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Biochimica et Biophysica Acta 1240 (1995) 75–82

BBA Biochimica et Biophysica Acta

# $^2$H-NMR study of two probe-labelled glycosphingolipid-derived signalling modulators in bilayer membranes

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Received 23 March 1995; accepted 6 June 1995

**Abstract**

We describe here the first report of sphingoid bases bearing non-perturbing $^2$H probe nuclei. These were produced, by two different routes of partial synthesis, to permit direct assessment of their arrangement and behaviour as minor components in membrane systems. Wideline $^2$H-NMR spectra of *N,N*-dimethylsphingosine with deuterated amino-methyl groups ([$^2H_6$]dimethylsphingosine), and of lyso-dihydrogalactosylceramide (lyso-GalCer) with deuterium nuclei at $C_4$,$C_5$ of the sphingosine backbone and at $C_3$,$C_4$ of the galactose ring ([$^2H_4$]lyso-GalCer), were recorded in unsonicated, cholesterol-containing fluid bilayer membranes. The sphingolipid metabolites behaved as single populations of lipid amphiphiles dispersed uniformly in the membrane and undergoing rapid symmetric motion about their long molecular axes. This was the case throughout the pH ranges examined, which included values generally considered for the cell cytoplasm. Spectra of [$^2H_6$]dimethyl sphingosine indicated that the methyl groups are equivalent on the NMR timescale, and that the molecule's orientation and behaviour are largely unaffected by pH over the range, 6 to 10.5. There was no spectral evidence of deprotonation of the tertiary amine function in this range. Similarly, variation of pH between 6.4 and 8.9 had virtually no effect on the average conformation and orientational order of lyso-GalCer at the level of $C_4$,$C_5$ in the sphingosine backbone. pH did, however, exert significant control over the orientation of the galactose residue – the effect being most marked in the region of the sphingoid base p$K_a$. The lyso-glycolipid showed some evidence of being less motionally ordered than the corresponding parent species, presumably as a result of removal of constraints imposed by the fatty acid.

*Keywords:* Dimethylsphingosine; Glycosphingolipid, lyso-; NMR, $^2$H-

## 1. Introduction

It has become apparent in recent years that sphingolipid metabolites serve as physiological modulators of signalling pathways in higher animal cells (reviewed in [1,2]). In particular, considerable attention has focused on the role of such species as inhibitors of protein kinase C [1–6]. However, there have been observations that the involvement of sphingolipid breakdown products in signal transduction extends to a wider range of major signalling elements – including other protein kinases [4,7], the EGF receptor [8–10], and phosphatidate phosphohydrolase [5,11]. Sphingolipid breakdown products considered as signalling pathway intermediates are amphipathic in nature, and suggested mechanisms for their roles in signalling have centred on events at the plasma membrane. It has been observed that in some cases there are highly specific structural requirements connected with sphingolipid derivative biochemical function [4,9]. In others, a considerable range of molecules sharing some common chemical feature give similar results [12,13]. Hypotheses as to how glycolipid metabolites might modulate signalling events range from specific interaction with enzymes, to electrostatic effects on surrounding lipids [4,7,12,14–16]. Given the complexity of the pathways, use of biochemical results to test these mechanisms has proven challenging [17]; and it has become highly desirable to characterise sphingoid base behaviour in membrane systems.

The phase behaviour of cationic sphingosine in bilayers of neutral and acidic phospholipids has been examined [18,19], as has its ability to influence electrostatic interaction of large molecules with acidic bilayer membranes [16]. Considerable information concerning the surface behaviour of related molecules is available from recent

Abbreviations: GSL, glycosphingolipid; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; Lyso-GalCer, lyso-dihydrogalactosylceramide.

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*SSDI* 0005-2736(95)00156-5

monolayer studies of amide derivatives of sphingosine and semisynthetic derivatives of gangliosides [20]. Workers noted early on that the amine group of sphingosine derivatives is the source of both a key set of physical properties and the potential for sensitivity to pH [1,14,15].

In the present study, we sought a method of recording, by deuteration and direct measurement, the physical behaviour and arrangement of several key sphingolipid breakdown products in membranes. $^2H$ wideline NMR offers a non-perturbing technique that has proven a particularly attractive source of information on intact membranes [21–23]; however, deuterated sphingolipid metabolites have not been reported previously. We chose dimethyl sphingosine for our initial studies, since it is a deglycosylated species that has been characterised with regard to structural detail as a modulator of protein kinases in an epidermoid cell line in which it is the predominant sphingosine [4,9], and as an affector of platelet [13] and neutrophil metabolism [6]. Lyso-galactosyl ceramide (lyso-GalCer), which has been demonstrated to inhibit protein kinase C [3], was selected as a carbohydrate-bearing breakdown product of a relatively simple glycosphingolipid. Both species were studied at low concentration in unsonicated bilayers of the fluid, naturally-occurring phospholipid, 1-palmitoyl-2-oleoylphosphatidylcholine, to which cholesterol was added to mimic plasma membrane fluidity characteristics.

## 2. Materials and methods

1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids, Birmingham, AL, and was used without further purification. *trans*-D-Erythro-2-amino-4-octadecene-1,3-diol-4-sphingenine (D-sphingosine) isolated from bovine brain cerebrosides was purchased from Sigma, St. Louis, MO (catalogue S6136 99% by TLC). Cholesterol was from the same supplier. Sodium borodeuteride (98.73 atom% $^2H$), methanol-$^2H_4$ (99.3 atom% $^2H$), and paraformaldehyde-$^2H_2$ (99.3 atom% $^2H$) were obtained from MSD Isotopes, Montreal, Canada and Cambridge Isotope Laboratories Woburn, MA. Non-deuterated *N,N*-dimethylsphingosine (TLC standard) was from Matreya, Pleasant Gap, PA.

The deuterated *N,N*-dimethyl derivative of D-sphingosine ([$^2H_6$]dimethylsphingosine) was synthesized by reductive methylation via a modification of the method of Igarashi et al. [9]. D-Sphingosine (50 mg, 167 μmol) was dissolved in 50 ml of a 1:1 mixture of pH 9.0 0.2 M borate buffer (made up in $^2H_2O$)/methanol-$^2H_4$, with the help of bath sonication. Following addition of sodium borodeuteride to the reaction vessel, deuterated paraformaldehyde was transferred in through a 1 cm (ID) glass tube 20 cm in length by sublimation above 150°C. Virtually complete conversion of the D-sphingosine to the faster-running deuterated dimethylsphingosine form could be achieved when the sodium borodeuteride was added in two separate aliquots spaced by 1 h during the sublimation of the paraformaldehyde. The flask was sealed and allowed to react for 24 h with constant stirring. The product was taken to dryness and extracted with chloroform. Thin-layer chromatography was on Silica Gel 60 (Merck) plates, eluting with $CHCl_3/CH_3OH/NH_4OH$ (80:20:2) against the dimethyl standard. In this TLC system the monomethyl derivative is slower running and migrates with unreacted sphingosine. Electron ionization and chemical ionization mass spectroscopy demonstrated the presence of a major product with the expected mass for dimethylsphingosine. This material was purified on an Iatrobead column (Iatron, Japan), eluting with 0–60% methanol in chloroform: it co-chromatographed with (non-deuterated) commercial standards.

Galactosylceramide (GalCer) from beef brain was purchased from Avanti Polar Lipids, Birmingham, AL. Natural beef brain GalCer (175 mg, 215 μmol) was deuterated over Raney nickel as described by Cioffi and Prestegard [24]. Raney nickel was predeuterated following a procedure by Koch and Stuart [25]. Following removal of paramagnetic impurities on a chelex 100 resin column (sodium mesh form, Bio-Rad), the product (10 mg, 30 μmol) was dissolved in dimethyl sulfoxide-$d_6$ and characterised by $^1H$-NMR. It was found to have complete deuteration of the double bond and partial deuteration at $C_3$ and $C_4$ of the sugar ring [24]. Lyso-GalCer (i.e., with the fatty acid removed) was produced from this material by hydrolysis in refluxing butanolic KOH [26]. The ninhydrin-positive product was applied to a $1.2 \times 12$ cm silicic acid column (Bio-Rad 200–400 mesh) and eluted with 65:15:4 $CHCl_3/CH_3OH/H_2O$. Thin-layer chromatography (TLC) plates were sprayed with sulfuric acid/ethanol and developed by charring.

Lipid bilayer membranes for these experiments were prepared by dissolving all components at the final desired ratio in $CHCl_3$, which was then removed under a stream of nitrogen. The resultant films were further dried in vacuum overnight. Initial hydration of dried lipid films was with deuterium-depleted water (MSD Isotopes) buffered with 30 mM Hepes, 20 mM NaCl and 5 mM EDTA at selected pH values. For subsequent repeated lyophilisations, 175 μl aliquots of deuterium depleted water were employed. Five freeze-thaw cycles followed the final hydration step. During thaw stages the samples were incubated 10°C above the transition temperature of the host matrix to assure diffusional equilibrium within the bilayer. Total lipid used per sample was typically 60–120 mg. Sample pH was checked after spectral analysis.

$^2H$-NMR spectra were acquired at 76.7 MHz on a Varian Unity spectrometer using a single-tuned Doty 5mm solenoid probe, with temperature regulation to ±0.1 °C. A quadrupolar echo sequence ('SSECHO') was employed with full phase cycling and $\pi/2$ pulse length of 5.2 μs. Pulse spacing was typically 30 μs. 25 000 transients were

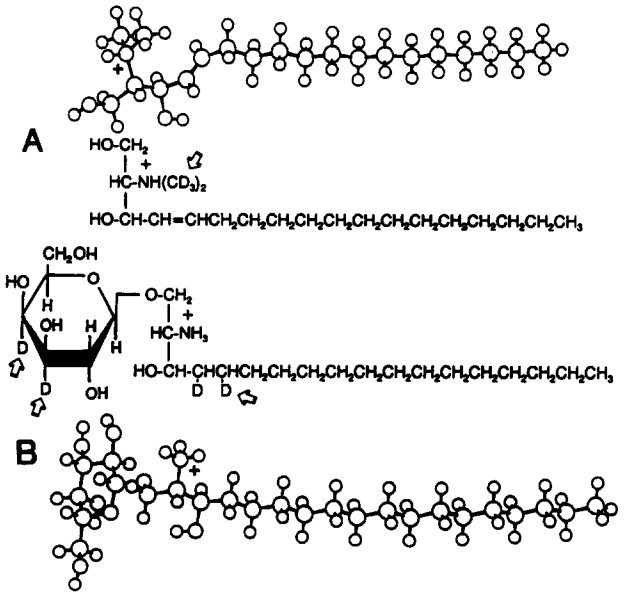

Fig. 1. Chemical structures of the selectively deuterated glycosphingolipid metabolites, [$^2H_6$]dimethylsphingosine (A) and [$^2H_4$]lyso-galactosylceramide (lyso-GalCer) (B). Structures arrived at by molecular modelling are included above and below the chemical structures respectively. Arrows indicate deuteration sites, 'D', considered in the present work.

generally acquired for spectra of [$^2H_6$]dimethylsphingosine, and 100000 in the case of [$^2H_4$]lyso-GalCer. DePakeing was performed by a non-iterative method utilising a non-negative least-squares algorithm [27] supplied by Dr. K. Whittall. Molecular modelling was via BIOSYM (Biosym Technologies, San Diego, CA), using the method of steepest descents and dielectric constant 1, in the absence of H-bonding. Input structures were derived from crystal coordinates of the precursor, dihydrogalactosylceramide (Cambridge Crystallographic Data Bank).

## 3. Results

Fig. 1 illustrates chemical structures for the GSL metabolites, [$^2H_6$]dimethylsphingosine and [$^2H_4$]lyso-GalCer, studied in the present work. Also presented are features arrived at by molecular modelling in vacuo. The molecules are shown protonated at the nitrogen nucleus, although this will be subject to a reversible equilibrium determined by pH and the $pK_a$ of the amino group. Selected 37°C $^2$H-NMR powder spectra of these compounds dispersed at low concentration in bilayer membranes of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC)/cholesterol (2:1 mol ratio) are presented in Fig. 2 (corresponding dePaked versions below). POPC is a highly fluid phospholipid with phase transition of −3°C [28].

### 3.1. Dimethylsphingosine

Powder spectra of [$^2H_6$]dimethylsphingosine in phospholipid bilayers were characterised by a single Pake

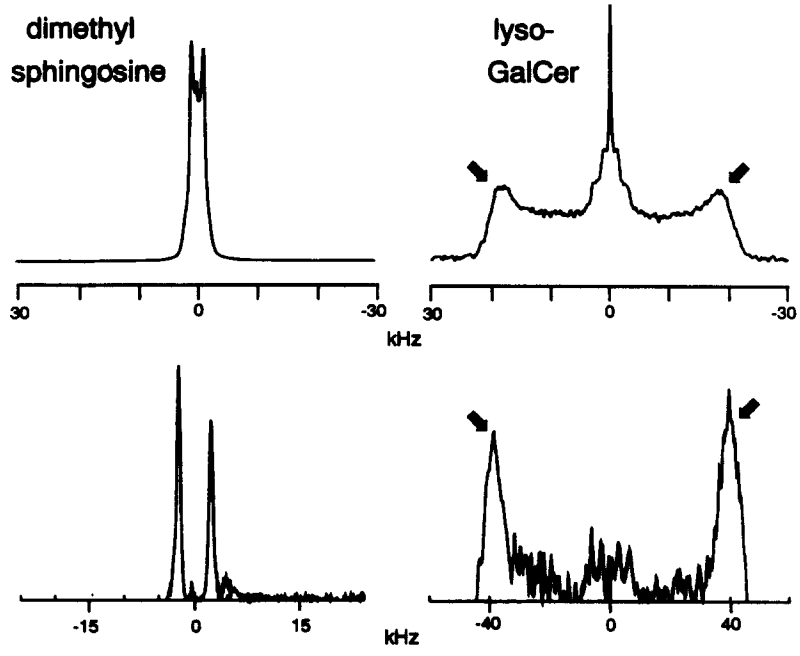


Fig. 2. Typical $^2$H-NMR spectra of [$^2H_6$]dimethylsphingosine and [$^2H_4$]lyso-GalCer. In each case the deuterated lipid was dispersed as a minor component (5 mol% in the case of dimethylsphingosine and 10 mol% for lyso-GalCer) in unsonicated fluid bilayer host matrices of POPC/cholesterol (2:1 mol ratio). Final sample pH, 7.1. Temperature 37°C. DePaked spectra are shown below their corresponding powder spectra. Note that dePaking produces the zero degree oriented spectrum by convention, so that quadrupole splittings are 'doubled': frequency axis scale was chosen for illustrative convenience. Arrows indicate the assigned $C_4$,$C_5$ sphingosine backbone deuteron peaks of [$^2H_4$]lyso-GalCer – only a minor degree of deuteration has occurred in the sugar ring (inner doublets).

doublet with quadrupole splitting about 2.3 kHz at neutral pH (Fig. 2). Such lineshapes are typical of molecules undergoing rapid symmetric motion about axes perpendicular to the plane of the bilayer. The sharp inner peak could be partially resolved on an expanded frequency axis (not shown here) into two components: the larger of the two being offset 0.1–0.2 kHz downfield from the powder spectrum centre and representing residual deuterated water; while the smaller peak, about which the powder pattern is symmetric, reflects the presence of some vesicles with high curvature for which the quadrupole splittings are motionally averaged to zero. Very similar spectra were obtained in POPC bilayers not containing cholesterol, although in the latter case the splitting was reduced (as expected in the absence of the ordering effect of cholesterol, see eq. 1 below) – being about 1.4 kHz at neutral pH and 37°C.

The spectra observed appear to be interpretable as follows. Examination of the dimethyl sphingosine structure in Fig. 1 shows that, in the absence of rapid inversion about the nitrogen centre, the deuteromethyl groups should be spectroscopically inequivalent due to asymmetry about $C_2$ (the sphingosine backbone carbon to which the amine group is attached). The well known rapid inversion about nitrogen centres does not occur for protonated amines. Hence one might have anticipated a pair of Pake doublets for [$^2H_6$]dimethyl sphingosine. However, the spectroscopic timescale of the present experiments is long – approximately the inverse of the spectral splitting or roughly $10^{-3}$ s. Relative to such a timescale, protonated and unprotonated forms of the molecule are expected to be in extremely rapid equilibrium, allowing for inversion while in the unprotonated form with resultant spectral equivalence of the deuterated methyl groups through rapid stereoisomer interconversion.

Typical spectral data collected as a function of pH (from 6.0 to 10.5) in POPC/cholesterol membranes are presented in Fig. 3. Considering the extensive pH range involved, these are remarkably similar. The measured quadrupole splittings are shown plotted in Fig. 4: they fall within experimental error ($\pm 0.1$ kHz) of one another. As suggested above, it is anticipated that the observed spectrum for a given set of sample conditions is the average of spectra arising from charged and uncharged species, with the relative contribution of each being in direct proportion to their relative quantities. Since the p$K_a$ of dimethylsphingosine has been reported to be greater than 10 [12], in the physiological pH range one would expect the spectrum to reflect only features associated with the protonated form. As the amount of unprotonated [$^2H_6$]dimethylsphingosine becomes significant (with increasing pH), there should be a continuous shift to features of the unprotonated form over approximately 1 pH unit on either side of the p$K_a$ for the tertiary amine group. Intuitively one would likely expect the average orientation and/or motional order of the deuterated methyl groups of dimethylsphingosine within the zwitterionic membrane to differ somewhat between protonated and unprotonated forms of the nitrogen nucleus to which the methyl groups are attached. In such a case, the splittings characterising charged and uncharged species would differ quantitatively. Since the pH range over which spectra were run covers values at which the amino function must be protonated, it would appear that the p$K_a$ of the amino function is beyond the range tested. This is in agreement with the titration measurements in detergent carried out previously by Merrill at al., who detected no dissociable protons as high as pH 10 for dimethylsphingosine [12].

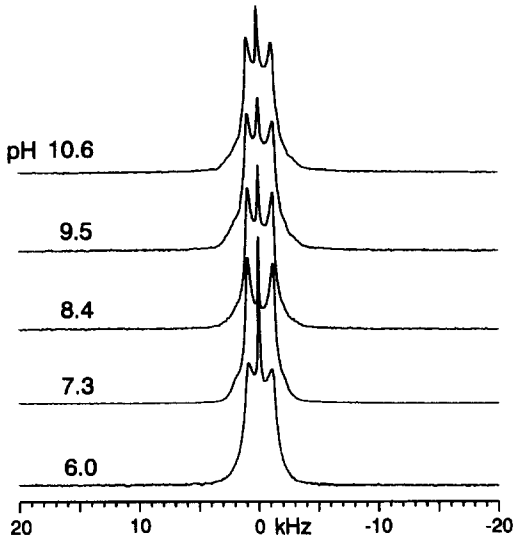


Fig. 3. pH dependence of $^2$H-NMR powder spectra for [$^2H_6$]dimethylsphingosine dispersed at 5 mol% in POPC/cholesterol (2:1 mol ratio) multilamellar vesicles. Temperature 37°C.

### 3.2. Lyso-GalCer

[$^2H_4$]lyso-GalCer provided an example of a carbohydrate-bearing sphingolipid metabolite. In this case the primary deuteration site was in the sphingosine backbone at $C_4$ and $C_5$. However the reaction used to introduce deuterium nuclei via catalytic reduction of the sphingenine $C_4C_5$ double bond of lyso-GalCer is also known to lead to some deuteron exchange of $H_3$ and $H_4$ in the sugar ring [24]. Thus, the [$^2H_4$]lyso-GalCer powder spectrum in Fig. 2 may be readily understood as follows. By far the most intense feature is the outer Pake doublet (quadrupole splitting some 39 kHz at 37°C). This must be associated with the deuterons at $C_4$,$C_5$, since no other intense peaks exist and the reductive process introduces equal numbers of deuterons at these two locations. Moreover they fall within what is considered the 'plateau region' of order profiles for alkyl chains within the membrane hydrophobic domain; and deuterons at these levels give overlapping peaks [21–23,29]. It would be expected that the C-$^2$H bonds at $C_4$,$C_5$ will have average orientations approaching 90° to

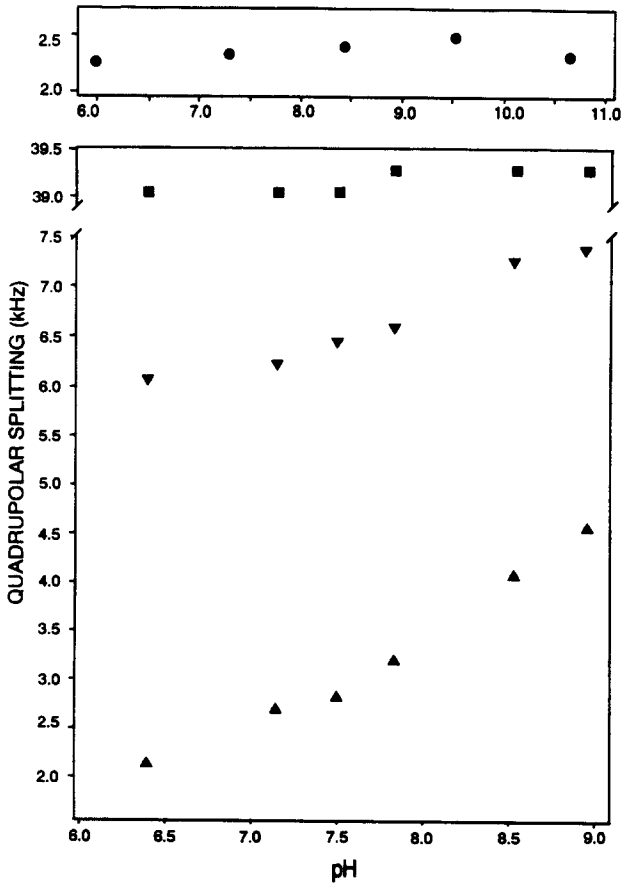


Fig. 4. Quadrupole splittings for spectra of [$^2H_6$]dimethylsphingosine and [$^2H_4$]lyso-GalCer plotted as a function of pH for the POPC/cholesterol membranes referred to in Figs. 3 and 5 at 37°C. Estimated experimental error is ±0.1 kHz. Circles represent the deuteromethyl groups of [$^2H_6$]dimethylsphingosine (upper plot). Squares in the lower plot refer to $C_4$,$C_5$ deuterons of the [$^2H_4$]lyso-GalCer sphingosine backbone (arrows in Fig. 2). Triangles in the lower plot refer to the outer and inner Pake doublets associated with the [$^2H_4$]lyso-GalCer carbohydrate moiety – note that the ratio of these latter two splittings clearly varies with pH since the larger splitting (inverted triangles) exhibits the smaller pH-induced change.

the axis of rotation (the membrane perpendicular), as is typical of methylene groups near the polar headgroup in free fatty acids and in acyl chains of glycerolipids and GSLs within highly ordered cholesterol-containing bilayer membranes [21–23,29]. From Eq. (1) below, the maximum 90°-edge quadrupole splitting ($\Delta\nu_Q$) such a deuteration site could exhibit would be 63 kHz [21–23].

$$\Delta\nu_Q = 3/8(e^2Qq/h)S_{mol}(3\cos^2\Theta_i - 1) \quad (1)$$

where $e^2Qq/h$ is the nuclear quadrupole coupling constant (165–170 kHz for an aliphatic C-D bond, $S_{mol}$ is the molecular order parameter (assuming axially symmetric order) describing orientational fluctuations of the lipid molecule relative to the bilayer normal, and $\Theta_i$ is the average orientation of each C-D bond relative to the molecular long axis (for $CD_3$ groups it is convenient to introduce a factor of 1/3 to the right hand side and consider $\Theta_i$ to be the angle between the C-$CD_3$ bond and the molecular long axis). The 63 kHz maximum splitting for methylene deuterons would be seen if there were perfect order and an all-*trans* chain conformation. Substituting into Eq. (1) a value of 90° for the angle the C-$^2$H bonds make with the molecular long axis, and the observed splitting of 39.2 kHz, should produce a lower limit for $S_{mol}$ at this position in the deuterated molecule. The value of 0.62 so obtained as a minimum estimate for $S_{mol}$ demonstrates that considerable order must be present in the whole-body motion of lyso-GalCer. The inner, less intense, Pake doublets with splittings of 2.7 and 6.2 kHz near pH 7 are assigned to the smaller numbers of deuterons known to be introduced at $C_3$ and $C_4$ of the galactose ring [24].

As was the case for the dimethylsphingosine already described, the protonated and unprotonated forms of lyso-GalCer are expected to be in rapid equilibrium on the NMR timescale. The spectra should represent averages of those for charged and uncharged forms, weighted in favour of the predominant species. However, since the p$K_a$ for a primary amine (lyso-GalCer) is considerably lower than that for a tertiary amine (dimethylsphingosine) [12], substantial fractions of each should exist at some of the pH values tested for the lyso-glycolipid. Thus it is interesting to examine spectra of the deuterated lyso-species as a function of pH (Fig. 5 – quadrupole splittings plotted in Fig. 4). Their similarity over the pH interval studied is noteworthy, given the sensitivity of $^2$H-NMR to orientation and order. Clearly the deuterons associated with the alkyl chain remain virtually unchanged – all displaying the same lineshape, and having measured spectral splittings essentially within the range 39.2 ± 0.1 kHz. The spectra associated with the sugar ring deuterons do however show a change of 1–2 kHz to larger values as a function of pH. Moreover, the ratio of the splittings for

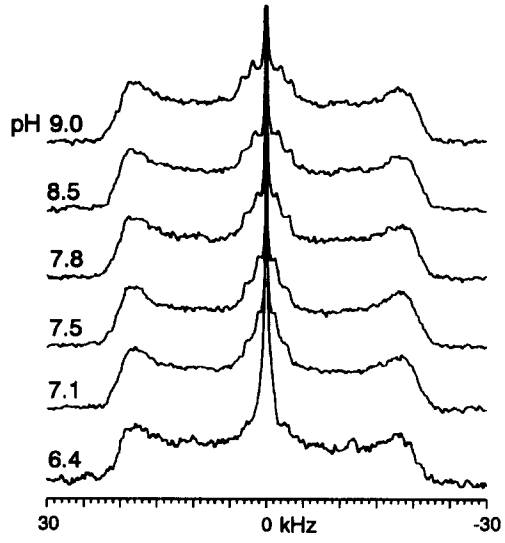


Fig. 5. pH dependence of $^2$H-NMR powder spectra for [$^2H_4$]lyso-galactosylceramide (GalCer) dispersed at 10 mol% in POPC/cholesterol (2:1 mol ratio) multilamellar vesicles. Temperature 37°C.

these deuterons (outer quadrupole splitting to inner quadrupole splitting) is not constant – varying from 2.85 at pH 6.4 to 1.61 at pH 8.9. This demonstrates that the pH-induced change arises from an orientational difference at the level of the sugar residue, since pH-induced changes in motional order alone would affect the spectral splittings of both sugar ring deuterons to the same fractional extent.

## 4. Discussion

In the present work we have recorded the first direct spectral measurements on two glycosphingolipid metabolites in membranes using the technique of wideline $^2$H-NMR. The presence of non-perturbing deuterium probes on the metabolites themselves made it possible to examine their physical characteristics when dispersed as minor components in unsonicated bilayer membranes mimicking fluidity characteristics of the eukaryote plasma membrane.

The spectral features for both [$^2H_6$]dimethylsphingosine and [$^2H_4$]lyso-GalCer must be seen as representing average conformations, reflecting statistically preferred occupation of available conformer energy wells. In the case of [$^2H_6$]dimethylsphingosine, there is the additional consideration that the single Pake doublet for the two -C$^2$H$_3$ groups presumably reflects rapid inversion at the chiral nitrogen centre in the unprotonated form of the molecule. Since the dominant forces in membrane structure are based on entropy-driven avoidance of hydrophobic group contact with water, removal of the sphingolipid fatty acid must eliminate an important constraint on the amino function and the molecule backbone. This could make it possible for the (charged) amino group to sit higher in the membrane – perhaps at the level of surrounding lipid phosphate groups. A number of studies have been made of $^2$H-NMR spectra associated with phospholipids deuterated in the headgroup region and incorporated into bilayer membranes [30–34]. As was observed here for the sphingosine methyl deuterons, in each case the quadrupole splittings reported for choline deuterons are small. Indeed, the spectra obtained in the present work for sphingosine with deuterated methyl groups are very similar to those reported for phosphatidylcholine with deuterated choline methyls [31]. In the latter case a narrow Pake doublet was recorded with quadrupole splitting of 0.9 kHz. Thus, while it may be that the small values (about 2.3 kHz) for dimethylsphingosine indicate fortuitous C-C$^2$H$_3$ bond average orientations near the magic angle (54.7°), it seems very possible that they also reflect significant disorder for the amino function (Eq. (1)).

Wideline $^2$H-NMR spectra for sphingolipids deuterated in the sphingosine chain itself have not been reported previously. However, such spectra obtained for [$^2H_4$]lyso-GalCer could be readily interpreted since there is a large literature base from experiments with deuterated fatty acids (e.g., [21–23,35,36]). With the exception of deuterons at $C_2$ of fatty acids attached to GSLs [37] or to the *sn*-2 position of glycerolipids [38], methylene deuterons near the membrane surface all give very similar splittings for a given lipid under a given set of conditions. The fact that the spectra for lyso-GalCer $C_4$,$C_5$ deuterons were not separately resolved indicates that the C-$^2$H bonds at these locations share the same average orientations and degree of orientational order. This in turn would be anticipated if the average orientation of the sphingosine chain long axis is perpendicular to the plane of the membrane at this depth, and would be consistent with the well-known existence of a 'plateau' of host phospholipid fatty acid order near the membrane surface [21–23,35] as also demonstrated for GSLs [36,39,40]. The splitting of 39.1–39.3 kHz seen for lyso-GalCer $C_4$,$C_5$ deuterons at 37°C is close to the 40 kHz reported for the analogous region of the acyl chain in GalCer dispersed in POPC/cholesterol at 40°C [36], although in the latter case the membranes contained only 23 mol% cholesterol. This suggests that the lyso-glycolipid is measurably less ordered under a given set of conditions. The spectra obtained from the deuterated sugar residue in [$^2H_4$]lyso-GalCer are analogous to ones first reported by Skarjune and Oldfield for deuterated glucosyl ceramide [41], and to those of glycerolipids [42–44], although splittings seen for lyso-GalCer are several times smaller than the largest ones reported by these earlier workers. Smaller splittings would be consistent with reduced carbohydrate motional order resulting from removal of the GSL fatty acid, although proof of this would require additional information to separate orientation effects from those of $S_{mol}$ (Eq. (1)).

It has been pointed out that sphingoid bases have the potential for important pH sensitivity in their actions, due to the presence of the protonatable amino function [12,14,15]. The internal pH of higher animal cells is generally considered more acidic than extracellular fluid. Values about 7.0 are often used, but numbers as low as 6.2 are quoted depending upon the metabolic state [45]. In the current experiments pH was varied through this range. The actual degree of protonation of the amino group for a particular sphingoid base under a given set of conditions has been a subject of some discussion in the literature. Merrill et al. noted that (intramolecular) H-bonding involving the -OH groups at $C_1$ and $C_3$ of the sphingosine chain might be expected to stabilise the unprotonated form of the amine, thus lowering its p$K_a$ [12]. They also cautioned that results may differ measurably for a molecule in a membrane environment. Using detergent micelles as a model membrane, Merrill et al. estimated the p$K_a$ for the primary amine group of sphingosine and for the secondary amine group of dimethylsphingosine to be 6.7 and greater than 10, respectively [12]. Bottega et al. [14] indicated that the value for sphingosine is higher, finding an apparent value of 7.7 [14]. In pure phosphatidylserine bilayers López-Garcia et al. measured a value of 8.9 for dimethylsphingosine [19].

Clearly, in the membrane environment provided by the present experiments, pH variation over the physiological range had no measurable effect on dimethylsphingosine conformation or dynamics as reflected in $^2$H-NMR spectra. Moreover, in agreement with Merrill et al. [12], no spectral evidence of deprotonation (or other change) was seen up to pH 10.5. It is interesting that for lyso-GalCer the deuterons at $C_4$,$C_5$ of the alkyl chain showed no evidence of altered geometry or disorder resulting from protonation/deprotonation when studied over a range that should include the $pK_a$. The conclusion would be that these aspects of the chain are insensitive to the presence or absence of a (+) charge on the amino group. The small amount of deuteration present in the sugar ring permits for the first time some insight into the effect of sphingoid base protonation on a carbohydrate headgroup. The spectral change was modest, but indicative of significant sugar headgroup reorientation. The region of greatest spectral change centred about pH 7.5, which is consistent with $pK_a$ expectations in the work mentioned above.

## 5. Conclusions

Wideline NMR spectroscopy of deuterated sphingolipid metabolites provides a useful mechanism for their detailed characterisation as minor components in membrane systems. The GSL metabolites examined in the present work appear to disperse randomly in fluid membranes of a monounsaturated phospholipid rich in cholesterol, and to behave and orient in the manner well known for more extensively-studied lipids. There was no evidence of pH effect on dimethylsphingosine conformation or behaviour over the physiological pH range of the cytoplasm, and as high as pH 10.5. There was also no evidence of pH effect within the hydrophobic chain of lyso-GalCer. However the carbohydrate headgroup was orientationally altered, particularly above the pH region of the cytoplasm. The $C_4$,$C_5$ portion of the lyso-GalCer sphingosine backbone showed qualitatively the same orientational behaviour expected for acyl chains of GSLs and glycerolipids.

## Acknowledgements

This research was supported by a grant to C.W.M.G. from the MRC of Canada. NMR spectroscopy and molecular modelling were carried out in the R.S. McLaughlin Macromolecular Structure Facility, established with grants to the department from the R. Samuel McLaughlin Foundation, the London Life Insurance Company, the MRC Development Program, and the Academic Development Fund of the University. A.C.R. was the holder of fellowships from the Ontario Graduate Student Program. We would like to thank Dr. G. Shaw for helpful discussions surrounding the experimental aspects of the work.

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