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Acyl chain length effects related to glycosphingolipid crypticity in phospholipid membranes: probed by ^{2}H-NMR

Katherine S. Hamilton [a], Kathleen Briere [b,1], Harold C. Jarrell [b,*], Chris W.M. Grant [a,*]

[a] *Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1*
[b] *Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6*

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Abstract

Wideline ^{2}H-NMR was used to consider the relationships amongst glycosphingolipid and phospholipid fatty acid chain length and glycosphingolipid receptor function, in a system classically associated with crypticity. Galactosyl ceramide (GalCer), having 18-or 24-carbon fatty acid, was deuterium labelled at the conformationally-restricted fatty acid α-carbon (C-2). ^{2}H-NMR spectra of *N*-[2,2-$^2H_2$]stearoyl and *N*-[2,2-$^2H_2$]lignoceroyl GalCer (GalCer with 18-vs. 24-carbon selectively deuterated fatty acid) were then compared over a range of temperatures in phosphatidylcholine/cholesterol membranes in which the host phospholipid had dimyristoyl, dipalmitoyl, or distearoyl fatty acid composition. Findings were evaluated in the light of known sensitivity of antibody interaction with GalCer to temperature and to both glycolipid fatty acid chain length and host matrix fatty acid chain length. Under the conditions of experimentation, spectra were not obtainable for glycolipids having rigid body motions that were slow on the NMR timescale (10^{-4}–10^{-5} s) – *i.e.*. motions typical of non-fluid (gel phase) membranes. The systems, DPPC/cholesterol and DSPC/cholesterol, in which the original observation was made of increased antibody binding to GalCer with long fatty acid, proved to be characterised by receptor motions that were in this slow timescale for both 18:0 and 24:0 GalCer at 22–24°C. Under conditions for which spectra could be obtained, those for GalCer with [2,2-$^2H_2$]lignoceroyl (24-carbon α-deuterated) fatty acid were qualitatively similar to those of its 18-carbon analogue in all (fluid) membranes examined. However, spectral splittings differed quantitatively between deuterated 18:0 and 24:0 GalCer at a given temperature, dependent upon host matrix. These differences were most marked at lower temperatures and in the longer chain (more ordered) matrices, DPPC/cholesterol and DSPC/cholesterol. This suggests that maximum effects of glycolipid chain length on glycolipid receptor function may be expected to occur in spatially and motionally constrained lipid environments. There was little effect of temperature on spectral splittings seen for a given sample containing deuterated 18:0 GalCer. The small differences seen could be adequately accounted for by relatively minor alterations in glycolipid order and backbone conformation. In contrast, 24:0 GalCer in DPPC/cholesterol and DSPC/cholesterol displayed significant variation in its spectral splittings as the temperature was reduced; and these proved to be the source of the quantitative differences between 18:0 and 24:0 GalCer referred to above. For 18:0 GalCer, the only spectral feature seen to be notably sensitive to temperature and to choice of host membrane phospholipid in the range, 22–65°C, was whole-body motion; which could be abruptly 'frozen out' with decreasing membrane fluidity. The 24:0 GalCer analogue was seen to undergo the same loss of motional freedom, in the same temperature range, as 18:0 GalCer. We suggest therefore that several previous observations of GalCer crypticity in phospholipid/cholesterol systems having longer vs. shorter chain phospholipids, may derive most importantly from reduced motional freedom of the glycolipid recognition sites.

Key words: Galactosyl ceramide; Antibody; Hapten

1. Introduction

Glycosphingolipids (GSLs), the carbohydrate-bearing lipids of eucaryotic cells, comprise a fundamental family of surface recognition sites. Their specific interactions with macromolecules appear to be modulated by GSL behaviour and arrangement. This

* Corresponding author. Fax: +1 (519) 6613175.
[1] Present address: Institute for Biodiagnostics, 435 Ellice Ave., Winnipeg, Canada R3B 1Y6.

Abbreviations: GSL, glycosphingolipid; DMPC, L-α-dimyristoyl phoshatidylcholine; DPPC, L-α-dipalmitoyl phosphatidylcholine; DSPC, L-α-distearoyl phosphatidylcholine; GalCer, Galβ1 → 1ceramide; PC, phosphatidylcholine.

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phenomenon, which has been identified in a variety of forms, has come to be known as receptor 'crypticity' [1]. A major literature observation relating to the concept involves galactosyl ceramide (GalCer) and its 'accessibility' to antibodies in phosphatidylcholine bilayer membranes rich in cholesterol [2–5]. Alving and co-workers made the interesting observation that GalCer, with a given fatty acid composition, is a more effective receptor for polyclonal antibodies when assembled into phosphatidylcholine/cholesterol bilayers whose phospholipids have 14-carbon fatty acids rather than 16- or 18-carbon analogues [2,3]. They observed a striking temperature dependence to this phenomenon: greater binding to the DMPC-containing membranes being particularly evident at about 23°C, but not at temperatures of 35°C and above [3]. The same workers also noted that GalCer having long chain (24:0) fatty acid could be a better receptor for specific antibodies than was GalCer with short chain (16:0 or 18:0) fatty acid in DPPC/cholesterol and DSPC/cholesterol liposomes at 23°C. Apparently-related observations have been made more recently for sulfate-substituted GalCer in the same phosphatidylcholine/cholesterol membranes at 37°C [6].

The considerable attention focused on this system – a very simple lipid hapten in liposomes – derives from its minimalist nature and resultant feasibility of analysis as a basic example of receptor control in membranes. Nevertheless, an understanding of the underlying phenomena has proven to be a challenging goal. In considering the molecular basis of observations relating to GalCer crypticity, Alving and co-workers suggested that the carbohydrate portion of a given glycolipid in a short chain host matrix (i.e., DMPC/cholesterol) might protrude farther than in a longer chain matrix (in which DMPC was replaced by DPPC or DSPC) and so be less spatially constrained and more accessible to macromolecules. Similarly, in a given host matrix, a short chain glycolipid might fail to protrude adequately to participate optimally in a binding event with a macromolecule [3]. An alternative concept has been proposed by Utsumi et al. [4] who, in recording that immune agglutination of liposomes containing GalCer was affected by temperature, cholesterol content and fatty acid chain length of the host matrix, noted that these factors could alter the lateral distribution of GalCer in the membrane and suggested a correlation between glycolipid distribution and the immune effects.

It is likely that these and other [1,7–10] examples of GSL recognition site control by intermolecular effects are significantly based upon spatial restrictions common to receptors at a membrane surface. It has, however, been difficult to isolate the relative importance of steric factors, lipid dynamics and lateral distribution as determinants of receptor 'accessibility' to specific macromolecules [4,5,7,11–13]. In this article we have directly measured the effect of host matrix and glycolipid fatty acid chain length on GalCer in the systems studied in the original experiments, using non-perturbing ^{2}H-NMR of nuclei located near the membrane surface in GalCer with 18:0 and 24:0 fatty acid.

2. Materials and methods

L-α-Dimyristoyl phosphatidylcholine (DMPC), L-α-distearoyl phosphatidylcholine (DSPC) and L-α-dipalmitoyl phosphatidylcholine (DPPC) were purchased from Avanti Polar Lipids, Birmingham, AL. Galactosyl ceramide (GalCer) and cholesterol were obtained from Sigma, St. Louis, MO. Deuterated solvents were from MSD Isotopes, Montreal, Canada. [2,2-$^2H_2$]Stearic acid and [2,2-$^2H_2$]lignoceric acid were synthesized from methyl stearate and methyl lignocerate, respectively (Aldrich, Milwaukee, WI.), using the method of Aasen et al. [14] for inserting deuterium at the C-2 position of fatty acids. Structures were confirmed by ^{1}H-NMR. Lyso intermediates of glycosphingolipids were synthesized via the method of Neuenhofer et al. [15]. The syntheses of *N*-[2,2-$^2H_2$]stearoyl GalCer and *N*-[2,2-$^2H_2$]lignoceroyl GalCer were performed by reacting the lyso intermediates with the acid chloride derivative of the appropriate fatty acid [16]. Lysoglycosphingolipid intermediates and final deuterated products were characterised and purified employing chromatography techniques described in previous work [17].

Sample preparation for ^{2}H-NMR and procedures for ^{2}H-NMR spectroscopy have been described elsewhere [17]. $\pi/2$ pulse length was between 5 and 6 μs (10 mm solenoid coil). Pulse spacing was 60 μs, with a recycle time of 100 ms. Spectral acquisition was for up to 60 h at a given temperature and was begun after pre-equilibration well above the host phospholipid main transition temperature. Samples typically consisted of 17–20 mg (23–27 μmol) of the stearoyl GalCer or 18–22 mg (22–27 μmol) of the lignoceroyl GalCer, which represented under 10 mol% of the total phospholipid (typical sample mol ratio of cholesterol/PC/GalCer, 32:61:7). Liposomes were suspended in 50 mM phosphate buffer (pH 7.4).

3. Results

Fig. 1 displays typical ^{2}H-NMR powder spectra for deuterated GalCer at 7 mol% in DMPC/cholesterol – representative of a key model system dealt with in previous studies of complement-mediated immune attack involving model membranes [2–4]. Samples were

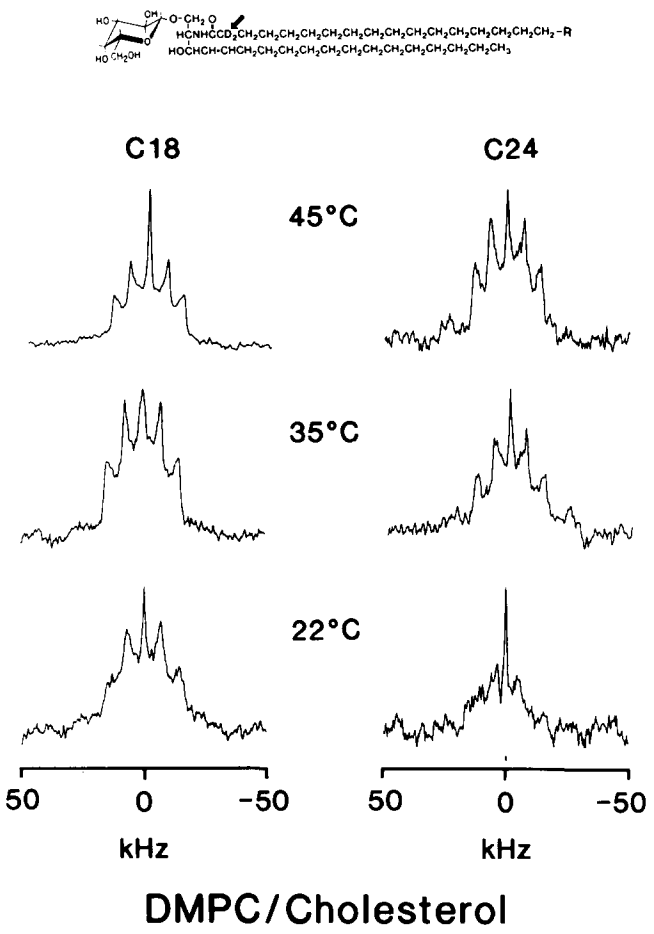

Fig. 1. ^{2}H-NMR (30.7 MHz) powder spectra for multilamellar vesicles of *N*-[2,2-$^2H_2$]stearoyl GalCer (left) and *N*-[2,2-$^2H_2$]lignoceroyl GalCer (right) at 7 mol% in DMPC bilayers containing 32 mol% cholesterol. The number of acquisitions at 22°C, 35°C and 45°C were: 400000, 150000 and 100000 (stearoyl GalCer) and 400000, 400000 and 100000 (lignoceroyl GalCer), respectively. Chemical structures of galactosyl ceramide species used in this study: *N*-[2,2-$^2H_2$]stearoyl GalCer (R = -CH_3) and *N*-[2,2-$^2H_2$]lignoceroyl GalCer (R = -CH_2-$(CH_2)_5CH_3$) are illustrated as an inset.

initially equilibrated at temperatures corresponding to highly fluid lipid bilayers and spectra were then run at various stages of cooling. Data are shown for the range, 45°C to 23°C. The left hand column presents spectra for the 18-carbon glycolipid, *N*-[2,2-$^2H_2$]stearoyl GalCer (18:0 GalCer); the right contains corresponding experimental results for the 24-carbon analogue, *N*-[2,2-$^2H_2$]lignoceroyl GalCer (24:0 GalCer). The deuterated glycolipid structures involved are illustrated as inserts: note the deuteron location at fatty acid C-2 (the α-carbon).

The spectra in Fig. 1 have an appearance typical of deuterated lipids undergoing axially symmetric motion in fluid membranes. In particular, they show Pake doublet structure, resulting from motional averaging with effective symmetry about an axis perpendicular to the bilayer [18–20]. The features seen for both *N*-[2,2-$^2H_2$]stearoyl and *N*-[2,2-$^2H_2$]lignoceroyl GalCer are those originally described by Skarjune and Oldfield [21] for pure *N*-palmitoyl GalCer in fully hydrated bilayer form. Two separate Pake doublets are resolved for the deuterons at this location in the fatty acid; indicating that the deuterons are magnetically inequivalent. This is considered to reflect the presence of a sharp bend in the fatty acid at C-2, away from the plane of the bilayer, such that the rest of the chain below this point extends perpendicular to the bilayer, as originally suggested by Skarjune and Oldfield [21]. The sharp central peak typically seen in wideline spectra of deuterated lipid samples (as here) may be due to the presence of some highly curved or rapidly tumbling vesicles in the sample, in addition to having a contribution from residual HOD. Measured spectral parameters are listed in Table 1. Changes in the ratio of outer to inner quadrupole splittings may be considered to reflect alterations in molecular geometry, as opposed to orientational order (i.e., alterations in Θ_i rather than S_{mol} in Eq. (1) below).

The observation of fluid phase spectra for GalCer at temperatures well below its main transition temperature range of 82°C–85°C [22–25], indicates that the glycolipid molecules are dispersed into the (lower melting) fluid host matrix [17,26,27]. Spatial orientation and motional order of the C-D bond in such systems are

Table 1
Quadrupole splittings, $\Delta\nu_Q$ (kHz), for galactosyl ceramide (GalCer) having either 18-carbon or 24-carbon fatty acid deuterated at C-2, at 7 mol% in lipid bilayers of DMPC, DPPC and DSPC in the presence of 32 mol% cholesterol

Samples	Quadrupolar splittings (±1 kHz)			
	23°C	35°C	45°C	65°C
DMPC/Chol: 18 carbon	15.6 32.1 (2.1)	16.4 30.8 (1.9)	16.4 29.6 (1.8)	–
24 carbon	–	14.2 28.5 (2.0)	15.4 28.0 (1.8)	–
DPPC/Chol: 18 carbon	–	15.6 28.8 (1.8)	16.2 29.6 (1.8)	17.4 28.0 (1.6)
24 carbon	–	11.7 25.9 (2.2)	12.5 26.9 (2.2)	15.4 26.1 (1.7)
DSPC/Chol: 18 carbon	–	–	15.6 27.3 (1.8)	17.9 26.5 (1.5)
24 carbon	–	–	10.9 22.6 (2.1)	15.4 25.3 (1.6)

Presented are the inner splittings, the outer splittings and (in brackets after each set of data) the ratios of outer peak splittings to inner splittings for a given temperature. Splittings were measured from powder spectra, and have an estimated uncertainty of up to ±1 kHz. The ratios have an estimated uncertainty of up to ±0.1. Values for 24:0 GalCer at 23°C in DMPC/cholesterol are not reported, as they were not accurately evaluable.

related via the observed quadrupole splitting, $\Delta\nu_Q$:

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

where e^2Qq/h is the nuclear quadrupole coupling constant, S_{mol} is the molecular order parameter (assuming axially symmetric order) describing orientational fluctuations of the lipid molecule relative to the bilayer normal and Θ_i is the average orientation of each C-D bond relative to the motional director axis (the molecular long axis) [18]. The relationship is highly sensitive to alterations in orientation and orientational order of the deuterated segment of a molecule.

Spectral splittings for the deuterated 18:0 GalCer (left hand column) are essentially within experimental error of one another over the temperature range studied. This includes the temperature range dealt with by Alving and co-workers [2,3]. Anticipated small changes in spectral splittings with temperature variation, which result from temperature-induced alteration in molecular order (S_{mol}), are probably being coincidentally offset by minor reorientation at C-2, as reflected in small changes in the ratio of outer to inner splittings.

Comparison of the spectra between left and right hand columns of Fig. 1 shows several noteworthy features of GalCer in the DMPC/cholesterol system. Firstly, the overall similarity amongst spectra for short and long chain glycolipids demonstrates clearly that in this 14-carbon fatty acid host matrix the intrinsic 18:0 and 24:0 GalCer backbone orientation and order are very similar throughout the range of temperatures studied in reported crypticity experiments, in spite of their very different fatty acid chain lengths. Secondly the spectral quadrupole couplings are quantitatively very similar: the values being marginally smaller for the longer chain species at a given temperature (Table 1). As already indicated, the quadrupolar splittings in such systems, particularly for the C-2 position, are sensitive to small alterations in orientation and order: hence the effects seen can be accounted for by relatively small alterations in angle – probably on the order of a few degrees. The poor signal-to-noise associated with the spectrum for the 24:0 GalCer did not permit accurate measurement of splittings at 23°C, however, key spectral features remain.

Primary comparisons made by investigators studying GalCer as a target for immune attack in model membranes have been amongst cholesterol-rich membranes based on the three saturated-fatty-acid phospholipids, DMPC, DPPC and DSPC. The fluid/gel phase transition temperature of pure DMPC in bilayer form is 23°C; DPPC and DSPC have main transition temperatures in their pure form of 41°C and 54°C–55°C, respectively [28–30]. Spectra for deuterated *N*-[2,2-$^2H_2$]stearoyl GalCer and *N*-[2,2-$^2H_2$]lignoceroyl GalCer in DPPC/cholesterol are shown in Fig. 2. DPPC/cholesterol provides a host matrix with saturated fatty acids longer by 2 methylene units than the 14-carbon DMPC/cholesterol matrix dealt with in Fig. 1. Spectra for the 18:0 GalCer (left hand column) over the range 35°C to 65°C are clearly similar to those already described with regard to Fig. 1. The measured parameters are listed in Table 1 for comparison. Interestingly, the spectral features for 18:0 GalCer in the DPPC-rich membranes are within experimental error of those seen above for the DMPC/cholesterol membranes.

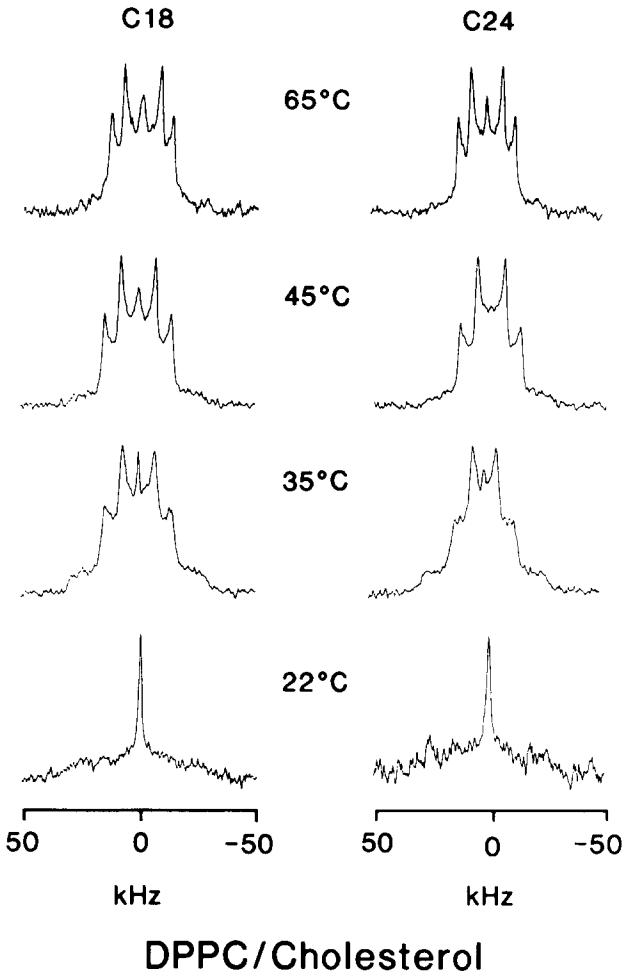

Fig. 2. ^{2}H-NMR (30.7 MHz) powder spectra for multilamellar vesicles of *N*-[2,2-$^2H_2$]stearoyl GalCer (left) and *N*-[2,2-$^2H_2$]lignoceroyl GalCer (right) at 7 mol% in DPPC bilayers containing 32 mol% cholesterol. The number of acquisitions at 22°C, 35°C, 45°C and 65°C for stearoyl GalCer and lignoceroyl GalCer were: 770000, 600000, 100000 and 50000, respectively.

In the DPPC/cholesterol matrix, the longer chain glycolipid manifests spectral splittings which differ measurably from those of its shorter chain analogue at a given temperature. The quadrupolar couplings are smaller for the 24:0 species and the ratio of inner to outer splittings is measurably different (2.2 vs. 1.8). Thus the only significant quantitative differences between GalCer spectra in the DPPC/cholesterol matrix

(Fig. 2) vs. the DMPC/cholesterol matrix (Fig. 1) are for the 24-carbon fatty acid glycolipid in DPPC/cholesterol – particularly at low temperatures, which would represent a more highly ordered membrane environment.

The spectra in Fig. 2 demonstrate a feature highly reminiscent of a phenomenon noted by Oldfield and colleagues [31] in a study of DPPC with deuterated fatty acids in cholesterol-containing bilayer membranes (without glycolipid): that the deuterons at C-2 of the *sn*-2 chain 'disappeared' selectively from the spectrum at low temperatures. These authors noted that, in phospholipid fatty acid chains, the C-2 position is uniquely restricted in conformational freedom, so that its motions are closely tied to those of the molecule as a whole. For both 18:0 and 24:0 GalCer in the DPPC/cholesterol matrix, spectra 'disappear' at 22°C.

Spectra of the of DSPC/cholesterol membranes containing *N*-[2,2-$^2H_2$]stearoyl and *N*-[2,2-$^2H_2$]lignoceroyl GalCer are shown in Fig. 3. In this case, the host matrix fatty acids are of the same (18-carbon) length as the short chain deuterated glycolipid. At 65°C and 45°C Pake doublets are seen for each of the two deuterons at C-2. The intrinsic spectral features in this case remain similar to those already described in Figs. 1 and 2. Indeed it can be seen from Table 1 that quantitatively the splittings for 18:0 GalCer in this matrix closely resemble those for the same glycolipid in the DPPC/cholesterol and DMPC/cholesterol membranes at comparable temperatures. However, there is clear evidence of quantitative difference between the long and short chain glycolipid in DSPC/cholesterol. Also there are differences between the 24:0 GalCer parameters in DSPC/cholesterol vs. those in DMPC/cholesterol and DPPC/cholesterol at a given temperature. These differences are particularly striking for the lower temperature (45°C) at which spectra were obtainable.

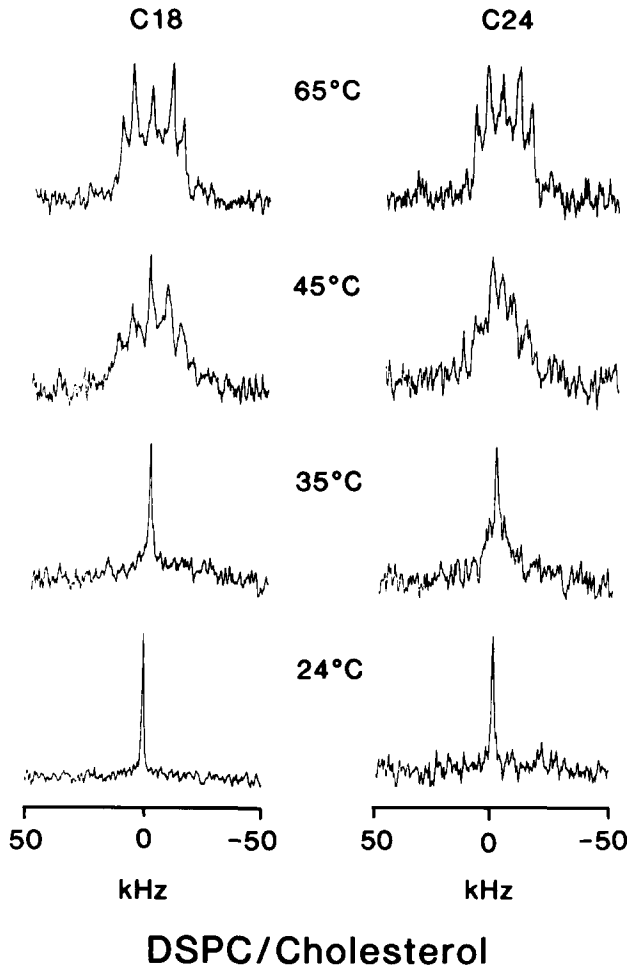

Fig. 3. ^{2}H-NMR (30.7 MHz) powder spectra for multilamellar vesicles of *N*-[2,2-$^2H_2$]stearoyl GalCer (left) and *N*-[2,2-$^2H_2$]lignoceroyl GalCer (right) at 7 mol% in DSPC bilayers containing 32 mol% cholesterol. The number of acquisitions at 24°C, 35°C, 45°C and 65°C for stearoyl Galcer and lignoceroyl GalCer were: 1 000000, 500000, 100000 and 50000, respectively.

The large numbers of spectral accumulations involved in DPPC/cholesterol at 22°C and in DSPC/cholesterol at 35°C, demonstrate the completeness of spectral 'disappearance' in these cases. For DPPC/cholesterol, both 18:0 and 24:0 GalCer show the same spectral intensity at 45°C and 65°C, but loss of about 50% of intensity at 35°C. For DSPC/cholesterol, both 18:0 and 24:0 GalCer show loss of about 50% of spectral intensity at 45°C. In the DMPC/cholesterol matrix (Fig. 1), there is seen to be loss of roughly 50% intensity for both GalCer species at 22°C, but no loss at the higher temperatures. Thus spectral 'disappearance' appears to occur at the same temperature for short and long chain species in a given matrix.

4. Discussion

Several key papers dealing with the phenomenon of receptor crypticity have focused on the glycolipid, GalCer, as a recognition site in model membranes rich in cholesterol. They present three basic observations: (i) that binding of antibody to receptor is importantly sensitive to host matrix fatty acid chain length (e.g., 14 vs. 16 or 18 carbons, (ii) that temperature can have a striking influence on antibody-mediated interactions (23°C vs. 35°C) and (iii) that in a given host matrix antibody binding can be sensitive to GSL fatty acid chain length [2–4]. Thus for instance, in 2:1.5:0.22 PC/cholesterol/dicetyl phosphate membranes containing 5–10 mol% GalCer at 23°C, considerably more antibody was bound when DMPC rather than DPPC was the phospholipid. The DPPC-based membranes in turn bound antibody somewhat better than did ones based on DSPC. In addition, GalCer with 24:0 fatty acid was a more effective recognition site than was 16:0 or 18:0 GalCer in DPPC/cholesterol and

DSPC/cholesterol [2,3]. These differences were absent or less marked at 35°C. In the present work, separate experiments are described with 18:0 and 24:0 species of GalCer which address the basis of these results in terms of GSL orientation and dynamics in membranes. Deuterium nuclei at C-2 of the glycolipid single fatty acid provide non-perturbing probes with high sensitivity to both spatial orientation of the surface backbone portion of the glycolipid and to glycolipid rigid body motions [17,21].

Given the responsiveness of deuterium quadrupolar splittings to C-D bond orientation and motional properties in fluid membranes, it is striking that the splittings measured in the three host matrices for 18:0 GalCer were extremely similar. These data certainly provide no indication that short chain GalCer displays differential orientation or dynamic behaviour near the membrane surface in fluid membranes of DMPC/cholesterol vs. DPPC/cholesterol or DSPC/cholesterol. One might hypothesize that reduced binding of antibodies to GalCer in DPPC/cholesterol and DSPC/cholesterol matrices at 23°C(vs. DMPC/cholesterol) could result from selective loss of binding to glycolipids with long chain fatty acids in the natural GalCer mixtures used for some of the original experiments – i.e., due to reorientation as reflected in the spectral splitting changes seen here for 24:0 GalCer. However, this would seem to directly contradict the observation that 24:0 GalCer bound antibodies more strongly at 23°C than did short-chain GalCer in DPPC/cholesterol and DSPC/cholesterol [2]. The explanation for loss of antibody binding to GalCer in DPPC/cholesterol and DSPC/cholesterol vs. DMPC/cholesterol noted in past at 23°C seems more likely to be related to the striking 'disappearance' of the glycolipid spectra as the temperature was lowered – observed here for the first time in ternary lipid mixtures.

Selective 'disappearance' of the spectral features associated with deuterons at C-2 of the *sn*-2 chain of phospholipid fatty acids, was first noted by Oldfield and colleagues upon lowering the temperature of lipid bilayer systems [31,32]. They demonstrated that, for 30% cholesterol in DPPC or DMPC, the peaks associated with deuterium nuclei at C-2 of the *sn*-2 chain (but not with other chain deuterons including C-2 of the *sn*-1 chain) broadened to become 'undetectable' below the phase transition temperature of the phospholipid involved. This was attributed to phospholipid immobilisation in the host matrix: the whole-body motion being fairly abruptly slowed on the NMR timescale of 10^{-4}–10^{-5} s. The authors noted that the C-2 methylene group of the *sn*-2 chain was apparently severely limited in its possibilities for internal motion as a result of a sharp bend in the chain at this position, consistent with X-ray structures of single crystals of phospholipids. A similar phenomenon has been described for pure deuterated glycerolipids in single-component bilayers as they underwent a fluid/gel phase transition; and explained in terms of changes in T_{2e} related to rigid body immobilisation [33,34]. Based upon X-ray studies of single crystals [35–37] and results of wideline ^{2}H-NMR spectroscopy [21,38], in glycosphingolipids the fatty acid attached in amide linkage to the sphingosine backbone might be considered analogous to the *sn*-2 fatty acid of phospholipids – having highly restricted conformational freedom at C-2. Thus, our observation that spectra of GalCer in DPPC/cholesterol and DSPC/cholesterol disappear when samples are cooled to 22–24°C, implies that, below 35°C, the rate of GSL whole body rotation about its long axis drops to less than the value of the spectral splittings – i.e., becomes less than about 30000 s^{-1}.

In binary mixtures, cholesterol is well known to suppress the sharply cooperative chain melting behaviour of pure phospholipids [39–42], and temperature effects on motional freedom occur over wider ranges. The temperature at which immobilisation occurred for GalCer in the samples studied here correlated with the phase transition temperatures of the host phospholipids (as observed by earlier workers for the deuterated glycerolipids described above [31–34]): mobile GalCer disappearing from spectra of DSPC-rich membranes about 35°C, those in the DPPC-rich samples disappearing below 35°C and those in the DMPC-rich membrane remaining significantly at room temperature. Although the lipid mixtures studied by Alving and co-workers [2,3] were typically somewhat higher in cholesterol content than our own (e.g., cholesterol/DMPC/DCP/GalCer mol ratio 6.7:9:1:1), deuterated GalCer in the latter mixture also remained mobile down to 23°C (spectra not shown).

Of particular relevance to our findings in the present work are past observations that membrane fluidity can be required for antibody binding and resultant immune damage to liposomes. For instance Kitagawa and Inoue recorded that complement-mediated immune damage of DPPC and DPPC/cholesterol liposomes bearing Forssman antigen "requires some fluid conditions of membranes" [7]. The same workers have made similar observations for GalCer in phospholipid membranes [4]. McConnell and co-workers, who have studied the factors underlying complement fixation by spin labelled phospholipids, noted, "The effect of increasing cholesterol concentration in enhancing antibody binding (and cellular activation) by spin labelled phospholipids is understandable in view of the enhanced rotational and perhaps lateral, mobility of the nitroxide group" [43] (see also [11]).

It is interesting to consider whether immobilisation of GalCer seen in the present work at reduced temperature, is a result of phase separation of domains en-

riched in glycolipid – or whether the matrix remains a homogeneous single phase. Utsumi et al. [4] have pointed out that phase separation alone could conceivably reduce macromolecule binding, as a direct result of receptor crowding. Unfortunately, proof of lipid lateral distribution in ternary (or more complex) lipid mixtures is an extremely challenging problem. In principle, examination of ^{2}H-NMR spectra over the range of spectral disappearance should provide insight into this question; however, this will require a system providing excellent signal-to-noise, given the small amount of deuterated lipid present in these samples. Also uncertainty regarding compositional inhomogeneity in a given population of liposomes would be of concern. It is clear that our observation of reduced GalCer rigid body motions at low temperature is closely analogous to earlier observations mentioned above involving deuterated DMPC or DPPC in binary mixtures with 30% cholesterol [31,32]. In this regard, recent phase diagrams for DMPC/cholesterol, DPPC/cholesterol and DSPC/cholesterol, typically show no coexisting phases above 20–25 mol% cholesterol [42,44–46] (see also [47]). Hence, given that the amount of glycolipid in our membranes is only 7 mol%, one might reasonably suggest that it will be no more than a minor perturbation on the existing binary phase diagrams for DMPC, DPPC and DSPC with cholesterol; and the bilayers can be expected to be a single phase throughout the temperature range examined. Nevertheless, there have been suggestions that domain coexistence may extend to high cholesterol concentrations in binary systems [48]. Also, there are indications that preferential lipid associations may lead to phase separation in ternary lipid mixtures containing concentrations of cholesterol well above 20% [49–51]. Thus we cannot exclude the possibility that GalCer phase separation forms the basis of immobilisation in our systems. With regard to the present study, Suzuki et al. found that egg PC liposomes containing natural GalCer at low mol ratio agglutinated just as well at 25°C with or without cholesterol; but that DPPC/GalCer liposomes did not agglutinate unless cholesterol had been added and at 33% cholesterol they agglutinated almost as well as did egg PC/GalCer liposomes [52]. These authors made the interesting suggestion that EPR spectra of spin labelled GalCer with 16 : 0 fatty acid in 1 : 1 DPPC/cholesterol could be interpreted in terms of a clustered/dispersed equilibrium – with the clustered GalCer being unable to bind antibody and dispersed GalCer capable of binding. However, in our hands, direct comparison of spin labelled GSL spectra to those of comparable spin labelled phosphatidylcholine as controls showed very little evidence of glycolipid phase separation up to 10% in PC/cholesterol mixtures [53]. Moreover Suzuki et al. also noted that good antibody/hapten interaction required addition of enough cholesterol to fluidize their lipid bilayers [52]. It will be crucially important to carefully follow up on above recent suggestions from the laboratories of Chapman [50] and Vaz and Thompson [51], that complex membranes with high cholesterol content may manifest phase separation, a concept put forward in the 1970's [48,49], but often discounted in more recent times.

The above discussion addresses primarily the mechanism underlying past experiments in which glycolipid fatty acid was fixed and host phospholipid chain length was varied. The present experiments also bear upon past observations on the same system while holding host matrix constant and varying the glycolipid fatty acid. In particular, Alving and co-workers found suggestions of superior antibody binding to GalCer with 24 : 0 fatty acid when compared to GalCer with 16 : 0 or 18 : 0 fatty acid in DSPC/cholesterol (and DPPC/cholesterol) at 23°C. They proposed that this might reflect greater extension above the surface for the longer chain GSL [2]. The data in Table 1 show evidence that the ceramide backbone geometry of 24 : 0 GalCer is indeed altered relative to that of 18 : 0 GalCer, at the lower temperatures in the more highly ordered fluid matrices, DPPC/ cholesterol and DSPC/cholesterol. At higher temperatures (i.e., in more disordered membranes), the differences between long and short chain fatty acids were much less marked, in agreement with NMR results for highly fluid host membranes of POPC [54]. As indicated above though, at no temperature does GalCer with a variety of fatty acids in DPPC/cholesterol or DSPC/cholesterol bind antibody better than it does in DMPC/cholesterol; hence the measured conformational change induced by the former two matrices seems unlikely to be a source of improved binding. Nevertheless, it would appear that their experiments involved relatively immobilised receptors, in highly ordered host matrices that emphasise 'fit' differences between GalCer with long vs. short fatty acid. 23°C is below the temperature at which our spectral features 'disappeared' due to slowing of whole body motion, so that we are unable to correlate spectral details with the system Alving and co-workers employed in comparing short and long chain GalCer.

5. Conclusions

Whole body motion of GalCer undergoes a thermally-induced transition between 'mobile' and 'immobile' in cholesterol-rich phosphatidylcholine membranes. The temperature at which the transition occurs is related to the host phospholipid fluid/gel transition temperature in DMPC/cholesterol, DPPC/cholesterol and DSPC/cholesterol bilayers. This was the only striking spectral effect exhibited by non-perturbing

deuterium probes at the backbone fatty acid C-2 position of GalCer in (fluid) membranes over the temperature range of previous crypticity measurements involving the systems studied. Reduction in whole body motion seems likely to be the key factor governing observations of reduced antibody binding to cholesterol-rich membranes containing high melting phospholipids. We have not excluded the possibility that phase separation is involved in the process; although such phase separation is often considered unlikely in membranes with high cholesterol content.

GSL fatty acid chain length was seen to influence backbone orientation of GalCer in the more highly ordered membranes. Hence it is certainly possible that headgroup orientation of the 24:0 glycolipid is different from that of 16:0 or 18:0 GalCer in the cholesterol-rich membranes studied at room temperature as examples of crypticity in past. However, this effect does not appear to be a primary source of improved macromolecule binding, since it does not correlate with known results of equally good, or better, binding to DMPC/cholesterol membranes containing natural GalCer. It seems conceivable that the longer chain GalCer presents a lower energy barrier to local rearrangement *subsequent* to binding (more favourable thermodynamics of binding) in membranes with gel phase characteristics, rather than a more effectively oriented target for initial antibody access.

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7. References

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