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A long chain spin label for glycosphingolipid studies: transbilayer fatty acid interdigitation of lactosyl ceramide

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16-Carbon and 18-carbon fatty acids with covalently attached nitroxide free radicals have seen wide usage in membrane studies of phospholipid dynamics, orientation, and associations. However, they are inadequate for dealing with some very important questions that relate to glycosphingolipids. We report here the synthesis of a long chain (24-carbon) spin-labelled fatty acid designed for such problems. We have used both the new 24-carbon and the more conventional 18-carbon spin-labelled fatty acids to replace the natural fatty acid of lactosyl ceramide so that we may begin to compare short and long chain derivatives to analyse the molecular basis of their functional differences. Spectra seen are consistent with the view that in a bilayer host matrix the methyl end of the long fatty acid crosses the hydrophobic membrane center and interdigitates with fatty acids of phospholipids of the opposing monolayer.

Introduction

Much of the current interest in glycosphingolipids derives from two factors: (a) their involvement as specific recognition sites for exogenous macrostructures ranging from antibodies to cells, and (b) their possible modulatory role in membrane structure and function. A certain amount of discussion regarding these roles has centered around a peculiar feature of the glycosphingolipid: the common great range of fatty acid chain length. The sphingosine backbone itself is generally 18-carbons long. Given that sphingolipids sit in the bilayer membrane in a fashion analogous to phospholipids, one would anticipate that a chain of 15 of these carbons would extend down into the membrane parallel to the fatty acids of the host matrix. Such a length of acyl chain is similar to the most abundant fatty acids of phospholipids (16- and 18-carbon). However, it is typical for glycolipids to have a high proportion of fatty acids with up to 24 carbons. Hence there is commonly an alkyl chain disparity of 9 carbons in depth of bilayer penetration within a given glycolipid (i.e., between the sphingosine portion and the fatty acid portion). Both the receptor function and membrane structure effects of glycosphingolipids have been suggested to be affected by this feature. Sen-Itiroh Hakomori, who introduced the concept of glycolipid crypticity, has raised the question of the role of glycolipid fatty acid composition in this regard (references in Ref. 1). Carl Alving, in establishing the basis for all current model membrane studies of the effect of fatty acid chain length on glycolipid receptor function, has demonstrated the increased 'accessibility' achieved by lengthening glycolipid fatty acids [2] or shortening host matrix fatty acids (Refs. 2, 3 and references

Abbreviation: EPR, electron paramagnetic resonance.

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therein). It has, however, proven difficult to differentiate amongst the possible roles of glycolipid dynamics, lateral distribution, and headgroup orientation or protrusion in determining receptor function [4–9]. A glycolipid with a very long fatty acid may well 'fit' in a characteristic fashion into a bilayer membrane (liquid) crystal lattice comprised of phospholipids with 16- and 18-carbon acyl chains [9]. This in turn would be expected to influence variables that affect its structural and receptor roles.

Lesser degrees of disparity in acyl chain length within a given lipid molecule are also a common feature of membrane phospholipids, especially the combination of a 16- and 18-carbon fatty acid. The effect of such chain length differences within a given pure phosphatidylcholine has been considered via studies of the phase transition behaviour and X-ray appearance of lipid bilayers (Reviewed in Refs. 10 and 11). Measurable structural effects are seen to occur, particularly when chain length disparity is a significant fraction of bilayer thickness. The question of 'interdigitation' has been raised: the concept that a longer alkyl chain may extend beyond its own monolayer to protrude amongst the shorter chains of the companion monolayer that comprises the other half of the bilayer (see for example, Refs. 10–12 and references therein). Such a concept is particularly attractive for bilayers made up totally of a population of phospholipids having one shorter and one longer chain. However, phospholipid bilayers containing only a small amount of glycolipid present a different situation, in that the host matrix is typically 16- to 18- carbon fatty acids while the odd molecule (the few percent glycolipid) may be vastly different. The very long chain fatty acid then has options that include extending amongst the acyl chains of its companion monolayer or collapsing into a highly localized nucleus of disorder. Presumably this situation also exists in cell membranes.

This paper describes the synthesis of a 24-carbon fatty acid spin label, and the result of experiments in which it was attached to the sphingosine backbone of lactosyl ceramide. Fatty acids bearing covalently attached nitroxide free radicals ('spin labels') have seen wide and successful application in membranes, although having been originally designed for use with phospholipids, nothing has been done previously with long-chain species. The long-chain fatty acid spin label introduced here provides a realistic method of examining many aspects of chain-length disparity in model and cell membranes. By comparison with the behaviour of the conventional 18-carbon fatty acid spin label attached to lactosyl ceramide we have been able to address for the first time the question of 'interdigitation' as it applies to glycolipids in a bilayer membrane.

Materials and Methods

L-α-Phosphatidylcholine from egg yolks was purchased from Avanti Polar Lipids, Birmingham, AL. L-α-Dimyristoyl- and L-α-dipalmitoyl-phosphatidylcholines were from Sigma, St. Louis, MO. Cholesterol was from Serdary, London, Canada. *N*-Lignoceroyldihydrolactocerebroside was from Sigma. *N*,*N*'-Dicyclohexylcarbodiimide (DCC) was from Aldrich, Milwaukee, WI. All lipids used were pure by the criterion of giving single spots on thin-layer chromatography plates (Merck silica gel 60) eluted with 65 : 25 : 4 (v/v) $CHCl_3/CH_3OH/H_2O$, and developed with 1 : 2.75 sulfuric acid/ethanol spray. Hexadecanedioic acid was from ICN/K and K, Plainsville, NY. Octyl bromide (1-bromooctane) was from Sigma. Silicic acid (Bio-Sil A 200–400 mesh) was from Bio-Rad, Richmond, CA.

Spin labelled fatty acids employed in this work were prepared following the general method of Hubbell and McConnell [13]. The approach involves deriving long-chain keto methyl esters of the formula $CH_3(CH_2)_mC(=O)(CH_2)_nC(=O)OCH_3$ via reaction of alkylcadmium compounds, $[CH_3(CH_2)_m]_2Cd$, with ω-carbalkoxyacyl chlorides, $ClC(=O)(CH_2)_nC(=O)OCH_3$. The spin label (*N*-oxyloxazolidine) ring is then produced at the location of the ketone by the method of Keana et al. [14]. The resultant spin-labelled fatty acids may then be named according to their (m,n) values as above, or according to the number of the fatty acid carbon carrying the spin label ring. Thus the long- and short-chain fatty acids synthesized for this work were the (7,14) and (1,14) spin labels, respectively, or the 16-nitroxylignoceric and 16-nitroxystearic acids, respectively. The (1,14) species

was first prepared by Philippe Devaux and Harden McConnell who communicated the procedural and analytical procedures to one of us (C.W.M.G.). Briefly the 16-carbon monomethyl ester of hexadecanedioic acid was linked to the 2-carbon fragment of ethyl bromide to produce $CH_3(CH_2)$-$C(=O)(CH_2)_{14}C(=O)OCH_3$, which was converted to a spin label [14]. For the new ((7,14)) spin label, $CH_3(CH_2)_7C(=O)(CH_2)_{14}C(=O)OCH_3$ was produced by replacing ethyl bromide in the above procedure with octyl bromide (1-bromooctane). Thus adjusting the procedure of Ref. 13, 21.33 g (0.108 moles) of octyl bromide was combined with 2.63 g of magnesium turnings in dry ether to prepare the appropriate Grignard reagent. This was then converted to the organocadmium reagent with 0.054 moles of anhydrous $CdCl_2$ and the result reacted with 24.5 g (0.073 moles) of the acid chloride of the hexadecanedioic acid monomethyl ester (again see Ref. 13 for conditions). The resultant long-chain fatty acid (methyl ester) with ketone at C-16 had the known IR features of keto-fatty acid esters. It was positively identified by proton NMR (Varian HA 200) and found to have integrated peak intensities within 3% of those expected from the formula.

Incorporation of spin-labelled fatty acids into the glycosphingolipid, lactosyl ceramide, followed the approach developed by our laboratory to synthesize spin-labelled glycolipids [15]. However, the base hydrolysis step which removes the original fatty acid prior to replacement with a spin-labelled fatty acid followed the general procedure of Neuenhofer et al. [16]. 25 mg (26 μmol) of lactosyl ceramide (Sigma) was dissolved in 10 ml of 1 M methanolic KOH in a 12 ml culture tube with screw cap (Kimble, Kimax 16×100 mm). The solution was briefly deoxygenated under a stream of N_2, and the cap firmly screwed on. The tube was stirred in an oil bath at 97°C for 24 h and reaction stopped with 0.75 ml acetic acid. TLC on Merck silica gel 60 plates eluting with 65 : 25 : 4 $CHCl_3/CH_3OH/H_2O$ (and developed with H_2SO_4/CH_3CH_2OH spray) showed that the original spot of R_F 0.64 had largely disappeared to be replaced by a ninhydrin-positive broad spot, R_F 0.06 and faster running free fatty acid. The sample was dried under nitrogen, freed of salt by 3 h dialysis against water, and lyso-lactosyl ceramide isolated using a column containing 1 g silicic acid poured in 5% $CH_3OH/CHCl_3$ and eluted first with 20% $CH_3OH/CHCl_3$ and then 60% $CH_3OH/CHCl_3$ (20 ml). In a typical reacylation of lyso-lactosyl ceramide, 13 mg of the latter was dissolved with 18–20 mg of spin-labelled fatty acid in 1 ml of calcium hydride-dried pyridine. 20 mg of dicyclohexylcarbodiimide was added and the solution stirred at 22°C in a sealed container over drying agent. Over a period of from 24 to 72 h a single new spot appeared with R_F indistinguishable from genuine lactosyl ceramide, while the slow-running lyso-compound (ninhydrin-positive spot) was correspondingly decreased. The spin labelled lactosyl ceramide was isolated on a silicic acid column (1.2×11 cm column packing) eluting with 5, 10 and 20% CH_3OH in $CHCl_3$. Overall yield based on native glycolipid was 26%.

Lipid bilayer membranes for these experiments were prepared by dissolving all components at the final desired ratio in 1 : 1 $CHCl_3/CH_3OH$, and removing the solvent under a N_2 atmosphere. Resultant films were further dried by pumping in vacuum (rotary pump) for 2 h at 22°C. Liposomes were prepared by hydration of dry films with appropriate buffer.

Results

Fig. 1 illustrates the long-chain spin-labelled fatty acid covalently attached to the sphingosine backbone of lactosyl ceramide. The conventional 18-carbon chain species is shown for comparison. For convenience we refer to these labelled glycolipids by reference to the long- and short-chain fatty acids as lignoceroyl-16-nitroxy- and stearoyl-16-nitroxylactosyl ceramide, respectively. The procedure for generating them followed a scheme similar to that which we originally developed to produce spectral probe labelled galactosyl ceramide [15]. It involved base hydrolysis of the native fatty acid/amide linkage and replacement via DCC activation of the labelled fatty acid (see Materials and Methods). To our knowledge this is the first report of spin-labelled lactosyl ceramide. Note that the methyl end of the fatty acyl chain extends a considerable distance beyond that of the sphingosine backbone in the case of the lignoceroyl species, while being only some 3 carbons longer in

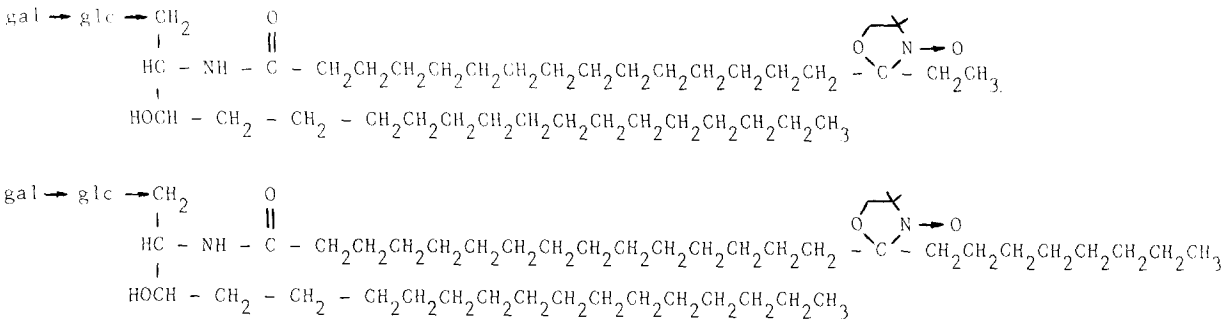

Fig. 1. Structures of the short-chain and long-chain spin-labelled glycolipids used in this work: stearoyl-16-nitroxylactosyl ceramide and lignoceroyl-16-nitroxylactosyl ceramide, respectively.

the stearoyl derivative. Yet the spin probe itself is at the same 'depth' in each case, and monitors the region very near the methyl terminus of a host bilayer matrix.

Since the opportunity for this type of comparison has not arisen previously, we show in Fig. 2 EPR spectra of lignoceroyl-16-nitroxylactosyl ceramide and stearoyl-16-nitroxylactosyl ceramide in various phospholipid bilayer host matrices. In particular we selected the fluid, natural matrix, egg phosphatidylcholine (primarily 16-carbon and 18-carbon fatty acids); as well as the synthetic matrices, dimyristoyl (14-carbon) and dipalmitoyl (16-carbon) phosphatidylcholines. The effect of

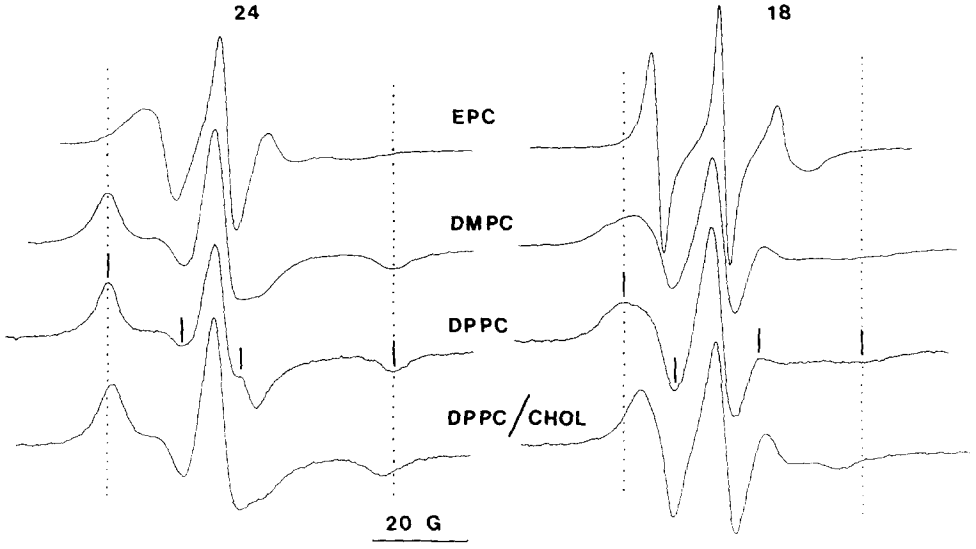

Fig. 2. EPR spectra of the long-chain ('24', left hand column) and short-chain ('18', right hand column) spin-labelled glycolipids in various phospholipid bilayers. EPC, egg phosphatidylcholine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DPPC/CHOL, 2:1 (mol ratio) dipalmitoylphosphatidylcholine/cholesterol. In each case the glycolipid comprised 2 mol% of the membrane lipid. Samples were prepared by hydrating dried films in 20 mM phosphate buffered saline (pH 7.4) at 51° C, and then allowed to cool slowly to 20° C. Sample suspensions were held in 50 μl glass capillary tubes sealed at one end. Spectra shown were recorded at 10° C using a Varian E12 spectrometer with variable temperature accessory and TM_{110} cavity. Dotted vertical lines mark the field location of the outer spectral extrema in the most rigid matrix (DPPC). The separation of these peaks is related to spin label correlation time (i.e., inversely related to spin label mobility) [19]. The locations of spectral features used for order parameter calculations such as those described in the caption to Table I are indicated by short vertical bars for the DPPC samples (outer spectral splitting corresponds to dotted vertical lines; inner spectral splitting is smaller).

cholesterol is also shown. In each case the concentration of glycolipid was kept low (2 mol%) so as to mimic the situation in cell membranes. Several points may be made about the appearance of these spectra. Firstly, the spectra show no obvious evidence of the phenomenon known as spin-exchange broadening [17]. This is a phenomenon whereby spin labels that collide with neighbouring spin labels broaden one another's spectral lines in a very characteristic way. The latter would certainly occur for instance if the labelled glycolipids were in pure clusters, rigorously phase-separated from the host phospholipid matrix. Indeed spin exchange broadening is readily detectable in homogeneous systems analogous to those described here when the concentration of labelled species is 15 mol% (data not shown). The question of lateral phase separation of glycolipids in membranes is an important one from both structural and functional viewpoints (for review, see Refs. 9, 18). However, the phenomenon is clearly only marginally understood at present. Currently it seems to be agreed that, in some model membranes at least, glycolipids tend to phase separate into domains of selective enrichment that coexist in a given bilayer. There is much debate about domain size and composition, the effect of fatty acid type, and especially about the likelihood of the phenomenon occurring in 'natural' lipid mixtures and cell membranes. The spin labels described here will lend themselves particularly well to answering certain such questions. Distinguishing between various subtle degrees of phase separation will, however, require systematic study, perhaps spectral simulation, and comparison with information on the same systems from other techniques such as immunology and electron microscopy; hence it is not appropriate to pursue the question further here. Secondly, and fairly trivially, comparisons within each vertical column of spectra in Fig. 2 show the relative features expected of the lipid matrices involved. Thus the spectra of glycolipids in the natural mixture, egg phosphatidylcholine, (which is not rigid at 10 °C) show the three relatively narrow lines so characteristic of spin labels tumbling with correlation times in the range of 10^{-9} s (see, for example, Ref. 19 and references therein). The rigid synthetic matrices, dimyristoyl- and dipalmitoylphosphatidylcholine, show spectral features such as the 'outer wings' (outer spectral extrema) marked by the dotted lines for the DPPC spectra, that approach those expected of immobile spin labels. The distance between these outer wings may be directly related to label immobility or correlation time, τ_c [19]. In accordance with this logic, note that the addition of cholesterol makes the spectral features measurably less rigid in keeping with its known tendency to disorder gel phase phospholipid bilayers [20]. The nitroxide spin label of course reflects in its EPR spectrum the mobility of that portion of a host molecule to which it is attached. Being in this case attached to a fatty acid carbon well down in the membrane, the fluidity gradient of phospholipid [21] and glycolipid [22] acyl chains would predict considerable mobility for the label at C-16 in a fluid host matrix. Thirdly, comparing between columns of spectra in Fig. 2, in each pair of spectra representing lignoceroyl-16-nitroxylactosyl ceramide vs. stearoyl-16-nitroxylactosyl ceramide in a given host matrix, the former shows distinct evidence of lower mobility. That is, the spectral appearance [19] indicates that the spin label on the long-chain fatty acid in the region of the host matrix terminal methyl group (the bilayer's most disordered and fluid region) is considerably less mobile than the label on the short-chain fatty acid. Such a result would be anticipated if the temporal oscillations of the C-16 carbon in lignoceroyl lactosyl ceramide were less than those in stearoyl lactosyl ceramide in a given bilayer. However, such a result might occur associated with local order, or local disorder, of the fatty acid segment involved.

The design of this pair of spin-labelled glycolipids makes it possible to consider a basic question which has until now not been addressed in the literature: the arrangement of the long-chain fatty acid in glycolipids relative to other membrane components. As already described, a closely related question has been addressed for pure phospholipids in which one fatty acid is longer than the other, and the suggestion has been made that the longer chains of each monolayer interdigitate with the shorter chains of the opposing monolayer. In the glycolipid case the chain-length discrepancy is considerably greater than that typical of natural phospholipids. Also glycolipids com-

prise only a small percentage of the lipids in membranes and are thought to be restricted to the outer surface, so that it is not obvious that an exceedingly long chain has an opposing slot to interdigitate into. The situation is illustrated diagramatically in Fig. 3: does the 'extra' length of alkyl chain of a glycolipid with 24-carbon fatty acid ball up to form a focus of disruption (situation A), or does it interdigitate with chains of the opposing monolayer (situation B)? The presumed arrangement of the glycolipid with short (18-carbon) fatty acid is also illustrated (situation C): it is shown fitting with minimal disruption amongst the 16-carbon and 18-carbon fatty acid phospholipids that comprise the bulk of membranes, as we originally determined for galactosyl ceramide [22]. Since the spin labels of lignoceroyl-16-nitroxylactosyl ceramide and stearoyl-16-nitroxylactosyl ceramide are localized to the region of 'crossover' for acyl chains interdigitating from one monolayer to the other, one may hope to gain some insight into whether situation A or B of Fig. 3 is the more common by examining the 'order parameters' associated with the spin-labelled portions of the acyl chains.

The concept of order parameter, S, in membranes arose out of early spin label studies in which people asked the question, "What is the degree of alignment of a given length of fatty acid chain perpendicular to the plane of the membrane?" [13,21,23]. Even in fluid membranes, alkyl chains tend to assume extended conformations perpendicular to this plane, and to pack in a hexagonal liquid-crystal lattice. The spin-label order parameter, S, has been defined in such a way that it varies between 0 and 1 according to how well the fatty acid segment to which the spin label is attached remains aligned as described above (perfect alignment gives $S = 1$, while total disorder gives $S = 0$) [13,23]. It has been pointed out that one should not confuse motional rate with degree of alignment, S: a segment of a given fatty acid may exhibit high mobility, while maintaining a high degree of overall alignment perpendicular to the bilayer plane (e.g., by pirouetting and wagging about its long axis). By the same token a given segment might be highly immobilized, but with random orientation. In Fig. 3, situation A would be expected to predispose toward an especially low order parameter, while situation B would do just the opposite. Note once again that since motional rate is not an obvious component of such considerations, the correlation time data alluded to in the previous paragraph will not help distinguish between these two possibilities.

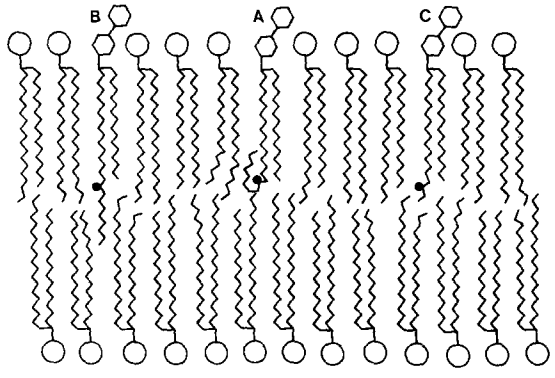

Fig. 3. Possible arrangements for the spin-labelled fatty acid of glycolipids described here in a bilayer membrane of 16- and 18-carbon fatty acid phosphatidylcholine. The headgroup of lactosyl ceramide is denoted by two linked hexagons: A, 24-carbon chain folded back upon itself to form a focus of disruption; B, 24-carbon chain extended so as to interdigitate with fatty acids of the opposing monolayer; C, 18-carbon chain in standard configuration. Note that the spin label is localized to a point roughly in the center of the hydrophobic interior.

Order parameter information is present in EPR spectra because the separation between spectral peaks is related to orientation of the nitroxide radical relative to the spectrometer magnetic field vector. A general and convenient technique for obtaining such information from sample spectra has been described [13,23,14] and comprehensively reviewed [25,26] by several groups. The basic approach is to identify a separation, $2T'_{\perp}$, of two particular inner peaks, and subtract this value from the separation, $2T'_{\parallel}$, of two outer peaks (illustrated for dipalmitoyl phosphatidylcholine spectra in Fig. 2). The ratio of the result to that found for the same spin label in a perfect crystal (order parameter 1) gives an approximate order parameter, S^{app}. This may then be refined by correcting for polarity of the spin-label environment and for slight differences between the measured splitting, $2T'_{\perp}$, and the desired matrix element, $2T_{\perp}$ (see caption to Table I).

Order parameter calculations for lactosyl ceramide with 18-carbon and 24-carbon fatty acid

TABLE I

ORDER PARAMETER DATA FOR LACTOSYL CERAMIDE WITH SPIN LABEL AT C-16 OF THE FATTY ACID CHAIN

Data are given for the long-chain and short-chain derivatives for comparison. Glycosphingolipid was incorporated at 2 mol% in liposomes of either egg phosphatidylcholine (EPC), dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), or DPPC/cholesterol (2:1 mol ratio). Sample preparation as in Materials and Methods. $T'_{\parallel}$ is half the measured outer spectral splitting (as shown for DPPC bilayers in Fig. 2), and is an accurate reflection of the desired matrix element, $T_{\parallel}$ [13,23,24]. $T'_{\perp}$ is half the measured inner spectral splitting (also shown as an example for DPPC in Fig. 2). A better estimate of the true value of the desired matrix element is $T_{\perp} = T'_{\perp} + 1.4\,(1 - S^{app})$ where

$$S^{app} = \left(T'_{\parallel} - T'_{\perp}\right) / \left(T^{c}_{zz} - \tfrac{1}{2}\left(T^{c}_{xx} + T^{c}_{yy}\right)\right)$$

is a first approximation to the order parameter ($T^{c}_{zz} = 32.9$ gauss, $T^{c}_{xx} = 5.9$ gauss, $T^{c}_{yy} = 5.4$ gauss are parameters from a crystal in which $S = 1$). A better value for the order parameter is then obtained as

$$S = a_0^{crystal}\left(T_{\parallel} - T_{\perp}\right) / a_0^{bilayer}\left(T^{c}_{zz} - \tfrac{1}{2}\left(T^{c}_{xx} + T^{c}_{yy}\right)\right)$$

where $a_0^{bilayer} = (T_{\parallel} + 2T_{\perp})/3$ and $a_0^{crystal} = (T^{c}_{xx} + T^{c}_{yy} + T^{c}_{zz})/3$ correct for polarity differences between crystal and bilayer.

Glycolipid fatty acid chain length (long = 24 carbons, short = 18 carbons) and host matrix phospholipid	Temperature (° C)	$T'_{\parallel}$ ($\simeq T_{\parallel}$) (gauss)	$T'_{\perp}$ (gauss)	S^{app}	$T_{\perp}$ (gauss)	S
Long in EPC (fluid)	10	21.0	9.7	0.41	10.5	0.40
Short in EPC (fluid)	10	16.5	11.9	0.17	13.1	0.13
Long in EPC (very fluid)	22	18.0	10.8	0.26	11.9	0.24
Short in EPC (very fluid)	22	15.9	12.5	0.12	13.8	0.08
Long in DMPC (rigid)	22	27.6	8.3	0.71	8.7	0.68
Short in DMPC (rigid)	22	19.8	10.4	0.34	11.3	0.32
Long in DPPC (rigid)	22	28.6	6.8	0.80	7.1	0.81
Short in DPPC (rigid)	22	20.8	9.4	0.42	10.2	0.42
Long in DPPC/CHOL (intermediate fluidity)	22	26.2	8.3	0.66	8.7	0.64
Short in DPPC/CHOL (intermediate fluidity)	22	19.7	10.2	0.35	11.1	0.33

spin label in four different lipid matrices are tabulated (Table I). These should be examined with regard to Fig. 3 in light of the possible fatty acid arrangement described. Note that in each case the degree of order of the long chain fatty acid at C-16 is considerably greater, rather than lower, in agreement with the concept of interdigitation.

Discussion

It seems that long chain fatty acid spin labels are likely to prove useful in dealing with problems relating to glycosphingolipids. It is known of course that the spin-label ring can represent a finite perturbation on the system being probed. However some of the most successful applications of spin labels have been in membrane systems very similar to those described here. In certain cases it has been possible to check EPR order parameter data with non-perturbing NMR probes, and the results have been found to be in basic agreement (see, for example, Refs. 27, 28). Quantitative differences between results from NMR and EPR almost certainly partly reflect the different timescales of the two techniques.

Clearly the spectra of the samples studied here are sensitive to factors related to dynamic and organizational differences between short and long chain glycolipids. It should be possible to use such spectral differences, in conjunction with immunological techniques and direct visualization by electron microscopy, to address current questions regarding glycolipid distribution and function in membranes. In this article we have taken advantage of the ability of the order parameter func-

tion, S to distinguish chain alignment from chain mobility in approaching the question of glycolipid interdigitation. We considered that situation A, of Fig. 3 (long-chain fatty acid forming a focus of local disorder) would lead to an S value similar to or less than that of the short-chain analogue. However, we observed that S values for the long-chain spin-labelled glycolipid are much greater than those of the short-chain analogue in fluid, rigid, and cholesterol-containing matrices. Such a result would be expected if interdigitation were occurring (situation B of Fig. 3). A third possibility, which is not shown, might also be considered for arrangement of the extra length of the 24-carbon fatty acid. The long chain might bend sharply by 90° at the depth of the surrounding phospholipid terminal methyl groups. The upper portion could then remain hexagonally packed with surrounding orderly acyl chains, while the extra length might lie in the plane of the membrane, extended amongst the phospholipid methyl termini. This is a significant possibility because, conceptually at least, if the 90° bend were below the spin label (i.e., at carbon No. 17 or greater) a higher S value might be predicted for the longer chain glycolipid fatty acid, as observed. However, the result with the dimyristoylphosphatidylcholine host matrix would seem to rule out such a possibility. The 14-carbon fatty acids in the latter case would dictate that the 90° bend be above the spin label, forcing the nitroxide ring to take on a relatively random arrangement with a low S value. In fact though the values of S observed for short and long chain lactosyl ceramide in dimyristoylphosphatidylcholine fit into the same pattern as the other host matrices.

Thus our results argue strongly that the 'extra' length of a 24-carbon glycolipid fatty acid (some 6–8 carbons) extends across the center of the membrane and interdigitates with the opposing monolayer, as opposed to 'balling up' to form a point of disruption. Bunow and Levin recorded evidence using Raman spectroscopy that in micelles of pure galactosyl ceramide some form of acyl chain interdigitation may occur. Other examples of interdigitation noted in the literature involve bilayers comprised of synthetic phospholipids with mismatched fatty acid chains. In such cases there is conceptually the possibility that long chains of one monolayer interdigitate by matching with short chains of the opposing monolayer [10–12]. However, in the example described here the chain length discrepancy involves only 2% of the total lipid (i.e., only the glycolipid), so that interdigitation must involve actual penetration of 'extra' chains into the opposing monolayer. Apparently this is possible in membranes of natural phospholipids, as well as in rigid, synthetic phospholipids.

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References

1 Lampio, A., Rauvala, H. and Gahmberg, C.G. (1986) Eur. J. Biochem. 157, 611–616
2 Alving, C.R. and Richards, R.L. (1977) Immunochemistry 14, 373–381
3 Schichijo, S. and Alving, C.R. (1986) Biochim. Biophys. Acta 858, 118–124
4 Balakrishnan, K., Mehdi, S.W. and McConnell, H.M. (1982) J. Biol. Chem. 257, 6434–6439
5 Endo, T., Nojima, S. and Inoue, K. (1982) J. Biochem. 92, 1883–1890
6 Thomas, P.D. and Podder, S.K. (1982) Biochim. Biophys. Acta 688, 453–459
7 Myers, M., Wortman, C. and Freire, E. (1984) Biochemistry 23, 1442–1448
8 Utsumi, H., Suzuki, T., Inoue, K. and Nojima, S. (1984) J. Biochem. 96, 97–105
9 Grant, C.W.M. (1987) in Gangliosides and Modulation of Neuronal Functions, (Rahmann, H., ed.), NATO ASI Series Cell Biology, Springer-Verlag Berlin, in the press
10 Davis, P.J. and Keough, K.M.W. (1985) Biophys. J. 48, 915–918
11 Huang, C. and Mason, J.T. (1986) Biochim. Biophys. Acta 864, 423–470
12 Keough, K.M.W. and Davis, P.J. (1979) Biochemistry 18, 1453–1459
13 Hubbell, W.L. and McConnell, H.M. (1971) J. Am. Chem. Soc. 93, 314–326
14 Keana, J.W., Keana, S.B. and Beetham, D. (1967) J. Am. Chem. Soc. 89, 3056–3065
15 Sharom, F.J. and Grant, C.W.M. (1975) Biochem. Biophys. Res. Commun. 67, 1501–1506
16 Neuenhofer, S., Schwarzmann, G., Egge, H. and Sandhoff, K. (1985) Biochemistry 24, 525–532
17 Devaux, P., Scandella, C.J. and McConnell, H.M. (1973) J. Magn. Reson. 9, 474–485
18 Thompson, T.E. and Tillack, T.W. (1985) Annu. Rev. Biophys. Chem. 14, 361–386

19 McCalley, R.C., Shimshick, E.J. and McConnell, H.M. (1972) Chem. Phys. Lett. 13, 115–119
20 Demel, R.A. and De Kruyff, B. (1976) Biochim. Biophys. Acta 457, 109–131
21 McConnell, H.M. and McFarland, B.G. (1972) Ann. N.Y. Acad. Sci. 195, 207–217
22 Sharom, F.J., Barratt, D.G., Thede, A.E. and Grant, C.W.M. (1976) Biochim. Biophys. Acta 455, 485–492
23 Seelig, J. (1970) J. Am. Chem. Soc. 92, 3881–3887
24 Gaffney, B.J. (1976) in Spin Labeling (Berliner, L.J., ed.), Academic Press, New York
25 Griffith, O.H. and Jost, P.C. (1976) in Spin Labeling (Berliner, L.J., ed.), Academic Press, New York
26 Marsh, D. (1981) in Mol. Biol. Biochem. Biophys., Vol. 31, Membrane Spectroscopy (Grell, E., ed.), Springer-Verlag, New York
27 Seelig, A. and Seelig, J. (1974) Biochemistry 13, 4839–4845
28 Stockton, G.W., Polnaszek, C.F., Tulloch, A.P., Hasen, F. and Smith, I.C.P. (1976) Biochemistry 15, 954–966
29 Bunow, M.R. and Levin, I.W. (1980) Biophys. J. 32, 1007–1022