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Lamellar-to-hexagonal H_{II} phase transitions in liposomes of rye plasma membrane lipids after osmotic dehydration *

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The plasma membrane of isolated protoplasts from non-acclimated but not from acclimated rye leaves undergoes lamellar-to-hexagonal H_{II} phase transitions after freeze-induced dehydration (Gordon-Kamm, W.J. and P.L. Steponkus (1984) Proc. Natl. Acad. Sci. USA 81, 6373–6377). An effort was made to determine whether plasma membrane lipid components show similar behavior when dehydrated. Liposomes were prepared from the total lipid mixture extracted from plasma membrane fractions isolated from non-acclimated (NA) or cold acclimated (ACC) rye leaves (*Secale cereale* L. cv Puma). Freeze-fracture electron microscopy was used to identify phase changes in liposomes after osmotic dehydration. When NA liposomes prepared in 1.3 wt% sorbitol were incubated at 4°C in 73 wt% sorbitol for 15 h (overnight), hexagonal H_{II} phase was observed in aggregates of apparently fused liposomes with H_{II} observed in approximately 75% of the fracture faces. In contrast, neither aggregates of apparently fused liposomes nor H_{II} phase was observed in ACC liposomes after dehydration under the same conditions. Dehydration of NA or ACC liposomes in 40 or 60 wt% sorbitol resulted in the formation of intermediate morphologies such as loosely ordered cylinders and ripple structures. Lipidic particles and pits of various sizes and morphologies were observed after dehydration of both NA and ACC liposomes in 40, 60, or 73 wt% sorbitol. These results indicate that lipid composition determines the propensity of the plasma membrane to undergo dehydration-induced phase transitions and that alterations in the lipid composition of the plasma membrane play a causal role in the process of cold acclimation.

A primary cause of freezing injury in plants is disruption of the semipermeable characteristics of the plasma membrane as a result of severe cell dehydration during the formation of ice in the suspending medium [1]. It has been suggested that lamellar-to-hexagonal H_{II} phase transitions in the plasma membrane may be, at least in part, responsible for this injury [1,2]. Recent evidence supporting this proposal is the documentation of lamellar-to-H_{II} phase transitions in the plasma membrane of isolated protoplasts after freeze- or osmotic-induced dehydration [3,4]. Importantly, H_{II} phase is observed in protoplasts isolated from non-acclimated (NA) rye leaves (*Secale cereale* L. cv Puma) but not in protoplasts isolated from rye leaves which have been acclimated to subzero

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Abbreviations: ACC, acclimated; H_{II}, hexagonal H_{II}; LN_2, liquid nitrogen; NA, non-acclimated; PC, phosphatidylcholine; PE, phosphatidylethanolamine; REV, reverse phase evaporation.

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temperatures. The minimal temperature for 50% survival of NA protoplasts is $-5\,^{\circ}C$; for acclimated (ACC) protoplasts, it is $-25\,^{\circ}C$ [5].

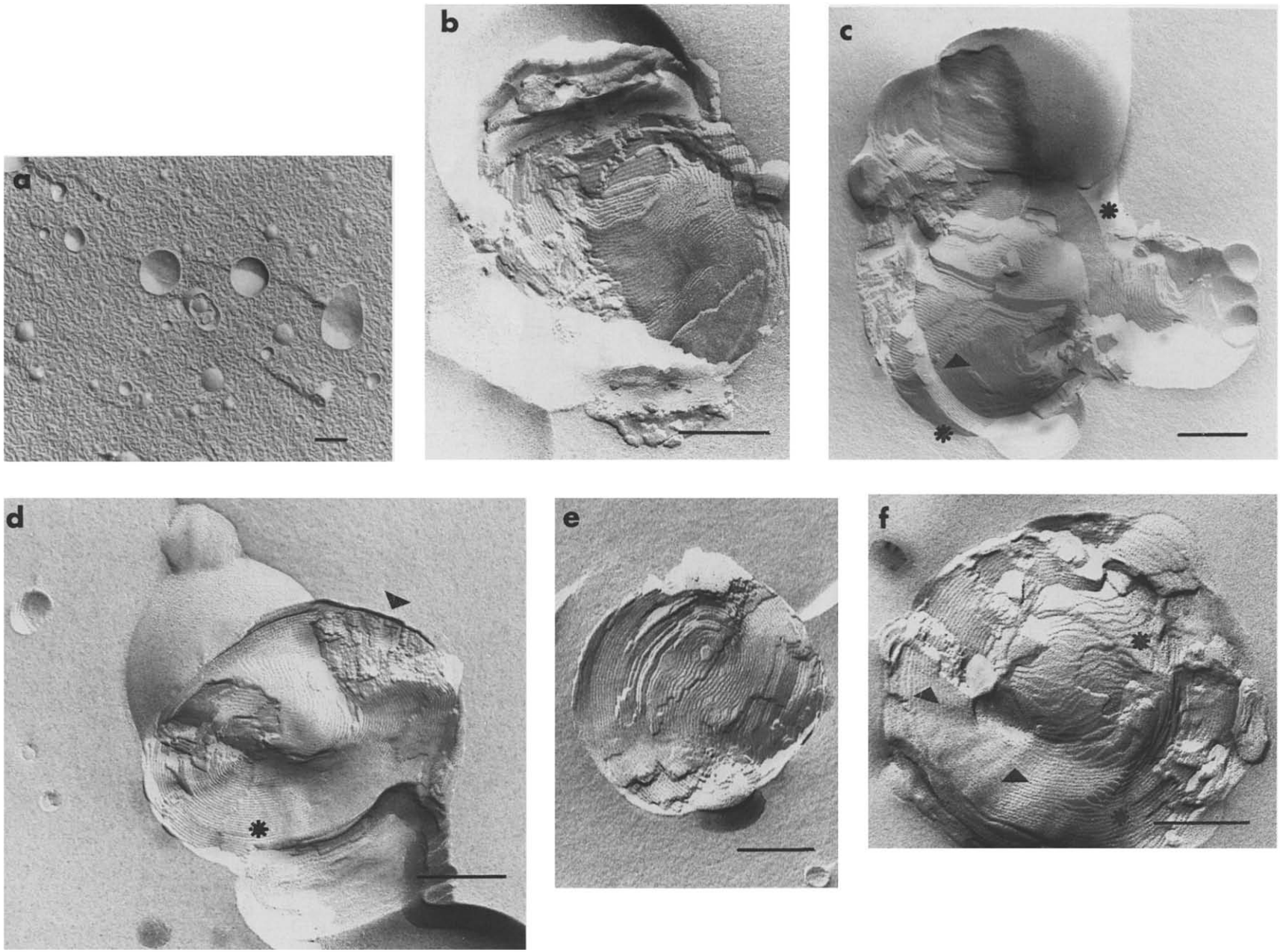
The plasma membrane of rye leaves is characterized by a high lipid to protein ratio (approx. 3.4 $\mu mol \cdot mg^{-1}$) and a lipid composition that is rather unique in comparison to other plant cell membranes [6]. Free sterols, steryl glucosides and acylated steryl glucosides comprise more than 50 mol% of the total lipids. The principal glycolipids (16 mol%) are glucocerebrosides. Phospholipids (predominantly phosphatidylcholine and phosphatidylethanolamine) comprise 32 mol% of the total lipids. (See Lynch and Steponkus [6] for a complete analysis at the molecular species level.) Cold acclimation alters the lipid composition of plasma membrane isolated from rye, resulting in a change in the proportion of virtually every lipid component [6]. These changes include increases in the mole percentages of free sterols and phospholipids, with decreases in the mole percentages of steryl glucosides, acylated steryl glucosides and glucocerebrosides. However, there is no single lipid molecular species that is unique to either NA or ACC plasma membranes. The lipid to protein ratio does not change dramatically with acclimation.

The objective of this study was to determine if the differential propensity for dehydration-induced lamellar-to-H_{II} phase transitions in the plasma membrane of NA and ACC protoplasts is a consequence of differences in the lipid composition of the plasma membrane. Although it is reasonable to assume that differences in the proportions of the various lipid components could influence the lyotropic phase behavior of the complex mixture of lipids found in the plasma membrane, other factors may also determine the differential behavior of the plasma membrane of NA and ACC protoplasts. For example, the decreased incidence of H_{II} formation in the plasma membrane of ACC protoplasts could be a consequence of alterations in plasma membrane proteins or cytoplasmic solutes such sucrose or proline. There are reports of the influence of proteins on lipid mesomorphic phase behavior (Cullis et al. 11) and a diverse array of carbohydrates preserve the structural and functional integrity of biological membranes during freezing and dehydration (see Crowe et al. [20]). Therefore, a comparison of the behavior of liposomes prepared from the lipid extracts of NA and ACC plasma membrane fractions (hereafter referred to as NA or ACC liposomes) would directly address the question whether or not the differential propensity of dehydration-induced H_{II} formation in NA and ACC protoplasts is attributable to alterations in the lipid composition per se.

Parts of these results were presented at the 24th Annual Meeting of the Society for Cryobiology, June 1987, Edmonton, Alberta, Canada.

Plasma membrane fractions were isolated from NA or ACC leaf tissue of winter rye (*Secale cereale* L. cv Puma) using the two-phase partition method of Uemura and Yoshida [7] as modified by Lynch and Steponkus [6]. Lipids were extracted from the plasma membrane fraction using the procedure of Bligh and Dyer [8] and were used within 3 days of isolation. A major problem in preparation of liposomes from the total lipid extracts was the incomplete incorporation of all components into the liposomes because of the high proportions of sterols and cerebrosides in the lipid extract. Unincorporated lipid appeared as long needle-like crystals suspended in solution with the liposomes. X-ray diffraction studies confirmed that these crystals were sterols (Lynch, D.V. and Caffrey, M., unpublished results). Another problem arose from the solubility properties of the glucocerebrosides, which comprise 15–16 mol% total lipid in the case of NA and 6–7 mol% total lipid in the case of ACC lipid extracts. The glucocerebrosides are insoluble in ethyl ether, the organic phase frequently used in preparing REV liposomes. Both of these solubility problems were solved if $CHCl_3$ was used as the organic phase and if the liposomes were prepared at elevated temperatures. A minimum temperature of $57\,^{\circ}C$ was required to incorporate all lipid in the NA liposomes. For ACC liposomes, the minimum temperature required was $95\,^{\circ}C$.

Liposomes were prepared by the reverse phase evaporation technique [9,10]. Lipids were dried under a stream of N_2 or by rotary evaporation. $CHCl_3$ was added to the dried lipids, 1.5 ml to plasma membrane lipid extracted from 70 g of leaf tissue (3–4 mg lipid). Then 0.5 ml of aqueous solution was added (either 0.07 osmolal sorbitol,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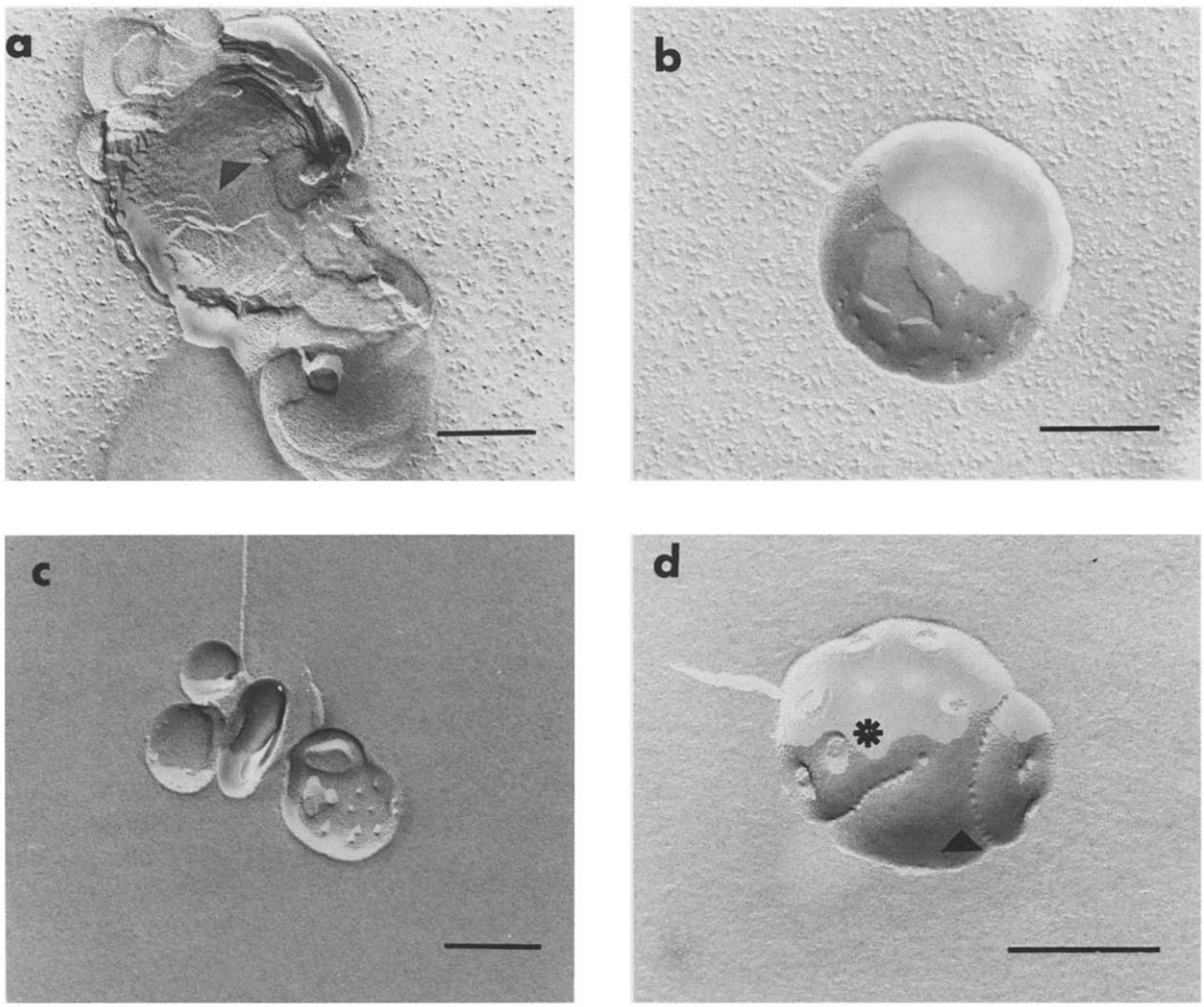
1.3 wt%; 0.04 osmolal sorbitol, 0.7 wt%; or 0.025 osmolal K_2HPO_4-KH_2PO_4 (pH 7.7), 0.01 M). The suspension was mixed by vortexing and then, in the case of NA lipids, warmed to 57°C in the rotary evaporator bath. $CHCl_3$ was slowly removed by rotary evaporation at 57°C. The solution was frequently mixed by vortexing during the gradual removal of the $CHCl_3$. The gel which was formed was broken by vortexing, and remaining $CHCl_3$ was removed by rotary evaporation, always at 57°C. Liposomes were examined by light microscopy after preparation. This procedure yielded a heterogeneous population of liposomes which were uni-, pauci-, and multilamellar with a median diameter of 84 nm in the case of NA liposomes. Fig. 1a shows a typical field of NA liposomes which were suspended in 0.07 osmolal sorbitol prior to freeze-fracture.

When these liposomes were incubated at 4°C in 73 wt% sorbitol for 15 h, H_{II} phase was observed in greater than 75% of the fracture faces. Examples of the H_{II} morphology adopted by these liposomes are shown in Figs. 1b–e. The repeat distance between H_{II} phase cylinder axes was 6–9 nm (Fig. 1c). In many cases, H_{II} phase was observed in aggregates of apparently fused liposomes. When liposomes were incubated in 60–70 wt% sorbitol at 4°C for 2 h, an unequivocal H_{II} phase was not adopted. Rather, a borderline in the transition from lamellar to H_{II} phase was observed. That is, lamellae (Fig. 1f, *) were often observed in the same liposome containing larger, loosely ordered cylinders as well as ordered areas of H_{II} (Fig. 1f, ▲). This suggests that the formation of lipid domains in the liposome bilayers may lead to the onset of the H_{II} phase transition.

Liposomes incubated in 40 or 60 wt% sorbitol at 4°C for 15 h showed intermediate morphologies. Fig. 2a is an example of the rippling stacked bilayers (▲) which frequently appeared after dehydration in both 40 and 60 wt% sorbitol. Lipidic particles and pits were observed in liposomes after overnight dehydration in 40, 60 or 73 wt% sorbitol. The shapes, sizes and organization of the particles were variable. Pits always occurred on convex faces and particles on concave faces (Figs. 2b and 2c). In Fig. 2d is shown both the small, well-defined pits (8 nm) forming rows (▲) and the large crater-like depressions which were frequently observed as well as their corresponding large protrusions (*). Neither lipidic particles nor H_{II} phase was observed for control liposomes incubated in 0.07 osmolal sorbitol at 4°C for 15 h.

To evaluate the possible influence of liposome-encapsulated sorbitol on H_{II} formation during dehydration, NA liposomes were prepared using the same REV method but having 0.025 osmolal K_2HPO_4-KH_2PO_4 (pH 7.7), 0.01 M as the

Fig. 1. Electron micrographs of NA liposomes after dehydration. REV liposomes comprised of the total lipid extracted from plasma membrane fractions of NA leaf tissue were dehydrated in sorbitol solutions for 15 h at 4°C. Liposome suspensions were analyzed by freeze-fracture electron microscopy. Liposomes were prepared using the reverse phase evaporation method (REV) [9,10]. The liposomes were concentrated by centrifugation at 300000 × *g* for 120 min. Fig. 1a shows a typical field of liposomes, suspended in 0.07 osmolal sorbitol prior to dehydration. Immediately after centrifugation 1–2 μl of the pellet was taken out in a capillary tube and dispersed in 60–75 μl of dehydrating solution. Concentrated sorbitol solutions (40, 60, 64 or 73 wt%) were used for dehydration. Dilution of the suspending sorbitol solutions by the added aliquot of liposome pellet (at most, 2 μl pellet in 60 μl sorbitol) could induce a maximum change of 2 wt% in the sorbitol solution. The liposome suspension was maintained at 4°C for either 2 h or 15 h (overnight). After dehydration, small drops of liposomes suspended in dehydrating medium were placed in gold freeze-fracture cups which were resting on a copper tube connected to a circulating ethanol bath. The tube was maintained at 0°C. Empty gold cups were equilibrated 5 min on the cold Cu tube, then the liposome suspension was added. The drops of suspension were then equilibrated 5 min on the Cu tube, then pushed off the tube into a brass cup of liquid propane cooled by liquid nitrogen (LN_2). The frozen samples were rapidly transferred to LN_2 and were stored under LN_2 until they were fractured. Freeze-fracture was carried out using a Balzers 360 M freeze-fracture apparatus. Fracturing was carried out at −107°C. Samples were etched for 1.5 min. C/Pt replicas were floated in distilled water and recovered immediately on Formvar-covered Cu grids. Replicas were viewed through a JEOL 300 electron microscope at 100 kV. Figs. 1b–e illustrate the H_{II} phase which was observed after dehydration in 73 wt% sorbitol for 15 h at 4°C. In Fig. 1c, the regular ribbed areas (*) and terraced fracture faces (▲) can be seen. In Fig. 1d, particulate cross fractures are clear (▲). Fig. 1f shows lamellae (*) in the same liposome with areas of H_{II} phase (▲), observed after dehydration in 60–70 wt% sorbitol at 4°C for 2 h. Bars represent 200 nm. Direction of shadowing is from bottom to top.

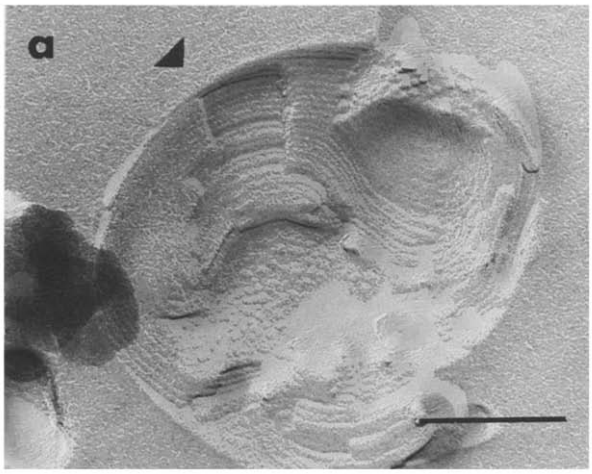


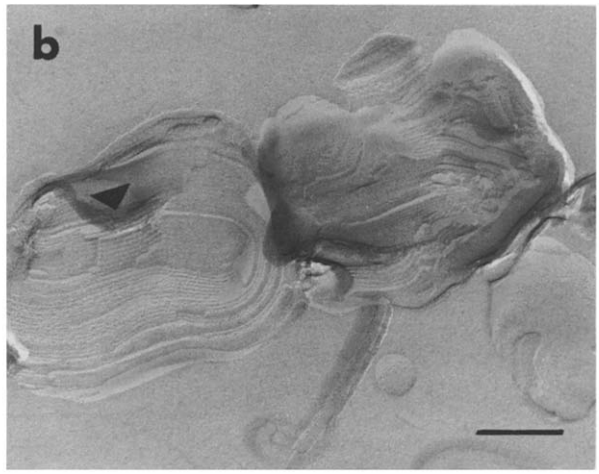
Figs. 2. Intermediate morphologies shown by NA liposomes after dehydration. Fig. 2a is an example of the rippling stacked bilayers (▲) observed after dehydration of NA liposomes in 40 wt% or 60 wt% sorbitol. Pits on convex faces (Fig. 2b) and lipidic particles on concave faces (Fig. 2c) as well as large crater-like depressions (*) in the same liposome with well defined pits (▲) (Fig. 2d) were observed after dehydration of NA liposomes in 40 wt%, 60 wt% or 73 wt% sorbitol. Fig. 2a was from a dehydration in 40 wt% sorbitol, Fig. 2b in 40 wt% sorbitol, and Figs. 2c,d in 60 wt% sorbitol, for 15 h at 4°C. Bars represent 200 nm. Direction of shadowing is from bottom to top.

encapsulated medium. When dehydrated osmotically, these liposomes behaved like those which encapsulated 0.07 osmolal sorbitol. That is, dehydration in 73 wt% sorbitol resulted in H_{II} phase formation (Fig. 3a, ▲; 3b, ▲). H_{II} phase was not observed after dehydration in 40 or 64 wt% sorbitol; however, intermediate morphologies were observed (Fig. 3c, *).

A comparison was then made of the phase behavior of liposomes comprised of the total lipid mix extracted from plasma membrane fractions isolated from ACC leaf tissue. Prior to dehydration, liposomes were uni-, pauci-, and multilamellar with a median diameter of 320 nm. Fig. 4a shows a typical field of these liposomes, suspended in 0.04 osmolal (0.7 wt%) sorbitol.

In contrast to NA liposomes, aggregates of apparently fused liposomes were not observed after dehydration of ACC liposomes. Instead, single liposomes or small, unfused aggregates of dehy-





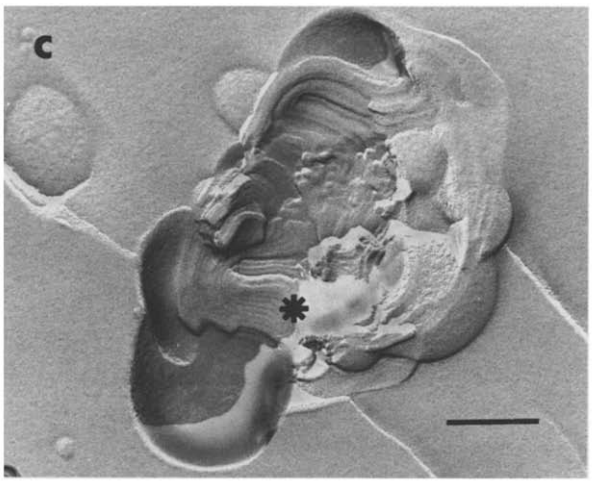


Fig. 3. H_{II} phase and intermediate morphologies of dehydrated NA liposomes encapsulating phosphate. REV liposomes prepared in 0.025 osmolal phosphate (KH_2PO_4-K_2HPO_4) rather than 0.07 osmolal sorbitol also adopted the H_{II} phase after dehydration in 73 wt% sorbitol at 4°C for 15 h, illustrated in Figs. 3a,b. Terraced breaks can be seen in the fracture faces in Fig. 3a (▲) and Fig. 3b (▲). Fig. 3c shows the tubular organizations often observed after dehydration in 40 wt% or 64 wt% sorbitol (*). Fig. 3c was from a dehydration in 64 wt% sorbitol for 15 h at 4°C. Bars represent 200 nm. Direction of shadowing is from bottom to top.

drated liposomes were typical (i.e., a cluster of individually distinguishable liposomes). Further, there was no evidence of significant H_{II} phase formation in ACC liposomes after dehydration under the same conditions which resulted in H_{II} phase formation in NA liposomes (73 wt% sorbitol, 4°C, 15 h). Tubular formations were apparent in an estimated 2–5% of the fracture faces. Lipidic particles of variable morphologies were frequently observed. Figs. 4b (▲) and 4c (▲) illustrate the tubular formations which were typical of the dehydrated liposomes. Dehydration of ACC liposomes in 40 or 60 wt% sorbitol resulted in the appearance of lipidic particles (Fig. 4d, *) and tubular structures which were qualitatively similar to those observed for NA liposomes under the same dehydrating conditions.

These studies indicate that the differential propensity for dehydration-induced lamellar-to-H_{II} phase transitions in the plasma membrane of NA and ACC protoplasts [3] is, in part, a consequence of differences in the lipid composition of the plasma membrane. Because there are no lipid species which are unique to either NA or ACC plasma membrane lipids [6], this differential behavior is the result of differences in the proportions of the various lipid species that occur after cold acclimation. For example, free sterols increase from 33 to 44 mol% of the total lipid, while steryl glucosides and acylated steryl glucosides

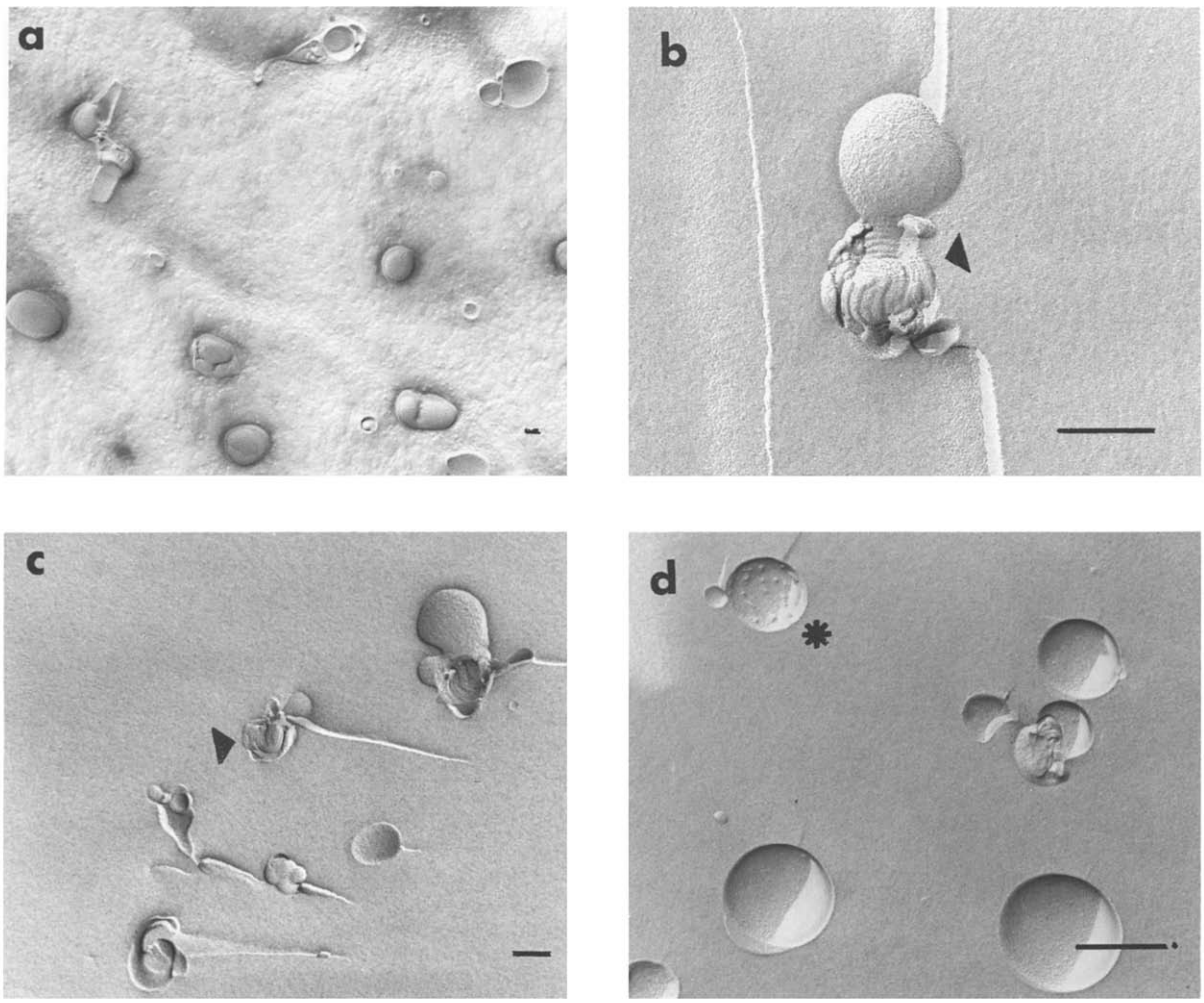


Fig. 4. Electron micrographs of ACC liposomes after dehydration. The aqueous/chloroform solutions containing extracted lipids were mixed by vortexing, then $CHCl_3$ was slowly removed during heating to 95° C. The solution was shaken during $CHCl_3$ removal and was mixed by frequent vortexing. Final traces of $CHCl_3$ were removed by rotary evaporation. Liposomes were concentrated by centrifugation as previously described. Liposomes were dehydrated in sorbitol solutions for 15 h at 4° C. Fig. 4a shows the liposomes suspended in 0.04 osmolal sorbitol prior to dehydration. Figs. 4b–d illustrate the tubular formations (▲) and lipid particles (*) which were typical of dehydrated liposomes. Figs. 4b,c were from dehydrations in 73 wt% sorbitol for 15 h at 4° C. Fig. 4d was in 60 wt% sorbitol for 15 h at 4° C. Bars represent 200 nm. Direction of shadowing is from bottom to top.

decrease from 15 to 6 mol% and 4 to 1 mol%, respectively; glucocerebrosides decrease from 16 to 7 mol%; and phospholipids increase from 32 to 42 mol%. Dehydration-induced lamellar-to-H_{II} phase transitions in the NA liposomes may be considered to be a consequence of the close approach of bilayers resulting from dehydration. This close approach of bilayers may induce segregation of membrane components [12], with lipid species which allow closest bilayer approach accumulating in the region of contact. It has been reported that egg PE allows closer bilayer approach than most of the lipids measured [13]. PE also prefers non-lamellar structures at 4° C [14,15]. A model has been drawn in which approach of bilayers leads to accumulation of PE in the area of contact, thus destabilizing the bilayer structure and leading to H_{II} phase formation [16], thought to be a trigger

for fusion [17]. Such a model explains the fact that we observed large aggregates of apparently fused liposomes only when H_{II} phase was also observed.

In all experiments reported here, we have dehydrated liposomes for a period of 15 h. Preliminary experiments of dehydration of liposomes comprised of NA total lipid in 73 wt% sorbitol indicated that only after such long times at 4°C would the H_{II} phase be adopted. For example, dehydration for 2 or 8 h in 73 wt% sorbitol resulted in the onset of intermediate morphologies but not an unequivocal H_{II} phase by NA liposomes. The requirement of long time periods for H_{II} phase formation has also been reported for the PC/cardiolipin/$CaCl_2$ system, where 26 h was required for the H_{II} phase to become predominant. An intermediate phase appeared at shorter incubation times [18].

In cases where H_{II} phase was observed after dehydration, it was easily identified by the large areas of lipid having a regular, ribbed appearance (Figs. 1c, *, 1d, *), by abrupt, terraced breaks in the fracture faces (Figs. 1c, ▲; 1f, ▲; 3a, ▲; 3b, ▲), and by the compressed particulate appearance of cross fractures (Fig. 1d, ▲). In some cases, the H_{II} phase tubes appeared to diverge gradually into stacked bilayers (Fig. 1f). This phenomenon has been observed in other lipid systems [18,19]; and, in mixed lipid systems, has been interpreted as arising from phase transitions of lipids that prefer the H_{II} phase and of lipids that prefer the bilayer phase, (i.e. a lateral phase separation is implied). However, in pure PE systems the same conversion of H_{II} tubes into smooth fracture faces has been observed upon freezing at a temperature just above the H_{II}-bilayer transition. Models involving H_{II} cylinders which lie parallel to the plane of the bilayer have been offered [19].

An array of lipidic particles and pits was observed after dehydration of both NA and ACC liposomes in any medium. It is thought that at least some of these particles could be evidence for inverted micelle formation between bilayers by non-bilayer forming lipids, implying a type of lateral phase separation. The large crater-like depressions and protrusions are similar to earlier reported morphologies, a model of which describes the structure as arising from the cross-fracture of an aqueous channel separating two membrane bound compartments [19]. In our case, this can be envisaged as the fracturing off of smaller lobes from a larger, central vesicle.

Another point which may be addressed regards the use of sorbitol in the aqueous phase during the preparation of the liposomes along with the use of sorbitol as an osmoticum in the dehydrating media. This concern arises from reports of the stabilization of lipid bilayer vesicles by various solutes during freezing [20–24]. Disaccharides such as trehalose and sucrose are more effective in suppressing fusion of vesicles than sugar alcohol solutes such as *myo*-inositol or sorbitol. However, at high concentrations, sorbitol is as relatively effective in preserving the structural and functional integrity of membrane vesicles during dehydration [24]. To determine the possible influence of sorbitol on our studies, we carried out dehydration experiments with liposomes comprised of the NA lipids containing a phosphate buffer. Had the encapsulated sorbitol exerted a protective effect, we would expect liposomes encapsulating phosphate to undergo H_{II} phase transitions under less strenuous dehydrating conditions (e.g., after dehydration in 40 or 60 wt% sorbitol). However, this did not occur. The liposomes encapsulating phosphate behaved like those encapsulating sorbitol, adopting intermediate morphologies such as loosely ordered cylinders, ripple structures and lipid particles and pits of various sizes and morphologies in 40 and 64 wt% sorbitol. Only after dehydration in 73 wt% sorbitol for 15 h at 4°C did the liposomes encapsulating phosphate undergo lamellar-to-H_{II} phase transitions. Thus we conclude that the protective effects of sorbitol were negligible in our experiments. In this context, it must also be noted that sorbitol was originally used in the preparation of liposomes because attempts to prepare liposomes in water or in aqueous solutions of NaCl were unsuccessful. Even at elevated temperatures, crystals of sterol components appeared in suspension with the liposomes.

In conclusion, we report a differential response of liposomes from NA or ACC plasma membrane lipids to dehydration, with only liposomes from NA lipids undergoing the lamellar-to-H_{II} phase transition. Thus, the decreased propensity for dehydration-induced H_{II} formation in the plasma membrane of protoplasts isolated from cold

acclimated rye leaves [3] is, in part, a consequence of alterations in the lipid composition of the plasma membrane.

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