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Effects of plant sterols on the hydration and phase behavior of DOPE/DOPC mixtures

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Abstract

Freeze-induced injury of protoplasts of non-acclimated rye and oat is associated with the formation of the inverted hexagonal (H_{II}) phase in regions where the plasma membrane and various endomembranes are brought into close apposition as a result of freeze-induced dehydration. The influence of lipid composition and hydration on the propensity of mixtures of DOPE:DOPC containing either sterols or acylated steryl glucosides to form the H_{II} phase was determined by DSC, freeze-fracture electron microscopy and X-ray diffraction. The addition of plant sterols to a mixture of DOPE/DOPC (either 1:1:1 or 1:1:2 mole ratio of DOPE/DOPC/sterols) reduced the total hydration of the mixture (expressed as wt% water) after desorption over a range of osmotic pressures of 2.8 to 286 MPa. However, most or all of the water remaining in the dehydrated lipid mixtures was associated predominantly with the phospholipids. Both sterols and acylated steryl glucosides significantly promoted both the dehydration-induced and thermally induced $L_\alpha \rightarrow H_{II}$ phase transitions in DOPE/DOPC mixtures however, acylated steryl glucosides were much more effective. In mixtures containing plant sterols, the H_{II} phase occurred after dehydration at 20 MPa (20°C), which resulted in a water content of 11.7 wt%. In contrast, mixtures containing acylated steryl glucosides were in the H_{II} phase in excess water, i.e., they did not require dehydration to effect the $L_\alpha \rightarrow H_{II}$ phase transition. The results indicate that genotypic differences in the lipid composition of the plasma membrane of rye and oat leaves have a significant influence on the propensity for formation of the H_{II} phase during freeze-induced dehydration.

Keywords: Sterol; Acylated steryl glucoside; Lipid hydration; Lipid phase behavior; Freezing; Hexagonal II phase transition; (Plasma membrane)

1. Introduction

Freezing tolerance is a primary factor limiting the geographic distribution of plants. Protoplasts isolated from the leaves of non-acclimated rye (NA protoplasts) are subjected to severe dehydration during freezing. At −10°C, the osmotic pressure of the unfrozen portion of the extracellular solution is 12 MPa, which results in a reduction in cell volume of more than 80% as a result of freeze-induced dehydration [1]. In NA protoplasts subjected to freeze-induced dehydration at −10°C, injury is manifested by the appearance of aparticulate domains in the plasma membrane, aparticulate lamellae subtending the plasma membrane, and the formation of the inverted hexagonal (H_{II}) phase in regions where the plasma membrane and subtending endomembranes are brought into close apposition [2]. These ultrastructural alterations are consequences of freeze-induced dehydration rather than low temperature per se [2]. The incidence of the H_{II} phase correlates with lethal injury to both protoplasts and leaves of non-acclimated seedlings as manifested by the loss of osmotic responsiveness of protoplasts and leakage of the intracellular contents of leaves [1].

The differential susceptibility of NA protoplasts of winter rye and spring oat to freeze-induced formation of the H_{II} phase (−6°C vs. −3°C, respectively) [3] is associated with significant differences in the lipid composition of the

Abbreviations: DOPC, dioleoylphosphatidylcholine; DOPE, dioleoylphosphatidylethanolamine; DPPC, dipalmitoylphosphatidylcholine; L_α phase, lamellar liquid-crystalline phase; L_β phase, lamellar gel phase; H_{II} phase, inverted hexagonal phase; $L_\alpha \rightarrow L_\beta$, liquid crystalline-to-gel phase transition; $L_\alpha \rightarrow H_{II}$, liquid crystalline-to-inverted hexagonal phase transition; DSC, differential scanning calorimetry; FFEM, freeze-fracture electron microscopy.

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plasma membrane [4]. In winter rye, the plasma membrane is composed of 36.6 mol% phospholipids, 16.4 mol% cerebrosides, 38.1 mol% free sterols, 5.6 mol% steryl glucosides, and 2.9 mol% acylated steryl glucosides. In contrast, the plasma membrane of spring oat is composed of 28.8 mol% phospholipids, 27.2 mol% cerebrosides, 8.4 mol% free sterols, 5.6 mol% steryl glucosides and 27.3 mol% acylated steryl glucosides [4]. (The chemical structures of the major plasma membrane lipids are shown in [5].) Therefore, a major difference between the lipid composition of the plasma membrane of winter rye and spring oat is in the proportion of free sterols and acylated steryl glucosides.

In protoplasts isolated from cold-acclimated rye leaves (ACC protoplasts), the H_{II} phase does not occur during freezing [2]. In both winter rye and spring oat, the acclimation-induced decrease in the propensity of the plasma membrane to form the H_{II} phase during freeze-induced dehydration is associated with alterations in the plasma membrane lipid composition [4–6]. In rye, the proportion of phospholipids, primarily phosphatidylcholine (PC) and phosphatidylethanolamine (PE), increases from 36.6 to 43.3 mol% of the total lipid, and the proportion of cerebrosides (CER) decreases from 16.4 to 10.5 mol% during cold acclimation. The proportion of free sterols increases slightly during acclimation (38.1 to 41.1 mol%), with a concomitant decrease in the steryl glucosides (5.6 to 3.5 mol%) and acylated steryl glucosides (2.9 to 1.4 mol%).

These studies indicate that membrane lipid composition is a primary factor that determines the cryostability of the plasma membrane to freeze-induced dehydration [5]. When the plasma membrane lipids purified from non-acclimated rye leaves were dispersed and then dehydrated in concentrated solutions of sorbitol, the H_{II} phase was formed [7]. In contrast, the H_{II} phase did not occur in aqueous dispersions of the plasma membrane lipids that were purified from cold-acclimated rye leaves and subjected to identical osmotic dehydration [7]. In addition, membrane bioengineering studies have demonstrated that enrichment of the plasma membrane of NA protoplasts with unsaturated species of PC by fusion of the protoplasts with liposomes of dilinoleoyl-PC prevents the formation of the H_{II} phase during freeze-induced dehydration [8]. Taken in sum, these and other studies [5] provide compelling evidence that plasma membrane lipid composition is a primary determinant of the cryostability of the plasma membrane during freeze-induced dehydration.

Of the lipids that comprise the plasma membrane, the unsaturated molecular species of PE are the most prone to undergo the $L_\alpha \rightarrow H_{II}$ phase transition [9]. Formation of the H_{II} phase in PE dispersions can be induced by increased temperature (thermally) and/or decreased hydration (lyotropically). The temperature at which the $L_\alpha \rightarrow H_{II}$ phase transition occurs (T_{bh}) is a function of both the unsaturation of the acyl chains and the water content of the PE dispersion. However, the proportions of PE present in the plasma membranes of rye and oat are sufficiently low (8.4 to 12.4 mol% of the total lipid) that they are stabilized in the lamellar phase by the other plasma membrane lipids. Nevertheless, we have recently demonstrated that the dehydration effected by freezing is sufficient to remove water hydrating phospholipid head groups [10] and sufficient to cause demixing (lateral fluid-fluid separation) of highly-hydrated PC from poorly-hydrated PE in binary mixtures of dioleoyl-PC (DOPC) and dioleoyl-PE (DOPE) [11]. The PE-enriched domains thus formed preferentially entered the H_{II} phase [11], and therefore, dehydration-induced lipid-lipid demixing promoted the $L_\alpha \rightarrow H_{II}$ phase transition.

Among the other major lipid components of the plasma membrane (sterols, acylated steryl glucosides and cerebrosides), the sterols are especially expected to influence the phase behavior of phospholipids during freeze-induced dehydration. As the $L_\alpha \rightarrow H_{II}$ phase transition is an interbilayer event that requires close bilayer-bilayer approach [9], poorly hydrated membrane lipids, such as the sterols, might be expected to reduce the interbilayer separation at a given osmotic pressure and, hence, increase the frequency of interbilayer interactions leading to formation of the H_{II} phase. In addition, the low hydration of sterols is likely to reduce lateral packing pressures in the headgroup domain of the lipid monolayers whereas the hydrophobic moiety of the sterols should increase packing pressures in the acyl domain. Consequently, sterols are expected to promote a monolayer curvature favoring the $L_\alpha \rightarrow H_{II}$ phase transition [12,13].

This study describes experiments to evaluate the influence of plant sterols on the lyotropic and thermotropic phase behavior of DOPE/DOPC/sterol mixtures. We report the influence of free sterols on: (1) the hydration of DOPE/DOPC mixtures; (2) the hydration dependence of the $L_\alpha \rightarrow L_\beta$ phase transition temperature (T_m); (3) the occurrence of fluid-fluid demixing between lipids of different hydration characteristics during dehydration; and (4) the occurrence of the $L_\alpha \rightarrow H_{II}$ phase transition during dehydration. In addition, we have compared the effect of free sterols on the propensity for the dehydration-induced vs. the thermally induced $L_\alpha \rightarrow H_{II}$ phase transition in fully hydrated mixtures of DOPE/DOPC and have also compared the effects of free sterols with those of acylated steryl glucosides on the propensity for both the dehydration-induced and the thermally induced $L_\alpha \rightarrow H_{II}$ phase transitions.

2. Materials and methods

2.1. Materials

DOPE, DOPC and DPPC were obtained from Avanti Polar Lipids. Acylated steryl glucosides (ASG) were obtained from Matreya Lipids. Free sterols, purified from

soybeans, were obtained from Sigma Chemical Company (S9889) and were composed primarily of β-sitosterol (approx. 60%) with the balance mostly campesterol. This sterol mixture is very similar to the mixture of free sterols in the plasma membrane of rye leaves [6] and will be referred to as β-sitosterol in this report. All lipids were more than 98% pure based on analytical chromatography of the lipids in $CHCl_3/CH_3OH/H_2O$ (65:25:4, v/v) and were used without further purification. Double-distilled, de-ionized water that was degassed under vacuum and then saturated with N_2 gas immediately prior to lipid dispersal was used in these studies.

2.2. Lipid dispersal

Lipids were mixed in $CHCl_3$ at the indicated mole ratios, solvent was removed under a stream of N_2 at 40°C, then trace solvent was removed under high vacuum for 16 h. Lipids were dispersed under N_2 at either 85 wt% or 50–60 wt% water by vortexing and sonicating as well as five to ten freeze/thaw cycles between −196°C and 50°C. The dispersions were allowed to equilibrate at 4°C for 16 h under an N_2 atmosphere before the experiments were started. DPPC was dispersed by extensive vortexing and brief sonication.

2.3. Desorption isotherms and differential scanning calorimetry

Lipids were desorbed for hydration measurements and differential scanning calorimetry (DSC) as described previously [11]. Briefly, aliquots containing 1–2 mg of lipid were placed in pre-weighed DSC pans and equilibrated over saturated salt solutions for 7 days at 20°C in an N_2 atmosphere. The saturated salt solutions were Na_2HPO_4, KNO_3, $BaCl_2$, KCl, NaCl, NaBr, $Mg(NO_3)_2$, $MgCl_2$ and LiCl with osmotic pressures of 2.8, 8.3, 12.8, 20.4, 38.9, 76.1, 88.5, 150 and 286 MPa, respectively, at 20°C. After equilibration, the pans were sealed and re-weighed and analyzed by DSC. After DSC analysis, the pans were re-opened and desiccated over P_2O_5 overnight at 70°C under vacuum for determination of the exact lipid weight. Water content of the equilibrated lipid dispersions was calculated as described previously [11].

Calorimetric analyses of the lipid dispersions were performed on a Perkin-Elmer DSC 7 instrument. Samples were scanned from 20°C to −50°C at 10 C° min^{-1} followed by heating to 80°C at the same rate. Transition temperatures (T_m) were calculated from the first heating scan using DSC 7 software; results were identical in subsequent heating scans. The peak T_m values obtained from heating scans were 4–5°C higher than those obtained from the cooling scans, however, the onset temperatures for the phase transitions were very similar in cooling and heating scans. Data obtained using a scan rate of 10 C° min^{-1} were very similar to those obtained using scan rates as low as 0.5 C° min^{-1} for dispersions having water contents ≥ 5 wt% [11].

2.4. Freeze-fracture electron microscopy

Lipid dispersions for freeze-fracture electron microscopy (FFEM) studies were prepared at approx. 15 wt% water as described above and then either concentrated by centrifugation or dispersed at a water content of 50–60 wt%. Aliquots of the concentrated dispersions were loaded on FFEM stubs and desorbed as described above. We have previously determined that the water contents of dispersions desorbed on FFEM stubs is identical to that of dispersions desorbed in DSC pans [11]. The equilibrated, lipid dispersions were quenched from 20°C by plunging into liquid propane supercooled 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 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 concentrated H_2SO_4 and examined in a Philips EM 300 electron microscope.

2.5. X-ray diffraction

X-ray diffraction powder patterns were obtained at the Cornell High Energy Synchrotron Source (CHESS). Most of the lipid dispersions were examined using the standard crystallography bench camera on the A1 station at CHESS. Monochromatic radiation from a 6-pole electromagnetic wiggle source ($\lambda = 0.156$ nm) was passed through a 0.3 mm collimator. 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 11–13 cm) using exposure times of 20 s to 1 min. Flux through this collimator was approx. $2 \cdot 10^{11}$ photons s^{-1}. Some of the experiments were performed on the C2 station at CHESS. Sagittally focused monochromatic radiation at 10 keV ($\lambda = 0.124$ nm) was passed through 0.3×2.0 mm slits (0.3 mm in the direction of lowest beam divergence). Diffraction patterns were recorded on film as described above but using exposure times of 2 to 5 min and a flux at the sample of approx. $2 \cdot 10^{10}$ photons s^{-1}.

Dehydrated lipid samples were rapidly transferred into a 1.0 or 1.5 mm (i.d.) capillary tube and mounted on the camera. The X-ray exposure was started within 2 min of opening the equilibrated sample. Diffraction patterns were collected at 20°C (±1°C); the sample temperature was measured using a thermocouple placed outside the capillary in a position immediately adjacent to the sample position. Sample temperature was regulated with an FTS air-jet cooling system. Fully hydrated samples were examined at temperatures between −5°C and 60°C. Sample temperature was 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. At these flux levels, artifactual phase transitions can occur during short exposure times [14]. Therefore, samples subjected to repeated exposure to the X-ray beam were moved slightly between exposures to minimize radiation-induced damage of the sample.

3. Results

3.1. Hydration characteristics of lipid mixtures

The water contents of the DOPE/DOPC (1:1, mole ratio) mixture and mixtures of DOPE/DOPC/β-sitosterol (1:1:1 and 1:1:2) as a function of osmotic pressure are shown in Fig. 1A. In all of the mixtures, the water content decreased linearly with the natural logarithm of the osmotic pressure. At all osmotic pressures, the DOPE/DOPC (1:1) mixture had the highest water content and the hydration of the lipid dispersions was decreased by the addition of β-sitosterol. For example, at an osmotic pressure of

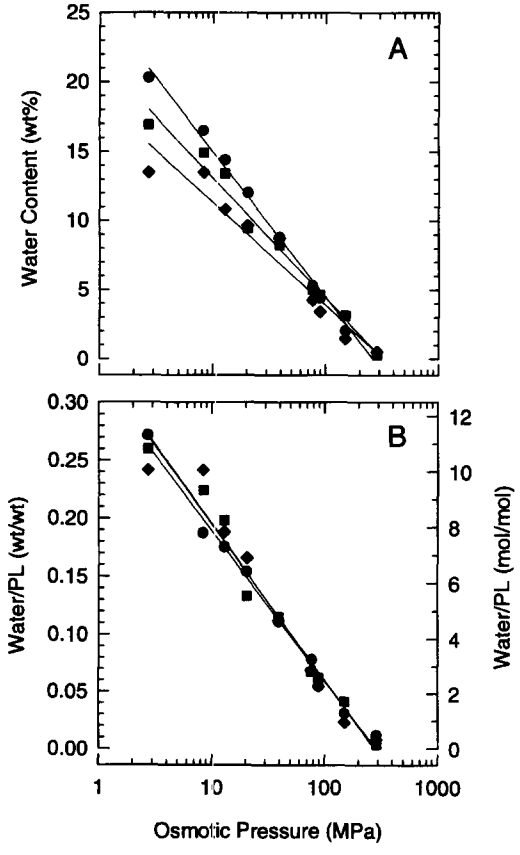

Fig. 1. Desorption isotherms for mixtures of DOPE/DOPC (1:1) (●), DOPE/DOPC/β-sitosterol (1:1:1) (■) and DOPE/DOPC/β-sitosterol (1:1:2) (◆). Water content of the samples is plotted as both (A) wt% of the total mixture and (B) normalized to the water/phospholipid (water/PL) ratio (wt/wt and mol/mol). Lines represent linear regressions; all regressions have $r^2 \geq 0.95$. Data represent the means ± S.D. from three experiments.

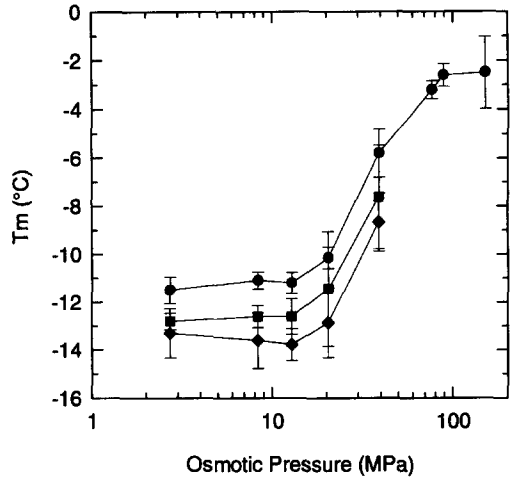

Fig. 2. Effect of dehydration on the $L_\beta \rightarrow L_\alpha$ phase transition temperature (T_m) of mixtures of DOPE/DOPC (1:1) (●), DOPE/DOPC/β-sitosterol (1:1:1) (■) and DOPE/DOPC/β-sitosterol (1:1:2) (◆). Transition temperature is plotted as a function of the osmotic pressure at which the samples were equilibrated. Data represent the means ± S.D. from three experiments.

12.8 MPa, the water contents of the DOPE/DOPC (1:1) and DOPE/DOPC/β-sitosterol (1:1:1 and 1:1:2) dispersions were 14.4, 13.4 and 10.8 wt% respectively. However, there were no significant differences between the mixtures when the water contents were expressed as water/phospholipid (wt/wt) ratios (Fig. 1B). Therefore, if it is assumed that the hydration of β-sitosterol was negligible and that the sterols did not alter phospholipid hydration, these results suggest that the water present in the desorbed lipid mixtures was predominantly associated with the phospholipids DOPE and DOPC.

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

The temperature of the $L_\alpha \rightarrow L_\beta$ phase transition (T_m) of the lipid mixtures in excess water was determined by DSC. The T_m of DOPE/DOPC (1:1) dispersions in excess water was −11°C [11]. In mixtures of DOPE/DOPC/β-sitosterol (1:1:1 and 1:1:2), the T_m of the fully hydrated dispersions was decreased to approx. −13°C (data not shown).

The T_m of dehydrated lipid mixtures was also determined by DSC. Dehydration of mixtures of DOPE/DOPC (1:1) at osmotic pressures over the range of 2.7 to 150 MPa resulted in an increase of T_m from −11°C to −2.5°C (Fig. 2). The first significant increase of T_m occurred after equilibration at an osmotic pressure of 20.4 MPa (Fig. 2). In mixtures containing β-sitosterol, the T_m increased from approx. −13°C at 2.7 MPa to approx. −8°C at 38.9 MPa (Fig. 2). As observed for the DOPE/DOPC (1:1) mixtures, the increase in T_m first occurred after equilibration at an osmotic pressure of 20.4 MPa. Over the range of 2.7 to 38.9 MPa, the T_m values for the 1:1:1 and 1:1:2 mixtures

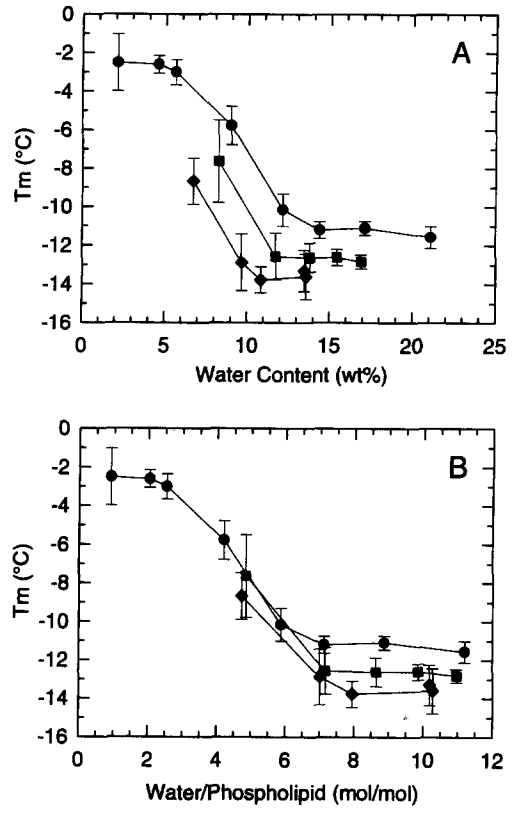

Fig. 3. Effect of dehydration on the $L_\beta \rightarrow L_\alpha$ phase transition temperature (T_m) of mixtures of DOPE/DOPC (1:1) (●), DOPE/DOPC/β-sitosterol (1:1:1) (■) and DOPE/DOPC/β-sitosterol (1:1:2) (◆). Transition temperature is plotted as a function of either (A) the water content measured for the total lipid mixture or (B) the water/phospholipid ratio (mol/mol) calculated for the desorbed dispersions. Data represent the means ± S.D. from three experiments.

of DOPE/DOPC/β-sitosterol were approx. 1.5°C and 2.5°C lower than those for the DOPE/DOPC (1:1) mixtures. At osmotic pressures greater than 38.9 MPa, the water contents of the DOPE/DOPC/β-sitosterol mixtures were less than 4.5–5 wt% and, therefore, were not accurately measured by DSC [11].

The effect of β-sitosterol on the dehydration-induced increase of the T_m of these mixtures was also observed when T_m, was expressed as a function of water content (Fig. 3A,B). The increase of the T_m of DOPE/DOPC (1:1) was observed in samples dehydrated to water contents below approx. 12 wt%. In mixtures of DOPE/DOPC/β-sitosterol, the T_m values also increased at water contents below 12 wt%, but were approx. 2–3 C° lower than those for the DOPE/DOPC (1:1) mixtures (Fig. 3A). The influence of β-sitosterol on the T_m of the mixtures was less apparent when the T_m was expressed as a function of the water/phospholipid mole ratio (Fig. 3B). This is consistent with the conclusion that most or all of the water present in the mixtures containing sterols was associated with the phospholipids DOPE and DOPC (Fig. 1B).

All of the DSC scans showed a single endothermic peak due to the lipid component of the samples; there was no evidence of a lipid-lipid phase separation. However, the presence of β-sitosterol in DOPE/DOPC mixtures effected a broadening of the temperature range over which the phase transition occurred. This effect was very similar to that observed for cholesterol on the $L_\alpha \rightarrow L_\beta$ phase transition of unsaturated phospholipids [15]. At the lower osmotic pressures (2.7 to 12.8 MPa), an additional sharp endothermic transition at 0°C was also observed. This transition was the result of the melting of ice formed from water that evaporated from the sample and subsequently condensed on the lid of the DSC pan, which had a lower temperature than the sample because of the temperature gradient in the DSC sample holder [10,16]. Representative DSC scans of the three mixtures dehydrated at 12.8 MPa are shown in Fig. 4. At water contents less than 4.5–5 wt%, the phase transition temperatures were not accurately measured by the scan rate employed in this study [11] and are not reported.

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

Dispersions of the DOPE/DOPC/β-sitosterol (1:1:1 and 1:1:2) mixtures, either in excess water or after equilibration at osmotic pressures between 2.8 and 286 MPa, were examined by FFEM and X-ray diffraction. In agreement with the DSC data presented 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. Dispersions of DPPC were examined by X-ray diffraction at 20°C as a control to confirm the position of the wide-angle reflections of the L_β phase; a sharp wide-angle reflection at 0.424 nm, characteristic of the L_β phase, was observed for DPPC (data not shown).

A summary of the phases present in these lipid mixtures as a function of osmotic pressure 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 approx. 0.46 nm and the occurrence of 3–5 diffraction orders in the small angle region indexing to ratios of 1:2:3.... At water contents between 21 wt% and 9 wt%, the DOPE/DOPC (1:1) mixture was present in either the L_α phase or as two separate L_α phases [11]. These water contents were typically achieved by desorption at osmotic pressures between approx. 2.7 and 38.9 MPa. The H_{II} phase was first observed at 76.1 MPa, equivalent to a water content of approx. 5.63 wt% and equivalent to a hydration of 2.53 mol H_2O/mol phospholipid (Table 1). In mixtures of DOPE/DOPC (1:1), both the lamellar and H_{II} phase were observed at 76.1 and 88.5 MPa, and the H_{II} phase was the only phase present at higher osmotic pressures [11].

The addition of β-sitosterol to the DOPE/DOPC mixture caused a significant reduction in the osmotic pressure required to elicit formation of the H_{II} phase. In the DOPE/DOPC/β-sitosterol (1:1:1) mixture, only the L_α

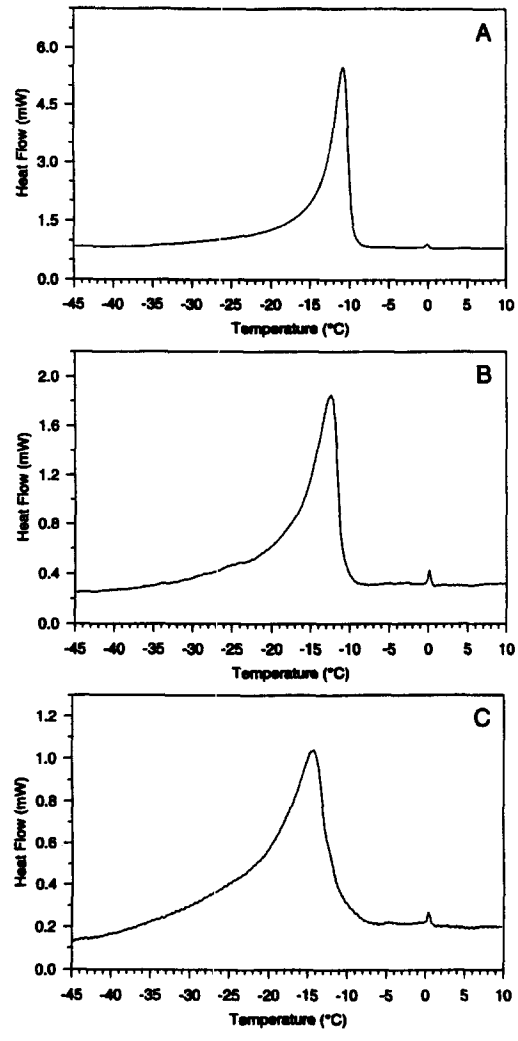

Fig. 4. DSC thermograms of (A) DOPE/DOPC (1:1), (B) DOPE/DOPC/β-sitosterol (1:1:1) and (C) DOPE/DOPC/β-sitosterol (1:1:2) after desorption at an osmotic pressure of 12.8 MPa. The T_m (peak) and onset of the peak (as calculated by the Perkin-Elmer DSC-7 software) were −10.7°C and −13.8°C for DOPE/DOPC (1:1), −12.6°C and −17.8°C for DOPE/DOPC/β-sitosterol (1:1:1) and −14.3°C and −21.3°C for DOPE/DOPC/β-sitosterol (1:1:2). The samples were scanned from −50°C to 30°C at 10 C° min^{-1}.

phase was observed after equilibration at 12.8 MPa (Table 1). A mixture of L_α and H_{II} phases was observed by both FFEM and X-ray diffraction after desorption at 20.4 MPa, corresponding to a hydration of 7.16 mol H_2O/mol phospholipid (Table 1 and Fig. 5a,b). At 38.9 MPa, both lamellar and H_{II} phases were observed by FFEM (Fig. 5c,d); the X-ray diffraction patterns were too complex to unambiguously assign (which we interpret as being indicative of the coexistence of multiple phases, possibly including a cubic phase). At this osmotic pressure, we also observed the occurrence of lamellae with a very regular rippled pattern that merged continuously with the H_{II} phase (Fig. 5d). At the higher osmotic pressures of 76.1 and 88.5 MPa, X-ray diffraction indicated only the presence of the H_{II} phase (Table 1); these samples were not examined by FFEM.

The dehydration-induced $L_\alpha \rightarrow H_{II}$ phase behavior of the 1:1:2 mixture of DOPE/DOPC/β-sitosterol was similar to that of the 1:1:1 mixture. The lamellar phase was the only phase present in dispersions of DOPE/DOPC/β-sitosterol (1:1:2) dehydrated at osmotic pressures ≤ 12.8 MPa at 20°C (Fig. 6a). However, at this osmotic pressure, ultrastructural alterations suggestive of bilayer destabilization preceding the $L_\alpha \rightarrow H_{II}$ phase transition were observed (Fig. 6b). Specifically, regions containing a high density of lipidic particles (Fig. 6b) indicative of close apposition of membranes, membrane joining and subsequent membrane fusion [17] were observed. The H_{II} phase was first observed in these mixtures after equilibration at 20.4 MPa, which resulted in a hydration of 6.99 mol H_2O/mol phospholipid (Table 1 and Fig. 6c,d). At this osmotic pressure, significant amounts of both lamellar (Fig. 6c) and H_{II} phases (Fig. 6d) were observed. As observed at 12.8 MPa, the lamellar domains were frequently observed to contain a rippled pattern similar to the H_{II} phase (Fig. 6c,d). At an osmotic pressure of 38.9 MPa, the H_{II} phase became the dominant phase; at 76.1 MPa, it was the only phase present (Table 1). As with the 1:1:1 mixture of DOPE/DOPC/β-sitosterol at 38.9 MPa, the small angle X-ray diffraction pattern was extremely complex and was interpreted as indicative of the co-existence of multiple phases; the possibility of a cubic phase in this mixture cannot be excluded.

In all lipid mixtures, the lamellar repeat spacing decreased with decreasing hydration. Repeat spacings for the DOPE/DOPC (1:1) mixture decreased from 5.8 nm at full hydration to 4.0–4.2 nm at hydrations just prior to the formation of the H_{II} phase [11]. In both 1:1:1 and 1:1:2 mixtures of DOPE/DOPC/β-sitosterol, the lamellar repeat spacings decreased from 6.75–6.80 nm at full hydra-

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

MPa	DOPE/DOPC	DOPE/DOPC/β-sitosterol	
	1:1	1:1:1	1:1:2
0	L_α	L_α	L_α
2.7	L_α (11.2)	–	–
8.3	L_α [a] (9.87)	–	L_α (10.27)
12.8	L_α [a] (7.13)	L_α (8.63)	L_α (7.94)
20.4	L_α [a] (5.87)	L_α/H_{II} (7.16)	L_α/H_{II} (6.99)
38.9	L_α [a] (4.21)	H_{II}/L_α (4.85)	H_{II}/L_α (4.72)
76.1	L_α/H_{II} (2.53)	H_{II} (2.99)	H_{II} (3.05)
88.5	H_{II}/L_α (2.03)	H_{II} (2.65)	

Phases were determined by freeze-fracture electron microscopy, X-ray diffraction and differential scanning calorimetry. The hydrations (mol H_2O/mol phospholipid) for the desorbed dispersions are given in parentheses; for samples at 0 MPa, the water contents were approx. 50 wt%. At osmotic pressures where two phases coexisted, the predominant phase is listed first

[a] Two coexisting fluid lamellar phases [11].

tion to approx. 5.2–5.5 nm at hydrations just prior to the formation of the H_{II} phase (not shown). Therefore, the lower water content of DOPE/DOPC mixtures containing β-sitosterol, compared to those lacking β-sitosterol (Fig. 1), was not reflected as a decreased lamellar repeat spacing in dehydrated mixtures. For the 1:1:1 and 1:1:2 mixtures of DOPE/DOPC/β-sitosterol present in the H_{II} phase, the inter-tube repeat distances decreased from 5.6 and 6.2 nm

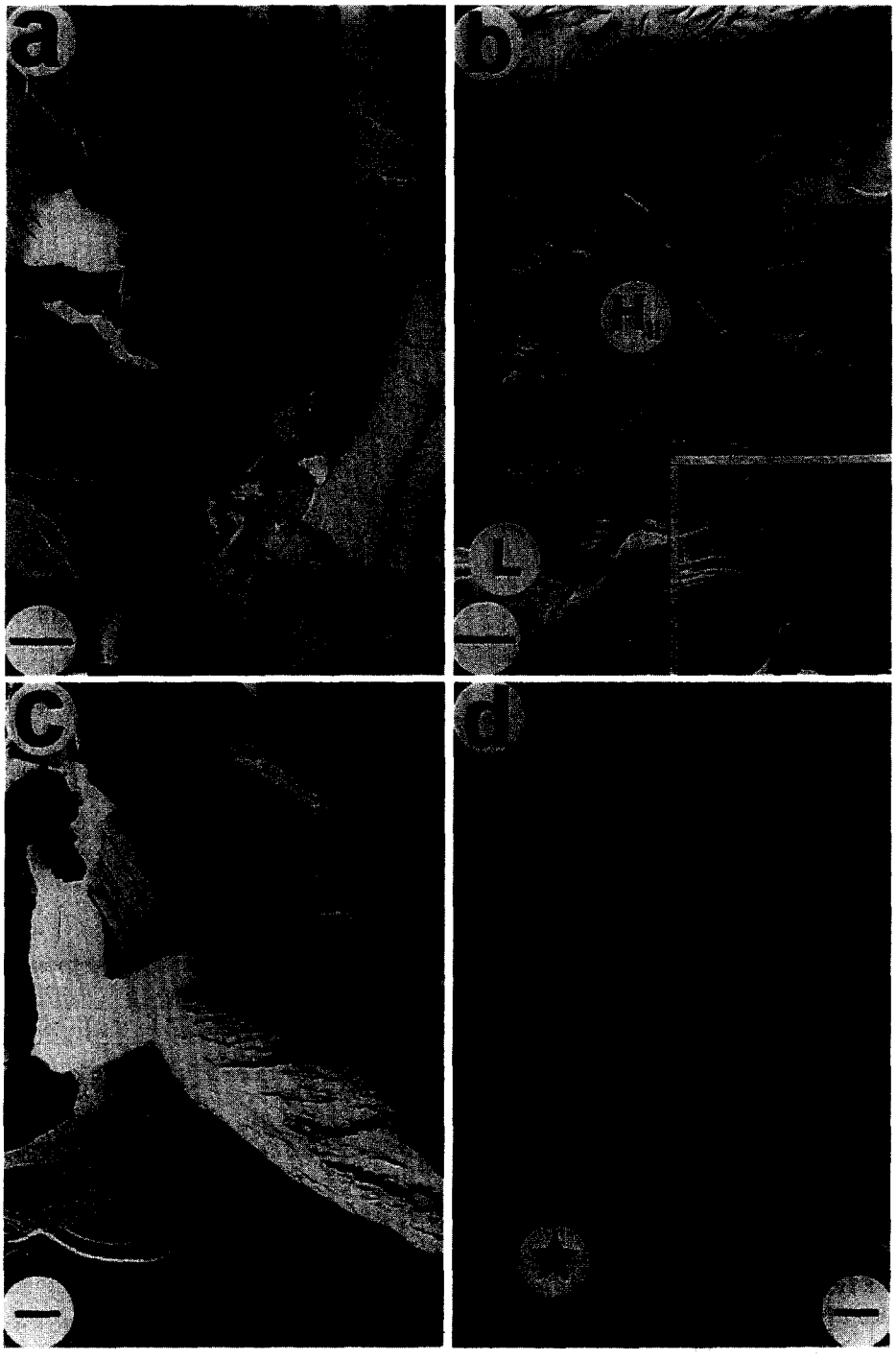

Fig. 5. Freeze-fracture electron micrographs of mixtures of DOPE/DOPC/β-sitosterol (1:1:1) desorbed at osmotic pressures of 20.4 (a,b) and 38.9 (c,d) MPa. At 20.4 MPa, the lamellar phase (L) was the predominant phase present (a) but regions of co-existing lamellar and inverted hexagonal (H_{II}) phase were also observed (b). At 38.9 MPa, larger domains of the H_{II} phase were observed (c) as well as regions where the H_{II} phase made a smooth transition to the lamellar phase via rippled intermediate structures (★, d). Inset in (b) shows the H_{II} phase region at higher magnification, indicating the presence of inverted cylindrical micelles. Bars represent 300 (a), 500 (b) and 200 (c,d) nm.

(respectively) at 20.4 MPa to approx. 5.10 nm at osmotic pressures $\geq$ 76.1 MPa (not shown).

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

Because freezing results in both thermal and dehydration stresses, the influence of the free sterols on the dehydration-induced formation of the H_{II} phase was compared with their influence on the thermally induced $L_\alpha \rightarrow H_{II}$ phase transition using X-ray diffraction.

Fully hydrated mixtures of DOPE/DOPC (1:1) were in the L_α phase at 20°C. During warming, the L_α phase existed from −2°C to 50°C (Fig. 7). The H_{II} phase in hydrated mixtures of DOPE/DOPC (1:1) was first ob-

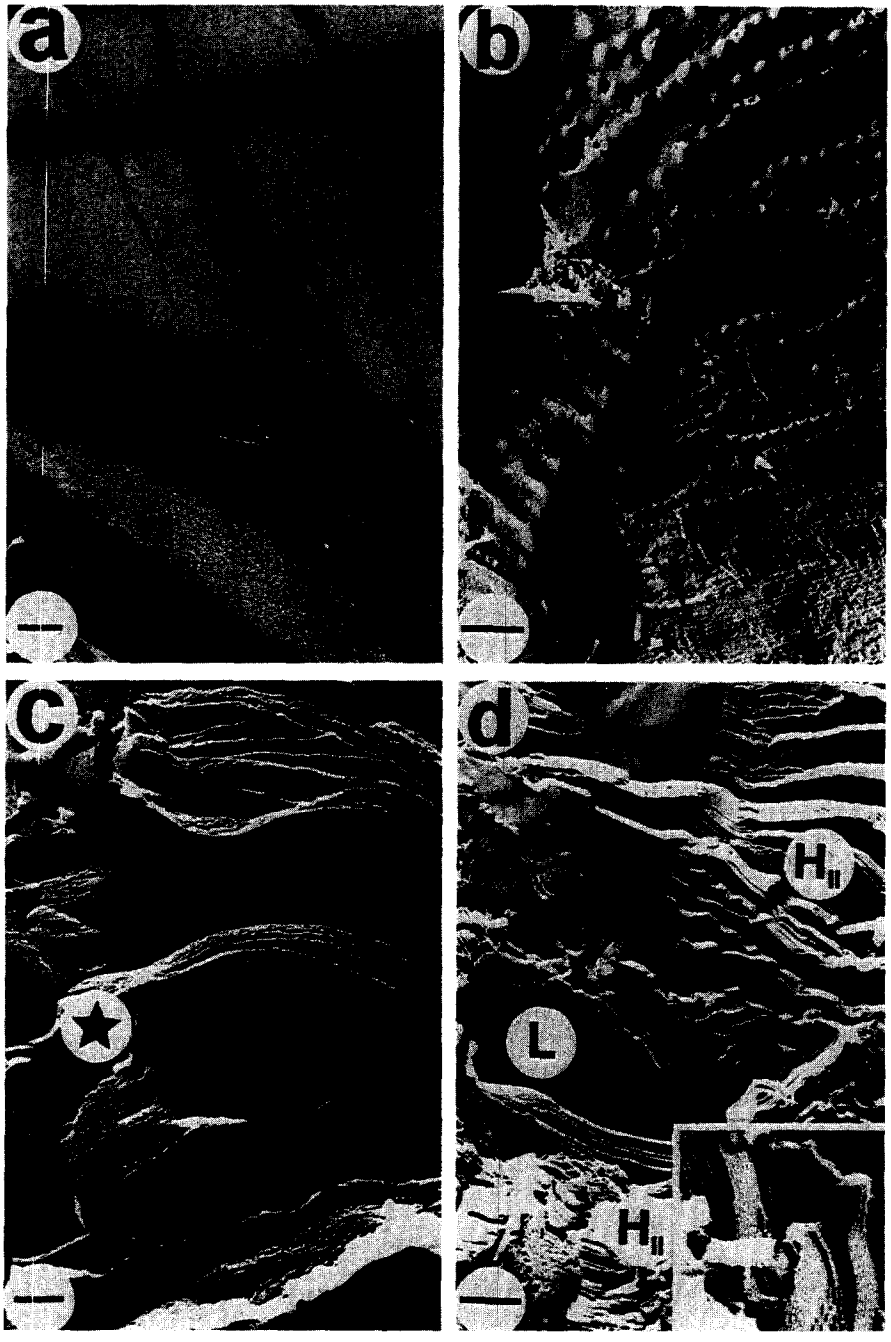

Fig. 6. Freeze-fracture electron micrographs of mixtures of DOPE/DOPC/β-sitosterol (1:1:2) desorbed at osmotic pressures of 12.8 (a,b) and 20.4 (c,d) MPa. At 12.8 MPa, the lamellar phase (L) was the only phase present (a), but domains containing lipidic particles indicative of bilayer destabilization preceding the $L_\alpha \rightarrow H_{II}$ phase transition were also observed (b). At 20.4 MPa, both lamellar (c) and H_{II} phases (d) were observed. The lamellar domains frequently contained a rippled pattern similar to the organization of the H_{II} phase (★, d). Inset in (d) shows the H_{II} phase region at higher magnification, indicating the presence of inverted cylindrical micelles. Bars represent 200 (a), 400 (b), 200 (c) and 300 (d) nm.

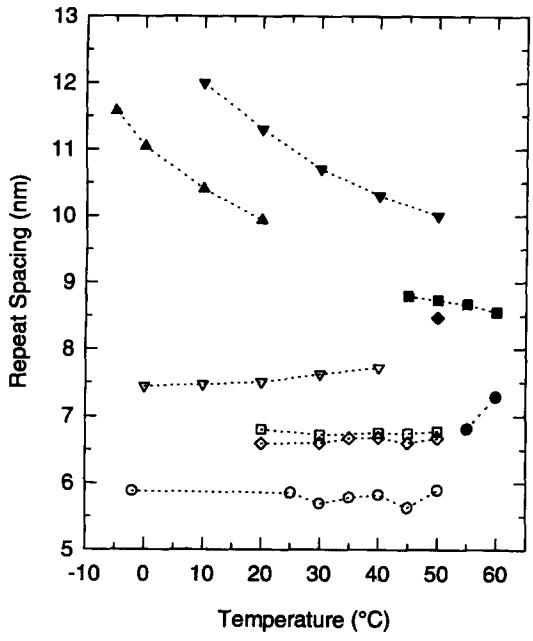

Fig. 7. Repeat spacings of the L_α and H_{II} phase of fully hydrated mixtures of DOPE/DOPC (1:1) (○, ●), DOPE/DOPC/β-sitosterol (1:1:1) (□, ■), DOPE/DOPC/β-sitosterol (1:1:2) (◇, ◆), DOPE/DOPC/ASG (1:1:1) (▲) and DOPE/DOPC/ASG (2:2:1) (▽, ▼) as a function of temperature. Repeat spacings for the lamellar phase are shown as open symbols, inter-tube distances for the H_{II} phase are shown as closed symbols. Temperatures at which both phases coexisted have both symbols. Samples at temperatures below 0°C were supercooled.

served at 55°C; therefore the inception of the $L_\alpha \rightarrow H_{II}$ phase transition in this dispersion was between 50 and 55°C. In a 1:1:1 mixture of DOPE/DOPC/β-sitosterol, the L_α phase was observed from 20°C to 50°C; the H_{II} phase was first observed at 45°C (Fig. 7 and Table 2). Similarly, the 1:1:2 mixture of DOPE/DOPC/β-sitosterol was in the L_α phase from 20°C to 50°C; the H_{II} phase was observed at 50°C (Fig. 7). Therefore, β-sitosterol promoted the thermally induced formation of the H_{II} phase in DOPE/DOPC mixtures; this was observed as a decrease in the T_{bh} of the fully hydrated lipid mixtures of approx. 5°C to 10°C.

Table 2
Summary of the phases and phase dimensions of fully hydrated DOPE/DOPC mixtures containing 0, 33 or 50 mol% β-sitosterol as a function of temperature

T (°C)	DOPE/DOPC	DOPE/DOPC/β-sitosterol	
	1:1	1:1:1	1:1:2
20	–	L_α	L_α
25	L_α	–	–
30	L_α	L_α	L_α
35	L_α	–	L_α
40	L_α	L_α	L_α
45	L_α	L_α/H_{II}	L_α
50	L_α	L_α/H_{II}	L_α/H_{II}
55	H_{II}	H_{II}	?
60	H_{II}	H_{II}	?

Data were obtained by X-ray diffraction as described in the Materials and Methods

In the DOPE/DOPC (1:1) mixture, the lamellar repeat spacing was relatively constant at approx. 5.8 nm between −2°C and 50°C (Fig. 7). After formation of the H_{II} phase at 55°C, the inter-tube spacing was approx. 7 nm at 55°C and 60°C. These measured values of T_{bh} and repeat spacing for DOPE/DOPC (1:1) mixtures are very similar to those reported previously for DOPE/DOPC (3.17:1) dispersions [18]. Dispersions of DOPE/DOPC/β-sitosterol had lamellar repeat spacings that remained constant at 6.7 nm between 20°C and 50°C. Upon formation of the H_{II} phase, the repeat spacing increased to 8.8 nm and then decreased to 8.5 nm with increasing temperature up to 60°C (Fig. 7). The repeat spacing of the H_{II} phase in the 1:1:2 mixture of DOPE/DOPC/β-sitosterol at 50°C was similar to that of the 1:1:1 mixture, but was not examined at temperatures above 50°C (Fig. 7).

3.5. Phase transitions of DOPE/DOPC/acylated steryl glucoside mixtures

The influence of acylated steryl glucosides (ASG) on both the dehydration-induced and thermally induced formation of the H_{II} phase in DOPE/DOPC mixtures was also examined. The influence of acylated steryl glucosides was of interest because they are present in much higher proportions in the plasma membrane of oat (27 mol% of the total lipids) than in rye (3 mol%) [4], and oat has a greater propensity to form the H_{II} phase during freeze-induced dehydration [3].

Mixtures of DOPE/DOPC/ASG (1:1:1) were equilibrated at osmotic pressures between 0 (excess water) and 76 MPa at 20°C and examined by X-ray diffraction. At 20°C the fully hydrated mixture of DOPE/DOPC/ASG (1:1:1) was in the H_{II} phase, with an inter-tube distance of 9.93 nm (Fig. 7). That is, no dehydration was required to elicit formation of the H_{II} phase. Dehydration of this mixture resulted in no phase change, but did cause a decrease in the inter-tube repeat spacing from 9.93 nm in excess water to 5.93, 5.67 and 5.19 nm at 20.4, 38.9 and 76.1 MPa, respectively (not shown). Therefore, ASG was significantly more effective than free sterols at promoting the dehydration-induced formation of the H_{II} phase. That is, the DOPE/DOPC/β-sitosterol (1:1:1) mixture required dehydration at an osmotic pressure of 20.4 MPa to elicit the $L_\alpha \rightarrow H_{II}$ phase transition, whereas the DOPE/DOPE/ASG (1:1:1) mixture required no dehydration for the same phase transition.

The extent to which ASG promoted the $L_\alpha \rightarrow H_{II}$ phase transition was evaluated by comparing the T_{bh} for fully hydrated DOPE/DOPC/ASG (1:1:1) with that of fully hydrated mixtures of DOPE/DOPC/β-sitosterol (1:1:1)

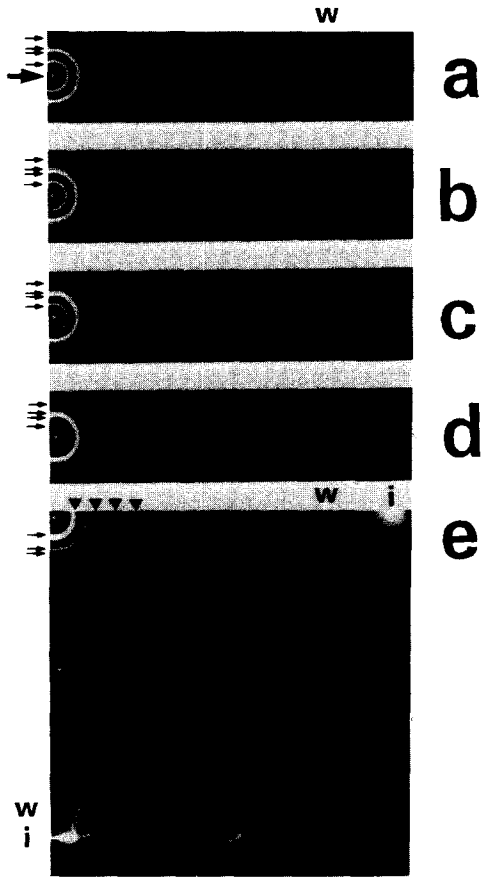

Fig. 8. X-ray diffraction powder patterns of fully hydrated mixtures of DOPE/DOPC/ASG (1:1:1) at 20°C (a), 10°C (b), 0°C (c), supercooled at −5°C (d), and same sample as (d) after ice nucleation and freeze-induced dehydration for 30 min at −5°C (e). The center of the X-ray beam is indicated by the large arrow in (a). In all plates, the small-angle 1;0, 1;1, 2;0 and 2;1 reflections of the H_{II} phase are indicated with small arrows (except for (e), in which the 2;1 reflection is not visible on this negative). In addition, in (e), the 1;0, 2;0, 3;0 and 4;0 reflections of the lamellar phase are indicated by arrowheads. The diffuse wide angle reflections indicative of disordered acyl chains are noted by 'w'; the speculate wide angle reflections arising from the presence of ice are indicated by 'i' in (e).

(45°C to 50°C). When fully hydrated mixtures of DOPE/DOPC/ASG (1:1:1) were cooled from 20°C to −5°C, the H_{II} phase inter-tube repeat spacing increased from 9.93 to 11.6 nm, but no transition to the L_α phase was observed – even in samples supercooled at −5°C (Figs. 7 and 8). However, in samples in which ice nucleation was effected at −5°C and then allowed to freeze-dehydrate for 30 min at −5°C, both the L_α and H_{II} phases having repeat spacings of 5.97 and 7.53 nm, respectively, were observed (Fig. 8).

In preliminary X-ray diffraction studies of fully hydrated mixtures of DOPE/DOPC/ASG containing a smaller proportion of ASG (2:2:1), only the L_α phase was observed at 0°C. At temperatures over the range of 10°C to 40°C, both L_α and H_{II} phases were observed. With increasing temperature between 10°C and 40°C, the proportion of lipid in the H_{II} phase increased (not shown), and the repeat spacing of the H_{II} phase decreased. At 50°C, only the H_{II} phase was observed (Fig. 7).

4. Discussion

During the freeze-induced dehydration of non-acclimated rye and oat protoplasts, localized regions of the plasma membrane that are in close apposition with subtending lamellae undergo the $L_\alpha \rightarrow H_{II}$ phase transition [2,3]. However, freeze-induced formation of the H_{II} phase occurs at significantly higher subzero temperatures and lower osmotic pressures in oat than in rye [5]. To determine the influence of specific lipids on the propensity of a lipid mixture to undergo the dehydration-induced $L_\alpha \rightarrow H_{II}$ phase transition, we have examined the effects of free sterols and acylated steryl glucosides on the hydration and phase characteristics of DOPE/DOPC mixtures.

The presence of β-sitosterol in equimolar mixtures of DOPE/DOPC reduced the bilayer water content (Fig. 1A). This result was consistent with the low hydration determined for sterols purified from non-acclimated rye leaves (Lynch, D.V. and Steponkus, P.L., unpublished results) and for cholesterol [19–21]. However, when the bilayer water content was expressed as a ratio of water/phospholipid (either wt/wt or mol/mol), there was no difference in the hydration of these three mixtures (Fig. 1B). These results indicate that hydration of β-sitosterol in the bilayer is negligible and that the water remaining in the desorbed mixtures was associated predominantly with the phospholipids. In contrast, it has been previously reported that the addition of 50 mol% cholesterol increased the hydration per phospholipid molecule of egg PC [19] but decreased the hydration of egg PE [20]. It should be noted that the decreased water content in mixtures containing β-sitosterol was not associated with a reduction of inter-bilayer separation. The repeat spacing (5.8 nm) of fully hydrated DOPE/DOPC (1:1) at 25°C was increased to approx. 6.7 to 6.8 nm in the 1:1:1 and 1:1:2 mixtures of DOPE/DOPC/β-sitosterol (Fig. 7). Similarly, at low osmotic pressures ($\leq$ 6 MPa), equimolar mixtures of cholesterol-egg PC had repeat spacings approx. 0.3 nm larger that did egg PC [22]. Since egg-PC-cholesterol dispersions undergo only small changes in bilayer thickness during dehydration [22], it is likely that the bilayer thickness was relatively constant in the DOPE/DOPC/β-sitosterol mixtures and that the decreased repeat spacings observed in these dispersions were predominantly due to dehydration. Therefore, the increased propensity of mixtures of DOPE/DOPC/β-sitosterol to undergo the dehydration-induced formation of the H_{II} phase was not associated with decreased interbilayer distances – despite the lower water content of these mixtures.

The presence of β-sitosterol lowered the $L_\beta \rightarrow L_\alpha$ phase

transition temperature by approx. 2°C at all hydrations in the range between full hydration and 5 wt% water. It is possible that β-sitosterol interferes with the decrease in lateral lipid-lipid spacing and the increase in lipid-lipid attractive forces that accompany the transition to the gel phase, thus lowering the transition temperature. β-Sitosterol and cholesterol have very similar effects on the $L_\beta \rightarrow L_\alpha$ phase transition of DPPC [23], orientational order and rotational diffusion in DOPC bilayers [24], and on increasing the order parameter of DMPC in soy PC bilayers as measured by ^{2}H-NMR [25]. The results reported here are consistent with the report that 30 mol% cholesterol slightly reduced the $L_\beta \rightarrow L_\alpha$ phase transition temperature of fully hydrated DOPC, but did not eliminate the phase transition [15]. Overall, these results indicate that, in membranes containing significant proportions of β-sitosterol, the $L_\alpha \rightarrow L_\beta$ phase transition is deferred to lower temperatures, although it is not deferred to higher osmotic pressures or lower water contents. Therefore, sterols may reduce the tendency of membranes to undergo the $L_\alpha \rightarrow L_\beta$ phase transition during freeze-induced dehydration [26].

In contrast to the effect of β-sitosterol on the dehydration-induced $L_\alpha \rightarrow L_\beta$ phase transition, the presence of β-sitosterol in mixtures of DOPE/DOPC promoted the dehydration-induced formation of the H_{II} phase at higher hydrations and water contents. In mixtures containing β-sitosterol, the threshold osmotic pressure for the first observation of the H_{II} phase was 20 MPa vs. 76 MPa in DOPE/DOPC (1:1) mixtures, which represents an increased hydration at which the H_{II} phase first occurred (expressed as both wt% water and water/phospholipid ratio) (Table 1). A primary influence of β-sitosterol is probably a reduction of lateral packing pressure in the surface region of the lipid monolayers, which is effected by reduced surface water content (Fig. 1a) and reduced steric interactions between phospholipid headgroups, consequently promoting a spontaneous monolayer curvature favoring formation of the H_{II} phase [12]. In addition, β-sitosterol is expected to increase the lateral packing pressure within the hydrophobic region of the bilayer, which also results in a monolayer curvature favoring the $L_\alpha \rightarrow H_{II}$ phase transition. Increased disorder in the acyl domain of the lipid monolayers caused by the presence of β-sitosterol is consistent with the observation of lower T_m values for the mixtures containing β-sitosterol (Fig. 2).

β-Sitosterol also promoted the thermally induced $L_\alpha \rightarrow H_{II}$ phase transition of fully hydrated lipid mixtures. That is, T_{bh} decreased from approx. 55°C in the 1:1 mixture of DOPE/DOPC to approx. 45°C to 50°C in the 1:1:1 and 1:1:2 mixtures of DOPE/DOPC/β-sitosterol (Table 2 and Fig. 7). Similarly, cholesterol promotes the thermally induced $L_\alpha \rightarrow H_{II}$ phase transition in a fully hydrated mixture of DOPE/DOPC at 40°C [27] and the addition of cholesterol at 50 mol% to fully hydrated DOPE/DOPC (1:1) results in the formation of the H_{II} phase at 20°C as detected by ^{31}P-NMR [28]. The absence of the H_{II} phase in a 1:1:2 mixture of DOPE/DOPC/β-sitosterol in excess water at 20°C, as detected by X-ray diffraction (Table 1 and Fig. 7), suggests that there may be a difference between β-sitosterol and cholesterol in their ability to promote the thermally induced $L_\alpha \rightarrow H_{II}$ phase transition.

It should be noted that we have occasionally observed inverted lipidic micelles in the freeze-fracture replicas of these dehydrated dispersions at osmotic pressures lower than those at which the $L_\alpha \rightarrow H_{II}$ phase transition occurred (Fig. 6b). Furthermore, X-ray diffraction patterns have regularly, but not invariably, suggested the presence of an inverted cubic phase at osmotic pressures close to those at which the H_{II} phase was first observed. Despite this, an unambiguous inverted cubic phase was not observed by freeze-fracture electron microscopy, and it could not be concluded whether the $L_\alpha \rightarrow H_{II}$ phase transition proceeded via an inverted cubic phase intermediate.

Alternatively, we previously demonstrated that the dehydration-induced formation of the H_{II} phase in mixtures of DOPE/DOPC was preceded and promoted by the fluid-fluid demixing of DOPE-and DOPC-enriched domains as a consequence of their different hydration characteristics [11]. However, in the DOPE/DOPC/β-sitosterol mixtures examined in this study, two separate lamellar phases were not observed at osmotic pressures lower than those at which the H_{II} phase was first observed. There are several possible explanations for this. First, demixing may occur at osmotic pressures intermediate between those at which the L_α (12.98 MPa) and the L_α/H_{II} (20.4 MPa) phases were observed. Alternatively, the absence of two fluid-lamellar phases in dehydrated DOPE/DOPC/β-sitosterol mixtures may indicate that the free sterols either promoted the miscibility of DOPE and DOPC during dehydration or altered the interactions between adjacent lamellae such that the long-range order necessary for the detection of discrete lamellar domains by X-ray diffraction did not occur. As we cannot distinguish between these possibilities, it is not possible to state whether fluid-fluid phase demixing occurred in these mixtures or if demixing promoted the dehydration-induced formation of the H_{II} phase.

Acylated steryl glucosides were much more effective than free sterols at promoting formation of the H_{II} phase: dehydration of the 1:1:1 mixture of DOPE/DOPC/ASG was not required to elicit the $L_\alpha \rightarrow H_{II}$ phase transition. Furthermore, the T_{bh} for the DOPE/DOPC/ASG (1:1:1) mixture in excess water (≤ -5°C) was significantly lower than that for the hydrated mixture of DOPE/DOPC/β-sitosterol (1:1:1) (45°C). Even when ASG was present at a lower proportion (i.e., 20 mol%), the H_{II} phase coexisted with the L_α phase to $\leq$ 10°C. It must be emphasized that the acylated steryl glucosides are present in significantly higher proportions in the plasma membrane of oat (27 mol%) than of rye (3 mol%) [4] and that a recent freeze-fracture analysis has shown that oat is significantly more

prone than rye to undergo the $L_\alpha \rightarrow H_{II}$ phase transition during freeze-induced dehydration [3].

DOPE/DOPC/ASG (1:1:1) supercooled at −5°C was present in the H_{II} phase, but formed co-existing L_α and H_{II} phases after ice formation and 30 min at −5°C to allow for freeze-induced dehydration. Since the inter-tube repeat distance of the H_{II} phase decreased from 11.6 nm to 7.5 nm after ice formation, freeze-induced dehydration effected the removal of water from the H_{II} phase lattice and consequently caused both a reduction of the size of the H_{II} phase and dehydration of the lipid headgroups. It is likely that freeze-induced dehydration caused the lateral separation of non-bilayer-forming lipids, which are poorly hydrated, from bilayer-forming lipids, which are more hydrated, and facilitated the subsequent phase separation of H_{II} and L_α. The former domain was probably composed predominantly of DOPE and ASG, while the latter was enriched in DOPC. Previously, ice formation has been implicated in small increases (approx. 3–6 C°) of the T_m of DOPE and dioleoylphosphatidylserine occurring as a consequence of freeze-induced dehydration [29,30] and with the formation of the H_{II} phase in rye protoplasts [2]. To our knowledge, this is the first demonstration of a lipid phase transition elicited by ice formation in a model membrane system.

In previous studies, we established that the dehydration-induced formation of the H_{II} phase in DOPE/DOPC mixtures occurred at an osmotic pressure much greater (approx. 76 MPa) than that at which freeze-induced formation of the H_{II} phase occurs in rye protoplasts isolated from non-acclimated leaves (i.e., approx. 12 MPa at −10°C) [11]. However, the addition of β-sitosterol to the DOPE/DOPC mixture in proportions that are similar to those in the plasma membrane of winter rye causes the occurrence of the $L_\alpha \rightarrow H_{II}$ phase transition at a much lower osmotic pressure (20 MPa). Addition of cerebrosides (another major component of the plasma membrane) to the DOPE/DOPC/β-sitosterol mixture further lowers the osmotic pressure required to elicit the $L_\alpha \rightarrow H_{II}$ phase transition (Webb, Irving and Steponkus; in preparation). Thus, these mixtures provide appropriate models for studies of the dehydration-induced phase transitions in the plasma membrane.

The data presented here indicates that the acylated steryl glucosides are much more effective than free sterols at promoting both the dehydration-induced and thermally induced formation of the H_{II} phase. This is of considerable significance in considering genotypic differences in the lipid composition of the plasma membrane of rye and oat in relation to the large difference in freezing tolerance of these two species. Spring oat is significantly more susceptible to freezing injury than rye, with a high incidence of freeze-induced formation of the H_{II} phase occurring at −5°C in spring oat and −10°C in winter rye [3]. Although the total amount of free sterols, steryl glucosides and acylated steryl glucosides is similar in the plasma membrane of oat (41 mol% of the total lipids) and rye (47 mol%), oat contains a much higher proportion of acylated steryl glucosides (27 mol%) than does rye (3 mol%) [4].

In both winter rye and spring oat, cold acclimation results in a decreased propensity for freeze-induced formation of the H_{II} phase and significant alterations in the lipid composition of the plasma membrane [5]. These lipid changes include an increased proportion of phospholipids, particularly the di-unsaturated phospholipids, and a decreased proportion of cerebrosides; however, there is only a relatively small change in the proportion of free sterols in rye and a relatively small decrease in the proportion of acylated steryl glucosides in oat. Thus, it is unlikely that the decreased propensity for freeze-induced formation of the H_{II} phase in cold-acclimated seedlings is associated with changes in the proportion of free sterols in rye or acylated steryl glucosides in oat – even though differences in free sterols and acylated steryl glucosides appear to be associated with genotypic differences in the propensity for freeze-induced formation of the H_{II} phase in non-acclimated seedlings. Rather, it is likely that the increased proportions of phospholipids coupled with the decreased proportions of cerebrosides that occur during cold acclimation in both rye and oat alter the spontaneous curvature of the monolayers that comprise the plasma membrane and stabilize the plasma membrane in the lamellar phase during freeze-induced dehydration. Recent results have demonstrated that lowering the proportion of rye cerebrosides in mixtures of DOPE/DOPC/β-sitosterol from 20 mol% to 5 mol% defers the dehydration-induced $L_\alpha \rightarrow H_{II}$ phase transition to higher osmotic pressures (Webb, Irving and Steponkus; in preparation). Collectively, these and other studies [5] indicate that the lipid composition of the plasma membrane is a primary determinant of the cryostability of the plasma membrane during freeze-induced dehydration. However, it must be emphasized that the propensity of the plasma membrane to participate in the freeze-induced $L_\alpha \rightarrow H_{II}$ phase transition is not determined solely by the proportion of a single lipid component, but by the summed effects of the lipid mixture on bilayer hydration and on the spontaneous curvature of the monolayers.

In summary, at molar proportions similar to those found in the plasma membrane of rye, free sterols significantly alter the osmotic pressures, water contents, and temperatures at which phase transitions occur in DOPE/DOPC mixtures. β-Sitosterol deferred the $L_\alpha \rightarrow L_\beta$ phase transition to lower temperatures, but promoted the dehydration-induced $L_\alpha \rightarrow H_{II}$ phase transition at higher water contents (lower osmotic pressures) and the thermally induced $L_\alpha \rightarrow H_{II}$ phase transition to lower temperatures. Furthermore, acylated steryl glucosides, which are present in a higher proportion in the plasma membrane of oat than in rye, are much more effective at promoting the $L_\alpha \rightarrow H_{II}$ phase transition than are free sterols. These results provide strong evidence that the difference in the lipid composition of the plasma membrane of oat and rye has a significant influ-

ence on the propensity of non-acclimated plants of these species to undergo injurious formation of the H_{II} phase during freeze-induced dehydration.

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