Structure and Phase Behavior of a Charged Glycolipid (1,2-*O*-Dialkyl-3-*O*-β-D-glucuronosyl-*sn*-glycerol)†

R. D. Koynova,‡ B. G. Tenchov,‡ H. Kuttenreich,§ and H.-J. Hinz*,§

Central Laboratory of Biophysics, Bulgarian Academy of Sciences, 1113 Sofia, Bulgaria, and Institut für Physikalische Chemie, Westfälische Wilhelms-Universität, D 48149 Münster, Germany

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ABSTRACT: In order to investigate the effects of a net surface charge on the properties of glycolipid membranes, we have synthesized a glyceroglycolipid, 1,2-*O*-dialkyl-3-*O*-β-D-glucuronosyl-*sn*-glycerol (GlcUA lipid), with saturated alkyl chains of varying length (14, 16, and 18 carbon atoms; 14-, 16-, and 18-GlcUA, respectively) and glucuronic acid with an ionizable 6-carboxyl group as polar residue. Aqueous dispersions of GlcUA lipids have been characterized by differential scanning calorimetry, densitometry, and X-ray diffraction methods as a function of pH. The carboxyl group deprotonation of apparent p*K* about 5.5 leads to a decrease of the melting temperatures by about 7 °C for all three compounds and to a chain-length-dependent reduction of the transition enthalpies by 0, 7, and 14% for 14-, 16-, and 18-GlcUA, respectively. The decrease of the transition temperature is consistent with current electrostatic concepts and models of charged membrane interfaces, but the chain-length-specific dependence of the enthalpy decrease with an increase of pH shows that the pH effects in GlcUA lipids are not of purely electrostatic origin. However, these effects appear to be simpler in some instances than corresponding effects in phospholipids with multiply ionizable head groups. For this reason, the lipids with the glucuronic acid head group appear to represent an appropriate model system for studies of net electric charge effects on the membrane properties. At pH 10.0, the lamellar gel phase of 18-GlcUA was found to display temperature-induced increases in specific volume and small-angle spacings, which we interpret as resulting from reversible swelling in heating scans and shrinking in cooling scans at temperatures between 40 and 50 °C. This process occurs therefore at a temperature well below the melting temperature of 68.6 °C. This is suggestive of the existence of an attractive (presumably short range) force dominating the electrostatic repulsion at low temperatures. The phase behavior of GlcUA lipids has been compared to that of the previously studied dialkylglucosylglycerols, or Glc lipids (Hinz, H. J., Kuttenreich, H., Meyer, R., Renner, M., Frund, R., Koynova, R., Boyanov, A., & Tenchov, B. et al. (1991) *Biochemistry 30,* 5125–5138), which have identical hydrophobic chains but a 6-hydroxymethyl group instead of the 6-carboxyl moiety in the sugar head group. The most interesting result of this comparison is that the thermodynamic parameters of the chain-melting transitions in Glc lipids and protonated GlcUA lipids are practically insensitive to the chemical difference in their head groups. This difference has, however, a profound effect on the nature of the high-temperature liquid-crystalline phase. While longer saturated chain (16 and 18 carbon atoms) Glc lipids display direct gel ↔ inverted hexagonal phase transitions, both protonated and deprotonated 18-GlcUA are characterized by a gel (L_β) ↔ lamellar liquid crystalline phase (L_α) transition. Thus, the 6-carboxyl group of GlcUA acts as a bilayer stabilizer suppressing formation of nonbilayer phases.

Glyceroglycolipids constitute one of the major glycolipid classes. They are found in plant and animal tissues (Sastry, 1974; Sweely & Siddiqui, 1987; Slomiany et al., 1987; Quinn & Williams, 1978) and many kinds of bacteria (Shaw & Stead, 1971; Oshima & Yamakawa, 1974; Ward, 1981). Mostly present as minor lipid components, their amount in some membranes can reach very high levels. Galactolipids, for example, comprise up to 75% of the total lipid content in chloroplast membranes (Nishihara et al., 1980). Various functions have been assigned to these lipids, among them mediation of cell surface recognition and interaction, maintenance of membrane structure and fluidity, and specific lipid–protein interactions (Ishizuka & Yamakawa, 1974). In comparison to glycerophospholipids, the properties of the glycroglycolipids are less well understood, although these two kinds of lipids have identical hydrophobic moieties. One factor that has so far impeded a better characterization is the extreme diversity of their carbohydrate composition, which results in a large variety of phase patterns with different thermodynamic, kinetic, and structural properties. Another major obstacle is the heterogeneity of natural glycolipid extracts which makes these systems unsuitable for detailed physical analysis. For these reasons, most of the recent progress in the characterization of the structures formed by these amphiphiles in water has been made possible by the development and improvement of procedures for synthesis of stereochemically pure glycolipid species of uniform fatty acid composition [e.g., Endo et al. (1982), Six et al. (1983), and Jarrell et al. (1987)]. In an attempt to single out the specific effects of the sugar residues, we have synthesized and investigated in our previous studies a series of glyceroglycolipids having identical saturated alkyl chains and different sugar head groups as well as a homologous series of glycolipids with identical head groups and varying alkyl chain lengths (Hinz et al., 1985, 1991; Koynova et al., 1988; Kuttenreich et al., 1988). We are particularly interested in ether-linked lipids because of their frequent occurrence in

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‡ Bulgarian Academy of Sciences.

§ Westfälische Wilhelms-Universität.

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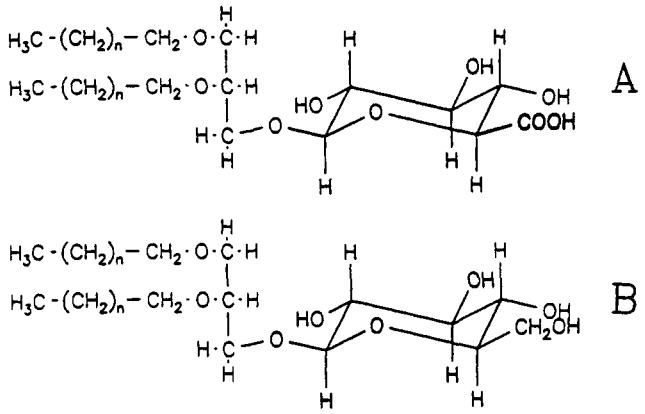

FIGURE 1: Structure of dialkylglucuronosylglycerols (A) and dialkylglucosylglycerols (B) with saturated alkyl chains of different length (n = 12, 14, and 16).

archaebacteria (Mangold and Paltauf, 1983). Characterization of the structure and phase behavior of these compounds by calorimetric, densitometric, X-ray diffraction, and monolayer techniques has facilitated the discrimination between the stereochemical and size effects of the sugar moieties, the effect of the glycerol stereoconfiguration, and the effects of variations in the chain length. A similar approach, adopted by Mannock et al., has recently revealed important facets of the phase behavior of synthetic glycolipids with diacylglycerol backbones (Mannock et al., 1987, 1988, 1990a,b; Sen et al., 1990).

So far, biophysical studies on synthetic glycolipids have employed sugar residues having no ionizable groups. In these compounds the interfacial effects, which are known to strongly modulate the lipid phase behavior, are related mainly to the hydration properties of the sugar groups and the ability of these groups to form inter- and intramolecular hydrogen bonds. A number of studies on phospholipids with ionizable head groups, such as phosphatidic acid (Jacobson & Papahadjopoulos, 1975; Träuble et al., 1976; Eibl & Woolley, 1979; Blume & Eible, 1979), phosphatidylserine (MacDonald et al., 1976; Cevc et al., 1981), and phosphatidylethanolamine (Cevc et al., 1986; Cevc, 1987; Yao et al., 1992), have demonstrated, however, that the lipid phase behavior is sensitive to electrical charge at the membrane surface. Our aim in this work was to evaluate the effects of a net surface charge on the properties of glycolipid membranes. Therefore, we have synthesized a glycolipid, 1,2-*O*-dialkyl-3-*O*-β-D-glucuronosyl-*sn*-glycerol, with saturated alkyl chains of varying length (14, 16, and 18 carbon atoms) and glucuronic acid as polar residue (Figure 1). Aqueous dispersions of GlcUA[1] lipids with different pH values have been characterized by differential scanning calorimetry, densitometry, and X-ray diffraction methods. The experimental results show distinct, albeit small, effects of the deprotonation of the carboxyl group on the glycolipid phase behavior. These effects have been analyzed on the basis of current theoretical models of charged membrane interfaces and compared with corresponding effects in phospholipids with ionizable head groups.

[1] Abbreviations: 14-GlcUA, 16-GlcUA, and 18-GlcUA, 1,2-*O*-dialkyl-3-*O*-β-D-glucuronosyl-*sn*-glycerol with 14, 16, and 18 carbon atoms per saturated alkyl chain, respectively; 10-Glc, 12-Glc, 14-Glc, 16-Glc, and 18-Glc, 1,2-*O*-dialkyl-3-*O*-β-D-glucosyl-*sn*-glycerol with 10, 12, 14, 16, and 18 carbon atoms per alkyl chain, respectively; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid; PG, phosphatidylglycerol; DSC, differential scanning calorimetry; DSD, differential scanning densitometry; L_α, lamellar liquid crystalline phase; L_β, lamellar gel phase; L_c, lamellar subgel (crystalline) phase; H_{II}, inverted hexagonal phase.

Since the only difference between the chemical structures of GlcUA lipids and the previously studied glucolipids, 1,2-*O*-dialkyl-3-*O*-β-D-glucosyl-*sn*-glycerols (Glc lipids) (Hinz et al., 1985, 1991; Koynova et al., 1988), is the replacement of the 6-hydroxymethyl group in Glc by a 6-carboxyl group in GlcUA (Figure 1), these GlcUA lipids provide us with very specific access to the study of charge effects on lipid phase behavior. pH decrease permits the head group charge to be abolished, thereby rendering the GlcUA group very similar to the uncharged Glc moiety.

A significant result of the present study, which arises from a comparison with the data on glycolipids published previously, is the finding that the thermodynamic parameters of the chain-melting transitions of Glc lipids and fully protonated GlcUA lipids are practically insensitive to the chemical difference in the head groups (COOH instead of CH_2OH), but that this difference has a profound effect on the nature of the high-temperature liquid-crystalline phase. Longer chain Glc lipids (16 and 18 carbon atoms) display direct gel to inverted hexagonal ($L_\beta \rightarrow H_{II}$) phase transitions, while both protonated and deprotonated 18-GlcUA are characterized by a gel to lamellar liquid-crystalline ($L_\beta \rightarrow L_\alpha$) phase transition.

MATERIALS AND METHODS

The synthesis of 1,2-*O*-dialkyl-3-*O*-β-D-glucuronosyl-*sn*-glycerols with saturated hydrocarbon chains with 14, 16, and 18 carbon atoms has been carried out according to procedures described elsewhere (Six et al., 1983; Kuttenreich, 1992). There are a few additional steps required for the introduction of the glucuronic acid group. D-Glucuronic acid γ-lactone is methylated before being peracetylated under acidic conditions. The tetra-*O*-acetyl-β-D-glucuronic acid methyl ester ($[\alpha]^{25}_D$ = +7.8° (c = 5, $CHCl_3$), mp = 178–179 °C) is then reacted using a filtered mixture of Br_2, glacial acetic acid, and red phosphorus to yield methyl tri-*O*-acetyl-α-D-glucuronatosyl bromide ($[\alpha]^{25}_D$ = +197° (c = 5, $CHCl_3$), mp = 105–106 °C). This compound is used in the Königs–Knorr reaction with 1,2-*O*-dialkyl-*sn*-glycerol to form 1,2-*O*-dialkyl-3-*O*-(methyl tri-*O*-acetyl-β-D-glucuronatosyl)-*sn*-glycerol as described previously for the glucolipids. The chromatographic purity of all compounds and intermediate products was assured by thin-layer chromatography on silica-gel plates (Merck), and the stereochemical purity was checked by 250-MHz ^1H and ^{13}C NMR as described previously (Hinz et al., 1985). The chemical shift of the $H^{1'}$ proton of glucose is 4.33 ppm. The doublet has a coupling constant $J_{1',2'}$ = 7.6 Hz indicative of a β-glycosidic bond. Deacetylation yields 1,2-*O*-dialkyl-3-*O*-(methyl β-D-glucuronatosyl)-*sn*-glycerol ($[\alpha]^{20}_D$ = −15.6° (c = 2, $CHCl_3/CH_3OH$ = 1:1), mp = 67–68 °C). Demethylation leads to the sodium salt of the final product ($[\alpha]^{20}_D$ = −16.8° ($CHCl_3/CH_3OH$ = 5:2), mp = 209–212 °C (dec)). Aqueous dispersions with lipid concentrations of ~0.5 mg/mL, 10–20 mg/mL, and 20 wt % (200 mg/mL) for the calorimetric, densitometric, and X-ray measurements, respectively, were prepared by sequential cycles of freezing and thawing in combination with vortex mixing for about 10 min at room temperature. This procedure avoids heating above the lipid melting transition and results in suspensions of rather good homogeneity. A 100 mM phosphate buffer was used at ambient pH, and low and high pH values were adjusted with sodium phosphate and diluted HCl or NaOH solutions, respectively. For the DSC measurements, dispersions of 18-Glc with different pH values were also prepared.

Calorimetric measurements were made using a high-sensitivity DASM-1M microcalorimeter (Hinz, 1986). Heat-

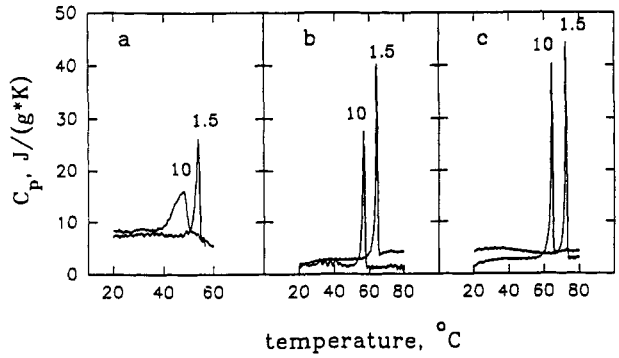

FIGURE 2: DSC scans of 14-GlcUA (a), 16-GlcUA (b), and 18-GlcUA (c) at pH 1.5 and 10. Transition temperatures and enthalpies for these lipids at different values of pH are given in Table I.

ing scans were performed in the range from 20 to 80 °C with a heating rate of 0.5 °C/min. The minimum number of samples used in each experiment was four. Density differences between the lipid suspensions and the respective electrolyte solutions were determined by using two DMA 602 HT external cells in combination with a DMA 60 measuring unit (A. Paar, Graz). Temperature scans between 20 and 90 °C were controlled with a Haake PG 20 temperature controller and a Haake F3 thermostated bath. The scanning rate was 0.5 °C/min for heating and cooling runs. Lipid specific volumes and temperature expansion coefficients were calculated as described elsewhere (Hinz et al., 1991). Low- and wide-angle X-ray studies were carried out using a Kratky compact camera and a pinhole camera, respectively (both from A. Paar, Graz) equipped with position-sensitive detectors and Peltier-regulated, temperature-controlled cells. Low-angle studies were performed with a detector from M. Braun, Garching, FRG; wide-angle measurements were made with a LETI detector from Inel, Buc, France. The temperature in the sample holder was determined with a Pt 100 resistance probe. Prior to each measurement, the samples were equilibrated at the respective temperature for 20 min.

Data obtained for 14-, 16-, and 18-GlcUA were compared with data for the respective dialkylglucosylglycerols 14-, 16-, and 18-Glc.

RESULTS

Differential Scanning Calorimetry Measurements. All three GlcUA lipids exhibited single endothermic transitions with temperatures and enthalpies depending on the pH of the medium. Typical thermograms of dispersions of 14-, 16-, and 18-GlcUA at pH 1.5 and 10 are presented in Figure 2. A decrease of the transition cooperativity at high pH, reported by Blume and Eibl (1979) for dimyristoyl-PA and by Yao et al. (1992) for dipalmitoyl-PE, was observed here only with samples of 14-GlcUA (Figure 2a), and not for 16- and 18-GlcUA in the first heating scans. The numerical values of the transition temperatures and enthalpies of the three glucuronides are given in Table I as a function of pH, and a graphical representation of these data is shown in Figure 3. It can be seen that the increase of pH from 1.5 to 10 results in a decrease of the transition temperature of about 7 °C for all three glycolipids. An inflection point at a pH value of about 5.5 is due to deprotonation of the carboxylic acid group in the glucuronic acid head group. That the sigmoidal curves reflect titration of the carboxylic acid group becomes quite obvious from a comparison of the DSC measurements of the glucuronic acid lipids with that of 18-Glc. From pH 1.5 to 10 the transition temperature of the glucolipid is independent of pH (Figure 3, top panel, solid squares).

Table I: pH Dependence of Calorimetric Parameters of Dialkylglucuronosylglycerols with Varying Chain Lengths[a]

	lipid					
	14-GlcUA		16-GlcUA		18-GlcUA	
pH	T_{tr} (°C) (±0.1)	ΔH (kJ/mol) (±0.3)	T_{tr} (°C) (±0.1)	ΔH (kJ/mol) (±0.3)	T_{tr} (°C) (±0.1)	ΔH (kJ/mol) (±0.3)
1.5	52.8	25.3	65.5	38.2	75.3	47.0
2.0	52.8	25.1	65.5	37.8	75.2	47.2
2.5	52.5	25.8	65.6	38.0	75.7	47.2
3.0	52.5	25.5	65.3	38.4	75.4	46.8
4.0	51.8	25.8	64.5	36.3	73.3	45.4
4.3	50.8	27.0	64.2	36.4	73.0	44.8
5.0	50.0	27.5	63.3	35.8	72.4	43.6
6.0	47.7	25.9	62.2	36.5	71.5	43.3
7.0	46.7	25.2	60.0	34.0	70.0	42.4
8.0	46.3	25.4	58.5	34.5	69.2	41.0
10.0	46.0	25.5	58.0	35.4	68.6	40.6
	glycolipids in twice-distilled water[b]					
	14-Glc $L_\beta \rightarrow L_\alpha$		16-Glc $L_\beta \rightarrow H_{II}$		18-Glc $L_\beta \rightarrow H_{II}$	
	T_{tr} (°C) (±0.1)	ΔH (kJ/mol) (±0.3)	T_{tr} (°C) (±0.1)	ΔH (kJ/mol) (±0.3)	T_{tr} (°C) (±0.1)	ΔH (kJ/mol) (±0.3)
	51.6	24.9	63.4	40.4	72.5	46.6

[a] For comparison, calorimetric data from Hinz et al. (1991) for the melting transitions of the respective dialkylglucosylglycerols 14-, 16-, and 18-Glc are given in the bottom part of the table. [b] Data from second and subsequent heating scans. In first heatings, 14-Glc displays an $L_c \rightarrow L_\alpha$ transition at 51.5 °C with ΔH = 55.3 kJ/mol; 16- and 18-Glc display $L_c \rightarrow L_\beta$ transitions at 57.1 and 56.7 °C with ΔH values of 22.9 and 23.3 kJ/mol, respectively. 14-Glc undergoes an $L_\alpha \rightarrow H_{II}$ transition at 56.4 °C with ΔH = 5.3 kJ/mol.

At alkaline pH, the transition enthalpies of the GlcUA lipids with 16- and 18-C alkyl chains decrease by about 7% and 14%, respectively, while the melting enthalpy of 14-GlcUA remains the same at the two ends of the pH scale (Figure 3, bottom panel, and Table I).

The transitions at low pH were reproducible in subsequent heating scans, while heating–cooling cycles through the melting transition of 18-GlcUA suspensions at pH 10 resulted in a pronounced decrease of the transition cooperativity, together with a slight decrease of the transition temperature (data not shown). The initial transition pattern was recovered, however, when the sample was kept for several hours at low temperature prior to the heating scan.

Differential Scanning Densitometry Measurements. The specific volume of 18-GlcUA and the volume changes during temperature scans in the range 20–90 °C were measured at pH 1.65 and 10. The results are summarized in Figure 4 and Table II. At low pH, when the glucuronic acid head group is fully protonated, 18-GlcUA exhibits a single cooperative transition at 73.6 °C, accompanied by a volume change of 69 μL/g (Figure 4a). This transition is reversible with about 1 °C hysteresis on cooling. At high pH, with an ionized head group, 18-GlcUA demonstrates a more complex behavior in the density measurements. The first heating after low-temperature sample preparation shows a single cooperative volume change at 68.2 °C (Figure 4b, curve 1). During subsequent heating–cooling cycles, however, reproducible anomalies occur in the density curves in the temperature range between 20 and 50 °C. The volume change associated with the transition at 68.2 °C was found to be larger than that observed in the first heating (Figure 4b). In heating scans, large density fluctuations take place at temperatures around 45 °C (Figure 4b, scans 3 and 7). In cooling scans, these anomalies are observed at about 35 °C and are accompanied

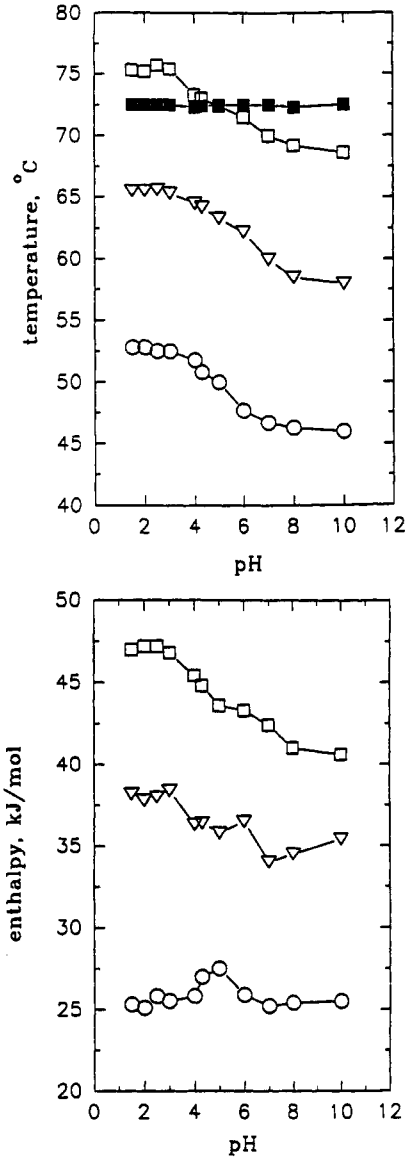

FIGURE 3: pH dependence of the phase transition temperature (top panel) and enthalpy (bottom panel) of 14-GlcUA (○), 16-GlcUA (▽), 18-GlcUA (□), and 18-Glc (■).

by poorly reproducible slow drifts of the specific volume in the range 20–40 °C (Figure 4b, scans 2 and 6). When the samples are not cooled below 50 °C, only one fully reversible transition at 68.2 °C is observed. The associated volume change is, however, almost twice as big as that observed in the first heating (Table II). Subsequent cooling scans down to 20 °C resulted again in the same irregular behavior of the specific volume of the lipids (Figure 4b, scans 6 and 7). While the cooperative volume change at 68.2 °C corresponds clearly to the endothermic phase transition of 18-GlcUA recorded by DSC, no heat capacity signal corresponding to the anomalous behavior of the specific volume at lower temperature is found. These observations appear to be rather unique, and we are not aware of another report of such anomalous density behavior in the literature. As shown in the next paragraph, we assume that these large volume fluctuations of 18-GlcUA below the melting temperature of the bilayer correspond to the reversible shifts in the low-angle reflections of the lamellar gel phase of 18-GlcUA observed by X-ray diffraction at the same temperatures.

Low- and Wide-Angle X-ray Diffraction Measurements. In order to correlate the enthalpy and volume changes observed

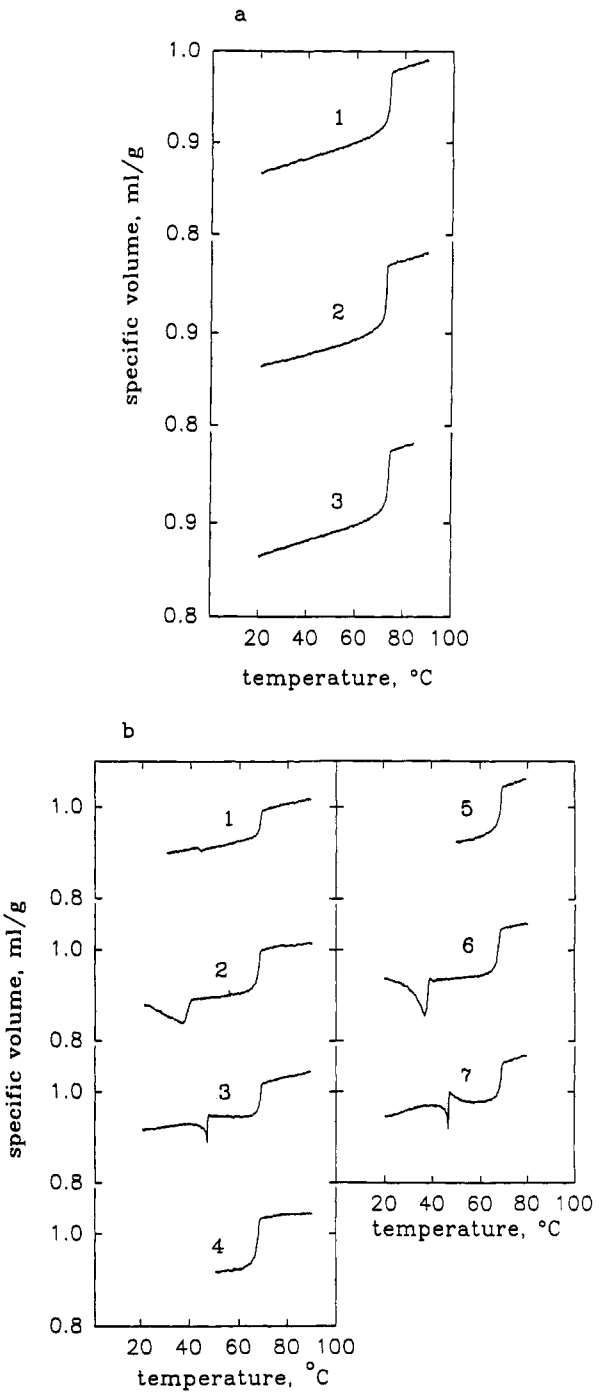

FIGURE 4: Successive heating and cooling scans of the specific volume of 18-GlcUA at pH 1.65 (a) and pH 10 (b). Odd numbers denote heating scans; even numbers, cooling scans. The lipid specific volumes and expansion coefficients at 20 and 80 °C are given in Table II.

by DSC and density measurements with structural rearrangements in 18-GlcUA dispersions, low- and wide-angle measurements were carried out at increasing and decreasing temperatures both at low and high pH. The diffraction patterns are shown in Figures 5 and 6. The low- and wide-angle spacings determined from these measurements are summarized in Table III. According to the data in Figure 5 and Table III, the temperature-induced phase transition in fully protonated 18-GlcUA at low pH, recorded by DSC and density measurements, is a reversible chain-melting transformation from a lamellar gel (L_β) to a lamellar liquid-crystalline (L_α) phase. This transformation is accompanied by a change of about 10 Å in the lamellar repeat distance,

Table II: Densitometric Characteristics of 18-GlcUA at Low and High pH[a]

pH	$\nu_{20°}$, mL/g (±0.005)	T_{tr}, °C (±0.2)	$\Delta\nu_{tr}$, mL/g	α_g, [mL/(g·K)] × 10^4 (±0.2)	α_f, [mL/(g·K)] × 10^4 (±0.2)
1.65	0.865 (L_β)	73.6, $L_\beta \rightarrow L_\alpha$	0.0690 ± 0.0020	8.2 (L_β phase)	8.8 (L_α phase)
10.0	0.912 (L_β)[b]	68.2, $L_\beta \rightarrow L_\alpha$	0.0591 ± 0.0020;[b]		11.9 (L_α phase)
	[c]		0.0980 ± 0.0100[d]	[c]	
			18-Glc in Twice-Distilled Water		
	0.895 (L_c)	56.7, $L_c \rightarrow L_\beta$	0.0610 ± 0.0020, $L_c \rightarrow L_\beta$	2.7 (L_c phase)	7.9 (H_{II} phase)
	0.937 (L_β)	72.5, $L_\beta \rightarrow H_{II}$	0.0650 ± 0.0020, $L_\beta \rightarrow H_{II}$	8.0 (L_β phase)	7.9 (H_{II} phase)

[a] $\nu_{20°}$ is the partial specific volume at 20 °C; $\Delta\nu_{tr}$ is the volume change during the phase transition; α_g and α_f are the temperature expansion coefficients of the gel phase at 20 °C and the fluid phase at 80 °C, respectively. Corresponding data of the glucoglycerolipid 18-Glc, according to Hinz et al. (1991), are given in the bottom part of the table. [b] Unheated low-temperature sample preparation. [c] The values of ν and α_g cannot be determined in this case due to the irregular behavior of the specific volume (Figure 5b). [d] $\Delta\nu_{tr}$ values were determined from scans 4 and 5 in Figure 5b.

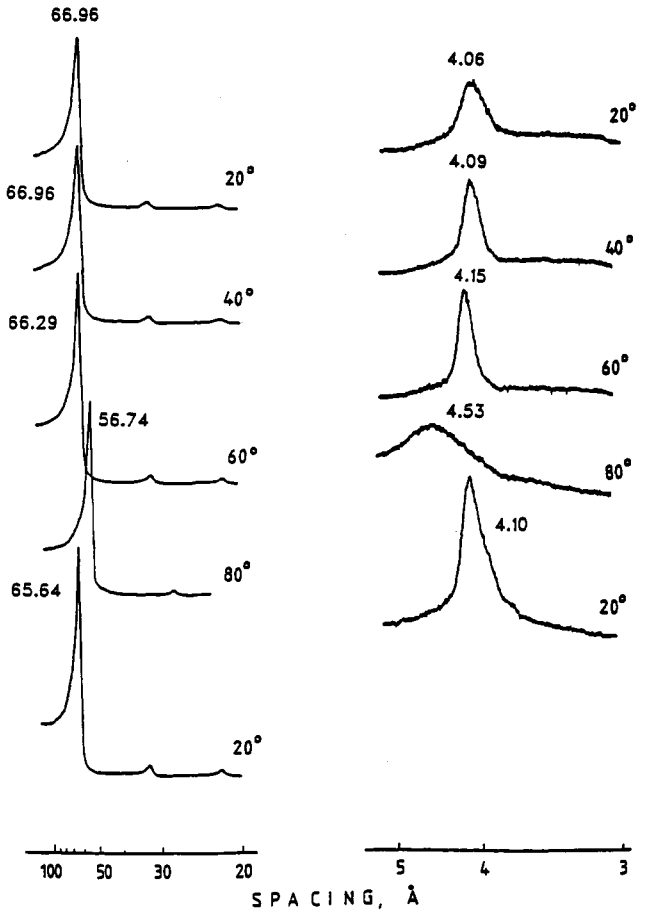

FIGURE 5: Successive low-angle (left) and wide-angle (right) X-ray diffraction patterns of 18-GlcUA at pH 1.6 recorded at different temperatures. The temperatures and the phases are indicated on each diffraction pattern. The values of the wide-angle and first-order low-angle spacings, determined from these patterns, are given in Table III.

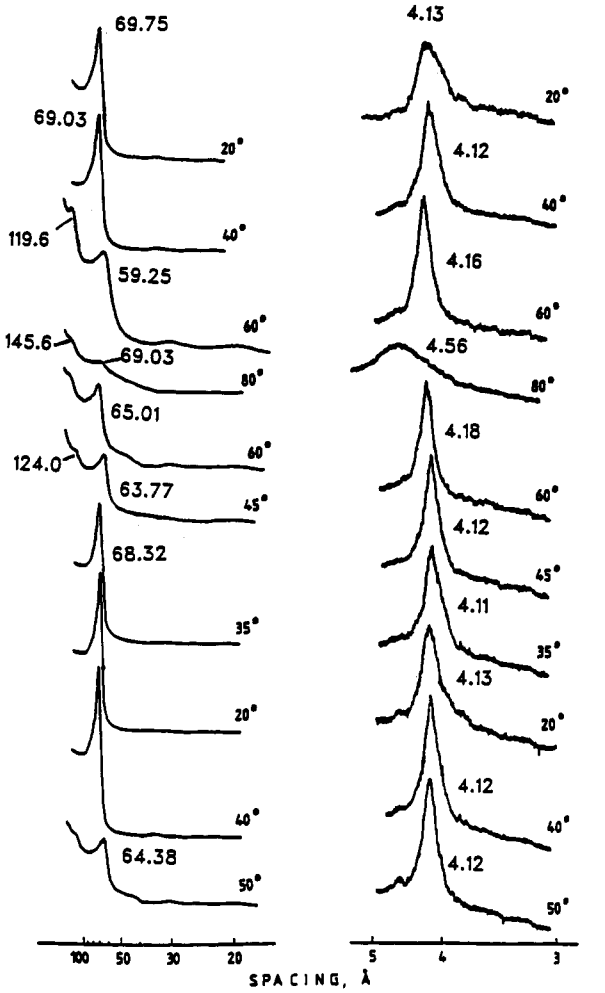

FIGURE 6: Successive low-angle (left and wide-angle (right) X-ray diffraction patterns of 18-GlcUA at pH 10 recorded at different temperatures during heating, cooling, and second heating. The temperatures and the phases are indicated on each diffraction pattern. The values of the wide-angle and first-order low-angle spacings, determined from these patterns, are given in Table III. The diffuse low-angle profiles recorded at 60, 80, 60, 45, and 50 °C (from top to bottom) indicate disordered swollen lamellar phases at these temperatures.

which decreases from 67 Å in the gel phase to 56.7 Å in the liquid-crystalline phase.

At 20 °C and high pH, when the 18-GlcUA head group is fully ionized, the lipid is in a well-stacked lamellar gel state, characterized by sharp low-angle reflections (Figure 6). The lamellar repeat distance is greater by 2.7 Å that that observed at low pH in the protonated state at the same temperature. This difference was maintained after a heating–cooling cycle, although both lamellar periods experienced a reduction by about 1.4 Å (Table III). Heating to temperatures above 40 °C results, however, in pronounced broadening and peak intensity decrease of the low-angle diffraction profile, together with a strong increase of the lamellar spacings. Such behavior of the low-angle diffraction pattern indicates a disorder in the bilayer stacking, which, in combination with the large increase of the lamellar repeat period, is suggestive of swelling of the lamellar phase. Most probably it results from electrostatic repulsion of the negatively charged membrane interfaces. We would like to emphasize that the assumed swelling takes place at temperatures well below the phase transition. This is evident from the corresponding wide-angle diffraction pattern at 60 °C, which still shows a single sharp reflection at 4.16 Å, characteristic of the gel state. A typical L_α pattern of the

Table III: Lamellar Repeat Distances and Wide-Angle Spacings in Aqueous Dispersions of 18-GlcUA at Low and High pH, Obtained from the Diffraction Patterns Shown in Figures 5 and 6[a]

glycolipid		first-order low- and (in parentheses) wide-angle spacings of 18-GlcUA at different temperatures, Å						
		20 °C	35 °C	40 °C	45 °C	50 °C	60 °C	80 °C
18-GlcUA	heating $L_\beta \rightarrow L_\alpha$	66.96 (4.06)		66.96 (4.09)			66.29 (4.15)	56.74 (4.53)
at pH 1.6	cooling, $L_\beta \leftarrow L_\alpha$	65.64 (4.10)						56.74 (4.53)
18-GlcUA	heating, $L_\beta \rightarrow L_\alpha$	69.75 (4.13)		69.03 (4.12)			~120 (4.16)	~140 (4.56)
at pH 10	cooling, $L_\beta \leftarrow L_\alpha$	68.32 (4.13)	68.32 (4.11)		~125 (4.12)		~130 (4.18)	~140 (4.56)
	2nd heating	68.32 (4.13)		68.32 (4.12)		~130 (4.12)		
18-Glc in twice-	heating, $L_\beta \rightarrow H_{II}$	62.00 (cryst)				62.00 (cryst)	66.96 (4.17)	56.26[b] (4.49)
distilled water	cooling, $L_\beta \leftarrow H_{II}$		67.23 (4.07)				67.23 (4.19)	56.26[b] (4.49)

[a] For comparison, corresponding data for the dialkylglucosylglycerol 18-Glc from Hinz et al. (1991) are given in the bottom part of the table. Approximate uncertainties: low-angle region, ±1.1 Å; wide-angle region, ±0.01 Å. [b] First-order low-angle reflection of the H_{II} phase of 18-Glc.

chains was obtained at 80 °C, together with further loss of lamellar order, as seen from the wide-angle diffraction patterns in Figure 6. This sequence of events is reversible on cooling and subsequent reheating (Figure 6, bottom; Table III).

According to these observations, the cooperative enthalpy and volume changes in 18-GlcUA at pH 10 reflect a chain-melting transition in strongly disordered bilayers. In connection with the density studies, they indicate also to us that reversible swelling and shrinking of the lamellar gel phase of 18-GlcUA takes place at temperatures between 40 and 50 °C, which are well below the chain-melting transition. Cooling to 35 °C results in shrinking of the disordered phase and recovery of the initial well-stacked lamellar gel phase, as the characteristic sharp low-angle reflections demonstrate. It is important to note that both the reversible shifts of the low-angle reflections observed by X-ray diffraction and the anomalous behavior of the specific volume of 18-GlcUA recorded by densitometry occur in the same relatively narrow temperature range between 35 and 50 °C.

DISCUSSION

Decrease of the Phase Transition Temperature in Ionized GlcUA Membranes. A decrease of the chain-melting transition temperature for ionized lipid bilayers in comparison to their uncharged state has been reported previously for different classes of phospholipids with ionizable head groups (see the introduction for references). An explanation proposed by Träuble et al. (1976) is based on the mutual repulsion of the negatively charged polar groups. The charge favors a lateral expansion of the membrane that destabilizes the ordered low-temperature phase. Consequently, the phase transition temperature, T_{tr}, is lowered, and its decrease is proportional to the increase in area per molecule during the transition, according to the equation

$$T_{tr} - T_{tr}^* = \Delta T_{tr\ max} = -2kT_{tr}(\Delta f/f)(N_A/\Delta S^*) \quad (1)$$

Here the uncharged state of the membrane is denoted by asterisks, T_{tr} is the transition temperature, $\Delta f/f$ is the relative expansion of the lipid molecular area during melting, ΔS^* is the entropy difference between fluid and ordered states of the uncharged membrane formed by protonated 18-GlcUA, k is the Boltzmann constant, and N_A is Avogadro's number. This equation has been derived by Träuble et al. (1976) under the conditions of full ionization and high surface potential, which apply to the state of 18-GlcUA membranes at pH 10. From the DSC data at pH 1.5 we determine $\Delta S^* = 135$ J/(mol K) and $T_{tr}^* = 348.7$ K, and from the wide-angle X-ray data the relative increase of molecular area during melting is $\Delta f/f = 0.177$ (from 40.2 Å^2 per molecule in the gel state at 60 °C to 48.0 Å^2 in the fluid state, giving $\Delta f = 7.8$ Å^2 and a mean value for $f = 44.1$ Å; the wide-angle reflections were indexed to a hexagonal lattice). With these parameters eq 1 predicts a decrease of the transition temperature of $\Delta T_{tr} = -7.4$ °C for the deprotonated state of 18-GlcUA relative to the protonated one, in rather good agreement with the experimental value of –6.7 °C given in Table I. The application of Träuble's theory can be extended further to calculate from the experimental data also the degree of ionization, α, as a function of pH. Such calculation shows that $\alpha = 0.5$ at pH 5.23 and approximates unity at pH 7.5 (Kuttenreich, 1992). Since it is obvious from Figure 3 that the apparent pK of GlcUA is about 5.5, we do not reproduce these calculations here. The above values are also in accord with the results of Cevc (1987) and Cevc et al. (1985, 1986). Their estimate of the electrostatically induced shift in transition temperature, which is only one among various mechanisms by which pH changes can cause T_{tr} shifts, is about –6 °C.

While theory and experiment agree well with respect to transition temperatures, the mere electrostatic argument is apparently not sufficient to explain the pH dependence of the transition enthalpies. The change in the transition enthalpy of the glucuronic acid lipids is also a function of the chain length, since the pH-induced enthalpy change is largest with 18-GlcUA and decreases with decreasing alkyl chain length (Figure 3; Table I). This effect cannot be accounted for by the above theoretical model for the obvious reason that this model considers electrostatic contributions to the membrane free energy which arise solely from ionization and which therefore by definition are independent of chain length (Träuble et al., 1976).

It is worth recalling in this context that also the studies on the phase behavior of ionizable phospholipids have shown that changes in pH often cause complex variations of transition temperatures and enthalpies that cannot be explained on the basis of purely electrostatic considerations. With respect to transition temperatures it is evident, however, from the analysis of Cevc (1987) that the main complication in the pH dependence of T_{tr} in phospholipids arises from the fact that their head groups usually contain more than one ionizable group. Except for the methylated derivatives (Träuble et al., 1976; Eibl & Woolley, 1979), the most frequently studied lipids, PA and PE, contain two ionizable groups, and PS contains three ionizable groups (phosphate, carboxyl, and amine). In pH titrations the complex behavior of the transition temperatures of these lipids is determined by the combined effects of all ionizable groups with respect to hydration, interlipid interactions, and the net surface electric charge (Cevc, 1987). To exclude such complex behavior, we synthesized the glucuronic acid lipids. The ionizable part of the head group is a single carboxylic acid, and its simple titration behavior is more easily amenable to analysis.

The phospholipids and the GlcUA lipids appear to differ also by the magnitude of the changes in transition enthalpy resulting from the increase of pH from 1.6 to 10. MacDonald et al. (1976) report a decrease from 9 to 3 kcal/mol in the transition enthalpy of dipalmitoyl-PS associated with the increase of pH to alkaline values. An enthalpy decrease from 5.2 to 2.9 kcal/mol occurs when dipalmitoyl-PA is titrated through pK_2 (Jacobson & Papahadjopoulos, 1975). Blume and Eibl (1979) observed contrasting and rather complex effects of pH on the transition enthalpies of dimyristoyl-PA and dihexadecyl-PA. In all cases the experimentally observed enthalpy differences exceed by far the possible contributions of the surface electrostatic interactions to the transition enthalpy.

The results reported here do not show such drastic pH effects on the transition enthalpies of the GlcUA lipids. Nevertheless, it is clear from the specific chain length dependence of these effects that any interpretation of the dependence of the enthalpy on pH must account for the influence of pH on both the interactions in the interfacial layer of the membrane and the change in the interactions in the hydrophobic core.

Temperature-Induced Swelling and Shrinking of the Ionized 18-GlcUA Lamellar Gel Phase: A Hypothesis To Explain the X-ray and Densitometric Data. It has been proposed by Hauser (1984) that unlimited swelling is a general phenomenon for charged lipid bilayers at low electrolyte concentrations, due to electrostatic repulsion between the bilayers. Screening of the surface charge by addition of electrolytes to the lipid dispersion reverses this process and makes the swollen phase shrink into ordered multilamellar arrays. In this respect, the hypothesis advanced in this study that ionized 18-GlcUA bilayers display temperature-induced reversible swelling and shrinking is new and interesting. These processes take place in the lamellar gel phase of the lipid at temperatures around 40–50 °C, which are well below the chain-melting transition at 68.6 °C. The reactions are accompanied by strong anomalies in the specific volume of the lipid. This behavior clearly indicates that an attractive force, counteracting the electrostatic repulsion, dominates at low temperatures, while the repulsive electrostatic interaction is dominant at higher temperatures. In principle, the van der Waals interaction between lipid bilayers could play the role of the attractive force that governs the low-temperature behavior. However, this is not very likely, since the processes which we assign to swelling and shrinking of the lipid phase occur within a relatively narrow temperature range of about 10–20 °C. A compensation of longe-range repulsive electrostatic and attractive van der Waals forces would be expected to extend over a much larger temperature interval and can hardly serve to explain why a temperature change of 10 °C should have such a drastic effect on the interbilayer separation.

A more likely explanation for the favorable interaction that could counteract the assumed swelling at low temperatures can be based on the known ability of the glycolipid polar residues to form intermolecular hydrogen bonds. Although ionization of 18-GlcUA results in a 2.7-Å increase of the lamellar repeat distance at 20 °C (Table III), this increase alone is no reason to exclude the hypothesis that at low temperature hydrogen bonds might occur between head groups in opposing bilayers, which would suppress swelling of the lamellar phase. Formation of interbilayer hydrogen bonds has been invoked to account for the lack of swelling in dielaidoyl-PE doped with small amounts of dielaidoyl-PA, whereas similarly doped dielaidoyl-PC exhibited the swelling phenomenon (Van der Kleij et al., 1988). The "hydrogen bond" hypothesis is preferable on the basis of the presently available data. Particularly it appears to account better for the temperature dependence of the hypothetical swelling behavior. Hydrogen bond networks can act as attractive forces between adjacent bilayers only at short distances. Their perturbation with increasing temperature will therefore induce a rather abrupt start of the swelling process. Anomalous volume decreases have been observed before in DSD studies on dipalmitoyl- and distearoyl-PEs. These volume decreases were found only in the first run with samples that had not been exposed to temperatures above T_m. They have been tentatively ascribed to an irreversible breakdown of a voluminous hydrogen bond network between neighboring bilayers (Koynova & Hinz, 1990). It should be emphasized that there is a difference between the reaction of PE and that observed with 18-GlcUA. The volume decrease found with PEs is irreversible, whereas that observed with 18-GlcUA is reversible. The interpretation of both processes as resulting from hydrogen bond dissociation (PE) and reversible formation–dissociation (18-GlcUA) is not rigorously proven but appears to be the best rationale for the experimental results in the absence of additional evidence.

The electrostatic repulsion between charged lipid bilayers is strongly dependent on the pH of the medium and on the electrolyte concentration. Increasing the electrolyte concentration results in effective screening of the charged interfaces, and decreasing the pH values leads to protonation of the lipid head groups and reduction of the surface charge density. It is reasonable to expect therefore that the assumed reversible swelling and shrinking of the lamellar gel phase of ionized 18-GlcUA lipids is limited to a certain range of electrolyte concentrations and pH values. Low enough electrolyte concentrations in combination with high pH may inhibit the shrinking of the bilayers at low temperatures, and high enough electrolyte concentrations and low pH values will suppress the swelling of the phase at high temperatures. Obviously the electrolyte conditions used in the present experiments (about 100 mM at pH 10) are in the range where both swelling and shrinking of the lamellar phase can be observed by increasing or decreasing the temperature, respectively. Another factor of importance for the hypothetical swelling–shrinking phenomenon is the lipid concentration in the sample. At high lipid concentrations swelling will be limited by the amount of free water available in the system, while at low lipid concentrations the shrinking of the bilayers can be kinetically inhibited by the larger interbilayer separations. For the 200 mg/mL samples employed in our X-ray experiments the maximum swelling corresponds to an average interbilayer separation of about 250 Å when one assumes that all interlamellar water layers are of equal thickness.

Although according to the X-ray data the hypothetical swelling–shrinking process is limited to a temperature range of 20 °C or smaller, it is not accompanied by calorimetrically observable anomalies of the specific heat. The absence of visible excess C_p effects could be explained by relatively low cooperativity of the process and also by the rather small enthalpy differences between the states. The process is, however, manifested by the anomalous behavior of the specific volume (Figure 4b). The nature of these anomalies is not clear, but there are some reasons to expect that they arise from restructuring of the lipid–water interface. Since the wide-angle X-ray patterns show a stable alkyl chain packing that is not affected by the hypothetical swelling or shrinking of the lamellar gel phase up to the chain-melting transition, we consider it highly improbable that the specific volume

anomalies might reflect rearrangements and slow density fluctuations in the hydrophobic core of the bilayers. Furthermore, following the analysis of Wiener et al. (1988), which suggests a negligible volume difference between interlamellar and bulk water, it is unlikely that the anomalous behavior of the specific volume results from the uptake or release of water by the lamellar phase. This view is also supported by the fact that in the swollen state the interbilayer separation is so large that no differences between interlamellar and bulk water can be envisaged. Therefore the simplest interpretation for the density of effects is the following: The anomalies seen in Figure 4b reflect transient rearrangements of low cooperativity in the interfacial layers of the membrane. They may indicate that, especially at small interbilayer distances, the conformation and the orientation of the charged head groups are sensitive to changes in the surface potential, to cation adsorption and desorption, and to dissociation and association of the hydrogen bond networks caused by the mutual approach or separation of the bilayers. The final verification of this interpretation could come from X-ray measurements, which can yield an electron-density profile for the bilayer and thereby provide a possibility to separate water layer thickness from the bilayer thickness. The few reflections we can observe do not permit a meaningful calculation of the electron-density profile.

Comparison of the Phase Properties of Glc Lipids and Protonated GlcUA Lipids. It is evident from both calorimetric and X-ray data that the phase behavior of protonated GlcUA lipids in aqueous dispersions is considerably less complex than that of the corresponding dialkylglucosylglycerols. All three lipids (14-, 16-, and 18-GlcUA) are characterized by a single, reproducible chain-melting phase transition. Its temperature and enthalpy display an increase with chain length typical for lipids with saturated hydrocarbon chains. The phase transition in 18-GlcUA has been identified by the X-ray measurements as a lamellar gel to lamellar liquid-crystalline ($L_\beta \rightarrow L_\alpha$) phase transformation. We did not perform such measurements with the shorter chain compounds 14- and 16-GlcUA, but we expect them to show the analogous transitions. It is known from studies on the chain-length dependence of the lipid phase behavior that increase in chain length destabilizes the L_α phase and favors formation of non-bilayer phases as a consequence of an increased hydrophobic/hydrophilic ratio of the lipid molecule. The effect of chain length on the phase behavior of Glc lipids can serve as an appropriate example. The shorter chain lipids 10-, 12-, and 14-Glc display $L_\beta \rightarrow L_\alpha$ transitions, and the longer chain lipids 16- and 18-Glc undergo direct $L_\beta \rightarrow H_{II}$ transformations without an intermediate L_α phase (Hinz et al., 1991; see also Table I). In view of these results it is reasonable to assume that the phase transitions recorded by DSC with the shorter chain compounds 14- and 16-GlcUA also reflect $L_\beta \rightarrow L_\alpha$ transformations. At least under the conditions of the present study, the protonated GlcUA lipids obviously do not form subgel phases, as judged from the reproducibility of the transitions in the first and following heating scans and also from the X-ray data of 18-GlcUA. These properties are quite different from the phase behavior of the corresponding Glc lipids which form a lamellar crystalline phase (L_c) after low-temperature sample preparation and resume it upon incubation at low temperature after heating (Koynova et al., 1988; Hinz et al., 1991).

The most surprising difference between 18-Glc and protonated 18-GlcUA is, however, seen in the chain-melting transitions. These transitions have almost identical thermodynamic parameters: Their enthalpy and volume changes coincide within error margins, and the melting temperature of 18-GlcUA is higher by only 2.8 °C. This amounts to a difference of less than 1% on the absolute temperature scale. Nevertheless, 18-Glc undergoes a direct lamellar gel to inverted hexagonal phase transition ($L_\beta \rightarrow H_{II}$), while 18-GlcUA undergoes a lamellar gel to lamellar liquid-crystalline phase transition ($L_\beta \rightarrow L_\alpha$) (Table I). As mentioned in the introduction, the only chemical difference between these two lipids is the exchange of the 6-hydroxymethyl group in the polar residue of 18-Glc for a 6-carboxyl group in 18-GlcUA (Figure 1). The difference amounts to the replacement of two hydrogen atoms at carbon atom 6 of the sugar ring by a doubly bonded oxygen. This minor change has no significant effect on the thermodynamic properties of the chain-melting transitions, yet it renders the stability of the L_α phase higher than that of the H_{II} phase. It is important to note that this effect cannot be attributed to the different electrolyte conditions, twice-distilled water in the case of 18-Glc and a solution containing sodium, phosphate, and chloride ions in the case of 18-GlcUA. On the contrary, on the basis of the studies of Harlos and Eibl (1981) and Seddon et al. (1983) in which it was demonstrated that increasing NaCl concentrations decrease the stability range of the L_α phase, it should be expected that the increased electrolyte concentration would rather suppress than favor the formation of the L_α phase in 18-GlcUA.

It is instructive to compare the properties of the 18-Glc/18-GlcUA pair with the properties of head-group-modified phospholipids. It is well known from the previous studies that monomethylation of the amine group of PE results in a large increase in the range of existence of the L_α phase at the expense of the H_{II} phase. This is accompanied by a downward shift by several degrees of the preceding $L_\beta \rightarrow L_\alpha$ transition. For example, monomethylation of dielaidoyl-PE results in an upward shift of the $L_\alpha \rightarrow H_{II}$ transition temperature from 63.5 to above 95 °C (where one could no longer measure) and a downward shift of the $L_\beta \rightarrow L_\alpha$ transition from 38.3 to 31.7 °C. These temperature shifts are associated with an enthalpy increase from 9.1 to 11.1 kcal/mol for the $L_\beta \rightarrow L_\alpha$ transition (Brown et al., 1986). Similar results have been obtained for monomethylated dioleoyl-PE (Gruner et al., 1988). In this respect the properties of PE and *N*-methylated PE are somewhat different from the properties of 18-Glc and 18-GlcUA: 18-Glc melts directly into H_{II} but at a slightly lower temperature than protonated 18-GlcUA.

The chain length of 18-Glc is well above the "stability boundary line" which divides between the appearance of L_α and H_{II} phases. The critical chain length is between 14 and 16 for the glycolipids. On the basis of the chain-length dependence one would therefore expect 18-GlcUA to assume an H_{II} phase at elevated temperature. The experiments show, however, that the chain-length effect can be outweighed by a very minor modification of the head group in favor of the L_α phase. This is a clear indication that the energetic balance and the relative stability of the lamellar and nonlamellar liquid-crystalline phases are extremely sensitive to specific contributions from the interfacial region and to small variations in head group–head group and head group–water interactions. Similar conclusions have been reached in a recent article by Mannock et al. (1992). These variations cannot be adequately explained in terms of the geometric shape–structure concept, which considers only the size of the head group in relation to the hydrophobic part of the molecule. Although it is clear that the 6-carboxyl group of GlcUA is more hydrophilic than the 6-hydroxymethyl group of Glc, its marked efficiency as

a bilayer stabilizer cannot be understood on the basis of the presently available data. One hypothetical mechanism is that it prevents interbilayer fusion, which is considered an obligatory step in the formation of an H_{II} phase (Cullis et al., 1980; Siegel, 1986; Ellens et al., 1986). Some support for this mechanism is given by calculations that were carried out to estimate the degree of hydration of the head group following the formalism of Nagle and Wiener (1988). Using the density and X-ray data given in Tables II and III, the average gel-phase hydrations at 20 °C of 18-Glc and 18-GlcUA are 5.3 water molecules per protonated 18-GlcUA molecule and 3.6 water molecules per 18-Glc molecule (Kuttenreich, 1992). Although the reliability of these numbers should not be overestimated because of the rather simplified geometric description of the hydrated bilayer structure involved in the model, they are in accord with the expected higher hydrophilicity of the carboxyl group. They suggest also that the interbilayer separation in the 18-GlcUA gel phase should be larger than that in the 18-Glc gel phase. This difference could be expected to reduce the fusogenic ability of 18-GlcUA bilayers and thus inhibit the formation of the H_{II} phase upon chain melting.

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