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Glycosphingolipid acyl chain order profiles: substituent effects

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Abstract

Fatty acid order parameter profiles were determined by ^{2}H-NMR in order to characterize the arrangement and behaviour of the hydrophobic region of glycosphingolipids (GSLs) dispersed as minor components in phosphatidylcholine/cholesterol membranes. Direct comparison was made amongst species with important fatty acid structural features found in natural glycosphingolipids. Galactosyl ceramides (GalCer) were prepared by partial synthesis having 18:0[d_{35}], D-α-OH 18:0[d_{34}], L-α-OH 18:0[d_{34}], 18:1[d_{33}], and 24:0[d_{47}] fatty acids. Unsonicated multilamellar liposomes of the common natural phospholipid, 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), containing 23% cholesterol, were employed as host matrix. Smoothed profiles of the order parameter, S_{CD}, for 18:0[d_{35}] GalCer proved to be very similar to profiles known for 16:0 and 18:0 fatty acids of glycerolipids in cholesterol-containing bilayers. In general, order along the GSL chain was slightly higher than anticipated for equivalent chain segments in phospholipids. Order parameter profiles for the GSL 18-carbon saturated fatty acids were strikingly similar. However, small quantitative differences were found for glycolipids having D- and L-α-hydroxylation at C-2 – the D-stereoisomer being marginally more ordered in the plateau region. Although order profiles have not been reported for unsaturated glycerolipid fatty acids in cholesterol-rich membranes, spectra of 18:1[d_{33}] GalCer appeared to be assignable by applying known ordering effects of cholesterol to existing data for unsaturated glycerolipids. The unsaturated chain was found to be less ordered than saturated 18-carbon chains toward the membrane surface, but more ordered in the region of the bilayer midplane. The ordering may result from cholesterol-induced restriction of isomerisation at the *cis*-double bond, and represents an apparent exaggeration of a phenomenon known for glycerolipids. Addition of an 'extra' 6 carbons to the fatty acid (24:0[d_{47}] GalCer) produced no significant effect on the order profile to a membrane depth of C-12–C-13. These results suggest that fluid membrane area requirements for GSLs with saturated fatty acids are not strongly influenced by the nature of that fatty acid when the GSL is a minor component. Order parameter profiles for the very long chain GSL deviated to higher order below this point, and formed a second 'plateau' of reduced negative slope toward the methyl terminus: this is characteristic of profiles for very long chain GSLs. These features were essentially unchanged over a range of temperatures providing different degrees of spatial constraint.

Keywords: Glycolipid; Phospholipid bilayer; Order parameter; NMR, ^{2}H-; Fatty acid

1. Introduction

Lipid arrangement and behaviour within the membrane hydrophobic interior are of interest as determinants of cell surface structure and function. A number of important features of this region are reflected in the arrangement and motional characteristics of the acyl chain methylene segments. These were first quantitated spectroscopically using nitroxide spin probes covalently attached to fatty acids [1,2], although potential advantages of wideline ^{2}H-NMR spectroscopy were quickly recognised [3,4]. It is now generally acknowledged that a variety of spectroscopic and thermodynamic techniques can provide valuable insight into the numerous processes with different timescales that characterize membrane environments [5,6]. However, ^{2}H-NMR has proven to be a particularly useful and non-perturbing approach [7–9]. It permits acquisition of data directly related to lipid arrangement and behaviour at any given depth within the membrane interior.

^{2}H-NMR studies of glycerolipid chains within membranes have typically focused on lipids comprising major or exclusive components of the systems considered. Complete 'order parameter profiles' have been described for

Abbreviations: GSL, glycosphingolipid; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl PC; GalCer, Galβ1 → 1ceramide; 18:0 GalCer, *N*-stearoyl GalCer; 18:1 GalCer, *N*-oleoyl GalCer; α-OH GalCer, *N*-(α-OH)stearoyl GalCer.

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glycerolipids in a wide range of membranes. For species with chain lengths in the 14- to 20-carbon range, such profiles typically display a 'plateau' of relatively high order having minimal negative slope from C-1 to about C-10/C-11, followed by an increasingly negative slope to the methyl terminus ([7–9]; see also [10–12] and references therein). The present work deals with glycosphingolipids (GSLs) – the carbohydrate-bearing lipids of higher animal cells. These generally exist as minor membrane constituents, and so might be expected to be importantly influenced by conditions and properties of the surrounding (other) lipids making up the bulk of the membrane.

Questions related to glycosphingolipid (GSL) fatty acid arrangement and motional properties have special significance. Firstly, GSLs have a much wider range of common natural fatty acids than do the glycerolipids which, with cholesterol, make up the bulk of membrane lipids. Notably, GSL fatty acids are often hydroxylated at C-2 and can be very long (up to 24 or 26 carbons) [13,14]. Secondly, the nature of GSL fatty acids has been widely considered to be an important contributor to the ability of GSLs to function as recognition sites and receptors, in addition to being a determinant of their structural role within membranes ([15]; see also references in [16,17]). In the present work we have systematically measured and compared order parameter profiles for a group of deuterated representative fatty acids attached to galactosyl ceramide (GalCer). GalCer has formed the basis of numerous important investigations of GSL characteristics and behaviour, including GSL structure by X-ray diffraction [18] and the original systematic studies of receptor crypticity [19] (reviewed in [13,14,20]). Where tested, its behaviour as a minor component in fluid membranes has been found to parallel that of a wide range of other common GSLs [21–24]. Measurements were made in bilayer membranes possessing three important features of cell membranes: GSL as minor component, *sn*-2-monounsaturated phospholipid (1-palmitoyl-2-oleoylphosphatidylcholine) as major component, and the presence of cholesterol.

2. Materials and methods

1-Palmitoyl-2-oleoylphosphatidylcholine (POPC), and cholesterol were obtained from Avanti Polar Lipids, Birmingham, AL; and were used without further purification. Galactosyl ceramides with perdeuterated fatty acid chains were prepared from beef brain GalCer (Avanti) as described previously [25,26]. Preparation of liposomes containing deuterated GalCer followed procedures described previously [26]. Each sample contained 7–8 mol% deuterated glycolipid, 68–69 mol% POPC, and 23 mol% cholesterol. Individual samples containing up to 15 mg of total deuterated lipid were hydrated in about 300 μl of 10 mM phosphate buffer at pH 7.4.

Spectra were acquired sequentially from high to low temperature after incubation at temperatures substantially above the fluidus curves of the systems studied (see Discussion), in order to facilitate equilibrium distribution of components in the bilayer. Technical details for the ^{2}H-NMR experiments have been described previously [26]. For each spectrum, 100000 to 300000 transients were collected with a repetition time of 0.4 s. Smoothed order parameter profiles were obtained using a modification of the approach outlined by Sternin et al. [27] and Lafleur et al. [28], in which an attempt was made to identify non-integral methylene areas as alpha carbon deuterons or alkene deuterons for isolation from the rest of the profile.

3. Results

Structures of the glycolipids investigated in this work are indicated in Fig. 1. They were produced by partial synthesis, replacing the natural fatty acids of beef brain GalCer with five different perdeuterated species. The surveyed group comprised 4 examples with 18-carbon fatty acids (stearic acid, the two α-OH isomers of stearic acid, and the *cis*-monounsaturated derivative of stearic acid); and one 24-carbon saturated species (lignoceric acid).

^{2}H-NMR spectra of lipids with perdeuterated acyl chains are composites of single Pake doublets corresponding to each non-equivalent deuteron in the chain. With the exception of C-2, deuterons associated with a given carbon contribute to a single Pake doublet, as each carbon represents a single average environment and average orientation. The 90° edges of each Pake doublet are split by

$$\Delta\nu_Q = \frac{3}{4}\frac{e^2qQ}{h}S_{CD} \quad (1)$$

where S_{CD} is the orientational order parameter of the carbon-deuterium (C-D) bond [7]:

$$S_{CD} = \tfrac{1}{2}\langle 3\cos^2\theta_{CD} - 1\rangle \quad (2)$$

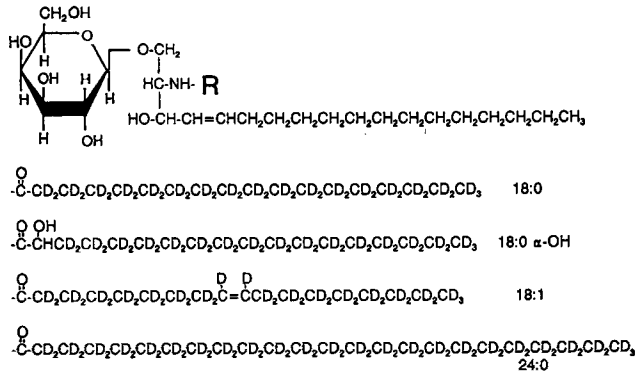

Fig. 1. Structures of the galactosyl ceramides (GalCer) studied in the present work. 'R' indicates the individual deuterated fatty acids, which are shown below the general structure in the following order: 18:0[d_{35}], D- and L-α-OH 18:0[d_{34}], *cis*-18:1[d_{33}], and 24:0[d_{47}] GalCer.

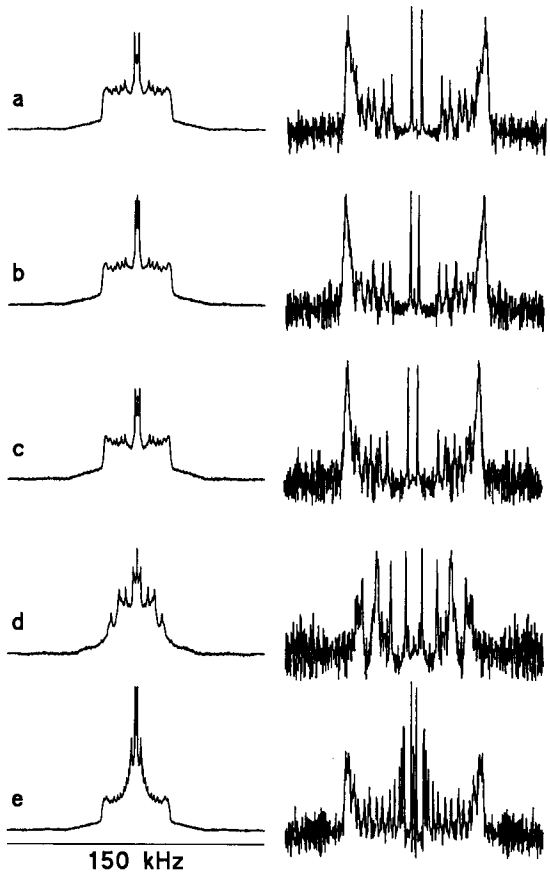

Fig. 2. Powder ^{2}H-NMR spectra at 40° C for 18:0[d_{35}] GalCer (a), D-α-OH 18:0[d_{34}] GalCer (b), L-α-OH 18:0[d_{34}] GalCer (c), 18:1[d_{33}] GalCer (d), and 24:0[d_{47}] GalCer (e) dispersed as minor components in unsonicated multilamellar liposomes of 1-palmitoyl-2-oleoyl-phosphatidylcholine (POPC), containing 23% cholesterol. Corresponding dePaked spectra are shown to the right of each powder spectrum. 120000 scans were acquired with oversampling by a factor of 4 to improve signal-to-noise by a factor of 2.

The average in Eq. (2) is over the time-dependent motions of the C-D bond, and θ_{CD} is the angle between this bond and the bilayer normal. Thus wideline ^{2}H-NMR spectra of perdeuterated lipids in membranes provide straightforward insight into both orientational and motional characteristics of the labelled species. In the present work, fatty acid substituent effects on GSL chain behaviour were examined by dispersing deuterated GalCer with controlled fatty acid alterations in cholesterol-containing bilayers of the common natural phospholipid, POPC.

Typical powder and dePaked spectra for the five glycolipids dispersed in POPC/cholesterol multilamellar liposomes are shown in Fig. 2 for the temperature, 40° C. All spectra appear to represent single populations of glycolipids undergoing rapid rotational diffusion about axes perpendicular to the bilayer. Spectra for the GalCer species with 18-carbon saturated fatty acid chains (Fig. 2a–c) are remarkably similar – displaying the distinctive intense outer edges familiar from extensive work on perdeuterated glycerolipids [7–9]. This feature is a reflection of there being slow decrease in order with progression from C-2 to a membrane depth of about C-10 or C-11. The spectrum corresponding to 18:1[d_{33}] GalCer (Fig. 2d) has a very different appearance; which in the case of glycerolipids has been demonstrated to result from the altered orientation of deuterons associated with the double bond, and from reduction in order due to the double bond [29–31]. The spectrum for 24:0[d_{47}] GalCer is unique, being characterised by outer edges resembling those associated with the 18-carbon saturated fatty acids, but also by considerable additional buildup of intensity in the central region. The spectrum for the same glycolipid deuterated selectively at C-22–C-24 demonstrated that the central buildup is associated with the 'extra' length of acyl chain.

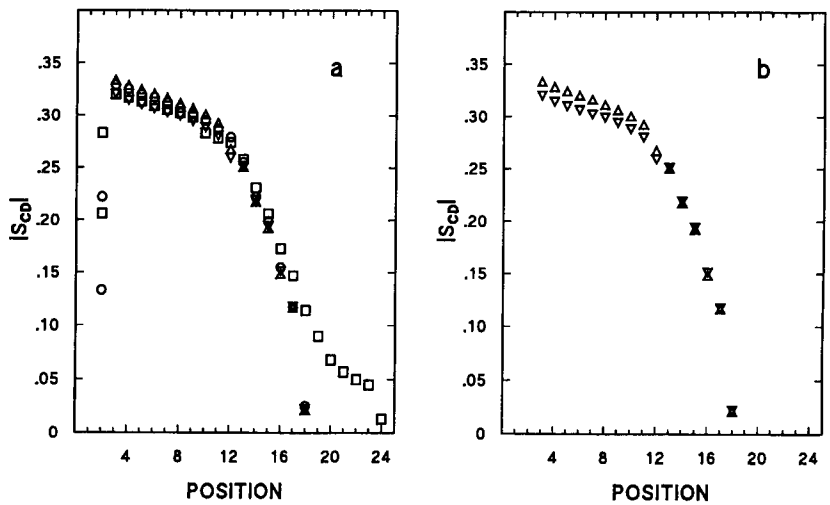

Fig. 3. Smoothed orientational order parameter profiles for 18:0[d_{35}] GalCer (○), D-α-OH 18:0[d_{34}] GalCer (△), L-α-OH 18:0[d_{34}] GalCer (▽), and 24:0[d_{47}] GalCer (□) in POPC/cholesterol at 40° C. In (a) all four have been superimposed. In (b) the profiles for the L-α-OH and (naturally-occurring) D-α-OH GalCer have been separately superimposed. 'Position' refers to carbon number of the fatty acid chain, based on the COOH terminus as C-1.

Bloom and coworkers have shown that, by assuming a monotonic decrease in order from C-1 to the methyl terminus, smoothed order parameter profiles may be extracted from oriented spectra of perdeuterated acyl chains [27,28]. There is some degree of arbitrariness inherent in the approach, since it may not deal adequately with 'nonconforming' deuterons (ones whose spectral splittings do not follow the assumed progressive diminution from C-1 to methyl terminus. This is particularly true if their spectral peak assignments are imperfectly known, as is the case for deuterons at C-2. However, these may often be reasonably identified by their contribution of only half the intensity expected of a methylene group; and such an approach has been taken in the present analysis. Oriented spectra were obtained from powder pattern spectra using the 'dePaking' algorithm [32]. For the saturated fatty acids, resultant smoothed order parameter profiles were calculated. Fig. 3 illustrates these profiles for GalCer with saturated fatty acids in the POPC/cholesterol matrix at 40° C. All four profiles have been superimposed to produce Fig. 3a. In Fig. 3b the profiles for the L-α-OH and (naturally-occurring) D-α-OH GalCer have been separately superimposed. The most striking aspect of Fig. 3a is similarity over the entire length for the 18-carbon species. The 24:0 GalCer curve is also very similar to a depth of C-13; but diverges steadily to higher order beyond that point, forming a secondary 'plateau' (i.e., a region of decreased slope). The order for the terminal methyl group of 24:0 GalCer is about half that associated with the methyl terminus of the GalCer species having 18-carbon saturated fatty acids. The D-α-OH glycolipid appears to have slightly higher order in the plateau region than does the L-α-OH derivative, although this is very close to the confidence limit for S_{CD} (± 0.01). In Fig. 3a the curve for D-α-OH GalCer begins above those for the other saturated fatty acids, but crosses them to have the smallest order parameter at the lower end of the plateau (C-11). Thus it displays a plateau region of steeper slope (in other words, less of a 'plateau').

In its simplest form, the order profile analysis described above presumes a smooth decrease in Pake doublet splitting (quadrupole coupling, $\Delta\nu_Q$) from membrane surface to methyl terminus. As indicated above, this is not a good approximation for C-2. It is inappropriate for unsaturated fatty acids in the region of the double bond. Hence it was more difficult to compare the data for the 18:1 GalCer with those for the other GSLs. However, it was possible to achieve this for key portions of the 18:1[d_{33}] GalCer profile by analysis of the spectral line assignment, as

Table 1
Predicted assignment of the 25° C ^{2}H-NMR spectrum for 18:1[d_{33}] GalCer in POPC/cholesterol

Carbon No.	$\Delta\nu_Q$ values (kHz) for ^{2}H in the oleate chain		
	A. laidlawii (25° C) (no cholesterol)	'expected' value with cholesterol	experimental value assigned [a]
2	20.0	*	*
	25.5	*	*
3	23.8	32.9	34.8
4	24.0	33.2	35.9
5	25.8	35.7 (30.8 [b])	37.0
6	22.0	30.4	34.1
7	22.0	30.4	33.5
8	15.6	21.6	28.7
9	14.8	20.5 (17.5 [b])	26.6
10	4.3	5.9 (5.3 [b])	4.3
11	7.8	10.8	16.1
12	13.5	21.4	26.6
13	13.3	21.1	26.6
14	13.0	20.6 (16.5 [b])	25.6
15	12.0	19.0	23.8
16	11.0	17.5	23.3
17	8.0	12.7	16.8
18	3.0	4.8 (3.1 [b])	6.2

The table is based upon literature assignments of ^{2}H-NMR data for deuterated oleic acid attached at the *sn*-2 position of glycerol based lipids in fluid membranes of *Acholeplasma laidlawii* [31] (see also closely corresponding data for POPC bilayers in [29,30]. Original data for *A. laidlawii* are presented in the left hand column as a function of ^{2}H location along the fatty acid chain ('Carbon No.') with the corresponding value of $\Delta\nu_Q$ (values for carbon Nos. 13 and 15 were interpolated by drawing a smooth curve through data points in [31]. The second column provides anticipated values for quadrupole splittings in the presence of 23 mol% cholesterol – estimated using literature data for cholesterol effects on order parameter profiles of palmitic acid in POPC/cholesterol [12], as adjusted to 23% cholesterol according to the data of Stockton and Smith [35] for stearic acid in egg PC. The membrane order adjustment factors employed in deriving column 2 from column 1 were 1.383 for C-3–C-11, and 1.587 for C-12–C-18.

* Values for C-2 were not included.

[a] Assignment based on matching relative magnitudes to predicted assignment.

[b] Values found by Rance et al. [34] for *A. laidlawii* grown on cholesterol and specifically deuterated oleate – final cholesterol content about 20 mol% – are included in column 2 in brackets. Compare to corresponding values in the 'experimental' column.

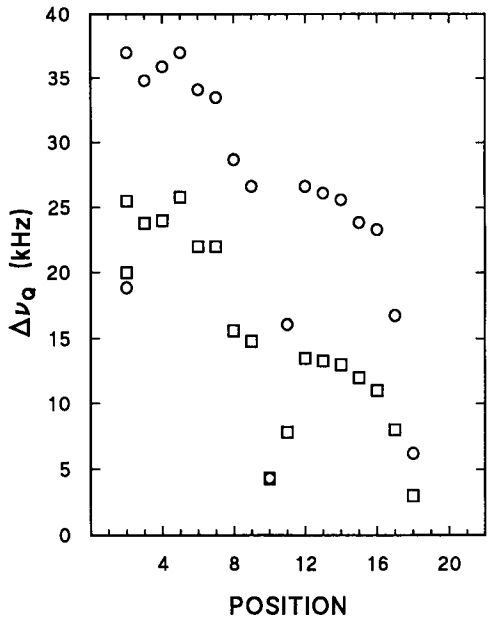

Fig. 4. Suggested spectral line assignments for 18:1[d_{33}] GalCer at 25° C (○), as drawn from Table 1. The data for deuterated 18:1 chains of glycerolipids in *A. laidlawii* membranes without cholesterol [31] are shown for comparison (□).

follows. A complete spectral assignment has been published for deuterated oleic acid chains at the *sn*-2 position of glycerolipids in *Acholeplasma laidlawii*, an organism that does not make cholesterol and which will incorporate exogenous fatty acids from the growth medium [31]. This result appears to have considerable generality since, with the exception of C-2, the spectral splittings were found to be the same, where tested, as those known for deuterated oleic acid chains in POPC bilayers [30]. In addition, we have demonstrated previously that ^{2}H-NMR spectra of GalCer with perdeuterated 18:1 chain in a variety of fluid membranes (including POPC without cholesterol), could be simulated by assuming direct correspondence with peak assignments from oleic acid chains on glycerolipids [33]. Thus, the 18:1 chain of Galcer is capable of closely mimicking the same chain at the *sn*-2 position of glycerolipids.

Complete spectral assignments do not exist for unsaturated fatty acids in cholesterol-containing glycerolipid membranes. However Rance et al. [34] have measured the spectral splittings for five selectively deuterated sites (C-5, C-9,10 (the double bond), C-14 and C-18) of 18:1 chains incorporated into *A. laidlawii* membranes containing 17–27% cholesterol. They noted that, at the locations probed, the overall order parameter profile features recorded for the same unsaturated fatty acid in the absence of cholesterol were preserved. In addition, the ordering effect of 30% cholesterol on the perdeuterated 16:0 fatty acid chain of POPC in bilayers has been quantitated [12], as has the effect of 17.4 vs. 33.4% cholesterol on perdeuterated stearic acid in egg PC bilayers [35]. The latter provide complete host matrix order profiles for membranes closely analogous to the POPC/cholesterol studied in the present work. This information was used to derive the likely assignments of spectral splittings in the 25° C 18:1[d_{33}] GalCer powder spectrum. The calculation is summarised in the caption to Table 1. The table lists, from left to right, literature quadrupole splittings for all carbon positions of the 18:1 chain in glycerolipids without cholesterol [30,31], the corresponding predicted values in similar (monounsaturated glycerolipid) membranes containing 23% cholesterol at 25° C, and the experimental values for 18:1[d_{33}] GalCer in POPC with 23% cholesterol at the same temperature. Available values from the literature for C-5, C-9,10, C-14 and C-18 of 18:1 chains in glycerolipids of *A. laidlawii* membranes are included in column 2. Fig. 4 shows the spectral splittings associated with cholesterol-free (*A. laidlawii*) membranes co-plotted with the assignments for 18:1[d_{33}] GalCer in the present work. It can be

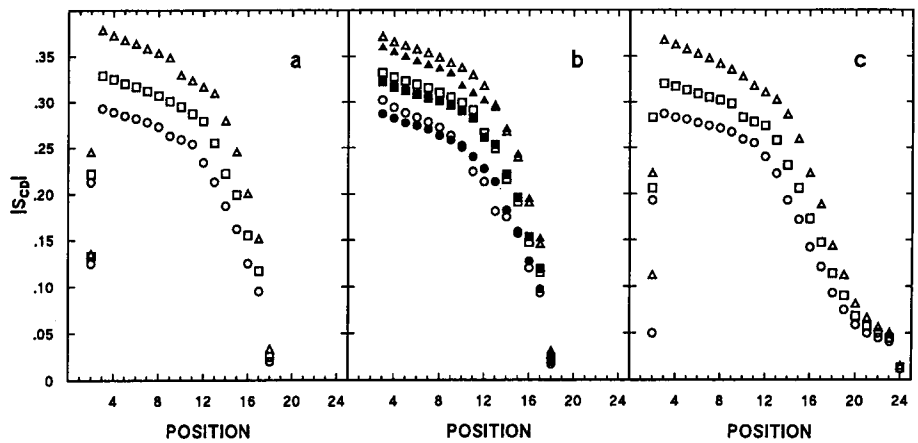

Fig. 5. Relative effects of host membrane compressibility on GSLs with different fatty acids. Smoothed orientational order parameter profiles at 55° C (○), 40° C (□), and 25° C (△) for 18:0[d_{35}] GalCer (a), D-α-OH 18:0[d_{34}] GalCer (b, open symbols), L-α-OH 18:0[d_{34}] GalCer (b, filled symbols), and 24:0[d_{47}] GalCer (c) in POPC/cholesterol.

seen that, (i) the experimental values for GalCer in POPC/cholesterol correspond to a spectrum having the same features observed for pure POPC and *A. laidlawii* (Fig. 4) [30,31], (ii) in the presence of cholesterol the quantitative splittings are always higher in the case of the GSL (Table 1, columns 2 and 3), (iii) the difference between predicted and assigned experimental values is much greater close to and below the double bond.

The relative effect of spatial constraint on the behavioural characteristics of the different fatty acid species was examined in the present work by varying the temperature. This is addressed in Fig. 5, in which smoothed order profiles of the GalCer species with saturated fatty acids are grouped by glycolipid for 25, 40, and 55° C. Over the temperature range considered, the order profile shapes and relationships described above were maintained. The basic features of the 18:1[d_{33}] GalCer spectrum (Figs. 2d and 4 upper curve) were also present at lower and higher temperatures (spectra not shown here).

4. Discussion

The experiments described here provide a general basis for considering the arrangement and behaviour of the hydrophobic portions of glycosphingolipids (GSLs) in fluid membranes. Information was derived for each position along the chain. Notable aspects of the present work which contrast with the design of past experiments were, dispersal of the glycolipids as minor components in monounsaturated-phospholipid/cholesterol bilayers, and use of the non-perturbing ^{2}H-NMR technique. In addition, data were obtained for fatty acids with a range of features of special significance to the roles of GSLs as structural elements and receptors. Fatty acid features tested included hydroxylation, unsaturation, and chain length.

The orientational order parameter curves derived demonstrated important correspondence with order profiles for fatty acids of phospholipids. This represents an extension and generalisation of several specific past observations involving GSLs. Firstly, spin labelled GSLs having 18-carbon fatty acids dispersed at concentrations as low as 1 mol% in phospholipid and phospholipid/cholesterol mixtures, have been shown to give spectra closely resembling those of corresponding phospholipids having spin labelled *sn*-2 palmitic or stearic acids (e.g., [23,36–39]). Secondly, Skarjune and Oldfield [40] noted the resemblance between acyl chain order parameters determined by ^{2}H-NMR for pure hydrated GalCer in fluid bilayer form (90° C) and those of pure phospholipids. They pointed out that the resemblance appeared to extend to the special characteristics of the motionally restricted C-2 position, and this has been confirmed for GSLs as minor components in phospholipid/cholesterol bilayers [16]. Further observations that ^{2}H-NMR spectra of GSLs (and plasmalogens [41]) mimic important characteristics of glycerolipid spectra have since been made for other membranes and more complex glycolipids [42–44]. In the present work it was found that close similarity to glycerolipids holds for a systematically varied set of GSLs in membranes containing cholesterol – a host matrix that displays limited fluidity/compressibility [45] and hence limited ability to accommodate structural differences.

An important finding in the present work was the striking quantitative spectral similarity amongst the three GSLs having saturated 18-carbon fatty acids over the full length of their hydrophobic regions. The only difference greater than experimental uncertainty was that the (natural) D-α-OH species showed some marginal evidence of higher order than L-α-OH at the membrane surface: the maximum difference was 5%. This did not appear to be attributable to the D-α-OH species 'sitting higher in the membrane', as its plateau region was if anything shorter than those of the other derivatives, and its order near the surface was comparable. Rather the D-α-OH GalCer appeared to display a plateau region of steeper (negative) slope than the L-α. The result is in keeping with earlier observations in saturated phospholipids without cholesterol [25]. The similarity in order profiles extended to GalCer with 24:0 fatty acid, to a membrane depth of C-13. In the plateau region the order profiles for 24:0 GalCer were superimposable upon those for the 18:0 species within the limits of experimental error. However, profiles for the longer analogue diverged to higher order at greater depths, producing an inflection point in the region of C-15–C-16, with S_{CD} values higher by some 15% and 27% at C-16 and C-17 respectively. S_{CD} at C-24 was half the value seen for the 18:0 terminal methyl. The overall result was a secondary 'plateau' of lower slope between C-18 and C-22.

The plateau regions for all four GSLs with saturated fatty acids demonstrated higher order than has been recorded in studies of phospholipids under comparable conditions. For instance, in the present experiments, at C-4 at 25° C we observed S_{CD} in the range 0.35–0.37 for samples containing 23% cholesterol. This compares to S_{CD} values of 0.34 for palmitic acid at the *sn*-2 position of POPC in POPC with considerably higher (33%) cholesterol content at the same temperature [12], (corresponding phospholipid value adjusted for the estimated effects of 23% cholesterol is 0.29). This finding is consistent with our previous observation that GSLs can exert an ordering effect on their local phospholipid-rich environment [36,37]. Others have also noted that GSLs can increase order parameters of phospholipid fatty acids in a given phospholipid host matrix [46–51].

Order parameter profiles found for the glycolipid with mono-unsaturated fatty acid were very different from those for the saturated species. This was expected, as the same phenomenon was originally described for glycerolipids [29–31,34]. Similar profiles have been found for 18:1 GalCer in fluid membranes without cholesterol [33]. The use of smoothed order profiles relies on the fact that

spectral peaks can be assigned to methylene groups along the length of the chain on the basis of continuously decreasing spectral splitting (and thus S_{CD}) from C-2 to methyl terminus. Since this relationship does not hold in the case of unsaturated fatty acids, interpretation of the 18:1 experimental data was more difficult. We took advantage of the fact that spectral peak positions for the oleic acid chain attached at the *sn*-2 position of glycerolipids have been assigned completely by selective substitution in *A. laidlawii* membranes [31] and in POPC bilayers [29,30] – both in the absence of cholesterol. Assignment has also been made for C-5, C-9, C-10, C-14 and C-18 of oleic acid at the *sn*-2 position of glycerolipids in *A. laidlawii* in the presence of some 17–27% cholesterol [34]. Because very close correspondence has been observed for quadrupole splittings of deuterated oleic acid in glycerolipids in a wide variety of fluid membranes [29–31,34], and because these in turn, where tested, accurately predicted the spectra of GSLs with deuterated 18:1 fatty acids in fluid membranes not containing cholesterol [33], an attempt was made to judge the correspondence between glycerolipids and GSLs in the present work. Existing literature values of splittings for oleic acid in *A. laidlawii* were adjusted for the effect of the presence of 23 mol% cholesterol (using measured effects of cholesterol on saturated chain order profiles [12,35]). The results were then compared to experimental data for 18:1[d_{33}] GalCer.

An interesting difference was apparent between cholesterol effects on the oleic acid chain of GalCer vs. the same chain at the *sn*-2 position of glycerolipids reported by Rance et al. [34]. The values from *A. laidlawii* containing 17–27% cholesterol are all lower than predicted by the approach described above (Table 1), while just the opposite is true for the same 18:1 chain on GalCer at 25° C. The discrepancy is particularly striking toward the methyl terminus. It is certainly possible that our spectral assignments are not all correct; although they adequately mimic the 25° C powder spectrum. However, the pattern of relatively much larger splittings for the GSL is a general one, and there can be no doubt about the peak assignment for the methyl terminus. This result recalls the work of Skarjune and Oldfield [40] in which cholesterol–GalCer interactions were found to differ markedly on the NMR timescale from those between cholesterol and phospholipids. Given the close correspondence amongst model systems and *A. laidlawii* lipids mentioned above, our result certainly suggests that cholesterol has a greater immobilising effect on GSLs with 18:1 fatty acids than it does on corresponding unsaturated glycerolipids. The quadrupole splitting for the 18:1 GalCer methyl terminus (6.2 kHz at 25° C) is particularly striking, being not only larger than predicted from literature effects of cholesterol, but noticeably larger in the powder spectra than those corresponding to the 18-carbon saturated fatty acids on GalCer (e.g., 3.5 kHz for 18:0 GalCer at 25° C). The maximum S_{CD} found for the 18:1 GalCer was 0.30 at 25° C in the present experiments. This compares to 0.34–0.37 for the saturated chains at the same temperature, and indicates that disorder has resulted from the presence of chain unsaturation. Seelig and Seelig [29] and Seelig and Waespe-Sarcevic [30] used ^{2}H-NMR to study POPC with perdeuterated palmitic acid chain and with oleic acid chain selectively deuterated at the double bond, in pure bilayer form. They observed that the overall effect of the double bond was one of disordering, but with maintenance of general fluidity gradient shape, and a relative stiffening of both the 18:1 chain itself and neighbouring saturated chains in the region of the double bond (see also [52]). Thus, the large CD_3 splittings seen uniquely in the case of 18:1 may suggest that cholesterol interacts with the *cis* double bond to restrict *trans / gauche* isomerisation, and that this effect is particularly large for GSLs.

Gally et al. [53] calculated a tilt of 7–8° from the bilayer normal for the double bond of POPC by assuming an order parameter, S_{mol}, of 0.37 from a closely related system. This causes the C-D bond at C-10 to be oriented at something approaching the magic angle of 54.7°, resulting in a very small quadrupole splitting (4.3 kHz for *A. laidlawii* without cholesterol). The assignment presumed in the present work retains this feature (splitting associated with C-10, 4.3 kHz – see Table 1).

^{2}H-NMR spectroscopy, as utilised here, cannot be considered highly sensitive to subtleties of lipid lateral distribution and translational diffusion. However, different phases in slow exchange would be expected to produce separately resolved overlapping spectra, and phases in rapid exchange would be expected to display temperature-induced spectral shape changes. These should have been detectable in the current experiments if over 10–20% of the lipid were involved. Such spectral features were not apparent in the samples studied over the range 25–55° C. On the one hand this result might have been expected for lipids in complex mixtures with cholesterol: 25° C is well above the −3° C phase transition temperature of POPC [65], and above even the range at which fluid/fluid phase coexistence might generally be considered for its mixtures with cholesterol (e.g., [54]). Nevertheless, there have been important indications that there may be phase coexistence in membranes containing high concentrations of cholesterol [52,55–57]. Johnston and Chapman studied 1:1:2 (mol ratio) mixtures of bovine brain GalCer/bovine brain PC/cholesterol by DSC [56]. They have pointed out that their data can be interpreted in terms of a strong tendency for the GalCer to self associate into domains of pure or almost pure GalCer in the case of a GalCer fraction rich in non-hydroxy fatty acids. Since this was not seen for the fraction with hydroxy fatty acids, they note that D-α-OH GalCer has an intrinsically lower transition temperature, which reflects importantly smaller GSL-GSL attractive forces and a consequently lower tendency to self-associate (see also [51]). The fact that we did not see evidence of phase separation, or major differences between α-OH and

non-hydroxy 18:0 GalCer may be a result of the fact that the GSL concentrations were quite different (7 vs. 25%). Another difference in the experimental protocols is that the hydroxy and non-hydroxy fractions used by Johnston and Chapman [56] were themselves complex natural mixtures. Subczynski et al. [52] have suggested that fluid phase micro-immiscibility is prevalent in mixtures of *cis*-unsaturated phosphatidylcholines with cholesterol as a direct result of structural non-conformability between cholesterol and the double bond. Such an arrangement likely would not have been expected to give rise to coexisting spectra in the present experiments as the authors suggest that the domains are small and/or very short lived.

The question of GSL involvement in H-bonding in fluid membranes has also arisen in a wider context. The chemical structure of GSLs indicates that they are intrinsically capable of making direct donor and acceptor H-bonds to other bilayer components; which has been suggested as a source of their relatively greater order and phase transition temperatures than glycerolipids in membranes [13,15,20,58]. Curatolo has reviewed theories relating to the possible role of (GSL) hydroxy fatty acids in intermolecular H-bonding effects at the surface of membranes [20]. The potential for inter- and intramolecular H-bonding via GSL-OH groups has been suggested particularly in association with X-ray crystallographic measurements ([59] and references therein). It is known too that fatty acid substitution in GalCer leads to different phase transition temperatures for hydrated bilayers formed from the pure material [14,60]. The extent of such forces for GSLs dispersed as minor components in complex fluid membranes is not known however. Sonnino et al. [51] have suggested that the GSL, GM_1, shows a slightly greater tendency to disperse in a fluid phospholipid bilayer, and less rigidifying effect, when its fatty acid is D-α-OH rather than non-OH. It has been proposed by Slater et al. [61] that phospholipid unsaturation weakens interlipid H-bonding involving water and that this influence is unaffected by lipid order. Cholesterol was claimed to have little effect on the H-bonding network, either through H-bonding effects of its own or through ordering effects. The experiments reported in the present work should reflect significantly on such considerations.

Complete order parameter profiles for α-OH fatty acids have not to our knowledge been reported previously, although partial profiles exist for perdeuterated 18:0 α-OH GalCer in dimyristoyl PC [25]. The virtually complete overlap of order profiles in the plateau region of the saturated GalCer's must be taken as evidence that the major forces controlling their behaviour and conformation are similar. There was slightly greater order at the surface in the case of the D-α-OH species compared to the L-α-OH. This might be considered to arise from (a) conformational effects, (b) greater intermolecular H-bonding in the D-α-OH case, (c) adverse steric effects in the L-α-OH case. However the profile for D-α-OH was similar to that of the non-hydroxy GalCer, which would argue against significantly increased H-bonding in the case of the D-α-OH. Thus, in spite of the potential for impact on lipid dynamics that one might anticipate from introduction of a hydroxyl group in place of a hydrogen atom of the fatty acid chain, the data provide little evidence of major effect in the membranes studied. This was also true of our earlier observation based on ^{2}H-NMR of the GalCer headgroup and freeze-etch electron microscopy: that the hydroxy fatty acid derivatives of GalCer as minor components in POPC showed less tendency to phase separate from fluid bilayers than did non-hydroxy GalCer [62]. The latter observation is consistent with the findings of Johnston and Chapman [56] and Sonnino et al. [51], and argues that the hydroxylated species manifest smaller intermolecular attractive forces as suggested by Johnston and Chapman [56] (see also [49]).

The quantitative similarity observed in the present work amongst order parameter profiles of the four different GalCer species having saturated fatty acids, bears upon glycolipid receptor function. In particular, it is known that certain glycolipids, including GalCer, can demonstrate apparent 'inaccessibility' ('crypticity') to specific binding events directed against them, based upon differences in fatty acid length and hydroxylation (reviewed in [20,63]). The explanation of this phenomenon has been elusive (see discussion in [16,44]). One possibility is that GSL fatty acid nature can determine area occupied by the GSL in the membrane – and thus availability for attack by macromolecules. However, Nagle [64] has indicated that the area occupied by a given lipid in a membrane is determined by the order parameters in the plateau region. In the present work it was clear that, to a very good approximation, these plateau region order parameters are the same. In this vein, Sonnino et al. [51] have measured a 2.5% greater area requirement for D-α-OH GM_1 than for non-OH GM_1 in pure GSL micelles.

Our spectral findings relate to the behaviour of GSLs at low concentration in semi-fluid phospholipid host matrices. Order parameter profiles for each fatty acid tested, including those estimated for the unsaturated GalCer, retained their relative features over the temperature range 25–65° C. Alteration of membrane spatial constraint by temperature variation should emphasise steric and conformational effects associated with fatty acid structure. The curve features already described were maintained throughout the set of fluidity conditions chosen. Hence it seems that the results discussed above have some generality – particularly given the use of a monounsaturated phospholipid with cholesterol as host matrix. The glycolipid, GalCer, studied here, although possessing the features characteristic of GSLs, is simple in having only one sugar residue. One might consider that a different result could be obtained for species with more complex headgroups. However, in past we have systematically probed the behaviour of 18:0 and 24:0 GalCer using spin labels attached cova-

lently at C-16, comparing the results for a series of other GSLs that included both neutral and acidic species (lactosyl ceramide, globoside, GM_1), and found no detectable differences [22,23]. Also, when probed by ^{2}H-NMR at C-2 of the fatty acid (i.e., at a motionally restricted location near the headgroup), very little difference was found for globoside, GlcCer, GalCer, and GM_1 [44]. Hence it seems very likely that these results can be extended to a wide range of GSLs.

5. Conclusions

The arrangement and behaviour of galactosyl ceramide acyl chains was measured on the NMR timescale for a variety of commonly-occurring fatty acid types. Dispersed as minor components in fluid bilayer membranes mimicking important features of cell membrane lipids, they demonstrated significant similarities and differences when compared to the known behaviour of glycerolipids. For GalCer with saturated fatty acid chain length approximating that of common phospholipids, behaviour and arrangement of the acyl chains were very similar to those known for glycerolipids. This was true for the GSLs with and without hydroxylation at C-2. The degree of order for the GSLs was however some 10% higher than is typical of similar chains on glycerolipids in the plateau region. The only measurable difference amongst the glycolipids with 18-carbon saturated chains occurred between C-3 and C-10, where the D-α-OH species showed slightly higher order than the L-α-OH. Interlipid attractive forces involving the α-OH group appeared not to play a major role in the systems studied. For GalCer with saturated 24-carbon fatty acid in the same host matrix, chain orientation and behaviour mimicked that of the 18-carbon analogue throughout the plateau region of the latter, and to a depth of C-13–C-14, before deviating to higher order, forming a secondary 'plateau' in the region C-18–C-22. This seems to be a characteristic feature of very long GSL fatty acid chains in fluid bilayers. GalCer with monounsaturated 18-carbon fatty acid displayed the anticipated (12–20%) lower order near the membrane surface, and evidence of relative stiffening at the depth of the double bond, but unexpectedly higher order at the methyl terminus. Differential effects of cholesterol on monounsaturated GSLs vs. corresponding glycerolipids such as POPC warrant further investigation to test the basis of the much larger GSL order parameters found in the present experiments. There was no evidence that variability in the glycolipid acyl chain conveys significantly different area requirements upon GSLs with saturated fatty acids in membranes.

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