Oligosaccharide Behavior of Complex Natural Glycosphingolipids in Multicomponent Model Membranes

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ABSTRACT: Wideline ^2H NMR of model membranes was used to consider the molecular consequences of factors often suggested as modulators of complex glycosphingolipid oligosaccharide arrangement and motional characteristics at cell surfaces. GM_1, asialo-GM_1, and globoside were studied as examples of plasma membrane recognition sites. The experimental approach involved substitution of deuterons (D) for protons at specific locations within the carbohydrate chains. Deuterated glycolipids were then dispersed at 7−10 mol % in unsonicated bilayers of 1-palmitoyl-2-oleoylphosphatidylcholine. Factors tested for their significance to carbohydrate chain conformation and dynamics included glycolipid natural alkyl and acyl chain variability, membrane fluidity, and the presence of cholesterol and a charged sugar residue (neuraminic acid). Effects of Ca^{2+} and membrane-associated protein were briefly considered. Two distinct strategies were employed in substituting deuterons for selected protons of carbohydrate residues. Neither approach necessitated alteration of the glycolipid natural fatty acid composition. (i) Protons of the exocyclic hydroxymethyl group on the terminal Gal residue of GM_1 and asialo-GM_1, and on the terminal *N*-acetylgalactosamine (GalNAc) residue of globoside, were replaced with deuterium (producing -CDHOH) by an enzymatic oxidation/reduction cycle. This represents the first application of such an approach to deuteration of complex neutral glycolipids. Spectral results were compared to those obtained for the similarly-deuterated monoglycosyl lipid, galactosylceramide (GalCer), with natural fatty acid composition. Efficacy of this labeling method may in principle be influenced by structural variations within a given glycolipid family. Also, asymmetric rotation of the deuterated group made it less attractive than the second method for relating spectral features to receptor geometry. (ii) A general synthetic, nonenzymatic method was investigated for replacing amino sugar *N*-acetyl groups with deuterated acetate (-$COCD_3$). The acetate group of the GalNAc residue of globoside, GM_1, and asialo-GM_1, as well as that on neuraminic acid in GM_1, was replaced with -$COCD_3$. This second method afforded better signal-to-noise—an important consideration for ^2H NMR. The NMR technique employed had the potential for detecting changes of as little as 10% in oligosaccharide orientation or motional order. Each glycolipid demonstrated clear evidence of preferred average oligosaccharide conformations in all (fluid) membrane environments examined. The most striking observation was that, in fluid matrices, conformation and motional order of the complex oligosaccharide chains were only modestly influenced by factors tested, including natural variation in the glycolipid hydrocarbon chains, membrane fluidity, temperature, and the presence of cholesterol or the *N*-acetylneuraminic acid (NeuAc) residue on GM_1. Thus the degree of conformational "information transfer" was minimal within a given complex glycolipid in fluid membranes. High concentrations of Ca^{2+} produced spectral changes in neutral as well as charged species, which may reflect generalized ionic interactions with the membrane. These results lend little support to suggestions that alterations in the hydrophobic segment of glycolipids in fluid membranes can induce sufficient change in headgroup conformation or degree of extension beyond the membrane surface to provide mechanisms for recognition site crypticity.

It is clear that certain fundamental mechanisms of cell growth control and metabolic regulation involve highly structured pathways of information transfer, triggered by specific contact events at the plasma membrane outer surface. The molecular mechanisms of these pathways remain the subject of speculation. One important associated concept is that conformation and behavior of recognition sites may be closely tied to inter- and intramolecular interactions: thus information transfer could potentially be initiated or modulated by subtle local alterations at or near the membrane surface. We have been attempting to devise experiments which test possible mechanisms of information transfer and its control by molecules in the membrane environment. Wideline ^2H NMR spectroscopy appears particularly appropriate to such studies, since it permits direct examination of membrane systems without the need to work in solution (Seelig, 1977; Davis, 1983; Smith, 1984). The approach is nonperturbing and is sensitive to details of molecular orientation and behavior. We describe here a group of experiments aimed at determining the extent to which receptor conformation and dynamics are linked to certain common structural variables within natural glycosphingolip-

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ids (GSLs)[1] as examples of complex oligosaccharide-bearing recognition sites. The monounsaturated phospholipid 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) provided a liposome-forming species widely representative of cell plasma membrane phospholipids, in which probe-labeled GSLs could be dispersed at the low concentrations common to cell membranes.

The application of wideline ^2H NMR to studies of membrane surface carbohydrate was first demonstrated by Eric Oldfield and colleagues for monoglycosyl glycosphingolipids (Skarjune & Oldfield, 1979, 1982); and it has been more recently applied to several monoglycosyl glycerolipids (Jarrell et al., 1986, 1987a,b) and one diglycosyl glycerolipid (Renou et al., 1989). Highly detailed information regarding membrane factors that may influence carbohydrate orientation and behavior is clearly available through application of this and other NMR techniques to simple glycolipids (Winsborrow et al., 1992; Aubin & Prestegard, 1993). However, the selective introduction of deuterium nuclei into larger biological molecules can be challenging. In the present work two synthetic approaches having very different characteristics were tested and applied to carbohydrate chains in a range of GSLs.

GM_1 is an important acidic glycolipid of neural tissue (Hakomori, 1981, Hakomori, 1986; Curatolo, 1987). It has a chain of four β-linked neutral carbohydrate residues, with a single *N*-acetylneuraminic acid (NeuAc) branch at the internal Gal residue. Hydrolytic removal of the NeuAc residue subsequent to deuteration offered a mechanism for testing the influence of this ((−)-charged) group. GM_1 from beef brain has homogeneous fatty acid composition, but is comprised of species with 18- and 20-carbon sphingosine backbones (Corti & Degiorgio, 1980; Sonnino et al., 1985). Thus, it served as a test for effects of alkyl chain length.

Globoside from erythrocytes provided an unbranched neutral tetraglycosyl glycosphingolipid (GSL) that has been the subject of considerable interest with regard to possible mechanisms of recognition site modulation in plasma membranes. Globoside in erythrocytes was the first example specifically described of membrane recognition site "cryptic ity" (Koscielak et al., 1968)—the phenomenon whereby cell surface receptors can be present at a cell surface, but inaccessible to specific macromolecular ligands (Hakomori, 1981, 1986; Lampio et al., 1986; Curatolo, 1987). Among the mechanisms entertained for altering GSL "recognition" by specific macromolecules, particular attention has focused on the role of the (single) GSL fatty acid, and the lipid nature of the host membrane itself. While the phospholipids of cell membranes tend to be composed of species with (nonhydroxylated) fatty acids having chain lengths of predominantly 16 or 18 carbons, GSLs commonly possess fatty acids that are up to 26 carbons in length and in which hydroxylation at C_2 is frequent (Hakomori, 1981; Thompson & Tillack, 1985; Curatolo, 1987). Examples which have been systematically examined in bilayer model systems are the simple GSLs, GalCer (Alving et al., 1980), and, more recently, sulfated GalCer (Crook et al., 1986; Stewart & Boggs, 1993a,b). A triglycosyl globo-series GSL from kidney has been clearly demonstrated to exhibit recognition site modulation by structural alterations not directly involving the carbohydrate portion when probed in hydrated lipid films on solid supports (Kiarash et al., 1994). In the present work, ^2H NMR of asialo-GM_1, globoside, and GalCer offered insight into the effect of a wide range of hydrophobic domain alterations on neutral GSL carbohydrate at the surface of fluid membranes.

MATERIALS AND METHODS

DMPC (dimyristoyl-PC) and POPC were obtained from Avanti Polar Lipids, Birmingham, AL, and used without further purification. Cholesterol was from Sigma. GM_1 was the kind gift of Fidia Pharmaceuticals, Italy. Globoside was isolated from pig's blood as previously described (Mehlhorn et al., 1988).

The -$COCD_3$ derivatives of complex glycolipids were made by partial synthesis, the natural fatty acid composition being preserved, following a method outlined by Higashi and Basu for incorporating ^{14}C radiotracers into amino sugars (Higashi & Basu, 1982). Briefly, the respective dry glycolipids were dissolved in anhydrous hydrazine, sealed in glass ampules, and heated at 105 °C for 8 h. Degree of hydrazinolysis was followed by silicic acid thin layer chromatography (TLC), eluting with 55:45:10 $CHCl_3/CH_3OH/0.2\%$ $CaCl_2$. Hydrazinolysates were dried down and left under vacuum for 6 h. They were subsequently dissolved in methanol with warming and the addition of benzene and reacetylated using acetic anhydride-d_6 (MSD Isotopes) in benzene. This material was purified on an Iatrobead (Iatron Laboratories Inc.) column eluted with linear gradients of methanol and water in chloroform/methanol/water (Momoi et al., 1976) and identified by TLC. GM_1 has two possible sites for acetate replacement: in the NeuAc residue, and in the GalNAc residue. The degree of acetate substitution was found to differ between the two positions by analysis with proton NMR, following the assignment outlined by Koerner et al. (1983). NeuAc and GalNAc residues were approximately 60% and 28% deuterated, respectively.

Asialo-GM_1 was generated by hydrolytic removal of the NeuAc residue from acetate-deuterated GM_1 using formic acid (Mansson, (1973). The reaction mixture was subsequently dialyzed against distilled water at 4 °C with frequent changes of the water. Dialyzed material was lyophilized and purified on a silicic acid column eluted with a $CH_3OH/CHCl_3$ gradient. The product was identified by TLC.

Deuterium labeling in the exocyclic hydroxymethyl group of GSL terminal Gal or GalNAc residues was performed using galactose oxidase and $NaBD_4$ as previously described (Jarrell et al., 1992). It typically produced a yield of 75−85%.

Lipid samples were multilamellar vesicles (MLV) prepared with vortexing above the host transition temperature. Hydration was with buffer containing 10 mM phosphate, pH 7.4, or 5 mM Hepes, pH 7.4, with added salts and chelating agents described in the text and table footnotes. Following repeated lyophilization from deuterium-depleted water, samples were rehydrated with deuterium-depleted water and freeze-thawed repeatedly. Binary lipid mixtures contained 10 mol

[1] Abbreviations: GSL, glycosphingolipid; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-PC; DMPC, dimyristoyl-PC; GalCer, Galβ1→1ceramide; GalNAc, *N*-acetylgalactosamine; NeuAc, *N*-acetylneuraminic acid; GM_1, Galβ1→3GalNAcβ1→4Gal(3←2αNeuAc)-β1→4Glcβ1→1ceramide; globoside, GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1ceramide; asialo-GM_1, Galβ1→3GalNAcβ1→4Galβ1→4Glcβ1→1ceramide.

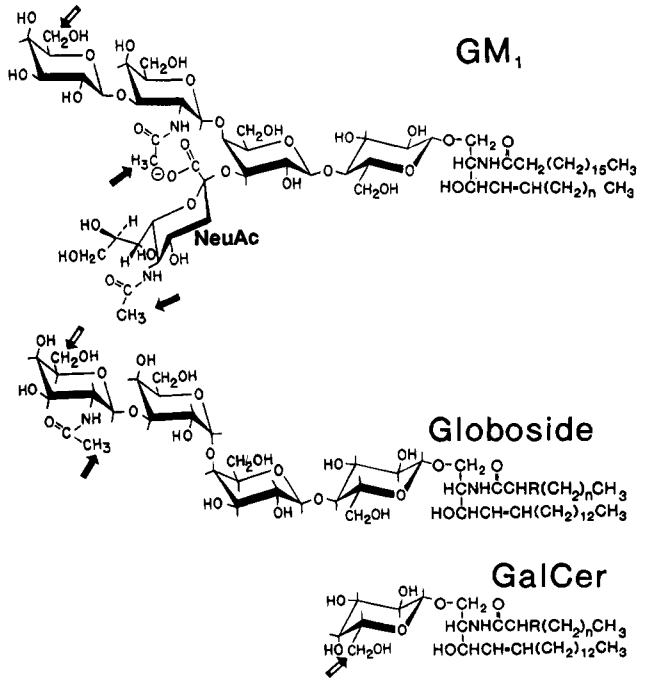

FIGURE 1: Structures for the glycosphingolipids GM_1, globoside, and galactocerebroside (GalCer): arrows indicate deuteron locations. Deuteration of exocyclic hydroxymethyl groups (open arrows) involved the terminal Gal of GM_1, asialo-GM_1, and GalCer, and the terminal GalNAc of globoside. Deuteration of the acetate group (filled arrows) involved NeuAc and GalNAc residues of GM_1, or GalNAc of globoside and asialo-GM_1. Hydrolytic removal of NeuAc from GM_1 yielded the asialo species studied. Natural variability in the hydrophobic portions of the molecules is indicated: the sphingosine chain for GM_1 (and asialo-GM_1) is a mixture of C18 and C20 species (n = 12 or 14), and the fatty acids for globoside and GalCer are a diverse mixture (R = H or OH, and n = 15−23).

% glycolipid, while ternary mixtures contained 7.7 mol % labeled lipid and 22.9 mol % cholesterol. The amount of labeled lipid used in each sample varied from 5 to 16 μM. Protein incorporation is described in the footnotes to Table 1. Sample volumes were 400−700 μL. Details of NMR instrumentation and data handling have been described elsewhere (Singh et al., 1992a; Barber et al., 1994). Spectra were acquired from high to low temperature, after preequilibration in the spectrometer well above the host matrix gel−fluid transition temperature.

RESULTS

Structures of the deuterated glycosphingolipids (GSLs) studied in the present work are illustrated in Figure 1. In one group of samples, deuterons were incorporated into the exocyclic hydroxymethyl groups of terminal sugars, yielding -CDHOH on the terminal GalNAc residue of globoside, or on the terminal Gal residue of GM_1, asialo-GM_1, and GalCer. In the other, *N*-acetate groups of all amino sugars were replaced with -$COCD_3$: this involved the globoside terminal GalNAc residue, the nonterminal GalNAc of GM_1 and asialo-GM_1, and the NeuAc residue of GM_1.

For molecules undergoing rapid symmetric rotation about axes perpendicular to the plane of the bilayer, the following equation describes the relationship between ^{2}H NMR powder spectra 90° edge splittings ($\Delta\nu_Q$), and molecular orientation

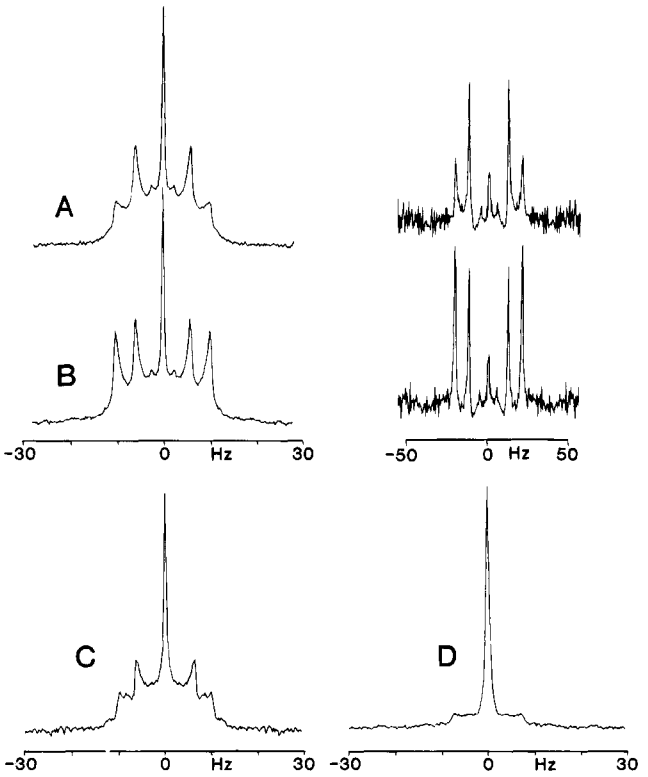

FIGURE 2: Selected ^{2}H NMR powder spectra for glycolipids deuterated in sugar exocyclic hydroxymethyl groups to give -CDHOH: [*d*Gal]GM_1 (A, B), [*d*Gal]asialo-GM_1 (C), and [*d*GalNAc]globoside (D). The GM_1 in (B) was subjected to a second round of oxidation and reduction such that both protons of -CH_2-OH were replaced with deuterium. DePaked spectra are included to the right of (A) and (B). In each case the glycolipid was dispersed at 10 mol % in unsonicated POPC bilayers at 40 °C. Buffer details are given in the table footnotes. Typically, 140 000−200 000 transients were averaged.

and motional order:

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

where e^2Qq/h is the nuclear quadrupole coupling constant (170 kHz for an aliphatic C−D bond) (Seelig, 1977; Davis, 1983; Smith, 1984), 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.

General Features Observed in Spectra of GSLs Deuterated in Specific Sugar Exocyclic Hydroxymethyl Groups. Figure 2 presents selected ^{2}H NMR powder and dePaked spectra for [*d*Gal]GM_1 (Figure 2A,B) and powder spectra for [*d*Gal]-asialo-GM_1 (Figure 2C) and [*d*GalNAc]globoside (Figure 2D) at 40 °C. DePakeing selects an oriented component from a powder spectrum, which can simplify measurements. In each case the glycolipids contain deuterium as a -CDHOH group on the fourth (terminal) carbohydrate residue from the membrane surface. At this temperature the basic feature of each powder spectrum is a pair of Pake doublets. A comprehensive set of measured quadrupole splittings ($\Delta\nu_Q$), and where appropriate, outer/inner splitting ratios corresponding to these and related samples studied, are listed in Table 1. The splittings are comparable in magnitude to those observed for similarly labeled Gal residues in the mono- and diglycosyl glycolipids that have been studied

Table 1: ^{2}H NMR Spectral Data for GSL Sugars Deuterated in Hydroxymethyl Groups[a]

-CDHOH labeled GSL and host matrix	temp (°C)	outer, inner quadrupole splittings (±0.5−1 kHz)	outer/inner ratio (±0.05−0.1)
GM_1 in POPC[d] (values in parentheses are with cholesterol)	40	21.0, 12.4	1.7
		21.0, 12.4[g]	1.7
		(21.3, 12.2)	(1.7)
	65	17.6, 11.4	1.5
		17.6, 11.5[g]	1.5
		(17.1, 10.8)	(1.6)
GM_1 in POPC[f], 20 mM Ca^{2+}	40	21.5, 11.9	1.8
	65	19.1, 11.5	1.7
GM_1[c] in POPC/cholesterol[d] with glycophorin	40	21.0, 11.8[g]	1.8
GM_1[h] in POPC[e] with cerebroside SO_4	40	22.5, 12.9	1.7
asialo-GM_1 in POPC[d] (values in parentheses are with cholesterol)	40	20.5, 13.2	1.6
		(19.9, 12.3)	(1.6)
	65	16.6, 11.4	1.4
		(16.1, 10.8)	(1.5)
globoside in POPC[e]	40	15.3, 1	
globoside (non-OH FA) in POPC[e]	40	15.1, 1	
		(16.0, 1)	
globoside (-OH FA) in POPC[e]	40	16.2, 1	
		(16.7, 1)	
globoside (non-OH FA) in DMPC[e]	15	*b*	
	25	17.8, 1	
GalCer in POPC[e]	40	18.8, 10.5	1.8
GalCer (18:0 FA) in POPC[e]	40	17.7, 9.7	1.8

[a] Temperature and acyl chain dependencies of quadrupolar splittings ($\Delta\nu_Q$) for deuterated GM_1, asialo-GM_1, globoside, and GalCer (all with complete natural fatty acid composition except where indicated otherwise), dispersed in fluid bilayers of POPC, POPC/cholesterol, or DMPC, and buffered at pH 7.4. Two types of sugar deuteration site are represented (Figure 1): -CDHOH (Table 1) and -$COCD_3$ (Table 2). Unless stated otherwise, cholesterol concentration was 23 mol % in samples with cholesterol. Outer/inner splitting ratios were not calculated for hydroxymethyl-deuterated globoside due to excessive sensitivity to measurement uncertainty in the small central splitting. [b] Oligosaccharide motion asymmetric (host T_t 23 °C). [c] These samples contained protein (40 wt % glycophorin or 50 wt % BSA), introduced by exhaustive dialysis (Ketis et al., 1980) of detergent solutions at 4 °C (octyl glucoside, 10:1 mol ratio detergent/lipid). [d] 100 mM NaCl, 5 mM EGTA, 5 mM Hepes, pH 7.4. [e] 10 mM PO_4, pH 7.4. [f] 100 mM NaCl, 20 mM $CaCl_2$, 5 mM Hepes, pH 7.4. [g] Doubly deuterated in hydroxymethyl function. [h] This sample was 70:23:7:20 mol ratio POPC/cholesterol/GM_1/GalCer sulfate [i.e., a typical POPC/cholesterol/GM_1 sample to which was added a large amount of (−)-charged lipid to see the effect of charge close to the surface].

previously, for which S_{mol} values of 0.3−0.5 have been recorded (Skarjune & Oldfield, 1979; Renou et al., 1989; Singh et al., 1992b). The central sharp peak is a common spectral feature of deuterated lipids in membranes—representing contributions from membranes with high curvature, and traces of residual HOD. Any population of deuterated carbohydrate residues which are undergoing rapid isotropic motion on the NMR time scale would also give rise to a sharp central peak. However, given the long time scale for the NMR measurements involved (10^{-3}−10^{-5} s), the latter possibility seems likely only if resulting from a subpopulation of highly curved vesicles. The spectral effect of high vesicle curvature has been well described for phosphatidylcholine with deuterated choline methyl groups in fluid bilayers by Curatolo and Neuringer (1986).

All spectra obtained for the lipids investigated here with deuterium in the -CH_2OH group were reminiscent of ones originally described by Skarjune and Oldfield (1979) for bilayers of the pure monoglycosyl species, [*d*Gal]GalCer (with 16:0 fatty acid) in fluid bilayer form. These workers determined that the two Pake doublets seen were of equal intensity and demonstrated that they were readily accounted for by spectral inequivalence of the *pro-R* and *pro-S* deuterons in the -CDHOH group. They pointed out that the spectra could be explained in terms of slow rotation about the C−CDHOH bond, resulting from hydrogen bonding involving -CDHOH. However, they went on to note that subsequent measurements indicate rapid rotation of this group attached to a galactose ring (Skarjune & Oldfield, 1982). Hence it has generally been considered that the equal intensity Pake doublets seen reflect the different average spatial orientations for the two stereoisomeric deuterons as they undergo rapid interconversion among rotamers [gauche(+), gauche(−) and trans] about the C−CDHOH bond (Renou et al., 1989; Singh et al., 1992b).

Direct assignment of these spectral splittings to specific (*pro-R* or *pro-S*) deuterons has not been reported for glycolipids. However, the behavior of the -CDHOH group has been considered in detail by Renou et al. (1989) for the galactose residue of the glycero glycolipid, lactosyl-1,2-di-*O*-tetradecyl-*sn*-glycerol. The latter species also gave a pair of Pake doublets of equal intensity when studied in fluid bilayer form: assignment of the inner splitting to the *pro-R* position was consistent with independently-derived orientation of the Gal residue, presuming rapid reorientation about the C−CDHOH bond. We demonstrate below that spectral interpretation in terms of rapid -CDHOH reorientation for the Gal residue is equally possible for the complex oligosaccharide chain of GM_1.

Figure 2A shows a typical powder spectrum of GM_1 labeled in the -CH_2OH group of the Gal residue *via* a single exposure to galactose oxidase followed by $NaBD_4$ reduction. The intensities of the Pake doublets are not equal (more clearly seen in the dePaked spectrum to the right of the powder spectrum). In contrast, the correspondingly deuterated Gal residue of GalCer (Skarjune & Oldfield, 1979; Singh et al., 1992b; Jarrell et al., 1992) and lactosyl-1,2-di-*O*-tetradecyl-*sn*-glycerol (Renou et al., 1989) produce paired Pake doublets of equal intensity (see also Figure 4E below). Either of two possibilities could explain this observation:

(i) the -CDHOH group in GM_1 is rotating slowly on the NMR time scale among 2 or more conformers whose corresponding spectral peaks happen to be hidden under one another, and/or (ii) there is unequal deuteration of the (two) spectrally inequivalent positions on the carbon.

The first of these possibilities presumes that, in contrast to literature expectations for galactose (Hayes et al., 1982), and contrary to previous ^2H NMR observations on Gal residues (Skarjune & Oldfield, 1979; Renou et al., 1989), rotation of -CDHOH in the GM_1 terminal Gal residue is not rapid—as a result of the additional sugars present in the molecule or different interactions with the surface environment for the more complex GSL. If two or more rotamers associated with the C−CDHOH bond were in slow exchange as has been reported to be the case for this group in glucose residues of several simple glycolipids (Jarrell et al., 1986, 1987a,b; Renou et al., 1989), the spectral intensities would be functions of the relative rotamer populations. This possibility would require that fortuitous peak overlap occur, resulting in merging of at least 8 spectral peaks to the 4 actually observed, with the additional coincidence that the ratio of inner-to-outer peak intensity fits the pattern described below following a second round of oxidation/reduction.

The second possibility may be understood in terms of the reaction stereochemistry, as follows. The process used in the present work for introduction of ^2H nuclei into GM_1 entails enzymatic oxidation of the $-CH_2OH$ on the terminal Gal moiety to produce an aldehyde. The subsequent step is reduction of the aldehyde with $NaBD_4$. This process would be expected to result in the introduction of equal amounts of *pro-R* and *pro-S* deuterons if there were no stereoselectivity involved in the reactions. If, however, there is selectivity, the relative amounts of the isomers would differ and could be a source of the observed inequality of their peak intensities. The stereoselectivity of this process has been considered in detail by Maradufu et al. (1971) for the monosaccharides galactose and β-methyl galactoside, dissolved in water. In work with lipids these reactions are carried out in 1:1 THF/water to improve solubility and presumably involve large micelles for the neutral lipids.

Maradufu et al. (1971) indicate that the initial oxidation of free galactose in aqueous solution may be expected to specifically remove the *pro-S* hydrogen, producing a (symmetric) nondeuterated aldehyde. Subsequent reduction of this compound with $NaBD_4$ leads preferentially to the *pro-R* (mono)deuterated product in a 3.6:1 ratio: i.e., after an initial oxidation/reduction process, the *pro-R* (mono)deuterated Gal should be the predominant product, with considerably less *pro-S*. GM_1 is the only water soluble glycolipid studied in the present work. Hence the fact that the GM_1 spectrum in Figure 2A displays an inner/outer intensity ratio of about 2:1 might suggest that the inner Pake doublet is associated with the *pro-R* deuteron and the outer doublet with the *pro-S*. According to Maradufu et al., ^1H nuclei at the *pro-S* position should be selectively removed 7.7 times more rapidly than ^2H nuclei at the same location due to a kinetic isotope effect. They also found that oxidative removal of the nucleus at the *pro-R* position does not occur, as mentioned above. Hence, under our reaction conditions, *pro-S* ^2H nuclei may be expected to be unaffected by a second round of enzymatic oxidation. Neither ^1H nuclei nor ^2H nuclei at the *pro-R* position should be affected. As a result, subsequent reduction with $NaBD_4$ should produce essentially complete deuteration of the $-CH_2OH$ group, with the exception of the residual small amount of *pro-R* left undeuterated after the first round of reaction. If one may assume that the resultant $-CD_2OH$ group is undergoing rapid rotation such that each Pake doublet corresponds to either a *pro-S* or *pro-R* deuteron, the stereoselectivity described would be expected to produce almost equal intensity for the two Pake doublets, but with a slight preponderance of that associated with the *pro-S* position—in agreement with the observation of reversal of the inner/outer intensity ratio in Figure 2B. The results are highly consistent with the inner Pake doublet's being associated with the *pro-R* deuteron, and rapid rotation of the hydroxymethyl group.

It is difficult to accurately judge relative peak intensities for [*d*GalNAc]globoside since, with the natural fatty acid mixture, the inner Pake doublet is not distinctly resolved from the central peak (Figure 2D) (see below). However, the basic features are readily understood in terms of the logic described for GM_1 and simpler glycolipids.

General Features Observed in Spectra of GSLs Deuterated in Amino Sugar Acetate Groups. For (symmetrically rotating) deuterated methyl groups, it is convenient to consider the projection of the C−D bond vectors directed along the $C-CD_3$ 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. Independent motion of the rigid, planar, $-NHCOCD_3$ group about the C−N bond linking it to the sugar ring is highly restricted (Yadav & Luger, 1980; Acquotti et al., 1990; Poppe et al., 1990), and the ring C−N bond is directed approximately parallel to the acetate $C-CD_3$ bond; so that rotation about C−N does not importantly 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. Hence, a given amino sugar deuterated in this fashion and associated with a glycolipid in a fluid membrane should produce one Pake doublet. The existence of different glycolipid populations long-lived on the NMR time scale would be expected to produce multiple Pake doublets.

Figure 3 presents typical ^2H NMR spectra for [d_3NeuAc,d_3-GalNAc]GM_1 (Figure 3A−C), [d_3GalNAc]asialo-GM_1 (Figure 3D), and [d_3GalNAc]globoside (Figure 3E), dispersed as minor components in bilayers of POPC. Each spectrum is characterized by a predominant single Pake doublet—although in the case of the globoside with natural fatty acids (Figure 3E) the small doublet splitting is poorly resolved from the central peak (see below). For asialo-GM_1 and globoside, only one Pake doublet is expected per glycolipid average orientation and environment, as there is only one amino sugar. For GM_1, high resolution analysis indicated significant deuteration at the NeuAc and GalNAc positions (3:1 ratio); so that two doublets were expected in the wideline study. It would appear that the quadrupole splittings of these doublets are similar and that as a result the GalNAc doublet is not well resolved (although small shoulders exist in Figure 3A,B).

The maximum splitting that might be observed for a rapidly rotating $-CD_3$ group on a lipid in fluid membranes is about 42 kHz. This would be the case if S_{mol} were 1.0 and the methyl group were oriented perpendicular to the

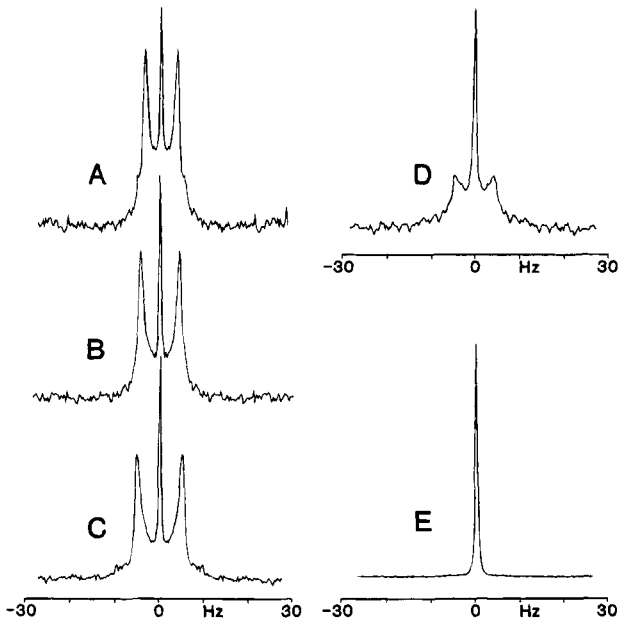

FIGURE 3: Selected ^2H NMR powder spectra for glycolipids deuterated in amino sugar acetate groups to give -$COCD_3$: [d_3-NeuAc,d_3GalNAc]GM_1 at 65 °C (A), 40 °C (B), and 25 °C (C); [d_3GalNAc]asialo-GM_1 at 40 °C (D); and [d_3GalNAc]globoside at 40 °C (E). All samples contain GSL dispersed at 10 mol % in POPC bilayers. Buffer details are given in the table footnotes. Typically, 60 000 transients were averaged.

plane of the membrane. Hence, the 10 kHz splittings seen for GM_1 indicate considerable preservation of orientational order. In the case of globoside, the splittings are in the 1 kHz range and might suggest just the opposite—i.e., a small value for S_{mol} (Table 1). However, it is clear that, for globoside with -CDHOH group in the terminal sugar residue (Table 1 and Figure 2D), the spectra show outer splittings of magnitude comparable to those seen for GM_1 labeled in the same fashion (e.g., 15−16 kHz *vs* 21 kHz at 40 °C). Given the existence of three significant rotamer populations for a Gal -CDHOH group calculated by Renou et al. (1989), the maximum splitting that might be observed for such a deuteron is probably in the range of 60 kHz, assuming no independent wobble of the labeled sugar. Hence S_{mol} for the globoside chain cannot be close to 0.0, and one may rule out the suggestion that the small quadrupole splittings obtained for acetate-labeled globoside result solely from very high disorder of the oligosaccharide chain.

Effect of GSL Hydrophobic Portion. All GSLs studied in the present work were homogeneous in having 90−96% sphingosine base (dihydrosphingosine comprising the remainder) (Abrahamsson et al., 1972; Thompson & Tillack, 1985). The natural fatty acid composition of GM_1 from the source studied is primarily 18:0 (Corti & Degiorgio, 1980; Sonnino et al., 1985). However, in GM_1 the sphingosine chain length is divided between two families: 60−70% is C18 and 30−40% is C20 (Corti & Degiorgio, 1980; Sonnino et al., 1985)—as distinct from the situation for globoside and GalCer which are almost totally C18 sphingosine (Sweeley, 1989). Thus, while the GM_1 studied had homogeneous acyl chain length, its alkyl chain length was divided between two large fractions. Examination of the hydroxymethyl-deuterated [*d*Gal]GM_1 examples in Figure 2A,B shows that, apart from the sharp central component, well over 90% of the spectral intensity is localized to the single, fairly homogeneous, two-doublet spectrum already described for a -CDHOH group in one average environment. Similarly, in the spectra of [d_3NeuAc,d_3GalNAc]GM_1 in Figure 3A−C, greater than 90% of the signal intensity can be accounted for by invoking a single population of molecules. Clearly the two-carbon length difference in the alkyl chain can have very little effect on headgroup behavior in the systems represented in Figures 2 and 3. Similar results were obtained for asialo-GM_1 (Figures 2C and 3D).

The fatty acids of globoside from pig erythrocytes are highly heterogeneous (some 90% are C20 or longer and just over half are α-OH) (Hanahan et al., 1971; Bunow & Levin, 1980). It may be separated on silicic acid into faster and slower running fractions enriched in non-hydroxy fatty acids and more polar α-OH species, respectively. Chain length and unsaturation are reportedly similar for the non-hydroxy and α-OH species (Hanahan et al., 1971). A typical spectrum of natural globoside deuterated with the sensitive -CDHOH probe ([*d*GalNAc]globoside) is shown in Figure 2D. It displays an outer Pake doublet with large quadrupole splitting, and an inner Pake doublet whose splitting is poorly resolved from the central peak. These doublets were more distinctly resolved in separate experiments with the faster and slower running (TLC) fractions; but the largest differences (in the outer splitting) are close to experimental error (Table 1). Thus, although spectral differences among the subpopulations may be significant, the variation actually measured in the current experiments can be accounted for by modest alterations in S_{mol} and/or orientation of the carbohydrate portion. The same was true in the case of the -CD_3 labeled [d_3GalNAc]globoside (Figure 3E). The one acetate label per globoside headgroup should result in a single Pake doublet. This was seen for globoside with only 18:0 fatty acid; however, the splitting was barely resolvable (Barber et al., 1994). If globoside fatty acid nature determines headgroup conformation or order, one might expect the spectrum in Figure 3E to show coexisting pairs of Pake doublets, reflecting the wide range of natural fatty acids present. Instead, the spectral lines are simply broadened. Powder spectra of the faster and slower running fractions displayed single Pake doublets with very small splittings (Table 2).

Further insight into this phenomenon was obtained from the monoglycosyl species, [*d*Gal]GalCer having natural fatty acid composition. A spectrum of this species in POPC is included as Figure 4E. It retains the features demonstrated originally for fluid bilayers of pure [*d*Gal]GalCer with only 16:0 fatty acid (Skarjune & Oldfield, 1979). The natural fatty acids of GalCer from beef brain comprise many α-OH species and significant amounts of material with both C18 and longer acyl chains [>75% of the fatty acids are C20 or longer, and roughly half are α-OH fatty acids (Bunow & Levin, 1980)]. The spectrum in Figure 4E demonstrates quadrupole splittings of 10.5 and 18.8 kHz at 40 °C. These values are within experimental error of those previously recorded for [*d*Gal]GalCer with single pure fatty acids (18:0, 18:1, 24:0, 18:0 α-OH) in fluid POPC bilayers (Singh et al., 1992b). They are also within experimental error of the major splittings measured for the natural α-OH and non-hydroxy fractions which had been separately isolated prior to enzymatic oxidation and subsequent reduction with $NaBD_4$ in the present work (Table 1).

A variety of influences were subsequently imposed on the membranes studied, in an attempt to identify factors that

Table 2: ^{2}H NMR Spectral Data for GSL Sugars Deuterated in Acetate Methyl Groups[a]

-$COCD_3$ labeled GSL and host matrix	temp (°C)	quadrupole splitting (± 0.5−1 kHz)
GM_1 in POPC[d] (values in parentheses are with cholesterol)	15	10.7
	25	10.5 (10.1)
	35	9.7 (9.7)
	40	9.3
	65	7.8
GM_1 in POPC[f]	15	11.0
20 mM Ca^{2+}	40	9.9
	65	8.2
2 mM Ca^{2+}	23	10.5
asialo-GM_1 in POPC[d]	40	9.6
globoside in POPC[e] (values in parentheses are with cholesterol)	15	1 (1)
	25	1 (1)
	40	1 (1)
	65	(<1)
globoside (18:0 FA) in POPC[e]	25	1.6 (1.6)
	35	1 (1.6)
globoside (non-OH FA) in POPC[e]	40	1.6 (1.6)
globoside (-OH FA) in POPC[e]	40	1.6 (1.6)
globoside in DMPC[e]	15	*b*
globoside[c] in POPC/cholesterol[g] with glycophorin	25	(1.5)
globoside[c] in POPC[g] with BSA, 2 mM Ca^{2+}	23	1

[a] Temperature and acyl chain dependencies of quadrupolar splittings ($\Delta\nu_Q$) for deuterated GM_1, asialo-GM_1, globoside, and GalCer (all with complete natural fatty acid composition except where indicated otherwise), dispersed in fluid bilayers of POPC, POPC/cholesterol, or DMPC, and buffered at pH 7.4. Two types of sugar deuteration site are represented (Figure 1): -CDHOH (Table 1) and -$COCD_3$ (Table 2). Unless stated otherwise, cholesterol concentration was 23 mol % in samples with cholesterol. [b] Oligosaccharide motion asymmetric (host T_t 23 °C). [c] These samples contained protein (40 wt % glycophorin or 50 wt % BSA), introduced by exhaustive dialysis (Ketis et al., 1980) of detergent solutions at 4 °C (octyl glucoside, 10:1 mol ratio detergent/lipid). [d] 100 mM NaCl, 5 mM EGTA, 5 mM Hepes, pH 7.4. [e] 10 mM PO_4, pH 7.4. [f] 100 mM NaCl, 20 mM $CaCl_2$, 5 mM Hepes, pH 7.4. [g] 20 mM NaCl, 5 mM $CaCl_2$, 5 mM Hepes, pH 7.4.

might directly alter orientation and behavior of the recognition sites, or modify their susceptibility to elements described above.

Effect of Cholesterol. Tables 1 and 2 provide data for samples similar to those containing the complex GSLs described above, but with cholesterol in the membrane. In general, 23−30% cholesterol did not change the quadrupole splittings within experimental error in the fluid membranes studied. Examples of the spectra obtained are presented for -$COCD_3$ labeled globoside (Figure 4A, dePaked version included) and -CDHOH labeled asialo-GM_1 (Figure 4B).

Effect of Temperature/Membrane Fluidity. Only very modest temperature variation was apparent in spectra of glycolipids studied in fluid membranes (e.g., Figure 3A−C and Tables 1 and 2). Alteration of the host matrix to a gel phase did however produce dramatic spectral changes. This is apparent in Figure 4C,D which displays spectra for [d_3-GalNAc]globoside (Figure 4C, compare to Figure 3E) and [*d*GalNAc]globoside (Figure 4D, compare to Figure 2D) with natural fatty acid composition at 10 mol % in DMPC below the 23 °C phase transition of the host matrix. It is clear that, in a gel phase host matrix, the spectrum changes drastically to reflect altered (slowed) motional properties. These results in DMPC at 15 °C contrast with spectra obtained for globoside with natural fatty acid composition in POPC, which retained the (fluid phase) features already discussed to well below 10 °C.

Effect of Desialylation. Under a given set of conditions, there was striking quantitative similarity between spectra for [*d*Gal]asialo-GM_1 (Figure 2C) and those corresponding to the identically deuterated [*d*Gal]GM_1 from which it was derived (Figure 2A). This was also true for the -$COCD_3$ labeled species (Figure 3). The -$COCD_3$ spectra shown for GM_1 in Figure 3A−C should display one Pake doublet for the NeuAc residue and a second doublet associated with deuterons on the GalNAc residue (Figure 1). Based upon ^{1}H NMR characterization of the product of hydrazinolysis in the present work, the GalNAc Pake doublet is expected to be 1/3 the intensity of that associated with the NeuAc residue. A second Pake doublet is not obviously present, in this or in samples more extensively hydrazinolyzed prior to reacetylation (although a shoulder can be seen at the high temperature). It appears that the peaks associated with -$COCD_3$ on the NeuAc of GM_1 closely overlap those associated with -$COCD_3$ on the GalNAc residue. Indeed, selective removal of the d_3NeuAc residue from GM_1, so that only the GalNAc deuterons remain, leaves much the same spectrum: a splitting of 9.6 kHz for asialo-GM_1 at 40 °C compared to a value of 9.3 kHz for GM_1 (panels D and B of Figure 3, respectively, Table 2). Note too that introduction of an additional (−) charge near to the membrane surface also had little effect: when sulfatide (GalCer sulfate) was incorporated at 20 mol % into POPC/cholesterol membranes containing GM_1, the alteration in the spectrum was close to experimental uncertainty (Table 1).

Other Factors. A subset of the systems dealt with above were further examined in the presence of ionic calcium—generally at the relatively high concentration of 20 mM. Figure 4F shows the spectrum of -CDHOH labeled GM_1 in the presence of 20 mM Ca^{2+}, and Figure 4G shows that for -$COCD_3$ labeled GM_1 in the presence of the same high Ca^{2+} concentration. The features already described remain, and the quadrupole splittings are similar to values without divalent cation (Tables 1 and 2). In general, we have observed that addition of 20 mM Ca^{2+} could induce some broadening of the original features with minimal effect on the measured splittings. Ca^{2+} was also seen to induce generalized and reversible broadening of the spectral features of asialo-GM_1, while the 90° edges remained close to the values measured without the divalent cation (Tables 1 and

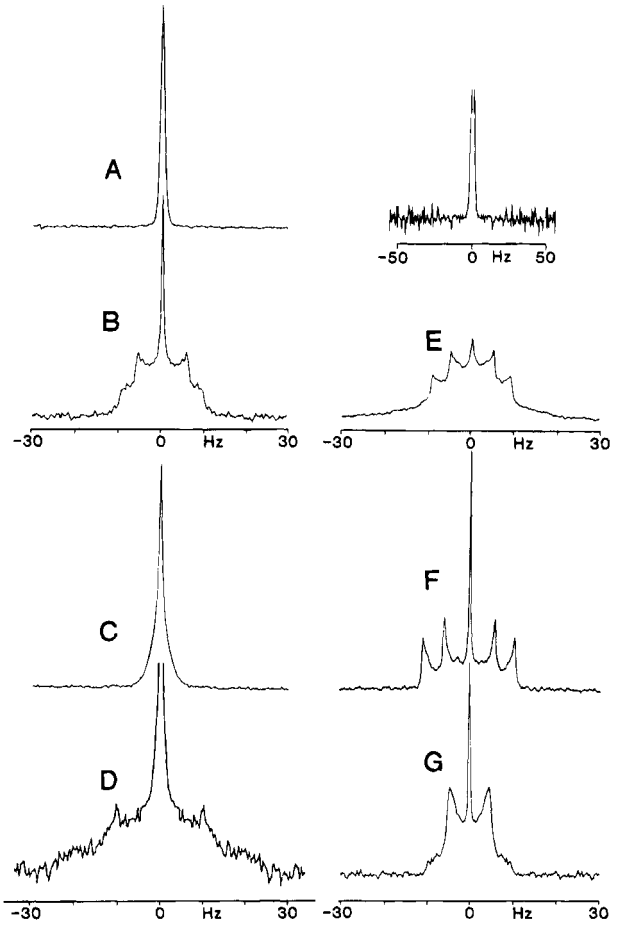

FIGURE 4: ^{2}H NMR powder spectra for the following: (A) [d_3-GalNAc]globoside at 40 °C in POPC/cholesterol (depaked spectrum to right); (B) [*d*Gal]asialo-GM_1 at 40 °C in POPC/cholesterol; (C) [d_3GalNAc]globoside at 15 °C in DMPC (phase transition temperature 23 °C, compare to fluid matrix in Figure 3E); (D) [*d*GalNAc]globoside at 15 °C in DMPC (phase transition temperature 23 °C, compare to fluid matrix in Figure 2D); (E) [*d*Gal]GalCer with natural fatty acid mixture at 40 °C in POPC; (F) [*d*Gal]GM_1 (two cycles of oxidation/reduction) at 40 °C in POPC with 20 mM Ca^{2+}; (G) [d_3NeuAc,d_3GalNAc]GM_1 at 40 °C in POPC with 20 mM Ca^{2+}.

2). We have observed similar Ca^{2+}-induced behavior in spectra of (+)-charged sphingolipid degradation products (deuterated in the polar portion and dispersed in fluid POPC membranes)—while the quadrupole splittings remained unchanged (unpublished results).

High concentrations of the integral glycoprotein, glycophorin, were incorporated into POPC/cholesterol liposomes containing GM_1 deuterated in the terminal Gal -CH_2OH group or in the amino sugar -$COCH_3$ groups. The spectral splittings were unchanged within experimental error at 15 and 25 °C (Table 1, spectra not shown). Similarly, neither glycophorin nor a very high surface concentration of serum albumin had appreciable effect on [d_3GalNAc]globoside spectral characteristics (Tables 1 and 2).

DISCUSSION

In a brief communication, we reported earlier that spectra of GM_1 deuterated in the -CH_2OH group of the terminal Gal residue demonstrated the existence of a preferred average oligosaccharide conformation at a POPC bilayer surface (Jarrell et al., 1992). In the present systematic study this was found to be true in a number of systems, and the same approach to deuteration introduced by Skarjune and Oldfield (1979) was extended to complex neutral glycolipids. Spectral interpretations demonstrated previously for glycerolipids (Renou et al., 1989) were shown to be appropriate for the more complex chain of GM_1. Spectral inequivalence of the two deuteron locations in -CDHOH provides considerable potential for sensitivity to alterations in headgroup orientation and behavior. Unfortunately, the existence of incompletely-characterized rotational conformers about the C−C bond attaching the group to the sugar ring adds a difficult-to-handle factor for considerations of carbohydrate headgroup orientation. Also, the fact that separate spectral peaks are observed for each deuteron minimizes the signal intensity when this approach is used (although if the oxidation/reduction cycle is repeated, it can be improved by more fully labeling both sites). Another possible source of concern when using only this (enzymatic) labeling technique is that, theoretically, certain subpopulations of a given GSL could be preferentially labeled.

The second technique for probe introduction employed in the present work involved deuteration of acetate groups on amino sugars. We recently reported a different mechanism of achieving this (Barber et al., 1994), but it failed to label the GalNAc residue, and was tedious and produced low yields since it involved consequent removal of the GSL fatty acid (Neuenhofer et al., 1985). The hydrazinolysis method used here led to substitution of each acetate and did not cleave the amide linkage attaching the (single) fatty acid to the GSL ceramide backbone [presumably because of its sequestration within some form of lipid aggregate (Higashi & Basu, 1982)]. As a result, the reaction was a simple two-step procedure offering high yields of deuterated GSLs with natural fatty acids. -$COCD_3$ groups gave a 3-fold improvement in signal-to-noise ratio as a result of spectral equivalence of the 3 deuterons, and made possible more direct correlation with spatial orientation of the carbohydrate portion.

Wideline ^{2}H NMR is a nonperturbing and sensitive method of probing spatial orientation and degree of motional order within deuterated molecules. Thus it is noteworthy that each of the oligosaccharide recognition sites studied, dispersed as a minor component at the surfaces of fluid membranes, was seen to have a preferred average conformation. Perhaps more remarkable was preservation of these average conformations in the face of alterations in hydrophobic structure, temperature, and surroundings. One might suggest that this resulted from the fact that the carbohydrate chains and/or probe sites chosen are extremely disordered and flexible such that their motions are insensitive to local occurrences. However, this is not the case. The maximum 90° edge spectral splitting anticipated for a rapidly rotating -CD_3 group, attached to a molecule which is itself undergoing rapid rotation about a molecular axis, is 42 kHz; and for a -CDHOH group it is likely in the neighbourhood of 60 kHz (depending on the number and statistical likelihood of conformers about C−CDHOH). Hence the quadrupole splittings observed in the present work suggest that values of S_{mol} for the deuterated segments of GM_1, asialo-GM_1, and globoside cannot have been less than 0.25−0.5. Interestingly, S_{mol} values reported for β-linked monoglycosyl species fall within this range (Skarjune & Oldfield, 1982; Jarrell et al., 1986, 1987a,b). Independent wobble of individual sugar residues in the chain must be limited. Thus the approach

used here was adequate to detect phenomena affecting conformation and order of the GSL oligosaccharide chains. We cannot exclude the possibility that more significant headgroup alterations may be occurring involving small (undetected) populations of the glycolipids studied.

A number of workers have noted that glycolipid participation in recognition events can be importantly modulated by changes in lipid structure, and by membrane fluidity or cholesterol and protein content. The concept of receptor "crypticity" derives from such observations [Alving et al., 1980; Kannagi et al., 1983; Shichijo & Alving, 1985; Hakomori, 1986; Lampio et al., 1986; Curatolo & Neuringer, 1986; Mehlhorn et al., 1988; Stromberg et al., 1991; Stewart & Boggs, 1993a; Kiarash et al. (1994) and references therein]. The underlying mechanisms warrant consideration for their implications to triggering events connected with initiation of specific signaling pathways at cell surfaces. Some such examples of oligosaccharide "communication" with the hydrophobic region may be dependent on properties of the systems in which the measurement was made—e.g., gel phase nature of the bilayer membranes (Hamilton et al., 1994) or the presence of membrane proteins. Lingwood and colleagues have observed that toxin binding differences for GSLs based on fatty acid chain length can be more marked in lipids dried down and rehydrated on plastic surfaces than in liposomes [Kiarash et al. (1994) and personal communication]. X-ray studies form an important basis for considering oligosaccharide control by lipid structure, as they suggest effects of GSL backbone on headgroup orientation. For instance, in single crystals of GalCer there is evidence that fatty acid α-hydroxylation could alter carbohydrate conformation *via* H-bonding [Pascher & Sundell, 1977; see also Nyholm et al. (1990)]. A question that remains is the extent to which these basic forces identified in single crystals and model systems may be altered for receptors dispersed in (often fluid) membranes [e.g., Bunow and Levin (1980) and Curatolo (1987)]. In recent years, improved molecular modeling algorithms have made it possible to extend earlier (Maggio et al., 1981) efforts at calculation of GSL conformation (Poppe et al., 1990; Scarsdale et al., 1990; Nyholm & Pascher; 1993), but these remain dependent upon experimental input.

Parameters suggested to be capable of inducing receptor crypticity include lateral distribution, motional properties, orientation, and degree of protrusion from the membrane surface. The technique of ^{2}H NMR, as employed in the present work, has particular application to a subset of these. The presence of deuterated GSL in a gel phase—a condition such that both lateral and rotational diffusion were strongly reduced—led to a very distinctive spectrum. Thus we should have detected the formation of domains highly enriched in glycolipid, since these would be gel phase at the temperatures studied [Maggio et al., 1985; Bunow amd Levin (1988) and references therein]. However, our approach would not be sensitive to minor receptor enrichment leading only to increased local order in fluid membranes, based on our observations of the minor effect of cholesterol. Hence the present experiments say little about the role of moderate changes in GSL lateral distribution in the systems examined. Where briefly tested (Figure 4C,D), host matrix rigidification induced striking spectral alterations—interpretable in terms of loss of rapid symmetrical rotation of the GSL molecule. This likely indicates that the carbohydrate portion is importantly influenced, at least in its axial diffusion, by immobilization of the membrane-inserted portion. The experiment did not rule out the possibility that GSL clustering in the gel phase, with resultant steric hindrance, was a contributory factor to the oligosaccharide motional changes. Altered headgroup order and orientation were sensitively tested for, as was headgroup protrusion if it influences either of these parameters. Lateral diffusion in fluid membranes may be expected to alter NMR relaxation times, but not the spectral parameters measured here.

Chain length and hydroxylation are key factors identified as possible sources of recognition site modulation in studies of crypticity. With regard to the GSLs examined, it is known that a sphingosine chain length difference of 2 carbons is sufficient to influence the size of pure ganglioside micelles (Yohe et al., 1976). Thus it is noteworthy that a 2-carbon alteration in the sphingosine chain length of GM_1 and asialo-GM_1 had no obvious spectral effect, as measured by deuterons in the terminal NeuAc or Gal residues and in the internal GalNAc. The faster and slower running fractions of globoside have also been reported to show differences in micelle size related to fatty acid composition (Tinker et al., 1976). However, our spectra of globoside with a wide variety of natural fatty acids showed evidence of only minor spectral differences in the headgroup region. The maximum quantitative change in spectral splittings associated with fatty acid alteration was some 1−2 kHz, which is no more than 5−10% of the range over which splittings could have varied for orientation changes between 1° and 90°. These small differences must reflect correspondingly small changes in orientation or S_{mol}. The above findings appear to be generalizations of our earlier observation that ^{2}H NMR spectra of [*d*Gal]GalCer dispersed in fluid POPC bilayers were the same for 5 different synthetic fatty acids (Singh et al., 1992b). As mentioned above, a concern when studying GSLs deuterated *via* the enzymatic oxidation/reduction cycle is that subfractions might selectively fail to react. This is probably minimized in the organic solvent mixture used when oxidation times are long (as here). We have not observed failure to react for any of the natural fractions or for more defined fatty acids [Singh et al., 1992b; see also Stewart and Boggs (1993a)]. The acetate labeling procedure does not rely on enzymatic oxidation and gave results consistent with the enzymatic approach.

It is also noteworthy that the effect of temperature on the above observations was very modest, in spite of the fact that sample warming could potentially alter intermolecular associations and collisions as well as the effects of intramolecular forces. The temperatures chosen were influenced to some extent by the fact that, above 40 °C, up to 10 mol % in POPC (without cholesterol), GalCer remains dispersed in a fluid matrix. Below 40 °C, depending on the GSL fatty acid, there can be measurable separation of a GSL-enriched phase (Singh et al., 1992b). We have not seen evidence of phase separation in POPC near 40 °C with GM_1 and globoside—which have considerably lower main transition temperatures than GalCer (Maggio et al., 1985) (unpublished observations). The small temperature-induced changes seen in the current work are inconsistent with any major structural alterations in the carbohydrate headgroup. One form of intermolecular association that has attracted interest with regard to oligosaccharide chains is carbohydrate association with the membrane surface. Little is known about the forces

governing such interactions [see for instance Stromberg et al. (1991) and Aubin and Prestegard (1993)]. However, binding of soluble carbohydrates to liposomes is well-known; and it has been postulated that effects on water of hydration are involved (Crowe et al., 1987). Hence it is conceivable that long carbohydrate chains could associate with bilayer surfaces. Such associations would probably involve conformational distortion and would be expected to be temperature sensitive. However, it would appear that, in fluid membranes, the effect of increased temperature on the complex GSL oligosaccharide was limited to a modest decrease in order. This result is consistent with the observation of Bechinger et al. (1988) that simple neutral glycolipids induced no measurable alteration in the ^2H NMR spectrum of the choline headgroup of POPC.

Pure POPC bilayers exhibit a main transition temperature of −3 °C (Davis & Keough, 1985) and have low chain order at the temperatures investigated. High cholesterol concentration is known to greatly restrict chain flexibility in fluid phospholipid matrices, while having much less effect on rotational (whole body) diffusion (Oldfield & Chapman, 1972; Demel & De Kruyff, 1976; Yeagle, 1985; Vist & Davis, 1990). In addition, cholesterol increases fluid bilayer thickness. One might suggest that the oligosaccharide of GSLs should be more sensitive to variations in the hydrophobic region in cholesterol-rich membranes, where compressibility is greatly reduced (Needham et al., 1988). We recently observed that deuterons at C_2 of GalCer fatty acids showed evidence of greater orientational differences between the 18:0 and 24:0 species when the membranes contained cholesterol, particularly at low temperature (Hamilton et al., 1994). Some of the earliest systematic observations concerning glycolipid (and phospholipid) crypticity noted important effects of cholesterol (Brulet & McConnell, 1977; Balakrishnan et al., 1982; Utsumi et al., 1984; Stanton et al., 1984; Mehlhorn et al., 1988; Stewart & Boggs, 1990). Nevertheless, in the present work the spectral effect of cholesterol in membranes containing GSLs with natural fatty acids was found to be close to or within experimental error. Presumably, this is a further reflection of lack of oligosaccharide sensitivity to even quite large structural and conformational alterations within the membrane-inserted portions of GSLs dispersed in fluid membranes.

Neuraminic acidic (NeuAc) residues are important features of cell surfaces. They have been implicated in recognition events and in membrane structure. As a result, natural extracellular neuraminidase activity has attracted interest and is suggested to be of significance in some signaling events [reviewed in Sweeley (1993)]. Selective removal of NeuAc from GM_1 left the uncharged tetrameric chain Galβ1→3GalNAcβ1→4Galβ1→4Glc (asialo-GM_1). Hence, the design of the current experiments made it possible to test the relationship among conformation, behavior, and the presence of a (−)-charged NeuAc residue in a surface-attached oligosaccharide chain. One might have anticipated a measurable spectral effect of removing NeuAc from GM_1 since the sugar is attached in the middle of the chain and is the only source of charge in the molecule. High resolution NMR of GM_1, GM_{1b}, and GD_{1a} in DMSO/2% H_2O solution have indicated conformational effects related to placement of the NeuAc residue (Scarsdale et al., 1990). However, under a given set of conditions, there was striking quantitative similarity between spectra for asialo-GM_1 deuterated in the terminal Gal residue ([*d*Gal]asialo-GM_1) and those corresponding to the identically deuterated GM_1 from which it was derived, the spectral differences between the two glycolipids being within experimental error. The same result was obtained when asialo-GM_1 was probed with -CD_3 on the next-to-terminal GalNAc ([d_3GalNAc]asialo-GM_1): that the spectral values were within experimental error of those for GM_1 under a given set of conditions. It seems therefore that GM_1 and asialo-GM_1 have very similar conformations and order parameters at the (fluid) membrane surface. This apparent insensitivity to removal of the charged NeuAc residue on GM_1 led us to test the opposite experiment: addition of GalCer SO_4^{2-} to form a layer of (−)-charged groups close to the membrane surface. A cholesterol-containing membrane was chosen to minimize the anticipated "stiffening" effect of added (high-melting) GSL. There was no significant effect on spectra of deuterons in the terminal Gal residue of GM_1.

The above findings were tested briefly and nonsystematically after the incorporation of several additional factors that might be expected to influence the observations described: the divalent cation, Ca^{2+}, and protein. There is an extensive literature on metallic ion interactions with membranes (Rand & Parsegian, 1989; McLaughlin, 1989), and on ^2H NMR spectroscopy of phospholipid headgroup deuterium probes in the presence of ions (Akutsu & Seelig, 1981; Seelig & MacDonald, 1987; Seelig et al., 1987; Roux & Bloom, 1990). For instance, Akutsu and Seelig (1981) recorded that 10 mM Ca^{2+} induced quadrupole splitting changes of up to 10−20% in wideline NMR spectra of deuterons in the choline headgroups of DPPC bilayers. In recent years there has developed a specific interest surrounding the fact that divalent cations have been observed to encourage adhesion of carbohydrate-bearing liposomes, including those containing gangliosides or GalCer (Brewer & Thomas, 1984; Webb et al., 1988; Kojima & Hakomori, 1989; Hakomori et al., 1991; Stewart & Boggs, 1993b). The mechanism of this effect is unknown, and it represents too complex an area to consider in detail in the present work; however, the examples tested may provide insight into the design of future experiments. It is interesting that the spectral locations of the acetate peaks of GM_1 were found in the present work to be only modestly affected by Ca^{2+} concentrations that were up to 10 times physiological. Some broadening of spectral features was noted. However, since related spectral alterations were seen for asialo-GM_1 and even (+)-charged sphingolipid derivatives, it is clear that the effect is not simply related to the presence of the (−)-charged NeuAc residue.

In spite of early suggestions to the contrary, the affinity of Ca^{2+} ions for gangliosides is now known to be modest [McDaniel & McLaughlin, 1985; and see Felgner et al. (1982)]. The overall Ca^{2+} binding constant is roughly the same for gangliosides as it is for anionic phospholipids, but the strong Ca^{2+}-mediated drive to lateral rearrangement often seen in the latter case has not been observed for gangliosides [reviewed in Raudino and Bianciardi (1991)]. Raudino and Bianciardi (1991) have discussed this in terms of "diffuse" *vs* "compact" interfaces. Our observations seem to indicate that, for high concentrations of Ca^{2+}, there is some general alteration in GSL behavior as minor components in fluid membranes, perhaps mediated through Ca^{2+} effects on the host matrix rather than through a specific effect on acidic

glycolipids. The effect may be related to cation-induced alterations in hydration forces between the membranes studied (Rand & Parsegian, 1989). The situation is complicated by the fact that neutralization of the GM_1 net negative charge by Ca^{2+} could alter membrane spacing, as could binding of cations to an otherwise uncharged bilayer. Interestingly, the same conclusion—that Ca^{2+} had no measurable effect on preferred carbohydrate orientation—was reached in a recent detailed NMR study of a simple model glycolipid containing a NeuAc residue (Aubin & Prestegard, 1993). Goins et al. (1986) reported that 5 mM Ca^{2+} had no effect upon the lateral mobility of GM_1 labeled with a fluorescent probe on the NeuAc residue when dispersed in fluid DMPC. Ca^{2+} effects on GSLs have been considered by Maggio et al. (1987), who concluded from DSC studies that variation in intermolecular packing was not involved, but that ion binding for both neutral and charged complex GSLs in DPPC was accompanied by modification in dipolar properties of the interface or in its state of hydration.

Membrane protein effects have been suggested by some workers as possible contributors to the phenomenon of GSL crypticity in cell membranes [reviewed in Hakomori (1981)]. Such effects could be considered to arise from (i) specific interactions and (ii) simple macromolecular "crowding" [reviewed in Zimmerman and Minton (1993)]. Unfortunately, the physical nature of lipid/protein systems, and their homogeneity, can be difficult to assess; so that it may prove extremely difficult to isolate contributory factors. Glycophorin was chosen in the present work as an example of a membrane-spanning species with extensive carbohydrate (Marchesi et al., 1976). Bilayers containing glycophorin have been extensively characterized by us and other workers (Ketis & Grant, 1980; Ruppel et al., 1982; Grant & Peters 1984; MacDonald & Pink, 1987), and it is known to distribute uniformly as small oligomers in such systems. Glycophorin A is a 30 kDa integral membrane protein that contains 16 (−)-charged oligosaccharide chains (Vitala & Jarnefelt, 1985). Hence this species might be expected to alter GSL spectra by a number of mechanisms. It has been suggested that GM_1 may associate in bilayers with glycophorin (Umeda et al., 1984; Terzaghi et al., 1993). Serum albumin was selected as a common protein present at the outer plasma membrane surface [e.g., Rechfeld et al. (1975)] through its very high concentration in extracellular fluid. Spectral splittings were not seen to be altered in the presence of either protein. However, the glycophorin sample showed broader spectral lines. Whether this effect reflected some GSL/protein direct interaction or an indirect effect mediated through bilayer changes will be very difficult to determine unambiguously.

CONCLUSIONS

Methods were developed for selectively replacing carbohydrate hydrogen nuclei with deuterons in three important complex natural glycosphingolipids: GM_1, asialo-GM_1, and globoside. In a variety of systems, without exception, the oligosaccharide chains had preferred average conformations at fluid membrane surfaces. A 2-carbon alteration in the sphingosine chain length of GM_1 and asialo-GM_1 had no effect within the limits of experimental sensitivity in the fluid membranes studied. The range of common natural fatty acid structures found in globoside and GalCer, which includes wide variation in chain length and hydroxylation, had only very modest effects. Removal of the (−)-charged neuraminic acid from GM_1 had no significant effect on headgroup conformation or motional order; nor did introduction of additional (−) charges at the membrane surface in the fluid membrane environment. These observations were not changed by the temperature variations tested, or by the presence of cholesterol. Temperature variation demonstrated no evidence of having altered headgroup conformation through effects on GSL collision frequency or inter- and intramolecular forces. Although host matrix transition to a gel phase clearly influenced headgroup motion, relative stiffening of fluid membranes by temperature reduction or cholesterol did not.

It appears that, when dispersed as minor components in fluid membranes (as is typically the case in cells), GSL oligosaccharide conformation and degree of freedom are not greatly influenced by the changes studied, or by the conformation of hydrocarbon chains. These results provide little evidence that alterations in the GSL hydrophobic portion lead to changes in headgroup conformation or extension from the surface as a mechanism of receptor crypticity in fluid membranes. Similarly, they offer little support for conformational change as a mode of information transfer (signaling) between the (fluid) membrane surface and interior. Where very briefly tested, the presence of membrane proteins was not seen to change the basic observations, but it is certainly possible that specific protein−carbohydrate interactions could play an important role. High concentrations of ionic calcium introduced general spectral broadening which may reflect nonspecific interaction with the membrane surface. The techniques used in the present work would be relatively insensitive to changes in lateral diffusion rates in the fast exchange limit and might not reflect the presence of phase separated fluid domains.

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REFERENCES

Abrahamsson, S., Pascher, I., Larsson, K., & Karlsson, K. (1972) *Chem. Phys. Lipids 8*, 152−179.

Acquotti, D., Poppe, L., Dabrowski, J., von der Leith, C.-W., Sonnino, S., & Tettamanti, G. (1990) *J. Am. Chem. Soc. 112*, 7772−7778.

Adebodun, F., Chung, J., Montez, B., Oldfield, E, & Shan, X. (1992) *Biochemistry 31*, 4502−4509.

Akutsu, H., & Seelig, J. (1981) *Biochemistry 20*, 7366−7373.

Alving, C. R., Urban, K. A., & Richards, R. L. (1980) *Biochim. Biophys. Acta 600*, 117−125.

Aubin, Y., & Prestegard, J. H. (1993) *Biochemistry 32*, 3422−3428.

Barber, K. R., Hamilton, K. S., Rigby, A. C., & Grant, C. W. M. (1994) *Biochim. Biophys. Acta 1190*, 376−384.

Bechinger, B., MacDonald, P. M., & Seelig, J. (1988) *Biochim. Biophys. Acta 943*, 381−385.

Brewer, G. J., & Thomas, P. D. (1984) *Biochim. Biophys. Acta 776*, 279−287.

Bunow, M. R., & Levin, I. W. (1980) *Biophys. J. 32*, 1007−1022.

Bunow, M. R., & Levin, I. W. (1988) *Biochim. Biophys. Acta 939*, 577−586.

Corti, M., & Degiorgio, V. (1980) *Chem. Phys. Lipids 26*, 225–238.
Crook, S. J., Boggs, J. M., Vistnes, A. I., & Koshy, K. M. (1986) *Biochemistry 25*, 7488–7494.
Crowe, J. H., Crowe, L. M., Carpenter, J. F., & Aurell-Wistrom, C. (1987) *Biochem. J. 242*, 1–10.
Cumar, F. A., Maggio, B., & Caputto, R. (1980) *Biochim. Biophys. Acta 597*, 174–182.
Curatolo, W. (1986) *Biochim. Biophys. Acta 861*, 373–376.
Curatolo, W. (1987) *Biochim. Biophys. Acta 906*, 137–160.
Curatolo, W., & Neuringer, L. J. (1986) *J. Biol. Chem. 261*, 17177–17182.
Davis, J. H. (1983) *Biochim. Biophys. Acta 737*, 117–171.
Davis, P. J., & Keough, K. M. W. (1985) *Biophys. J. 48*, 915–918.
Demel, R. A., & De Kruyff, B. (1976) *Biochim. Biophys. Acta 457*, 109–131.
Dreyfus, H., Aunis, D., Harth, S., & Mandel, P. (1977) *Biochim. Biophys. Acta 489*, 89–97.
Felgner, P., Thompson, T. E., Beach, J., Barenholz, Y., & Wong, M. (1982) *Biophys. J. 37*, 209a.
Goins, B., Masserini, M., Barisas, B. G., & Freire, E. (1986) *Biophys. J. 49*, 849–856.
Grant, C. W. M., & Peters, M. W. (1984) *Biochim. Biophys. Acta 779*, 403–422.
Hakomori, S.-I. (1981) *Annu. Rev. Biochem. 50*, 733–764.
Hakomori, S.-I. (1986) *Chem. Phys. Lipids 42*, 209–233.
Hakomori, S.-I., Igarashi, Y., Kojima, N., Okoshi, K., Handa, B., & Fenderson, B. (1991) *Glycoconjugate J. 8*, 178.
Hamilton, K. S., Briere, K., Jarrell, H. C., & Grant, C. W. M. (1994) *Biochim. Biophys. Acta 1190*, 367–375.
Hanahan, D. J., Ekholm, J. E., & Benson, B. (1971) *Biochim. Biophys. Acta 231*, 343–359.
Harris, P. L., & Thornton, P. L. (1978) *J. Am. Chem. Soc. 100*, 6738–6745.
Hayes, M. L., Serianni, A. S., & Barker, R. (1982) *Carbohydr. Res. 100*, 87–101.
Higashi, H., & Basu, S. (1982) *Anal. Biochem. 120*, 159–164.
Jarrell, H. C., Giziewicz, J. B., & Smith, I. C. P. (1986) *Biochemistry 25*, 3950–3957.
Jarrell, H. C., Jovall, P. A., Giziewicz, J. B., Turner, L. A., & Smith, I. C. P. (1987a) *Biochemistry 26*, 1805–1811.
Jarrell, H. C., Wand, A. J., Giziewicz, J. B., & Smith, I. C. P. (1987b) *Biochim. Biophys. Acta 897*, 69–82.
Jarrell, H., Singh, D., & Grant, C. W. M. (1992) *Biochim. Biophys. Acta 1103*, 331–334.
Ketis, N. V., & Grant, C. W. M. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3788–3790.
Kiarash, A., Boyd, B., & Lingwood, C. A. (1994) *J. Biol. Chem. 269*, 11138–11146.
Koerner, T. A. W., Jr., Prestegard, J. H., Demou, P. C., & Yu, R. K. (1983) *Biochemistry 22*, 2676–2687.
Kojima, N., & Hakomori, S.-I. (1989) *J. Biol. Chem. 264*, 20159–20162.
Koscielak, J., Hakomori, S.-I., & Jeanloz, R. W. (1968) *Immunochemistry 5*, 441–455.
Lampio, A., Rauvala, H., & Gahmberg, C. G. (1986) *Eur. J. Biochem. 157*, 611–616.
MacDonald, A. L., & Pink, D. A. (1987) *Biochemistry 26*, 1909–1917.
Maggio, B., Cumar, F. A., & Caputto, R. (1978) *FEBS Lett. 90*, 149–152.
Maggio, B., Cumar, F. A., & Caputto, R. (1981) *Biochim. Biophys. Acta 650*, 69–87.
Maggio, B., Ariga, T., Sturtevant, J. M., & Yu, R. K. (1985) *Biochim. Biophys. Acta 818*, 1–12.
Maggio, B., Sturtevant, J. M., & Yu, R. K. (1987) *Biochim. Biophys. Acta 901*, 173–182.
Mansson, S. (1973) *J. Biochem. 248*, 740–742.
Maradufu, A., Cree, G. M., & Perlin, A. S. (1971) *Can. J. Chem. 49*, 3429–3437.
Marchesi, V. T., Furthmayer, H., & Tomita, M. (1976) *Annu. Rev. Biochem. 45*, 667–698.
McDaniel, R., & McLaughlin, S. (1985) *Biochim. Biophys. Acta 819*, 153–160.
McLaughlin, S. (1989) *Annu. Rev. Biophys. Biophys. Chem. 18*, 113–136.
Mehlhorn, I. E., Florio, E., Barber, K. R., Lordo, C., & Grant C. W. M. (1988) *Biochim. Biophys. Acta 939*, 151–159.
Momoi, T., Ando, S., & Magai, Y. (1976) *Biochim. Biophys. Acta 441*, 488–497.
Needham, D., McIntosh, T. J., & Evans, E. (1988) *Biochemistry 27*, 4688–4673.
Neuenhofer, S., Schwarzmann, G., Egge, H., & Sandhoff, K. (1985) *Biochemistry 24*, 525–532.
Nyholm, P.-G., & Pascher, I. (1993) *Biochemistry 32*, 1225–1234.
Nyholm, P.-G., Pascher, I., & Sundell, S. (1990) *Chem. Phys. Lipids 52*, 1–10.
Oldfield, E., & Chapman, D. (1972) *FEBS Lett. 23*, 285–297.
Pascher, I., & Sundell, S. (1977) *Chem. Phys. Lipids 20*, 175–191.
Poppe, L., von der Leith, C.-W., & Dabrowski, J. (1990) *J. Am. Chem. Soc. 112*, 7762–7771.
Radin, N. S. (1972) *Methods Enzymol. 28*, 300–306.
Rand, R. P., & Parsegian, V. A. (1989) *Biochim. Biphys. Acta 988*, 351–376.
Raudino, A., & Bianciardi, P. (1991) *Biochem. Bioenergetics 26*, 63–67.
Rechfeld, S. J., Eatough, D. J., & Hansen, L. D. (1975) *Biochem. Biophys. Res. Commun. 66*, 586–590.
Renou, J.-P., Giziewicz, J. B., Smith, I. C. P., & Jarrell, H. C. (1989) *Biochemistry 28*, 1804–1814.
Roux, M., & Bloom, M. (1990) *Biochemistry 29*, 7077–7089.
Ruppel, D., Kapitza, H.-G., Galla, H. J., Sixl, F., & Sackmann, E. (1982) *Biochim. Biophys. Acta 692*, 1–17.
Sankaram, M. B., & Thompson, T. E. (1990) *Biochemistry 29*, 10676–10684.
Scarsdale, J. N., Prestegard, J. H., & Yu, R. K. (1990) *Biochemistry 29*, 9843–9855.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.
Seelig, J., & MacDonald, P. M. (1987) *Acc. Chem. Res. 20*, 221–228.
Seelig, J., MacDonald, P. M., & Scherer, P. G. (1987) *Biochemistry 26*, 7535–7541.
Shichijo, S., & Alving, C. R. (1985) *Biochim. Biophys. Acta 820*, 289–294.
Singh, D., Davis, J. H., & Grant, C. W. M. (1992a) *Biochim. Biophys. Acta 1107*, 23–30.
Singh, D., Jarrell, H. C., Barber, K. R., & Grant, C. W. M. (1992b) *Biochemistry 31*, 2662–2669.
Skarjune, R., & Oldfield, E. (1979) *Biochim. Biophys. Acta 556*, 208–218.
Skarjune, R., & Oldfield, E. (1982) *Biochemistry 21*, 3154–3160.
Smith, I. C. P. (1984) *Biomembranes 12*, 133–168.
Sonnino, S., Kirschner, G., Ghidoni, R., Acquotti, D., & Tettamanti, G. (1985) *J. Lipid Res. 26*, 248–257.
Stewart, R. J., & Boggs, J. M. (1993a) *Biochemistry 32*, 5605–5614.
Stewart, R. J., & Boggs, J. M. (1993b) *Biochemistry 32*, 10666–10674.
Stromberg, N., Nyholm, P.-G., Pascher, I., & Normark, S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9340–9344.
Sweeley, C. C. (1989) *Pure Appl. Chem. 61*, 1307–1312.
Sweeley, C. C. (1993) *Adv. Lipid Res. (B) 26*, 235–252.

erzaghi, G., Tettamanti, G., & Masserini, M. (1993) *Biochemistry 32*, 9722−9725.
hewalt, J. L., & Bloom, M. (1992) *Biophys. J. 63*, 1176−1181.
hompson, T. E. & Tillack, T. W. (1985) *Annu. Rev. Biophys. Biophys. Chem. 14*, 361−386.
inker, D. O., Pinteric, L., Hsia, J. C., & Rand, R. P. (1976) *Can. J. Biochem. 54*, 209−218.
meda, M., Kanda, S., Nojima, S., Wiegandt, H., & Inoue, K. (1984) *J. Biochem. 96*, 229−235.
ist, M. R., & Davis, J. H. (1990) *Biochemistry 29*, 451−464.
itala, J., & Jarnefelt, J. (1985) *Trends Biochem. Sci. 10*, 392−395.
Webb, M. S., Tilcock, C. P. S., & Green, B. R. (1988) *Biochim. Biophys. Acta 938*, 323−333.
Winsborrow, B. G., Brisson, J., Smith, I. C. P., & Jarrell, H. C. (1992) *Biophys. J. 63*, 428−437.
Yadav, J. S., & Luger, P. (1983) *Carbohydr. Res. 119*, 57−73.
Yeagle, P. L. (1985) *Biochim. Biophys. Acta 822*, 267−287.
Yohe, H. C, Roark, D. E., & Rosenberg, A. (1976) *J. Biol. Chem. 251*, 7083−7087.
Zimmerman, S. B., & Minton, A. P. (1993) *Annu. Rev. Biophys. Biomol. Struct. 22*, 27−65.

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