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Order and Dynamics in Mixtures of Membrane Glucolipids from *Acholeplasma laidlawii* Studied by ^{2}H NMR†

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ABSTRACT: The two dominant glucolipids in *Acholeplasma laidlawii*, viz., 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol (MGlcDG) and 1,2-diacyl-3-*O*-[α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl]-*sn*-glycerol (DGlcDG), have markedly different phase behavior. MGlcDG has an ability to form nonlamellar phases, whereas DGlcDG only forms lamellar phases. For maintenance of a stable lipid bilayer, the polar headgroup composition in *A. laidlawii* is metabolically regulated in vivo, in response to changes in the growth conditions [Wieslander et al. (1980) *Biochemistry 19*, 3650; Lindblom et al. (1986) *Biochemistry 25*, 7502]. To investigate the mechanism behind the lipid regulation, we have here studied bilayers of mixtures of unsaturated MGlcDG and DGlcDG, containing a small fraction of biosynthetically incorporated perdeuterated palmitic acid, with ^{2}H NMR. The order-parameter profile of the acyl chains and an apparent transverse spin relaxation rate (R_2) were determined from dePaked quadrupole-echo spectra. The order of the acyl chains in DGlcDG-d_{31} increases upon addition of protonated MGlcDG, whereas the order of MGlcDG-d_{31} decreases when DGlcDG is added. The variation of order with lipid composition is rationalized from simple packing constraints. R_2 increases linearly with the square of the order parameter (S^2) up to $S \approx 0.14$; then, R_2 goes through a maximum and decreases. The increase in R_2 with S^2, as well as the magnitude of R_2, is largest for pure MGlcDG-d_{31}, smallest for DGlcDG-d_{31}, and similar for mixtures with the same molar ratio of MGlcDG/DGlcDG but with the deuterium label on different lipids. The relaxation data indicate the presence of slow reorientational motions, such as collective bilayer fluctuations and/or lipid lateral diffusion over a curved bilayer surface. The variation of acyl-chain order and bilayer curvature and/or fluctuations with sample composition are discussed in relation to the tendency of MGlcDG to form nonlamellar phases in vitro and in relation to the lipid regulation in vivo.

The presence of a lipid bilayer in several biological membranes of both eukaryotic and prokaryotic origin has been demonstrated by X-ray diffraction studies [reviewed by Shipley (1973)]. The hydrocarbon chains of the lipids in a biological membrane, or at least a major fraction of them, are usually in a disordered liquid-like state (Melchior, 1982). Investigations of the cell-wall-less prokaryote *Acholeplasma laidlawii* have shown that these structural conditions of the membrane lipids are actively maintained by metabolic regulation of the polar headgroup and, to a certain extent, the hydrocarbon chain composition. A minimum fraction of the hydrocarbon

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chains must be in a disordered state in order for the organism to grow and divide (McElhaney, 1974), and the formation of nonbilayer structures of the bulk membrane lipids is avoided (Wieslander et al., 1980, 1986; Lindblom et al., 1986).

Most lipids present in a biological membrane form a lamellar phase (built up of stacked bilayers) at physiological temperatures when they are isolated and mixed with large amounts of water. However, all membranes contain at least one lipid that has the ability to form nonbilayer structures and thus nonlamellar phases (Rilfors et al., 1984; Lindblom & Rilfors, 1989; Seddon, 1990). Phosphatidylethanolamine (PE),[1] monogalactosyldiacylglycerol, and monoglucosyldiacylglycerol are examples of the latter lipids. Information has been obtained about the structural importance and the functions of especially PE in model membranes, and some investigations have dealt with the influence of PE on bilayer permeability (Noordam et al., 1980; Taraschi et al., 1982), membrane fusion (Siegel, 1986), orientational order and dynamics of the acyl chains (Blume et al., 1982; Marsh et al., 1983; Perly et al., 1985a,b; Cullis et al., 1986; Lafleur et al., 1990a,b), and the activity of membrane-bound enzymes (Jensen & Schutzbach, 1989).

The major membrane lipid in *A. laidlawii* that is able to form nonlamellar phases is, under most conditions, 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol (MGlcDG) (Wieslander et al., 1978; Lindblom et al., 1986). The organism varies the fraction of MGlcDG as a function of the growth temperature, the incorporation of different fatty acids into the lipids, and the incorporation of sterols, hydrocarbons, alcohols, and detergents into the membrane (Wieslander et al., 1980, 1986; Rilfors, 1985; Lindblom & Rilfors, 1989). MGlcDG has similar physicochemical properties as monogalactosyldiacylglycerol, present in chloroplast membranes, and PE, present in prokaryotic and eukaryotic cell membranes (Brentel et al., 1985; Hinz et al., 1985; Kuttenreich et al., 1988; Mannock et al., 1988, 1990; Lewis et al., 1989).

The present work deals with the influence of MGlcDG on the orientational order and dynamics of the acyl chains of a lipid bilayer. The other major glucolipid present in the *A. laidlawii* membrane (Wieslander & Rilfors, 1977), 1,2-diacyl-3-*O*-[α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl]-*sn*-glycerol (DGlcDG), was chosen as the bilayer-forming lipid. Perdeuterated or unlabeled palmitic acid, in mixtures with a majority of oleic acid, were biosynthetically incorporated into the membrane lipids during growth of different *A. laidlawii* cell batches. MGlcDG and DGlcDG with unlabeled and perdeuterated (MGlcDG-d_{31} and DGlcDG-d_{31}) palmitoyl chains were isolated from these cells, and the orientational order and dynamics of the acyl chains were investigated by ^{2}H NMR in bilayers of MGlcDG-d_{31}, DGlcDG-d_{31}, and in mixtures of the two glucolipids in which one of the lipids contained a perdeuterated palmitoyl chain. Significant differences in both order and dynamics between DGlcDG-d_{31} and MGlcDG-d_{31} were found and are rationalized in terms of packing constraints on the lipid molecules and curvature and/or fluctuations of the lipid bilayer, respectively.

[1] Abbreviations: NMR, nuclear magnetic resonance; MGlcDG, 1,2-diacyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol; DGlcDG, 1,2-diacyl-3-*O*-[α-D-glucopyranosyl-(1→2)-*O*-α-D-glucopyranosyl]-*sn*-glycerol; MGlcDG-d_{31} and DGlcDG-d_{31}, MGlcDG and DGlcDG with perdeuterated palmitoyl chains; DPMGlcDG, 1,2-dipalmitoyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; L_β, lamellar gel phase; L_α, lamellar liquid-crystalline phase; T_m, the L_β-to-L_α transition temperature; T_u, the temperature at which the last trace of the L_β phase disappears; H_{II}, reversed hexagonal phase; SAC, saturated acyl chains other than palmitic acid; rf, radio frequency.

Table I: Acyl-Chain Composition (mol %) in Glucolipids Isolated from *A. laidlawii*[a]

acyl chain	12:0	13:0	14:0	15:0	16:0	16:0-d_{31}	18:0	18:1cis9
MGlcDG	2.3	1.3	6.1	2.2	26.1		0.9	61.1
MGlcDG-d_{31}	4.2	2.7	11.2	4.3	14.1	15.6	1.3	46.6
DGlcDG		0.1	1.4	0.9	8.2		0.3	89.1
DGlcDG-d_{31}	0.4	0.5	3.0	1.9	8.1	8.2	0.9	77.0

[a] The acyl chain is denoted as *n*:*k* where *n* is the number of carbon atoms and *k* is the number of double bonds.

Materials and Methods

Cell Growth. A. laidlawii, strain A, EF22 (Wieslander & Rilfors, 1977), was grown statically in 15-L bottles in a medium containing, per liter; 20 g of lipid-depleted tryptose (Difco Laboratories, Detroit, MI) (Christiansson & Wieslander, 1980), 4 g of fatty acid poor bovine serum albumin, fraction V (Boehringer Mannheim, Mannheim, FRG), 7 g of glucose, 5 g of NaCl, 5 g of Tris-HCl, and 100 000 IU of penicillin G. The growth medium was supplemented with 100 μM oleic acid (18:1c) plus either 25 μM perdeuterated palmitic acid (16:0-d_{31}) or 25 μM unlabeled palmitic acid (16:0). The media were inoculated with 7% (v/v) of a 24 h old cell culture, and the cells were grown for 24 h at 28 °C. A total of 120 L of each medium was harvested at 5 °C by continuous-flow centrifugation with a Beckman JCF-Z rotor; the flow rate was 100–120 mL/min, and the relative centrifugal force was 28000*g* at the rotor wall. The cells were washed once in β buffer (Christiansson & Wieslander, 1978) and frozen at −80 °C.

Isolation and Purification of Lipids. The cells were extracted twice with chloroform–methanol, 2:1 (v/v), and once with methanol on a magnetic stirrer at room temperature, followed by centrifugation. Nonlipid contaminants were removed by chromatography on Sephadex G-25 Fine (Wells & Dittmer, 1963). The lipids were then applied to a silicic acid column (Bio-Sil HA minus 325 mesh, Bio-Rad Laboratories, Richmond, CA) (Hirsch & Ahrens, 1958) and separated into three different classes. Pigments and neutral lipids (mostly free fatty acids and glycerides) were eluted with chloroform, MGlcDG and DGlcDG were eluted with acetone, and the anionic lipids were eluted with methanol. A slight N_2 pressure was maintained over the Sephadex and silicic acid columns in order to prevent lipid oxidation and to secure constant flow rates. The glucolipids were separated by thin-layer chromatography (TLC), and the lipids were eluted from the gel as described previously (Lindblom et al., 1986). A final purification of DGlcDG and DGlcDG-d_{31} was performed on a Bio-Sil HA column (see above). Chloroform–acetone mixtures with an increasing fraction of acetone were used as eluents, and the two lipids were eluted with chloroform–acetone, 1:9 (v/v). About 0.8–1.0 g each of MGlcDG, MGlcDG-d_{31}, DGlcDG, and DGlcDG-d_{31} was obtained; the purity of MGlcDG and MGlcDG-d_{31} was 95–96%, and the purity of DGlcDG and DGlcDG-d_{31} was 98–99%, as judged by TLC. The lipids were stored in chloroform–methanol, 2:1 (v/v), at −80 °C. The acyl-chain composition of the lipids (Table I) was determined by gas–liquid chromatography as described previously (Rilfors, 1985).

Sample Preparation. The lipids were first dried to a film with a stream of N_2 in an 8-mm o.d. glass tube (or in a tube with a larger diameter for the samples containing more than about 150 mg of lipid) and then dried to constant weight in a vacuum ($<10^{-7}$ Torr). A total of 9 mol of deuterium-de-

pleted water was added per mole of lipid, which is between 15 and 18% (w/w) of water depending on the ratio of MGlcDG/DGlcDG. This amount of water corresponds approximately to the maximum hydration of the glucolipids (Lindblom et al., 1986) and is too low to allow for the formation of liposomes. Rather, a powder sample of hydrated microcrystallites is formed. The tubes were flame sealed, and the components were mixed by extended centrifugation at 40 °C. When the sample was prepared in a larger tube, the sample was transferred to an 8-mm tube after the centrifugation procedure. Repeated NMR measurements after several freeze/thaw cycles gave reproducible results, indicating that the size of the microcrystallites was large enough not to influence the results.

NMR Spectroscopy. ^{2}H NMR spectra at 76.776 MHz were acquired with the quadrupole-echo technique (Davis et al., 1976) with a Bruker AM 500 spectrometer and a standard broad-band high-resolution probe. The temperature was controlled with a Bruker BV-T unit and was measured before and after the acquisition of a spectrum with a sensor inserted into the probe. The 90° pulse width, determined on a cubic liquid-crystalline sample with 30% (w/w) of ^2H_2O, was 14 μs, which is short enough to obtain sufficient coverage over the spectral region of interest here (+/−25 kHz) (Bloom et al., 1980) (see further below). The delay time between the two pulses in the echo sequence was 80 μs, and the spectral width was 166 kHz. The spectrum frequency was carefully adjusted to the center of the powder spectrum, and the pulses were digitally phase shifted to give a pure absorption signal in one of the quadrature channels. The imaginary part of the FID was not discarded, since at this field strength the chemical shift difference between the methyl doublet and the methylenes is significant. Between 10000 and 30000 scans were collected with a recycle time of 1.5 s. For detection of the presence of a gel phase component, spectra were also acquired with 45° pulses (7 μs) and a recycle time between 0.5 and 1.0 s. The transverse spin relaxation was studied by recording quadrupole-echo spectra as a function of the time, τ, between the pulses in the quadrupole-echo sequence.

Computational Procedures. All processing and calculations were performed on a personal IRIS 4D/25 workstation. The time-domain signals were transferred from the Aspect 3000 computer over the local ethernet via a SUN 3/60 to the IRIS workstation. The files were converted to the format of FTNMR (Dr. D. Hare, Hare Research, Woodinwille, WA) with the program BRIRIS (Dr. P. Kraulis, University of Uppsala, Sweden, unpublished). Care was taken to start the Fourier transform exactly at the top of the quadrupole echo (Davis, 1983). The baseline offset in the spectra was eliminated, and the spectral amplitude was corrected (unless otherwise stated) for the limited spectral coverage of the radio-frequency pulses (Bloom et al., 1980). At a spectral offset of 25 kHz, the maximum offset for spectra from lamellar liquid-crystalline (L_α) phases in this study, the attenuation factor due to the limited spectral coverage of 90° pulses of 14 μs is 0.457, and with 45° pulses of 7 μs at an offset of 65 kHz, typical for the lamellar gel (L_β) phase, the attenuation factor is 0.314 (Bloom et al., 1980).

The ^{2}H NMR spectrum of an unoriented sample of an L_α phase, formed by a lipid with perdeuterated acyl chains, consists of a superposition of powder patterns ("Pake doublets"), each characteristic of the residual quadrupolar splitting of separate deuterium nuclei in the acyl chain (Seelig, 1977). The spectrum corresponding to an oriented sample can be calculated from the powder-pattern lineshape if all interactions that are responsible for the lineshape, including the line-broadening effects, scale as $(3\cos^2\beta - 1)$, where β is the angle between the bilayer normal and the magnetic filed, and if the orientational distribution of the bilayers is isotropic (Bloom et al., 1981; Sternin et al., 1983). The technique, named "dePakeing", offers a convenient way to resolve overlapping powder patterns that otherwise would require time-consuming lineshape simulations.

With a FORTRAN program for iterative dePakeing in the frequency domain (Sternin, 1982), five iterations of dePakeing were performed on each spectrum, followed by a five-point binomial smooth. From the resolved splittings in the dePaked spectrum, six or seven of the smallest splittings could be directly determined (Figures 2 and 3). Estimates of the remaining nine or eight splittings were obtained by integrating the region with nonresolved splittings in the dePaked spectrum and dividing it into intervals with an area corresponding to one methylene group each. The midpoint in each of these intervals was used for estimating the magnitude of the nonresolved splittings (Lafleur et al., 1989). The order parameter, S, corresponding to each quadrupole splitting, Δ, was calculated from

$$\Delta = \frac{3}{4} S\chi \qquad (1)$$

(Seelig, 1977), where χ is the quadrupole coupling constant of ^{2}H in a methylene segment, 167 kHz. The splittings were assigned to the methylene groups by assuming a monotonic decrease of the order along the acyl chain (Lafleur et al., 1989). This ignores the oscillatory variation of the order parameter for the methylene groups near the polar headgroup that has been observed for bilayers of selectively deuterated membrane lipids (Seelig & Seelig, 1977; Stockton et al., 1977). Furthermore, the α-methylene deuterons of an acyl chain in the *sn*-2-position in phospholipids have previously been found to give two different quadrupolar splittings (Seelig & Seelig, 1975). However, since the deuterated palmitic acid is expected to be primarily incorporated into the *sn*-1 position (Saito et al., 1977), we have assumed that the α-methylene segment gives rise to only one splitting. Furthermore, as indicated from Rance et al. (1983), the α-methylene in the *sn*-2 position of *glucolipids* only give rise to one splitting.

For the purpose of quantification, the "smoothed" order profile, up to position 15, was fitted by the equation

$$S(x) = S_0(1 - \mu x^\upsilon) \qquad (2)$$

where $x = n/16$ is a continuous variable associated with the chain position, n, and S_0, μ, and υ are fitting parameters (Davis et al., 1980; Lafleur et al., 1989); S_0 is the maximum order, υ is the curvature of the order profile, and $\mu = (1 - S_t/S_0)$ where S_t is the order of the end of the acyl chain.

The quadrupole-echo spectra from the relaxation experiments were dePaked and subsequently symmetrized. The slice corresponding to each resolved splitting was integrated in each spectrum, and the amplitude was fitted with a single exponential decay by using a nonlinear least-squares regression program. The nonresolved splittings were arbitrarily divided into three parts that were integrated separately. Generally, the decays were single exponential within the accuracy of the measurements.

RESULTS

Phase Equilibria. Six samples with different mixtures of unsaturated MGlcDG and DGlcDG, with one of the lipids containing perdeuterated palmitoyl chains, were prepared. In all samples the water content was 9 mol of ^1H_2O per mole of lipid. The composition of the samples and their net acyl-chain

Table II: The Polar Headgroup Composition, the Acyl-Chain Composition, and the Endpoint of the Gel-to-Liquid-Crystalline Phase Transition Region of Samples Prepared from *A. laidlawii* Glucolipids[a]

DGlcDG-d_{31}	MGlcDG	SAC	$C_{16:0}$	$C_{18:1}$	$\langle n \rangle$	T_u (°C)	S_0	μ	ν
100	0	6.7	16.3	77.0	17.4	34	0.187	0.817	2.28
71	29	8.5	19.1	72.4	17.3	34	0.208	0.796	2.57
24	76	11.3	23.7	65.0	17.1	17	0.205	0.809	2.63
DGlcDG	MGlcDG-d_{31}	SAC	$C_{16:0}$	$C_{18:1}$	$\langle n \rangle$	T_u (°C)	S_0	μ	ν
0	100	23.7	29.7	46.6	16.4	47	0.202	0.819	2.39
23	77	18.9	24.8	56.3	16.7	42	0.194	0.846	2.11
75	25	8.0	13.6	78.5	17.4	32	0.191	0.851	2.47

[a] The water content was 9 mol of ^1H_2O per mole of lipid in all samples. The lipid and acyl-chain composition are given as mol % of the total polar headgroup content and as mol % of the total acyl-chain content, respectively. SAC, saturated acyl chains other than $C_{16:0}$ (acyl chains are abbreviated as in Table I); $\langle n \rangle$, the average acyl-chain length (from gas–liquid chromatography data); T_u, the temperature at which the last trace of the gel phase has disappeared. S_0, μ, and ν are the parameters obtained from the fit of eq 2 to the order profiles. S_0 is a measure of the maximum order parameter, and μ and ν define the shape of the order profile (cf. the text for further details).

composition are given in Table II. It has been shown previously that the saturated and unsaturated fatty acids are preferentially incorporated into the *sn*-1 and *sn*-2 positions of these lipids, respectively (Saito et al., 1977).

The presence of an L_β phase component is possible to detect in quadrupole-echo spectra recorded with 45° pulses of 7 μs, and in Figure 1 such spectra are shown for the sample with pure MGlcDG-d_{31} as a function of the temperature. The spectral component with a maximum width of 125 kHz is typical for an L_β phase of membrane lipids, and the central component with a maximum spectral width of approximately 50 kHz is typical for an L_α phase [see, for example, Smith (1984)]. Thus, up to 46 °C an L_β phase is in equilibrium with an L_α phase (Figure 1). The L_β phase gradually disappears with increasing temperature, and at 47 °C no trace of the L_β phase remains.

The temperature (T_u) at which the last trace of the gel phase disappears is given in Table II. For MGlcDG-d_{31}, T_u decreases when protonated DGlcDG is intermixed, as expected from the amount of saturated acyl chains in the sample (Table II). For pure DGlcDG-d_{31}, the L_β phase disappears at lower temperature than for pure MGlcDG-d_{31}, which can be expected from the higher degree of unsaturation of the acyl chains in DGlcDG-d_{31}. Unexpectedly, T_u decreased to 17 °C when 76 mol % of MGlcDG, which has a higher degree of saturation than DGlcDG-d_{31}, was added. This might be due to a complicated phase behavior of the multicomponent system below 17 °C. At 48 °C the gel-phase component has disappeared in all six samples. Therefore, this temperature was selected for studying the order-parameter profile in the six samples.

At 44 °C an isotropic spectral component appears in the center of the spectrum of MGlcDG-d_{31} (Figure 1). The relative amount of the isotropic component increases with temperature, and between 60 and 70 °C the anisotropic component disappears. The sample then becomes optically isotropic. The presence of an isotropic component in ^2H as well as in ^{31}P NMR spectra in liquid-crystalline systems have in several cases been shown to arise from a cubic liquid-crystalline phase [see, for example, Wieslander et al. (1981); Brentel et al. (1985); Lindblom et al. (1986); Rilfors et al. (1986); and Lindblom and Rilfors (1989)]. In a previous study (Lindblom et al., 1986), we showed that dioleoyl-MGlcDG forms a bicontinuous cubic phase at low water content and room temperature. In view of the higher degree of acyl-chain saturation of MGlcDG-d_{31} in this study (Table I), it is reasonable that the cubic phase, as observed here, appears at higher temperature than for dioleoyl-MGlcDG. In contrast to the phase behavior of MGlcDG-d_{31}, pure DGlcDG-d_{31} exclusively forms an L_α phase up to at least 70 °C (spectra not shown). This is in agreement with previous observations that the di-

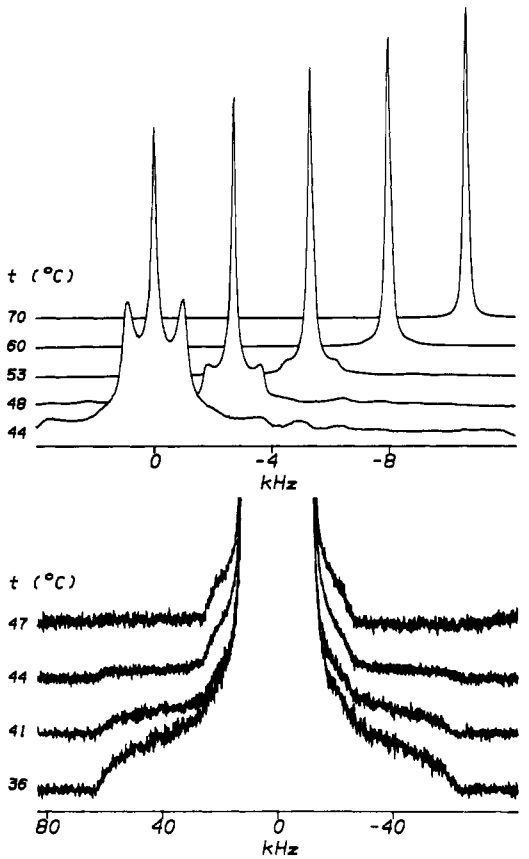

FIGURE 1: The quadrupole-echo spectra of MGlcDG-d_{31} as a function of temperature, showing the transition from the L_β phase to the L_α phase between 36 and 47 °C (bottom) and the transition from the L_α phase to the cubic phase between 44 and 70 °C (top). The water content was 9 mol of ^1H_2O per mole of lipid. Below 48 °C the spectra were acquired with 45° pulses (7 μs), and at 48 °C and above the spectra were acquired with 90° pulses (14 μs). In the upper spectra only the central region, with the methyl doublet and the isotropic signal from the cubic phase, is shown. The spectra are not corrected for the limited spectral coverage of the rf pulses.

glycosyldiacylglycerols generally form lamellar phases only (Wieslander et al., 1978; Brentel et al., 1985; Lindblom et al., 1986).

Order-Parameter Profiles. The ^2H quadrupole-echo spectra of the pure lipids and the mixtures, the dePaked spectra, and the order-parameter profiles, are given in Figures 2 and 3. The parameters S_0, μ, and ν, obtained from fits of eq 2 to the order profiles, are given in Table II. The acyl-chain order of pure DGlcDG-d_{31} is lower than in pure MGlcDG-d_{31}. When 29 mol % (of total lipid) of protonated MGlcDG is added to a bilayer of DGlcDG-d_{31}, the order of DGlcDG-d_{31} increases by about 10% (Figure 2). Increasing the amount of MGlcDG

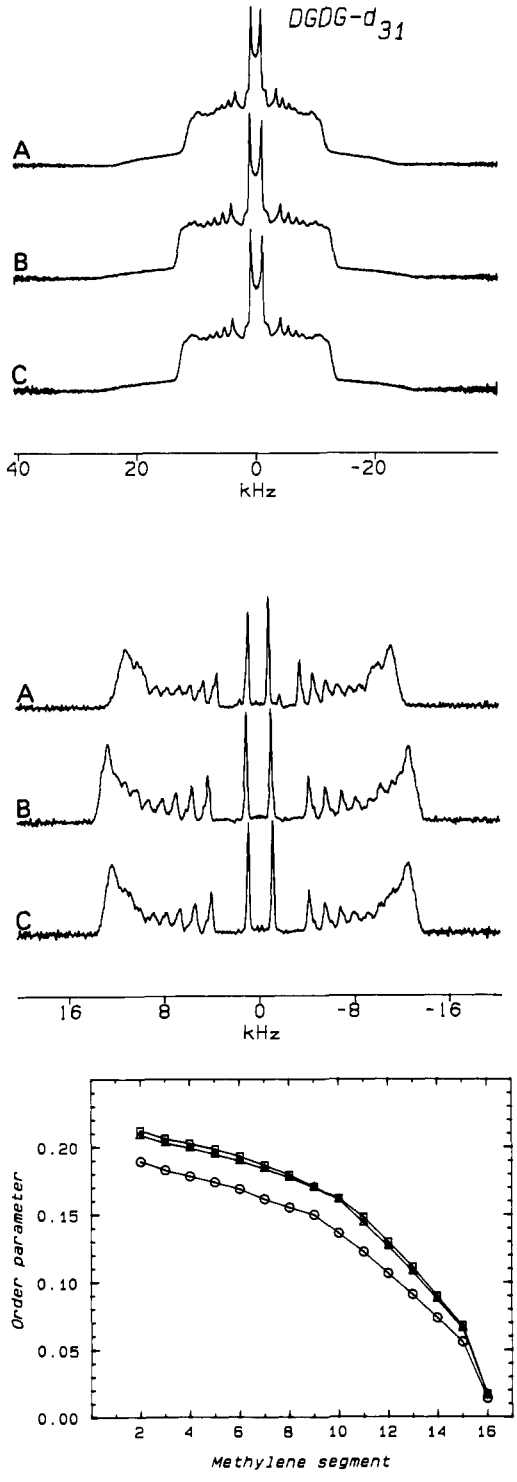

FIGURE 2: The quadrupole-echo spectra (top), the corresponding dePaked spectra (middle), and the order-parameter profile (bottom) of DGlcDG-d_{31}, with a varying amount of protonated MGlcDG added. The water content was 9 mol of ^1H_2O per mole of lipid and the temperature 48 °C. The spectra are corrected for the limited spectral coverage of the rf pulses. The samples are (A and ○) pure DGlcDG-d_{31}; (B and □) DGlcDG-d_{31}/MGlcDG (71:29 mol/mol); and (C and △) DGlcDG-d_{31}/MGlcDG (24:76 mol/mol).

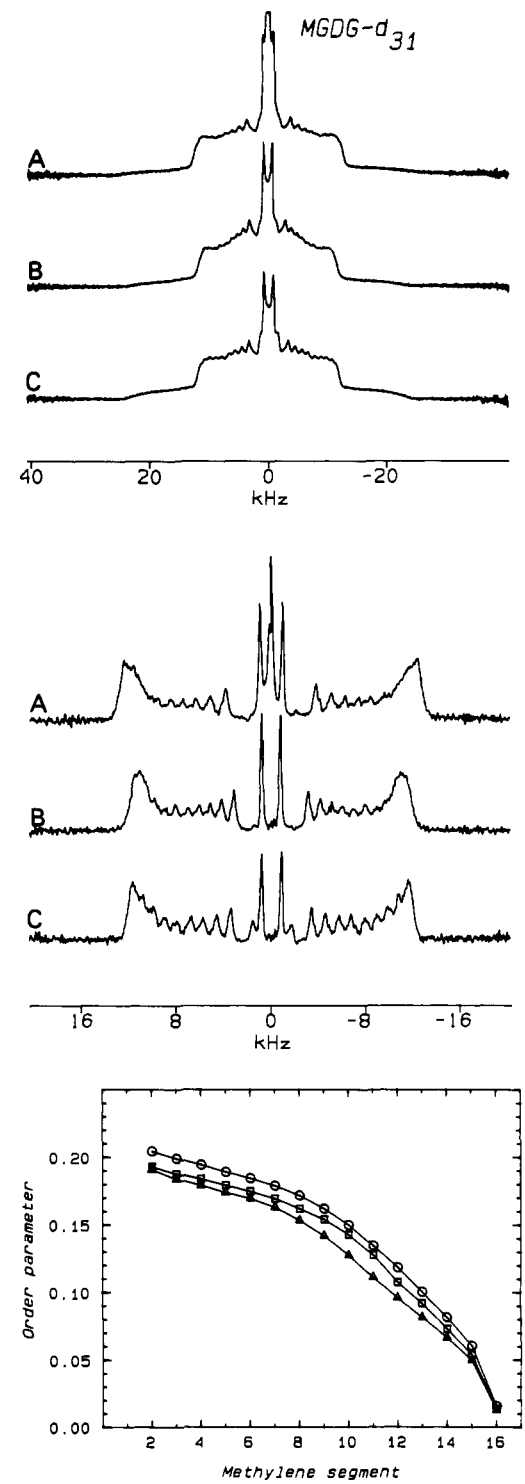

FIGURE 3: The quadrupole-echo spectra (top), the corresponding dePaked spectra (middle), and the order-parameter profile (bottom) of MGlcDG-d_{31}, with varying amounts of protonated DGlcDG added. The water content was 9 mol of ^1H_2O per mole of lipid and the temperature 48 °C. The spectra are corrected for the limited spectral coverage of the rf pulses. The samples are (A and ○) pure MGlcDG-d_{31}; (B and △) MGlcDG-d_{31}/DGlcDG (77:23 mol/mol); and (C and □) MGlcDG-d_{31}/DGlcDG (25:75 mol/mol).

to 76 mol % has no further influence on the order of the acyl chains. When 23 mol % of protonated DGlcDG is added to MGlcDG-d_{31}, the acyl-chain order decreases (Figure 3). However, when the amount of protonated DGlcDG is further increased to 75 mol %, the acyl-chain order, especially of the methylenes further down the chain, increases again. For samples with a similar molar ratio of MGlcDG/DGlcDG, the acyl-chain order profile depends on which of the lipids is deuterium labeled. For example, for a mixture with MGlcDG/DGlcDG ≈3:1 (mol/mol), the order of the acyl chains of DGlcDG-d_{31} is significantly higher than the order of MGlcDG-d_{31} (triangles in Figures 2 and 3, Table II). The acyl-chain composition differs between the two samples (Table II). However, the degree of saturation is larger for the MGlcDG-d_{31}/DGlcDG mixture than for the MGlcDG/DGlcDG-d_{31} mixture, which would affect the order in the

opposite direction to what is observed. Thus, the order-parameter profile is not correlated with T_u (Table II). A further argument for this conclusion is the fact that when the amount of MGlcDG is increased from 29 to 76% in DGlcDG-d_{31}/MGlcDG mixtures, T_u is lowered (Table II) while the order of DGlcDG-d_{31} is unchanged (Figure 2, Table II).

In the dePaked spectra, extra peaks of lower intensity can be distinguished close to the peaks of the smaller splittings (Figures 2 and 3). These are probably due to perdeuterated acyl chains in the *sn*-2 position. Since the glycerol backbone can be expected to be oriented perpendicular to the membrane surface (Jarrell et al., 1987), the *sn*-1 chain projects further into the lipid bilayer than the *sn*-2 chain. Due to packing constraints, the last methylene segments of the *sn*-1 chain are therefore less ordered. In these glucolipids, the palmitic acid has been found to have preference for the *sn*-1 position (Saito et al., 1977). Therefore, the intensity is smaller for the peak corresponding to the *sn*-2 position.

Transverse Spin Relaxation. The line width of the individual components in the dePaked spectra increases with increasing quadrupole splitting (Figures 2 and 3). This is also reflected in the original powder spectra where the distinct edges of the superimposed powder patterns, usually observed for phospholipids [see for example Lafleur et al. (1990a,b)], are absent. This characteristic of the ^2H quadrupole-echo spectrum has previously been observed for membranes from *A. laidlawii* (Davis et al., 1980).

The apparent transverse relaxation rate (R_2) of the individual components in the dePaked spectra is given as a function of the square of the order parameter, S^2, in Figure 4. R_2 increases linearly with S^2 up to $S \approx 0.14$ for all six samples, after which R_2 decreases. Both the magnitude of R_2 and the initial slope are largest for pure MGlcDG-d_{31} and smallest for pure DGlcDG-d_{31}. When MGlcDG is added to DGlcDG-d_{31}, both R_2 and the initial slope increase; and when DGlcDG is added to MGlcDG-d_{31}, R_2 and the initial slope decrease. In mixtures with a similar molar ratio of MGlcDG/DGlcDG, the R_2 values are similar, irrespective of which of the two lipids is labeled with deuterium. Thus, the results indicate that the transverse spin relaxation is primarily determined by the lipid headgroup composition of the bilayer. This is in contrast to the difference in order between samples with the same molar ratio of MGlcDG/DGlcDG but with different deuterium labeling (Figures 2 and 3) (see above).

Determination of R_2 of individual spectral components from dePaked powder spectra is probably not fully justified due to the angular dependence of R_2, which can be expected for lipid bilayers (Perly et al., 1985b; Bloom & Sternin, 1987). However, if the bilayer surface is slightly curved, the anisotropy of R_2 is partially averaged by the lateral diffusion of the lipids. Also, it can be expected that the dePakeing procedure primarily picks up the intensity of the peaks due to the 90° orientation in the powder spectrum.

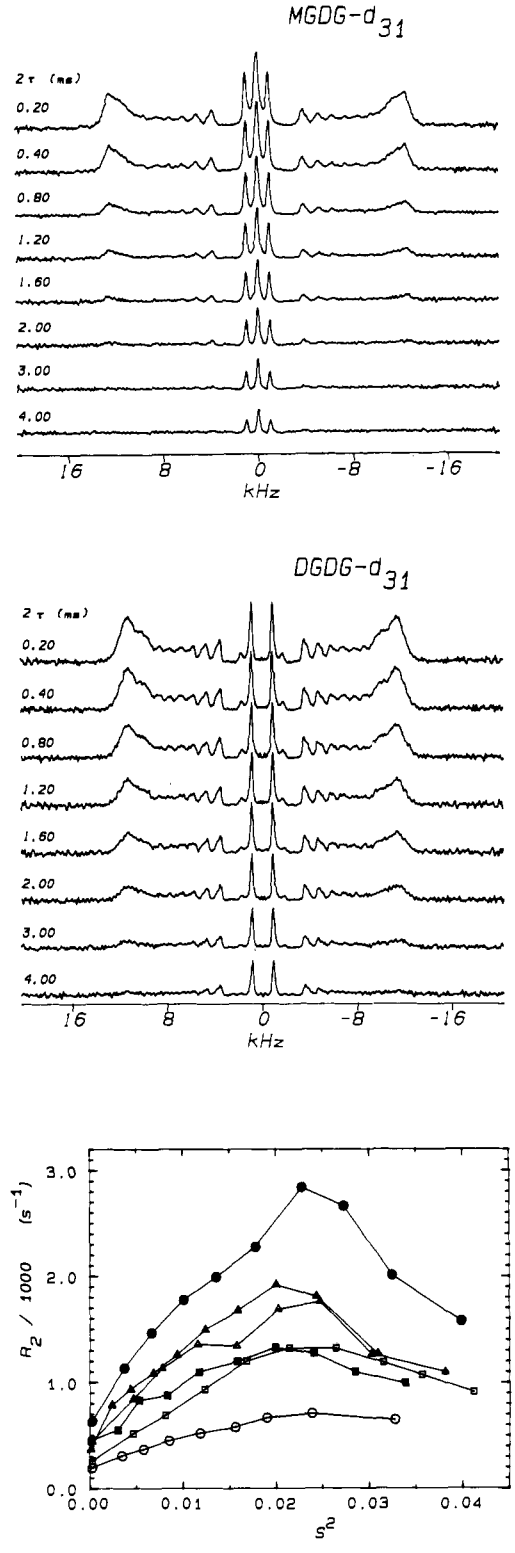

FIGURE 4: The dePaked spectra as a function of the interpulse delay, τ, for MGlcDG-d_{31} (top) and for DGlcDG-d_{31} (middle); and the apparent transverse relaxation rate, R_2, as a function of the square of the order parameter, S^2 (bottom), obtained from the decay of the spectra components in the dePaked spectra; for DGlcDG-d_{31} with varying amounts of MGlcDG added (open symbols), and for MGlcDG-d_{31} with varying amounts of DGlcDG added (filled symbols). The sample composition is (○) pure DGlcDG-d_{31}; (□) DGlcDG-d_{31}/MGlcDG (71/29, mol/mol); (△) DGlcDG-d_{31}/MGlcDG (24:76 mol/mol); (●) pure MGlcDG-d_{31}; (▲) MGlcDG-d_{31}/DGlcDG (77:23 mol/mol); (■) MGlcDG-d_{31}/DGlcDG (25:75 mol/mol). The water content was 9 mol of ^1H_2O per mole of lipid and temperature 48 °C.

Discussion

Phase Equilibria. The transition between L_β and L_α phases of glucosyldiacylglycerols of biological as well as synthetic origin has been investigated previously (De Kruijff et al., 1973; Wieslander et al., 1978; Silvius et al., 1980; Lindblom et al., 1986; Mannock et al., 1988, 1990). Synthetic 1,2-dipalmitoyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol (DPMGlcDG) has a gel-to-liquid-crystalline transition temperature (T_m) of 57 °C (Mannock et al., 1990). The MGlcDG-d_{31} studied in this work has a heterogeneous acyl-chain composition (Tables I and II), and therefore T_m is not well-defined. Lipid species with two palmitoyl chains probably constitute a very small fraction of the lipid preparation (Saito et al., 1977; see above). Still, the presence of these lipids can explain the

observed high end point temperature, T_u, of the L_β-to-L_α transition interval (Table II). Even though oleoyl chains make up nearly 50 mol % of the acyl chains, T_u is about 20 °C above the growth temperature of the cells. The T_m for MGlcDG and DGlcDG, with 99 mol % of isopalmitoyl chains, isolated from *A. laidlawii*, has previously been determined by differential thermal analysis (Silvius et al., 1980). In the heating mode, T_m for DGlcDG was higher than for MGlcDG. When *A. laidlawii* strain A is grown in the presence of both oleic and palmitic acid, oleic acid is incorporated to a higher extent in DGlcDG than in MGlcDG (Wieslander & Rilfors, 1977; Wieslander et al., 1978, 1981) (Table I). This could explain why T_u is considerably lower for DGlcDG-d_{31} than for MGlcDG-d_{31} (Table II).

The transitions between lamellar and nonlamellar phases of glucosyldiacylglycerols have also been studied previously (Wieslander et al., 1978, 1981; Lindblom et al., 1986; Mannock et al., 1988, 1990). Three preparations of MGlcDG, previously isolated from *A. laidlawii*, formed reversed hexagonal (H_{II}) phases at maximum hydration and physiological temperatures. The acyl-chain compositions of these three preparations were (1) 92 mol % of elaidoyl chains; (2) 52 mol % of palmitoyl and 46 mol % of oleoyl chains; and (3) 97 mol % of oleoyl chains, respectively (Wieslander et al., 1978; Lindblom et al., 1986). The MGlcDG-d_{31} studied in this work contains the same amount of oleoyl chains as the MGlcDG in the second preparation above, but 22 mol % of the palmitoyl chains have been replaced by shorter saturated acyl chains (Table I). In contrast to the previous preparations of MGlcDG, the MGlcDG-d_{31} isolated here forms a mixture of L_β and L_α phases up to 46 °C; above this temperature the L_β phase disappears and a cubic phase begins to form (Figure 1). Membrane lipids often form a cubic phase between the lamellar phase and the H_{II} phase (Lindblom & Rilfors, 1989). It has also been noted previously for PE that shorter acyl chains shift the phase equilibria from the H_{II} phase toward the L_α phase (Seddon, 1990; Lewis et al., 1989). Consequently, MGlcDG does not always form an H_{II} phase under physiological conditions. However, its phase behavior differs greatly from that of DGlcDG since the latter lipid has not been found to form nonlamellar phases (Wieslander et al., 1978, 1981; Lindblom et al., 1986). Preliminary studies show that the protonated MGlcDG used in this work (Table I) forms a cubic phase at room temperature, and at about 55 °C an H_{II} phase appears (unpublished observations). It can also be noted that synthetic DPMGlcDG forms a cubic phase at 79 °C (Mannock et al., 1990).

Order-Parameter Profiles. The orientational order of the acyl chains of *A. laidlawii* membrane lipids has been investigated above all by Smith and co-workers (Smith, 1984, and references therein). Their studies were mostly performed on intact membranes and total membrane lipids, and they determined the first complete acyl-chain order-parameter profile for a biological membrane (Stockton et al., 1977). In one study, the membrane lipids were divided into glucolipids and phospholipids, and the two lipid classes were compared; in this case just the C-2 and C-3 positions of the acyl chains were studied (Rance et al., 1983). The present work is the first one reporting measurements of the orientational order and dynamics of the acyl chains of individual glycosylglycerolipids.

The order of the acyl chains is significantly larger in pure MGlcDG-d_{31} than in pure DGlcDG-d_{31} (Figures 2 and 3). The acyl-chain order of DGlcDG-d_{31} increases when 29 mol % of MGlcDG is added to the bilayer (Figure 2). Further addition of MGlcDG, up to 76 mol %, has no effect on the order. These observations can be rationalized by considering the packing constraints on the lipid molecules, specifically the match between the effective cross section of the lipid headgroup and the two acyl chains. A larger area per polar headgroup (A) creates a looser packing of the acyl chains and a lower order. Small-angle X-ray diffraction studies of MGlcDG-d_{31} and DGlcDG-d_{31} have recently been performed (unpublished observations). At 20 °C, A are 61 and 71 Å^2 for MGlcDG-d_{31} and DGlcDG-d_{31}, respectively; the corresponding values at 47 °C are 63 and 74 Å^2. Consequently, in DGlcDG-d_{31} the order of the acyl chains is decreased when packed to fill up the space in the hydrocarbon region created by the packing of the larger headgroups. When MGlcDG is added, with its smaller headgroup, the match between the average cross section of the headgroups and the acyl chains is improved and the order of the acyl chains is increased. Addition of 26 mol % of MGlcDG is enough to create "optimal packing" for the acyl chains of DGlcDG-d_{31}, since when increasing the amount of MGlcDG to 76 mol %, the order is not further increased. The order of MGlcDG-d_{31} decreases somewhat when adding DGlcDG, which thus seems to loosen up the packing of the acyl chains in MGlcDG-d_{31}.

The higher acyl-chain order in MGlcDG-d_{31} compared to DGlcDG-d_{31}, and the effect of the intermixing of MGlcDG into bilayers of DGlcDG, can be related to the phase behavior of the lipids. MGlcDG has an ability to form a nonlamellar phase (Wieslander et al., 1978; Lindblom et al., 1986; Figure 1). This can be rationalized from the "wedge-like" shape of MGlcDG (a large hydrophobic volume and a small headgroup), which favors the formation of a reversed-type nonlamellar phase structure, like an H_{II} phase or a reversed cubic phase (Wieslander et al., 1980; Israelachvili et al., 1980). When MGlcDG forms bilayers, the acyl chains are forced into a more ordered state. Similarly, the introduction of the wedge-shaped MGlcDG into a bilayer of DGlcDG-d_{31} increases the order of the acyl chains. These remarks have previously been made about analogous results obtained from synthetic phospholipids (Lafleur et al., 1990a; see below).

In membranes of *A. laidlawii* strain A, DGlcDG is usually the dominant lipid, but there is always some MGlcDG present. The increase in the acyl-chain order of DGlcDG-d_{31} with the addition of MGlcDG stops at less than 26 mol % of MGlcDG. This fraction of MGlcDG is roughly what is synthesized by the cells when grown under the present conditions (acyl-chain composition and temperature) (Lindblom et al., 1986; Clementz et al., 1987).

Previously, in both experimental and theoretical works, several authors have pointed out the connection between A and the order-parameter profile of the acyl chains. In an early study of Mely et al. (1975), the lamellar liquid crystal of an amphiphile was investigated. A direct relationship was established between an increased A and a decreased hydrocarbon chain order. For two lamellar systems of amphiphile and water, with the same A but at different water content and temperature, the experimentally determined order profiles were very similar. The role of A in determining the acyl-chain order in L_α phases has also been confirmed by theoretical calculations (Dill & Flory 1980; Meraldi & Schlitter 1981a,b). Generally, short-range repulsive interactions, i.e., packing constraints, are considered to govern the conformations of the acyl chains (cf. Salmon et al., 1987).

Analogous results to those presented here have previously been obtained from investigations of the acyl-chain order in synthetic PE and phosphatidylcholine (PC) (Blume et al., 1982; Marsh et al., 1983; Perly et al., 1985a; Cullis et al., 1986;

Lafleur et al., 1990a). It was generally found that the order of the acyl chains is larger in PE than in PC. This has been rationalized (Blume et al., 1982; Cullis et al., 1986; Lafleur et al., 1990a) from the smaller A (Gruner et al., 1988) of PE than of PC, which imposes tighter packing constraints on the acyl chains in PE. It has also been related to the tendency of PE to form nonlamellar structures (Cullis et al., 1986; Lafleur et al., 1990a). These findings are in line with our observation of a higher acyl-chain order in MGlcDG-d_{31} than in DGlcDG-d_{31}. Furthermore, it was found (Cullis et al., 1986) that the order parameter for the methylene group in position 11 of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) increases monotonically when 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (DOPE) is added, up to a DOPE content of 50 mol %. This is in line with our observation that the order of DGlcDG-d_{31} increases upon addition of MGlcDG up to 26 mol%; however, the order does not increase further between 26 and 76 mol % of MGlcDG. In addition, the difference in acyl-chain order between PC and PE is larger (Blume et al., 1982) than between DGlcDG and MGlcDG.

Transverse Spin Relaxation. The S^2 dependence of R_2 (Figure 4) indicates the presence of slow reorientational motions that modulate the residual interaction, remaining after the averaging by fast local motions (e.g., trans–gauche isomerisations of the acyl chain, rotational diffusion of the molecule around the long axis, and local restricted reorientation of the long molecular axis relative to the bilayer normal). The slower motions can, for example, be collective motions like bilayer fluctuations (e.g., twist, bend, and splay) (Brown, 1982, 1984a,b; Marqusee et al., 1984; Stohrer et al., 1990) or reorientation due to lateral diffusion over a curved bilayer surface (Bloom & Sternin, 1987). The amplitude of these slowly fluctuating interactions is proportional to S and gives a contribution to R_2, which is proportional to S^2 (Brown, 1982; Marqusee et al., 1984). The low water content of the samples in this study (15–18 wt %) makes the existence of collective bilayer fluctuations less probable than for liposomes formed at excess water. Thus, reorientation due to lateral diffusion over a curved membrane surface is left as a more probable explanation for the S^2-dependent R_2.

A quantitative interpretation of the relaxation data presented here is not possible due to the use of nonoriented samples and the expected angular dependence in R_2. Use of oriented samples, together with a full lineshape analysis (Stohrer et al., 1990), would give a more detailed picture of the molecular and collective motions of the bilayer. However, at the present stage, it is possible to draw conclusions from the qualitative differences and similarities in R_2 between the investigated samples.

The slope of R_2 versus S^2 is largest for pure MGlcDG-d_{31} and smallest for pure DGlcDG-d_{31} (Figure 4). The addition of DGlcDG to MGlcDG-d_{31} decreases the slope, and the addition of MGlcDG to DGlcDG-d_{31} increases the slope. For mixtures of the two lipids with the same molar ratio between MGlcDG and DGlcDG, R_2 is very similar, irrespective of which lipid is deuterium labeled, indicating that R_2 is governed by collective motions and/or aggregate structure such as bilayer curvature. Thus, the relaxation data could indicate that the bilayer curvature increases with an increasing amount of MGlcDG. [Alternatively a decreasing lateral diffusion coefficient with increasing MGlcDG content (cf. Wieslander et al., 1981) could explain the results.] The reason for the maximum in R_2 as a function of S^2, observed for all samples (Figure 4), is unclear. A similar maximum in R_2 has previously been observed for chain-perdeuterated synthetic phospholipids, in powder samples (Davis, 1979), and in oriented samples (T. Trouard, personal communication).

The variations of R_2 with the sample composition can be related to the ability of the lipids to form nonlamellar phases. For MGlcDG-d_{31} [which forms a cubic phase at increasing temperature (Figure 1)] the bilayer curvature and/or fluctuations in the L_α phase gives a larger contribution to R_2 than for pure DGlcDG-d_{31}. Furthermore, the curvature and/or fluctuations in the lipid mixtures is determined by the relative amount of the lipid with an ability to form a nonlamellar phase (MGlcDG). Thus, the closer the bilayer is to the formation of nonbilayer structures, the larger the contribution to R_2 from bilayer curvature and/or fluctuations. On the basis of electron microscopy studies of multilayered liposomes, it has been proposed that the formation of bulges and invaginations on the lipid bilayer is the first stage in the transition between L_α and H_{II} phases (Borovjagin et al., 1982).

As previously shown, *A. laidlawii* regulates its membrane lipid composition in such a way that there is a nearly constant difference between the growth temperature and the temperature for the transition to nonlamellar phases in total lipid extracts (Lindblom et al., 1986). In order for the lipid-synthesizing enzymes to maintain an appropriate lipid composition, the closeness to the transition to nonbilayer structures must be reflected in the properties of the bilayer itself. This study has demonstrated that the packing properties of the acyl chains and the bilayer curvature/fluctuations vary with the lipid composition and thus with the tendency to form nonbilayer structures. These properties of the bilayer could therefore possibly be the signal to the lipid-synthesizing enzymes about how far the membrane is from the nonbilayer state.

Conclusions. As shown here, the acyl-chain order in binary mixtures of membrane glucolipids from *A. laidlawii* depends on the headgroup composition of the lipid mixtures. An increased amount of the lipid with an ability to form nonbilayer structures (MGlcDG) gives an increased acyl-chain order. This can be rationalized from the tighter packing imposed by the smaller headgroup area of MGlcDG and is analogous to previous findings for mixtures of synthetic PE and PC. Furthermore, the relaxation data indicate that the contribution from bilayer curvature and/or collective bilayer fluctuations to R_2 increases with an increasing amount of MGlcDG. These properties of the bilayer could possibly point toward a mechanism for the lipid-synthesizing enzymes to sense the stability of the bilayer with respect to the formation of nonbilayer structures.

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