Glycosphingolipid Fatty Acid Arrangement in Phospholipid Bilayers: Cholesterol Effects

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ABSTRACT Deuterium wide line NMR spectroscopy was used to study cholesterol effects on the ceramide portions of two glycosphingolipids (GSLs) distributed as minor components in fluid membranes. The common existence of very long fatty acids on GSLs was taken into account by including one glycolipid species with fatty acid chain length matching that of the host matrix, and one longer by 6 carbons. *N*-stearoyl and *N*-lignoceroyl galactosyl ceramide with perdeuterated fatty acid (18:0[d_{35}] GalCer and 24:0[d_{47}] GalCer) were prepared by partial synthesis. They were dispersed in bilayer membranes having the 18-carbon-fatty-acid phospholipid, 1-stearoyl-2-oleoyl-phosphatidylcholine (SOPC), as major component. Glycolipid fatty acid chain behavior and arrangement were analyzed using order profiles derived from their ^{2}H-NMR spectra. Cholesterol effects on order parameter profiles for 18:0[d_{35}] GalCer, with chain length equal to that of the host matrix, followed the pattern known for acyl chains of phospholipids. The presence of sterol led to restriction of *trans/gauche* isomerization along the length of the chain, with the largest absolute increase in order parameters being toward the surface, but somewhat greater relative effect just below the "plateau" region. In cholesterol-containing membranes, order parameter profiles for the long chain species, 24:0[d_{47}] GalCer, showed a characteristic secondary "plateau" associated with carbon atoms C_{14} to C_{23}, a feature also present in SOPC bilayers without cholesterol and in pure hydrated 24:0[d_{47}] GalCer. Cholesterol-induced ordering effects on the long chain glycolipid were similar to those described for the shorter chain species, but were minimal at the methyl terminus. Within a given membrane, S_{CD} profiles for 18:0[d_{35}] GalCer and 24:0[d_{47}] GalCer were quantitatively similar to a membrane depth of C_{13} to C_{14}. S_{CD} values at C_{16} and C_{17} were about 15% and 28% higher, respectively, for the long chain GSL than for its short chain analogue in SOPC/cholesterol (compared to 21 and 31%, respectively, in membranes without cholesterol). Nitroxide spin labels attached rigidly to C_{16} of the long chain glycolipid gave EPR order parameters that were twice as high as for the same spin label at C_{16} on the shorter chain glycolipid in both matrices. It would appear that the above factors impose a tendency for the "extra" portion of the 24-carbon chain to cross the bilayer midplane where it may interact with terminal portions of acyl chains in the opposing monolayer; however, steric constraints, and probably collision events associated with lateral diffusion, induce wide orientation fluctuations in the segment involved.

INTRODUCTION

The importance of cholesterol to membrane structure and function has been recognized for many years. A number of reviews have dealt with its effects on glycerolipids, as both are major components of cell plasma membranes. Fluid glycerolipid bilayers that contain cholesterol are characterized by lipid lateral and rotational diffusion rates similar to those in their cholesterol-free fluid counterparts, while displaying greatly increased conformational order (Demel and de Kruyff, 1976; Yeagle, 1985; Vist and Davis, 1990; Ispen et al., 1987). In addition, cholesterol increases fluid bilayer thickness, the gel-to-liquid-crystalline phase transition is suppressed, and mean cross-sectional area per molecule is decreased. These effects of cholesterol on glycerolipid membranes lend themselves particularly well to investigation by ^{2}H-NMR spectroscopy. In the present work, we have extended this approach to glycosphingolipids (GSLs), the carbohydrate-bearing lipids of higher animal cells, dispersed as minor components in glycerolipid membranes. The subject has special relevance to GSLs, since they commonly possess very long fatty acids that might be expected to be uniquely susceptible to cholesterol effects. Moreover, the nature of the characteristic GSL hydrophobic portion and its arrangement as a minor inclusion in phospholipid- and cholesterol-rich plasma membranes, has often been considered an important modulator of GSL function (Curatolo, 1987a; Thompson and Tillack, 1985; Hamilton et al., 1994).

GSLs are thought to serve as both structural elements and specific recognition sites in animal cells (Thompson and Tillack, 1985; Hamilton et al., 1994; Curatolo, 1987b). They are generally minor membrane components, although in a few cases they make up a large fraction of the membrane lipids. The single fatty acid of the GSL ceramide backbone typically varies in length between 18 and 26 carbons. The remainder of the ceramide backbone is an 18-carbon sphingosine chain which extends some 15 carbons into the membrane hydrophobic interior. Thus GSLs with long chain fatty acids are natural examples of "mixed chain" lipids in that there are very different chain lengths within the same molecule. In cell membranes, the majority of the host matrix

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Abbreviations used: GSL, glycosphingolipid; PC, phosphatidylcholine; SOPC, 1-stearoyl-2-oleoyl PC; 18:0 GalCer, *N*-stearoyl galactosyl ceramide; 24:0 GalCer, *N*-lignoceroyl galactosyl ceramide.

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phospholipids have 16- or 18-carbon fatty acids. Molecular arrangement at the midplane of fluid membranes, particularly for species mismatched with the host matrix, is currently the subject of conjecture. The present experiments permitted a direct consideration of the role of cholesterol in such questions.

The use of ^{2}H-NMR spectroscopy permitted consideration of the influence of cholesterol at each point along a glycolipid acyl chain. For this purpose, galactosyl ceramide (GalCer) was produced by partial synthesis having a perdeuterated fatty acid of 18 or 24 carbons length. 18:0[d_{35}] GalCer and 24:0[d_{47}] GalCer were assembled as minor components into unsonicated lipid bilayer membranes formed from 1-stearoyl-2-oleoyl phosphatidylcholine (SOPC) or SOPC mixed with cholesterol. Thus comparisons could be made between glycolipid species with chain lengths which matched or were longer than that of the host matrix. SOPC is a common natural phospholipid, having a main transition temperature of 6°C (Davis and Keough, 1985) and resultant very high disorder of the lipid chains at the temperatures investigated.

MATERIALS AND METHODS

SOPC and GalCer with natural fatty acid mixture from beef brain were obtained from Avanti Polar Lipids, Birmingham, AL, and were used without further purification. *N*-stearoyl GalCer (18:0[d_{35}] GalCer), N-lignoceroyl-[d_{47}] GalCer (24:0[d_{47}] GalCer) and corresponding derivatives spin labeled at C_{16}, were made by linking the labeled fatty acids to lyso-GalCer following general procedures described previously (Singh et al., 1992b; Morrow et al., 1992; Mehlhorn et al., 1988). Reactions involving the long chain species often required longer times or harsher conditions, presumably because of their different aggregation characteristics in solution. This was particularly evident in the degree of difficulty of hydrolyzing the long chain spin label fatty acid methyl ester, but was also reflected in the yields of reactions that linked fatty acids to sphingosine backbones. Preparation of liposomes containing 18:0[d_{35}] GalCer and 24:0[d_{47}] GalCer followed procedures described previously (Morrow et al., 1992). Individual samples containing up to 15 mg of deuterated lipid were hydrated in about 300 μl of 50 mM phosphate buffer at pH 7.0. Samples contained glycolipid at 10 mol % relative to phospholipid (7% relative to total lipid in cholesterol-containing samples). Cholesterol content was 23 mol % of total lipid. Lipid bilayer membranes for EPR experiments were prepared and their spectra recorded as described elsewhere (Mehlhorn et al., 1988), hydrating with 10 mM HEPES buffer pH 7.4.

Spectra were acquired after incubation at temperatures substantially above the fluidus curves of the systems studied to facilitate equilibrium distribution of components in the bilayer. Technical details for the ^{2}H-NMR experiments have been described previously (Morrow et al., 1992). For each spectrum, 100,000 to 300,000 transients were collected with a repetition time of 0.4 s. Smoothed order parameter profiles were obtained using a modification of the approach outlined by Sternin et al. (1988) and Lafleur et al. (1989), in which an attempt was made to identify unresolved peaks associated with C_2 and alkene carbons based on their not having integrated intensities corresponding to two deuterons (one methylene group). Spectra were recorded sequentially from high to low temperature.

RESULTS

The NMR spectral feature associated with a given deuteron is a doublet with 90° edges split by

$$\Delta\nu_Q = \frac{3}{4}\frac{e^2qQ}{h}S_{CD} \qquad (1)$$

where S_{CD} is the orientation order parameter of the carbon-deuterium (C-D) bond:

$$S_{CD} = \tfrac{1}{2}\langle 3\cos^2\theta_{CD} - 1\rangle. \qquad (2)$$

The average in Eq. 2 is over the time-dependent motions of the C-D bond, and Θ_{CD} is the angle between this bond and the bilayer normal. These relationships (Seelig, 1977) underpin the interpretations recorded here.

Fig. 1 presents typical powder spectra (left column) and dePaked spectra (right column) for 18:0[d_{35}] GalCer (*a, b*) and 24:0[d_{47}] GalCer (*c, e*), dispersed as minor components in fluid bilayers of SOPC and SOPC/cholesterol at 40°C. Deuterated lipid structures are shown as inserts. Spectra for the long chain GSL deuterated selectively toward the methyl end of the fatty acid (24:0[d_7] GalCer) are included as *d, f* below those for corresponding perdeuterated samples: these permit verification of peak assignments at positions C_{22}, C_{23}, and C_{24}. The spectra are characteristic of axially symmetric chain motion in a liquid crystalline phase (Seelig, 1977; Davis, 1983; Smith, 1984; Mantsch et al., 1977). Initial examination of the data in Fig. 1, *a* and *b*, suggests that the effect of cholesterol on 18:0[d_{35}] GalCer, whose chain length

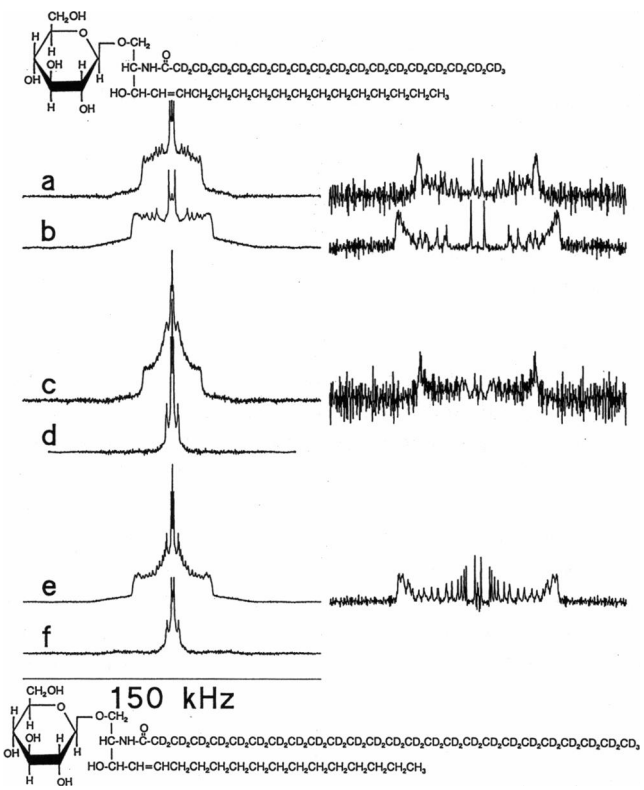

FIGURE 1 Representative ^{2}H-NMR powder spectra for 18:0[d_{35}] GalCer (*a, b*) and 24:0[d_{47}] GalCer (*c, e*) dispersed as minor components in fluid bilayers of SOPC (*a, c*) and SOPC/cholesterol (*b, e*). DePaked spectra (zero degree orientation) are shown to the right of the corresponding powder spectra. Spectra for 24:0 GalCer deuterated selectively on the methyl terminal three carbons are included as *d, f* for SOPC and SOPC/cholesterol, respectively. Structures of the perdeuterated glycosphingolipids studied are indicated as inserts: upper, 18:0[d_{35}]GalCer; lower, 24:0[d_{47}]GalCer. All spectra run on unsonicated multilamellar vesicles at 40°C.

matches closely that of the host matrix, is to increase spectral splitting along the entire length of the acyl chain. This is more obvious in the depaked spectra shown to the right: all quadrupole splittings appear to be increased, reflecting increased orientation order as described by the relationships in Eqs. 1 and 2 above. It appears that a similar cholesterol effect is operating in the case of 24:0[d_{47}] GalCer, and that the degree to which the longer fatty acid is influenced may be reduced toward the central region of the spectrum corresponding to the most distal end of the chain (see below).

In comparing powder spectra for 18:0[d_{35}] GalCer and 24:0[d_{47}] GalCer without cholesterol (*a* vs. *c*), the most striking difference is that there is relatively greater spectral intensity buildup in the central region for the longer chain species. This has been shown to arise from deuterons associated with the "extra" length of the 24-carbon chain (Lu et al., 1993). Comparison of *b* and *e* demonstrates that the same feature occurs in the presence of cholesterol. The powder spectra *d* and *f* represent the portions of the spectra in *c* and *e*, respectively, that are associated with the methyl terminal three carbons of the 24:0 chain (C_{22}, C_{23}, C_{24}). Comparison of *f* with *e* (and *d* with *c*) demonstrates that the unusual central intensity buildup in the 24:0[d_{47}] GalCer spectra is also associated with deuterons toward the methyl end of the long chain in cholesterol-containing membranes.

Bloom and co-workers have noted that a smoothed order parameter profile may be extracted from the oriented spectrum of a perdeuterated acyl chain by assuming a monotonic decrease in orientation order from carboxyl terminus to methyl terminus (Sternin et al., 1988; Lafleur et al., 1989). This has been done in Fig. 2 for the perdeuterated samples described in the present work at 40°C. The analysis permits detailed comparison of chain arrangement and behavior at each point along their length. Fig. 2 *a* shows smoothed order parameter profiles for 18:0[d_{35}] GalCer in the absence and presence of cholesterol, while Fig. 2 *b* shows corresponding plots for 24:0[d_{47}] GalCer. The curves in *a* have very similar appearances to data that exist in the literature for glycerolipids of chain length 14–18 carbons in bilayers with and without cholesterol (Vist and Davis, 1990; Nezil and Bloom, 1992; Sankaram and Thompson, 1990; Stockton and Smith, 1976). Experiments corresponding to the comparison in *b* have not been reported previously for any lipid family. The upper regions of the curves in *b* resemble those in *a;* however, the lower portions are highly distinctive. The shape of the 24:0[d_{47}] GalCer spectrum suggests that orientation order for this long chain glycosphingolipid in a shorter chain host matrix is unusual toward the methyl terminus. There is an inflection point at C_{16}, followed by a second "plateau" of less abruptly decreasing order. However, at no point does the slope of the curve become positive i.e., at no point does the order of a lower segment become greater than that of chain segments above it. It is striking that the relative arrangement and behavior of the 18:0 glycolipid fatty acid were seen to be primarily the same in membranes with and without cholesterol. This qualitative similarity in profiles between membranes in the presence and absence of sterol was also apparent for the very long glycolipid. In the case of the long chain species, at the methyl terminus itself the absolute values of the order parameters in the presence of cholesterol approach or equal those found in the same membrane without cholesterol.

A more extensive comparison of orientation and behavioral effects corresponding to the 18:0 and 24:0 chains is possible through examination of Figs. 3 and 4. Anticipating that alteration of spatial constraint within the host matrix could have significant impact on the relative arrangements of short versus long chain GSLs, particularly in membranes containing cholesterol, samples were examined over a range of temperatures that represent considerable variation in lipid conformational order and fluidity. Fig. 3 displays stacked spectra for cholesterol-rich membranes bearing 18:0[d_{35}] GalCer and 24:0[d_{47}] GalCer at 25, 40, and 55°C. An important observation is that, intrinsic features of spectra for 18:0[d_{35}] GalCer and 24:0[d_{47}] GalCer did not change throughout the temperature range studied. The effects of decreasing temperature were highly analogous to the effects of

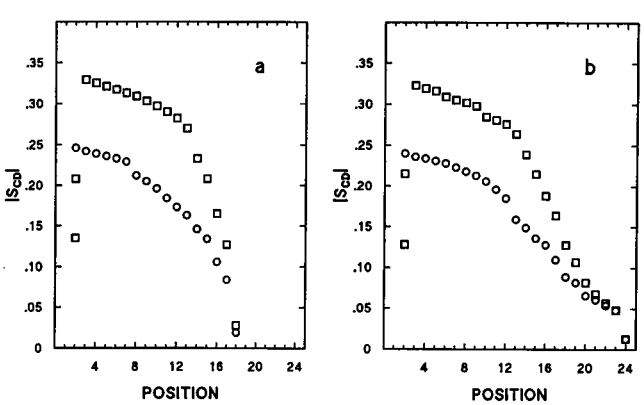

FIGURE 2 Smoothed order parameter profiles for 18:0[d_{35}] GalCer (*a*), and 24:0[d_{47}] GalCer (*b*), at 40°C in bilayers of SOPC (○) and SOPC/cholesterol (□).

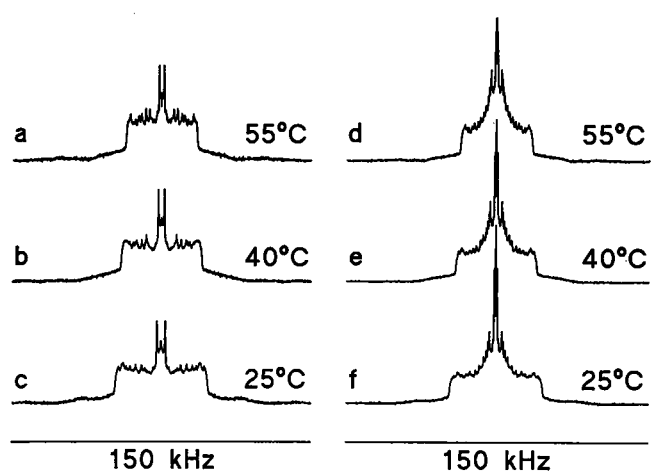

FIGURE 3 Stacked ^{2}H-NMR powder spectra corresponding to 18:0[d_{35}] GalCer (*a-c*) and 24:0[d_{47}] GalCer (*d-f*) dispersed as minor components in bilayers of SOPC/cholesterol at 55, 40 and 25°C, respectively.

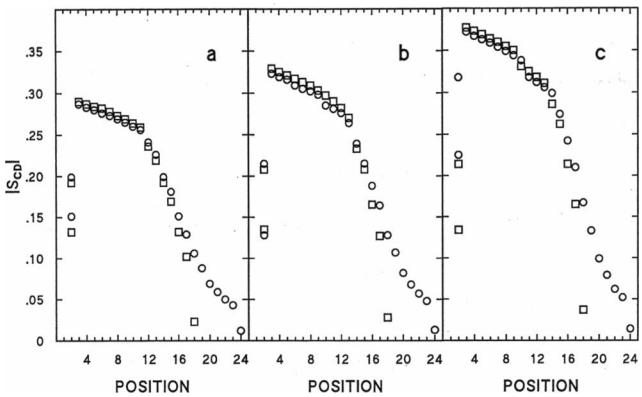

FIGURE 4 Superimposed smoothed order parameter profiles for 18:0-[d_{35}] GalCer (□) and 24:0[d_{47}] GalCer (○) in SOPC/cholesterol at 55°C (*a*), 40°C (*b*), and 25°C (*c*).

cholesterol addition described in Figs. 1 and 2: spectral splitting increased due to the greater conformational constraint. Once again, there is the appearance that the methyl terminus of the very long chain is less subject to change.

Fig. 4 displays superimposed smoothed order parameter profiles for 18:0[d_{35}] and 24:0[d_{47}] GalCer at 55°C (*a*), 40°C (*b*), and 25°C (*c*) in SOPC/cholesterol, to permit detailed evaluation of the points made above. Features to note are: 1) The profiles for 18:0 and 24:0 GalCer species remain virtually superimposable to a membrane depth of C_{13}-C_{14}. 2) At depths greater than C_{13}-C_{14} the 24:0 profiles diverge as a result of reduced slope, producing a feature resembling a second plateau region. 3) As was the case with added cholesterol, lowering the temperature increased the degree of order relatively little at the 24:0 methyl terminus. 4) There was no discontinuous change in relationship between the order profiles corresponding to 18:0 and 24:0 GSLs over the temperature range studied. At each temperature, for 24:0[d_{47}] GalCer, methylene groups in the region C_{15} to C_{18} were significantly more ordered than corresponding chain segments in 18:0[d_{35}] GalCer. C_{19} to C_{23} in the long chain were (progressively) more disordered than C_{17} of the short chain. This difference did not change with temperature-induced alterations in host matrix order. For instance at C_{16} the 24:0 chain was 14–15% more ordered over the temperature range studied; at C_{17} the difference was 27–29%. In the absence of cholesterol these differences at C_{16} and C_{17} were 21% and 31%, respectively, at 40°C. The result was that in cholesterol-containing membranes the C_{17} methylene group of the long chain had the same degree of order as C_{16} of the short acyl chain, and C_{18} of the long chain had the same degree of order as C_{17} in the short chain glycolipid. In the absence of cholesterol at 40°C these relationships were also true.

The temperature study represented by Figs. 3 and 4 shows no evidence of cholesterol-induced glycolipid separation into coexisting phases. As was true for cholesterol addition, temperature reduction was seen to have less effect close to the methyl terminus of the 24:0 chain. The sterol did not measurably increase the order of the long chain terminal methyl group (most clearly seen in Fig. 2 *b*). The degree of order for the terminal methyl of 24:0 GalCer was about half that of the 18:0 terminal methyl group in membranes containing cholesterol over the temperature range studied. In the absence of cholesterol, order of the methyl terminus was some 66% that of the short chain methyl terminus over the same temperature range (Morrow et al., 1993).

In separate experiments, spin label orientation order parameters S_{mol} ($=2S_{CD}$ under the present conditions), were determined (Gaffney, 1976) by EPR spectroscopy for 18:0 and 24:0 GalCer having a nitroxide radical attached rigidly at C_{16} of the glycolipid fatty acid. These measurements were carried out on spin labeled lipids dispersed at 2 mol % in fluid membranes of SOPC and SOPC with 30 mol % cholesterol. The values for (spin labeled) methylene group order at C_{16} of the long chain species were found to be consistently twice those for the same (spin labeled) carbon of 18:0 GalCer: S_{mol} values of 0.09 and 0.17 were found for 18:0 GalCer and 24:0 GalCer, respectively, in SOPC, and 0.20 and 0.40, respectively, in the presence of cholesterol.

Quantitative analysis of data derived from these experiments was employed to consider the implications for long chain penetration across the bilayer midplane. Schindler and Seelig (1975) demonstrated that it is possible to use order parameters of chain methylene segments, in conjunction with the known C-C bond length, to arrive at an estimate of the time-average extension of the chain as a whole into the fluid membrane interior. Their approach involves assigning as 0 the probability of any conformation with $\langle \cos \beta \rangle$ negative, where β is the angle between the normal to the plane formed by the CD_2 bonds and the bilayer normal. Such an assumption could break down significantly for the methyl terminal few groups, leading to an overestimation of contribution to overall length for small order parameters. In the present case, the relationship used was

$$\langle \text{Length} \rangle = 1.25\ \text{Å}(n \times 0.5 + \Sigma S_{CDi}) \qquad (3)$$

where $n = 16$ or 22, for 18:0 and 24:0 chains, respectively. For 18:0[d_{35}] GalCer in SOPC, the time-averaged length of the deuterated segment without methyl group would be 13.8 Å at 40°C, and the presence of cholesterol increased this by 1.5 Å to 15.3 Å. For 24:0[d_{47}] GalCer in SOPC, the time-averaged length of the deuterated segment without methyl group would be 18.1 Å at 40°C, and the presence of cholesterol increased this by 1.6 Å to 19.7 Å. Thus, the effect of cholesterol was similar for each GSL. The 24:0 chain is calculated to be some 4.3 Å longer than the 18:0 chain in the absence of cholesterol, and some 4.4 Å longer in the cholesterol-containing bilayers. Assuming that the length of the 18-carbon chains determine the bilayer thickness, this leads to the conclusion that the long chain fatty acid of 24:0[d_{47}] GalCer extends 4.3 to 4.4 Å farther than does the 18:0[d_{35}] analogue in the absence and in the presence of cholesterol. This suggests that the average location of the end of the long chain is at the level of C_{12} or C_{13} in the opposing monolayer acyl chains.

DISCUSSION

^{2}H-NMR spectroscopy offers a very useful non-perturbing technique for probing a number of aspects of molecular behavior in membranes. Its sensitivity to conformation, orientation, and motion characteristics of molecules was utilized in the present work to study the effect of cholesterol on two representative GSLs as minor components in fluid phospholipid membranes. Since the phase diagrams for 18:0 and 24:0 GalCer in SOPC have been demonstrated to possess essentially superimposable fluidus curves (Lu et al., 1993), it seems likely that distinctions between them reported here are not the result of differences in lateral association. Furthermore, at cholesterol concentrations of 20 mol % or more, phosphatidylcholines without polyunsaturated fatty acids are generally considered to form a homogeneous "liquid ordered" ("l_o") (Ipsen et al., 1990), or "β"-phase (Vist and Davis, 1990) (see also Thewalt and Bloom, 1992). In keeping with this logic, all spectra could be adequately interpreted in terms of a single population of GSL molecules undergoing rapid axial rotation.

Extensive ^{2}H-NMR data dealing with acyl chain order parameters of glycerol-based lipids and directly relevant to the present work are available from a number of laboratories (reviewed in Seelig, 1977; Davis, 1983; Smith, 1984; Mantsch et al., 1977). The technique has been a major tool employed in characterizing effects of cholesterol on glycerolipids in membrane architecture (Ipsen et al., 1990; Stockton and Smith, 1976; Oldfield et al., 1978; Rance et al., 1982; Vist and Davis, 1990; Nezil and Bloom, 1992; Sankaram and Thompson, 1990). In addition to laying a firm basis for model systems, this literature includes data for isolated membranes of *Acholeplasma laidlawii* (Rance et al., 1982) with and without cholesterol and for *Escherichia coli* (Gally et al., 1979). Thus it is noteworthy that orientation order profiles for the glycerolipids that have been studied all contain the same key features in the presence and absence of cholesterol (although such data are for systems of relatively homogeneous chain length). Furthermore, the profiles have shown remarkable similarities to one another throughout the wide range of single-component, mixed, and natural membranes studied. Where tested, *sn*-2 chain order parameters were also similar for plasmalogens (phospholipids with *sn*-1 ether linkages) to those of corresponding phospholipids (Malthaner et al., 1987). Such observations have been partly responsible for development of the concept of a generalized correlation length for decay of order along saturated alkyl chains in membranes, i.e., profiles for very different systems are often superimposable if temperature is adjusted to give similar order parameters in the plateau region (Lafleur et al., 1990). The same approach has been demonstrated to be applicable among certain chains of different length if position along the chain is scaled (Morrow and Lu, 1991).

^{2}H-NMR information related to acyl chain orientation order profiles is available in the literature for pure GalCer in bilayer form (Morrow et al., 1993; Skarjune and Oldfield, 1979), and for GalCer in phospholipid bilayers (Hamilton et al., 1994; Singh et al., 1992a, b; Lu et al., 1993; Morrow et al., 1993) or in a binary mixture with cholesterol (Skarjune and Oldfield, 1979). Order parameter profiles do not exist in the literature for GSLs in cholesterol-containing phospholipid membranes, although some data have been reported for the C_{16} position from experiments with nitroxide spin-labels rigidly attached at this location (Mehlhorn et al., 1988). It was noted early on that considerable similarity existed between order parameters for pure GSLs (Skarjune and Oldfield, 1979) or GSLs in PC bilayers (Sharom and Grant, 1977) and corresponding data for glycerolipids. The present work has demonstrated that cholesterol affects GSLs in bilayer membranes in the same fashion as it does glycerolipids. It should be noted too that spectra recorded here for 18:0[d_{35}] GalCer and 24:0[d_{47}] GalCer in phospholipid/cholesterol bilayers are very similar to profiles known for pure deuterated 16:0 GalCer (Skarjune and Oldfield, 1979) and 24:0 GalCer (Morrow et al., 1993), respectively in fluid bilayer form.

In the present work, order parameter profiles for 18:0 GalCer in both SOPC and in SOPC/cholesterol demonstrated a "plateau" of slowly decreasing S_{CD} values to a depth of C_{10}, with more rapidly decreasing order from C_{11} to the methyl terminus. The same pattern has been described for the perdeuterated palmitic acid chain of pure POPC and POPC/cholesterol (Nezil and Bloom, 1992), and for perdeuterated stearic acid dispersed in egg PC or egg PC/cholesterol (Stockton and Smith, 1976). For 18:0 GalCer, cholesterol raised the order at each point on the curve, with the greatest absolute effect being in the surface (plateau) region, and a somewhat larger relative effect immediately below that level. The same result has been specifically noted in the influence of cholesterol on glycerol-based lipids in egg PC (Stockton and Smith, 1976) and *A. laidlawii* (Rance et al., 1982) membranes. In both the presence and absence of cholesterol, order parameter profiles for the perdeuterated 24:0 fatty acid attached to GalCer were found to very closely track those of the corresponding 18:0 species to a membrane depth of C_{13} to C_{14}, suggesting that the acyl chains in these species share common isomerization amplitudes to this depth. Immediately below this point, profiles for the 24:0 chain deviated significantly to greater order. As a result, 24:0 profiles displayed a second, minor "plateau region" of reduced slope in this portion of the acyl chain. The same effect was present as measured by a nitroxide spin label at C_{16}. The condensing effect of cholesterol was reduced somewhat at the terminal methyl group of the 18:0 GalCer chain. This reduction was more pronounced in the 24:0 analogue to the point that no difference was measured at C_{24} between membranes with and without cholesterol.

It is interesting to consider implications of the present experiments to cholesterol effects on the probability of midplane crossing by the very long fatty acid of 24:0 GalCer in a shorter chain host matrix. It was originally suggested that midplane crossing may occur for GSLs as minor membrane components in shorter matrices, based upon the observation

that spin label order parameters measured at C_{16} of the glycolipid 24:0 fatty acid were larger than at the corresponding position of an 18:0 fatty acid (Mehlhorn et al., 1988). The term "interdigitation," has been used to refer to an analogous phenomenon whereby pure (single component) mixed chain lipids might interpose the methyl ends of one or both of the alkyl chains into the opposing monolayer (Jeffrey et al., 1989; Davis and Keough, 1985; Reed and Shipley, 1987; Bunow and Levin, 1980; Levin et al., 1985). Although there is some danger of semantic confusion, the same term has been borrowed to refer to the possibility of midplane crossing by GSLs (Mehlhorn et al., 1988) and cholesterol (Sankaram and Thompson, 1990, 1991) in more complex fluid membranes. Thompson and colleagues have specifically noted the possible critical relevance of cholesterol to interdigitation. In so doing, they emphasize the "average" nature of time-dependent lipid arrangements with regard to midplane crossing, suggesting that in the l_o phase there is partial interdigitation of the cholesterol molecule on a statistical basis. Both Sankaram and Thompson (1990, 1991) and Reinl et al. (1992) have suggested that vertical location of cholesterol relative to the midplane may be modulated by temperature under certain conditions, likely close to the main transition temperature of the host phospholipid. In the present work, for 18:0[d_{35}] GalCer and 24:0[d_{47}] GalCer the order parameter profile shapes and relative relationships (with and without cholesterol) were found to persist largely unchanged over the temperature range studied (25 to 55°C). This observation would argue against there being major changes in the average location of cholesterol under our conditions.

Order parameters determined for spin labels rigidly attached to C_{16} of 24:0 GalCer and dispersed in SOPC were about twice as large as those determined for the same labels at C_{16} of 18:0 GalCer under a given set of conditions. This was also quantitatively true for membranes with added cholesterol; which is the same result reported earlier for other phospholipids (Mehlhorn et al., 1988). As measured by ^{2}H-NMR, the order at C_{16} was 14–15% higher for the long chain GalCer (compared to 18:0 GalCer) in SOPC/cholesterol over the temperature range studied (the difference was 21% in SOPC alone). The difference between short and long chain order increased to 27–29% at C_{17} (31% in SOPC without sterol). The expectation of quantitatively different order parameters as measured by NMR and EPR has been discussed by others (Moser et al., 1989). Chain length calculations based upon observed NMR order parameters, making the approximation of no backfolding, suggest that the long chain species crosses the midplane by about 4 Å. The length calculation is essentially a quantitation of the observation that the 24:0 chain follows closely the order profiles of the 18:0 chain to a depth of C_{14}-C_{15}, has somewhat greater order to C_{18}, and non-zero order beyond that point. It must be taken as an upper limit for the mean chain extension. It is important to keep in mind that all such arguments deal with highly mobile systems, with the result that reference is to "average" lipid location over the (long) NMR time scale. It is interesting to note that the characteristic "double plateau" shape of the order profile seen in the present experiments for the long chain GalCer in SOPC and SOPC/cholesterol was also seen in fluid bilayers of pure 24:0 GalCer, a single component mixed chain system which has been suggested to interdigitate (Reed and Shipley, 1987).

Other observations clearly indicate that midplane crossing in fluid membranes such as those studied here is associated with adverse steric effects. A general framework developed by Morrow et al. for considering chain length extension (Morrow et al., 1993), indicated that the fatty acid methyl terminus of 24:0 GalCer in lipid bilayers, samples a wide conformational space. Also, it should be noted that at no point did the slope of plots of S_{CD} versus chain position become positive for 24:0[d_{47}] GalCer in the 18-carbon matrix. The likely interpretation of this is that at no point did methylene groups of the long fatty acid became more ordered toward the methyl terminus. Similarly, Jeffrey et al. (1989) calculated that the methyl group on sulfate-substituted 24:0[d_3] GalCer exhibited wide conformational swings in a fluid phase thought to be partially interdigitated. The chain ordering effect of cholesterol on 24:0[d_{47}] GalCer was found in the present work to be minimal near the long chain methyl terminus. Since cholesterol greatly reduces the compressibility of fluid membranes (Ipsen et al., 1990; Needham et al., 1988), an adequate rationale for our observations would appear to be that the presence of cholesterol exerts a marked generalized tendency to chain extension, but at the same time increases steric hindrance associated with midplane crossing. Steric hindrance might be particularly associated with lateral diffusion jumps, which are very fast on the NMR time scale.

The experiments reported here were limited to the simple GSL, GalCer. However, there is good reason to suppose that the results may be extended to GSLs in general. First, there is the fact that the overall profile shapes in the presence and absence of cholesterol are similar to ones known for acyl chains (and ether-linked chains) of glycerolipids (and plasmalogens) as described above, in a great variety of systems including cell membranes. Second, data recorded for a wide range of GSLs including complex charged and neutral species with spin labeled fatty acids specifically demonstrated quantitative correspondence for chain behavior among major GSL families (Mehlhorn et al., 1989). Recently work has been reported for globoside and GM_1 deuterated at C_2 (i.e., very close to the surface), which found great similarity to results for GalCer (Barber et al., 1994).

CONCLUSIONS

For a GSL having 18-carbon fatty acid comparable in length to those of surrounding fluid phospholipids, cholesterol effects upon acyl chain arrangement are very similar to those well known from previous work with glycerolipids. The absolute effect of cholesterol is highest near the surface (the "plateau" region), and the relative effect is slightly higher just below the plateau region. Essentially the same features and cholesterol effects exist for very long GSL fatty acids in common phospholipid membranes to a depth of C_{13}–C_{14}.

Molecular effects peculiar to the long chain GSL are centered about the "extra" portion of the acyl chain and are also seen for the pure glycolipid in bilayer form. A secondary plateau region, which demonstrates relatively higher order than corresponding segments of shorter chain species, is created. Cholesterol has minimal ordering effects on the methyl terminus of very long chain GSL fatty acids. It is likely that these factors operate in cell plasma membranes.

The fate of very long acyl chains on GSLs which are minor components in standard length PC and PC/cholesterol matrices, seems determined by two sets of competing forces. On the one hand the longer chains are constrained by surrounding bulk matrix to a common orientation order parameter profile through the (upper) plateau region. There appears to be higher long chain order below this point to the neighborhood of the midplane. The "extra" portion can thus spend significant time across the midplane, which may result in some favorable enthalpic gain from alignment with opposing chains. Opposing these factors are steric hindrance and the likely requirement for large chain distortions during lateral diffusion jumps. The result appears to be that the "extra" portion of the very long chain is subject to wide conformational swings.

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