Glycosphingolipids: ^2H NMR Study of the Influence of Ceramide Fatty Acid Characteristics on the Carbohydrate Headgroup in Phospholipid Bilayers†

Dev Singh,‡ Harold C. Jarrell,*,§ Kathryn R. Barber,‡ and Chris W. M. Grant*,‡

Department of Biochemistry, The University of Western Ontario, London, Ontario, Canada N6A 5C1, and Division for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6

Received October 25, 1991; Revised Manuscript Received December 23, 1991

ABSTRACT: Galactosylceramides bearing a variety of different pure fatty acid chains were ^2H labeled in the carbohydrate headgroup at C_6 of the terminal galactose residue, for study by ^2H NMR. Fatty acids investigated included the 24-carbon saturated lignoceric acid, 18-carbon saturated stearic acid, *cis*-9,10-unsaturated oleic acid, and D- and L-stereoisomers of α-hydroxystearic acid. Headgroup-deuterated glycolipids were incorporated at 10 mol % into unsonicated bilayers of 1-palmitoyl-2-oleoylphosphatidylcholine, and ^2H NMR spectra were recorded at 65 and 40 °C. Under these experimental conditions, the membranes studied were primarily in the liquid-crystalline phase. At a given temperature, spectra for deuterated galactosylceramides dispersed in the fluid phase were remarkably similar, regardless of the nature of the fatty acid attached to the glycolipid sphingosine backbone. In each case, the spectrum consisted of a superposition of two quadrupolar powder patterns of approximately equal intensity. The spectra may be interpreted as arising from equal populations of two stereoisomers (*pro-R* and *pro-S*) of the deuterated galactose hydroxymethyl function, which is undergoing rapid ($>10^6$ s^{-1}) interconversion among the possible rotamers about the C_5–C_6 bond of the sugar ring. Within experimental error, the only fatty-acid-induced spectral difference detected among these glycosphingolipids deuterated in the carbohydrate headgroup was in the species with α-hydroxy-substituted fatty acids. At 65 °C, *N*-(D-α-hydroxy)stearoyl- and *N*-(L-α-hydroxy)stearoylgalactosylceramide gave rise to the same quadrupole splittings, but these differed marginally from the splittings observed for the other glycolipids studied. At 40 °C, the $\Delta\nu_Q$ values, for the L-α-hydroxy species only, differed measurably from those of the other fatty acid derivatives. Our results would argue that, at least in the case of galactosylceramide in fluid phospholipid bilayers, very little change in the average glycosphingolipid carbohydrate headgroup orientation (probably less than a few degrees), and in the amplitude of orientational fluctuations about the bilayer normal, is induced by major alteration in the nature of the fatty acid attached to the sphingosine backbone. The effect of temperature on the carbohydrate portion, in the range studied, appeared to be confined largely to changes in motional order. At 65 °C, freeze–fracture electron microscopy of the liposomal preparations examined in this work demonstrated totally fluid bilayer membranes in all cases. At 40 °C, the phospholipid host matrices containing *N*-stearoylgalactosylceramide and those containing *N*-lignoceroylgalactosylceramide showed minor areas of gel-phase lipid, which seem likely to represent glycolipid-rich domains. This is a phenomenon not previously recorded by electron microscopy and was not seen for the other fatty acid derivatives at the same temperature.

Glycosphingolipids (GSLs)[1] are the carbohydrate-bearing lipids of higher animal cells. There has been considerable speculation concerning the involvement of the carbohydrate portion of this lipid family as structural elements of the membrane and as recognition sites [reviewed in Curatolo (1987)]. An interesting question that has arisen in this area of research has been how the single fatty acid of the GSL backbone may alter the disposition of the sugar headgroup and thus modulate GSL function in membranes. There is very significant latitude for variation in this parameter since, while the host phospholipid matrix of cell membranes tends to be composed of species with nonhydroxylated fatty acids whose chain lengths range predominantly between 16 and 18 carbons, GSLs commonly possess hydroxylated fatty acids and fatty acids that are up to 26 carbons in length (Hakomori, 1981; Thompson & Tillack, 1985; Curatolo, 1987a). *cis*-9,10-Unsaturated fatty acids occur frequently in both GSLs and phospholipids. The question of fatty acid control over glycolipid function has been most eloquently considered with regard to the receptor role of lipid-linked carbohydrate residues. Thus it has been recommended that glycolipid fatty acid chain length and hydroxylation be considered as contributors to the intriguing phenomenon of GSL crypticity in membranes—emphasizing, for instance, the potential of the fatty acid to control lateral arrangement in the membrane (Hakomori, 1986; Kannagi et al., 1983; Lampio et al., 1986; Curatolo & Neuringer, 1986). Carl Alving and his colleagues first demonstrated that a long-chain fatty acid on a sphingosine backbone could strikingly increase the antibody receptor function of the headgroup carbohydrate residue (Alving et al., 1980; Schichijo & Alving, 1985). They suggested that the presence of a long fatty acid might cause the headgroup to protrude above surrounding phospholipids (having shorter fatty acids), thus being more physically accessible to macromolecules [see also Crook et al. (1986)]. Analysis of the mechanism(s) underlying such observations is greatly complicated, however, by lack of structural information on the membranes studied. In addition, a variety of other factors may be involved in both cell and model membranes. For instance, there exists a clearly

† This work was supported by a grant from the Medical Research Council of Canada to C.W.M.G.

* To whom correspondence should be addressed.

‡ The University of Western Ontario.

§ National Research Council of Canada.

[1] Abbreviations: GSL, glycosphingolipid; GalCer, Galβ1→ceramide; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine.

0006-2960/92/0431-2662$03.00/0

demonstrated sensitivity of lipid-specific (including GalCer) binding macromolecules to membrane cholesterol content and fluidity, which strongly implies modulation by receptor dynamics (Brulet & McConnell, 1977; Balakrishnan et al., 1982; Utsumi et al., 1984; Stanton et al., 1984; Mehlhorn et al., 1988; Stewart & Boggs, 1990). Curatolo (1987) has reviewed GSL fatty acid effects as they may relate to membrane structural considerations.

In this article, we consider for the first time, by direct measurement of structural effects, the influence of common fatty acid alternatives on GSL headgroup disposition in membranes. GalCer with various selected pure fatty acids was modified by introduction of a nonperturbing deuterium probe in place of a proton at C_6 of the carbohydrate residue. The resultant species were assembled at low mole ratio into bilayers of POPC for examination by wide-line ^2H NMR. POPC was chosen as a common phospholipid of cell membranes. It has a gel-to-liquid-crystal transition temperature of −3 °C (Davis & Keough, 1985). GalCer was maintained as a minor component to reflect the low GSL content typical of cell membranes. Covalent attachment of the deuterium probe, with its known sensitivity to orientation and dynamics, to the sugar residue itself was felt to optimize the potential for detecting and measuring fatty acid influence on headgroup presentation at the membrane surface. GalCer is a species that has been clearly demonstrated to be importantly affected by its ceramide fatty acid nature, both in its bulk physical properties (Curatolo & Jungalwala, 1985; Reed & Shipley, 1989) and in its role as a recognition site for macromolecules (Alving et al., 1980; Utsumi et al., 1984; Stanton et al., 1984; Mehlhorn et al., 1988).

Materials and Methods

1-Palmitoyl-2-oleoyl-3-*sn*-phosphatidylcholine (POPC) was obtained from Avanti Polar Lipids, Birmingham, AL, and was used without further purification. Galactosylceramide (GalCer) from a beef brain source was also from Avanti Polar Lipids. Lyso-GalCer (i.e., with the fatty acid removed) was produced from the above material by hydrolysis in refluxing butanolic KOH (Taketomi & Yamakawa, 1963) and was ninhydrin positive. The lyso-GSL was applied to a column of Bio-Rad silicic acid 200–400 mesh and eluted with a gradient of methanol in chloroform. Isolated materials cochromatographed with known material on Merck silica gel 60 thin layer chromatography plates eluted with 65:15 $CHCl_3$/CH_3OH and developed with sulfuric acid/ethanol.

Galactosylceramides with α-hydroxy fatty acids were synthesized via their α-acetoxy analogues as follows. α-Acetoxystearic acids were prepared from the corresponding α-bromo intermediates, which had been generated by reacting octadecanoic acid (Aldrich) with liquid bromine in the presence of PCl_3, followed by hydrolysis of the acid chloride product (Allen & Kalm, 1967). Conversion to the corresponding α-acetoxy esters was accomplished by refluxing with anhydrous sodium acetate in glacial acetic acid (Guest, 1947). The mixture of stearic acid α-acetoxy esters was purified on a silicic acid column eluted with $CHCl_3$ and converted to the acid chloride form by refluxing with excess $SOCl_2$ (Kopaczyk & Radin, 1965). These reactions were followed by TLC on silicic acid plates eluted with hexane/diethyl ether/formic acid (70:30:1). The α-acetoxy ester was identified by ^1H NMR in C^2HCl_3. The fatty acid chlorides were coupled with lyso-GalCer, followed by hydrolysis of the acetate group (Pascher, 1974). D- and L-α-hydroxy stereoisomers of the GSL were separated using a silicic acid column eluted with a $CHCl_3$/CH_3OH gradient, the L-isomer being significantly faster-running in the solvent system described (Pascher, 1974). Semisynthetic nonhydroxy- and D-α-hydroxy-GalCer behaved similarly, but not identically on TLC, to natural beef brain GalCer non-hydroxy and D-α-hydroxy fractions, respectively, the semisynthetic species running slightly slower than their corresponding natural mixtures, which included longer fatty acids, as reported previously (Karlsson & Pascher, 1971).

N-Stearoyl-GalCer and *N*-lignoceroyl-GalCer were generated similarly to the above hydroxylated species, by hooking up the appropriate fatty acid (as its acid chloride) to lyso-GalCer. *N*-Oleoyl-GalCer was generated by coupling lyso-GalCer with oleic acid in the presence of dicyclohexylcarbodiimide and pyridine (Sharom & Grant, 1975).

Deuteration of the GalCer carbohydrate residue at C_6 was achieved by oxidation of the C_6 hydroxymethyl group on the various GalCer species with galactose oxidase/horse radish peroxidase, followed by reduction of the resulting aldehydes with $NaBD_4$ (Radin & Evangelatos, 1981). Purification was as previously described (Radin, 1972).

Lipid bilayer membranes for these experiments were prepared by dissolving all components at the final desired ratio (10 mol % glycolipid) in 3:1 $CHCl_3$/CH_3OH and removing the solvent using a rotary evaporator and a water bath at 50 °C. Resultant films were further dried under vacuum (rotary pump) for 3 h at 22 °C. Liposomes were generated by initial hydration of such films with deuterium-depleted water (MSD Isotopes, Ltd.) buffered with 10 mM phosphate at pH 7.4. Samples were subsequently lyophilized three times from a volume of 300 μL of deuterium-depleted water and subjected to eight freeze–thaw cycles following the final hydration step. The total lipid used per sample was typically 100–200 mg [of which 10–14 mg (11.6–16.2 μmol) was deuterated], and the total final volume was 500–700 μL. All samples were incubated at 50 °C to assure diffusional equilibrium within the bilayer.

^2H NMR spectra were acquired at 30.7 MHz on a "home-built" spectrometer operated by a Nicolet 1280 computer. The sample was enclosed in a glass Dewar, and the temperature was electronically regulated to within ±0.5 °C. Spectra were recorded using the quadrupolar echo pulse sequence (Davis et al., 1976) with full phase cycling of the rf pulses (Perly et al., 1985) and quadrature detection. The $\pi/2$ pulse length varied from 5 to 6 μs (10-mm solenoid coil), pulse spacing was 60 μs, and recycle time was 80 ms. Spectra were not folded about the Larmor frequency. The 90° oriented sample ("depaked") spectra were calculated from the powder spectra as described previously (Bloom et al., 1981).

Samples for freeze–fracture electron microscopy were withdrawn directly from the samples prepared for ^2H NMR and thus had the same history. The pure POPC control specimen was prepared without deuterated glycolipid and subjected to manipulations identical to those for the NMR samples. Prior to the "quenching" step that preserves sample structure for heavy-metal shadowing, samples were incubated under conditions designed to approximate as closely as possible the conditions under which NMR was performed. This involved placing small droplets of the liposome suspensions on gold alloy planchets in 6-mm holes drilled horizontally into a 6 × 6 × 22 cm brass block that was in turn immersed to just below the level of the sample hole in a water bath held several degrees above the NMR temperature to allow for slight cooling that occurs during sample transfer to the cryogenic bath. The bath itself was maintained in a temperature-controlled room at 35–37 °C. The atmosphere surrounding the brass block and samples was saturated with water vapor from

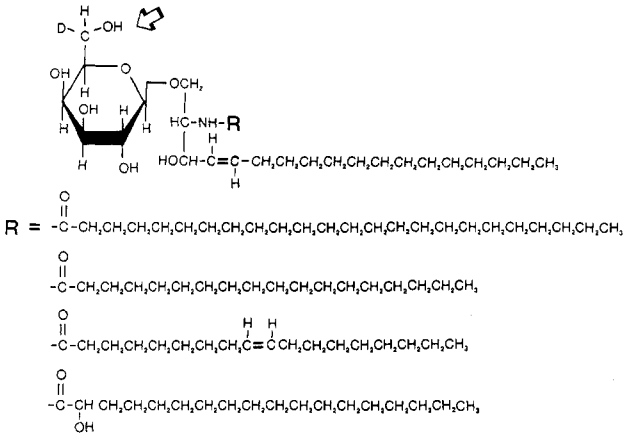

FIGURE 1: Chemical structures for the deuterated glycolipids studied in this work. The monodeuterated exocyclic hydroxymethyl group of the galactose headgroup is indicated (arrow); two stereoisomers are possible. "R" refers to the fatty acid attached in amide linkage: from top to bottom, the 24-carbon saturated species (lignoceric acid) and the 18-carbon species (stearic, oleic, and α-hydroxystearic acids, respectively).

the bath. Samples were removed from the drilled brass block with very fine forceps and frozen instantly in a slurry of Freon 22 cooled in liquid nitrogen. Frozen samples were freeze fractured at −110 °C and platinum shadowed in a Balzers BAF 301 apparatus equipped with electron beam guns. Replicas were cleaned initially in NaClO, rinsed with distilled water, and immersed in 1:1 acetone/ethanol for 1 h to remove residual lipid. Replicas were picked up on 400 mesh copper grids and viewed in a Phillips EM300 electron microscope.

RESULTS

The major question addressed for the first time in this work by direct structural assessment was "how the nature of the fatty acid attached to a GSL sphingosine backbone influences spatial arrangement and dynamics of the carbohydrate headgroup." ^{2}H NMR spectroscopy has been shown to be a technique of choice for dealing with questions related to lipid orientation and motional properties in membranes (Seelig, 1977; Davis, 1983; Smith, 1984). Replacement of ^{1}H nuclei by ^{2}H nuclei in the molecular region of interest (the GSL sugar headgroup in this case) represents a negligible perturbation. Anisotropic motion of a probe-labeled molecule (e.g., as a result of membrane association) results in incomplete averaging of the quadrupolar interaction, giving rise to a residual spectral splitting, $\Delta\nu_Q$, the value of which is dependent on the average orientation of the probe and the amplitude of motion about this direction. Thus $\Delta\nu_Q$ is directly related to the C–D bond order parameter, S_{CD}.

Figure 1 illustrates chemical structures of the galactosylceramides considered in this work. In each case, a single deuterium nucleus was introduced into the galactose exocyclic hydroxymethyl group to produce –CHDOH. The labeled carbon atom bears two stereochemically nonidentical protons normally (i.e., $-CH_2OH$); and the partial synthesis used to produce the deuterated glycolipids studied here replaces these randomly, so that two stereoisomeric monodeuterated products (the *R* and *S* diastereomers) result, in a 1:1 ratio (Skarjune & Oldfield, 1979). The ^{2}H NMR spectrum of pure GalCer labeled in this fashion and hydrated to form fluid lamellar structures (gel-to-fluid transition temperature, 82 °C) is known from the pioneering work of Oldfield and his colleagues (Skarjune & Oldfield, 1979) to give rise to a superposition of two quadrupolar powder spectra of equal intensity ($\Delta\nu_Q$ = 9.5 and 13.5 kHz at 90 °C) having line shapes representative of axially symmetric motion. Very similar spectra have been obtained from pure fluid bilayers of lactosyldiglyceride deuterated on the (terminal) galactose residue exocyclic hydroxymethyl group (Renou et al., 1989).

In the present study, interest was focused on lipid in the liquid-crystalline phase. Indeed, the limited quantities of sample available in general precluded the acquisition of gel-phase spectra. Samples studied consisted of the headgroup-deuterated GSLs shown in Figure 1, assembled at 10 mol % into unsonicated bilayers of the phospholipid, 1-palmitoyl-2-oleoylphosphatidylcholine. The physicochemical nature of these membranes may be inferred from previous studies in which a phase diagram for binary mixtures of POPC with natural GalCer from beef brain has been published (Curatolo, 1986). GalCer from this source contains about 60% hydroxylated fatty acids and a range of other saturated and unsaturated 16–24-carbon fatty acids (Bunow & Levin, 1980; Gambale et al., 1982). The fluidus in the published phase diagram occurs at about 30 °C for 10 mol % glycolipid. Thus, for the lipid mixtures included in the present study, homogeneous liquid-crystalline dispersions may be expected above 30–40 °C.

Representative ^{2}H NMR powder spectra of the various deuterated glycolipids in fluid POPC bilayers are shown in Figure 2 for the 65 °C temperature. Spectra are included for GalCer with 18-carbon saturated fatty acid (*N*-stearoyl-d_1-GalCer), its 24-carbon saturated fatty acid analogue (*N*-lignoceroyl-d_1-GalCer), the 18-carbon *cis*-9,10-unsaturated derivative (*N*-oleoyl-d_1-GalCer), and the D- and L-stereoisomers of *N*-(α-hydroxy)stearoyl-d_1-GalCer. A typical 90°-oriented sample ("depaked") spectrum is shown for *N*-stearoyl-d_1-GalCer. The spectra observed are similar to those recorded for pure hydrated GalCer from beef brain by Skarjune and Oldfield (1979) in that, in each case, two quadrupole splittings are manifest. Although the signal-to-noise ratios of the spectra do not permit a detailed analysis of the ^{2}H NMR line shapes, the spectra appear to be consistent with axially symmetric motional averaging. This conclusion is consistent with the results reported previously for pure GalCer bilayers (Skarjune & Oldfield, 1979). The overlapping powder patterns are of roughly equal intensity (readily seen by inspection of corresponding depaked spectra as for the example in Figure 2) and may be attributed to equal populations of the two stereoisomers at C_6 of the deuterated lipids studied. The presence of different $\Delta\nu_Q$ values (Table I) for the *pro-R* and *pro-S* deuterons at C_6 reflects the different average orientations of the associated C–^{2}H bonds relative to the axis of motional averaging (the molecular long axis).

Inspection of Figure 2 and the derived spectral data in Table I reveals that splittings for the glycolipids examined in the fluid host matrix at 65 °C appear, with the exception of the hydroxy fatty acyl lipids, to be equal (within experimental error). The $\Delta\nu_Q$ values for the D- and L-α-hydroxy fatty acid derivatives are also, within experimental error, equal.

The spectra obtained at 65 °C represent systems substantially above the physiological temperature range. Figure 3 illustrates spectra of the same samples upon temperature reduction to 40 °C. The same number of spectral acquisitions was obtained for any given sample at the different temperatures. As above, the depaked spectrum is shown for *N*-stearoyl-GalCer. The features seen are those already described for the higher temperature, although in each case the $\Delta\nu_Q$ values are larger at the lower temperature (Table I). As was the case at 65 °C, the similarity in values of $\Delta\nu_Q$ among GalCer molecules with very different fatty acids remains

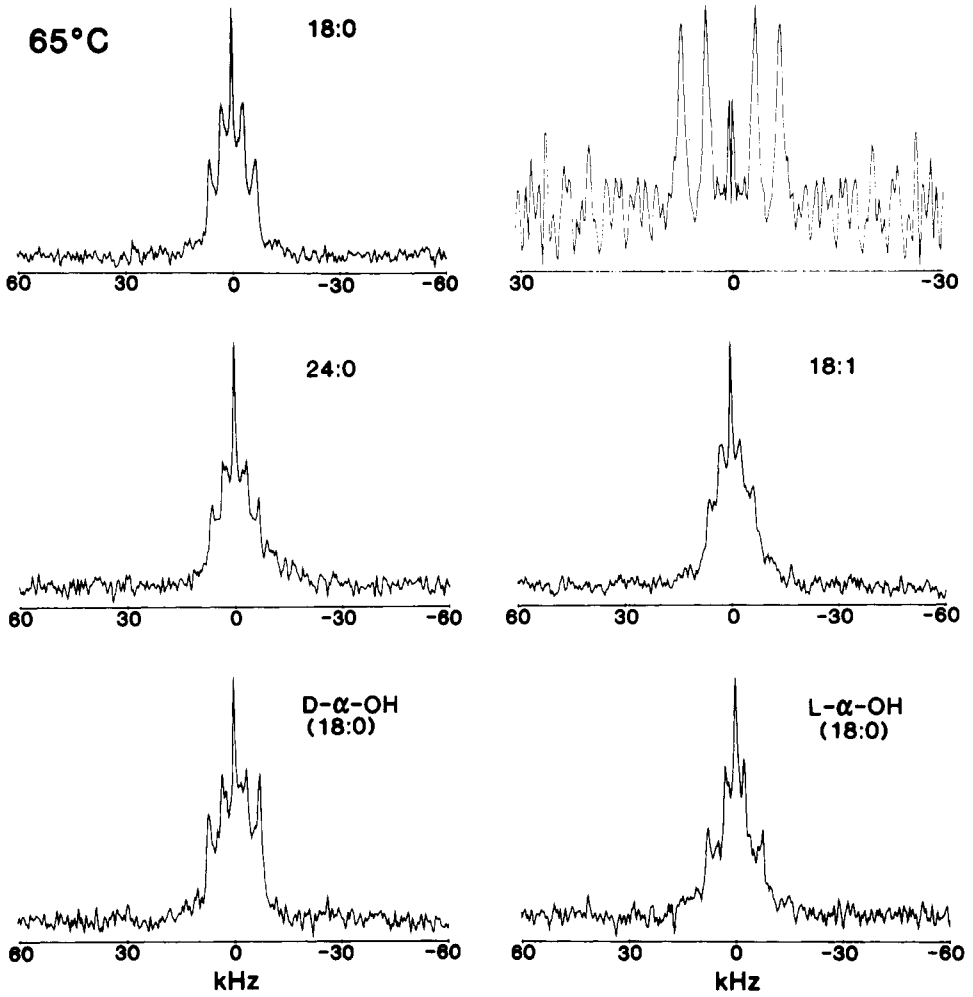

FIGURE 2: ^2H NMR powder spectra at 65 °C for low concentrations of headgroup-deuterated GalCer in phospholipid bilayers, as a function of ceramide fatty acid composition. Deuteration was at C_6 of the galactose residue as indicated in Figure 1. In each case, the glycolipid was dispersed at 10 mol % in unsonicated multilamellar vesicles of 1-palmitoyl-2-oleoylphosphatidylcholine. (A, top) *N*-Stearoyl-d_1-GalCer (depaked spectrum on right); (B, middle left) *N*-lignoceroyl-d_1-GalCer; (C, middle right) *N*-oleoyl-d_1-GalCer; (D, bottom left) *N*-(D-α-hydroxy)-stearoyl-d_1-GalCer; (E, bottom right) *N*-(L-α-hydroxy)stearoyl-d_1-GalCer. Each sample contained 10–14 mg (11.6–16.2 μmol) of deuterated GSL (ca. 50% labeling at each of the *pro-R* and *pro-S* C_6 positions).

Table I: Temperature and Acyl Chain Dependencies of Quadrupolar Splittings ($\Delta\nu_Q$) for Headgroup-Deuterated Galactosylceramide at Low Concentration in Fluid Phospholipid Bilayers[a]

T (°C)	glycolipid fatty acid	inner splitting (±0.5 kHz)	outer splitting (±0.5 kHz)	splitting ratio (O/I ● 0.1)
65	24:0	7.4	13.9	1.9
	18:0	7.0	13.8	2.0
	18:1	6.5	13.5	2.1
	18:0 D-α-OH	7.6	15.2	2.0
	18:0 L-α-OH	6.1	16.3	2.7
40	24:0	10.2	18.0	1.8
	18:0	9.7	17.7	1.8
	18:1	9.2	17.3	1.9
	18:0 D-α-OH	9.9	18.0	1.8
	18:0 L-α-OH	9.0	20.7	2.3

[a] In each case the deuteron was located on the exocyclic hydroxymethyl function of the glycolipid galactose residue (Figure 1). [$^2H_1$]-GalCer was assembled at 10 mol % into unsonicated bilayers of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC), suspended in 10 mM phosphate-buffered saline, pH 7.4.

striking. The only difference larger than experimental error is in the outer splitting of the L-α-hydroxy derivative, which also exhibited the largest difference at the higher temperature.

It has been suggested (Renou et al., 1989; Skarjune & Oldfield, 1979) that for galactose-containing lipids there is a conformational equilibrium among possible rotameric states about the carbohydrate C_5–C_6 bond [nominally, among gauche (+), gauche (−), and trans isomers]. Thus the change in $\Delta\nu_Q$ values with temperature could reflect a change(s) in the relative populations of the hydroxymethyl rotamer states, in headgroup orientation, and/or in the amplitude of motion about the bilayer normal. The data presented permit some insight into which of these possible mechanisms is most likely in operation. With the possible exception of *N*-(L-α-hydroxy)stearoyl-GalCer, if one considers the ratio of outer/inner $\Delta\nu_Q$ values at 65 and 40 °C for the stereoisomeric deuteron at C_6 of a given fatty acid derivative of GalCer, within experimental error there is virtually no change between the two temperatures (Table I). This is significant, since a change in either headgroup orientation or rotamer population would be expected to change the ratio, except in fortuitous circumstances. On the other hand, a change in molecular order (amplitude of motion) upon decreasing the temperature from 65 to 40 °C would alter the size of the residual quadrupolar interaction (increase it, as here) but leave the ratio unchanged. Thus, it would appear that lowering the temperature by 25 °C increases the orientational order, while the average headgroup orientation remains largely unchanged.

Inspection of Figures 2 and 3 reveals that for *N*-stearoyl- and *N*-lignoceroyl-GalCer, there is a significant reduction in spectral intensity as the temperature is lowered from 65 to 40 °C. This contrasts with the spectral behavior of the other lipids, for which less striking changes in intensity are seen.

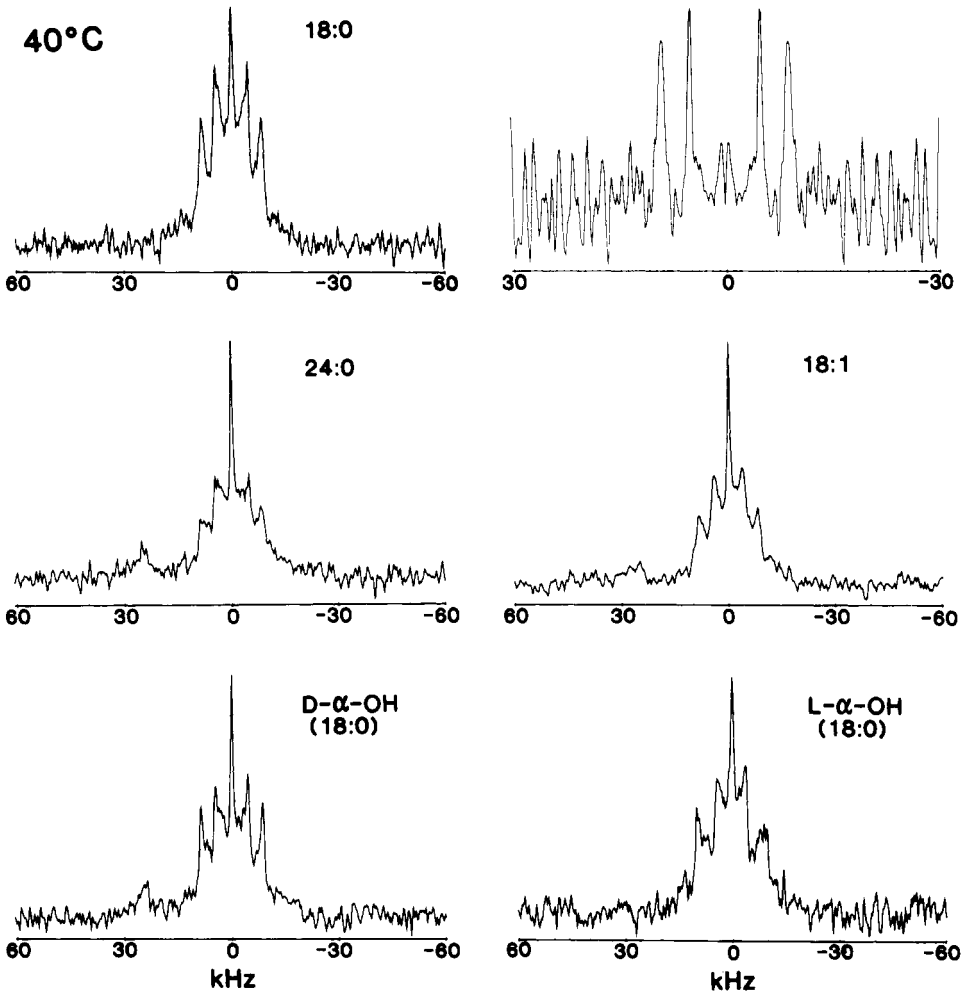

FIGURE 3: ^2H NMR powder spectra at 40 °C for low concentrations of headgroup-deuterated GalCer in phospholipid bilayers, as a function of ceramide fatty acid composition. Samples were those run to generate Figure 2. The number of spectral accumulations for each spectrum ranged from 308 000 to 374 000 and was the same for a given sample at 65 °C (Figure 2) and 40 °C (Figure 3). (A, top) *N*-Stearoyl-d_1-GalCer (depaked spectrum on right); (B, middle left) *N*-lignoceroyl-d_1-GalCer; (C, middle right) *N*-oleoyl-d_1-GalCer; (D, bottom left) *N*-(D-α-hydroxy)stearoyl-d_1-GalCer; (E, bottom right) *N*-(L-α-hydroxy)stearoyl-d_1-GalCer.

While a substantial increase in the transverse relaxation rate (resulting from changes in the rates of fluid phase molecular motion) cannot be discounted completely, the most likely origin of the intensity change is partial phase separation of the glycolipids involved. If, at 40 °C, some gel-phase glycolipid-rich domains are formed, the associated ^2H NMR spectrum would be substantially broader (Davis, 1983; Jarrell et al., 1986) and under the present conditions not readily observed. As a result, there would be an associated decrease in the amount of liquid-crystalline phase and a concomitant apparent decrease in spectral intensity.

A complementary examination of the POPC liposomes studied by ^2H NMR spectroscopy was performed by freeze–fracture electron microscopy, under conditions closely approximating those of the NMR experiments illustrated in Figures 2 and 3 (see Materials and Methods). Typical electron micrographs obtained are shown in Figure 4. In both temperature ranges investigated by ^2H NMR, they display overall features expected of multilamellar liposomes, without evidence of nonbilayer structures. Detailed examination of the exposed membrane surfaces provided additional insight into the nature of the membrane properties. Sample preparation for freeze–fracture involves incubation of the aqueous liposome suspension being studied at some temperature of interest prior to "instantaneous" freezing ("quenching") to very low temperatures. This quenching process is designed to be sufficiently rapid to preserve molecular features present in the membrane at the temperature of incubation, including, within limits, molecular spatial relationships. Since the frozen sample is subsequently maintained below −100 °C until after it has been replicated with heavy-metal shadow, these features may be seen in the electron microscope. Bilayer membranes of highly homogeneous phosphatidylcholines (such as POPC), which are in the gel phase immediately prior to quenching, typically manifest a highly regular rippled appearance reflecting their orderly molecular packing [e.g., the $P_{\beta'}$ phase (Ververgaert et al., 1973; Grant et al., 1974)], while the same membranes frozen from a fluid state do not. Closely related features can often be identified in more complex multicomponent membranes (Grant et al., 1974; Peters et al., 1984; Mehlhorn et al., 1986; Rock et al., 1990) and indeed are seen in the micrographs of Figure 4. Thus none of the 65 °C samples (A, D, G, and J) display orderly ripples characteristic of rigid domains but rather have a smooth or jumbled-ripple appearance to their membranes. Theoretically, the liquid-crystal membrane appearance should be smooth if the freezing process were infinitely rapid. The jumbled appearance more typically seen for such preparations in standard freeze–fracture preparations has been described previously for related systems, including ones containing GSLs in phosphatidylcholine matrices (Grant et al., 1974; Peters et al., 1984). These are features of fluid bilayers in which, as quenching occurred from the temperature of interest, lipids diffused laterally over short distances seeking to assume orderly gel-phase packing during

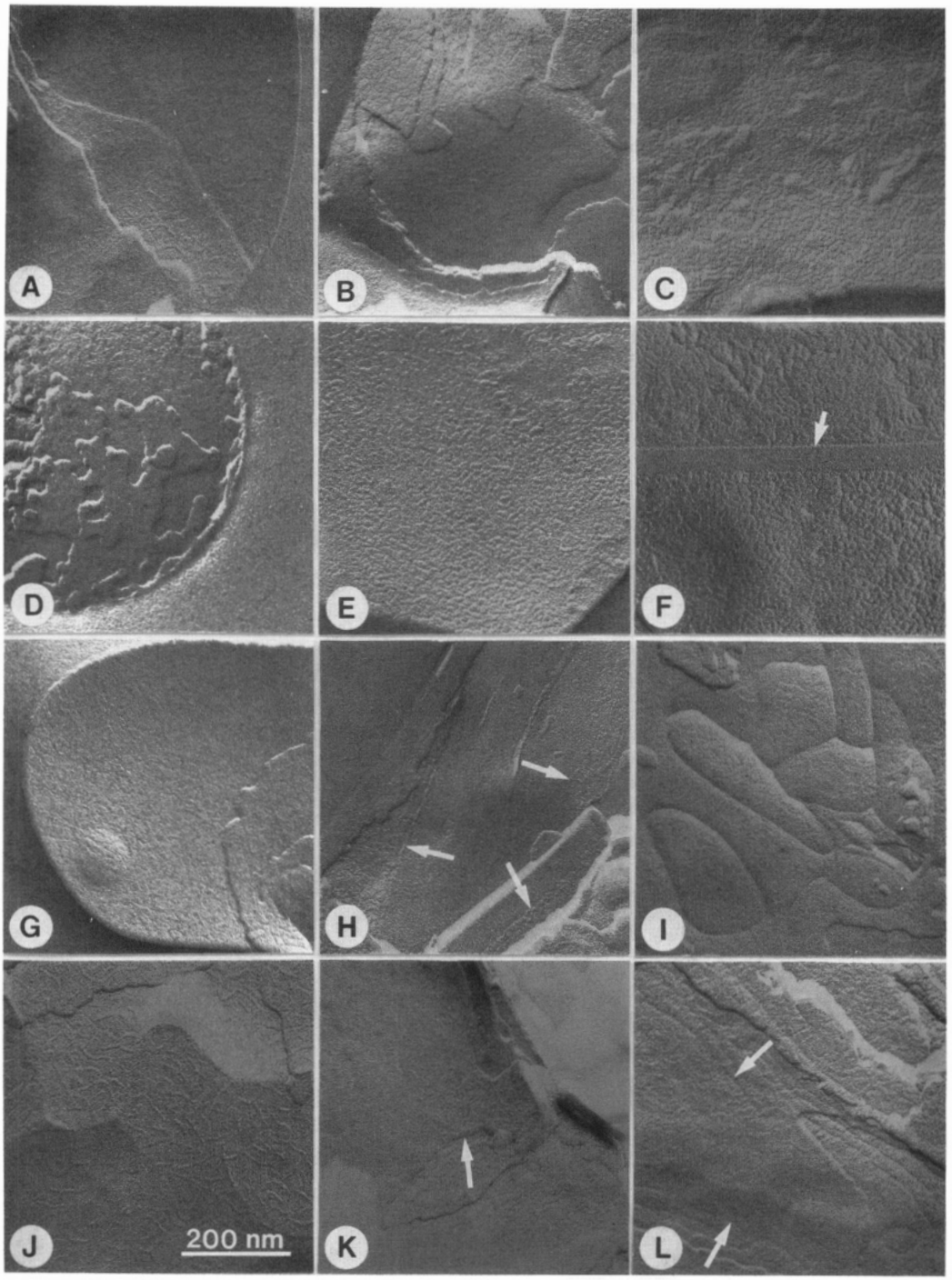

FIGURE 4: Freeze–fracture electron micrographs of samples studied by ^2H NMR. Procedures were chosen so that sample conditions immediately preceding freezing were those present during the NMR experiments at 65 and 40 °C (see Materials and Methods). 10 mol % *N*-(D-α-hydroxy)stearoyl-d_1-GalCer in POPC, corresponding to 65 °C (A) and 40 °C (B). Pure POPC control without glycolipid (40 °C) (C). 10 mol % *N*-oleoyl-d_1-GalCer in POPC, corresponding to 65 °C (D) and 40 °C (E). 10 mol % *N*-stearoyl-d_1-GalCer in POPC, corresponding to 65 °C (G) and 40 °C (H). The 40 °C micrograph was selected to show a membrane region with a rigid domain in equilibrium with (more typical) liquid-crystal domains: the arrows point from rigid membrane to the domain boundaries. 10 mol % *N*-lignoceroyl-d_1-GalCer in POPC, corresponding to 65 °C (J) and 40 °C (panels I, K, and L). Panels K and L were selected to display obviously rigid domains enriched in the 24-carbon fatty acid GSL: arrows point from rigid membrane to the domain boundaries, while the micrograph in panel I shows a potentially domain-related feature (sharply demarcated circular patches) without obviously rigid nature. Panel F illustrates the appearance of rigid domains (arrow) which can be found in binary mixtures of POPC with the other glycolipids studied at lower temperatures (in this case 5 mol % *N*-oleoyl-GalCer in POPC at 20 °C). Magnification, ×100000; the bar indicates 200 nm. The shadow direction is from bottom to top of the page.

the fraction of a second taken for sample heat transfer to the cryogenic bath. The maximum distances of lipid lateral reorganization during quenching in the samples displayed in Figure 4 can be estimated from the jumbled-ripple maximum dimensions of 10–20 nm. This phenomenon has been shown to be one of the determinants of a functional limit on the resolution of the freeze–fracture technique, although ultrafast freezing can reduce the magnitude of the effect (Mehlhorn et al., 1986, 1988; Rock et al., 1990). The same homogeneous fluid bilayer appearance is seen in the micrographs corresponding to POPC liposomes containing 10 mol % GalCer with either the monounsaturated 18-carbon fatty acid or the α-hydroxy 18-carbon fatty acid at 40 °C, and in the liposomes of pure POPC (main transition, −3 °C) (Figure 4, panels B,

E, and C, respectively). However, liposomes containing *N*-stearoyl-GalCer or the 24-carbon species, *N*-lignoceroyl-GalCer, at 40 °C (Figure 4, panels H, I, K, and L) show a strikingly different feature: the presence of significant domains of highly ordered bilayer membrane that is clearly in the gel phase at 40 °C (Figure 4, panels H, K, and L). On the basis of the published phase diagram for beef brain GalCer in POPC (Curatolo, 1986), these seem likely to represent phase-separated domains of gel-phase lipid selectively enriched in GalCer, in equilibrium with fluid domains enriched in POPC. Coexisting domains have been directly visualized in the past in mixtures of phospholipids and cholesterol (Ververgaert et al., 1973; Grant et al., 1974; Verkleij et al., 1974; Luna & McConnell, 1977) and for binary mixtures of phospholipids containing smaller amounts of glycoproteins or glycolipids (Grant & McConnell, 1974; Peters et al., 1984; Rock et al., 1991). To our knowledge this is the first report of such a phenomenon in a binary glycolipid/phospholipid membrane. These results are fully consistent with the conclusion that the reduction in spectral intensity for *N*-lignoceroyl- and *N*-stearoyl-GalCer upon decreasing the temperature from 65 to 40 °C arises from line broadening associated with segregation of glycolipid into gel-phase domains. It was also possible to induce rigid domain formation in the unsaturated GSL membranes and in those with hydroxy fatty acids by going to lower temperatures, as expected (e.g., Figure 4F). The binary mixtures of POPC with the 24-carbon fatty acid glycolipid displayed an unusual feature: apparently sharply demarcated semicircular domains that were not obviously related to fluid/gel phase separations (Figure 4I).

Discussion

In studies by Carl Alving and co-workers of the effect of GSL fatty acid chain length on (GalCer) recognition site function in phospholipid bilayers, it was noted that a greater quantity of (nonmonoclonal) anti-GalCer antibody bound specifically to liposomes when the glycolipid fatty acid was considerably longer than those of the phosphatidylcholine host matrix (Alving & Richards, 1977; Alving et al., 1980). Thus, greater antibody binding was seen by GalCer bearing a 24-carbon (lignoceric) fatty acid than by GalCer with 18-carbon (stearic) fatty acid. The same phenomenon has since been reported for the sulfated derivative of GalCer (Crook et al., 1986). An observation which seems likely to be related to those above is that, under certain conditions, GalCer with a given fatty acid chain length bound more antibody when incorporated in a short chain phospholipid matrix than it did in a long-chain host matrix (Alving et al., 1980). Alving has suggested that the longer chain glycolipids might protrude farther from the membrane and thus be more accessible to macromolecular attachment. Our experimental data bear upon the mechanism behind such observations. Clearly the long-chain fatty acid in our hands caused no change in orientation of the carbohydrate residue in POPC at 65 or 40 °C; hence, if there is greater protrusion of the long-chain species, it is without orientational effect on the carbohydrate portion. Conformational potential energy calculations have suggested that the membrane surface can influence both glycolipid headgroup conformation and orientation in glycosphingolipid systems (Nyholm et al., 1989) and in glyceroglycolipids (Winsborrow and Jarrell, unpublished results). Such calculations also show that headgroup conformation and orientation (and thus associated residual quadrupolar splittings) are sensitive to both the lipid cross-sectional area and to the distance of the carbohydrate from the membrane surface (Winsborrow and Jarrell, unpublished results). Since the residual quadrupolar interaction is very sensitive to even small orientational changes relative to the bilayer surface, and to alterations in both amplitude and rate of motion (Skarjune & Oldfield, 1979; Davis, 1983; Seelig & MacDonald, 1987; Jarrell et al., 1987), the insensitivity of the $\Delta\nu_Q$ values to acyl chain length seen here at both 40 and 65 °C suggests that the headgroup position relative to the bilayer surface is not significantly influenced by chain length.

The role of inter- and intramolecular hydrogen bonding in determination of GSL conformation and dynamics (and hence function) within the membrane has been a topic of considerable discussion in the literature. Curatolo (1987b) has reviewed theories relating to the possible role of hydroxy fatty acids in intermolecular hydrogen-bonding effects at the surface of membranes. Data suggestive of such a hydrogen-bonding role has been reported, although the strongest evidence is associated with X-ray crystallographic measurements. In this regard, it has been reported that the D-α-hydroxy group of GalCer can form a hydrogen bond to the carbohydrate residue (Pascher & Sundell, 1977). If this were the case, one would probably expect a significant alteration in molecular conformation: Nyholm et al. (1990) have recently used X-ray diffraction to consider the possibilities for intramolecular hydrogen bonding in GalCer and its effect on carbohydrate presentation. However, it is not clear that the same forces will be equally important for glycolipids in single crystals and glycolipids dispersed at low concentration in fluid membranes, particularly given the highly hydrated nature of the latter. Indeed Bunow and Levin (1980) demonstrated via Raman spectroscopy that intramolecular hydrogen bonding in fully hydrated GalCer seems to differ from that seen via X-ray crystallography. A dominant result in our experiments is that, in spite of the very different fatty acid composition of the glycolipids studied, in all cases there is marked similarity in spectral features which are extremely sensitive to structural alterations. The observation that there was some evidence of alteration upon fatty acid hydroxylation is interesting, in the light of speculation as to the role of α-hydroxylation in GSL binding events and the finding by Kannagi et al. (1983) that hydroxylation of GSL fatty acids seemed to be connected with GSL crypticity in some cells. However, one must emphasize the potentially minimal nature of the orientational and/or conformational change that would be consistent with the spectral effects seen here.

It is known that fatty acid substitution in GalCer leads to different phase transition temperatures for hydrated bilayers formed from the pure material. Thus the main transition temperature for *N*-oleoyl-GalCer is 44.8 °C (to a metastable phase that changes with time to a new gel phase having a transition temperature of 55.5 °C) (Reed & Shipley, 1989); and for *N*-α-hydroxystearoyl-, *N*-stearoyl-, and *N*-lignoceroyl-GalCer, it is approximately 70, 83, and 83 °C, respectively (Curatolo & Jungalwala, 1985). It seems likely that the gel-phase domains seen for *N*-stearoyl- and *N*-lignoceroyl-GalCer in fluid POPC at 40 °C reflect their higher transition temperatures and correspondingly greater tendencies to phase separate from the fluid host matrix.

Acknowledgments

We thank Dr. Kathy Briere for assistance in obtaining ^2H NMR spectra and Dr. R. Shivers for the use of his freeze–fracture facility.

References

Allen, C. F., & Kalm, M. J. (1967) in *Organic Synthesis*

(Rabjohn, N., Ed.) Vol. IV, pp 398–400, Wiley, New York.
Alving, C. R., & Richards, R. L. (1977) *Immunochemistry 14*, 373–381.
Alving, C. R., Urban, K. A., & Richards, R. L. (1980) *Biochim. Biophys. Acta 600*, 117–125.
Balakrishnan, K., Mehdi, S. W., & McConnell, H. M. (1982) *J. Biol. Chem. 257*, 6434–6439.
Bloom, M., Davis, J. H., & MacKay, A. I. (1981) *Chem. Phys. Lett. 80*, 198–202.
Brulet, P., & McConnell, H. M. (1977) *Biochemistry 16*, 1209–1217.
Bunow, M. R., & Levin, I. W. (1980) *Biophys. J. 32*, 1007–1021.
Crook, S. J., Boggs, J. M., Vistnes, A. I., & Koshy, K. M. (1986) *Biochemistry 25*, 7488–7494.
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., & Jungalwala, F. B. (1985) *Biochemistry 24*, 6608–6613.
Curatolo, W., & Neuringer, L. J. (1986) *J. Biol. Chem. 261*, 17177–17182.
Davis, J. H. (1983) *Biochim. Biophys. Acta 737*, 117–171.
Davis, J. H., Jeffrey, K. R., Bloom, M., Valic, M. I., & Higgs, T. P. (1976) *Chem. Phys. Lett. 42*, 390–394.
Davis, P. J., & Keough, K. M. W. (1985) *Biophys. J. 48*, 915–918.
Gambale, F., Robello, M., Usai, C., & Marchetti, C. (1982) *Biochim. Biophys. Acta 693*, 165–172.
Grant, C. W. M., Wu, S. H., & McConnell, H. M. (1974) *Biochim. Biophys. Acta 363*, 151–158.
Guest, H. H. (1947) *J. Am. Chem. Soc. 69*, 300–302.
Hakomori, S.-I. (1981) *Annu. Rev. Biochem. 50*, 733–764.
Hakomori, S.-I. (1986) *Chem. Phys. Lipids 42*, 209–233.
Jarrell, H. J., Giziewicz, J. B., & Smith, I. C. P. (1986) *Biochemistry 25*, 3950–3957.
Kannagi, R., Stroup, R., Cochan, N. A., Urdal, D. L., Young, W. W., Jr., & Hakomori, S. (1983) *Cancer Res. 43*, 4997–5005.
Karlsson, K. A., & Pascher, I. (1971) *J. Lipid Res. 12*, 446–471.
Kopaczyk, K. C., & Radin, N. S. (1965) *J. Lipid Res. 6*, 140–145.
Lampio, A., Rauvala, H., & Gahmberg, C. G. (1986) *Eur. J. Biochem. 157*, 611–616.
Luna, B., & McConnell, H. M. (1977) *Biochim. Biophys. Acta 470*, 303–316.
Mehlhorn, I. E., Parraga, G., Barber, K. R., & Grant, C. W. M. (1986) *Biochim. Biophys. Acta 863*, 139–155.
Mehlhorn, I. E., Barber, K. R., & Grant, C. W. M. (1988) *Biochim. Biophys. Acta 943*, 389–404.
Mehlhorn, I. E., Barber, K. R., Florio, E., & Grant, C. W. M. (1989) *Biochim. Biophys. Acta 986*, 281–289.
Nyholm, P. G., Samuelsson, B. E., Breimer, M., & Pascher, I. (1989) *J. Mol. Recognit. 2*, 103–113.
Nyholm, P.-G., Pascher, I., & Sundell, S. (1990) *Chem. Phys. Lipids 52*, 1–10.
Pascher, I. (1974) *Chem. Phys. Lipids 12*, 303–315.
Pascher, I., & Sundell, S. (1977) *Chem. Phys. Lipids 20*, 175–191.
Perly, B., Smith, I. C. P., & Jarrell, H. C. (1985) *Biochemistry 24*, 1055–1063.
Peters, M. W., Mehlhorn, I. E., Barber, K. R., & Grant, C. W. M. (1984) *Biochim. Biophys. Acta 778*, 419–428.
Radin, N. S. (1972) *Methods Enzymol. 28*, 300–306.
Radin, N. S., & Evangelatos, G. P. (1981) *J. Lipid Res. 22*, 536–541.
Reed, R. A., & Shipley, G. G. (1989) *Biophys. J. 55*, 281–292.
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.
Rock, P., Allietta, M., Young, W. W., Jr., Thompson, T. E., & Tillack, T. W. (1991) *Biochemistry 30*, 19–25.
Schichijo, S., & Alving, C. R. (1985) *Biochim. Biophys. Acta 820*, 289–294.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.
Seelig, J., & MacDonald, P. (1987) *Acc. Chem. Res. 20*, 221–228.
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.
Taketomi, T., & Yamakawa, T. (1963) *J. Biochem. (Tokyo) 54*, 444–451.
Thompson, T. E., & Tillack, T. W. (1985) *Annu. Rev. Biophys. Biophys. Chem. 14*, 361–386.
Utsumi, H., Suzuki, T., Inoue, K., & Nojima, S. (1984) *J. Biochem. (Tokyo) 96*, 97–105.
Ververgaert, P. H. J. T., Verkleij, A. J., Elbers, P. F., & van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta 311*, 320–329.
Verkleij, A. J., Ververgaert, P. H. J. T., de Kruyff, B., & van Deenen, L. L. M. (1974) *Biochim. Biophys. Acta 373*, 495–501.