Glycosphingolipid acyl chain orientational order in unsaturated phosphatidylcholine bilayers

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ABSTRACT The glycosphingolipid, galactosyl ceramide (GalCer), was studied by ^2H nuclear magnetic resonance (NMR) in fluid phospholipid bilayer membranes, with regard to arrangement of its acyl chain. For this purpose, species with perdeuterated 18-carbon fatty acid (18:0[d_{35}] GalCer) or with perdeuterated 24-carbon fatty acid (24:0[d_{47}] GalCer) were dispersed in bilayers of the 18-carbon phospholipid, 1-stearoyl-2-oleoyl-phosphatidylcholine (SOPC). For 18:0[d_{35}] GalCer, smoothed profiles of the order parameter, S_{CD}, were found to be very similar to one another over the range of glycolipid concentration, 5–40 mol%. In addition, they were very similar to orientational order parameter profiles well known from the literature on phospholipid and glycolipid acyl chains (which deals in general with membranes of homogeneous chain length in the range 14–18 carbons). Corresponding order parameter profiles for the long-chain species, 24:0[d_{47}] GalCer, were also similar to one another for glycolipid concentrations between 5 and 40 mol%. Their shapes, however, were distinctly different from those of the shorter chain analogues. S_{CD} profiles for the two species were quantitatively similar to a membrane depth of C_{15}. S_{CD} values at C_{16} and C_{17} were ~20 and 30%, respectively, higher for the long-chain glycosphingolipid than for its short-chain analogue in SOPC. Nitroxide spin labels attached rigidly to C_{16} of the long-chain glycolipid in SOPC gave electron paramagnetic resonance (EPR) order parameters that were twice as high as for a spin label at C_{16} on the shorter chain glycolipid.

Comparison was made between spectra of 24:0[d_{47}] GalCer in SOPC and fully hydrated bilayers of the pure 24:0[d_{47}] GalCer, a system that is considered to be partially interdigitated in fluid and gel phases. The resultant ^2H NMR order parameter profiles displayed similar features, indicating that related organizational properties exist in these fluid systems. Effective chain length of 24:0[d_{47}] GalCer within the SOPC membrane was calculated using the method of Schindler and Seelig (1975. *Biochemistry,* 14:2283–2287). The result suggested that the long-chain fatty acid should protrude roughly one third of the host matrix chain length across the bilayer midplane. However, a treatment of the same order parameters making very few assumptions about chain conformation indicated a high degree of orientational flexibility for the "extra" length of the long chain fatty acid. It seems likely that a realistic treatment of the long-chain fatty acid in a shorter chain fluid host matrix considers interdigitation as a subset of the conformational possibilities, many of which are rapidly interconverting on the NMR timescale of 10^{-4}–10^{-5} s and longer lived on the EPR timescale of 10^{-8}–10^{-9} s.

INTRODUCTION

The concept of phospholipid arrangement in bilayer membranes involves two planar lipid monolayers with hydrophobic domains in close apposition. The bilayer midplane is customarily viewed as a planar "potential space" between two flat surfaces composed of fatty acid terminal methyl groups associated with these monolayers. However, there is a significant range of lipid lengths within a given natural membrane (and in cell membranes this region is well known to be traversed by numerous structures). In higher animal cells, lipid length inequality is particularly striking for glycosphingolipids (GSLs)[1]: the single fatty acid of the ceramide backbone is commonly 18–24 or 26 carbons in length, whereas host membrane phospholipids typically have fatty acids of 16 or 18 carbons, and the glycolipid sphingosine alkyl chain extends some 15 carbons into the hydrophobic interior. In general, GSLs are minor membrane components, although in some cases they make up a sizeable fraction of the membrane lipids.

A considerable literature exists on lipids having medium length (14–18 carbon) acyl chains, with regard to membrane structure. In contrast, the arrangement and dynamics of lipids with very long chains in medium length host matrices is largely unknown. It has been suggested that fatty acid length may have important impact on GSL roles as membrane recognition sites and structural elements (1–3). We have studied previously this phenomenon spectroscopically by attaching spin-label probes at C_{16} of GSLs incorporated into phospholipid bilayers (4). ^2H nuclear magnetic resonance (NMR) offers a nonperturbing technique for probing lipid arrangement and behavior at all membrane depths. "Order parameters" associated with motion of lipids in fluid membranes are readily calculated from wideline ^2H NMR spectra and have proven useful in such studies (5–7). Orientational order parameter refers to tensor elements that describe the time average orientation of portions of a given molecule. NMR of lipids with deuterated methylene groups and electron paramagnetic resonance (EPR) of spin-labeled derivatives have provided distinctive orientational order parameter profiles for lipids in a wide variety of fluid membranes (5–10). Such studies have almost all been carried out in membranes of homogeneous chain length.

In the present work we have considered the physical arrangement and dynamics of GSL acyl chains in fluid

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[1] *Abbreviations used in this article:* DPPC, dipalmitoyl phosphatidylcholine; EPR, electron paramagnetic resonance; 18:0 GalCer, *N*-stearoyl galactosyl ceramide; 24:0 GalCer, *N*-lignoceroyl galactosyl ceramide; GSL, glycosphingolipid; NMR, nuclear magnetic resonance; SOPC, 1-stearoyl-2-oleoyl phosphatidylcholine.

0006-3495/93/03/654/11 $2.00

matrices formed from the phospholipid 1-stearoyl-2-oleoyl phosphatidylcholine (SOPC), which is representative of species commonly found in cell membranes. The GSL selected for study, galactosyl ceramide (GalCer), was deuterated by replacement of its natural fatty acid mixture rich in 18- and 24-carbon chain lengths, with perdeuterated 18:0 or 24:0 species ([d_{35}]stearic acid and [d_{47}]lignoceric acid, respectively).

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 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 after general procedures described previously (11, 12, and 4, respectively). 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 hydrolysing the long-chain spin-label fatty acid methyl ester but was also reflected in the yields of reactions that linked fatty acid to sphingosine backbones. Preparation of liposomes containing 18:0[d_{35}] GalCer and 24:0[d_{47}] GalCer followed procedures described previously (12). Individual samples containing up to 15 mg of deuterated lipid were hydrated in ~300 μl of 50 mM phosphate buffer at pH 7.0. Lipid bilayer membranes for EPR experiments were prepared and their spectra recorded as described elsewhere (4), hydrating with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid 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 ^2H NMR experiments have been described previously (12). For each spectrum, 12,000–48,000 transients were collected with a repetition time of 0.6 s. Smoothed order parameter profiles were obtained as outlined by Sternin et al. (13) and Lafleur et al. (14).

RESULTS

Data are presented for 18:0[d_{35}] GalCer and 24:0[d_{47}] GalCer as examples of GSLs possessing common extremes in fatty acid chain length. In most samples, deuterated glycolipid was dispersed in fluid bilayers of the monounsaturated phospholipid, SOPC (fluid/gel transition temperature 6°C [15]). Fig. 1 *a* displays observed powder spectra of the short-chain (18:0[d_{35}]) species for a series of glycolipid mole fractions from 0.05 to 0.40. Spectra were run at temperatures such that similar plateau value order parameters should exist; this involved choosing temperatures and membrane compositions that reflect points describing a curve parallel to and above the fluidus of the GalCer/SOPC phase diagram (Lu, D., D. Singh, M. R. Morrow, and C. W. M. Grant, unpublished results). As anticipated, all spectra show typical liquid crystal features (5–7).

A useful property of deuterium liquid crystal spectra is that the effects of axially symmetric motions are well understood. The ^2H NMR spectrum associated with a particular deuteron is a doublet with 90° edges split by

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

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

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

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. Bloom and co-workers (13, 14) have demonstrated that by assuming a monotonic decrease in order along the chain, a smoothed orientational order parameter profile may be extracted from the oriented spectrum of a perdeuterated acyl chain. (The smoothed order parameter approach [13, 14] essentially disperses over the whole profile errors introduced by ignoring the anomalous nature of splittings associated with conformational peculiarities at C_2.) Oriented spectra may be obtained from powder pattern spectra using the "dePakeing" algorithm (16, 17). This was done for the spectra in Fig. 1 *a* (dePaked spectra not shown), and the resultant smoothed order parameters were calculated. Fig. 1 *b* shows derived orientational order parameter profiles for the four highest concentration spectra in Fig. 1. The $x = 0.05$ sample gave qualitatively the same sort of profile, but this curve has been omitted to simplify the figure. Each smoothed profile displays a plateau region near the bilayer surface, with decreasing order toward the methyl terminus. It is clear that the shape of the profiles is largely independent of glycolipid concentration within the membrane.

Fig. 2 permits comparison of the results in Fig. 1, with relevant data for phospholipids. It shows the profile for the sample having 40% 18:0[d_{35}] GalCer in SOPC at 73°C, together with data obtained by scaling the smoothed profile for liquid crystal-phase pure dipalmitoyl phosphatidylcholine perdeuterated in the *sn*-2 chain ([d_{31}] DPPC) at 42°C (DPPC data from reference 18). Although the profile for the glycolipid has a relatively shorter plateau than that for the phosphatidylcholine, they are very similar overall.

Fig. 3 *a* shows spectra for a series of concentrations of the long-chain glycolipid, 24:0[d_{47}] GalCer, in SOPC, which is analogous to that displayed in Fig. 1 *a* for the 18:0[d_{35}] analogue. As in the case of the shorter chain GSL, temperatures were chosen such that the samples correspond to points along a line parallel to and above the liquidus of the corresponding 24:0 GalCer/SOPC phase diagram (12). As anticipated from the choice of sample temperature relative to the known phase diagram, the spectra are all liquid crystal in nature. There is relatively greater spectral intensity buildup in the central region than was seen in the spectra of the shorter chain analogue (Fig. 1). Fig. 3 *b* shows the corresponding smoothed order parameter profiles for 24:0[d_{47}] GalCer in SOPC. Once again the data for the $x = 0.05$ mole

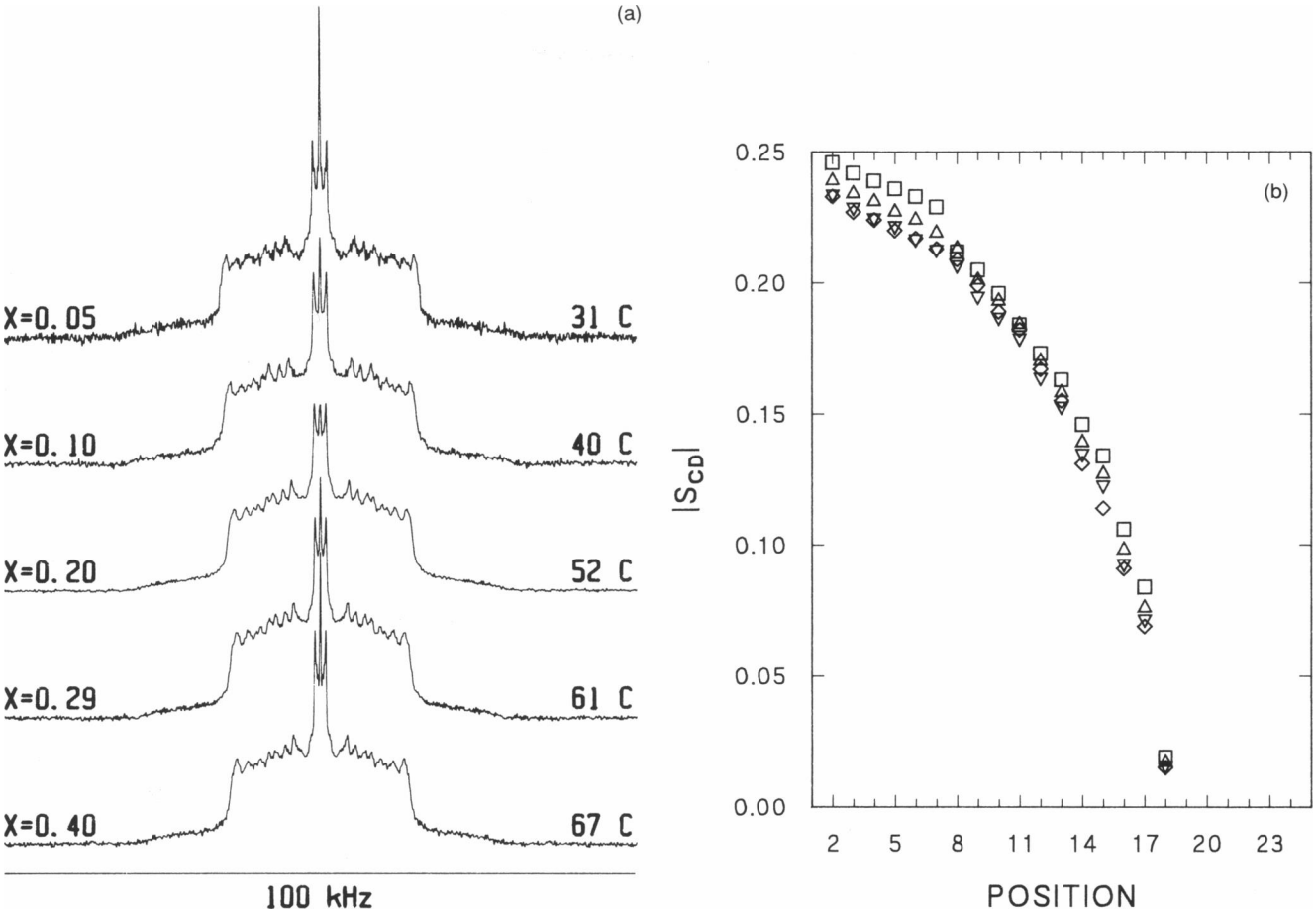

FIGURE 1 (*a*) ^{2}H NMR spectra for various concentrations of 18:0[d_{35}] GalCer in fluid phosphatidylcholine bilayers. For a given concentration of GalCer in SOPC, temperatures were chosen from examination of the 18:0 GalCer/SOPC phase diagram (Lu, D., D. Singh, M. R. Morrow, and C. W. M. Grant, unpublished data) to correspond to points describing a line parallel to the liquidus curve, with the intention of providing approximately equal values of $|S_{CD}|$ in the plateau region of the acyl chains. Fractional concentration of glycolipid is indicated. (*b*) Smoothed order parameter profiles derived from the four highest concentration spectra in *a* above: (□) $x = 0.10$ (10°C); (△) $x = 0.20$ (52°C); (▽) $x = 0.29$ (61°); (◇) $x = 0.40$ (67°C).

fraction sample have been omitted from the plots to simplify data presentation. There is some evidence that the flatness of the plateau region of the profile for 24:0 GalCer in SOPC depends slightly on concentration of the long acyl chain. There are basic differences in the order parameter profiles for the long- and short-chain glycolipids.

Fig. 4 displays superimposed profiles for 24:0[d_{47}] and 18:0[d_{35}] GalCer. Data displayed are from an experiment in which each glycolipid was dispersed at 10 mol% and spectra were recorded at 40°C. Similar results were seen at other temperatures. The peak assignments in the longer chain case were checked at positions C_{22}, C_{23}, and C_{24} by specific deuteration (spectra not shown). Features to note in Fig. 4 are as follows: (*a*) the profiles have very similar shapes to a membrane depth of C_{15} and (*b*) at depths greater than C_{15} the 24:0 profile diverges markedly to produce a feature resembling a second plateau region. For the longer chain glycolipid in SOPC, the C_{16} and C_{17} deuterons are significantly more ordered than in the short-chain case (~20 and 30%, respectively). The deuterons of the C_{18} methylene group of the long chain are slightly more ordered than the C_{17} deuterons of the short acyl chain, whereas C_{19} has the same degree of order as C_{17} in the short chain glycolipid. C_{20} to C_{23} in the long chain are all less ordered than C_{17} of the short chain, and the rate at which order decreases with position is much lower for the (methyl) end of the long chain than is the case in homogeneous chain length bilayers. In a separate experiment, spin-label order parameters were determined (8) by EPR spectroscopy for 18:0 and 24:0 GalCer having a nitroxide radical covalently attached at C_{16} of the glycolipid fatty acid. These measurements were carried out for spin-labeled lipids dispersed at 2 mol% in fluid membranes of SOPC. The values were found to be 0.09 and 0.17, respectively, at 25°C and 0.06 and 0.14, respectively, at 37°C.

The order parameter profile of the long-chain GSL was examined in more detail for two samples: the 10 mol% 24:0[d_{47}] GalCer sample and a sample of pure

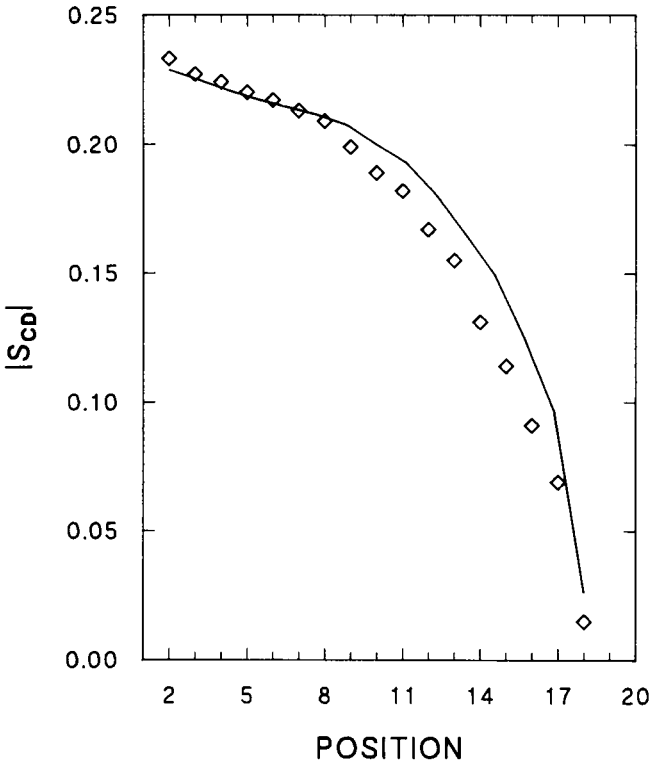

FIGURE 2 Comparison of glycosphingolipid and phospholipid orientational order parameter profiles. The profile for 40 mol% 18:0[d_{35}] GalCer in SOPC at 73°C is presented (◇), along with data obtained by scaling the smoothed profile for pure *sn*-2[d_{31}] DPPC at 42°C (18).

24:0[d_{47}] GalCer for comparison. Fig. 5 shows observed and dePaked spectra for 24:0[d_{47}] GalCer at mole fraction 0.10 in SOPC at 52°C. Fig. 6 shows the observed and dePaked spectra for pure 24:0[d_{47}] GalCer at 85°C. These spectra were obtained with large numbers of scans (120,000 and 140,000, respectively), using oversampling by a factor of 4 to further improve signal to noise (19). Both samples represent situations in which the deuterated chain was incorporated into a membrane having a large fraction of shorter chains: the effective fraction of long chains in the pure sample being 0.5 and in the mixture 0.05.

Fig. 7 shows smoothed orientational order parameter profiles corresponding to pure 24:0[d_{47}] GalCer (*upper curve*) and to the same material dispersed at 10 mol% in SOPC, as derived from the spectra presented in Figs. 6 and 5, respectively. The buildup of intensity in the central regions of the original spectra is reflected in flattening of the profiles toward the methyl end. The basic shapes are importantly similar. Quantitative differences seen, particularly in the high order parameters corresponding to the headgroup end of the acyl chain, may be attributable partially to differences in overall membrane fluidity, which is difficult to control in such an experiment. For the pure glycolipid, 85°C is a few degrees above the gel–liquid crystal transition (the fluid/gel transition temperature of 24:0 GalCer is reported to be 82–85°C [20, 21]), whereas 52°C is more than 20° above the liquidus line for the 10 mol% mixture with SOPC (12). The plateau region for the pure glycolipid is flatter than that observed for the mixture. Although this may reflect relative proximity to the transition in the pure glycolipid, the possibility that the presence of the shorter chain is suppressing the order of the longer chain also should be considered.

Two analyses of the order parameter data derived from experiments summarized in Fig. 4 (18:0[d_{35}] and 24:0[d_{47}] GalCer dispersed at 10 mol% in SOPC) have been used to consider the implications for chain organization. In the first case, the emphasis is on average distance of chain extension into the fluid membrane interior; in the second case, the calculation is of relative probabilities of the C—C bond being oriented at 90° to a direction normal to the membrane surface (reflecting chain disorder). The first treatment follows an approach outlined by Schindler and Seelig (22), which relates chain extension to order parameter. This approach involves assigning as zero 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. Thus, $\beta = 0°$ is defined as the CD_2 orientation having maximum contribution to extension toward the bilayer midplane. Such an assumption could break down significantly for the methyl terminal few CD_2 groups, leading to an overestimation of contribution to overall length for small order parameters. Nevertheless, application to the orientational order parameters, S_{CD}, for 18:0[d_{35}] GalCer and 24:0[d_{47}] GalCer in fluid SOPC (Fig. 4), leads to the conclusion that the long-chain fatty acid of 24:0[d_{47}] GalCer extends 4.3 Å farther than does the 18:0[d_{35}] analogue. If the bilayer midplane is ~14.6 Å and the methyl length is ~0.9 Å, then the calculation 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.

A potentially more general analytical framework for dealing with observed values of S_{CD} may be attempted by considering a wider range of possible orientations about carbon atoms of the acyl chain. Unfortunately, once the restriction on backfolding is relaxed, it is impossible to extract the projection of the chain along the bilayer normal using only $|S_{CD}|$. It is, however, possible to extract the probability that a given C—C bond is perpendicular to the bilayer normal from order parameter data if such probabilities are available for neighboring segments. As shown by Jeffrey et al. (23), rapid rotation of the methyl group about its C—C bond, on the NMR timescale, makes it possible to determine the probability that the rotation axis of the terminal methyl group is perpendicular to the bilayer normal. Assuming that *trans*-gauche isomerization is the mechanism of chain distortion, the angle ϕ between the bilayer normal and any C—C bond

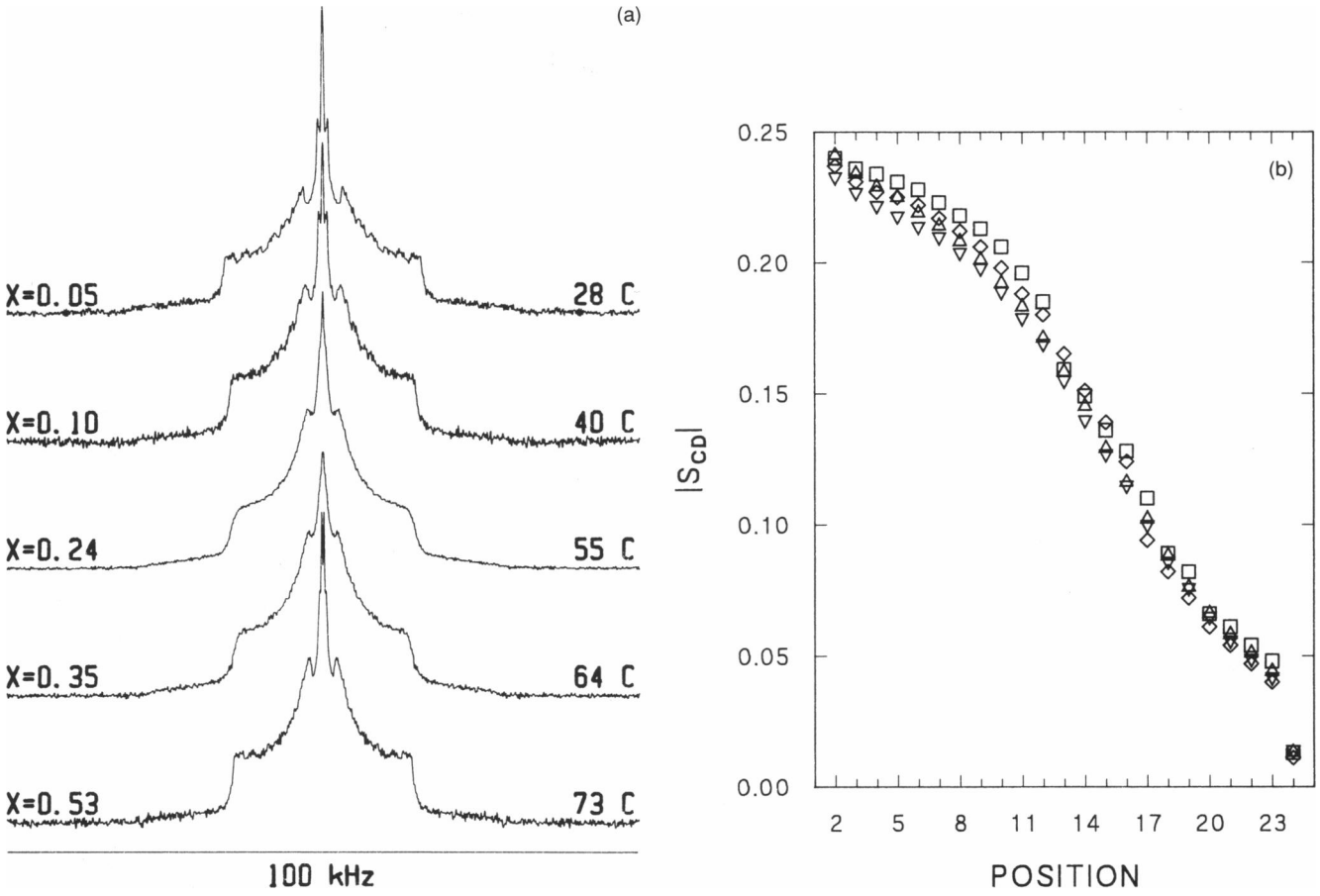

FIGURE 3 (*a*) ^{2}H NMR spectra for various concentrations of 24:0[d_{47}] GalCer in fluid phosphatidylcholine bilayers. For a given concentration of GalCer in SOPC, temperatures were chosen from examination of the 24:0 GalCer/SOPC phase diagram (12) to correspond to points describing a line parallel to the liquidus curve, as described for Figure 1 *a*. Fractional concentration of glycolipid is indicated. (*b*) Smoothed order parameter profiles derived from the four highest concentration spectra in *a* above: (□) $x = 0.10$ (40°C); (△) $x = 0.24$ (55°C); (▽) $x = 0.35$ (64°C); (◇) $x = 0.53$ (73°C).

is most likely to have values of 35.3, 90, or 144.7°. For reorientation of the methyl group rotational axis among these three values of ϕ, the deuteron orientational order parameter is

$$S_{CD} = \left(\frac{3\cos^2\psi - 1}{2}\right) \sum_{\phi} p_{\phi}^{m} \left(\frac{3\cos^2\phi - 1}{2}\right), \qquad (3)$$

where ψ is the angle between the C—D bond and the methyl rotation axis and p_{ϕ}^{m} is the probability that the methyl rotation axis is inclined at an angle ϕ to the bilayer normal. Using $\psi = 109.5°$ and $p_{35.3}^{m} + p_{90}^{m} + p_{144.7}^{m} = 1$, the mean orientational order parameter can be written as

$$S_{CD} = -1/6\,(1 - 2p_{90}^{m}). \qquad (4)$$

For cerebroside sulfate in the liquid crystalline phase, Jeffrey et al. (23) used this approach to show that the terminal methyl group of the acyl chain is oriented at 90° to the bilayer normal nearly 50% of the time. We have adapted this approach to the other C—C bonds in the chain by working up the linkages toward the polar headgroup, using the probability that a particular C—C bond is oriented perpendicular to the bilayer normal to calculate the corresponding probability for the next C—C bond.

Fig. 8 shows the possible ways in which C—C and C—D bonds can be arranged around a particular carbon in the chain, assuming only *trans*-gauche isomerization. If carbon C_2 is taken to be the nth carbon from the backbone end of the acyl chain, then carbon C_1 and C_3 are positions $n - 1$ and $n + 1$, respectively, along the chain. The sequence 1–2–3 thus represents moving along the chain toward the methyl terminus.

We will take β to be the angle between the bilayer normal and the normal to the plane defined by the two C—D bonds and θ_{CD} to be the angle between a C—D bond and the bilayer normal. Following Schindler and Seelig (22), we use the abbreviations $S_s = 1/2(3\cos^2 s - 1)$. The probability for conformation i, will be labelled p_i. For conformations b through f, a mirror image can be obtained by reflection in the plane of the page, and p_i is defined to be the probability that the conformation is either i or its mirror image.

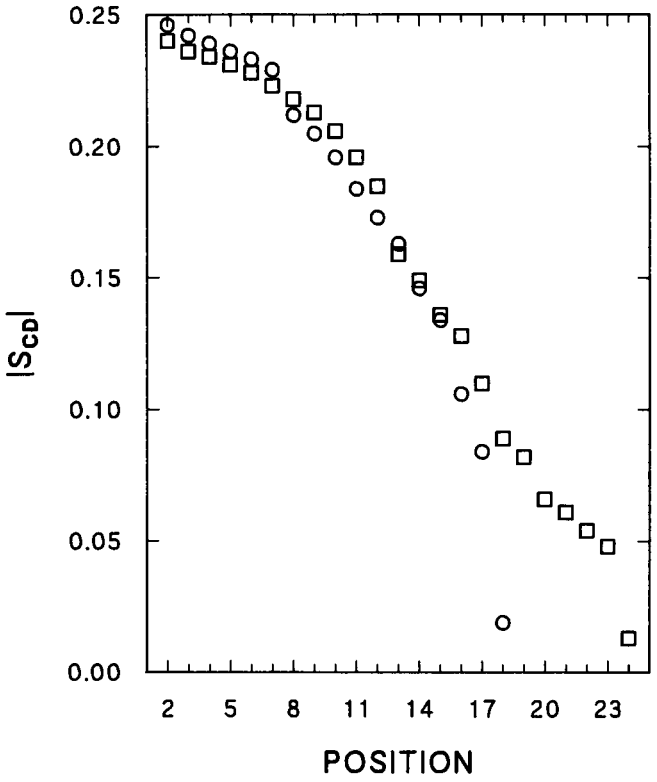

FIGURE 4 Comparison of smoothed order parameter profiles for 24:0[d_{47}] GalCer (□) and 18:0[d_{35}] GalCer (○) dispersed in fluid bilayers of SOPC. In each case, glycolipid concentration was 10 mol% and temperature was 40°C.

For conformation a, we have $\beta = 0°$ and $S^{a}_{CD} = p_a S_{90} = -\frac{1}{2}p_a$, where S^{a}_{CD} is the contribution to S_{CD} for the particular methylene group in question from the conformation a. For conformations b and c, $\beta = 60°$ and

$$S^{b,c}_{CD} = p_{b,c}\left(\frac{S_{90} + S_{35.3}}{2}\right) = 0. \quad (5)$$

For conformation d, we have $\beta = 90°$ and

$$S^{d}_{CD} = p_d\left(\frac{S_{144.7} + S_{35.3}}{2}\right) = \frac{1}{2}p_d. \quad (6)$$

For conformations e and f, we have $\beta = 120°$ and $S^{e,f}_{CD} = 0$. Finally, for conformation g, $\beta = 180°$ and $S^{g}_{CD} = p_g S_{90} = -\frac{1}{2}p_g$. The order parameter for the deuterons attached to C_2 is thus

$$S_{CD} = \frac{1}{2}(p_d - p_a - p_g). \quad (7)$$

In the notation used by Schindler and Seelig (22), $p_o = p_a$, $p_{60} = p_b + p_c$, and $p_{90} = p_d$, and the probabilities p_e, p_f, and p_g are assumed to be zero.

Having calculated the probability p^{m}_{90} that the methyl rotation axis is perpendicular to the bilayer normal, this formalism provides the means to repeat that calculation at successive positions up the chain toward the headgroup. This is a consequence of the fact that $p^{m}_{90} = p_{bdf}$,

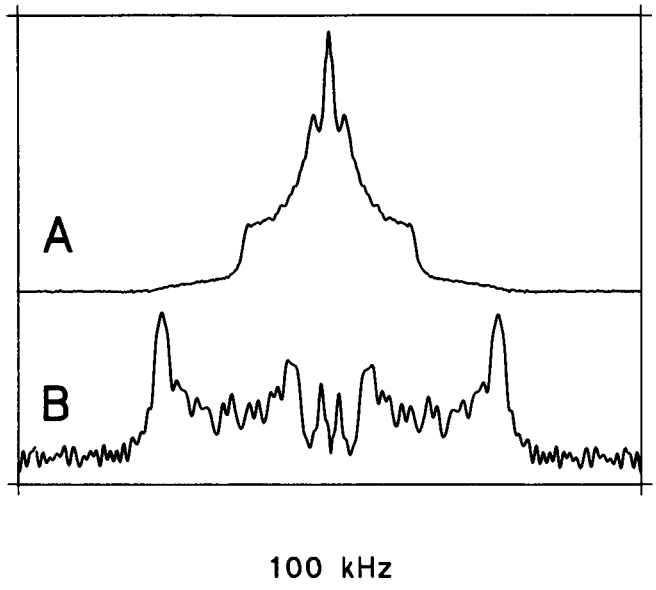

FIGURE 5 Observed (*A*) and dePaked (*B*) ^{2}H NMR spectra for 10 mol% 24:0[d_{47}] GalCer dispersed in bilayers of SOPC at 52°C. 120,000 scans were acquired with oversampling by a factor of 4 to improve signal-to-noise ratio by a factor of 2.

where $p_{bdf} = p_b + p_d + p_f$ and these probabilities apply to the next carbon up the chain from the methyl carbon. Using the order parameter for deuterons on that carbon and the fact that $p_a + p_b + \cdots + p_f + p_g = 1$, it is possible to calculate $p_{cde} = p_c + p_d + p_e$ and $p_{abgf} = p_a + p_b + p_f + p_g$ for that carbon. This process is extended further up the chain by recognizing that knowledge of p_{cde} for a given carbon is equivalent to knowing p_{bdf} for the next carbon

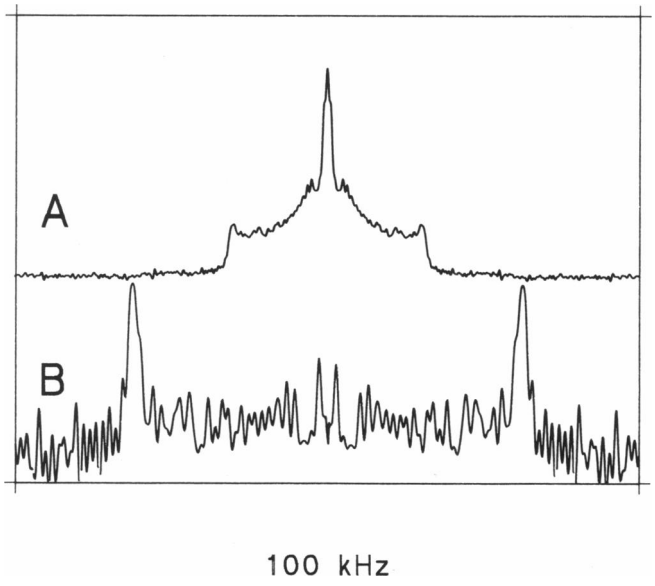

FIGURE 6 Observed (*A*) and dePaked (*B*) ^{2}H NMR spectra for pure fully hydrated 24:0[d_{47}] GalCer at 85°C. 140,000 scans were acquired with oversampling by a factor of 4.

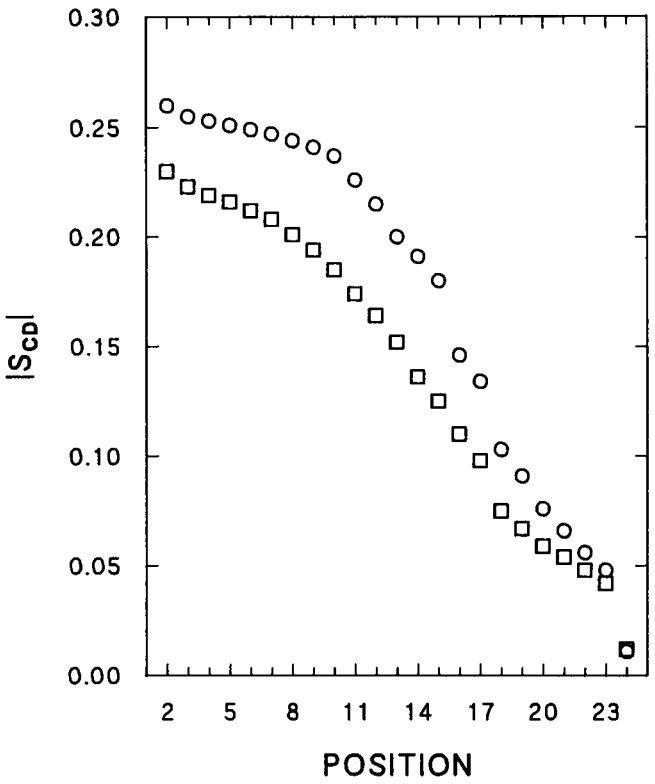

FIGURE 7 Smoothed orientational order parameter profiles corresponding to the spectral data presented in Figures 5 (□) and 6 (○).

up the chain. In principle, then, it should be possible to calculate the probability that any given C—C bond in the chain is perpendicular to the bilayer normal.

Fig. 9 shows p_{bdf} and p_{abgf} for 10 mol% 24:0[d_{47}] GalCer in SOPC and 10 mol% 18:0[d_{35}] GalCer in SOPC both at 40°C. Because of the recursive nature of the calculation, small errors in order parameters near the methyl end of the chain can lead to oscillation of the calculated probabilities, but the overall trend of the probabilities is probably represented accurately. Such oscillations are not as serious in the longer chain data.

It can be seen that from C_2 to C_{17}, the behavior of the longer chain is similar to that of the short chain in corresponding mixtures. For the long-chain glycolipid, the probability that a given C—C bond is perpendicular to the normal goes from ~0.25 near the headgroup end up to ~0.4 for C_{18} (the methylene carbon "corresponding to" the 18:0 GalCer terminal methyl group). For the remaining C—C bonds of the long chain, the probability of being oriented perpendicular to the bilayer normal increases slowly with position from ~0.43 to ~0.46. The significance of this result can be appreciated by recognizing that for an isotropically reorienting methylene group, all conformations, including the mirror images of b through f, are equally likely. In the case of such isotropic reorientation, the value of p_{bdf} is 0.5. We thus see that the last several C—C bonds of the long chain, in this mixture, are undergoing reorientation that is about as isotropic as that of the last C—C bond in the short chain mixture.

DISCUSSION

It might be expected that GSL acyl chain order, in mixtures with other lipids, would be influenced by the nature of the sphingosine backbone and by the extent of molecular mismatch within the membrane. Of special interest in the latter regard is the question of long-chain fatty acid behavior, since the frequent occurrence of very long GSL fatty acids has been implicated in their roles as receptors and structural elements of the cell membrane. Differences found between the short- and long-chain glycolipid species in SOPC cannot be attributed to different lateral associations, since we previously have demon-

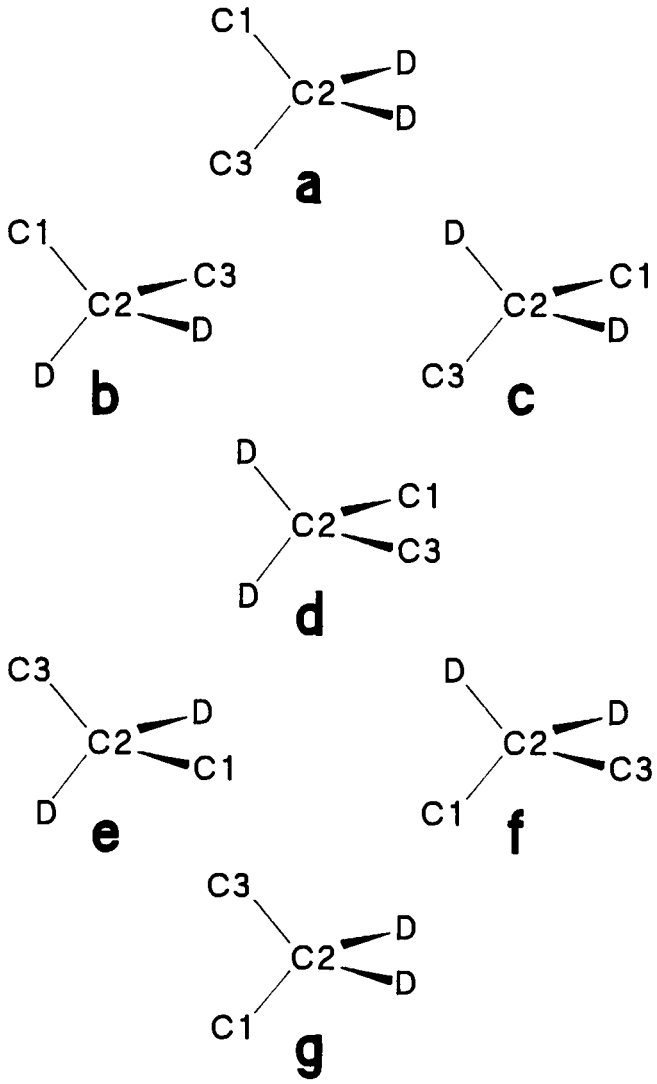

FIGURE 8 Pictorial representation of methylene conformations accessible through *trans*-gauche isomerization, which may contribute to the deuteron spectrum associated with a given position along the acyl chain.

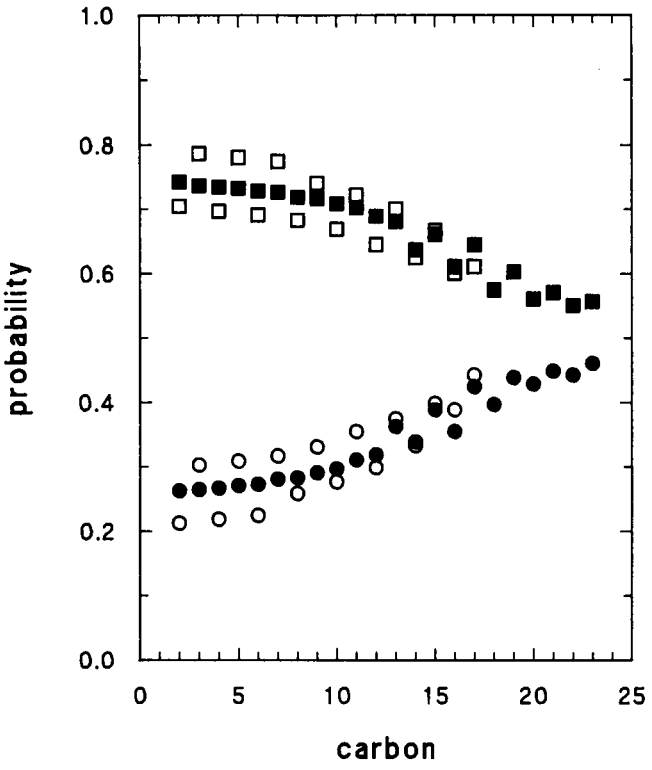

FIGURE 9 Probabilities, as a function of position along the chain, for specified sets of the methylene conformations shown in Figure 8, as calculated recursively from the order parameter profiles for 24:0[d_{47}] GalCer (*solid symbols*) and 18:0[d_{35}] GalCer (*open symbols*), both dispersed at 10 mol% in SOPC at 40°C, as described in the text. Circles show $p_{bdf} = p_b + p_d + p_f$, which is the sum of the probabilities for conformations in which the $C_2—C_3$ bond is perpendicular to the bilayer normal. Square symbols show $p_{abfg} = p_a + p_b + p_f + p_g$.

strated that the phase diagrams for 18:0 and 24:0 GalCer in SOPC have essentially superimposable fluidus curves (Lu, D., D. Singh, M. R. Morrow, and C. W. M. Grant, unpublished result).

Order parameter profiles for 18:0 GalCer in SOPC showed a plateau region near the bilayer surface, with rapidly increasing disorder toward the methyl terminus. The results were very similar to well-known literature profiles for phospholipids and glyceroglycolipids in pure and mixed lipid systems (5–7). This is in agreement with previous NMR and EPR studies of GSL fatty acid organization in pure form (24) and dispersed in phospholipid membranes (25, 26). For a variety of non-GSL systems, this profile has been reported to be determined adequately by the value of S_{CD} in the plateau region (27). When position along the chain is normalized, this simple dependence on maximum order parameter can be extended to diacyl phosphatidylcholines with a range of acyl chain lengths (18). Such observations seem to imply the existence of a somewhat generalized correlation length for decay of order along the chain and suggest that chain order may be relatively insensitive to significant structural differences. Although the discrete nature of the available set of temperatures makes precise matching impossible, the similarity of order parameter profiles for 18:0[d_{35}] GalCer, for all glycolipid concentrations studied, to known phospholipid profiles seems to be a further example of this interesting phenomenon.

Order parameter profiles for the 24-carbon GSL showed a distinctive shape. Because data were collected at finite temperature intervals and only a subset of spectra could be collected with sufficient numbers of scans to permit very accurate determination of the order parameter profiles, it was not possible to match the maximum splittings precisely. Nevertheless, for closely comparable maximum spectral splittings, the curves for 24:0 GalCer in SOPC were nearly superimposable. The similarity of profile shapes for bilayers having similar mismatch (i.e., 24:0[d_{47}] GalCer and its mixtures with SOPC) illustrates a novel extension of the observation for the phospholipid systems referred to above (18, 27) that the value of order parameter in the plateau region largely can be sufficient to determine profile shape within a group of structurally similar lipids.

Very little is known about the arrangement and behavior of the "extra" 6–10 carbon length of long-chain GSLs in membranes composed primarily of phospholipids having common chain lengths. However, pure single component mixed chain phospholipids and sphingolipids in bilayer form have been the subject of extensive characterization with regard to possible chain arrangement. "Interdigitation" refers to the phenomenon whereby pure phospholipids and sphingolipids with intramolecular alkyl chain length mismatch, when hydrated to form bilayers, may interpose the methyl ends of one or both of the alkyl chains into the opposing monolayer. The term has been used to describe possible chain arrangements in single component bilayer phases having chain length mismatch (15, 21, 28–33). It was initially applied to certain ordered phase systems, for which three forms of interdigitation have now been described: partial (long chains of one monolayer opposite short chains of the opposing monolayer), mixed (short chains of one monolayer opposite short chains of the opposing monolayer with the long chains spanning the bilayer), and complete (the membrane is in fact a "monolayer" with both polar groups and methyl termini of acyl chains near each surface). Partial interdigitation in fluid phase systems has been suggested in some mixed chain phospholipids (31, 33) and sphingolipids including 24:0 GalCer (21, 23, 34, 35) on the basis of x-ray measurements of bilayer thickness and other considerations. In some of the mixed chain phospholipids (C[18]:C[10] phosphatidylcholine and C[18]:C[12] phosphatidylcholine) studied by x-ray diffraction, the degree of interdigitation in the fluid phase is described as small (30). The nature and extent of interdigitation in the fluid phase systems is not as well understood as in the gel phase systems.

The term "interdigitation" has been borrowed to refer to the possibility of bilayer midplane crossing by glycolipid long fatty acids in lipid mixtures (4, 36). It has been suggested that interdigitation of the terminal portion of GSL 24:0 acyl chains in fluid phospholipid bilayers having shorter fatty acids could explain the observation that spin-labeled GSL order parameters measured at C_{16} were larger for 24:0 GSL fatty acids than for 18:0 fatty acids of GSLs dispersed in the same membranes (4). In the present work, the spin-label order parameters for C_{16} on 24:0 GalCer dispersed in SOPC were about twice as large as on 18:0 GalCer under the same conditions. It should be noted that the positioning of the nitroxide ring in these experiments (i.e., at C_{16}) did not force extension of the spin label itself across the bilayer midplane, a scenario that might be sterically less favorable. As measured by ^2H NMR, the order at C_{16} was ~20% higher for the long-chain GalCer. It has been argued that a significant contribution to such quantitative differences between NMR and EPR order parameters may be expected to arise from the combination of perturbation by the spin label and the very different timescales of molecular motions to which they are sensitive (10^{-4}–10^{-5} and 10^{-8}–10^{-9} s, respectively) (37, 38) and that the NMR values should be smaller (37). In general, NMR order parameters are often larger than corresponding EPR values. We observed here that order parameter profiles for the pure 24:0[d_{47}] GalCer and those for the same GSL in SOPC are qualitatively similar, which strongly suggests that analogous glycolipid chain arrangements exist in the pure system and in mixtures with SOPC. This is significant since x-ray measurements of the bilayer thickness of pure 24:0 GalCer in the fluid phase have been reported to be consistent with a partially interdigitated arrangement of the long fatty acid and short sphingosine chain (21). Pure 24:0 GalCer membranes and its mixtures in SOPC represent situations in which a large fraction of the fatty acid chains in the membrane are considerably shorter than the labeled chain.

Given the transient nature of the inter- and intramolecular relationships involved in fluid membranes, it is obviously inappropriate to consider only models in which the extra chain length is constrained to exist in a primarily extended state in the opposing monolayer. In fluid membranes, acyl chains undergo rapid conformational fluctuations by *trans/gauche* isomerization and lipid molecules translate laterally in the membrane (37). In pure single component mixed chain species (e.g., 24:0 GalCer), partial interdigitation has been pictured as a matching of the short and long chains of opposing monolayers so that, in effect, the extra length of the long chain is located beyond the plane of the opposing monolayer long chain methyls and between planes formed by methyls of the short chains. For small quantities of the 24:0 GSL dispersed in fluid bilayers of SOPC, the range of possibilities must be extended to include penetration of the long chain beyond the plane of phospholipid methyls associated with the opposite monolayer. The possibilities for the behavior of the long chain in these systems fall along a continuum between two extremes. At one extreme is an arrangement in which the methyl end of the long chain is primarily extended deep into the opposing monolayer. At the other extreme is a picture in which the extra chain length forms a highly fluid layer at the bilayer midplane without significantly penetrating beyond the methyl groups of the opposing monolayer.

The possibility of significant crossing of the membrane midplane seems to be supported by the mean chain length calculation following the approach of Schindler and Seelig (22), since this indicated average interdigitation by ~4.3 Å. Because of the assumption of no backfolding of the chain, this must be taken as an upper limit for the mean chain extension. In particular, the assumption of no backfolding might be expected to break down to some extent in highly disordered regions, although some conformations would remain largely inaccessible on the basis of steric hindrance. The same order parameter data and an extended set of allowed conformations, however, gave probabilities for C—C bonds to be oriented perpendicular to the bilayer normal, which suggested considerable chain disorder toward the methyl terminus. It is clear, in any case, that the end of the long chain is in a dynamic state.

The observation of increased order in the long chain, relative to the short chain at C_{16}, indicates a relative restriction of the long-chain motion at this point. This, however, does not distinguish clearly the possibilities described above, since even a highly fluid extra chain segment might have some restricting effect on motion of regions farther up the chain. Boggs and Rangaraj (39), using ESR, noted that complete interdigitation of phospholipids with 16:0 fatty acids, induced by glycerol and polymyxin, raised the order at C_{16} relative to that at C_5, in effect abolishing the fluidity gradient. The flattening of the 24:0[d_{47}]GalCer order parameter profiles, without consideration of the magnitude of the order parameter in the flattened region, might thus be taken as indicative of interdigitation as well as of a very fluid midplane region. On the other hand, one might question whether, if extensive interdigitation of long-chain GalCer in SOPC were the dominant conformational mode, the order should not actually rise again after reaching a minimum near C_{18}. This does not occur. If interdigitation were dominant, then one might also expect the order parameter of the terminal methyl on the 24:0 GalCer in SOPC to be higher than on 18:0 GalCer in SOPC under corresponding conditions. In fact, the opposite situation was observed, although it should be noted that Jeffrey et al. (23) also observed a highly disordered methyl group on sulfate-substituted 24:0[d_3] GalCer in a fluid phase thought to be partially interdigitated. Although the latter considerations of overall profile shape do not appear to favor a picture in which interdigitation is the dominant mode, it should be noted that the profiles for the 24:0

GalCer and 18:0 GalCer, both in SOPC, correspond closely from C_2 to C_{15}, which indicates that their chains extend, on average, to the same depth at this point. In the region of C_{16} and C_{17}, backfolding should not be significant, so the mean extension of the long chain should be at least the same, if not greater, in this region. Although calculation of mean extension of the highly disordered segment of the chain cannot be done reliably, it is true that this segment is not completely disordered and that a consideration of steric hindrance leads one to expect that the contribution to mean chain extension must continue to be in the direction away from the polar headgroup end of the chain, although possibly to only a small degree. The mean extension of the 24:0 chain must thus be at least slightly greater than that of the 18:0 chain under similar conditions. It is thus likely that, on average, the methyl group of the long chain is situated across the bilayer midplane. This, however, does not necessarily rule out a picture involving only short distance penetration of the opposing monolayer, since, if the long-chain methyl ends are highly fluid, it is not inconceivable that the bilayer thickness, locally or on average, might depart from the value for a pure bilayer. In such a situation, the bilayer midplane might not be congruent with the plane formed by the chain methyls in the monolayer opposite the side containing a specific long chain. Thus, the observed order profiles do not permit one to categorically distinguish between the extremes considered.

CONCLUSIONS

For a GSL with fatty acid chain length comparable with that of surrounding fluid phospholipids, acyl chain arrangement is very similar over a wide range of membrane compositions to that well known from previous work with glycerolipids. Chain arrangement of a long-chain GSL in phospholipid membranes with considerably shorter fatty acids differs significantly. On the NMR timescale, there is considerable quantitative analogy to a depth of C_{15}. The molecular effects peculiar to the long-chain GSL appear to be centered around the extra portion of the acyl chain.

High spin-label order parameters, observed previously at C_{16} of 24-carbon fatty acids born by GSLs in shorter length host matrices, occur also for GalCer in SOPC. A similar result was seen in the deuterium NMR data at C_{16} (and C_{17}), although the magnitude of the difference between short- and long-chain species was considerably less as measured by ^2H NMR. Chain length calculations via the method of Schindler and Seelig (22) indicate that, on average, the conformation of the long chain is such that it extends across the bilayer midplane of the fluid membrane. However, an analysis of the observed order parameters in terms of probability of orientation of a given C—C bond demonstrates that the long-chain carbons, C_{20}–C_{23}, are undergoing motion as highly disordered as that characterizing the methyl end of the 18-carbon chain. Clearly, the system studied is a dynamic one, encompassing a range of conformational possibilities. Hence, it would appear that interdigitation occurring in this highly fluid system should be viewed as a subgroup of conformational possibilities.

We thank Dr. J. R. Bolton (Department of Chemistry, University of Western Ontario) for the use of his EPR spectrometer.

This research was supported by a grant from the Medical Research Council of Canada to C. W. M. Grant and from the Natural Science and Engineering Research Council of Canada to M. R. Morrow.

Received for publication 17 September 1992 and in final form 16 November 1992.

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