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Cerebrosides alter the lyotropic and thermotropic phase transitions of DOPE:DOPC and DOPE:DOPC:sterol mixtures

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Abstract

Freezing injury in rye and oat is a consequence of the formation of the inverted hexagonal (H_{II}) phase in regions where the plasma membrane is brought into close proximity with cytoplasmic membranes during freeze-induced dehydration. Susceptibility to plasma membrane destabilization and H_{II} phase formation during freezing is associated with alterations in plasma membrane lipid composition. This paper examines the influence of lipid composition and hydration on the propensity of lipid mixtures of DOPE:DOPC and DOPE:DOPC:sterols with added cerebrosides (CER) to form the H_{II} phase during dehydration. The addition of CER to DOPE:DOPC:β-sitosterol mixtures decreased the water content of the dispersions in a manner suggesting that most or all of the water in the dehydrated mixtures was associated with the phospholipids. The addition of CER significantly decreased the osmotic pressure at which the $L_\alpha \rightarrow H_{II}$ phase transition occurred from an osmotic pressure of 76.1 MPa for DOPE:DOPC (50:50) to 20 MPa in DOPE:DOPC:β-sitosterol:CER (22.5:22.5:50:5) and 8 MPa in DOPE:DOPC:β-sitosterol:CER (15:15:50:20). Experiments examining the effects of CER on the thermally-induced formation of the H_{II} phase in fully hydrated mixtures and examining the influence of CER on the formation of the H_{II} phase in DOPE:DOPC mixtures lacking β-sitosterol suggested that CER facilitated the $L_\alpha \rightarrow H_{II}$ phase transition by effecting a decrease in bilayer hydration and by increased lateral packing pressures within the acyl domain of the bilayer. Taken in sum, these data indicate that the differential propensity of the rye and oat plasma membranes to undergo freeze-induced formation of the $L_\alpha \rightarrow H_{II}$ phase cannot be attributed to one lipid species. Rather, the propensity towards freeze-induced membrane destabilization is a consequence of the summation of physical characteristics of the membrane lipid components that included bilayer hydration, packing pressures within the hydrophobic domain of the membrane, the propensity of the lipid components to demix, and the relative proportions of the various lipid components.

Keywords: Hexagonal II phase; Phase transition; Freezing; Dehydration; Differential scanning calorimetry; X-ray diffraction

Abbreviations: CER, rye cerebrosides; DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine

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1. Introduction

Freezing injury in winter annuals, such as rye and oat, is primarily a consequence of membrane destabilization resulting from freeze-induced dehydration rather than exposure to low temperature per se [1]. In the leaves and protoplasts of both winter rye and spring oat, the ultrastructural manifestations of freezing injury include the appearance of particulate domains in the plasma membrane, particulate lamellae subtending the plasma membrane and by the occurrence of the inverted hexagonal (H_{II}) phase in regions where the plasma membrane is brought into close apposition with subtending endomembranes [1]. The incidence of the H_{II} phase correlates with lethal injury to both protoplasts and leaf tissue as indicated by loss of osmotic responsiveness of protoplasts and leakage of the intracellular contents of leaves [2].

The temperature-dependence for the onset of the freeze-induced formation of the H_{II} phase is significantly different in protoplasts of winter rye and of spring oat ($-6°$ and -3°C, respectively) and is associated with vast differences in the lipid composition of the plasma membrane [3]. The plasma membrane lipids of winter rye are comprised of the phospholipids phosphatidylcholine (PC) and phosphatidylethanolamine (PE) (37 mol% of the total lipid), cerebrosides (CER; primarily 1-*O*-*β*-D-glucopyranosyl-*N*-2-hydroxynervonoyl-4-hydroxy-8-*cis*-phingenine; see Ref. [3] for chemical structures) (16 mol%) and free sterols (38 mol%), steryl glycosides (6 mol%) and acylated sterylglycosides (3 mol%). In contrast, the plasma membrane of spring oat contains significantly lower proportions of the phospholipids and free sterols (29 mol% and 8 mol% of the total lipid, respectively), and considerably greater proportions of the cerebrosides (27 mol%) and the acylated sterylglycosides (27 mol%). In both winter rye and spring oat, freeze-induced formation of the H_{II} phase does not occur after cold acclimation [4]. Cold acclimation also results in alterations in the lipid composition of the plasma membrane [5]. Specifically, the lipid composition of the plasma membrane of winter rye and of spring oat is altered to include higher proportions of the phospholipids and lower proportions of the cerebrosides, sterylglycosides and acylated sterylglycosides [5]. These, and other [3,6–8] studies indicate that both genotypic and environmentally-induced alterations in the plasma membranes of winter rye and spring oat that increase the proportions of the phospholipids PC and PE, and simultaneously decrease the proportions of the free sterols, cerebrosides and acylated sterylglycosides, act to ameliorate the occurrence of the H_{II} phase during freezing.

Our efforts have focused on the characterization of the dehydration-induced (lyotropic) and thermally-induced (thermotropic) phase behavior of bilayers containing the lipids that comprise the plasma membranes of rye and oat. Our intent is to determine which lipid species facilitate, or alleviate, the $L_\alpha \rightarrow H_{II}$ phase transition during freezing-induced dehydration. In previous studies, we demonstrated that the $L_\alpha \rightarrow H_{II}$ phase transition in 1:1 mixtures of dioleoyl-PE (DOPE) and dioleoyl-PC (DOPC) occurred at 6–7 wt% water at 20°C [9]. X-ray diffraction data showed that fluid-fluid phase demixing of highly-hydrated DOPC from poorly-hydrated DOPE occurred during dehydration [9]. Further, these studies suggested that the DOPE-enriched lamellar domain preferentially underwent the lyotropic $L_\alpha \rightarrow H_{II}$ phase transition. In subsequent studies [10], it was demonstrated that the addition of free plant sterols to DOPE:DOPC (1:1) mixtures lowered the hydration of the lipid mixture and facilitated both the lyotropic and thermotropic transitions from the lamellar to the hexagonal II phase. In the present study, we have examined the lyotropic phase behavior of dispersions of DOPE and DOPC that also contain various proportions of plant sterols and rye cerebrosides. The experimental objectives of this study were to determine the effects of the addition of rye cerebrosides to mixtures of DOPE:DOPC and of DOPE:DOPC:sterols on: (1) bilayer hydration, (2) the lyotropic increase of the $L_\beta \rightarrow L_\alpha$ phase transition temperature (T_m), (3) fluid-fluid phase separation, and (4) the propensity of the lipid mixtures to undergo the lyotropic and thermotropic $L_\alpha \rightarrow H_{II}$ phase transition.

2. Materials and methods

2.1. Materials

DOPE, DOPC and DPPC were obtained from Avanti Polar Lipids and were used without further

purification. Plant sterols, purified from soybeans, were obtained from Sigma Chemical Co. and were composed primarily of β-sitosterol and campesterol, which are the two predominant free sterols in the plasma membrane of rye (64% β-sitosterol, 27% campesterol) [11] and will be referred to as β-sitosterol in this report. Cerebrosides (CER) were purified from the leaves of non-acclimated rye as described previously [11]. All lipids were $>98\%$ pure based on analytical chromatography of the lipids in $CHCl_3$:CH_3OH:H_2O (65:25:4, v:v:v). Butyl-Sepharose 4B was obtained from Pharmacia. All water used in this study was double-distilled and de-ionized and was degassed under vacuum then saturated with nitrogen gas before lipid dispersal.

2.2. Lipid dispersal and desorption

Lipids and lipid mixtures were dissolved in $CHCl_3$ at the indicated mol ratios; the solvent was then removed under a stream of N_2 at 40°C and then trace solvent removed under high vacuum for 16 h. Lipids were dispersed at 85 wt% in water under N_2 gas. Hydration of the lipid mixtures was achieved by vortexing and mild sonication in a bath sonicator as well as with thermal cycling between $-196°$ and 20°C. Pure DPPC or cerebrosides dispersions were hydrated in water by extensive vortexing and mild sonication and by brief heating to 50° or 70°C respectively. All dispersions were allowed to equilibrate under N_2 at 4°C for 16 h before the experiments were started.

Lipids were desorbed for hydration measurements and differential scanning calorimetry (DSC) as described previously [3,9]. Briefly, aliquots containing 1–2 mg of lipid were placed in pre-weighed DSC pans and were allowed to equilibrate in an N_2 atmosphere above saturated salt solutions for 7 days at 20°C. After equilibration, the pans were sealed and re-weighed; after the DSC studies the pans were re-opened and desiccated over P_2O_5 at 70°C under vacuum for determination of the exact lipid weight. The saturated salt solutions were: Na_2HPO_4, KNO_3, $BaCl_2$, KCl, NaCl, NaBr, $Mg(NO_3)_2$, $MgCl_2$ and LiCl. At 20°C these solutions had osmotic pressures of 2.8, 8.3, 12.8, 20.4, 38.9, 76.1, 88.5, 150 and 286 MPa, respectively [3,9].

Lipid dispersions for freeze-fracture electron microscopy were either prepared as described above and concentrated by centrifugation or were dispersed at a starting water content of approx. 50 wt%. Aliquots were loaded on freeze-fracture electron microscopy stubs and desorbed as described above and as described in Refs. [9,10]. Mixtures prepared for analysis by X-ray diffraction were dispersed at 85 wt% water and desorbed over saturated salt solutions as described above except that the samples were equilibrated for 10 days at 20°C in 1.5 ml conical Eppendorf centrifuge tubes.

2.3. Differential scanning calorimetry (DSC)

Calorimetric analysis of the lipid dispersions was performed on a Perkin-Elmer DSC7 instrument. Samples were inserted into the DSC7 instrument at 20°C then data was collected continuously during cooling from 20° to −50°C, then during heating from −50°C to 80°C, re-cooling from 80° to −50°C, and finally during heating from −50° to 20°C, all at $10°C \cdot min^{-1}$. Results obtained during the first and second heating scans, and during the first and second cooling scans, were identical. Transition temperatures (T_m) were calculated from the first heating scan using DSC7 software. For dehydrated lipid dispersions having water contents below 5 wt%, this DSC procedure does not yield accurate estimates of the phase transition temperature, as described previously [9], and consequently these are not reported.

2.4. Freeze fracture electron microscopy

Desorbed lipid dispersions were quenched from 20°C by plunging into liquid propane cooled by liquid nitrogen. Fracture and replication were performed using either a Balzers 360 freeze-fracture device at −102°C and $<2 \cdot 10^{-6}$ torr or using a Balzers 400K device at −107°C and $<1 \cdot 10^{-7}$ torr. Replicas were washed in either $CHCl_3$:CH_3OH (1:2, v:v) or in concentrated sulfuric acid, then examined in a Philips EM 300 electron microscope.

2.5. X-ray diffraction

Desorbed lipid dispersions were rapidly transferred to 1.0 or 1.5 mm (i.d.) quartz capillary tubes as described previously [10]. X-ray diffraction powder

patterns were obtained using the crystallography bench camera on the A1 station at the Cornell High Energy Synchrotron Source (CHESS). Wiggler-enhanced monochromatic radiation ($\lambda = 0.156$ nm) was passed through a 0.3-mm collimator with a flux of approx. $2 \cdot 10^{11}$ photons $\cdot s^{-1}$. Powder patterns were recorded on a stack of three or four Kodak DEF-5 films placed at a carefully measured specimen-to-film distance (usually around 12 cm) using exposure times of 20 s to 1 min. Desorbed lipid dispersions were individually loaded into X-ray capillaries and examined individually such that the total time from the end of the sample equilibration period to the completion of film exposure was less than 3 min. Hydrated lipid dispersions were loaded into X-ray capillaries and then several capillaries were simultaneously mounted on the bench camera for those experiments examining the phase behavior as a function of temperature. For experiments at different temperatures, the sample temperatures were changed in 5°C increments and the dispersions were allowed to equilibrate for at least 5 min at each temperature before either recording the powder pattern or changing to the next temperature. Sample temperature was determined with a thermocouple placed outside the capillary in a position immediately adjacent to the position of the sample. For all diffraction experiments, the sample temperature was maintained (± 1°C) using an FTS air-jet cooling system. Those samples subjected to repeated exposure to the X-ray beam were moved slightly between exposures to minimize radiation-induced damage. The lamellar and H_{II} phases were identified by small angle reflections with reciprocal spacing ratios of 1:2:3... or 1:$\sqrt{3}$:2:$\sqrt{7}$:3... respectively from the first 3–5 diffraction orders.

3. Results

3.1. Hydration characteristics of lipid mixtures

The water contents of the lipid mixtures as a function of osmotic pressure in the vapor phase are shown in Fig. 1A. In all of the mixtures, the water content decreased linearly with the log of the osmotic pressure. At all examined osmotic pressures, the DOPE:DOPC (50:50) mixture had the highest water content. The total hydration of the lipid dispersions was decreased by the addition of rye CER. For example, at an osmotic pressure of 10 MPa, the interpolated water contents of the DOPE:DOPC (50:50), DOPE:DOPC:β-sitosterol (25:25:50), as well as of DOPE:DOPC:β-sitosterol:CER (22.5:22.5:50:5 and 15:15:50:20) mixtures were 14.4, 10.8, 10.7 and 6.82 wt% respectively.

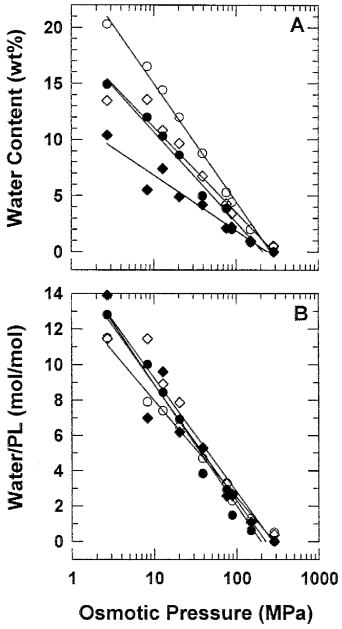

Fig. 1. Plot of water content as wt% water (A) or water/phospholipid molar ratio (B) vs. osmotic pressure for dispersions of DOPE:DOPC, 50:50 (○); DOPE:DOPC:β-sitosterol, 25:25:50 (◇); DOPE:DOPC:β-sitosterol:CER, 22.5:22.5:50:5 (●); and DOPE:DOPC:β-sitosterol:CER, 15:15:50:20 (♦) dehydrated at 20°C. Data for DOPE:DOPC 50:50 from Ref. [9], data for DOPE:DOPC:sterols 25:25:50 from Ref. [10] for comparison. Lines represent linear regressions.

When the water content of the lipid dispersions was normalized to the quantity of the phospholipid (DOPE + DOPC), there was no significant differences in the molar water/phospholipid ratio between DOPE:DOPC (50:50) and those mixtures containing DOPE, DOPC, sterols and 5 mol% or 20 mol% of CER (Fig. 1B). These results suggest that the water present in the desorbed lipid mixtures was associated predominantly with the phospholipids DOPE and DOPC. That is, if it is assumed that the hydration of β-sitosterol and cerebrosides in the bilayer was negligible and that the sterols and cerebrosides did not alter phospholipid hydration, then the amount of water present in these dehydrated mixtures could be

accounted for entirely by the hydration of the phospholipids. Decreased bilayer hydrations due to the presence of both β-sitosterol and CER are consistent with the low hydrations observed for both the free sterols and the cerebrosides purified from rye leaves (Lynch, D.V. and Steponkus, P.L., unpublished results).

3.2. Dehydration-induced $L_\beta \rightarrow L_\alpha$ phase transitions

The temperature of the $L_\beta \rightarrow L_\alpha$ phase transition (T_m) of lipid mixtures at full hydration was determined by DSC. The T_m of DOPE:DOPC (50:50) dispersions in excess water was −11°C [9]; addition of 50 mol% of β-sitosterol in the equimolar DOPE:DOPC mixtures lowered the T_m of the fully hydrated dispersions to −13°C [10]. The addition of 5 mol% CER (DOPE:DOPC:β-sitosterol:CER; 22.5:22.5:50:5) had no effect on the T_m of the mixture in excess water (−13°C) while the addition of 20 mol% CER (DOPE:DOPC:β-sitosterol:CER; 15:15:50:20) lowered the T_m of the mixture in excess water to −18°C (data not shown). The mechanism by which CER lowered the T_m of the mixtures is not clear.

The T_m of dehydrated lipid mixtures was also determined by DSC. Dehydration of DOPE:DOPC (50:50) at osmotic pressures greater than 20 MPa (i.e., to water contents below approx. 15 wt%) resulted in an increase of the T_m from −11° to −2°C [9] (Fig. 2). In the presence of 50 mol% β-sitosterol (DOPE:DOPC:β-sitosterol, 25:25:50) the increase of T_m at higher osmotic pressures was also observed [9] (Fig. 2). In the mixture containing 5 mol% CER (DOPE:DOPC:β-sitosterol:CER, 22.5:22.5:50:5), the T_m increased by approx. 2°C after dehydration at an osmotic pressure of 38.9 MPa (Fig. 2). In contrast, with dispersions containing 50 mol% β-sitosterol and 20 mol% CER (DOPE:DOPC:β-sitosterol:CER, 15:15:50:20) there were no changes in T_m as a consequence of dehydration at osmotic pressures in the range between 2.7 to 38.9 MPa. The absence of a dehydration-induced increase of T_m for those mixtures containing 20 mol% of CER was also observed when the T_m was expressed as a function of the water content (Fig. 3A) or the water/phospholipid molar ratio (Fig. 3B). At an osmotic pressure of 38.9 MPa,

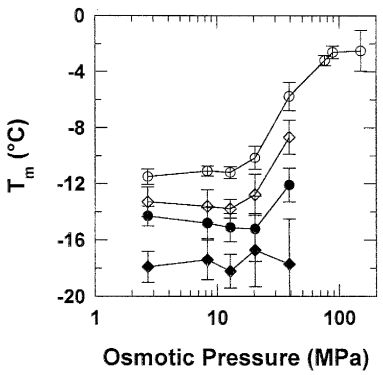

Fig. 2. Variation of the temperature of the $L_\beta \rightarrow L_\alpha$ phase transition observed by DSC with the osmotic pressure at which the lipid dispersions were equilibrated. Temperatures represent the peak of the endotherm observed during the first heating scan from −50°C to +80°C. Symbols and data as described in Fig. 1; data represent the means ± standard deviation from three experiments.

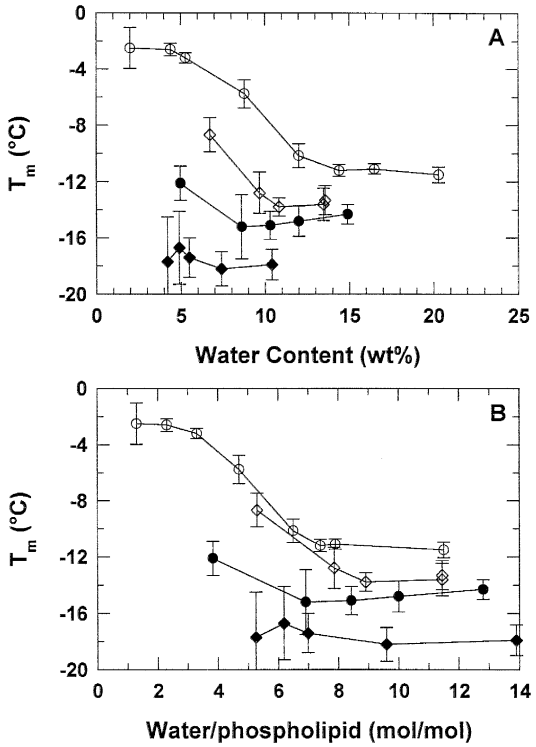

Fig. 3. Variation of the temperature of the $L_\beta \rightarrow L_\alpha$ phase transition observed by DSC with the water content (A) and molar water/phospholipid ratio (B) of the lipid dispersions. Temperatures represent the peak of the endotherm observed during the first heating scan from −50°C to +80°C. Symbols and data as described in Fig. 1; data represent the means ± standard deviation from three experiments.

the lipid mixtures had water contents varying between 4 and 9 wt% (Fig. 1A), but all of the dispersions had phospholipid hydrations of approx. 5 H_2O/phospholipid (Fig. 1B). At this osmotic pressure and phospholipid hydration, DOPE:DOPC:β-sitosterol:CER (15:15:50:20) had a T_m of -18°C, compared to approx. -12°, -9° and -6°C for the DOPE:DOPC:β-sitosterol:CER (22.5:22.5:50:5), DOPE:DOPC:β-sitosterol (25:25:50) and DOPE:DOPC (50:50) mixtures, respectively (Fig. 1B and Fig. 3B).

It should be noted that the DSC data presented here indicates that all of the dehydrated lipid mixtures were exclusively in the fluid phase (either L_α or H_{II}) at 20°C at all examined hydrations. That is, DSC scans of these lipid mixtures between -50° and 80°C revealed no evidence for the existence of an L_β phase at temperatures higher than 20°C that be would be comprised of a phase-separated domain enriched in high-melting point cerebrosides [12]. Examination of the dehydrated mixtures by wide-angle X-ray diffraction confirmed the absence of either the $L_ß$ or L_c phases at 20°C (next section). Therefore, rye cerebrosides were fully miscible in the DOPE:DOPC:β-sitosterol mixtures at proportions up to 20 mol%.

3.3. Dehydration-induced $L_\alpha \rightarrow H_{II}$ phase transitions

Dispersions of DOPE:DOPC:β-sitosterol:CER (22.5:22.5:50:5 and 15:15:50:20) that were equilibrated at osmotic pressures between 2.7 and 88.5 MPa were examined by freeze-fracture electron microscopy and X-ray diffraction. The results obtained by freeze-fracture electron microscopy were consistent with those determined by X-ray diffraction and, therefore, will not be presented in detail. However, it is important to note that the samples analyzed by freeze-fracture electron microscopy had water contents identical to those desorbed in DSC pans [9], were desorbed at very high surface area/volume ratios and were rapidly quenched by plunging into liquid propane. Since these samples had phases very similar to those observed in samples analyzed by X-ray diffraction, this indicates that the latter samples had reached equilibrium at the indicated osmotic pressures prior to their analysis by X-ray diffraction.

Fully hydrated dispersions of DPPC and CER were also examined by X-ray diffraction at 20°C to confirm the expected positions of the wide-angle reflections for the L_β and L_c phases. DPPC had a sharp wide-angle diffraction at 0.424 nm, characteristic of the L_β phase (data not shown). Rye CER had a series of weak wide-angle reflections, at spacings identical to those reported previously [12], characteristic of a cerebroside L_c phase (data not shown). In agreement with the DSC data described above, examination of the wide angle reflections of the X-ray powder patterns revealed no evidence for the occurrence of the L_β phase in any of these lipid mixtures at any hydration.

A summary of the phases present in these lipid mixtures as a function of hydration is presented in Table 1. At full hydration and 20°C, all of the lipid mixtures were exclusively in the L_α phase, as indicated by the presence of a diffuse wide-angle X-ray reflection at 0.46 nm and the occurrence of 3–5 diffraction orders in the small angle region indexing to ratios of 1:2:3.... Previous work demonstrated that DOPE:DOPC (50:50) was present in either the L_α phase or as two demixed L_α phases at osmotic pressures in the range between 2.7 and 38.9 MPa [9]. The H_{II} phase was first observed at approx. 6 wt% water, a hydration equivalent to approx. 3 H_2O/phospholipid and after desorption at 76 MPa [9]. Addition of β-sitosterol to 50 mol% (DOPE:DOPC:β-sitosterol, 25:25:50) caused a sig-

Table 1
Summary of the phases formed by dispersions containing DOPE, DOPC, β-sitosterol and cerebrosides at 20°C as a function of osmotic pressure (MPa)

MPa	DOPE:DOPC:β-sitosterol:CER	
	22.5:22.5:50:5	15:15:50:20
0	L_α	L_α
2.7	-	L_α (12.6)
8.3	L_α (9.45)	L_α and H_{II} (9.40)
12.8	L_α (8.16)	H_{II} and L_α (8.17)
20.4	H_{II} and L_α (6.77)	H_{II} and L_α (6.84)
38.9	H_{II} and L_α (4.85)	H_{II} and L_α (5.00)
76.1	H_{II} (2.85)	-

Phases were determined by freeze-fracture electron microscopy and X-ray diffraction. The hydrations of the desorbed dispersions are given in parentheses (in mol H_2O/mol phospholipid) and represent interpolations of the linear regressions plotted in Fig. 1B (all r^2 values for these regressions were ≥ 0.93).

nificant increase in the water content at which the H_{II} phase was first observed (7.3 H_2O/phospholipid) and significantly decreased the osmotic pressure at which this lyotropic phase transition occurred from 76.1 to 20.4 MPa [10]. The addition of small proportions of CER (DOPE:DOPC:β-sitosterol:CER, 22.5:22.5:50:5) had no additional influence on either the osmotic pressure at which the H_{II} phase was first observed (20.4 MPa) or on the hydration at which the lyotropic transition to the H_{II} phase occurred (6.8 H_2O/phospholipid) (Table 1). With further increases in the proportions of CER in the mixtures (DOPE:DOPC:β-sitosterol:CER, 15:15:50:20), the H_{II} phase was first observed at a significantly lower osmotic pressure (8.3 MPa) and at a significantly higher phospholipid hydration (9.4 H_2O/phospholipid) (Table 1). At an osmotic pressure of 12.8 MPa, the H_{II} phase was the predominant phase present in the DOPE:DOPC:β-sitosterol:CER (15:15:50:20) mixture, but was not present in the DOPE:DOPC:β-sitosterol:CER (22.5:22.5:50:5) mixture (Table 1). In the DOPE:DOPC:β-sitosterol:CER (15:15:50:20) mixture, both the H_{II} phase and the L_α phases were observed at all osmotic pressures in the range between 8.3 and 38.9 MPa (Table 1).

The repeat spacings for the lamellar and H_{II} phases described in Table 1 are reported in Table 2. To facilitate the comparisons, the spacings for the lamellar and H_{II} phases of dehydrated mixtures of DOPE:DOPC:β-sitosterol (25:25:50) are also included [10]. In the fully hydrated dispersions, increasing proportions of CER were associated with a decrease in the lamellar repeat spacing from 6.73 nm to 5.89 nm (Table 2). This result is consistent with the decreased water contents of the lipid mixtures containing higher proportions of CER (Fig. 1A). That is, even in the presence of excess water, the amount of water hydrating the surface of the bilayers comprised of significant proportions of CER was reduced, resulting in lower bilayer hydration and smaller interbilayer separations. After these mixtures were equilibrated at osmotic pressures in the range between 2.7 and 76.1 MPa, the lamellar phase repeat spacing decreased with increasing osmotic pressure (Table 2). This decreased repeat spacing was likely due to the removal of water from the interbilayer spaces occurring as a consequence of decreased water contents and phospholipid hydration (Fig. 1A,B). Similarly, after the dehydration-induced formation of the H_{II} phase, the inter-tube spacings of this phase decreased with increasing osmotic pressure (Table 2). For example, the inter-tube spacing of the H_{II} phase present in the DOPE:DOPC:β-sitosterol:CER (15:15:50:20) mixture decreased from 8.52 nm at 8.3 MPa to 5.76 nm at 38.9 MPa (Table 2). It should also be noted that the dimensions of the H_{II} phase at the osmotic pressure at which this phase was first observed were significantly different. Specifically, the inter-tube spacing of the H_{II} phase in the DOPE:DOPC:β-sitosterol (25:25:50) at 38.9 MPa was 5.42 nm, com-

Table 2
Summary of the dimensions of the lamellar and H_{II} phases formed by dispersions containing DOPE, DOPC, β-sitosterol and rye cerebrosides (CER) as a function of the osmotic pressure at which the dispersions were equilibrated

Osmotic pressure (MPa)	Repeat spacing (nm)					
	DOPE:DOPC:β-sitosterol 25:25:50		DOPE:DOPC:β-sitosterol:CER 22.5:22.5:50:5		DOPE:DOPC:β-sitosterol:CER 15:15:50:20	
	L_α	H_{II}	L_α	H_{II}	L_α	H_{II}
0	6.73	-	6.09	-	5.89	-
2.7	-	-	-	-	6.16	-
8.3	6.69	-	6.49	-	6.00	8.52
12.8	6.49	-	6.26	-	5.58	8.43
20.4	5.98	5.60	[a]	5.80	5.77	5.91
38.9	5.76	5.42	5.66	5.46	5.57	5.76
76.1	-	5.38	-	5.38	ND	ND

Values represent the lamellar repeat spacing or the inter-tube spacing of the H_{II} phase using the phase assignments in Table 1 and from [10] for DOPE:DOPC:β-sitosterol (25:25:50).
[a] Not determined.

pared to 5.80 nm for the DOPE:DOPC:β-sitosterol:CER (22.5:22.5:50:5) mixture at 20.4 MPa and 8.52 nm for DOPE:DOPC:β-sitosterol:CER (15:15:50:20) at 8.3 MPa (Table 2).

The increased propensity of the DOPE:DOPC:β-sitosterol:CER (15:15:50:20) mixtures to undergo the $L_\alpha \rightarrow H_{II}$ phase transition during dehydration could be attributed to the high sterol:phospholipid ratio in this mixture, rather than to a direct effect of the higher proportions of CER. To evaluate this possibility, the lyotropic phase behavior of dispersions of DOPE:DOPC:CER in the absence of β-sitosterol was also examined by X-ray diffraction. Cerebrosides present at 20 mol% in equimolar DOPE:DOPC (DOPE:DOPC:CER 40:40:20) did not facilitate the formation of the H_{II} phase, and the mixture remained in the L_α phase at osmotic pressures up to 76.1 MPa (data not shown). At 33 mol% in fully hydrated DOPE:DOPC mixtures (DOPE:DOPC:CER, 33.3:33.3:33.3) at 20°C, the cerebrosides were completely miscible. However, during dehydration of this lipid mixture, a phase separation of L_α and L_c phases was observed (data not shown). The H_{II} phase was not observed in this mixture as a consequence of dehydration at osmotic pressures up to 76.1 MPa. These results indicate that the cerebrosides alone at 20 mol% of the total lipid are insufficient to facilitate the lyotropic transition to the H_{II} phase in DOPE:DOPC dispersions at osmotic pressures less than 76.1 MPa.

3.4. Thermotropic $L_\alpha \rightarrow H_{II}$ phase transitions

The preceding results show that the presence of 20 mol% of CER in DOPE:DOPC:β-sitosterol:CER dispersions facilitated the dehydration-induced formation of the H_{II} phase. To determine if the low hydration of the CER head-group influences the propensity of these mixtures to undergo the $L_\alpha \rightarrow H_{II}$ phase transition, the thermotropic phase transitions of fully hydrated lipid dispersions were also examined by X-ray diffraction. Mixtures of DOPE:DOPC (50:50) undergo the $L_\alpha \rightarrow H_{II}$ phase transition at 50–55°C [9] (Table 3). In the presence of 33 mol% β-sitosterol (DOPE:DOPC:β-sitosterol, 33.3:33.3:33.3) the $L_\alpha \rightarrow H_{II}$ phase transition occurred between 40° and 45°C [10] (Table 3). However, in the presence of either 20 or 33 mol% CER (DOPE:DOPC:CER, 40:40:20 or 33.3:33.3:33.3) the H_{II} phase was not observed at any temperature in the range between 20° and 60°C (Table 3). In the complex lipid mixtures composed of DOPE:DOPC:β-sitosterol:CER (22.5:22.5:50:5 and 15:15:50:20), the $L_\alpha \rightarrow H_{II}$ phase transition occurred at significantly lower temperatures. Specifically, in the DOPE:DOPC:β-sitosterol:CER (22.5:22.5:50:5) mixture the first occurrence of the H_{II} phase was at 40°C (Table 3). This value is very similar to that at which the first occurrence of the $L_\alpha \rightarrow H_{II}$ phase transition at 40–45°C in DOPE:DOPC:β-sitosterol 25:25:50 was observed [10]. However, when the cerebrosides

Table 3
Summary of the phases present in fully hydrated dispersions of DOPE, DOPC, cerebrosides and β-sitosterol as a function of temperature

°C	DOPE:DOPC	DOPE:DOPC:β-sitosterol	DOPE:DOPC:CER		DOPE:DOPC:β-sitosterol:CER	
	50:50	33.3:33.3:33.3	40:40:20	33.3:33.3:33.3	22.5:22.5:50:5	15:15:50:20
20	-	L_α	L_α	L_α	L_α	L_α
25	L_α	-	-	-	-	-
30	L_α	L_α	L_α	L_α	L_α	L_α
35	L_α	-	-	-	L_α	H_{II} and L_α
40	L_α	L_α	L_α	L_α	H_{II} and L_α	H_{II}
45	L_α	L_α and H_{II}	-	-	H_{II}	-
50	L_α	L_α and H_{II}	L_α	L_α	H_{II}	-
55	H_{II}	H_{II}	-	-	-	-
60	H_{II}	H_{II}	L_α	L_α	H_{II}	-

Dispersions were examined by X-ray diffraction as described in Section 2. Samples were in excess water, typically a water content of approximately 50 wt%. At temperatures where two phases co-existed, the predominant phase is listed first. Data for DOPE:DOPC (50:50) are from Ref. [9] and data for DOPE:DOPC:β-sitosterol (33.3:33.3:33.3) are from Ref. [10] and are provided for comparison.

represented 20 mol% of the total lipid (DOPE:DOPC:β-sitosterol:CER, 15:15:50:20), the temperature at which the H_{II} phase was first observed was decreased to 35°C (Table 3).

4. Discussion

Freezing injury of non-acclimated protoplasts of both rye and oat is associated with the occurrence of the $L_\alpha \rightarrow H_{II}$ phase transition in localized regions where the plasma membrane is brought into close apposition with various endomembranes, especially the chloroplast envelope, as a result of freeze-induced dehydration [1]. However, the formation of the H_{II} phase occurs at significantly higher subzero temperatures, and lower osmotic pressures, in oat than in rye. These differences in the propensity for freeze-induced formation of the H_{II} phase between rye and oat are associated with significant differences in plasma membrane lipid composition [3,6–8]. As part of our continuing efforts to determine the influence of lipid composition on the propensity of lipid mixtures to undergo the lyotropic $L_\alpha \rightarrow H_{II}$ phase transition (see Ref. [3]), we have examined the effects of rye cerebrosides (CER) on the hydration and phase transitions of DOPE:DOPC:CER and DOPE:DOPC:β-sitosterol:CER mixtures.

Small amounts (5 mol%) of cerebrosides, proportions similar to those of the cold-acclimated rye plasma membrane [11], had no detectable effect on either the water content (Fig. 1A) or the phospholipid hydration (Fig. 1B) of DOPE:DOPC:β-sitosterol:CER mixtures. The addition of 5 mol% of CER to mixtures of DOPE:DOPC:β-sitosterol also had a negligible effect on the temperature of the $L_\beta \rightarrow L_\alpha$ phase transition temperature (T_m) when expressed as a function of osmotic pressure (Fig. 2). However, when expressed as function of bilayer water content (Fig. 3A) or phospholipid hydration (Fig. 3B), 5 mol% of CER caused a small reduction in the dehydration-induced increase of T_m, but only at the most extensive dehydrations examined. At proportions (20 mol%) intermediate between those of the non-acclimated rye (16.4 mol%) and oat (27.2 mol%) plasma membranes [3], CER addition resulted in significantly decreased bilayer water content (Fig. 1A). The decreased hydration of these lipid dispersions is consistent with the low hydration of pure rye cerebrosides (Lynch, D.V. and Steponkus, P.L., unpublished results) and are interpreted to indicate that the water present in the desorbed lipid mixtures was associated predominantly with the phospholipids (Fig. 1B). It should be pointed out that the phospholipid hydration results presented in Fig. 1B do not indicate that the CER were present in a phase-separated state in these mixtures, resulting in independent hydration of the CER and phospholipid-enriched domains. This conclusion is based on the absence of small-angle X-ray reflections indicative of demixed phases in these mixtures that were equilibrated at the lower osmotic pressures (Tables 1 and 2). The lower bilayer hydrations observed in the DOPE:DOPC:β-sitosterol:CER mixtures (15:15:50:20) is consistent with smaller lamellar repeat spacings in this mixture than in either the DOPE:DOPC:β-sitosterol:CER (22.5:22.5:50:5) or DOPE:DOPC:β-sitosterol (25:25:50) mixtures at full hydration (Table 2). In the DOPE:DOPC:β-sitosterol:CER (15:15:50:20) mixture, the dehydration-induced increase of T_m observed in the other lipid mixtures at osmotic pressures of 38.9 MPa was not observed (Fig. 3), despite the similarities in the hydration of the phospholipids in the presence or absence of CER (Fig. 1B). This was best observed when the T_m was expressed as a function of the water/phospholipid ratio (Fig. 3B); the T_m of the DOPE:DOPC:β-sitosterol:CER (15:15:50:20) mixtures at 5–6 H_2O/phospholipid was identical to that at ≥ 11 H_2O/phospholipid. This is in contrast to the increase of T_m in the DOPE:DOPC:β-sitosterol (25:25:50) mixture (from $-13.5°$ to $-9°$C) during dehydration over the range from ≥ 11 to 5–6 H_2O/phospholipid (Fig. 3B). Overall, these results indicate that the addition of CER to mixtures of DOPE:DOPC:β-sitosterol decreased the total bilayer hydration but did not alter the phospholipid hydration. The presence of higher proportions of CER (20 mol%) in these mixtures precluded the lyotropic increase of the T_m.

The addition of 5 mol% of CER to DOPE:DOPC:β-sitosterol had little, if any, effect on either the hydration-dependent isothermal phase behavior (i.e., osmotic pressure at which the lyotropic $L_\alpha \rightarrow H_{II}$ phase transitions occurred (Table 2) or the T_{bh} of the fully hydrated mixtures (Table 3). An increase in the proportion of CER from 5 mol% to 20

mol% of the total lipid did, however, promote the occurrence of the dehydration-induced $L_\alpha \rightarrow H_{II}$ phase transition at lower osmotic pressures and higher phospholipid hydrations (Tables 1 and 2) and lowered the T_{bh} of the fully hydrated dispersions (Table 3). It should be noted that we have not observed the thermotropic $L_\alpha \rightarrow H_{II}$ transition that was reported to occur in fully hydrated 33.3:33.3:33.3 DOPE:DOPC:bovine brain CER dispersions (Table 3 and Ref. [13]) or the L_β phase in dehydrated 33.3:33.3:33.3 DOPE:DOPC:bovine brain CER dispersions at 15 wt% water (data not shown and Ref. [13]). However, the rye glucocerebrosides are distinct from the bovine galactocerebrosides in terms of both head-group and acyl composition [3] as well as phase behavior [12].

The formation of the H_{II} phase at lower osmotic pressures in the DOPE:DOPC:β-sitosterol:CER (15:15:50:20) mixture may have occurred as a consequence of the low hydration of the CER head-group. The reduced water content of this mixture (Fig. 1A) is consistent with the observed decrease in interbilayer separation (Table 2) and would be expected to bring juxtaposed lamellae into close apposition at lower osmotic pressures than in the lipid mixtures with higher water contents. In addition, a secondary effect of low hydration of CER (Fig. 1A) on the lyotropic $L_\alpha \rightarrow H_{II}$ phase transition may be effected via a reduction of lateral pressures at the bilayer surface. A reduction in lateral packing pressures at the bilayer surface will favor a monolayer curvature leading to the formation of the H_{II} phase [14,15]. However, these considerations presuppose that the primary effect of CER on the $L_\alpha \rightarrow H_{II}$ phase transition results from the low head-group hydration of this lipid. The observation that fully hydrated lipid dispersion containing 20 mol% CER (DOPE:DOPC:β-sitosterol:CER, 15:15:50:20) underwent the thermotropic $L_\alpha \rightarrow H_{II}$ phase transition at temperatures lower than in the lipid mixtures with lower proportions of CER (i.e., 5 mol% in DOPE:DOPC:β-sitosterol:CER, 22.5:22.5:50:5) (Table 3) suggests that CER may also promote the formation of the H_{II} phase by effects within the acyl domain. The cerebrosides have acyl chains that are significantly longer than those of the phospholipids of the rye plasma membrane [16]. Therefore, it is possible that the cerebroside acyl chains may preferentially extend into the interstitial areas of the H_{II} lattice and relieve packing stresses within the hydrophobic domain of the H_{II} phase and, thus, stabilize the H_{II} phase [14,15].

It could be argued that the greater propensity of dispersions containing 20 mol% of CER to undergo the $L_\alpha \rightarrow H_{II}$ phase transition was not due to direct effects of CER described above, but was a consequence of the increased sterol/phospholipid ratio in the DOPE:DOPC:β-sitosterol:CER (15:15:50:20) mixtures. However, in a previous study we established that an increase in the sterol/phospholipid ratio from 0.5/1.0 to 1.0/1.0 did not increase the propensity of DOPE:DOPC:sterol dispersions to undergo either the dehydration- or temperature-induced $L_\alpha \rightarrow H_{II}$ phase transition [10]. Therefore, it unlikely that the higher β-sitosterol/phospholipid molar ratio in the DOPE:DOPC:β-sitosterol:CER (15:15:50:20) mixtures studied here was solely responsible for the greater propensity of this lipid mixture to undergo the $L_\alpha \rightarrow H_{II}$ phase transition. Similarly, it is unlikely that the higher CER content of this mixture was solely responsible for the increased tendency towards the formation of the H_{II} phase. This conclusion is based on the observation that CER proportions up to 20 or 33 mol% in equimolar DOPE:DOPC mixtures lacking sterols did not facilitate the lyotropic formation of the H_{II} phase in these mixtures (data not shown). In addition, fully hydrated dispersions comprised of 33 mol% CER in equimolar DOPE:DOPC mixtures lacking sterols also did not undergo the $L_\alpha \rightarrow H_{II}$ phase transition at temperatures less than 60°C (Table 3).

It is concluded that the increased propensity for the dehydration- and temperature-induced $L_\alpha \rightarrow H_{II}$ phase transitions in DOPE:DOPC:β-sitosterol:CER (15:15:50:20) mixtures is the result of synergistic interactions between the phospholipids, sterols and cerebrosides. Overall, these results, and the results from a previous study [10], clearly show that the propensity of a lipid mixture to undergo the lyotropic or thermotropic $L_\alpha \rightarrow H_{II}$ phase transition cannot be predicted solely on the basis of the content of a single lipid species (i.e., the unsaturated species of PE). The mixture containing lower proportions of DOPE (15 mol% in DOPE:DOPC:β-sitosterol:CER, 15:15:50:20) was more prone to both the lyotropic (Table 1) and thermotropic (Table 3) $L_\alpha \rightarrow H_{II}$ phase

transitions than the mixture containing higher proportions of DOPE (22.5 mol% in DOPE:DOPC:β-sitosterol:CER, 22.5:22.5:50:5). Therefore, the differential propensity of the plasma membranes of rye and oat to undergo the $L_\alpha \rightarrow H_{II}$ phase transition during freeze-induced dehydration appears to be a consequence of the summation of physical characteristics of the membrane lipid components – including bilayer surface hydration, packing pressures within the hydrophobic domain of the membrane, and the tendency of the lipid components to demix – which are influenced by the relative proportions of the various lipid components. Future experiments will determine the influence of intracellular solutes on the differential propensities of the plasma membranes of rye and oat to undergo freeze-induced destabilization.

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