Calorimetric and x-ray diffraction studies of rye glucocerebroside mesomorphism

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ABSTRACT Glucocerebrosides (GlcCer) isolated from the leaves of winter rye (*Secale cereale* L. cv Puma) differ from the more commonly investigated natural and synthetic cerebrosides, in that greater than 95% of the fatty acids are saturated and monounsaturated hydroxy fatty acids. Isomers of the trihydroxy long chain base hydroxysphingenine (t18:1$^{8\ cis\ or\ trans}$) and isomers of sphingadienine (d18:2$^{4trans,\ 8\ cis\ or\ trans}$) comprise 77% and 17%, respectively, of the total long chain bases. The phase behavior of fully hydrated and dry rye leaf GlcCer was investigated using differential scanning calorimetry (DSC) and x-ray diffraction. On initial heating, aqueous dispersions of GlcCer exhibit a single endothermic transition at 56°C and have an enthalpy (ΔH) of 46 J/g. Cooling to 0°C is accompanied by a small exothermic transition ($\Delta H = -8$ J/g) at 8°C. On immediate reheating, a broad exothermic transition ($\Delta H = -39$ J/g) is observed between 10 and 20°C in addition to a transition at 56°C. These transitions are not reversible, and the exothermic transition rapidly diminishes when the sample is held at low temperature. Using x-ray diffraction, it was determined that the endotherm at 56°C represents a transition from a highly ordered lamellar crystalline phase (L_c) with a *d*-spacing of 57 Å and a series of wide-angle reflections in the 3–10 Å range, to a lamellar liquid crystalline (L_α) phase having a *d*-spacing of 55 Å and a diffuse wide-angle scattering peak centered at 4.7 Å. Cooling leads to the formation of a metastable gel phase (L_β) with a *d*-spacing of 64.0 Å and a single broad reflection at 4.28 Å. Subsequent warming to above 15°C restores the original L_c phase. Thus, rye GlcCer in excess water exhibit a series of irreversible transitions and gel phase metastability. Dry GlcCer undergo an initial heating endothermic transition at 130°C, which is ascribed to a transformation into the H_{II} phase from a two phase state characterized by the coexistence of phases with disordered (α) and helical (δ) type chain conformations but of unknown lattice identity. An exotherm at 67.5°C observed upon subsequent cooling is of unknown origin. Since an undercooled H_{II} phase persists down to 19°C, the exotherm may derive in part from an α-to-δ type chain packing conformational change especially under slow cooling conditions. Upon reheating from low temperatures to 65°C, a phase with a two-dimensional, primitive rectangular lattice and δ-like chain packing (R_δ phase) in coexistence with the H_{II} phase emerges. With continued heating to 90°C these coexisting phases give way to a phase with a two-dimensional, centered rectangular lattice and δ-like chain packing (P_δ phase) which again coexists with the H_{II} phase. Above 130 °C, the P_δ phase disappears and the sample converts completely to the H_{II} phase as observed upon initial heating. These results indicate that the mesomorphic behavior of rye leaf GlcCer is distinct from that of other cerebrosides.

INTRODUCTION

Cerebrosides and related sphingolipids are derived from *N*-acyl long chain bases. A variety of long chain bases differing in the chain length, the number of hydroxyl groups, and the number, position, and stereochemical configuration of double bonds occur in nature (Karlsson, 1970). To date, the majority of cerebroside species investigated have been derived from mammalian tissue and contain sphingosine (d18:1$^{4\ trans}$) with which is associated an amide-linked, long chain fatty acid. Hydroxy fatty acids are common constituents of cerebrosides from bovine brain myelin and other tissues (see Curatolo, 1982). Non-hydroxy fatty acids are common to glucocerebrosides (GlcCer) from Gaucher's spleen (Correa-Freire et al., 1979) and constitute 40% of acyl chains in bovine brain galactocerebrosides (GalCer) (Curatolo, 1982).

Physical studies of synthetic cerebrosides (Freire et al., 1980; Ruocco et al., 1981; Reed and Shipley, 1987, 1989), GalCer from bovine brain (Bunow, 1979; Curatolo, 1982; Curatolo and Jungalwala, 1985; Fernandez-Bermudez et al., 1977; Hosemann et al., 1979), and GlcCer from Gaucher's spleen (Freire et al., 1980) have demonstrated that these lipids have distinctive physical properties and mesomorphism related to their molecular structure (see Curatolo, 1987a, b). All have high gel to liquid-crystal phase transition temperatures. Those having non-hydroxy fatty acids exhibit complex phase behavior and gel phase metastability, whereas hydroxy fatty acid-containing cerebrosides, with one exception (Curatolo and Jungalwala, 1985), exhibit a simpler mesomorphism. Metastability has also been observed in

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preparations of sphingomyelin containing stearic acid (Barenholz et al., 1976; Estep et al., 1980) but not in those containing palmitic acid or lignoceric acid (Barenholz et al., 1976). Gel phase metastability of cerebrosides and related sphingolipids is characterized by an exothermic transition on heating following cooling from the liquid crystalline phase. X-ray diffraction studies have demonstrated structural changes from a less to a more ordered lamellar phase accompanying the exothermic transition. This behavior has been attributed to a hydration–dehydration process (Ruocco et al., 1981) and to differences in hydrogen bonding (Lee et al., 1986; Pink et al., 1988).

Cerebrosides are found in high concentrations in certain tissues such as brain, kidney, and intestine and, at the subcellular level, are localized in the plasma membrane of animal cells (Karlsson, 1982). Although GlcCer, the predominant glycosphingolipids in plants, are only minor components of plant tissue lipid extracts, analyses of highly purified plasma membrane and tonoplast (vacuolar membrane) preparations have demonstrated that GlcCer are major components of these two membranes (Haschke et al., 1990; Lynch and Steponkus, 1987; Rochester et al., 1987; Sandstrom and Cleland, 1989; Yoshida and Uemura, 1986). Rye plasma membrane GlcCer contain hydroxy fatty acids and are enriched in trihydroxy long chain bases having a *cis* double bond (Cahoon and Lynch, 1990). Although cerebrosides containing trihydroxy bases are common in leaf tissue (Ohnishi et al., 1983, 1985, 1988), trihydroxy long chain base-containing sphingolipids are also prevalent in kidney (Karlsson, et al., 1973), intestine (Dahiya and Brasitus, 1986), and thyrocytes (Bouchon et al., 1987). The primary goal of this study was to determine if the physical properties of rye GlcCer differ from the sphingosine-containing cerebrosides previously investigated. A secondary objective was to gain insight into GlcCer properties in relation to freezing injury and plasma membrane behavior. After cold acclimation of rye plants, a 50% decrease in plasma membrane GlcCer content is observed (Lynch and Steponkus, 1987). Studies of the cryobehavior of liposomes (Steponkus and Lynch, 1989) and protoplasts (Steponkus et al., 1988) have provided circumstantial evidence that GlcCer may play a role in determining plasma membrane cryostability. The results of physical studies employing differential scanning calorimetry (DSC) and x-ray diffraction reported here demonstrate that rye GlcCer exhibit complex thermotropic and lyotropic behavior.

MATERIALS AND METHODS

Isolation and purification of glucocerebrosides. Leaf tissue of winter rye (*Secale cereale* L. cv Puma) was harvested, submersed in liquid nitrogen and ground with a cold mortar and pestle. The resulting powder (100 g) was extracted immediately with 300 ml chloroform: methanol, 1:2 (vol/vol) (Bligh and Dyer, 1959). After allowing the extract to reach room temperature, 100 ml chloroform and 80 ml water were added sequentially while mixing. The extract was filtered through cheesecloth and glass wool before transferring to a separatory funnel. The chloroform phase was subsequently removed and taken to dryness by rotary evaporation.

Aliquots of the lipid extract equivalent to 30–35 g starting material were separated into lipid classes using silica Sep Pak cartridges (Millipore, Bedford, MA) as previously described (Lynch and Steponkus, 1987). The combined glycolipid fractions (eluted with acetone and acetone:acetic acid, 100:1 (vol/vol)) were hydrolyzed in 0.3N NaOH in methanol:chloroform, 1:1 (vol/vol) for 1 h (Kates, 1972) to degrade the sugar-containing glycerolipids. GlcCer and sterylglucoside (SG) were separated from hydrolysis products using a silica Sep Pak cartridge, eluting free fatty acids with 16–20 ml chloroform:acetic acid, 100:1 (vol/vol) and intact glycolipids with 6 ml acetone and 6 ml acetone:acetic acid, 100:1 (vol/vol). The two acetone fractions were combined and GlcCer and SG were then separated by silica Sep Pak chromotography, eluting sequentially with (*a*) 5 ml chloroform:acetic acid, 100:1 (vol/vol); (*b*) 5 ml chloroform:acetone, 80:20 (vol/vol); (*c, d, e*) 5 ml chloroform:acetone, 50:50 (vol/vol); (*f*) 5 ml acetone, and (*g*) 5 ml acetone:acetic acid, 100:1 (vol/vol). Sterylglucosides were eluted in fractions *c*, *d* and *e*. Glucocerebrosides were eluted in fractions *f* and *g*. In some instances fraction *e* contained traces of GlcCer in addition to SG. In these cases the fraction was rechromatographed using the same protocol. The GlcCer fractions thus obtained were checked for purity by thin-layer chromatography in chloroform: methanol:acetic acid:water, 85:15:15:3 (by vol) on silica gel 60 (E. Merck, Darmstadt, Germany). Isolated GlcCer was free of any colored impurities by acetone precipitation (Kates, 1972). The resulting pellet was dissolved in chloroform:methanol, 1:1 (vol/vol) and partitioned against 0.4 vol water. The chloroform phase containing pure GlcCer was used for all subsequent experiments. The yield of GlcCer from 100 g leaf tissue was ~10 mg.

Analysis of glucocerebrosides. An aliquot of GlcCer was hydrolyzed in 0.5 ml 3N methanolic HCl in sealed ampoules at 60°C for 3 h. Hydrolysis products were recovered after addition of 1.5 ml 1N NaOH:methanol, 1:1 (vol/vol) and 1 ml chloroform. Alternatively, the lipid was hydrolyzed in 0.5 ml HCl in aqueous acetonitrile in sealed ampules at 75°C for 3 h. Hydrolysis products were recovered after drying and extraction with chloroform:methanol:water (Bligh and Dyer, 1959). Methyl esters (or fatty acids) and long chain bases were separated by thin-layer chromatography using the same solvent system as given above and recovered from silica gel as previously described (Lynch and Steponkus, 1987). Fatty acids were analyzed as *O*-trimethylsilyl methyl esters (Lynch and Steponkus, 1987) and long chain bases were analyzed as *N*-acetyl-*O*-trimethylsilyl derivatives (Polito et al., 1968) by gas chromatography and coupled GC-MS (Cahoon and Lynch, 1990).

Differential scanning calorimetry. Aliquots of GlcCer (1–4 mg) in chloroform:methanol 6:1 (vol/vol) were dried in small test tubes under N_2 and, subsequently, in vacuo for 2–4 h. The dried lipid was then dispersed in excess water (15–20 μl) by vortexing at 70°C and allowed to equilibrate overnight at 20°C under N_2 before transferring to aluminum DSC volatile-sample pans. For measurements of enthalpies requiring exact lipid weight, aliquots of chloroform:methanol solution containing GlcCer were transferred directly into preweighed sample pans, dried in vacuo, and weighed before adding excess water and sealing. Anhydrous GlcCer samples were prepared by drying lipid samples in preweighed pans in vacuo over P_2O_5 at 90°C for ≥5 h, then sealed under a dry nitrogen atmosphere.

Heating and cooling scans over the range of −20 to 150°C were performed using a Perkin-Elmer DSC-7 calorimeter (Norwalk, CT) equipped with liquid nitrogen cooling. Cyclohexane and indium were used for calibration. Heating and cooling rates ranged from 1 to 30°C/min. Transition temperatures given are the temperatures of peak maxima/minima, in as much as transitions were broad and usually asymmetric. Transition enthalpies were calculated using the computer software program available on the DSC-7.

X-ray diffraction. Aqueous dispersions of GlcCer were prepared in small test tubes by dispersing dried lipids in excess water with vortexing at 70°C and then equilibrating overnight at 20°C under nitrogen before transferring into 1–2-mm quartz capillary tubes. For anhydrous samples, lipid was dried as described above, powdered, and transferred to quartz capillary tubes. Samples were held over P_2O_5 in a nitrogen atmosphere before sealing. X-ray diffraction measurements were performed using synchrotron (Cornell High Energy Synchrotron Source) radiation as previously described (Caffrey, 1985).

The phases encountered in this study included the one-dimensional periodic lamellar (L) phase and two-dimensional periodic inverted hexagonal (H), rectangular centered (P), and rectangular primitive (R) phases. Indexing the various lattices made use $1/d$ the following equations relating the reciprocal spacing (s) of the reflection of indices h, k, and l to the dimensions of the reciprocal unit cell ($1/d$, a^*, b^*) as follows:

lamellar: $s_l = 1/d$

rectangular (primitive): $s_{hk}^2 = h^2a^{*2} + k^2b^{*2}$

rectangular (centered): $s_{hk}^2 = h^2a^{*2} + k^2b^{*2}$ $(h + k = 2n)$

hexagonal: $s_{hk}^2 = a^{*2}(h^2 + k^2 + hk)$.

Where reflections from coexisting phases (lattices) overlap both phases are indicated in the indexing system (Table 1). On a few occasions a reflection could not be indexed to within 2% of the calculated s value. These exceptions are carefully noted in the text.

RESULTS

Analysis of rye GlcCer composition. Of the fatty acids contained in rye leaf GlcCer, >95% were hydroxy fatty acids. Hydroxytetracosenoic acid (C24:1(h)), hydroxytetracosanoic acid (C24:0(h)), hydroxydocosenoic acid (C22:1(h)), hydroxydocosanoic acid (C22:0(h)), hydroxyeicosanoic acid (C20:0(h)), and hydroxyhexadecanoic acid (C16:0(h)) were the major constituents and accounted for 52, 12, 9, 9, 4, and 3%, respectively, of the total fatty acids. High performance liquid chromatography and coupled GC-MS analyses demonstrated that trihydroxy and dihydroxy long chain bases comprised ~80 and 20%, respectively, of the total long chain base composition. The *cis* isomer of hydroxysphingenine (t18:$1^{8\,cis}$) accounted for over 70% of the total long chain bases, whereas the *trans* isomer of t18:1 comprised 6% of the total. The major dihydroxy long chain base constituents were isomers of sphingadienine (d18:$2^{4\,trans,\,8\,cis\ or\ trans}$), which accounted for ~17% of the total. The major form of sphingadienine was d18:$2^{4\,trans,\,8\,cis}$, comprising 14% of the total. Separation of the underivatized intact molecular species by HPLC demonstrated that GlcCer containing hydroxysphingenine paired with hydroxytetracosenoic acid was the predominant species, constituting ~40% of the total species. Although there were more than 30 molecular species of GlcCer, three species, namely C24:0(h), C22:0(h), and C22:1(h) coupled with hydroxysphingenine comprised 9, 7, and 7%, respectively, of the total. GlcCer from whole leaf extracts and plasma membrane extracts were qualitatively and quantitatively very similar. A detailed analysis of rye leaf GlcCer composition is presented in a separate publication (Cahoon and Lynch, 1991).

Differential scanning calorimetry studies of hydrated GlcCer. Dispersions of GlcCer in excess water displayed a broad endothermic transition over the range of 40 to 60°C with a peak maximum (T_m) at 56°C and a transition enthalpy (ΔH) of 46 J/g or ~9.2 kcal/mol (molecular weight assumed, 841; Fig. 1 *A*). Although a single endothermic transition was observed during the initial heating scan, more complex phase behavior was observed during successive heating/cooling scans. After heating above 56°C, the subsequent cooling scan displayed a small ($\Delta H = -5$ J/g) exotherm at ~8°C and a smaller exotherm ($\Delta H = -0.7$ J/g) at ~0°C (Fig. 1 *B*). These two minor exotherms were not entirely resolved. Calculation of enthalpy under the two merged peaks involved redrawing the baseline and resulted in a value of $\Delta H = -8$ J/g. Heating the sample immediately after cooling to −5°C resulted in a minor endotherm and a large exotherm in addition to the major endotherm at 56°C (Fig. 1 *C*). Under normal calorimeter operating conditions, i.e., heating at 10°C/min from −5°C, the minor endotherm occurred at 8°C and the exotherm occurred at ~18°C. The transition enthalpy of the exotherm varied from −30 to −39 J/g. This latter value is roughly equivalent in magnitude but opposite in sign to the sum of the enthalpies of the heating endotherm and the cooling exotherm. Such variability was not related in an obvious way to DSC operating conditions (heating/cooling rate and maximum/minimum temperature), because these were held constant, nor to different GlcCer preparations. It may be attributed in part to ambiguities in defining the baseline between the small endotherm and the exotherm. The respective enthalpies of the high temperature transition were similar for the first and subsequent scans. The thermal behavior exhibited during the second heating cycle was observed for all successive scans of the sample, provided that the cooling/heating regime was not punctuated by isothermal halts and the sample was scanned from below 0°C to above the T_m of 56°C. These results suggest that a metastable phase is formed on cooling and undergoes an exothermic transition to a more stable phase during subsequent heating.

The reversibility of the transitions was examined by

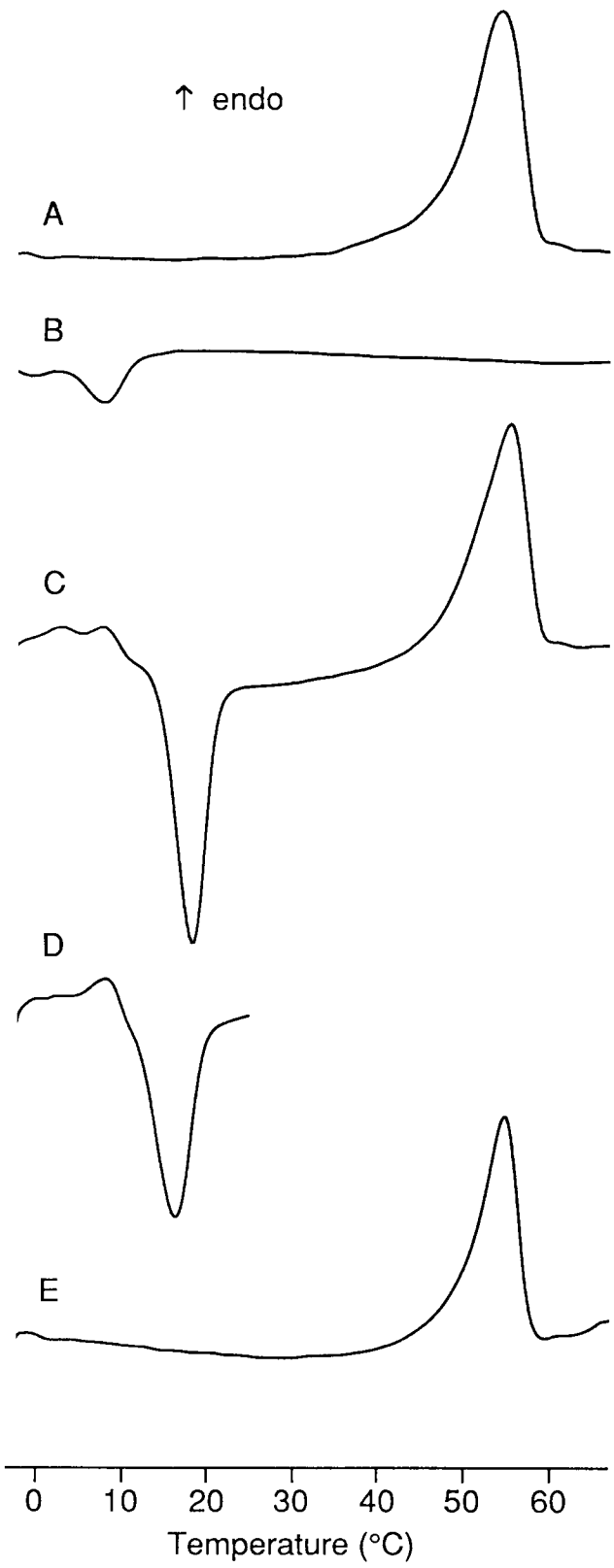
TABLE 1 X-ray diffraction data and phase designation for dry and hydrated rye glucocerebrosides

	Dry									Hydrated				
T (°C)§	↑ 34	↑ 144.5	↓ 29	↑ 64	↑ 90	↑ 121	↑ 146.5	↓ 80	↓ 19	↑ 27.3	↑ 55.7	↓ 0	↑ 125.6	↓ 31
Low-angle Scattering (Å)‖	41.5S	38.1S‡‡	45.0‡S	44.7‡S	62.6*W	59.0M*	37.9S	40.3S	44.8S‡	56.7S	55.0S	63.8S	52.9S	57.3S
	14.2M	21.9W	39.1S	39.1S	39.7S	39.7S	21.7W	37.1W	39.2W	28.1S	27.8M	34.2W	25.6S	29.2W
		19.0W	27.4M‡	27.5M‡	34.4*W§§	32.8M*	18.8W	23.5W	27.4W‡	19.0W	18.5S	21.4W	17.5M	19.3S
		14.4M	23.9M‡	23.7M‡	22.9M	25.4W*	14.3M	20.3W	22.6M‡	14.2M	14.0M	16.0S	13.1W	14.4M
		12.7W	22.6W‡	22.4M‡	19.9M	22.6W	12.6W	15.5M	15.1W	11.3M		12.8W		
			17.6M‡	17.3W‡	14.9S	19.7W*		13.5W	11.4W‡					
			14.6W	14.7M‡	13.0M	14.7S*								
			12.9W‡	12.8W‡	11.1W	13.0M*								
			11.3W‡	11.8W‡	10.9W	11.3W*								
				11.1W‡		10.8W*								
Wide-angle Scattering (Å)¶										9.09W				
										5.09W				
										4.79W				
										4.65W				
										4.53W				
						D5.13S				4.33W				
	4.75S		4.80W	4.80W	4.79S	D4.79S				4.17W				
	B4.51M	D4.7S	D4.51S	D4.5S	D4.54S	D4.54W	D4.74S	D4.5S	D4.51S	3.50W	D4.54S	4.28S	D4.62S	D4.55S
Phase designation	δ + α	H_{II}	$R_\delta + H_{II}$	$R_\delta + H_{II}$	$P_\delta + H_{II}$	$H_{II} + P_\delta$	H_{II}	$H_{II}+t$¶¶	$R_\delta + H_{II}$	L_c	L_α	L_β	L_α	L_α***
d_{001} (Å)										56.7	55.5	64.0	52.2	57.5
$d_{01}H_{II}$ (Å)		38.0	39.1	38.9	39.7‖‖	38.6	37.8	40.6	39.3					
aH_{II} (Å)		43.9	45.1	44.9	45.9	44.6	43.7	46.9	45.4					
a** (Å)			45.0	44.7	125.2‖‖	117.9			44.8					
b** (Å)			27.4	27.5	35.8‖‖	34.1			27.4					

§Measurements made upon sample heating and cooling are indicated by ↑ and ↓, respectively.
‖Intensity ratings are S, strong; M, medium; and W, weak.
¶D, denotes that the peak is diffuse; B, denotes that the peak is broad; all others are sharp.
**Values of a and b refer to the unit cell dimensions of the primitive rectangular (R) or centered rectangular lattice (P) as indicated under Phase designation.
‡‡Underlined reflections index on a hexagonal lattice, ‡denotes reflections indexing on a primitive rectangular lattice, while * indicates reflections indexing on a centered lattice. Some reflections satisfy the indexing criteria of both the hexagonal and the rectangular lattices. As an indication of the reliability of phase assignments we list pairwise the observed and calculated reciprocal lattice coordinates, respectively, for the low-angle reflections of the dry lipid at 64°C as follows: 224, 224; 256, 257; 364, 364; 422, 426; 447, 447R, 445H; 577, 577; 679, 671R, 680H; 781, 763R, 771H; 847, 854; 899, 895 ($\cdot 10^{-4}$ Å^{-1}). R and H denote the R_δ and H_{II} phases, respectively.
§§This reflection appears as a halo around the 39.7 Å reflection.
‖‖The calculated value of a is based on a single reflection (20) at 62.6 Å and the value of b on a single reflection (11) at 34.4 Å.
¶¶t denotes that some other phase is present as evidenced by additional reflections that do not index on the lattice indicated.
***This sample corresponds to the undercooled L_α phase.

performing heating/cooling scans over intermediate temperature ranges. When a dispersion of GlcCer in the metastable phase (cooled from above the T_m to −5°C) was heated to 25°C, thus undergoing an exothermic transition (Fig. 1 *D*), then cooled again to −5°C and subsequently heated again, an exotherm was not detected (Fig. 1 *E*), indicating that the transition is not reversible. The exothermic transition was only observed if the sample was heated above the temperature of the major endotherm before cooling/rewarming. The transition at 56°C was not observed in samples cooled from above 60°C to 30°C and subsequently held for up to 3 h before heating; however, it was observed in samples cooled from above 60°C and held at least 24 h at 25°C (not shown) or cooled below the temperature of the cooling exotherm (to 0°C) before reheating. This behavior is consistent with the notion that a metastable phase is formed from the undercooled liquid crystalline phase which subsequently converts to a stable gel or crystalline phase.

The kinetic behavior of GlcCer dispersions was examined by performing temperature scans at different heating/cooling rates. The transition temperature of the major high temperature endotherm was independent of



heating rate between 3 and 30°C/min (Fig. 2). In contrast, the observed temperatures for the minor endothermic transition and the exothermic transition during heating were dependent on heating rate, suggesting that these transitions are slow, kinetically limited processes. Extrapolating to an infinitely slow heating rate, the minor endothermic transition and the major exothermic transition are predicted to occur at ~3 and 12°C, respectively. In related experiments, slower cooling rates increased slightly the observed temperature of the associated exothermic transition, from 4°C at a cooling rate of 30°C/min to 8°C at a cooling rate of 2°C/min (data not shown). Varying the cooling rate did not alter the enthalpy of the cooling exotherm, nor did it influence the temperature and associated enthalpy of the exothermic transition observed subsequently on heating (data not shown). However, the absolute enthalpy of the exothermic transition observed on heating diminished as a function of time when the sample was held at low temperatures after cooling. Thus, an exotherm was observed upon heating immediately after cooling from above 60°C but no exotherm was observed on a subsequent scan after cooling from above 60°C when the sample was held at 0°C for 60 min before heating.

X-ray diffraction studies of hydrated GlcCer. In order to identify and to structurally characterize the phases associated with the transitions observed using DSC, low- and wide-angle x-ray diffraction measurements were made. To this end, a synchrotron source was used, which provided indispensible in "catching" the shorter lived metastable phase. The results are presented in Figs. 3 and 4 and in Table 1. The low temperature phase observed for fully hydrated GlcCer prepared in the same way as for DSC was identified as being of the lamellar crystalline (L_c) type. It is characterized by a series of sharp, low-angle reflections indexing on a one-dimensional lamellar lattice. The corresponding *d*-spacing at 27.3°C is 56.7 Å. The wide-angle region was replete with weak, sharp and broad reflections in the 3–10 Å region (Table 1, Figs. 3 *A*, 4 *C*, 4 *D*). In the L_c phase, the low- and wide-angle reflections were relatively insensitive to temperature in the 27 to 45°C range although a small, negative thermal lattice coefficient of −0.00035/°C was determined for the lamellar *d*-spacing at 40°C. Raising

FIGURE 1 Differential scanning calorimetry traces of aqueous dispersions of rye leaf glucocerebrosides: (*A*) initial heating scan; (*B*) subsequent cooling scan; (*C*) heating scan immediately following cooling scan; (*D*) heating scan immediately following cooling scan, halted at 25°C; (*E*) subsequent heating scan immediately following cooling of sample from 25°C after heating as in *D* above. For all scans, the heating/cooling rate was 10°C/min.

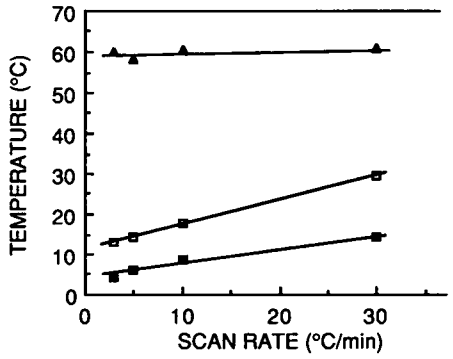


FIGURE 2 Heating rate dependence of the temperatures of the minor endothermic (*closed squares*), exothermic (*open squares*), and major endothermic (*closed triangles*) transitions of fully hydrated rye leaf glucocerebrosides.

the sample temperature to 55.7°C witnessed the complete transformation from the L_c to the lamellar liquid crystalline (L_α) phase. The latter is characterized at 55.7°C by four sharp, low-angle reflections with a lamellar repeat of 55.5 Å and a diffuse band centered at 4.54 Å (Figs. 3 *B*, 4). Thus, the calorimetric endotherm at 56°C in Fig. 1 *A* corresponds to the L_c-to-L_α transition. As observed with the L_c phase, both low- and wide-angle scattering from the L_α phase was relatively insensitive to temperature in the range 56 to 76°C (Fig. 4).

Cooling the sample from 76 to 31°C gave rise to an undercooled L_α phase (Table 1). This phase exhibited four sharp low-angle reflections with a lamellar repeat of 57.5 Å and a diffuse band centered at 4.55 Å. Continued cooling to 0°C produced a metastable lamellar gel phase identified as L_β. The low-angle diffraction pattern from the L_β phase showed five sharp reflections with a *d*-spacing of 64.0 Å, while a single, slightly broadened reflection at 4.28 Å was seen in the wide-angle region of the pattern (Figs. 3 *C*, 4 *B*, 4 *D*, Table 1). It seems likely, therefore, that the exotherm at 8°C in Fig. 1 *B* arises from the L_α-to-L_β transition.

Warming the sample to 4.9°C witnessed the emergence of an unidentified wide-angle reflection at 4.88 Å without much additional change in the L_β pattern compared with that observed at 0°C. However, upon continued warming to 10.3°C, wide- and low-angle reflections characteristic of the original L_c phase became apparent. At 15.1°C the original L_c phase was fully developed without a trace of precurser L_β remaining. This condition persisted up to 40°C (Fig. 4 *B*, 4 *D*). The L_c phase observed in the first and second heating scans were identical, based on their low- and wide-angle powder patterns (Table 1, Figs. 3 *A*, 3 *D*, 4). The results indicate that the exotherm at 18°C in Fig. 1 *C* derives from a metastable L_β-to-L_c transition. Differences in sample thermal history and temperature calibration likely account for the disparity in the metastable L_β-to-L_c transition temperatures observed by x-ray diffraction and DSC.

Continued heating of the sample to above the endothermic transition at 56°C regenerated the L_α phase (Fig. 4 *B*, *D*). This phase persisted up to 140.2°C. In the 75 to 140°C interval the thermal lattice coefficient was −0.00079/°C determined at 105°C (Fig. 4 *B*). The endotherm in Fig. 1 *C* at 56°C is thus ascribable to the L_c-to-L_α transition.

Differential scanning calorimetry studies of dry GlcCer. Dry GlcCer samples exhibited complex thermal behavior and metastability after the first heating/cooling excursion (Fig. 5). The first heating scan contained a major endotherm (ΔH = 12.5 J/g) at 130°C (Fig. 5 *A*). A small exotherm (ΔH = −1.9 J/g) at 67°C was observed on cooling (Fig. 5 *B*). Subsequent heating scans (Fig. 5 *C*) exhibited a minor endotherm ($\Delta H \cong$ 1 J/g) at 40°C that apparently coincided with a change in specific heat of the sample or was preceded by a broad exothermic transition. Continued heating witnessed a second small endotherm (ΔH = 1.9 J/g) at 80°C, an exotherm (ΔH = −11.2 J/g) at 107°C, and an endotherm (ΔH = 12.5 J/g) at 130°C. The enthalpy values of the high temperature endotherm decreased slightly on successive runs, and the temperature of the endotherm was observed to increase by 2–4°C, suggesting perhaps some degradation of the lipid at these high temperatures. The thermal events observed below 130°C were not reversible in that heating to 120°C, cooling to 0°C, then reheating produced a thermogram similar to that observed for a first scan, with a single endotherm at 130°C.

X-ray diffraction studies of dry GlcCer. X-ray diffraction measurements were made on dry GlcCer which had not been previously heated. At 34°C an insufficient number of low-angle reflections were observed for an unambiguous phase determination (Table 1). The wide-angle region consisted of a sharp reflection at 4.74 Å with what appeared as a broad, wide-angle shoulder at 4.51 Å. The 4.51 Å peak is indicative of disordered α chains, while the sharp 4.75 Å reflection is reminiscent of the δ or helical chain conformation with chains packed on a square, two-dimensional lattice as described by Tardieu et al. (1973). Upon raising the temperature to 144.5°C the sample converted completely to the hexagonal (H_{II}) phase. At this temperature, the wide-angle region of the pattern consisted of a diffuse band at 4.5 Å, while the low-angle region contained five sharp reflections indexing on a hexagonal lattice with an a value of 43.9 Å.

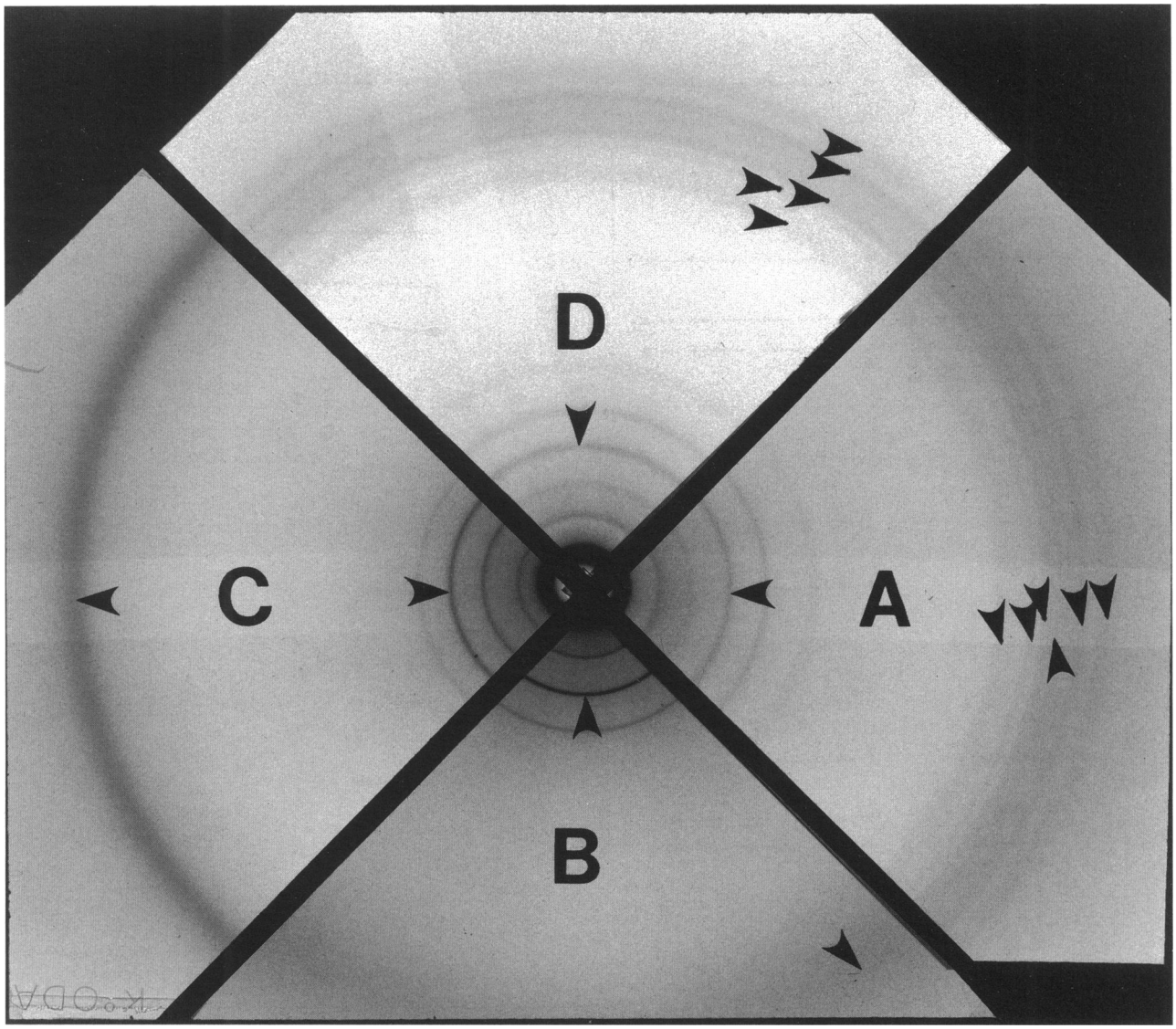


FIGURE 3 X-ray diffraction patterns of the lamellar phases observed with rye leaf glucocerebrosides in the presence of excess water: (*A*) lamellar crystalline (L_c) phase at 27.3°C, arrowed reflections are located at 14.2, 5.09, 4.79, 4.65, 4.53, 4.33, and 4.17 Å. This sample had not been previously heated; (*B*) lamellar liquid crystalline (L_α) phase at 55.7°C, arrowed reflection is located at 18.5 Å with a diffuse band centered at 4.54 Å; (*C*) lamellar gel (L_β) phase at 0°C, arrowed reflections are located at 16.0 and 4.28 Å; and (*D*) lamellar crystalline (L_c) phase at 40°C derived from the metastable L_β phase, arrowed reflections are located at 14.2, 5.08, 4.79, 4.65, 4.35, and 4.17 Å. Patterns were recorded on x-ray sensitive film at a sample-to-film distance of 9 cm using 1.559 Å x-rays and a 0.3 mm diameter collimator.

Cooling the sample to 29°C at a rate of ~20°C/min and subsequent incubation at 29°C for 20 min gave rise to a low-angle diffraction pattern, which can be indexed on a two-dimensional, primitive rectangular lattice (R) in coexistence with a hexagonal lattice. The wide-angle region consisted of a diffuse band at 4.51 Å and a faint but sharp reflection at 4.8 Å. The latter indicates chain packing on a square lattice and suggests that one of the phases is a primitive rectangular phase with helical chain conformation (R_δ) while the other is H_{II}. At 29°C, the lattice parameters of the R_δ phase were a = 45.0 Å and b = 27.4 Å, while the a value for the H_{II} phase was 45.1 Å (Table 1).

In the second heating cycle, diffraction measurements were made at 64, 90, 121, and 146.5°C. Heating the sample to 64°C did not dramatically alter the phase behavior compared with the condition at 29°C (Table 1). Further heating effected a change in the rectangular

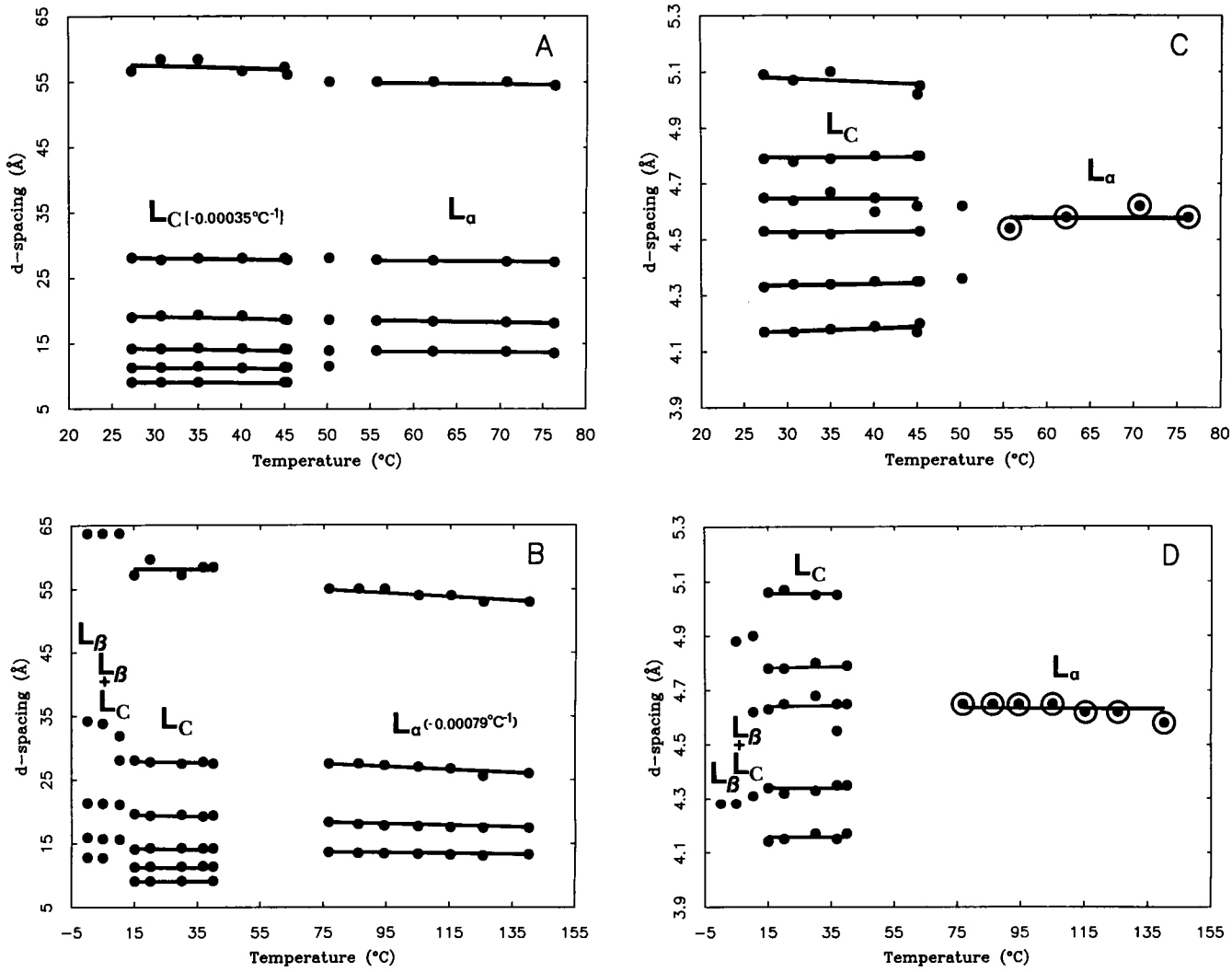


FIGURE 4 Changes in the x-ray diffraction patterns from rye leaf glucocerebrosides in the presence of excess water during the course of a temperature scan experiment. The *d*-spacing of the low-angle (*A*, *B*) and wide-angle (*C*, *D*) reflections are shown as a function of temperature during the first (*A*, *C*) and second (*B*, *D*) heating scans. Single phase and phase coexistence regions are indicated. The broad, diffuse nature of the scattering in the wide-angle region of the L_α phase is indicated by the encircled data points in *C* and *D*. The values in parenthesis correspond to thermal lattice coefficients $[(1/d)\partial d/\partial T]$ calculated from the slopes of the lines at 40°C (L_c) and 105°C (L_α).

phase from R_δ to a centered rectangular phase with helical chain conformation (P_δ), as indicated by the requirement for the centered lattice that $h + k = 2n$ (Table 1). The lattice parameters of the P_δ at 90°C were a = 125.2 Å and b = 35.8 Å. A sharp line at 4.8 Å accompanied the P_δ pattern. The P_δ phase at 90°C remained in coexistence with the H_{II} phase whose lattice parameter was similar to that seen at the lower temperatures. Continued heating to 122 °C did not alter the phase behavior of the lipid, although the relative amounts of the coexisting phases did change in the 90–120°C temperature interval. Further, the lattice parameters of the coexisting P_δ and H_{II} phases did not change dramatically in this range. When sample temperature was adjusted to 146.5°C the P_δ phase disappeared and the only phase in evidence was H_{II}, with an a value slightly reduced compared with that at low temperatures (Table 1).

Cooling the sample from 151°C at a rate of 20°C/min to 80°C give rise to an undercooled H_{II} phase. The pattern contained, in addition to the hexagonal lines, a faint line at 37.1 Å which could not be indexed on the hexagonal lattice. Further rapid cooling to 19°C witnessed the emergence of a primitive rectangular phase which coexisted with the undercooled H_{II} phase. The wide-angle region of the diffraction pattern did not

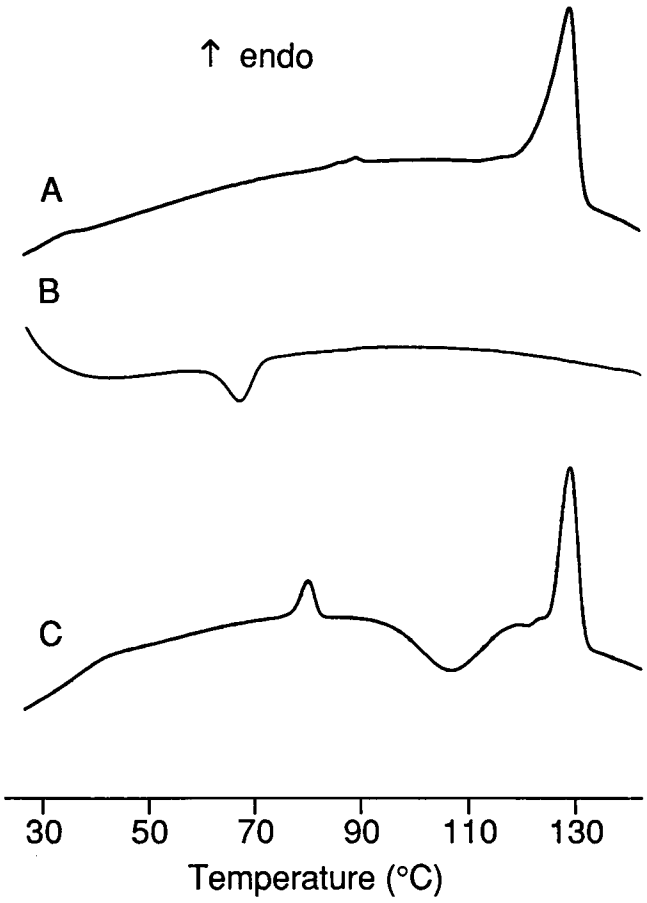


FIGURE 5 Differential scanning calorimetry traces of dry rye leaf glucocerebrosides: (*A*) initial heating scan; (*B*) subsequent cooling scan; (*C*) heating scan immediately following cooling scan. For all scans, the heating/cooling rate was 20°C/min.

contain a sharp reflection in the vicinity of 4.8 Å but rather a diffuse band at 4.5 Å, suggestive of a primitive rectangular phase with disordered chains (R_α).

DISCUSSION

Phase properties of hydrated GlcCer. A summary of the thermotropic phase behavior of hydrated GlcCer as determined by DSC and x-ray diffraction follows. Before being heated the lipid in excess water exists at 27°C in the L_c phase with a lamellar periodicity of 56.7 Å. The crystalline designation comes from the presence of multiple sharp reflections in the wide-angle region of the diffraction pattern in the range 3–10 Å. Upon heating to 56°C the lipid undergoes an endothermic transition to the L_α phase with a lamellar *d*-spacing of 55 Å and a diffuse wide-angle scattering peak centered at 4.54 Å. This phase persists up to a temperature of 126°C. Cooling the sample to 0°C from above the 56°C endotherm gives rise to a metastable L_β phase having a lamellar *d*-spacing of 64 Å and a single wide-angle reflection at 4.28 Å. The L_α-to-metastable L_β transition is evidenced by a small exotherm at 8°C. Reheating the sample gives rise to an exotherm between 10 and 20°C which derives from the metastable L_β-to-L_c phase transformation. This exotherm restores the original L_c phase. The degree of hydration of the three lamellar phases encountered in this study was not determined.

Phase properties of dry GlcCer. The wide-angle diffraction pattern of previously unheated, dry GlcCer recorded at 34°C suggests that the lipid exists in a phase with helical or δ type chain packing alongside a phase with disordered or α type chains. The long-range order of either phase could not be established because of a paucity of reflections in the low-angle region of the pattern. The first heating endotherm for the dry lipid at 130°C was accompanied by the emergence of a pure H_{II} with a lattice parameter (*a*) of 43.9 Å and a diffuse, scattering peak centered at 4.74 Å when recorded at 144.5°C. Upon cooling the sample, the H_{II} phase undercools down to 21°C. However, with slower cooling phase coexistence is observed. For example, at 19°C the H_{II} phase and a phase with a primitive, rectangular lattice (R) is present. Initially, the chains of the rectangular phase are disordered (R_α). However, upon incubation, a sharp reflection at 4.8 Å appears indicating a solidification of the fluid chains into the δ conformation (R_δ). Reheating the sample gives rise to a small endotherm at 80°C tentatively attributed to the R-to-P_δ transition, which under present conditions takes place with both rectangular phases in coexistence with the H_{II} phase. The high temperature endotherm at 130°C coincides with the loss of the P_δ phase and the complete conversion of the sample to the H_{II} phase as was observed during the final stage of the initial heating scan.

A perusal of the data in Table 1 and Figs. 1 and 5 indicates that the presence of water profoundly affects the thermotropic properties of GlcCer and points to its lyotropic character. In excess water three lamellar phases, L_c, L_β, and L_α, appear in the temperature range examined. In contrast, in the "anhydrous" state over a similar temperature range a lamellar phase was not in evidence, while mesophases with two-dimensional periodicity, namely, the R_δ, P_δ and H_{II} phases, dominated. The H_{II} and P_δ phases are common features in the low hydration region of lipid/water phase diagrams (Tardieu et al., 1973; Seddon, 1990). The R_δ phase, on the other hand, is a rarity. Like the H_{II} and P_δ phases it tends to be stabilized in the high temperature, low hydration region of polar lipid/water phase diagrams (Luzzati, 1968; Tardieu et al., 1973; Hogan, 1989). Interestingly, neither

of these two-dimensionally periodic phases has been described previously for cerebrosides (Abrahamsson et al., 1972; Pascher and Sundell, 1977; Reiss-Husson, 1967).

Comparison with other cerebrosides. The general scheme of rye GlcCer phase behavior in excess water is grossly similar to that for GlcCer from Gaucher's spleen (Freire et al., 1980), nonhydroxy fatty acid-containing bovine brain cerebrosides (Curatolo, 1982), and synthetic *N*-palmitoyl galactosylsphingosine (Ruocco et al., 1981). However, comparison of the results from the present and previous calorimetry and x-ray diffraction studies indicate that the phase behavior of GlcCer isolated from rye tissue differs in several respects from that of other cerebrosides investigated to date. These differences are discussed below.

The temperature of the major endothermic transition at 56°C is the lowest of any naturally-occurring cerebroside. The enthalpy of the major endotherm of rye GlcCer is intermediate between that of cerebrosides forming a highly ordered stable gel phase, e.g., GlcCer from Gaucher's spleen (Freire et al., 1980) and that of cerebrosides which exhibit more typical phase behavior, e.g., unfractionated bovine cerebrosides (Curatolo, 1982). It was concluded from studies of fractionated bovine GalCer that the presence of hydroxy fatty acids prevents the formation of the L_c phase (Curatolo, 1982). Only hydroxytetracosanoic acid-containing GalCer exhibits gel-phase metastability and a major endotherm with a high transition enthalpy, suggesting that the long acyl chain may overcome disruptive effects of the hydroxyl group, thus decreasing the kinetic barrier to reaching a stable, highly ordered phase (Curatolo and Jungalwala, 1985). The results presented here for rye GlcCer containing predominantly hydroxy fatty acids are not entirely consistent with these previous suggestions, in that the stable L_c phase formed readily, with the conversion from the metastable L_β phase occurring within 15–60 min at 0°C.

Comparison of the thermodynamic parameters (transition temperatures and enthalpies) of rye GlcCer with those of other cerebrosides that exhibit gel phase metastability reveals some unique characteristics of the phase behavior of the plant sphingolipid (Table 2): whereas the heating endotherm and heating exotherm of rye GlcCer are ~30°C lower than the corresponding transitions of other cerebrosides, the cooling exotherm of rye GlcCer is over 50°C lower than those of other cerebrosides. For rye GlcCer, the heating exotherm occurs at a higher temperature than the cooling exotherm. In contrast, the heating exotherms occur at lower temperatures than the cooling exotherms of the other cerebrosides, and different cooling rates alter the cooling/heating exotherms. Differences in the relative magnitudes of the respective transition enthalpies also exist. Whereas the enthalpies of the heating and cooling exotherms of other cerebrosides are roughly equivalent, the enthalpy of the heating exotherm of the rye GlcCer is much greater than that of the cooling exotherm. The respective enthalpies of the cooling exotherms for nonhydroxy fatty acid-containing cerebrosides are of sufficient magnitude to account for the ordering of the hydrocarbon chains (Freire et al., 1980; Ruocco et al., 1981). In contrast, the enthalpy of the cooling exotherm for rye GlcCer is relatively small (−1.6 kcal/mol) and so apparently does not represent a recrystallization of hydrocarbon chains. Consistent with this is the x-ray diffraction data that demonstrate a wide-angle reflection at 4.28 Å for the metastable L_β phase, not indicative of highly ordered chain packing. In contrast, the metastable "gel" phase of synthetic *N*-palmitoyl-galactosylsphingosine is characterized by wide-angle reflections at 4.55 and 4.16 Å and the stable L_c phase by reflections at 4.6 and 4.1 Å, indicating highly ordered chain packing in both phases (Ruocco et al., 1981). In this study, the magnitude of the heating exotherm and the x-ray diffraction data indicate that crystallization of the hydrocarbon chains of rye GlcCer occurs during warming. Hence, it appears that the nature of the metastable phase differs from that of previously characterized cerebrosides.

The biological consequences of L_c phase-forming lipids in membranes are not understood. Ignoring interactions with other membrane lipid constituents that may modulate cerebroside behavior, it is possible that GlcCer exist in the thermodynamically favorable L_c phase under

TABLE 2 Comparison of calorimetric parameters (transition temperature and enthalpy) of hydrated cerebrosides which exhibit metastability

Cerebroside	Heating endotherm		Cooling exotherm		Heating exotherm	
	T (°C)	Δ*H* (*kcal/mol*)	*T* (°C)	Δ*H* (*kcal/mol*)	*T* (°C)	Δ*H* (*kcal/mol*)
Gaucher's Spleen*	83	13.6	63	−7.1	61	−6.3
NPGS‡	82	17.5	62	−8.5	51	−8.0
18:0 NFA-CER§	83	9.7	61	−4.9	55	−3.6
Rye GlcCer	56	9.2	8	−1.6	20	−7.6

*Freire et al., 1980. ‡Ruocco et al., 1981. §Curatolo and Jungalwala, 1985.

in vivo conditions. Although Curatolo (1982) has proposed that the presence of hydroxy fatty acids in GalCer of myelin may serve to prevent crystallization, i.e., formation of L_c phase, it is not clear that fatty acid hydroxylation is a requirement for functional myelin (see Curatolo, 1987b). Given that increased hydroxylation is thought to be associated with increased membrane stability and decreased permeability (Karlsson, 1982), it is possible that the high proportions of hydroxy fatty acids and trihydroxy long chain bases in GlcCer of rye and other plants (Ohnishi et al., 1983, 1985, 1988) contribute to the overall integrity of the plasma membrane and tonoplast.

Preliminary DSC studies indicate that fully hydrated, HPLC-purified species of GlcCer containing the *cis* isomer of t18:1 and C24:1(h), C22:1(h), C24:0(h), or C22:0(h) also exhibit metastability (Cahoon, 1990). That is, during cooling from the liquid crystalline phase, the purified lipids exhibit undercooling and, subsequently, exothermic transitions during reheating. Species of GlcCer containing C_{16} to C_{24} saturated hydroxy fatty acids and d18:1, d18:2, or t18:1 long chain base isomers exhibited markedly different behavior during cooling, depending on the configuration (*cis* and *trans*) of the double bond at the C-8 of the long chain base (Ohnishi et al., 1988): whereas the cooling exotherms for purified GlcCer species containing a *trans* double bond occur at ~60°C, the species containing a *cis* double bond have cooling exotherms in the range of 18 to 38°C, the specific temperature being a function of fatty acid chain length. Note that only cooling DSC scans were reported, so a complete picture of the possible metastable behavior is lacking.

The biological relevance of the presence of *cis* double bonds in the long chain base of GlcCer requires further study. Of the plant species examined to date, only the cold hardy cereals, rye and wheat, contain predominantly (≥90%) *cis* isomers of t18:1, and a relationship between chilling sensitivity and increasing proportions of *trans* isomers of unsaturated long chain bases has been proposed, based on limited DSC studies of purified GlcCer species (Ohnishi et al., 1988). Although these correlative approaches do not provide an understanding of the role played by the various lipid species in membrane stability, it is apparent that the presence of the *cis* double bond in the long chain base allows for modification of the mesomorphic behavior (at least in regard to undercooling ability) of the parent GlcCer simply by altering the fatty acid moiety. Thus, the physical properties of GlcCer with *cis* unsaturated long chain bases, as found in rye, may be adjusted through metabolic or enzymatic changes in the fatty acyl moiety of the lipid. The ability to modulate membrane lipid properties is thought to be important in plants and other organisms subject to wide variations in environmental conditions. Testing this hypothesis will require further comparative biochemical studies of plant GlcCer and detailed physical studies of individual GlcCer species purified from natural sources.

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