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Behaviour of complex oligosaccharides at a bilayer membrane surface: probed by ^{2}H-NMR

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Abstract

Deuterium wideline NMR was used in an attempt to directly assess oligosaccharide arrangement and motional characteristics of complex glycosphingolipids dispersed as minor components in phospholipid membranes. A convenient, general synthetic approach was developed which involved replacement of the acetate group of amido sugars with deuteroacetate (-$COCD_3$). This provided excellent signal-to-noise when applied to the terminal GalNAc residue of globoside, and the terminal NANA residue of GM_1. Simultaneously, globoside and GM_1 fatty acids were replaced with stearic acid deuterated at C-2 – a probe location sensitive to glycolipid hydrophobic backbone orientation and rigid body motion. Deuterated GM_1 and globoside were studied by ^{2}H-NMR in bilayers of 1-palmitoyl-2-oleoyl phosphatidylcholine, in the presence and absence of physiological quantities of cholesterol. The monoglycosyl glycosphingolipid, glucosyl ceramide, which is the common skeleton of many complex glycosphingolipids including those studied here, was also deuterated at fatty acid C-2 for comparative study in the same matrices. Correlation with spectra of the complex glycolipids demonstrated that, for a given temperature and membrane composition, ceramide backbone conformation was very similar amongst the species studied. Spectral features of GM_1 deuterated on terminal NANA and assembled at a membrane surface, were found to be highly consistent with the oligosaccharide conformation determined in studies of GM_1 in solution. In contrast, globoside deuterated in the terminal GalNAc residue gave spectra very different from those predicted on the basis of the conformation considered to exist in solution. It seems likely that this result reflects a combination of greater oligosaccharide chain flexibility relative to GM_1, and the presence of the membrane environment. Interestingly, although there was highly significant spatial geometry associated with the complex oligosaccharide chains, and although temperature and the presence of cholesterol exert measurable effects on the membrane-inserted portion, these factors had very little impact on the measured spectral parameters associated with the NANA residue of GM_1 or the terminal GalNAc residue of globoside. This seems to indicate lack of sensitivity of the complex oligosaccharide chains to conformation and internal motions of the hydrophobic chain segments in these fluid and semi-fluid membranes; and has important implications for mechanisms of crypticity.

Key words: Glycosphingolipid; GM_1; Globoside; NMR, ^{2}H-

1. Introduction

There is widespread interest in the spatial arrangement and behaviour of cell surface recognition sites, as factors that regulate communication and adhesion. A significant aspect of such considerations is the anticipated influence of membrane environment on receptor conformation and interaction. It seems a reasonable expectation that, in the crowded two-dimensionally-constrained membrane surface milieu, conformation will be particularly susceptible to inter- and intramolecular interactions. Indeed peptide conformation has recently come to be recognised as highly sensitive to surface effects [1]. Moreover, it has been demonstrated that carbohydrate residues attached to lipids in membranes can be measurably altered in their orientation and dynamics [2–8]. As a general rule, molecules associated with membrane surfaces tumble slowly and anisotropically. Hence, of the spectroscopic techniques that one might apply to the problem, wideline ^{2}H-NMR

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Abbreviations: GSL, glycosphingolipid; POPC, 1-palmitoyl-2-oleoyl phosphatidylcholine; GalCer, Galβ1 → 1ceramide; GlcCer, Glcβ → 1ceramide; globoside, GalNAcβ1 → 3Galα1 → 4Galβ1 → 4Glcβ1 → 1ceramide; GM_1, Galβ1 → 3GalNAcβ1 → 4Gal(3 ← 2αNeuAc)β1 → 4Glcβ1 → 1ceramide.

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has special potential. The utility of this technique in studies of membrane surface carbohydrate was first demonstrated with deuterated monoglycosyl glycosphingolipids (GSLs) by Oldfield and colleagues [2,9]; and more recently it has been applied to several deuterated monoglycosyl glycerolipids [3–5,7], and the diglycosyl glycerolipid, lactosyl diglyceride [6]. However, the introduction of deuterium nuclei selectively at appropriate locations in complex systems is a challenging problem that must be dealt with as a preliminary to any such study. For more complex GSLs typical of the carbohydrate-bearing lipids in higher animal cells, these problems have not been surmounted to date.

By incorporation of deuterium nuclei in the exocyclic hydroxymethyl group of the terminal Gal residue on GM_1, we were recently able to demonstrate using ^{2}H-NMR that this ganglioside, when incorporated at low concentration into multibilayers of 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC), shows substantial restriction of motion about the glycosidic and sugar-ceramide bonds of the complex headgroup [10]. This result was the first direct demonstration that headgroup orientational order can exist for a complex glycolipid incorporated into bilayer membranes. An important advance for such studies will be to develop deuterium probes whose motions more simplistically correlate with those of the sugar to which they are attached, and which can be located in well characterized positions on complex oligosaccharide chains. In the present article, we have investigated such a method in generating by partial synthesis two complex GSLs, globoside and GM_1, with deuterium on amino sugar acetamido groups. By virtue of having three equivalent deuterium nuclei, this approach also should optimize the spectral signal-to-noise ratio, which is characteristically limiting in ^{2}H-NMR of biological systems. Globoside is a neutral species having an unbranched tetrasaccharide chain, and is the major GSL of human erythrocytes. It is a recognition site used for blood group typing, and has been associated with crypticity phenomena [11–14]. GM_1 is an important acidic glycolipid of neural tissue – having a branched pentasaccharide chain with a terminal neuraminic acid [11–13]. In order to reproduce constraints imposed by membrane surfaces, glycolipids to be studied were assembled at low concentration into unsonicated lipid bilayers of controllable fluidity.

Given the difficulties of analysis associated with complex surface recognition sites, the most realistic approach is to use as a starting point findings in related systems. Such information is becoming widely available through application of modern high resolution NMR techniques to membrane components in solution, and from X-ray diffraction studies. Recent analyses, employing two-dimensional high resolution NMR in combination with conformational energy calculations, have provided key information regarding headgroup arrangement for intact gangliosides [15–16] and globoside [17,18] in polar organic solvent (see also [19,20] and references therein). The approach has been extended to detergent micelles in the case of globoside [18]. These studies of GM_1 [15,16] and its oligosaccharide headgroup [21] have indicated that there is a relatively well-defined average structure to the carbohydrate portion, but that this may be less the case for globoside [17,18].

2. Materials and methods

1-Palmitoyl-2-oleoyl phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids, Birmingham, AL; and was used without further purification. Globoside was isolated from pig erythrocytes as described elsewhere [22]. GM_1 was a kind gift of Fidia Research Laboratories, Abano Terme, Italy, or was prepared from beef brain gangliosides as described previously [23]. Lyso glucosyl ceramide was from Sigma, St. Louis, MO. Deuterated acetic anhydride (99.6 atom% ^{2}H) was purchased from MSD Isotopes, Montreal, Canada; as were deuterated solvents and deuterium depleted water. Iatrobeads 6RS-8060 were from Iatron Lab, Tokyo, Japan. Methyl stearate and 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride were purchased from Aldrich, Milwaukee, WI. [2,2-$^2H_2$]Stearic acid was synthesized from methyl stearate as described previously [24].

The initial GSL hydrolysis step was the same for GM_1 and globoside, and followed the procedure developed by Neuenhofer et al. [25]: typically 100 mg quantities of starting material were used. Hydrolysis in methanolic KOH at 100°C in tightly sealed Kimax (Kimble) culture tubes for 20–22 h produced one major (slower running R_F value 0.08) spot, that was ninhydrin-positive on TLC plates eluted with 55:40:2:8 $CHCl_3/CH_3OH/NH_4OH/H_2O$ for GM_1 or 65:25:4 $CHCl_3/CH_3OH/H_2O$ for globoside. Following neutralization of excess KOH, the material was briefly dialysed and then dried in vacuo.

N-[2,2-$^2H_2$]Stearoyl GalNAc *N'*-[$^2H_3$]acetyl globoside and *N*-[2,2-$^2H_2$]stearoyl NANA *N'*-[$^2H_3$]acetyl GM_1 were synthesized following procedures previously described for corresponding spin-labelled derivatives [22]. However, replacement of the *N'*-acetyl group of GalNAc (in globoside) or of NANA (in GM_1) was with [$^2H_3$]$_2$acetic anhydride rather than [$^1H_3$]$_2$acetic anhydride, and stearic acid was added as the (α-deuterated) acid chloride. The latter step followed quantitatively the procedure below for GlcCer. Purification was on 'Bio Sil A' silicic acid columns eluting with a 20–60% gradient of CH_3OH in $CHCl_3$ for GM_1 and a 10–50% gradient for globoside.

N-[2,2-$^2H_2$]Stearoyl glucosyl ceramide (GlcCer) was synthesized from lyso-GlcCer (1-β-D-glucosyl sphingosine) by reacting 30 mg lyso-GlcCer with a mol excess of [2,2-$^2H_2$] stearic acid chloride in 1 ml THF with 1.5 ml of 50% aqueous sodium acetate for 2 h with stirring at room temperature [26]. Purificaton was on a 'Bio Sil A' silicic acid column eluting with a 5–15% gradient of CH_3OH in $CHCl_3$. Yield of deuterated GlcCer was 50% based on starting glycolipid.

GM_1 with deuterium in the acetyl group of NANA and bearing either stearic acid or [2,2-$^2H_2$]stearic acid was also synthesized by a second method [27]. Initial alkaline hydrolysis was the same as in the Neuenhofer et al. procedure. However, instead of the series of reactions prior to specific re-*N*-acylation with stearic acid, reacylation was performed directly in the presence of 1-(3-dimethylaminopropyl)-3-ethyl-carbodiimide hydrochloride (DEC). Subsequently, re-*N*-acetylation of the sialic acid was accomplished using acetic anhydride-d_6. For example, 150 ml aqueous solution of 4.5 mM [2,2-$^2H_2$]stearic acid and 5 mM DEC were stirred together for 2 h at 50°C followed by the addition of 80 mg lyso-GM_1, and further reacted for 5 h at 50°C – the mixture was dialysed against H_2O for 2 days at 4°C, then taken to dryness and re-dissolved in 9 ml 0.5 M $NaHCO_3$. 450 μl acetic anhydride-d_6 (approx. 70 mole excess) was added in 50 μl aliquots over 1 h at 20°C with vigorous stirring, and the mixture dialysed against H_2O at 4°C overnight. Two spots were visible on TLC – even after the acetic anhydride treatment was repeated, although Sonnino et al. [27] suggest that a spot running slightly slower than GM_1 may be due to non-re-acetylated material. In certain cases, to test that *O*-acyl groups had not been added during the reaction, following the dialysis step, 2–3 ml 6 N NaOH was added with stirring at 23°C for 5 h; and the material was re-dialysed. The final deuterium-labelled GM_1, regardless of labelling method, was further purified on an Iatrobeads column eluted with a linear gradient of methanol and water in $CHCl_3/CH_3OH/H_2O$ [28]. Identification of the deuterated GM_1 and globoside was by ^{1}H-NMR at 500 MHz, and by TLC co-migration with commercially available standards. ^{1}H-NMR of *N*-[2,2-$^2H_2$]stearoyl NANA *N'*-[$^2H_3$] acetyl GM_1 in DMSO/H_2O (98/2) with comparison to unmodified GM_1 and spectral assignment listed in reference 26 showed disappearance of the acetate methyl from the NANA residue only. The GalNAc

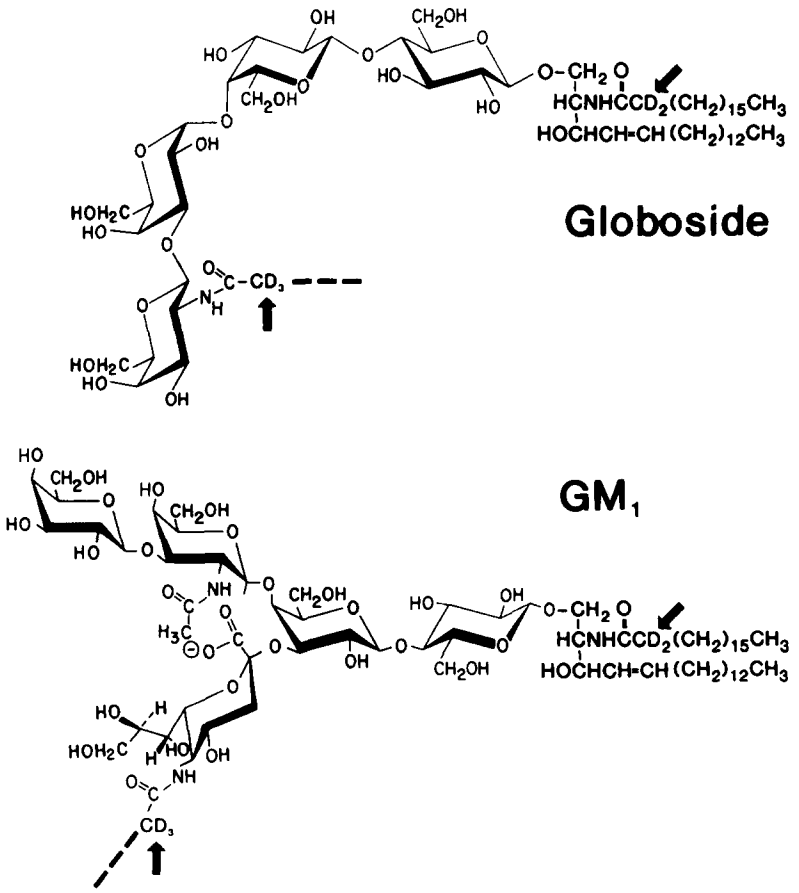

Fig. 1. Structures for GM_1 and globoside, with sites of deuteration indicated (arrows). Note particularly the deuteroacetate (-$COCD_3$) group on the NANA residue of GM_1 and on GalNAc of globoside. The dashed lines indicate pictorially the resultant CD bond vectors associated with C-CD_3. The other deuteration site illustrated is the α-carbon (C-2) of the fatty acid.

acetate methyl group was seen to be absent from the NMR spectrum of *N*-[2,2-$^2H_2$]stearoyl GalNAc *N'*-[$^2H_3$]acetyl globoside. Overall yield of deuterated GM_1 and globoside was 10–20% based upon starting glycolipid.

Preparation of fully hydrated lipid bilayer membranes for these experiments was as described in previous work [24]. In all cases initial sample hydration was with deuterium depleted water buffered at pH 7.4 with phosphate buffer, and subsequent hydration was with deuterium depleted water. Wideline ^{2}H-NMR spectra were acquired at 30.7 MHz on a 'home-built' instrument, or at 46 MHz on a Bruker MSL spectrometer, or at 76.7 MHz on a Varian Unity spectrometer with Doty 5 mm solenoid probe. Details of spectrometer setup and data acquisition and handling were similar to those described previously [24]. Molecular modelling of globoside and GM_1 was via molecular mechanics algorithms in BIOSYM (Biogen) and PCMODEL (Serena Software) and was based on crystallographic structures of GalCer, NANA, GalNAc, and β-lactose from the Cambridge Data Bank.

3. Results and discussion

The procedure described by Neuenhofer et al. for alkaline hydrolysis of fatty acyl linkages in glycosphingolipids, results in high yield de-*N*-acylation of the ceramide backbone [25]. It also results in simultaneous de-*N*-acetylation of the neuraminic acid residue on GM_1 (but not of the GalNAc residue) [25], and of the GalNAc residue on globoside [22]. Use of a two-phase water/diethyl ether system permits subsequent selective blocking of the sphingosine amino group formed [22,25], leaving the carbohydrate amino groups available for independent modification. The primary approach we have investigated for generating the deuterated complex glycolipids required for the present study took advantage of these features by reacetylating using acetic anhydride-d_6, followed by re-*N*-acylation of sphingosine with [2,2-$^2H_2$]stearic acid. This is fairly straightforward for globoside, which has only one amino sugar. Partly to confirm the validity of the approach in the more complicated case of GM_1, which has two *N*-acetylated sugars, the deuterated product was also arrived at *via* the method of Sonnino et al. [27]. Locations of deuterium nuclei are indicated in Fig. 1.

Molecules in fluid membranes tend to undergo symmetric rotation about axes perpendicular to the bilayer. In such situations, the following relationship is useful for relating deuterium spectral quadrupolar splittings, $\Delta\nu_Q$, to molecular orientation and motional characteristics:

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

where e^2Qq/h is the nuclear quadrupole coupling constant (170 kHz for an aliphatic C-D bond [29–31]), 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 bilayer normal. For (rapidly rotating) deuterated methyl groups, it is convenient to consider a 'resultant' C–D bond vector directed along the C–C bond attaching the methyl group: this can be dealt with in Eq. (1) by considering Θ_i to be the angle between the C–CD_3 vector and the molecular long axis, and introducing an additional factor of 1/3.

As a point of departure for the present work, globoside and GM_1 oligosaccharide chains were subjected to molecular modelling; and subsequently these structures were matched, via rotation about anomeric linkages, to structures recently derived by other workers using molecular dynamics calculations in concert with high resolution NMR constraints for GM_1 and globoside in solution ([15–18] and references therein). This permitted computer-assisted angle interpolation from structures thought to exist in the absence of membrane effects (Section 2). Independent motion of the rigid, planar, –$NHCOCH_3$ group about the C–N bond linking it to sugar rings is highly restricted [15,18,32]. This should result in the wideline ^{2}H-NMR spectral features of the –$NHCOCD_3$ groups attached to the NANA residue of GM_1 and the GalNAc residue of globoside being dominated by orientation of the carbohydrate residue in question, and by motional properties of the oligosaccharide chain (Fig. 1). In the present work we have used the approximation that the dihedral angle defined by H_2C_2NH in GalNAc and by H_5C_5NH in NANA is centred about 180°. In actuality, there is expected to be some libration of the rigid, planar acetamido group about this orientation. In the case of the GalNAc residue in globoside, solution studies indicate that low energy conformers corresponding to −140° and +120° should be primarily populated (60 : 40 ratio) [18]. In the case of the GalNAc residue in GM_1, similar solution considerations indicated that wobble of ±30° about the 180° conformer occurs, and the H_2C_2NH angle was held at 180° by Acquotti et al. for purposes of calculation [15]. The same authors considered that a torsional angle of 150° was consistent with their data for the acetamide group of NANA. Note, however, that, within the planar acetamido group, the C-2–N bond is directed parallel to the C–CD_3 bond; so that conservative libration about C-2–N does not sensitively alter the orientation of the C–CD_3 vector relative to the sugar ring. Moreover, the effect of rapid rotation parallel to this axis (i.e., about the C–CD_3 bond) is already explicit in the calculation used when a factor of 1/3 is introduced to Eq. (1) as described above.

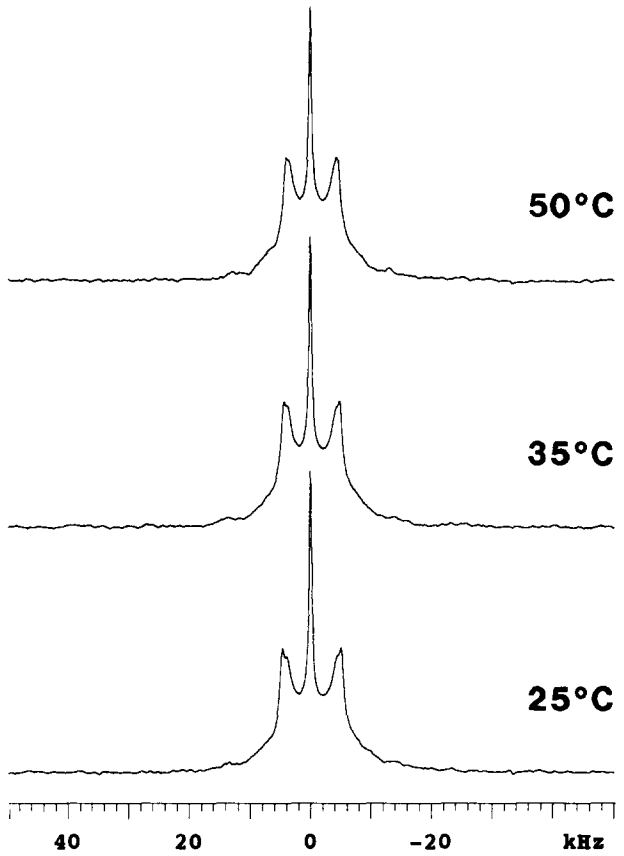

Fig. 2. Illustrative ^{2}H-NMR spectra for *N*-[2,2-$^2H_2$]stearoyl NANA *N'*-[$^2H_3$]acetyl GM_1 at 7 mol% in POPC/cholesterol bilayers. Lipid samples were dispersed as unsonicated bilayers in pH 7.4 phosphate buffer. The Pake doublet associated with C-CD_3 in the NANA acetamide function (intense doublet) is seen spanning the sharp central peak. Spectra displayed were run on the Varian Unity 500 instrument with $\pi/2$ pulse length of 5.5 μs at 300 watts transmitter power, using the 'SSECHO' pulse sequence (quadrupolar echo sequence with phase cycling). Each spectrum represents 12000 accumulated transients. Recycle time was 200 ms.

Fig. 2 illustrates the spectral features found for deuterated GM_1 dispersed as a minor component in semi-fluid POPC/cholesterol membranes (POPC/cholesterol/GM_1 mol ratio 63:30:7) at temperatures that include the physiological range. Fig. 3 presents analogous spectra for globoside in POPC/cholesterol (POPC/cholesterol/globoside mol ratio 62:31:6.3). The spectra shown are closely representative of spectra seen for all GM_1 and globoside samples described in this work. They are indicative of rapid axially symmetric motion for the deuterated glycolipids. Each is characterized by a predominant single Pake doublet. The additional central sharp peak is a well known spectral feature of deuterated lipids in membranes, likely representing contributions from small vesicles, highly curved membrane regions, and also any residual HOD. It is particularly intense for the GM_1 samples – comprising 15% of the integrated intensity. We have observed the GM_1 vesicles to have a greater tendency to disperse, perhaps due to their negative charge. Any population of deuterated glycolipid undergoing rapid random motion on the NMR timescale, but in slow exchange with the pool of ordered GSL, would also give rise to a sharp central peak. Given the long NMR timescale (10^{-3}–10^{-5} s), the latter possibility seems likely only if resulting from a subpopulation of highly curved vesicles. An apparently closely related phenomenon has been well described for phosphatidylcholine with deuterated choline methyl groups in fluid bilayers by Curatolo and Neuringer [33]. Measurement of the intense doublet gives the quadrupole splitting to be used in Eq. (1) in dealing with the -$COCD_3$ group – on the NANA acetamide in the case of GM_1 and on the GalNAc acetamide in the case of globoside (Fig. 1). These splittings are listed in Table 1, along with corresponding values for the same glycolipids in POPC multibilayer membranes without cholesterol. It will be seen that all the values fall within a very narrow range about 10 kHz for GM_1 and about 1 kHz for globoside.

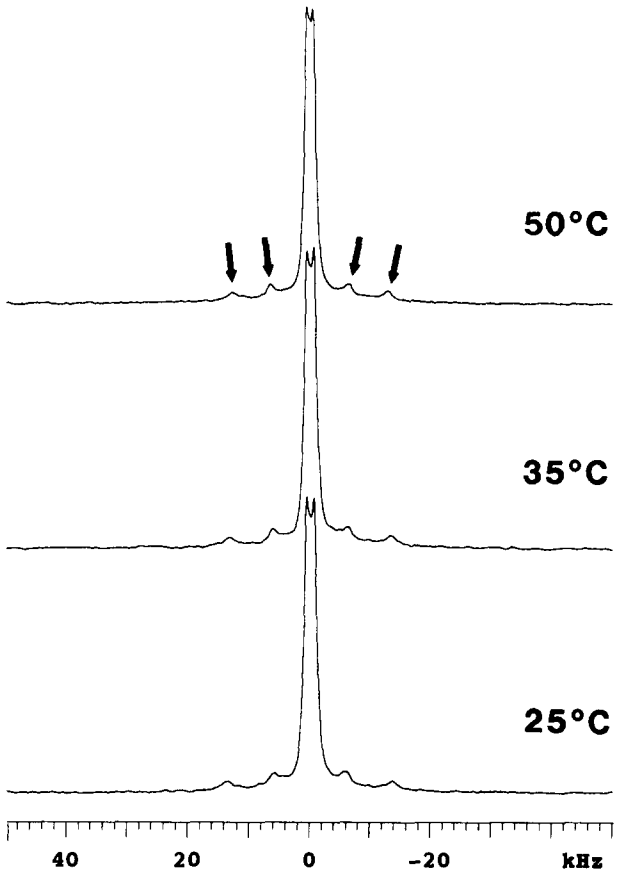

Fig. 3. Illustrative ^{2}H-NMR spectra for *N*-[2,2-$^2H_2$]stearoyl GalNAc *N'*-[$^2H_3$]acetyl globoside at 6.3 mol% in POPC/cholesterol bilayers. Lipid samples were dispersed as unsonicated bilayers in pH 7.4 phosphate buffer. The intense narrow Pake doublet is associated with C-CD_3 in the GalNAc acetamide function of globoside. Minor Pake doublets are associated with the non-equivalent deuterium sites at C-2 of the fatty acid (arrows and Fig. 4). NMR instrument parameters were as in Fig. 2, with 8000 accumulated transients.

Table 1
^{2}H-NMR quadrupolar splittings ($\Delta\nu_Q$) for *N*-[2,2-$^2H_2$]stearoyl NANA *N'*-[$^2H_3$]acetyl GM_1, *N*-[2,2-$^2H_2$]stearoyl GalNAc *N'*-[$^2H_3$]acetyl globoside and *N*-[2,2-$^2H_2$]stearoyl GlcCer, dispersed at 6–10 mol% in unsonicated fluid bilayers

Glycosphingolipid and deuterium location	T (°C)	*N*-Acetyl splitting (±0.5–1 kHz)		α-$^2H_2$ splitting (±1 kHz)			
		POPC	POPC/Chol	POPC		POPC/Chol	
Globoside:	25	1.6	1.6	15.1	24.9	12.8	28.4
N-acetate	35	~1	1.6	15.0	23.8	13.2	27.7
α-$^2H_2$	50	<1	1.4	14.7	22.2	13.6	26.5
GM_1:	25	10.5	10	16.7	25.7	15.4	29.0
N-acetate	25 *	10.5	–	16.5	26.4	–	–
α-$^2H_2$	35	9.7	9.7	16.2	25.2	15.3	28.7
	35 *	9.7	–	16.0	24.5	–	–
	50	9.4	9.2	16.7	24.7	14.5	26.7
	50 *	9.3	–	15.7	23.8	–	–
GlcCer:	35	–	–	17.5	25.2	16.7	29.6
α-$^2H_2$	50	–	–	17.0	23.6	16.8	27.9

Data are included for GSLs in host matrices of pure 1-palmitoyl-2-oleoyl phosphatidylcholine (POPC) and for POPC containing 30–31 mol% cholesterol. All samples were suspended in 10 mM phosphate-buffered saline (pH 7.4). Several sites of deuteration are represented (see text and Fig. 1): the acetate (-$COCD_3$) of NANA on GM_1 and of GalNAc on globoside and C-2 of the fatty acid in each case.
* Refers to GM_1 made via the route of Neuenhofer et al. [25].

Other features include low intensity peaks associated with the α-deuterons at C-2 of the fatty acid (vida infra).

It is interesting that the spectral splittings associated with the deuteroacetate peaks of a given complex oligosaccharide chain in POPC (at 10 mol%) vs. POPC/cholesterol membranes are so similar, given the very different fluidity of the matrices involved. POPC is a highly fluid phospholipid, having a main transition temperature of −3°C [34], and resultant high disorder of the lipid chains at the temperatures investigated. High cholesterol concentration on the other hand is known to greatly restrict the conformational flexibility of a fluid phospholipid matrix – to the extent that the individual lipids rotate as rigid units and membrane thickness is altered [35–38]. The result seen here can be understood by considering that the effect of cholesterol on whole-body (rigid body) motions of lipids in fluid membranes is markedly less than its effect on *trans / gauche* isomerisation of the acyl chains ('fluidity'): values of $S_{mol} = 0.6–0.7$ having been found for rigid body order parameters in cholesterol-free membranes and 0.8–1.0 for the same membranes with > 25% cholesterol [4,29,39]. Presumably the observation of relative headgroup insensitivity to the presence of cholesterol, reflects lack of oligosaccharide sensitivity to conformation and length of the sphingosine and fatty acid chains.

Analysis of the measured quadrupolar splittings in Figs. 2 and 3, and Table 1, for consideration of their implications to oligosaccharide orientation and dynamics at the membrane surface, was undertaken as follows using Eq. (1). Skarjune and Oldfield found a value of 0.32 for S_{mol} of the glucose residue of the monoglycosyl GSL, GlcCer, at 17 mol% in fluid DPPC at 90°C [2]. Although DPPC was some 50C° above its main transition at this temperature, very similar values of S_{mol} were also found by the same authors for pure GlcCer whose transition temperature is 83°C. Somewhat higher values have been recorded for sugars on glycerolipids: up to 0.51–0.53 for S_{mol} of the Glc and Gal residues of lactosyl diglyceride [3–6]. Hence, as a first approximation, $S_{mol} = 0.32$ was used in Eq. (1). In arriving at estimated values of Θ_i and corresponding predicted values of $\Delta\nu_Q$ for comparison with experimental quadrupole splittings, existing solution structures were examined. Previous workers have considered the conformation of the oligosaccharide chains attached to GM_1 and globoside in dimethyl sulphoxide (detergent micelles have also been studied in the case of globoside [18]) ([15–18] and references therein). In the studies referenced, spatial relationships established by two-dimensional high resolution NMR were superimposed as constraints in an energy minimization process using molecular mechanics algorithms. The results were interpreted as indicating a well defined average spatial geometry for the carbohydrate portion of GM_1 as an extended chain [15,16]. For globoside, it was suggested that the headgroup exists in a conformation having a distinctive 'L'-shape with greater potential for internal mobility ([17,18] and references therein). Our approach to molecular modelling in these systems was to mimic the most likely predicted structures delineated by the above workers (particularly reference [18] for globoside and references [15,16] for GM_1), and to interpolate from these the spatial orientation of the acetamide $C–CD_3$ bonds (in the absence of a membrane surface) (Fig. 1).

Very little is known about oligosaccharide chain interaction with membrane surfaces [8,40]. In determining 'predicted' headgroup orientation of the complex species studied here, account was taken of the fact that, to date, all NMR data on glycolipids having β-linked sugar headgroups in fluid membranes have shown extension of the carbohydrate portions away from the membrane surface (i.e., more or less parallel to the molecular long axis). Data exist for several monoglycosyl glycerolipids [3–5], for one monoglycosyl GSL (GlcCer [2]), and for one diglycosyl glycerolipid (lactosyl diglyceride [6]). No doubt a contributory factor in this extended orientation is steric surface constraint imposed at the bilayer surface upon the rapidly rotating elongated molecules involved. Thus it seems likely that the first two residues in the carbohydrate

chains of GM_1 and globoside (which are Galβ1 → 4Glc in β linkage to the membrane-inserted portion, as in lactosyl diglyceride) also extend away from the membrane surface. Furthermore, since GlcCer (the only GSL carbohydrate structure to have been orientationally determined in membranes) is the primary structural building block of both GM_1 and globoside, it was possible to orient their solution structures such that the Glcβ1 → 1cer structure common to all three lipids, matched the orientation determined by Skarjune and Oldfield [2]. The resultant C–CD_3 vector orientation could then be interpolated relative to the director axis (the membrane perpendicular), and values of Θ_i directly derived. The result of this approach for GM_1 is $\Theta_i = 26°$, giving a predicted spectral splitting, $\Delta\nu_Q = 9.7$ kHz. A range of $\pm 5°$ about this angle, as an estimate of the uncertainty involved in such a calculation, would produce values of $\Delta\nu_Q$ between 8.2 and 11 kHz. This range in fact covers the observed values (Table 1). The same calculation for globoside results in $\Theta_i = 9°$, giving a predicted spectral splitting, $\Delta\nu_Q = 13.1$ kHz. A $\pm 5°$ uncertainty about this angle would produce values of $\Delta\nu_Q$ between 12.4 and 13.4 kHz. In contrast to the result for GM_1, this range is far from the observed spectral splittings. Values of $\Theta_i = 0°$, 54.7°, and 90° would be predicted to produce splittings of 13.6, 0, and −6.8 kHz, respectively.

In addressing the molecular basis of GSL receptor function, it is important to consider how the above values may relate to orientation and order of the hydrophobic portions of the glycolipids involved. For this purpose, certain of the glycolipids used in this study were also deuterated at C-2 of the (18-carbon saturated) fatty acid chain. This double labelling permitted correlation of backbone and headgroup sites in the molecule to help localize the level of conformational and dynamic differences, and effects induced by sample conditions. C-2 is a backbone location having restricted internal motion, and is sensitive to orientation of the membrane-inserted portion [9,24]. Definitive assignment of the double labelled globoside and GM_1 spectra was made possible by comparison with the spectrum of *N*-[2,2-$^2H_2$]stearoyl GlcCer (Fig. 4), and by separate selective deuteration of GM_1 and globoside (spectra not shown). It is readily seen that, as indicated with regard to Figs. 2 and 3, the fatty acid α-deuterons are responsible for small spectral features lateral to the intense peaks associated with the deuterated acetate peaks (arrows). ^{2}H-NMR spectral data for the fatty acid deuterons associated with GlcCer, globoside, and GM_1, at low concentration in POPC bilayers with and without cholesterol, are presented for 25°C, 35°C and 50°C in Table 1. Given the uncertainty of up to ± 1 kHz in measurement of these less intense features, the values for labels at the fatty acid C-2 position must be considered within experimental error of one another for a given temperature and membrane composition. Thus they permit confident localization of the spectral effects discussed above to the oligosaccharide portion. Note that, while cholesterol addition had remarkably little effect on the oligosaccharide spectra (and therefore on oligosaccharide arrangement and behaviour), it had a distinct effect on the GSL backbone as reflected in splitting differences between the matrices with and without cholesterol. By implication then, significant changes in the membrane-inserted portion of GSLs can have little impact on oligosaccharide orientation and independent dynamics, as we have indicated in past based upon studies of spin labelled glycolipids and glycoproteins in a variety of membranes [41–43], and upon ^{2}H-NMR studies of a simple glycolipid in pure POPC [44]. This has important implications for proposed mechanisms of GSL crypticity, since GSL receptor function for macromolecules including globoside has been strongly tied to GSL fatty acid characteristics and membrane cholesterol content [12,22,45–49].

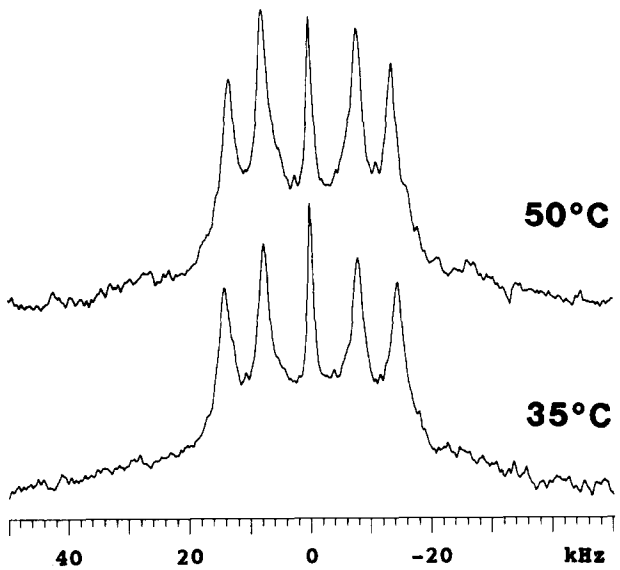

Fig. 4. Illustrative ^{2}H-NMR spectra for *N*-[2,2-$^2H_2$]stearoyl GlcCer at 6.6 mol% in POPC/cholesterol bilayers. Lipid samples were dispersed as unsonicated bilayers in pH 7.4 phosphate buffer. Two Pake doublets are evident – associated with the non-equivalent deuterium locations at C-2 of the fatty acid. Their positions correlate with those of the minor spectral peaks in Figs. 2 and 3. NMR instrument parameters were as in Fig. 2, with 100000 accumulated transients and 100 ms recycle time.

The value of S_{mol} (0.32) taken from the literature and applied to the deuterated carbohydrate residues in this work was based upon the value reported for the single carbohydrate residue of GlcCer (the common backbone of GM_1 and globoside as well as many other complex GSLs) [2]. This is tantamount to making the

approximation that there is zero independent wobble about sugar-sugar anomeric linkages (i.e., that the orientational order of each sugar residue is the same within the complex GSL). It is relevant to this approximation that S_{mol} has been found to be the same for each sugar in the disaccharide headgroup of lactosyl diglyceride in fluid membranes [6], and that NMR relaxation times have been reported to be roughly the same for all sugar residues of GM_1 in micelles [50]. Such an approximation might be considered more likely to be accurate for the NANA residue of GM_1, which is at a branch point in the oligosaccharide chain and closer to the membrane origin, than for the terminal GalNAc of globoside. Indeed, the high resolution studies referenced for GM_1 and globoside in solution suggested that there is greater conformational freedom in globoside [15–18]. If there is significant libration about sugar-sugar anomeric linkages in the oligosaccharide chain, over and above the wobble associated with the glucose residue attached directly to ceramide, the actual splitting observed would be expected to be smaller than the calculated value. Thus, in the case of GM_1, the range of quadrupole coupling values predicted based upon solution studies, is highly consistent with the 8.8–10.5 kHz range of splittings observed in the sample studied, provided that S_{mol} is similar for the deuterated NANA residue and the Glc residue in the chain. Scenarios consistent with the observed splitting for globoside would be $C–CD_3$ vector orientations in the range of 50° or 59° (as opposed to 9°), provided there is little independent wobble of the GalNAc residue relative to Glc in the chain. Alternatively, if one considers that the $C–CD_3$ orientation in globoside is in fact as predicted from the solution structures (9°), the spectral splitting could be explained by assuming a low order parameter, S_{mol}, of about 0.04 (9-times smaller than that for the Glc residue). Thus one must presume that either the oligosaccharide orientation in the case of globoside is very different from the solution case ($C–CD_3$ orientation approaching the magic angle of 54.7°), and/or that the independent wobble of the terminal portion of the globoside oligosaccharide is virtually isotropic. Indeed, as indicated above, there was evidence from the solution NMR studies of globoside that the oligosaccharide chain may present greater potential for conformational mobility than was seen for GM_1 [17,18]. However, spectra of globoside in which the deuterium label was in the exocyclic hydroxymethyl group of the same GalNAc residue deuterated in the present work (Singh, D., unpublished observation), have demonstrated splittings of comparable magnitude to those found for the monoglycosyl GSLs, GalCer and GlcCer, deuterated in the hydroxymethyl function [2,9,10]. Hence, it seems unlikely that the much smaller quadrupole splittings seen here for globoside than predicted, can be attributed solely to carbohydrate disorder. It seems possible that maintenance of an acute 'L'-shape at the spatially-constrained membrane surface is sterically difficult for an oligosaccharide chain rotating rapidly about the membrane perpendicular. It is also interesting to speculate that greater oligosaccharide flexibility in globoside vs. GM_1 might provide for greater susceptibility to conformation modification by the membrane environment.

4. Conclusions

The ideal method of investigating oligosaccharide orientation and dynamics at a membrane surface would be to have multiple deuteration at known sites on all the sugar residues. This would permit independent conformational and dynamic information to be obtained for each residue. Technically such labelling is extremely difficult to achieve for any but the simplest systems, and unlikely to be realised as a general method. Moreover, multiple sites of deuteration result in peak overlap and difficulties of assignment in wide-line techniques. However, in many cases it should be possible to make realistic assumptions in order to reduce a given problem to a manageable level of complexity, and to focus on restricted regions. The selective methyl deuteration used in the present work made it possible to investigate in situ the conformation and behaviour of two complex glycolipids containing *N*-acetylated sugar residues. The technique provided relatively good signal-to-noise and employed a chemical group whose motion is effectively constrained to reflect that of the sugar ring involved, and whose spectra could in principle be simply related to sugar orientation and dynamics. Each complex glycolipid showed evidence of significant average geometry preservation in the oligosaccharide chain. Significant changes in this geometry could be very sensitively tested for – in this case as a function of conditions of temperature and membrane fluidity – but were not seen. With regard to mechanisms of crypticity of complex GSLs, this argues against mechanisms that invoke reorientation of complex oligosaccharide chains based on changes within a fluid bilayer. Ceramide backbone conformation at the level of the fatty acid linkage was shown to be importantly similar for both complex species and for their common building block, GlcCer, under a given set of sample conditions – but was distinctly altered by changes in these conditions. It seems very likely that the GM_1 headgroup geometry recorded previously in solution is preserved at the membrane surface, at least at the level of the NANA residue. Spectral features of the terminal GalNAc residue on globoside were consistent with greater independent motion, and likely a different orientation than in solution.

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6. References

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