Globoside as a Membrane Receptor: A Consideration of Oligosaccharide Communication with the Hydrophobic Domain†

David H. Jones,‡ Clifford A. Lingwood,§ Kathryn R. Barber,‡ and Chris W. M. Grant*,‡

Department of Biochemistry, University of Western Ontario, London, Ontario, Canada N6A 5C1, and Departments of Microbiology & Biochemistry, Research Institute, The Hospital for Sick Children, Toronto, Ontario, Canada M5G 1X8

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ABSTRACT: Recognition of macromolecules by glycosphingolipids is closely correlated with the nature of the glycolipid carbohydrate; however, it is also thought to be secondarily modulated by the structure of the single fatty acid. In the present work, we sought insight into what physical effect a change in this fatty acid has on the extramembranous portion of globosides at liposomal surfaces mimicking systems for which modulated receptor function has been recorded in the past. Protons of the exocyclic hydroxymethyl group on the terminal Gal residue of globotriaosylceramide (Gb_3) were replaced with deuterium. In this location, the nonperturbing probe nuclei sampled cumulative conformational and orientational characteristics of the oligosaccharide chain at a sugar residue that is critical in specific binding of verotoxins. Deuterated Gb_3 having 18:1 fatty acid was compared to the same species having 22:1 fatty acid, at 6.3 mol % in unsonicated bilayers of dipalmitoylphosphatidylcholine/cholesterol. Both produced narrow, apparently axially asymmetric ^2H NMR spectra over a wide temperature range. Motional properties of the terminal sugar were measurably influenced by the fluidity of the host matrix; however, evidence was not found for conformational or orientational variation in this sugar brought about by the fatty acid alteration. In related experiments, acetate protons on the terminal *N*-acetyl galactosamine (GalNAc) residue of globotetraosylceramide (Gb_4) were substituted with deuterium, and the natural fatty acid was replaced with 18:0 or 24:0 species deuterated at C2. Once again, species with short *vs* long fatty acid were examined for evidence of headgroup differences. Spectra of Gb_4 were compared at 10 mol % in unsonicated fluid bilayers of 1-palmitoyl-2-oleoylphosphatidylcholine, and at 5 mol % in membranes containing 33 mol% cholesterol. Spectral splittings reflecting cumulative effects on conformation and order at the terminal deuterated sugar remained unchanged between species having 18:0 *vs* 24:0 fatty acid in POPC/cholesterol. In a pure POPC host matrix, there was clear evidence of a motional difference between the two—the longer chain Gb_4 demonstrating spectral asymmetry—but the spectral width was unchanged. Transverse relaxation times, T_2, were measured. Our findings appear to help correlate the conclusions of a number of workers dealing with the molecular basis of crypticity. We suggest that changes in glycolipid receptor function based on ceramide fatty acid variation have a major origin in the fatty acid's ability to determine the thermodynamics of interaction with the host matrix, as reflected in such parameters as glycolipid motional properties, local membrane curvature, and likely glycolipid time-dependent lateral associations. The result at low concentrations of glycolipid may often be only a subtly altered collective surface epitope, best detected by a specific recognition event.

Glycosphingolipids of higher animal cells represent an important class of surface receptor [reviewed in Hakomori (1981, 1989), Thompson and Tillack (1985), and Curatolo (1987)]. As such, they have been a source of insight into how cells regulate participation in recognition events. Clearly one mechanism is *via* expression of receptors possessing a particular primary sequence. However, in membrane environments, receptor density, orientation, location relative to the surface, and motional characteristics become potential determinants of macromolecule binding. These additional factors raise the question of how the spatial/temporal arrangement of surface receptors might be related to structural modifications within the membrane, which in turn has important implications for the nature of early events in signal transduction.

The phenomenon examined in the present work is the observation that a glycolipid's receptor function can be modulated by the nature of its single fatty acid. This has been most clearly demonstrated in model membranes using the simple species galactosylceramide and its sulfated analogue (Alving et al., 1980; Stewart & Boggs, 1993), and for the complex glycolipid Gb_3[1] (Pellizzari et al., 1992; Kiarash et al., 1994). The same concept has been invoked to explain observations of certain inaccessible (i.e., "cryptic") sites on cell membranes (Koscielak et al., 1968; Hakomori, 1981; Kannagi et al., 1983; Lampio et al., 1986; Kiarash et

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* To whom correspondence should be addressed.
‡ University of Western Ontario.
§ Research Institute, The Hospital for Sick Children.

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al., 1994), noting that, 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, glycosphingolipids commonly possess hydroxylated fatty acids and ones that are 18−26 carbons in length. Systematic studies of model membranes containing galactosylceramide originated the somewhat general observation that glycolipids with longer fatty acids tend to interact more effectively with macromolecules (Alving et al., 1980). However, the sum of literature observations on crypticity is extremely complex. In the current experiments, we sought direct evidence of an association between glycolipid fatty acid chain length and conformational or dynamic behavior in globo-series species. The results appear to bear upon the field in general.

Forssman glycolipid and gangliosides have been considered with regard to the influence of hydrophobic domain modifications on receptor function (Uemura et al., 1979; Bunow & Bunow, 1979; Maggio et al., 1988; Masserini et al., 1988, 1989; Palestini et al., 1995). However, the only complex glycolipid for which crypticity has been extensively documented in model membranes as a function of its fatty acid is Gb_3, a triaosyl globoside found in kidney (Lingwood, 1993). Association of *Escherichia coli* verotoxins with Gb_3 has provided striking examples of receptor modulation by fatty acid in a variety of model and cell systems (Pellizzari et al., 1992; Kiarash et al., 1994). This family of pentavalent proteins binds with high discrimination to the terminal Galα1−4Gal sequence of glycosphingolipids in bilayer membranes (Strömberg et al., 1991) [but not to corresponding lysosphingolipids (Pellizzari et al., 1992)]. Verotoxin binding to human endothelial cells forms the basis for vascular pathology in hemorrhagic colitis and hemolytic uremic syndrome (Karmali, 1989; Lingwood, 1993). An important finding was that Gb_3 with longer chain fatty acids tended to be a better recognition site for the verotoxin VT1. Interestingly though, 18:1 Gb_3 was a better site for VT2c than was the 22:1 species, a quite unusual observation (Boyd et al., 1994; Arab & Lingwood, 1996).

Another globoside, the tetraglycosylated Gb_4 of erythrocytes, represents one of the earliest examples of recognition site sensitivity to structural perturbations. Indeed, the term "receptor crypticity" appears to have been coined by Hakomori and colleagues surrounding the observation that Gb_4 in adult human erythrocytes was relatively inaccessible to antibodies when compared to Gb_4 in fetal or trypsinized cells (Koscielak et al., 1968; Hakomori, 1981; Lampio et al., 1986).

The approach we have used in addressing the basis of these observations surrounding receptor function for Gb_3 and Gb_4 is wideline ^2H NMR spectroscopy. This technique permits direct examination of intact bilayer membrane systems comprising even quite complex groups of different molecules (Seelig, 1977; Davis, 1983; Smith, 1984). It is nonperturbing, and highly sensitive to molecular orientation and behavior. Somewhat to our surprise, in related studies involving other glycolipids, we have found little evidence of conformational sensitivity at one site to structural changes induced at others (Fenske et al., 1991; Singh et al., 1992a, 1995; Hamilton et al., 1994; Morrow et al., 1995; Jones et al., 1996), although motional properties appear to be affected in some cases (Morrow et al., 1995a).

Gb_3 having 18:1 or 22:1 fatty acid was prepared by partial synthesis, and deuterated in the exocyclic hydroxymethyl group of the terminal carbohydrate residue. ^2H NMR spectra of these two analogues were compared in unsonicated liposomes of 1,2-dipalmitoylphosphatidylcholine (DPPC) containing cholesterol, to simulate the system described by Kiarash et al. (1994). Gb_4 having 18:0 or 24:0 fatty acid was prepared by partial synthesis and deuterated on the acetate group of the terminal sugar residue and at C2 of the fatty acid. The Gb_4 acetate probe afforded ease of spectral analysis (Singh et al., 1995), and also optimized signal-to-noise, which permitted some relaxation time measurements. The deuterons in the fatty acid made it possible to consider the relationship between headgroup and hydrophobic interior. For Gb_4, NMR spectra were compared in 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), and in POPC/cholesterol to approximate erythrocyte membrane lipids.

MATERIALS AND METHODS

POPC and DPPC were obtained from Avanti Polar Lipids, Birmingham, AL. Cholesterol was from Sigma, St. Louis, MO. Ten milligrams of globotriaosylceramide (Gb_3) with 18:1 cis ω9 fatty acid (oleic acid) and the 22:1 cis ω13 analogue was prepared by partial synthesis from natural Gb_3 isolated from human kidney as described elsewhere (Kiarash et al., 1994). Deuterium labeling in the exocyclic hydroxymethyl group of the terminal Gal residue in Gb_3 was performed using galactose oxidase and NaB^2H_4 as previously described for other glycosphingolipids (Skarjune & Oldfield, 1979; Jarrell et al., 1992). The yield was typically 75−85%, as demonstrated by ^1H NMR in 98% DMSO-d_6/2% ^2H_2O. Tetraosyl globoside (Gb_4) was isolated from porcine erythrocytes and the natural fatty acid replaced with stearic (18:0) or lignoceric (24:0) acid deuterated at C2 as described elsewhere (Fenske et al., 1991). Acetamido sugar deuteration of Gb_4 and isolation and deuteration of GM_1 have been described previously (Singh et al., 1995).

Lipid samples were unsonicated multilamellar vesicles (MLV) prepared by hydration of films derived by rotary evaporation of 2:1 $CHCl_3/CH_3OH$ from lipid solutions at 50 °C and subsequent trace solvent removal under high vacuum. Hydration was carried out at 50 °C with 30 mM HEPES buffer, pH 7.4, containing 20 mM NaCl and 5 mM EDTA. Following repeated lyophilization from deuterium-depleted water, samples were rehydrated with deuterium-depleted water and freeze-thawed 5 times. The amount of labeled lipid used in each sample varied from 5 to 16 μmol in a total volume of 200 μL. For liposomes containing cholesterol, the composition was a 7.5:7.5:1 mole ratio of DPPC/cholesterol/Gb_3, or 12.7:6.3:1 of POPC/cholesterol/glycolipid. Samples not containing cholesterol were a 9:1 to 49:1 mole ratio phospholipid/glycolipid as indicated. Wideline ^2H NMR spectra were acquired at 76.7 MHz on a Varian Unity spectrometer with a Doty 5 mm solenoid probe. The $\pi/2$ pulse width was 5.2 μs. Pulse spacing in the quad echo sequence was 20 μs, with a repetition time of 100−200 ms. Spectra were acquired from high to low temperature after

[1] Abbreviations: PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-PC; DPPC, dipalmitoyl-PC; Gb_3, globotriaosylceramide (Galα1→4Galβ1→4Glcβ1→1ceramide); Gb_4, globotetraosylceramide (GalNAcβ1→3Galα1→4Galβ1→4Glcβ1→1ceramide); GM_1, Galβ1→3GalNAcβ1→4Gal(3←2αNeuAc)β1→4Glcβ1→1ceramide.

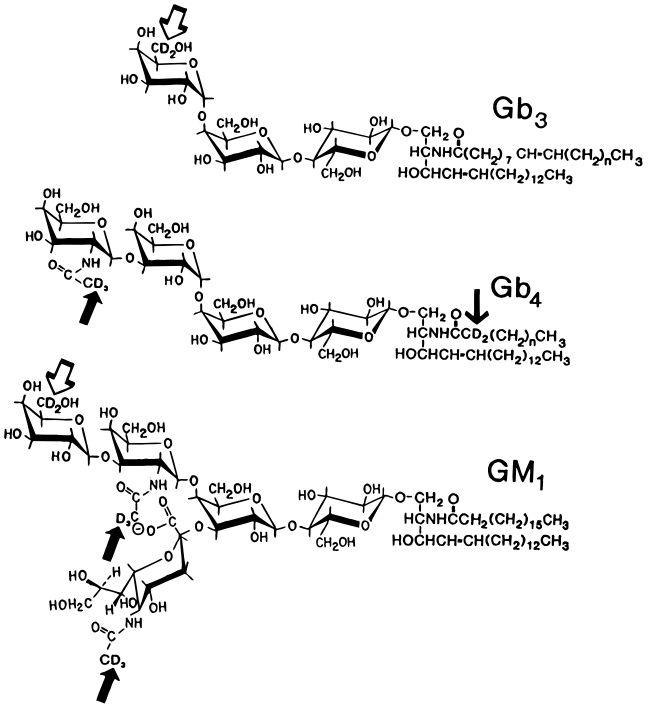

FIGURE 1: Glycolipid structures: locations of deuterium nuclei. Deuteration sites ("D") on terminal galactose exocyclic hydroxymethyl groups (hollow arrows) are indicated for the globotriaosylceramide, Gb_3, and for the ganglioside, GM_1. Nuclei at this sugar location have either *pro-R* or *pro-S* stereochemistry, and the hydroxymethyl group has been suggested to show restricted rotation (see text). Deuteration sites within amino sugar acetate groups are indicated (thick solid arrows) for the tetraosylceramide, Gb_4, and for GM_1. Independent motion of the rigid, planar, $-NHCOCD_3$ group about the C−N bond linking it to the sugar ring has been claimed to be highly restricted (see text). *pro-R* and *pro-S* stereochemistry exist for deuterons at fatty acid C2 in globotetraosyl ceramide (Gb_4) (thin solid arrow). For Gb_3, $n = 7$ or 11; for Gb_4, $n = 15$ or 21.

preequilibration in the spectrometer for 30 min. For T_2 measurements, powder spectrum peak heights were plotted as a function of $2\tau_1$ (where τ_1 is the delay between $\pi/2$ pulses), and "T_2 quadrupolar echo" was calculated using linear regression (Davis, 1979; Bloom & Sternin, 1987).

RESULTS

Figure 1 illustrates glycolipid structures and deuterium probe locations. The two deuterons ("D" in figures) on the exocyclic hydroxymethyl group (hollow arrows) of terminal galactose in Gb_3 and GM_1 have either *pro-R* or *pro-S* stereochemistry, and hence are spectrally inequivalent. This is also true for the deuterons at fatty acid C2 in Gb_4 (thin solid arrow). Evidence has been presented that rotation of the hydroxymethyl group about the bond attaching it to the sugar ring is restricted (Skarjune & Oldfield, 1979; Renou et al., 1989; Morrow et al., 1995a). Independent motion of the rigid, planar, $-NHCOC^2H_3$ group about the C−N bond linking it to amino sugars in Gb_4 and GM_1 (thick solid arrows) is considered to be highly restricted (Yadav & Luger, 1980; Acquotti et al., 1990; Poppe et al., 1990). The three methyl deuterons of this group are spectrally equivalent.

For molecules undergoing axially symmetric rapid rotation, ^2H NMR spectra are characterized by "Pake doublets". Spectral splittings measured at the point corresponding to 90° field orientation obey the simple relationship:

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

where e^2Qq/h is the nuclear quadrupole coupling constant [165−170 kHz for an aliphatic C−^{2}H 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−^{2}H bond relative to the molecular rotation axis. Superimposed unsplit very sharp central peaks are generally seen for membrane samples, reflecting the presence of some highly curved vesicles and traces of natural deuterated water (molecules reorienting isotropically on the NMR time scale). Broader centrally-peaked nondoublet spectra are common for molecules undergoing axially asymmetric rotation in membranes (Huang et al., 1980; Meier et al., 1986; Siminovitch et al., 1988; Auger et al., 1990). We have previously recorded the latter phenomenon for deuterated neutral glycosphingolipids in fluid membranes (Morrow et al., 1995a).

Deuterated globotriaosylceramides (Gb_3) have not been described previously. However, workers have employed sequential treatment with galactose oxidase and NaB^2H_4 to introduce ^2H in place of ^1H in the terminal galactose (or galactosamine) residue of other neutral glycolipids (Skarjune & Oldfield, 1979; Singh et al., 1995). The procedure appears to modify the *pro-R* and *pro-S* sites without appreciable stereoselectivity in the case of globotetraosylceramides (Gb_4) (Singh et al., 1995) and galactosylceramide (Skarjune & Oldfield, 1979; Morrow et al., 1995a). In the present work, we found that the terminal galactose of 18:1 and 22:1 Gb_3 could be efficiently deuterated *via* the same route. High-resolution ^1H NMR spectroscopy was used to characterize the products (Figure 2). Relevant ^1H NMR spectral peak assignments for globosides have been determined by previous workers (Alvarez et al., 1992): in Gb_3 from bovine spermatozoa, a doublet at 4.798 ppm was assigned to the proton at C′1 of the terminal Gal residue; a corresponding doublet can be seen at 4.78 ppm in Figure 2. They assigned the proton at C′5 of the same sugar in Gb_3 to a degenerate quartet (triplet) at 4.082 ppm. In agreement with this interpretation, for the Gb_3 from kidney used in the present work a triplet at 4.094 ppm collapsed toward a doublet and then toward a singlet with successive rounds of deuteration (Figure 2). However, the same workers also tentatively assigned a triplet at 3.875 ppm to one of the C′6 protons on terminal Gal. In our hands, the apparently corresponding spectral feature at 3.86 ppm (downfield of the methylene envelope) remained unaltered in splitting and intensity after deuteration, while intensity loss occurred in the region near 3.5 ppm in a pattern consistent with sugar hydroxymethyl protons (Figure 2). We suggest that in fact one C′6 proton is near 3.5 ppm, while the other is partially obscured by solvent and methylene peaks further upfield of this location. Connectivity between the peaks at 4.094 ppm (C′5) and the peaks near 3.5 ppm, and the lack of connectivity to the triplet at 3.86 ppm, was readily confirmed by 2-D NMR COSY experiments (data not shown).

Typical wideline ^2H NMR spectra for deuterated Gb_3 in DPPC/cholesterol bilayers are presented in Figure 3 for a range of temperatures. At high temperatures spectra of the short-chain species, 18:1 Gb_3, were approximated by a narrow doublet-like pattern. Thus, at 55 °C there is a 0.9

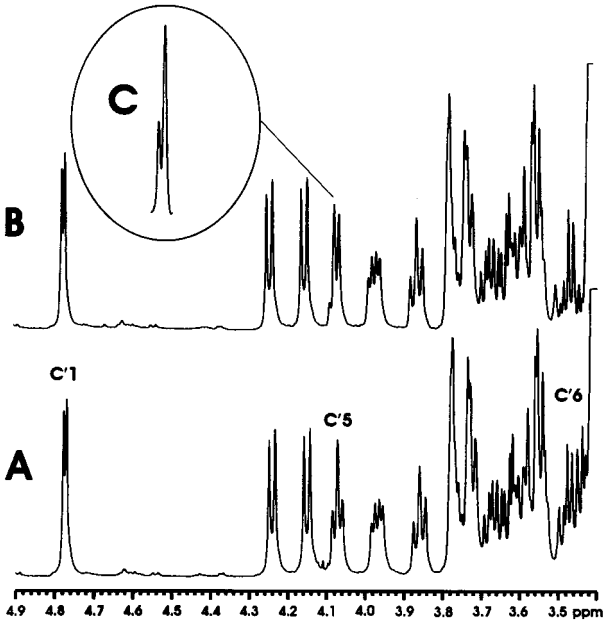

FIGURE 2: Deuteration of Gb_3. High resolution ^1H NMR spectra of pure Gb_3 dissolved in DMSO-d_6/2% ^2H_2O are shown for the 18:1 species prior to deuteration (A), after one round of oxidation/reduction (B), and after two rounds (insert C). Spectral locations of the terminal galactose protons at C′1, C′5, and C′6 are indicated in (A): note the progression of C′5 toward a singlet.

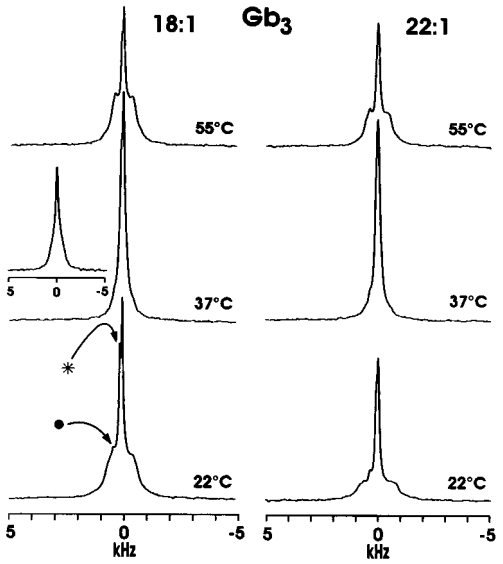

FIGURE 3: ^2H NMR spectra of Gb_3 in DPPC/cholesterol membranes. Stacked wideline spectra are presented for deuterated Gb_3 species with 18- *vs* 22-carbon fatty acids (left and right columns, respectively), at three temperatures. In each case, the glycolipid was 6.3 mol% in 1:1 DPPC/cholesterol liposomes, and probe nuclei were in the terminal Gal hydroxymethyl group. Very sharp minor component peaks are indicated arising from traces of ^2H in the water (*) and in the sphingolipid amide (●). Each spectrum represents 172 000 accumulated transients. The result of host matrix fluidization by the addition of POPC (final host membrane composition, 2:1:1 mole ratio POPC/DPPC/cholesterol) is indicated by the insert to the 18:1 spectrum at 37 °C.

kHz identifiable splitting (as measured at the 7/8 peak height outer shoulders generally taken to represent 90° orientation to the field). This temperature is well above the DPPC phase transition of 41.5 °C. For the longer-chain analogue, the spectrum remains similar (width at 7/8 height, 1.0 kHz); however, the presence of an identifiable splitting is questionable. As the temperature is lowered through the DPPC phase

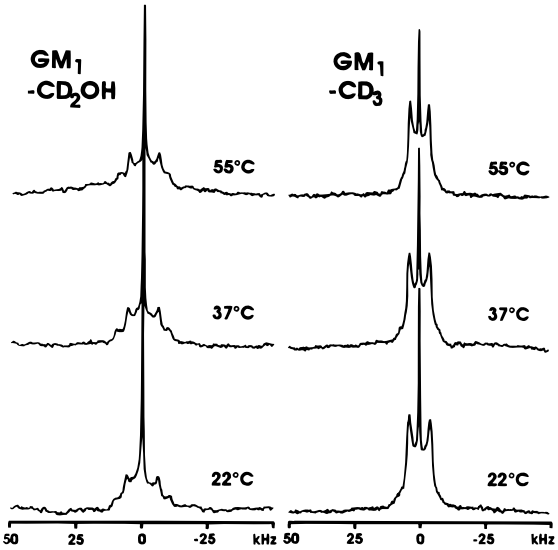

FIGURE 4: ^2H NMR spectra of GM_1 in DPPC/cholesterol membranes. Stacked wideline spectra are presented for 18:0 GM_1 deuterated in the terminal Gal hydroxymethyl group (left column) and in acetamide sugars (right column). GM_1 was dispersed at 6.3 mol% in 1:1 DPPC/cholesterol liposomes. Spectra have been normalised within each stack and represent 400 000 and 500 000 accumulated transients for the left- and right-hand spectra, respectively.

transition region, the spectrum of 18:1 Gb_3 displays markedly increased central buildup, suggesting asymmetric motion (Figure 3). The spectral appearance and changes seen as a function of temperature are virtually identical for 22:1 Gb_3. At 22 °C, with increased host matrix order, the spectrum has collapsed to a clearly axially asymmetric result—width 1.2 kHz for 18:1 Gb_3 and 1.6 kHz for 22:1 Gb_3—without intensity loss. Modifications in sample history such as heating to 75 °C with various cooling and rewarming schedules did not alter the results shown.

The above spectral results were checked by reisolating the glycolipid, subjecting it to a second round of oxidation/reduction, and reassembling it into DPPC/cholesterol bilayers. Resultant spectra were not significantly altered from those presented in Figure 3. In order to verify that the narrowed spectrum did not reflect some artifactual peculiarity of the host matrix such as glycolipid exclusion from very stiff 1:1 DPPC/cholesterol bilayers, the 18:1 sample was remade in a more fluid form. The sample was dried and redissolved in $CHCl_3/CH_3OH$, and a 2-fold ratio of POPC to DPPC was added prior to rotary evaporation and rehydration. Once again, the spectral result was similar (Figure 3, insert). Limited sample availability precluded a wider range of experiments, and reisolation of material was associated with substantial losses.

In an attempt to pursue the implications of the spectral changes seen for Gb_3, another complex glycosphingolipid, GM_1, was examined in the same DPPC/cholesterol membranes at the same concentration and temperatures since it has a better characterized degree of conformational order at membrane surfaces (Jarrell et al., 1992; Aubin et al., 1993; Jones et al., 1996). Typical results of such experiments are presented for this ganglioside deuterated in terminal and internal sugar residues (Figure 4). Temperature reduction did not lead to either shape changes or intensity losses for any of the three probe locations. Results were confirmed with samples from separate syntheses and lipid lots. The

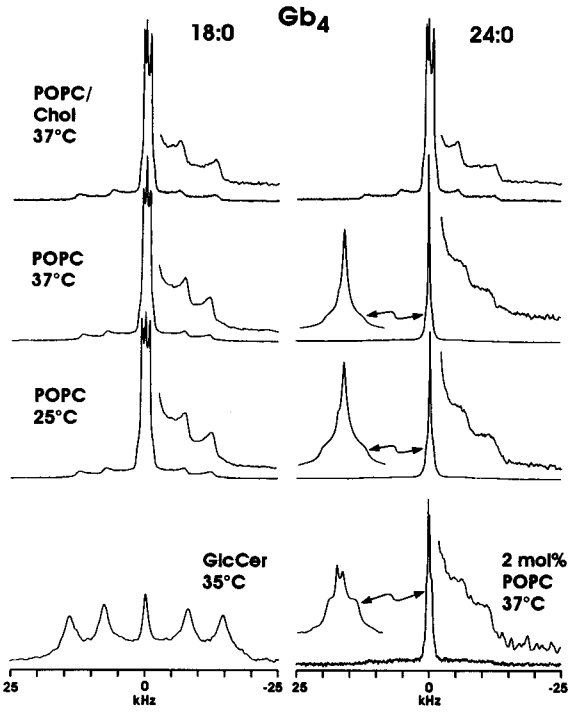

FIGURE 5: ^{2}H NMR spectra of Gb_4 in POPC and POPC/cholesterol membranes. Wideline spectra are presented for deuterated Gb_4 species with 18- *vs* 24-carbon fatty acids (left and right sides, respectively). Probe nuclei are in the terminal acetamido sugar, and at C2 of the fatty acid. The uppermost two spectra represent 5 mol% Gb_4 in 2:1 POPC/cholesterol. The middle four spectra represent 10 mol% Gb_4 in POPC without cholesterol. The lowest two spectra show glucosylceramide with fatty acid deuterated at C2 (bottom left) for peak assignment purposes, and deuterated Gb_4 (with 24-carbon fatty acid) diluted to 2 mol% in POPC (bottom right). At the right-hand side of each Gb_4 spectrum, the peaks representing the C2 (fatty acid) deuterons have been shown on vertically expanded scales. For Gb_4 with 18-carbon fatty acid, the sugar acetate methyl deuterons give rise to a single clearly resolved more central doublet with a small splitting. This is also true for the 24-carbon Gb_4 in POPC/cholesterol membranes. In contrast, for Gb_4 with 24-carbon fatty acid in POPC alone, this doublet is infilled to a broad single peak, likely as a result of different motional properties. This is demonstrated on an expanded frequency axis of 3.15 kHz to the left in each case (double arrows). Note that the same infilling phenomenon is also present in the C2 peaks for the 24-carbon species in POPC alone. Each spectrum represents 300 000 accumulated transients.

Pake "doublet" arising from acetate-labeled GM_1 (right-hand column) can be resolved upon axis expansion into two closely overlapping components from the acetate residues of NANA and GalNAc (Jones et al., 1996). The corresponding quadrupole splittings were 8.5, 9.1, and 9.6 kHz (inner Pake doublet: GalNAc) and 10.7, 11.0, and 11.0 kHz (outer doublet: NANA) at 55, 37, and 22 °C, respectively. For the better resolved Pake doublets of deuterons in the terminal Gal hydroxymethyl group (left column), quadrupole splittings were 11.7, 12.3, and 12.8 kHz (inner) and 19.9, 20.2, and 21.4 kHz (outer) at 55, 37, and 22 °C, respectively.

Spectra related to the question of fatty acid/oligosaccharide communication in globotetraosyl species (Gb_4) are presented in Figure 5. Results in POPC membranes are importantly different for short- *vs* long-chain Gb_4. For 18:0 Gb_4 there is a very intense inner Pake doublet associated with the $-C^2H_3$ group, and two outer Pake doublets of low intensity associated with the two spectrally inequivalent deuterons at C2 of the fatty acid. The assignment of the fatty acid deuteron spectral features has been demonstrated in Figure 5 using the monoglycosyl species glucosylceramide, which comprises the ceramide portion and first sugar residue of globosides and which was deuterated only in the fatty acid (Fenske et al., 1991). 24:0 Gb_4 clearly exhibits features corresponding to those observed for the shorter chain analogue, and almost identical spectral width. But in the long-chain case, the central doublet displays central intensity buildup that obscures the splitting, suggesting asymmetric motion. This is reminiscent of the phenomenon recorded above for Gb_3 with long-fatty acid chain, but more striking. Note that central buildup still occurs with reduction of the Gb_4 content from 10 mol% to only 2% of total lipid (Figure 5), although there appears to be some return of spectral shape toward Pake nature in that the shoulders are more defined at 2 mol%. Addition of cholesterol to the POPC host matrix largely eliminated spectral asymmetry from the long-chain Gb_4 spectrum (Figure 5). As was found above for Gb_3, the spectral widths for long- and short-chain species were within experimental error of one another for a given host matrix and temperature (Table 1). Temperature effects on this system were modest, as expected since POPC has a phase transition temperature of −3 °C (Davis & Keough, 1985).

Table 1: Gb_4 Quadrupole Splittings, $\Delta\nu_Q$

	$\Delta\nu_Q$ in kHz (±0.2 for $-C^2H_3$; ±0.5 for $>C^2H_2$)					
	25 °C			37 °C		
host matrix	$-C^2H_3$	$>C^2H_2$		$-C^2H_3$	$>C^2H_2$	
C18 POPC	1.7	15.3	25.5	1.5	15.0	24.4
C18 POPC/chol	–	–	–	1.4	12.9	26.3
C24 POPC	1.6	12.7	24.2	1.3	12.9	21.0
C24 POPC/chol	–	–	–	1.5	11.1	25.1

Table 2: T_2 Quadrupolar Echo Values for Gb_4[a]

	T_2 in μs (±10% for $-C^2H_3$; ±20% for $>C^2H_2$)					
	25 °C			37 °C		
host matrix	$-C^2H_3$	$>C^2H_2$		$-C^2H_3$	$>C^2H_2$	
C18 POPC	1179	477	342	1229	510	350
C18 POPC/chol	–	–	–	1333	518	440
C24 POPC	701	326	223	828	364	217
C24 POPC/chol	–	–	–	1196	362	403

[a] T_2 values listed are "T_2 quadrupolar echo" (Davis, 1979; Bloom & Sternin, 1987). T_2 was calculated by linear regression, and in view of the limited signal-to-noise, measurements of two investigators were averaged for this purpose.

^{2}H NMR transverse relaxation times (T_2) offer the potential of sensitivity to a wide range of processes, including slow ones that often typify membrane components (Davis, 1979; Bloom & Sternin, 1987; Weisz et al., 1992; Köchy & Bayerl, 1993). To our knowledge, this measurement has not been previously reported for glycosphingolipids. In the present work, we tested the approach on the Gb_4 samples. Results are listed in Table 2. 24:0 Gb_4 had significantly shorter relaxation times than 18:0 Gb_4 in POPC without cholesterol. The differences were not outside experimental error in the presence of cholesterol. Probe nuclei at C2 within the fatty acid had significantly shorter relaxation times in both matrices than did carbohydrate probes.

DISCUSSION

Spectral asymmetry and narrow width impose limitations on the interpretation of the Gb_3 experiments. However,

examination of Figure 3 shows that the features seen for short- and long-chain fatty acid species as a function of temperature are similar to the point that they appear inconsistent with the existence of global conformational differences. In particular, at 55 °C the longer chain (22:1) Gb_3 species demonstrated the same spectrum as the 18:1 species, but with greater evidence of axial asymmetry (i.e., greater central buildup, see below). Both displayed identical axially asymmetric centrally-peaked spectra at 37 °C. This is remarkable, given the sensitivity of such asymmetric spectra to subtleties of probe motion. Spectra for short- and long-chain species collapsed to very similar, clearly axially asymmetric, patterns in the highly ordered DPPC/cholesterol host matrix at 22 °C. At 55 and 37 °C, the measured spectral widths for short- and long-chain species are well within experimental error of one another. At 22 °C, the 22:1 Gb_3 spectral width was marginally greater than for 18:1 Gb_3.

The narrow spectra seen for Gb_3 with short- or long-chain fatty acid could potentially be associated simply with highly disordered motion of each deuterated sugar residue. However, the similarly deuterated terminal sugar of Gb_4 produced quadrupole splittings of almost 20 kHz even in very fluid membranes (Singh et al., 1995). Thus, if the narrow spectrum for Gb_3 results from disorder alone, the terminal sugar residue of the trisaccharide Gb_3 headgroup is considerably more disordered than is the terminal sugar of the longer, but otherwise identical, Gb_4 headgroup. We suggest a more likely explanation of the Gb_3 spectral narrowness is that motion of the terminal sugar in Gb_3 is intrinsically asymmetric, with resultant collapse of quadrupole splittings (Huang et al., 1980; Meier et al., 1986; Siminovitch et al., 1988; Auger et al., 1990). We have demonstrated by spectral simulation that similar narrow, centrally-peaked spectra can arise from asymmetric motion of a simpler glycolipid, galactosylceramide, deuterated (as here) in the sugar exocyclic hydroxymethyl group (Morrow et al., 1995a). The suggestion of greater spectral asymmetry for Gb_3 with a longer fatty acid is also reminiscent of previous findings for galactosylceramide (Morrow et al., 1995a). We cannot exclude a third possibility: that the terminal sugar residue of Gb_3 is by chance oriented in such a fashion that both C−^{2}H bonds on galactose approach the magic angle of 54.7° to the axis of motion (resulting in vanishingly small splittings). However, if so, the conclusion must be once again that the conformation adopted by the carbohydrate chain of Gb_3 is identical in 18:1 and 22:1 species.

DPPC has a phase transition temperature of 41.5 °C (Hinz & Sturtevant, 1972; Shimshick & McConnell, 1973). In the presence of cholesterol, it exhibits complex and somewhat controversial phase behavior [reviewed recently in Vist and Davis (1990), Sankaram and Thompson (1990), Reinl et al. (1992), Silvius (1992), and McMullen and McElhaney (1995)]. However, it seems reasonable to anticipate a significantly stiffened matrix 20 °C below the phase transition temperature of pure DPPC, and a considerably more fluid membrane above 41.5 °C. In previous work, using 32 mol% cholesterol in DPPC as host matrix, rotational diffusion of the glycosphingolipid galactosylceramide was found to be severely restricted at 22 °C (Hamilton et al., 1994). Hence, we were not surprised to see evidence of slowed rotational motion and spectral asymmetry at temperatures well below 41.5 °C. Indeed, it is more noteworthy that the Gb_3 headgroup retained enough motion to present a narrow spectrum in DPPC/cholesterol at 22 °C. The experiments with deuterated GM_1 in DPPC/cholesterol were originally performed to elaborate on this result. GM_1 (with an 18-carbon fatty acid) was chosen for its anticipated high oligosaccharide conformational order based on previous measurements in membranes containing GM_3 (Aubin et al., 1993) and GM_1 (Jarrell et al., 1992; Singh et al., 1995). GM_1, which had almost exclusively 18-carbon fatty acid, showed no tendency to asymmetry, or to intensity loss at low temperature. Pake powder spectra were obtained, and splittings increased slightly, consistent with increasing motional order as the temperature was reduced (Figure 4). This was true for both types of probe location (hydroxymethyl and acetate). The conclusion would seem to be that in 1:1 DPPC/cholesterol globosides and GM_1 retain considerable rotational freedom. The greater spectral effect on Gb_3 than on GM_1 may reflect the exceptional sensitivity of asymmetric spectra to subtle motional alterations.

The globoside Gb_4 from erythrocytes was studied in a system intended to approximate erythrocyte lipids. Probe nuclei existed in the terminal sugar and at C2 of the fatty acid. Although Gb_4 receptor function has not been examined by previous workers in such a systematic study of fatty acid influence as has Gb_3, it possesses the same chemical structure—the only difference being the addition of a fourth sugar residue (Figure 1). For each probe location, spectral widths at a given temperature in a given host matrix were the same for 18:0 and 24:0 Gb_4 within experimental error. Thus, as has been our experience in the past with glycosphingolipids, a key result seems to be similarity of carbohydrate conformation and order parameter among species having different fatty acids (Singh et al., 1992a, 1995; Morrow et al., 1995). The lack of headgroup conformational sensitivity is also in agreement with our recent findings in ganglioseries lipids: that even removal of sialic acid residues resulted in at most very modest change in conformation or order (Jones et al., 1996).

There was, however, some evidence of greater spectral asymmetry for Gb_4 with longer fatty acid, as also suggested above for Gb_3 at 55 °C. This was again manifest as greater central spectral intensity buildup, which partially obscured (infilled) the quadrupole splittings. It was seen most clearly in POPC membranes without cholesterol (Figure 5). Temperature increase did not reduce the effect, which rules out the possibility that it reflects intermediate time scale axial rotation. We have in the past recorded differences in phase behavior for long- *vs* short-chain glycolipids in phospholipid bilayers near a gel phase boundary (Lu et al., 1993). Hence, one might suggest greater phase separation of the long-chain species as a cause of axially asymmetric rotation for 24:0 Gb_4. Certainly in the absence of cholesterol, one might anticipate some phase separation of glycolipid-enriched domains in POPC at temperatures below 30 °C (Curatolo, 1986; Rock et al., 1990; Lu et al., 1993). However, persistence of the spectral asymmetry at high temperature in the present work seems to rule out simple phase separation as the underlying phenomenon, just as it rules out restricted rotation.

The fact that Gb_4 spectral asymmetry was seen in POPC even with only 2 mol% glycolipid (Figure 5) suggests that the longer chain species has a strong intrinsic tendency to move differently. The fact that spectral asymmetry was simultaneously observed at fatty acid C2 and in the distal

sugar portion suggests that the motion involved was whole body motion (e.g., glycolipid axial diffusion). It is not solely due to formation of small vesicles (see below) because the spectral width of the C2 deuterons was some 26 kHz, and their line shape was affected in the same manner as was that of the acetate group. As mentioned above with regard to Gb_3, we have previously noted spectral features associated with the asymmetry of motion for GalCer whose fatty acid chain is significantly different from host matrix fatty acids (Morrow et al., 1995a). In the case of Gb_4 in POPC, this effect is striking. Furthermore, in agreement with the finding that the long-chain species demonstrated infilling of the Pake powder pattern, our previous studies of Gb_4 with natural (long) fatty acid mixture also showed unresolved acetate group splitting (Singh et al., 1995). It is interesting that addition of cholesterol to the POPC host matrix largely removed spectral asymmetry from the long-chain Gb_4 spectrum (Figure 5).

Although we do not favor simple phase separation as the primary source of the differences recorded here between glycolipids with short- *vs* long-chain fatty acids, there is a highly relevant literature surrounding glycolipid phase behavior in phospholipid membranes. Bunow and Bunow (1979) suggested a thermodynamic rationale whereby longer chain fatty acids on glycolipids could be expected to lead to greater phase separation in cell membranes, with possible significance to GM_1/toxin interactions. Bunow and Levin (1988) found phase behavior differences for GalCer in SOPC based on structural differences in the hydrophobic portion of the glycolipid. Masserini et al. (1988, 1989) have measured phase separation differences by DSC for gangliosides in DPPC based on glycolipid fatty acid chain length and unsaturation. They found that the protein *Vibrio cholerae* sialidase, can differentially recognize populations of GD_{1A} having different sphingosine chain length, and they suggested that this is a reflection of lateral distribution differences. Differential phase partitioning of complex glycolipids having different fatty acid chain lengths at very low concentrations in two-component phospholipid host matrices has been demonstrated [Rock et al., 1990; Palestini et al., 1995 (see also Ali et al., 1991; Singh et al., 1992b; and Palestini et al., 1995)]. Nevertheless, although there clearly appear to be forces that predispose toward glycolipid distribution differences based on fatty acid structure, several groups including our own have cautioned that such distribution differences need not be large (Bunow & Levin, 1988; Gardam & Silvius, 1989; Mehlhorn et al., 1989).

In seeking commonality between our results and the literature, we would emphasise a discovery by Curatolo and Neuringer (1986). They studied galactosylceramide in membranes of DPPC and POPC, noting that 10−20% concentrations of glycolipid could increase bilayer curvature markedly. Typically, the effect was more pronounced for species with longer chain fatty acids, and was reversed by addition of 10% cholesterol. In a closely related vein, Maggio et al. (1988) have remarked that small changes in concentration of a wide variety of natural glycolipids can bring about "remarkable variations" in the radius of curvature of DPPC bilayers. Uemura et al. (1979) measured effects of modifications to the Forssman glycolipid hydrophobic domain on its receptor function and concluded that "the physicochemical nature {of the overall epitope} may be modified". As early as 1981, Hinz et al. suggested that differences in thermodynamic and structural properties of sonicated DPPC vesicles containing GM_1 *vs* GD_{1A} might reflect the higher content of longer chain sphingosine in the latter. Such observations apparently reflect the presence of thermodynamic forces that differentiate short- from long-chain fatty acid species in terms of physical behavior, perhaps based on fit/solubility within a given matrix (Alving et al., 1980). A seeming basis for this exists in that midplane crossing by long-chain glycolipid fatty acids even in fluid membranes can be associated with adverse steric effects (Jeffrey et al., 1989; Morrow et al., 1993).

The question arises as to how forces based on fatty acid variation might be translated into modulation of receptor function if there is no general carbohydrate conformational effect. A mechanism that seems consistent with our observations and those in the literature is that these forces—which in some cases are sufficient to grossly alter lipid lateral distribution, motional properties, and membrane curvature—result in an overall modification of the multi-glycolipid epitope seen by macromolecules as suggested by Masserini et al. (1988, 1989). Such a scenario is particularly likely to have relevance for multivalent macromolecules, whose binding events are extremely sensitive to receptor spacing and orientation. This concept has its basis in early work demonstrating the importance of lipid hapten density and mobility in determining the epitope for macromolecule binding [see especially Brulet and McConnell (1977), Suzuki et al. (1981), Podder et al. (1981), Balakrishnan et al. (1982), Utsumi et al. (1984), and Grant and Peters (1984)]. The same workers and others (Stanton et al., 1984; Mehlhorn et al., 1988; Stewart & Boggs, 1990) also specifically emphasized the importance of cholesterol. This logic would explain the interesting verotoxin binding properties of Gb_3 (Kiarash et al., 1994), including the fact that Gb_3 with heterogeneous fatty acids showed a marked preference for VT1 (*vs* VT2c) that was not shown by any of the pure species . A related issue is that optimally favorable situations for binding may be limited at any moment in time to a subpopulation of receptors, and that fatty acid nature influences subpopulation size. This would be particularly likely if binding were improved by local deviations from a more general physical array (e.g., transient increase in receptor density). Such logic may explain why macromolecule binding events to lipid haptens are commonly improved by fatty acid disparities.

^{2}H wideline NMR relaxation times, T_2, have not been measured previously for receptors. Partly this reflects the fact that their measurement and interpretation in membrane systems is a complex subject (Davis, 1979; Bloom & Sternin, 1987; Köchy & Bayerl, 1993). In the present work, we tested T_2 sensitivity to receptor behavioral differences based on fatty acid nature. The values determined essentially underscore the spectral line shape observations described above. Thus, the longer chain species had shorter measured values of T_2, and the differences were really only significant in POPC bilayers without cholesterol. Shorter T_2 values could arise from higher membrane curvature (Bloom & Sternin, 1987), although as already mentioned the spectral width associated with C2 deuterons argues against very high curvature: altered diffusion is a more likely cause. It seems reasonable to suggest that the shorter T_2 values observed for the fatty acid C2 deuterons are a reflection of restricted motional properties typical of fatty acid methylene groups near the membrane surface.

CONCLUSIONS

A number of observations related to effects of glycosphingolipid fatty acids on recognition of the carbohydrate portion by macromolecules are explainable in terms of a phenomenon noted in the work of Curatolo and Neuringer (1986) [see also Maggio et al. (1988)]: that the thermodynamics of phospholipid bilayer membranes are subject to control by the fatty acid nature of contained glycolipids and that this can be modified by the presence of cholesterol. In extreme cases, effects include grossly altered membrane curvature. More subtle effects include variations in lipid arrangement and dynamics. We propose that the resultant overall epitope variations are often most sensitively distinguished by specific recognition events, which place high demands on intermolecular relationships at constraining (membrane) surfaces. Such a proposal has a basis in much early work by other groups (Brulet & McConnell, 1977; Suzuki et al., 1981; Podder et al., 1981; Balakrishnan et al., 1982; Utsumi et al., 1984). Multidentate macromolecules may be particularly sensitive to subtle differences based on their need for favorable receptor density (Podder et al., 1981; Utsumi et al., 1984; Grant & Peters, 1984).

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REFERENCES

Acquotti, D., Poppe, L., Dabrowski, J., von der Leith, C.-W., Sonnino, S., & Tettamanti, G. (1990) *J. Am. Chem. Soc. 112*, 7772−7778.
Ali, S., Brockman, H., & Brown, R. (1991) *Biochemistry 30*, 7488−7494.
Alvarez, J. G., Storey, B. T., Hemling, M. L., & Grob, R. L. (1992) *J. Liquid Chromatogr. 15*, 1621−1638.
Alving, C. R., Urban, K. A., & Richards, R. L. (1980) *Biochim. Biophys. Acta 600*, 117−125.
Arab, S., & Lingwood, C. A. (1996) *Glycoconjugate J. 13*, 159−166.
Aubin, Y., Ito, Y., Paulson, J. C., & Prestegard, J. H. (1993) *Biochemistry 32*, 13405−13413.
Auger, M., Van Calsteren, M.-R., Smith, I. C. P., & Jarrell, H. (1990) *Biochemistry, 29*, 5815−5821.
Balakrishnan, K., Mehdi, S. Q., & McConnell, H. M. (1982) *J. Biol. Chem. 257*, 6434−6439.
Bloom, M., & Sternin, E. (1987) *Biochemistry 26*, 2101−2105.
Boyd, B., Zhiuyan, Z., Magnusson, G., & Lingwood, C. A. (1994) *Eur. J. Biochem. 223*, 873−878.
Brulet, P., & McConnell, H. M. (1977) *Biochemistry 16*, 1209−1218.
Bunow, M. R., & Bunow, B. (1979) *Biophys. J. 27*, 325−337.
Bunow, M. R., & Levin, I. W. (1988) *Biochim. Biophys. Acta 939*, 577−586.
Curatolo, W. (1986) *Biochim. Biophys. Acta 861*, 373−376.
Curatolo, W. (1987a) *Biochim. Biophys. Acta 906*, 111−136.
Curatolo, W. (1987b) *Biochim. Biophys. Acta 906*, 137−160.
Curatolo, W., & Neuringer, L. J. (1986) *J. Biol. Chem. 261*, 17177−17182.
Davis, J. H. (1979) *Biophys. J. 27*, 339−358.
Davis, J. H. (1983) *Biochim. Biophys. Acta 737*, 117−171.
Davis, P. J., & Keough, K. M. W. (1985) *Biophys. J. 48*, 915−918.
Fenske, D. B., Hamilton, K., Jarrell, H. C., Florio, E., Barber, K. R., & Grant, C. W. M. (1991) *Biochemistry 30*, 4503−4509.
Gardam, M., & Silvius, J. R. (1989) *Biochim. Biophys. Acta 980*, 319−325.
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. (1989) *Adv. Cancer Res. 52*, 257−331.
Hamilton, K. S., Briere, K., Jarrell, H. C., & Grant, C. W. M. (1994) *Biochim. Biophys. Acta 1190*, 367−375.
Hinz, H.-J., & Sturtevant, J. M. (1972) *J. Biol. Chem. 247*, 6071−6075.
Hinz, H.-J., Körner, O., & Nicolau, C. (1981) *Biochim. Biophys. Acta 643*, 557−571.
Huang, T. H., Skarjune, R. P., Wittebort, R. J., Griffin, R. G., & Oldfield, E. (1980) *J. Am. Chem. Soc. 102*, 7377−7379.
Jarrell, H., Singh, D., & Grant, C. W. M. (1992) *Biochim. Biophys. Acta 1103*, 331−334.
Jeffrey, K. R., Boggs, J. M., Koshy, K. M., & Tulloch, A. P. (1989) *Biochim. Biophys. Acta 986*, 241−249.
Jones, D. H., Barber, K. R., & Grant, C. W. M. (1996) *Biochemistry 35*, 4803−4811.
Kannagi, R., Stroup, R., Cochran, N. A., Urdal, D. L., Young, W. W., Jr., & Hakomori, S.-I. (1983) *Cancer Res. 43*, 4997−5005.
Karmali, M. A. (1989) *Clin. Microbiol. Rev. 2*, 15−38.
Kiarash, A., Boyd, B., & Lingwood, C. A. (1994) *J. Biol. Chem. 269*, 11138−11146.
Köchy, T., & Bayerl, T. M. (1993) *Phys. Rev. E 47*, 2109−2116.
Koscielak, J., Hakomori, S., & Jeanoz, R. W. (1968) *Immunochemistry 5*, 441−455.
Lampio, A., Rauvala, H., & Gahmberg, C. G. (1986) *Eur. J. Biochem. 157*, 611−616.
Lingwood, C. A. (1993) *Adv. Lipid Res. 25*, 189−211.
Lu, D., Singh, D. M., Morrow, M. R., & Grant, C. W. M. (1993) *Bichemistry 32*, 290−297.
Maggio, B., Albert, J., & Yu, R. K. (1988) *Biochim. Biophys. Acta 945*, 145−160.
Masserini, M., Palestini, P., Venerando, B., Fiorilli, A., Acquotti, D., & Tettamanti, G. (1988) *Biochemistry 27*, 7973−7978.
Masserini, M., Palestini, P., & Freire, E. (1989) *Biochemistry 28*, 5029−5034.
McMullen, T. P. W., & McElhaney, R. N. (1995) *Biochim. Biophys. Acta 1234*, 90−98.
Mehlhorn, I. E., Florio, E., Barber, K. R., Lordo, C., & Grant C. W. M. (1988) *Biochim. Biophys. Acta 939*, 151−159.
Mehlhorn, I. E., Barber, K. R., Florio, E., & Grant C. W. M. (1989) *Biochim. Biophys. Acta 986*, 281−289.
Meier, P., Ohmes, E., & Kothe, G. (1986) *J. Chem. Phys. 85*, 3598−3617.
Morrow, M. R., Singh, D. M., Lu, D., & Grant, C. W. M. (1993) *Biophys. J. 64*, 654−664.
Morrow, M. R., Singh, D. M., & Grant, C. W. M. (1995a) *Biophys. J. 69*, 955−964.
Morrow, M. R., Singh, D. M., & Grant, C. W. M. (1995b) *Biochim. Biophys. Acta 1235*, 239−248.
Palestini, P., Allietta, M., Sonnino, S., Tettamanti, G., Thompson, T. E., & Tillack, T. W. (1995) *Biochim. Biophys. Acta 1235*, 221−230.
Pellizzari, A., Pang, H., & Lingwood, C. A. (1992) *Biochemistry 31*, 1363−1370.
Podder, S. K., Surolia, A., & Bachhawat, B. K. (1991) *Ind. J. Biochem. Biophys. 18*, 322−325.
Poppe, L., von der Leith, C.-W., & Dabrowski, J. (1990) *J. Am. Chem. Soc. 112*, 7762−7771.
Reinl, H., Brumm, T., & Bayerl, T. (1992) *Biophys. J. 61*, 1025−1035.
Renou, J.-P., Giziewicz, J. B., Smith, I. C. P., & Jarrell, H. C. (1989) *Biochemistry 28*, 1804−1814.
Rock, P., Allietta, M., Young, W. W., Jr., Thompson, T. E., & Tillack, T. W. (1990) *Biochemistry 29*, 8484−8490.
Sankaram, M., & Thompson, T. E. (1990) *Biochemistry 29*, 10676−10684.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353−418.
Shimshick, E. J., & McConnell, H. M. (1973) *Biochemistry 12*, 2351−2360.
Silvius, J. R. (1992) *Biochemistry 31*, 3398−3408.
Siminovitch, D. J., Ruocco, M. J., Olejniczak, E. T., Das Gupta, S. K., & Griffin, R. G. (1988) *Biophys. J. 54*, 373−381.
Singh, D., Jarrell, H. C., Barber, K. R., & Grant, C. W. M. (1992a) *Biochemistry 31*, 2662−2669.
Singh, D., Davis, J. H., & Grant, C. W. M. (1992b) *Biochim. Biophys. Acta 1107*, 23−30.

Singh, D. M., Shan, X., Davis, J. H., Jones, D., & Grant, C. W. M. (1995) *Biochemistry 34*, 451−463.
Skarjune, R., & Oldfield, E. (1979) *Biochim. Biophys. Acta 556*, 208−218.
Smith, I. C. P. (1984) *Biomembranes 12*, 133−168.
Stanton, S. G., Kantor, A. B., Petrossian, A., & Owicki, J. C. (1984) *Biochim. Biophys. Acta 776*, 228−236.
Stewart, R. J., & Boggs, J. M. (1990) *Biochemistry 29*, 3644−3653.
Stewart, R. J., & Boggs, J. M. (1993a) *Biochemistry 32*, 5605−5614.
Stewart, R. J., & Boggs, J. M. (1993b) *Biochemistry 32*, 10666−10674.
Strömberg, N., Nyholm, P.-G., Pascher, I., & Normark, S. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9340−9344.
Suzuki, T., Utsumi, H., Inoue, K., & Nojima, S. (1981) *Biochim. Biophys. Acta 644*, 183−191.
Thompson, T. E., & Tillack, T. W. (1985) *Annu. Rev. Biophys. Biophys. Chem. 14*, 361−386.
Uemura, K., Yuzawa, M., & Taketomi, T. (1979) *Jpn J. Exp. Med. 49*, 1−8.
Utsumi, H., Suzuki, T., Inoue, K., & Nojima, S. (1984) *J. Biochem. 96*, 97−105.
Vist, M. R., & Davis, J. H. (1990) *Biochemistry 29*, 451−464.
Waddell, T., Head, S., Petric, M., Cohen, A., & Lingwood, C. A. (1988) *Biochem. Biophys. Res. Commun. 152*, 674−679.
Weisz, K., Gröbner, G., Mayer, C., Stohrer, J., & Kothe, G. (1992) *Biochemistry 31*, 1100−1112.
Yadav, J. S., & Luger, P. (1983) *Carbohydr. Res. 119*, 57−73.

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