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Thermotropic Phase Properties of 1,2-Di-*O*-Tetradecyl-3-*O*-(3-*O*-Methyl-*β*-D-Glucopyranosyl)-*sn*-Glycerol

Theodore P. Trouard,* David A. Mannock,‡ Göran Lindblom,* Leif Rilfors,* Morio Akiyama,§ and Ronald N. McElhaney‡

*Department of Physical Chemistry, University of Umeå, S-901 87 Umeå, Sweden; ‡Department of Biochemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2H7; and §Department of Physics, Sapporo Medical College, S.1, W.17, Chuo-Ku, Sapporo 060, Japan

ABSTRACT The hydration properties and the phase structure of 1,2-di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-*β*-D-glucopyranosyl)-*sn*-glycerol (3-*O*-Me-*β*-D-GlcDAlG) in water have been studied via differential scanning calorimetry, ^{1}H-NMR and ^{2}H-NMR spectroscopy, and x-ray diffraction. Results indicate that this lipid forms a crystalline (L_c) phase up to temperatures of 60–70°C, where a transition through a metastable reversed hexagonal (H_{II}) phase to a reversed micellar solution (L_2) phase occurs. Experiments were carried out at water concentrations in a range from 0 to 35 wt %, which indicate that all phases are poorly hydrated, taking up <5 mol water/mol lipid. The absence of a lamellar liquid crystalline (L_α) phase and the low levels of hydration measured in the discernible phases suggest that the methylation of the saccharide moiety alters the hydrogen bonding properties of the headgroup in such a way that the 3-*O*-Me-*β*-D-GlcDAlG headgroup cannot achieve the same level of hydration as the unmethylated form. Thus, in spite of the small increase in steric bulk resulting from methylation, there is an increase in the tendency of 3-*O*-Me-*β*-D-GlcDAlG to form nonlamellar structures. A similar phase behavior has previously been observed for the *Acholeplasma laidlawii* A membrane lipid 1,2-diacyl-3-*O*-(6-*O*-acyl-*α*-D-glucopyranosyl)-*sn*-glycerol in water (Lindblom et al. 1993. *J. Biol. Chem.* 268:16198–16207). The phase behavior of the two lipids suggests that hydrophobic substitution of a hydroxyl group in the sugar ring of the glucopyranosylglycerols has a very strong effect on their physicochemical properties, i.e., headgroup hydration and the formation of different lipid aggregate structures.

INTRODUCTION

Amphipathic lipid molecules in biological membranes are typically organized in bilayer structures with the hydrocarbon chains of opposing monolayers arranged end-to-end and their polar headgroups extending into the aqueous region. However, many naturally occurring lipids do not form bilayer structures when dispersed in water at physiological temperature. These nonbilayer structures can be separated into normal (oil-in-water) structures and reversed or inverted (water-in-oil) structures. In each of these structures the lipid headgroups point toward the aqueous phase while their chains make up the hydrocarbon core. The most common normal arrangements include the cubic phase (I_I), the hexagonal phase (H_I), and the micellar solution phase (L_1). These phases occur in dispersions of fatty acid soaps (Ekwall, 1975; Tiddy, 1980), lysophospholipids (Arvidson et al., 1985; Delacroix et al., 1993; Eriksson et al., 1987; Lindblom et al., 1992; Tilcock et al., 1986), and complex membrane lipids such as gangliosides (Ulrich-Bott and Wiegandt, 1984). The corresponding reversed structures are the reversed cubic phase (I_{II}), the reversed hexagonal phase (H_{II}), and the reversed micellar solution phase (L_2). Although I_{II} and H_{II} phases have been frequently observed in dispersions of monoacylglycerols (Caffrey, 1987; Gutman et al., 1984; Lutton, 1965) and simple phospho- and glycolipids (Lindblom and Rilfors, 1989; Mariani et al., 1988; Seddon and Templer, 1993; Seddon et al., 1984, 1990), L_2 phases have been observed almost exclusively in ionic surfactant systems (Ekwall, 1975; Hauser et al., 1989; Lindblom et al., 1970).

There have been many attempts to rationalize the origins of the structural diversity in aqueous lipid dispersions. In general, these explanations include the molecular shape (Israelachvili et al., 1980) and the intrinsic curvature concepts (Gruner, 1985; Helfrich, 1973; Rand et al., 1990). The basis of these hypotheses include several investigations of the physical properties of many synthetic lipid species, including the phosphatidylcholines (PCs) (see Lewis and McElhaney, 1992, for references; Silvius and McElhaney, 1979, 1980; Sjölund et al., 1989), phosphatidylethanolamines (PEs) (Lewis and McElhaney, 1993; Shyamsunder et al., 1988; Tenchov et al., 1984, 1988; Thurmond et al., 1993), lysophospatidylcholines (lysoPCs) (Arvidson et al., 1985; Eriksson et al., 1987), and some native glucocyl diacylglycerols (Brentel et al., 1985; Lindblom et al., 1986, 1993; Mannock et al., 1985; Shipley et al., 1973; Wieslander

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Address reprint requests to Göran Lindblom, Department of Physical Chemistry, University of Umeå, S-901 87 Umeå, Sweden. Tel.: 46 90 165228; Fax: 46 90 167779; E-mail: goran.lindblom@physchem.umu.se.

Abbreviations used: I_I, normal cubic; H_I, normal hexagonal; L_1, normal micellar; I_{II}, reversed cubic; H_{II}, reversed hexagonal; L_2, reversed micellar; L_α, lamellar liquid crystalline; $L_{\beta'}$, lamellar gel; L_c, lamellar crystalline; Glc, glucose; Gal, galactose; Man, mannose; PC, phosphatidylcholine; PE, phosphatidylethanolamine; lysoPC, lysophosphatidylcholine; MAcMGlcDAcG, 1,2-diacyl-3-*O*-[6-*O*-acyl-(*α*-D-glucopyranosyl)]-*sn*-glycerol; 3-*O*-Me-*β*-D-GlcDAlG, 1,2-di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-*β*-D-glucopyranosyl)-*sn*-glycerol; *β*-D-GalDAlG, 1,2-di-*O*-alkyl-3-*O*-(*β*-D-galactopyranosyl)-*sn*-glycerol; *β*-D-GlcDAlG 1,2-di-*O*-alkyl-3-*O*-(*β*-D-glucopyranosyl)-*sn*-glycerol; *α*-D-GlcDAcG, 1,2-di-*O*-acyl-3-*O*-(*α*-D-glucopyranosyl)-*sn*-glycerol; DGlcDAcG, 1,2-di-*O*-acyl-3-*O*-[*α*-D-glucopyranosyl-(1→2)-*O*-*α*-D-glucopyranosyl]-*sn*-glycerol, PAG, 1-*O*-polyprenyl, 2-*O*-acyl-*α*-D-glucopyranoside; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance spectroscopy; PFG, pulsed field gradient.

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et al., 1978, 1981). Several investigations of the thermotropic phase properties of synthetic diacyl and dialkylglycosylglycerols containing a single hexopyranose headgroup have recently appeared, in which both the hydrocarbon chain length and the anomeric configuration and stereochemistry of the headgroup have been systematically varied (see Hinz et al., 1991; Mannock et al., 1992 for refs.). The DSC and x-ray diffraction studies of these glycosyl glycerolipids reveal a similar pattern of phase behavior which consists of a lamellar gel to liquid crystalline (L_β/L_α) phase transition at lower temperature followed by a lamellar/nonlamellar phase transition at higher temperature (either L_α/H_{II} or L_α/I_{II}). The temperature and enthalpy of the L_β/L_α event increase, whereas the transition temperature of the lamellar/nonlamellar event decreases, with increasing chain length. The nature of the nonlamellar phase is also chain-length-dependent, and at short chain lengths I_{II} phases are preferred over H_{II} phases. In contrast, the temperature of the L_β/L_α phase transition is not greatly affected by changes in the headgroup anomeric configuration (α- versus β-anomer), stereochemistry (Glc versus Gal versus Man), or the chirality of the glycerol backbone (see Hinz et al., 1991; Mannock et al., 1992, 1993 for refs.). However, each of these factors play a significant role in determining the nature of the nonlamellar phase, its phase transition temperature, and also the rate of formation of highly ordered lamellar crystalline (L_c) phases from the gel phase.

There has been considerable effort to explain the possible role of nonbilayer forming lipids in the biological membrane (Gruner, 1985, 1992; Lindblom et al., 1986, 1993; Lindblom and Rilfors, 1989; Quinn and Williams, 1983; Wieslander et al., 1980). Some workers have postulated that the transient formation of nonlamellar structures could play a key role in processes such as membrane fusion (Siegel, 1986; Verkleij et al., 1979). Others argue that the actual formation of nonlamellar lipid structures in biological membranes is unlikely and that the role of nonbilayer-forming lipids is to impart some special (though as yet undefined) properties to the lipid bilayer phase (Gruner, 1985, 1992; Hui, 1987).

The ability of a lipid to form nonbilayer structures has been connected with its regulation in vivo in *Acholeplasma laidlawii* (see Rilfors et al., 1993 for a review). The regulation of the membrane lipid composition has been investigated predominantly in the strains A-EF22, B-JU, and B-PG9 of *A. laidlawii*. The strains A-EF22 and B-JU appear to consistently regulate the proportion of lipids with a tendency to form nonbilayer structures. In an early investigation it was concluded that strain B-PG9 does not consistently regulate this quantity (Bhakoo and McElhaney, 1988). However, when strains A-EF22 and B-PG9 were grown in the two media normally used to grow these bacteria, it was observed that both strains regulate the balance between bilayer- forming and nonbilayer-forming lipids in a similar way (Rilfors et al., 1993; Wieslander et al., 1993). When *A. laidlawii* B-PG9 is grown on a medium containing a large excess of a high melting point fatty acid, an additional lipid, 1-*O*-polyprenyl-2-*O*-acyl-α-D-glucopyranoside (PAcG) is produced at the expense of the 1,2-di-*O*-acyl-3-*O*-(α-D-glucopyranosyl)-*sn*-glycerol (α-D-GlcDAcG). Both the L_β/L_α and L_α/H_{II} phase transitions of PAcG are lower than those in the corresponding α-D-GlcDAcG, suggesting that the organism is attempting to regulate its membrane fluidity as well as the ratio of bilayer/nonbilayer-forming lipids (Lewis et al., 1990). In strain A-EF22, 1,2-diacyl-3-*O*-[6-*O*-acyl-(α-D-glucopyranosyl)]-*sn*-glycerol (MAcMGlcDAcG) (Hauksson et al., 1994) is synthesized under similar growth conditions. This lipid has recently been shown to have unusual equilibrium phase properties, forming no liquid crystalline phases but rather a gel/crystalline phase below 80°C and an L_2 phase at higher temperatures (Lindblom et al., 1993). This phase behavior is especially interesting in view of the link between lipid biosynthetic regulation in *A. laidlawii* and the phase properties of individual membrane lipids (Lindblom et al., 1986, 1993; Rilfors et al., 1993).

In the present paper we report the phase and hydration properties of 1,2-ditetradecyl-3-*O*-(3-*O*-methyl-β-D-glucopyranosyl)-*sn*-glycerol (3-*O*-Me-β-D-GlcDAlG). This lipid is similar in structure to other glucosyldialkylglycerols whose phase properties have been recently reported (Hinz et al., 1985, 1991; Mannock et al., 1992), save for an *O*-methyl group substitution of the 3-OH of the sugar ring. As is shown, this alteration significantly affects the physicochemical properties of the lipid, which are surprisingly similar to those of the MAcMGlcDAcG found in *A. laidlawii* A.

MATERIALS AND METHODS

Synthesis and sample preparation

1,2-Di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-β-D-glucopyranosyl)-*sn*-glycerol (3-*O*-Me-β-D-GlcDAlG) was synthesized from the acetobromosugar and 1,2-di-*O*-tetradecyl-*sn*-glycerol according to previously published procedures (Glew et al., 1991; Ogawa and Beppu, 1982; van Boeckel et al., 1985). The α- and β-anomers of the 3-*O*-Me-D-GlcDAlGs were separated as their peracetates by column chromatography on silica gel (Davisil, 200–425 mesh), which was eluted with a gradient of hexane and ethyl acetate. The subsequent deprotection and purification steps are identical to those reported for the corresponding β-D-galactosyl compounds (Mannock et al., 1993). All analytical measurements were consistent with the defined structures. Melting points (uncorrected, °C) and optical rotations ($\alpha^{20}{}_{D}$, c = 3.8, chloroform) are as follows: 3-*O*-Me-β-D-GlcDAlG; mp 70–71°C, $\alpha^{20}{}_{D}$, = −1.5 (see also Mannock et al., 1993). The purity of these compounds is at least 98% as estimated by elemental analysis and NMR spectroscopy techniques. All solvents were reagent grade and were distilled before use. Dipalmitoylphosphatidylcholine (DPPC) was purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

Calorimetry

DSC measurements were performed with a Perkin Elmer DSC-2C calorimeter equipped with a thermal analysis data station. Lipid samples for DSC were prepared and quantified as reported earlier (Mannock et al., 1990a, 1992). DSC curves were recorded between −3°C and 97°C.

X-ray diffraction

Samples for x-ray diffraction were prepared by transferring 3–5 mg of dry lipid into a thin-walled quartz capillary (1.5 mm). Deionized water (1–2 times the lipid weight) was added and the two components were mechanically mixed. The capillary was then sealed using 5-min epoxy, and the sample was repeatedly heated and cooled between 20 and 90°C.

The equipment and conditions for both wide angle and low angle x-ray diffraction measurements were as previously reported (Mannock et al., 1992).

NMR spectroscopy

All lipid samples for spectroscopy were dried under vacuum to constant weight and then hydrated in 8-mm test tubes with appropriate amounts of ^2H_2O (>99.8% ^2H) and flame-sealed to ensure constant hydration. The 3-*O*-Me-β-D-GlcDAlG dispersions were heated to 80°C and cooled to room temperature before spectroscopy (unless specifically noted). DPPC containing 35 wt % ^2H_2O was vortexed and taken through several freeze-thaw cycles before spectroscopy to ensure equilibration. The 3-*O*-Me-β-D-GlcDAlG NMR samples were checked for purity after spectroscopy via TLC (chloroform/methanol/water, 65:25:4; $R_f = 0.75$).

^1H-NMR spectroscopy on lipid dispersions were carried out at 100.1 MHz on a Bruker MSL-100 employing a dipolar echo pulse sequence (Janes et al., 1990). The delay between successive $\pi/2$ pulses was 30 μs and other experimental parameters were analogous to those described elsewhere (Lindblom et al., 1993). ^2H-NMR on lipid/^2H_2O mixtures were carried out at 38.4 MHz on a Bruker ACP-250 spectrometer. A quadrupolar echo pulse sequence (Davis, 1979) was used using a spectral width of 50 kHz and an interpulse spacing of 100 μs. All other experimental parameters were analogous to those described elsewhere (Lindblom et al., 1993). Sample temperature in each case was maintained by blowing heated air over the sample, which was allowed to equilibrate for 30 min at a given temperature. The apparent second moment M_2 of ^1H-NMR spectra which are related to the amount of orientational order in the lipid molecules (Bloom et al., 1978) were calculated from Fourier transformed ^1H-NMR spectra as previously described (Lindblom et al., 1993).

Pulsed field gradient (PFG) NMR for measuring diffusion coefficients of 3-*O*-Me-β-D-GlcDAlG dispersed in ^2H_2O was carried out at 100.13 MHz on a Bruker MSL 100 spectrometer equipped with an external, home-built gradient field supply. A modified stimulated echo experiment was used (Gibbs and Johnson, 1991)

$$\pi/2 - \tau - \pi/2 - T - \pi/2 - \tau - \pi/2 - T_e - \pi/2 - \text{acquire} \quad (1)$$

where two gradient pulses of duration ∂ and strength g are applied 500 μs after the first and third $\pi/2$ pulses. A 64 step phase cycling routine was used to suppress unwanted signals. In the above experiment, magnetization is stored as Zeeman order during the duration T and T_e. The delay T enables large separation (Δ) of the gradient pulses without losing signal due to T_2 decay and T_e allows eddy currents to decay before acquisition. In addition to the pulses mentioned above, a pulse train of three identical gradient pulses were applied before the experiment with a repetition time of $T + t$. This pulse sequence has been demonstrated to minimize spectral distortion due to stray eddy currents and long-lasting gradient tails (Gibbs and Johnson, 1991). The magnitude of the acquired signal in this pulse sequence is affected by translational diffusion according to

$$A = A_0 \exp\{-(\gamma\delta g)^2(\Delta - \delta/3)D\} \quad (2)$$

where A and A_0 are the observed intensities in the presence and absence of the gradient pulses, respectively, γ is the gyromagnetic ratio for protons, Δ is the separation in time between the beginning of the two gradient pulses ($T + t$), and D is the diffusion coefficient. These coefficients were determined by varying δ from 1 to 10 ms and fitting the obtained data via nonlinear least squares method to Eq. 2. Experimental parameters were as follows: $t = 50$ ms, $T = 50$ ms, $T_e = 100$ ms, $g = 0.5\ T_m^{-1}$. The gradient field strength was calibrated as described previously (Eriksson and Lindblom, 1993; Stilbs, 1987) using the known diffusion of water for low gradient fields and oleic acid as an intermediate field standard.

RESULTS

Calorimetry and x-ray diffraction

DSC thermograms of 3-*O*-Me-β-D-GlcDAlG are shown in Fig. 1. Initial heating of an anhydrous sample of 3-*O*-Me-β-D-GlcDAlG shows a broad irreversible exotherm event between 15 and 45°C (≈247 kJ/mol) and a sharp, strongly endothermic transition at ≈73°C (Table 1). The latter agrees with the capillary melting point determinations and identifies this high temperature phase as a transition from a crystalline phase to an isotropic liquid. On cooling, this event is seen to be reversible, however, the transition temperature and enthalpy are markedly altered by the cooling rate (1°C·min^{-1}, 56.89°C, 77.3 kJ/mol; 10°C·min^{-1}, 46.0°C, 64.4 kJ/mol, where the data are written in the order: cooling rate, transition temperature, enthalpy, respectively) suggesting that solid state polymorphism may exist even in the anhydrous lipid, although no other phase transitions were detected by DSC.

The thermal events observed by DSC in the samples of 3-*O*-Me-β-D-GlcDAlG dispersed in excess water and heated

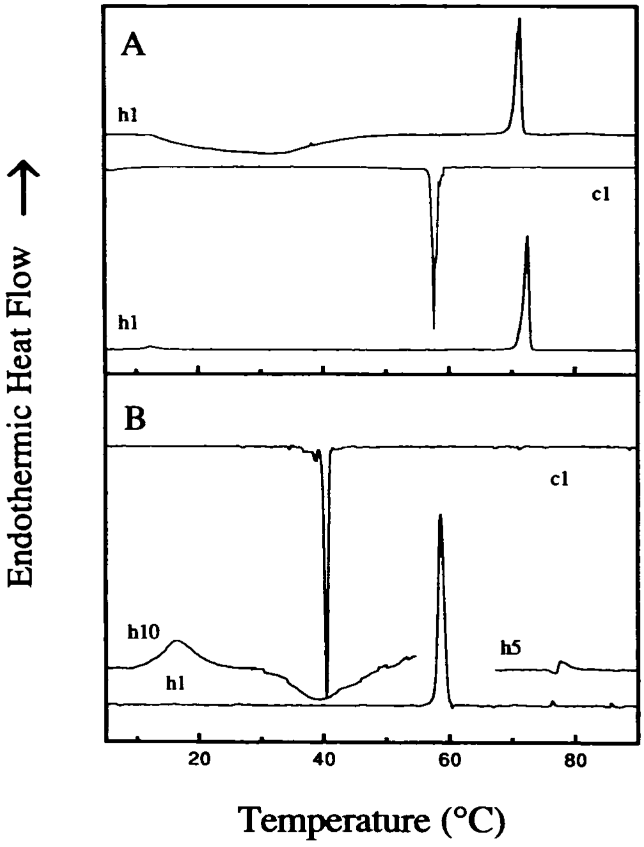

FIGURE 1 DSC thermograms of anhydrous 1,2-di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-β-D-glucopyranosyl)-*sn*-glycerol (*A*) and hydrated 1,2-di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-β-D-glucopyranosyl)-*sn*-glycerol (*B*) dispersed in excess water. In each panel the heating and cooling traces are denoted by h and c, and the rate of temperature change in 1°C min^{-1} is added as a suffix. Thus, h10 signifies a heating trace obtained at a rate of 10°C min^{-1}. The lower curve in *A* is the second heating trace. See Table 1 *A* for thermodynamic data.

TABLE 1 Phase transition temperature (T_m, °C) and enthalpy (ΔH, kJ/mol) values and x-ray diffraction spacings (nm) for 1,2-di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-β-D-glucopyranosyl)-sn-glycerol (3-*O*-Me-β-D-GlcDAlG)

		L_c/Liquid		Liquid/L_c			
A)	H_2O (wt %)	T_m	ΔH	T_m	ΔH		
	0	72.8 ± 0.3 46.0 ± 0.9[*]	80 ± 2 −64.7 ± 0.4	56.9 ± 0.7	−77 ± 2		
		L_c/H_{II}		H_{II}/L_2		H_{II}/L_c	
	H_2O (wt %)	T_m	ΔH	T_m	ΔH	T_m	ΔH
	Excess	59.8 ± 0.2	78 ± 2	78.1 ± 0.8	4.8 ± 0.4	42.1 ± 0.3	−73 ± 3[‡]
B)	H_2O (wt %)	L_c *d* (nm)	H_{II} *d* (nm)	L_2 *d* (nm)			
	Excess 1.7, 1.3	5.0 at 25.4°C 2.2, 1.9	3.8 at 69.5°C	3.7 at 79°C no other reflections observed			

All data are obtained at scan rates of 1°C/min unless otherwise stated.

[*] Scan rate was 10°C/min.

[‡] One would normally expect reversible transitions to exhibit a slightly larger ΔH on cooling than on heating because of errors originating from baseline estimates.

at 1°C·min^{-1} are shown in Fig. 1 *B*. Typically they consist of a highly energetic, chain-melting phase transition at 57–58°C with a higher temperature, weakly energetic phase transition at ~77°C (see also Table 1). On cooling at 1°C min^{-1}, the higher temperature event is not detected by DSC and the lower temperature transition is supercooled by ~20°C. Here the enthalpy is also 6–8 kJ/mol smaller than the corresponding event seen on heating. At faster scan rates (5 and 10°C·min^{-1}, Fig. 1 *B*) a broad, weakly energetic endotherm is observed on heating at ~14°C (~5.4 kJ/mol), followed by an exotherm, whose transition minimum varies from ≈37 to ~49°C (~7.5 kJ/mol), depending on the scan rate. These events are not reversible on cooling; nevertheless, the enthalpy difference between the heating and cooling transitions suggests the existence of metastable phases in aqueous dispersions of this lipid. However, we have been unable to define the experimental conditions that would allow us to isolate and characterize those metastable phases by DSC because of their relative instability.

X-ray diffraction measurements of samples of 3-*O*-Me-β-D-GlcDAlG dispersed in water taken as a function of temperature (Fig. 2) confirm the pattern of events seen in the DSC experiments obtained at a scan rate of 1°C·min^{-1} and demonstrate that both the chain-melting and the higher temperature phase transitions are reversible on cooling. Representative x-ray diffraction intensity profiles are shown in Fig. 3. The low-angle reflections observed for the sample measured at 25.4°C are characteristic of an L_c phase. The corresponding wide-angle diffraction pattern shows a large number of peaks between 0.51 nm and 0.38 nm, suggestive of a highly ordered hydrocarbon chain packing similar to that observed for L_c phases of the β-D-GlcDAlGs and β-D-GalDAlGs (Hinz et al., 1985, 1991; Mannock et al., 1993). Indeed, the magnitude of the first-order spacing (5.00 nm at 25°C; 5.04 nm at 39°C) is close to that of the L_c phase in the di-14:0-β-D-GlcDAlG (5.19 nm at 38.5°C) rather than that of the L_β phase (5.47 nm at 38.5°C; M. Akiyama, unpublished results). This pattern persists up to the transition between 56 and 61°C, at which point there is a decrease in the first-order spacing from 5.05 nm to 3.85 nm, with a corresponding change in the ratio of the higher-order peaks to 1:$\sqrt{3}$:$\sqrt{4}$. This latter ratio is characteristic of an H_{II} phase and identifies the event centered at ~60°C as an L_c/H_{II} phase transition. The H_{II} phase persists up to ~75°C when the first-order spacing at 3.85 nm is replaced by a much broader reflection at 3.7 nm. At the same time the higher order spacings typical of the H_{II} phase disappear and are not replaced. The absence of higher-order reflections is a distinguishing feature of this new phase and is indicative of a lack of long-range order. Furthermore, the magnitude of the Bragg spacing for both these phases is unusually small compared, for example, with the values of 5.6 nm and 5.5 nm obtained for the H_{II} phases of the di-14:0-β-D-Glc- (M. Akiyama, unpublished observations; Hinz et al., 1991) and di-14:0-β-D-GalDAlGs (Hinz et al., 1991; Mannock et al., 1993), respectively. This suggests that the H_{II} and higher temperature phases seen in 3-*O*-Me-β-D-GlcDAlG are poorly hydrated relative to those seen in the corresponding unmethylated compounds.

NMR spectroscopy

Representative ^{1}H-NMR spectra of 3-*O*-Me-β-D-GlcDAlG hydrated to 35 wt % at various temperatures are shown in Fig. 4. At the lowest temperatures studied, a very broad, almost featureless spectrum is observed that has appreciable intensity out to ± 40 kHz. This broad spectrum is indicative of non-averaged dipolar interactions from lipid molecules with crystal-like properties, consistent with the wide-angle diffraction results. Upon heating, little change in the spectra are observed until a temperature of approximately 60°C, where the intensity of the broad component begins to disappear, accompanied by the appearance of a strong isotropic peak.

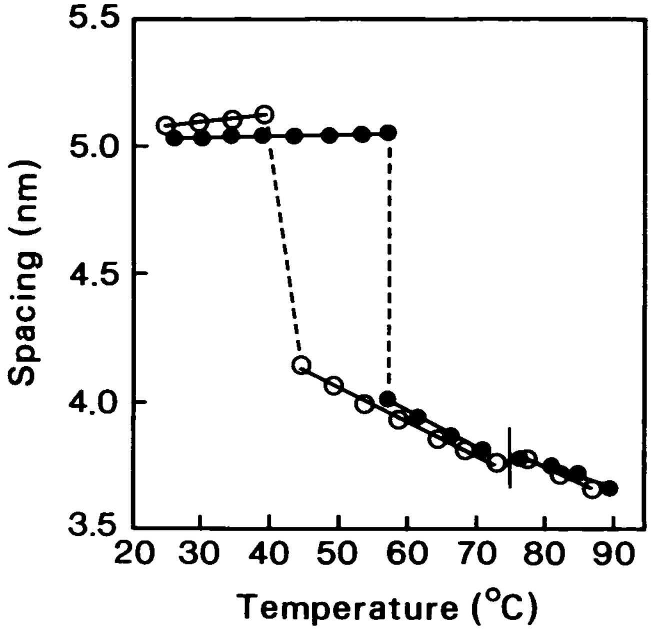

FIGURE 2 Plots of the first order spacing (nm) as a function of temperature (°C) for 1,2-di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-β-D-glucopyranosyl)-*sn*-glycerol dispersed in excess water. In each case the solid circles represent heating measurements from the L_c phase. The open circles represent cooling measurements. The solid lines through the symbols are lines of regression. The remaining lines mark the phase transitions and are merely a guide to the eye. The measurements were performed stepwise at a heating/cooling rate of 1°C min^{-1}. The exposure time was 60 s for each measurement.

The disappearance of the broad component and appearance of the relatively narrow peak is indicative of a phase transition from a crystalline-like structure to an isotropic phase where molecules are highly mobile and flexible. In this high temperature phase, at all levels of hydration, there is visual phase separation of bulk water. Also, the sample was relatively non-viscous, having the ability to flow. Spectra from the water-free lipid at the similar temperatures (not shown) are identical to those obtained from the sample hydrated to 35 wt % ^2H_2O.

The apparent second moment M_2 of ^{1}H-NMR spectra is related to the amount of orientational order in lipid molecules and is useful in quantifying spectral differences in ^{1}H-NMR (Bloom et al., 1978). Values of M_2 from the ^{1}H-NMR spectra of 3-*O*-Me-β-D-GlcDAlG versus temperature are given in Table 2. The M_2 for 3-*O*-Me-β-D-Glc-DAlG decreases from $5.8 \cdot 10^9$ s^{-2} at 30°C to $3.82 \cdot 10^9 s^{-2}$ at 60°C to a value of $0.043 \cdot 10^9 s^{-2}$ at 70°C. The large change in the moment with increasing temperature is indicative of a transition from a well-ordered state (rigid acyl chains and headgroup) to a fluid state with significant orientational freedom.

For comparison, ^{1}H-NMR spectra of DPPC in 35 wt % ^2H_2O at 30, 40, and 50°C are shown in Fig. 5 along with spectra from 3-*O*-Me-β-D-GlcDAlG with the same hydration at temperatures of 30 and 80°C. At 30°C, DPPC is in the $L_{\beta'}$gel phase characterized by mostly rigid acyl chains and the presence of whole-molecule rotation about the lipid long axis (Davis, 1979; Mackay, 1981; Ulmius et al., 1977). The spectrum from the low temperature phase of 3-*O*-Me-β-D-GlcDAlG is much broader than that from the $L_{\beta'}$ phase of DPPC, indicating 3-*O*-Me-β-D-GlcDAlG in its crystalline phase is experiencing much less motion averaging than DPPC in its $L_{\beta'}$ phase. A smaller amount of rotation about a preferred motion axis for 3-*O*-Me-β-D-GlcDAlG is the most reasonable explanation for the observed spectral difference. At 40°C, DPPC is in the $P_{\beta'}$ phase that is characterized by rippled bilayers, semi-rigid acyl chains and some long axis rotation (Janiak et al., 1979; Ulmius et al., 1977). The increased rotation of lipids about their long axis is observed in the narrowing of the spectra. The acyl chains in both $L_{\beta'}$ and $P_{\beta'}$ gel phases experience only a small amount of *trans-gauche* isomerization (Mendelsohn et al., 1989;

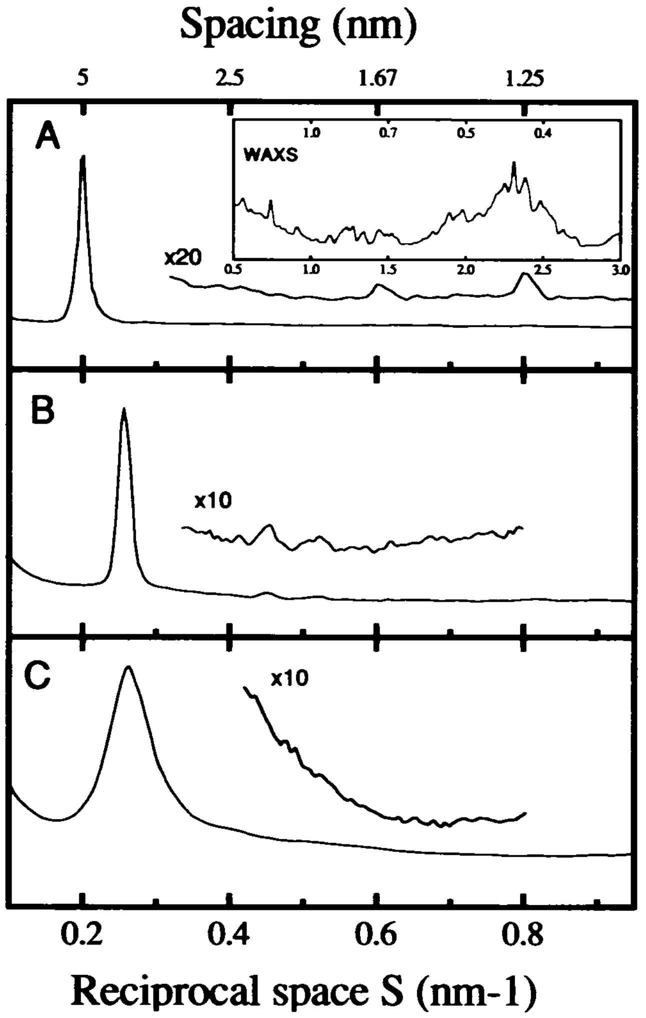

FIGURE 3 Representative low angle (SAXS) and wide angle (WAXS) x-ray diffraction intensity profiles [intensity vs. reciprocal space, S (nm^{-1})] of 1,2-di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-β-D-glucopyranosyl)-*sn*-glycerol dispersed in excess water. S is defined as 2sin θ/λ, where 2θ is equal to the scattering angle and λ is the wavelength. The intensity profiles shown are taken directly from the temperature profile in Fig. 2. The aqueous sample was heated to 90°C for 1 h to ensure uniformity before obtaining the initial heating profile. (*A*) L_c phase; (*B*) H_{II} phase; (*C*) higher temperature (L_2) phase. The top trace in each panel represents a magnification of the bottom trace by the specified amount. The inset in *A* contains wide-angle data for the L_c phase. See Table 1 *B* for numerical data.

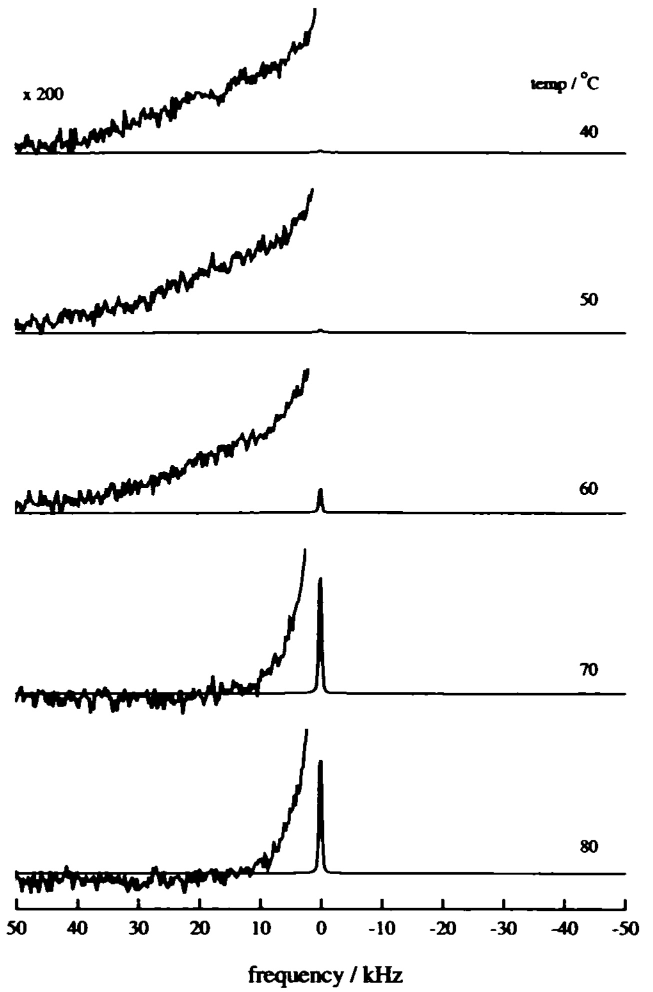

FIGURE 4 High power ^{1}H-NMR spectra of 1,2-di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-β-D-glucopyranosyl)-*sn*-glycerol in 35 wt % ^2H_2O at various temperatures. Expansions of spectra are included to show relative intensities at large spectral frequencies. The reversed micellar solution phase starts to form at ~60°C from the L_c phase.

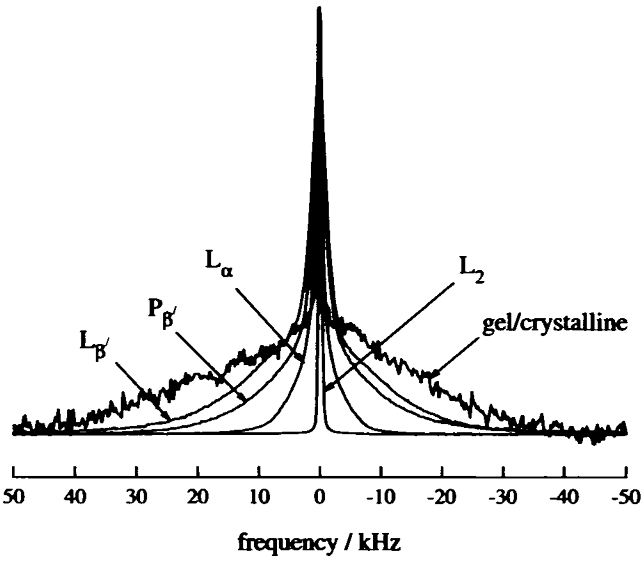

FIGURE 5 High power ^{1}H-NMR spectra from various lipid aggregate structures. The crystalline and L_2 structures are 1,2-di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-β-D-glucopyranosyl)-*sn*-glycerol in 35 wt % ^2H_2O at 30 and 80°C, respectively; the $L_{\beta'}$, $P_{\beta'}$, and L_α structures are dipalmitoylphosphatidylcholine in 35 wt % ^2H_2O at 30, 40, and 50°C, respectively. All samples were heated or cooled to the appropriate temperature, at which they were left for ~0.5 h to equilibrate before recording the NMR spectra.

TABLE 2 Apparent second moments M_2 from high power ^{1}H-NMR spectra of 1,2-di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-β-D-glucopyranosyl)-sn-glycerol (3-*O*-Me-β-D-GlcDAlG) and DPPC at various temperatures

	$M_2 \times 10^9$ s^{-2}	
T(°C)	3-*O*-Me-β-D-GlcDAlG	DPPC
30	5.8	2.9
40	5.6	2.3
50	5.6	0.33
60	3.8	0.28
70	0.04	
80	0.04	

Casal and McElhaney, 1990). At 50°C, DPPC is in the L_α phase characterized by melted, highly flexible acyl chains, fast rotational diffusion about the lipid long axis and fast lateral diffusion about the bilayer surface (Ulmius et al., 1975). The spectrum corresponding to this phase displays the typical super-Lorentzian line shape due to fast motion averaging about the bilayer normal (Ulmius et al., 1975; Wennerström, 1973). The spectrum from the melted phase of 3-*O*-Me-β-D-GlcDAlG is much narrower than that of the super-Lorentzian line shape from DPPC in the L_α phase due to the increased motion averaging of 3-*O*-Me-β-D-GlcDAlG in this phase.

Apparent second moments from the DPPC spectra are included in Table 2 and quantify the observed spectral differences seen in Fig. 5. At the lowest temperatures studied (30°C), the M_2 of DPPC is nearly half that of 3-*O*-Me-β-D-GlcDAlG. The M_2 of DPPC at 50°C is significantly larger than that of 3-*O*-Me-β-D-GlcDAlG in the high temperature phase (70°C). At 50°C DPPC is in the L_α phase and intermolecular dipolar interactions are effectively removed by fast lateral diffusion and do not contribute to M_2. The difference in M_2 is due, then, to a smaller amount of intramolecular interactions in 3-*O*-Me-β-D-GlcDAlG due to increased flexibility or fast reorientational averaging.

To determine the hydration properties of 3-*O*-Me-β-D-GlcDAlG, ^{2}H-NMR was performed at 5, 18, and 33 mol ^2H_2O/mol lipid (13, 35, and 70 wt % ^2H_2O, respectively). An isotropic signal is observed under all hydration conditions and temperatures studied. Only between 60 and 70°C is there an additional quadrupolar splitting of ~1 kHz that is a small fraction of the total signal. A representative spectrum is shown in Fig. 6 for 5 mol ^2H_2O/mol lipid at 65°C. The presence of a quadrupolar splitting indicates the existence of a liquid crystalline phase in at least part of the sample. While the observed splitting is compatible with x-ray data that shows the lipid is in a hexagonal phase, it is impossible from the observed spectra alone to determine what type of liquid

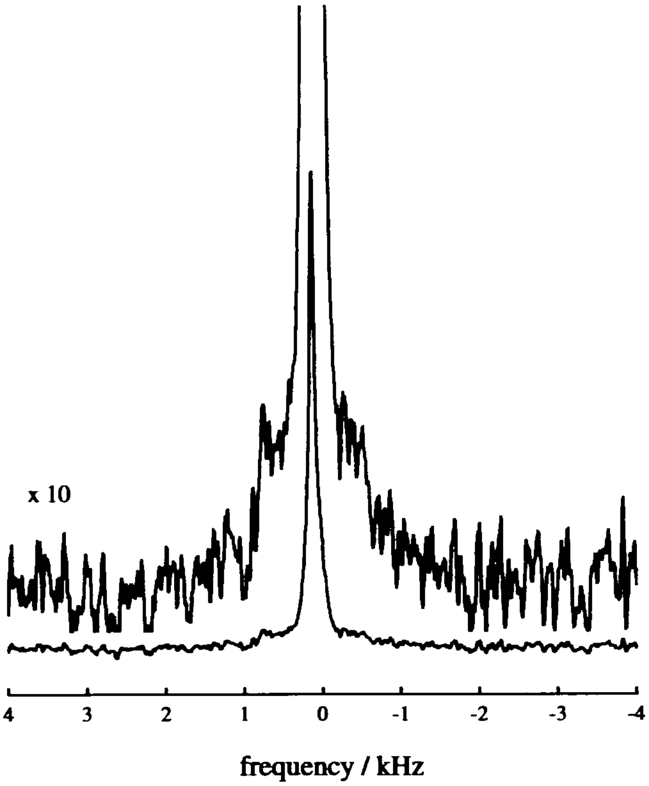

FIGURE 6 ^{2}H-NMR spectrum of 1,2-di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-β-D-glucopyranosyl)-*sn*-glycerol hydrated with 5 mol ^2H_2O/mol lipid at 65°C. A 10-fold expansion is included to accentuate the low intensity quadrupolar splitting. The sample was first heated to ~80°C and then cooled to 65°C, at which temperature it was left for ~30 min before the spectrum was recorded.

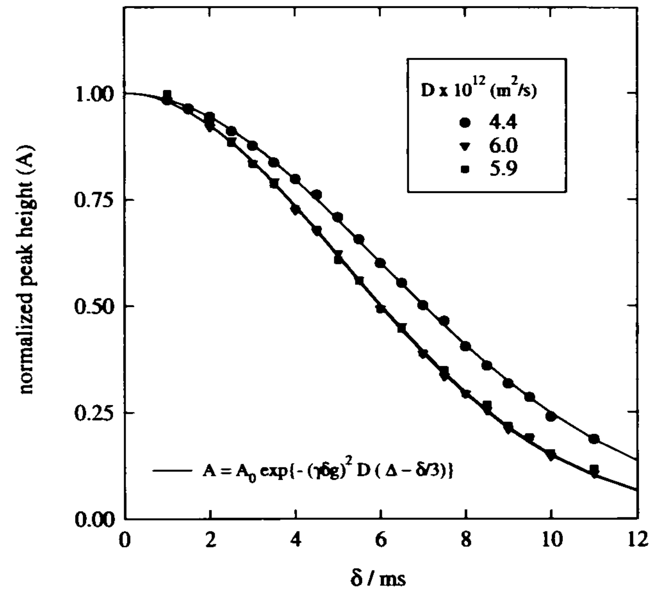

FIGURE 7 Peak height versus the gradient pulse length δ for 1,2-di-*O*-tetradecyl-3-*O*-(3-*O*-methyl-β-D-glucopyranosyl)-*sn*-glycerol under various levels of hydration: dry (●), 5 mol ^2H_2O/mol lipid (▼), and 35 wt % (~18 mol ^2H_2O/mol lipid) (■). Peak heights were normalized to their calculated gradient absent values. Solid lines through symbols are nonlinear least squares fits of data to Eq. 2. Calculated diffusion rates from fitting are included for reference. All samples were studied at an equilibrium temperature of 75°C.

crystalline phase this is. The magnitude of the splitting remains essentially constant with increasing hydration while its relative intensity (compared with the isotropic signal) decreases. This suggests that this intermediate phase is fully hydrated at or below 5 mol water/mol lipid. By simulation of the spectrum in Fig. 6, the hydration of this intermediate phase is estimated to be 1–2 mol water/mol lipid. It should be noted that the appearance of this quadrupolar splitting is somewhat dependent of the thermal history of the sample, consistent with the hysteretic behavior of this class of lipids. Outside of this temperature range, only an isotropic ^{2}H-NMR signal is observed and, as mentioned previously, phase separation of water and lipid melt are observed visually above 70°C at all levels of hydration.

Pulsed field gradient NMR experiments were carried out on 3-*O*-Me-β-D-GlcDAlG at 75°C and at three levels of hydration (0, 5, and 18 mol ^2H_2O/mol lipid) to investigate the lipid diffusion in the high temperature phase. Only in the melted phase is a ^{1}H spin echo observable with the experimental parameters used. Results from these experiments are shown in Fig. 7. The diffusion rate of water-free 3-*O*-Me-β-D-GlcDAlG is $4.4 \cdot 10^{-12}$ m^2 s^{-1}. The diffusion rate of 3-*O*-Me-β-D-GlcDAlG with 5 mol ^2H_2O/mol lipid is slightly larger ($6.0 \cdot 10^{-12}$ m^2 s^{-1}) and approximately equal to that with 35 wt % water or 18 mol ^2H_2O/mol lipid ($5.9 \cdot 10^{-12}$ m^2 s^{-1}). An increase in lipid diffusion is observed on the inclusion of a small amount of water, but more than 5 mol ^2H_2O/mol lipid has no additional effect.

DISCUSSION

The measurements using DSC, x-ray diffraction, and NMR spectroscopy all confirm the pattern of thermal events observed in aqueous dispersions of 3-*O*-Me-β-D-GlcDAlG. At temperatures below 60°C, 3-*O*-Me-β-D-GlcDAlG forms a very rigid L_c phase which is very poorly hydrated. Between 60 and 70°C, the lipid melts to a liquid crystalline phase, which x-ray diffraction shows is a reversed hexagonal phase (H_{II}). An H_I phase can be ruled out by the low level of hydration. The H_{II} phase is replaced above 70°C by a poorly hydrated, isotropic solution phase containing <5 mol water/mol lipid. However, the NMR measurements of samples containing different quantities of water suggest that the reversed hexagonal phase is metastable and that consequently the precise nature of the phase transition from the L_c phase may be dependent both on the thermal history of the sample and on the hydration.

Although we have not been able to define the exact structure of the phase above 70°C using x-ray diffraction, our other results strongly indicate that this high temperature phase is an L_2 phase. First, this lipid forms an isotropic phase above 70°C at all levels of hydration, shown clearly by the ^{1}H-NMR spectra and the magnitude of the M_2 values (cf. the M_2 values obtained for reversed micellar solutions in the work by Lindblom et al., 1993). The sample is also visually isotropic when viewed in between crossed polarizers. Second, this phase takes up very little water as evidenced by isotropic ^{2}H-NMR signals from hydrating ^2H_2O, visual phase separation between water and lipid at a low level of hydration (5 mol ^2H_2O/mol lipid), lipid diffusion properties, and x-ray diffraction patterns. The increase of lipid diffusion with the addition of 5 mol ^2H_2O/mol lipid to a water-free sample, without further change at higher hydration levels, indicates that the lipid matrix takes up no more than 5 mol water/mol lipid and probably less. X-ray results show only a small first-order repeat, which suggests that the amount of water of hydration in this phase is small and that there are strong headgroup interactions. The inclusion of a small amount of water rules out any normal phases which require more hydration. Third, this sample has the ability to flow. At all levels of hydration, this phase is fairly non-viscous which is compatible with reversed micellar aggregates, but not typically observed in other poorly hydrated, isotropic phases which might be suspected (i.e., reversed cubic phases).

Comparison with other glycerolipids

The phase properties of 3-*O*-Me-β-D-GlcDAlG are significantly different from those of other glycosyldialkylglycerols studied to date. DSC and low angle x-ray diffraction measurements of fully hydrated 1,2-di-*O*-tetradecyl-3-*O*-(β-D-glucopyranosyl)-*sn*-glycerol (di-14:0-β-D-GlcDAlG which is similar to 3-*O*-Me-β-D-GlcDAlG except for the *O*-methyl substitution) show the existence of an L_β/L_α phase transition at 52°C with a transition to an H_{II} phase at 57.3°C which persists up to 100°C (M. Akiyama, unpublished observations; Hinz et al., 1985). ^{2}H-NMR experiments on samples of di-14:0-β-D-GlcDAlG hydrated with ^2H_2O exhibit quadrupolar splitting consistent with the pattern of phase behavior outlined above (T. Trouard, unpublished observations). On annealing at room temperature, the L_β phase of di-14:0-β-D-GlcDAlG converts to an L_c phase over a period of ~24 h. A similar pattern of events is observed for the corresponding di-14:0-β-D-GalDAlG (Mannock et al., 1993), although the conversion time to the L_c phase is only a few minutes. In addition, the temperature range over which the L_α phase is stable in these glycosyldialkylglycerols has been shown to depend on two factors: hydrocarbon chain length, where the L_α window becomes smaller as the chain length increases, and headgroup stereochemistry, where the L_α window decreases in the following order: Gal > Glc >> Man (M. Akiyama, unpublished observations; Hinz et al., 1991; Jarrell et al., 1987; Mannock et al., 1993) and α > β (Mannock et al., 1988, 1990b; Mannock and McElhaney, 1991; D. Mannock, unpublished observations). On the basis of these observations, it has been suggested (Mannock et al., 1992, 1993) that both the L_c phase conversion kinetics and the L_α/H_{II} phase transition temperature may be regulated by differences in headgroup hydration, which are dependent on both sugar stereochemistry and anomeric configuration (Galema and Hoiland, 1991; Hoiland and Holvik, 1978; Levine and Slade, 1988). This proposed mechanism is congruent with the intrinsic curvature hypothesis suggested by Gruner (1985), since the monolayer curvature free energy term must contain an interfacial hydration component (Seddon and Templer, 1993).

The addition of a methyl group at position 3 of the β-D-Glc ring could have several effects on the physicochemical properties of the lipid. At the simplest level, it can be considered as an increase in the steric bulk of the headgroup, which can be expected to both lower the L_β/L_α and raise the L_α/H_{II} phase transition temperatures. Surface area measurements of the di-14:0-β-D-GlcDAlG and the corresponding 3-*O*-Me-β-D-GlcDAlG give at 30 mN/m surface pressure, pH = 7.4 and 22°C values of 41 and 52 Å^2/molecule, respectively (R. Demel, personal communication). The larger area/molecule and the greater nonbilayer tendency of the 3-*O*-Me-β-D-GlcDAlG cannot be easily explained in terms of a dimensionless packing parameter as in earlier measurements of monolayer films of DPPE and the corresponding α- and β-D-GlcDAcGs (Asgharian et al., 1989). Thus, the observed phase behavior shows that the addition of a methyl group at position 3 of the sugar ring cannot be treated simply as an increase in the steric bulk or "size" of the headgroup compared with the unmethylated lipid, but rather reflects a significant alteration in the hydrophobic/hydrophilic balance. Furthermore, the replacement of the hydroxyl group with a methyl group will also change the hydrogen bonding properties and lead to a decrease in the headgroup hydration. It is also possible that the methyl substitution could alter the orientation of the headgroup through changes in the hydrogen bonding properties or steric hindrances. This has been demonstrated in single crystal studies of the β-D-Gal ceramide and its permethylated derivative (Nyholm et al., 1990). Comparison of the di-14:0-β-D-GlcDAlG and the 3-*O*-Me-β-D-GlcDAlG strongly indicates that it is the chemical constituents which comprise the headgroup and the way in which they interact with water and/or each other that plays the major role in determining the lipid phase behavior. The importance of the chemical properties of the lipid headgroup is strongly supported by a comparison of PE and its methylated analogues with β-D-GlcDAlG and 3-*O*-Me-β-D-GlcDAlG. Sequential methylation of PE (MePE, diMePE, and PC) lowers the L_β/L_α and raises the L_α/H_{II} phase transition temperatures and thus shifts the phase equilibria toward an L_α phase. This is probably due to weakened headgroup/headgroup attractive forces allowing more water to penetrate into the interfacial region (Rand and Parsegian, 1992). As a consequence, the tendency of the lipid to form nonbilayer structures decreases. Evidently, the same chemical modification of a lipid headgroup can have opposite effects on the bilayer/nonbilayer phase preference in different lipid classes.

Comparison with MAcMGlcDAcG

The physical properties of 3-*O*-Me-β-D-GlcDAlG are particularly interesting when compared with the similar behavior observed in MAcMGlcDAcG isolated from *A. laidlawii* A. MAcMGlcDAcG is synthesized when large amounts of saturated, straight-chain fatty acids are incorporated into the membrane lipids (Rilfors et al., 1993). In MAcMGlcDAcG, the 6-hydroxyl group of the sugar ring is esterified with a saturated, long-chain fatty acid (Lindblom et al., 1993). The increase in the number of acyl chains from two to three while the number of glucose units remains constant adds to the hydrophobicity of the lipid and therefore should increase the tendency of MAcMGlcDAcG to form nonlamellar structures compared with α-D-GlcDAcG. It can be speculated that a hydrophobic, long-chain acyl group may be necessary to overcome the tendency of α-D-GlcDAcG to form bilayer structures. By virtue of its probable headgroup orientation relative to the bilayer surface (Jarrell et al., 1987; Sanders and Prestegard, 1992), α-D-GlcDAcG has a lower tendency to form nonbilayer structures than the corresponding β-D-GlcDAcG (Mannock et al., 1988, 1990b). As a regulatory mechanism, synthesis and incorporation of MAcMGlcDAcG into the membrane counters the tendency of saturated straight chain fatty acids to decrease the nonlamellar-forming potential of the lipid mixture. The underlying reasons why *A. laidlawii* A has chosen to modify α-D-GlcDAcG with a long acyl chain, to keep the balance between the lamellar/nonlamellar-forming lipids, may be due to the fact that this modification represents a metabolically efficient method of converting α-D-GlcDAcG to the more potent nonlamellar-forming MAcMGlcDAcG using a fatty acid from the supplemented growth medium.

The physicochemical properties of the synthetic lipid 3-*O*-Me-β-D-GlcDAlG closely resemble those of MAcMGlcDAcG despite the differences in their anomeric conformation and the addition of a larger, more hydrophobic acyl chain to position 6 of the sugar ring of MAcMGlcDAcG. Water-free MAcMGlcDAcG is in a gel/crystalline phase up to ~80°C, where it melts to a liquid phase. With 5 and 10 mol ^2H_2O/mol lipid, MAcMGlcDAcG is in a gel/crystalline phase up to 80°C, where an L_2 phase is formed. While the phase behavior of MAcMGlcDAcG and 3-*O*-Me-β-D-GlcDAlG are similar, they are both very dissimilar to the phase behavior of their unsubstituted counterparts (α-D-GlcDAcG and di-14:0-β-D-GlcDAlG, respectively) (Mannock et al., 1990b). In a water-free sample of MAcMGlcDAcG at 85°C, the translational diffusion coefficient of the lipids was measured to be $2.6 \cdot 10^{-12}$ m^2 s^{-1} compared with $4.4 \cdot 10^{-12}$ m^2 s^{-1} measured for 3-*O*-Me-β-D-GlcDAlG at 75°C. Diffusion rates normally increase with temperature so the difference in the measured diffusion could be due to the greater hindrance to motion of the esterified acyl chain in MAcMGlcDAcG when compared with the relatively small methyl group in 3-*O*-Me-β-D-GlcDAlG. Upon inclusion of 5 mol ^2H_2O/mol lipid, the diffusion rate of MAMGlcDAG increases to $6.2 \cdot 10^{-12}$ m^2 s^{-1} (Lindblom et al., 1993) compared with $6.0 \cdot 10^{-12}$ m^2 s^{-1} in 3-*O*-Me-β-D-GlcDAlG. This increase in the diffusion rate on incorporation of water into the L_2 phase probably arises from an increase in headgroup hydration, which reduces headgroup-headgroup interactions and allows less hindered movement of the lipid about the surface of the newly formed micelles. An alternative explanation could be the actual formation of micelles themselves, since these permit lateral diffusion on a preferred surface rather than being a three-dimensional diffusion process as in the water-free lipid melt. The pronounced increase in the diffusion rate of MAcMGlcDAcG may be due to the inclusion of a larger amount of water than in 3-*O*-Me-β-D-GlcDAlG. Addition of 5 mol ^2H_2O/mol lipid to MAcMGlcDAcG increases the ^1H-NMR line width by more than a factor of two (Lindblom et al., 1993) while virtually no change in the line width of 3-*O*-Me-β-D-GlcDAlG is observed in the present study. This may be attributable to the fact that the hydrophobic group attached to the sugar ring of MAcMGlcDAcG is an ester and therefore may be more favorable for the formation of hydrogen bonds with water molecules relative to the *O*-methyl group in 3-*O*-Me-β-D-GlcDAlG.

While both 3-*O*-Me-β-D-GlcDAlG and MAcMGlcDAcG contain a hydrophobic substitution of a sugar ring hydroxyl group, it is somewhat surprising that their phase and hydration properties resemble one another in view of the difference in the relative size of the substitutions at the sugar ring. Their similar phase behavior may indicate that it is the loss of a hydroxyl group as opposed to the presence of a hydrophobic moiety that has the greatest affect on the phase behavior of these lipids.

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