that trans-bilayer interdigitation of glycosphingolipid long chain fatty acids may be a general phenomenon. *Biochim. Biophys. Acta.* 939:151–159.

Meier, P., E. Ohmes, and G. Kothe. 1986. Multipulse dynamic NMR of phospholipid membranes. *J. Chem. Phys.* 85:3598–3617.

Morrow, M. R., D. Singh, D. Lu, and C. W. M. Grant. 1992. Glycosphingolipid phase behaviour in unsaturated phosphatidylcholine bilayers: a ^{2}H NMR study. *Biochim. Biophys. Acta.* 1106:85–93.

Morrow, M. R., D. Singh, D. Lu, and C. W. M. Grant. 1993. Glycosphingolipid acyl chain orientational order in unsaturated PC bilayers. *Biophys. J.* 64:654–664.

Needham, D., T. J. McIntosh, and E. Evans. 1988. Thermochemical and transition properties of DMPC/cholesterol bilayers. *Biochemistry.* 27: 4688–4673.

Nyholm, P.-G., I. Pascher, and S. Sundell. 1990. The effect of H-bonds on the conformation of glycosphingolipids. *Chem. Phys. Lipids.* 52:1–10.

Oldfield, E., M. Meadows, D. Rice, and R. Jacobs. 1978. Spectroscopic studies of ^{2}H labeled membrane systems. NMR investigation of the effects of cholesterol in model systems. *Biochemistry.* 17:2727–2740.

Opella, S. J. 1986. Protein dynamics by solid state NMR. *Methods Enzymol.* 131:327–361.

Prosser, R. S., J. H. Davis, F. W. Dahlquist, and M. A. Lindorfer. 1991. ^{2}H NMR of the gramicidin A backbone in a phospholipid bilayer. *Biochemistry.* 30:4687–4696.

Qiu, X., P. A. Mirau, and C. Pidgeon. 1993. Magnetically induced orientation of phosphatidylcholine membranes. *Biochim. Biophys. Acta.* 1147:59–72.

Reed, R. A., and G. G. Shipley. 1989. Effect of chain unsaturation on the structure and thermodynamic properties of galactocerebrosides. *Biophys. J.* 55:281–292.

Reinl, H., T. Brumm, and T. Bayerl. 1992. Changes of the physical properties of the liquid-ordered phase with temperature in binary mixtures of DPPC with cholesterol. A 2H NMR, FT-IR, DSC, and neutron scattering study. *Biophys. J.* 61:1025–1035.

Renou, J. P., J. B. Giziewicz, I. C. P. Smith, and H. C. Jarrell. 1989. Glycolipid membrane surface structure: orientation, conformation, and motion of a disaccharide headgroup. *Biochemistry.* 28:1804–1814.

Seelig, J. 1977. Deuterium magnetic resonance: theory and application to lipid membranes. *Q. Rev. Biophys.* 10:353–418.

Seelig, J., F. Borle, and T. A. Cross. 1985. Magnetic ordering of phospholipid membranes. *Biochim. Biophys. Acta.* 814:195–198.

Silvius, J. R. 1992. Cholesterol modulation of lipid intermixing in phospholipid and glycosphingolipid mixtures. Evaluation using fluorescent lipid probes and brominated lipid quenchers. *Biochemistry.* 31: 3398–3408.

Simatos, G. A., K. B. Forward, M. R. Morrow, and K. M. W. Keough. 1990. Interaction between perdeuterated DMPC and low molecular weight pulmonary surfactant protein SP-C. *Biochemistry.* 29: 5807–5814.

Singh, D., H. C. Jarrell, K. R. Barber, and C. W. M. Grant. 1992a. Glycosphingolipids: ^{2}H NMR study of the influence of fatty acid characteristics on the carbohydrate headgroup in phospholipid bilayers. *Biochemistry.* 31:2662–2669.

Singh, D., H. C. Jarrell, E. Florio, D. B. Fenske, and C. W. M. Grant. 1992b. Effects of fatty acid alpha-hydroxylation on glycosphingolipid properties in phosphatidylcholine bilayers. *Biochim. Biophys. Acta.* 1103:268–274.

Singh, D. M., X. Shan, J. H. Davis, D. Jones, and C. W. M. Grant. 1995. Oligosaccharide behavior of complex natural glycosphingolipids in multicomponent model membranes. *Biochemistry.* 34:451–463.

Skarjune, R., and E. Oldfield. 1979. Physical studies of cell surface and cell membrane structure: ^{2}H NMR of cerebrosides. *Biochim. Biophys. Acta.* 556:208–218.

Smith, I. C. P. 1984. Conformational and motional properties of lipids in biological membranes as determined by deuterium magnetic resonance. *Biomembranes.* 12:133–168.

Speyer, J. B., P. K. Sripada, S. K. Das Gupta, G. G. Shipley, and R. G. Griffin. 1987. Magnetic orientation of sphingomyelin-lecithin bilayers. *Biophys. J.* 51:687–691.

Stanton, S. G., A. B. Kantor, A. Petrossian, and J. C. Owicki. 1984. *Biochim. Biophys. Acta.* 776, 228–236.

Stewart, R. J., and J. M. Boggs. 1993. Exposure of galactosyl ceramide to galactose oxidase in liposomes. *Biochemistry.* 32:5605–5614.

Stromberg, N., P.-G. Nyholm, I. Pascher, and S. Normark. 1991. Saccharide orientation at the cell surface affects glycolipid receptor function. *Proc. Natl. Acad. Sci. USA.* 88:9340–9344.

Thewalt, J. L., and M. Bloom. 1992. Phosphatidylcholine:cholesterol phase diagrams. *Biophys. J.* 63:1176–1181.

Thompson, T. E., and T. W. Tillack. 1985. Organization of glycosphingolipids in bilayers and plasma membranes of mammalian cells. *Annu. Rev. Biophys. Chem.* 14:361–386.

Utsumi, H., T. Suzuki, K. Inoue, and S. Nojima. 1984. Haptenic activity of galactosyl ceramide and its topographical distribution on liposomal membranes. *J. Biochem.* 96:97–105.

Wittebort, R. J., E. T. Olejniczak, and R. G. Griffin. 1987. Analysis of 2^H NMR lineshapes in anisotropic media. *J. Chem. Phys.* 86: 5411–5420.