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Lamellar-to-hexagonal$_{II}$ phase transitions in the plasma membrane of isolated protoplasts after freeze-induced dehydration

(rye/cold acclimation/freeze-fracture/membrane morphology/nonbilayer lipid structures)

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ABSTRACT **In protoplasts isolated from nonacclimated rye leaves (*Secale cereale* L. cultivar Puma), cooling to −10°C at a rate of 1°C/min results in extensive freeze-induced dehydration (osmotic contraction), and injury is manifested as the loss of osmotic responsiveness during warming. Under these conditions, several changes were observed in the freeze-fracture morphology of the plasma membrane. These included (*i*) lateral phase separations in the plasma membrane, (*ii*) aparticulate lamellae lying next to the plasma membrane, and (*iii*) regions of the plasma membrane and associated lamellae in various stages of lamellar-to-hexagonal$_{II}$ transition. These morphological changes also were observed after equilibration in 5.37 osmolal sorbitol at 0°C, which produced a similar extent of dehydration as did freezing to −10°C. In contrast, only small areas of lateral phase separation in the plasma membrane, with no observable aparticulate lamellae or hexagonal$_{II}$ configurations, were observed in protoplasts supercooled to −10°C. Therefore, freeze-induced lamellar-to-hexagonal$_{II}$ phase transitions in the plasma membrane are a consequence of dehydration rather than subzero temperature *per se*. When suspensions of protoplasts isolated from cold-acclimated leaves were frozen to −10°C, no injury was incurred, and hexagonal$_{II}$ phase transitions were not observed. No hexagonal$_{II}$ phase was observed even at −35°C, though acclimated protoplasts are injured at this temperature.**

The plasma membrane plays a central role in cellular behavior during a freeze–thaw cycle, and disruption of the semipermeable characteristics or lysis of the plasma membrane is a primary cause of freezing injury (1). Destabilization of the plasma membrane can occur at various times during a freeze–thaw cycle and may be manifested as any one of several distinctly different symptoms (2, 3). For protoplasts isolated from nonacclimated leaves and cooled to −5°C (the LT_{50}—minimal temperature for 50% survival), the predominant manifestation of injury is lysis of the protoplast during osmotic expansion after thawing of the suspending medium (4). This form of injury occurs because of irreversible endocytotic vesiculation of the plasma membrane during freeze-induced osmotic contraction (4–8). At lower subzero temperatures, freeze-induced dehydration becomes increasingly severe, and injury is manifested as a complete loss of osmotic responsiveness (4, 9, 10). This is the predominant form of injury for nonacclimated protoplasts cooled to −10°C but does not occur in acclimated protoplasts until much lower temperatures—i.e., −25 to −30°C (4).

Whereas expansion-induced lysis is the result of mechanical stresses incurred during freeze/thaw-induced osmotic contraction/expansion, loss of osmotic responsiveness occurs after severe freeze-induced dehydration when the membrane is flaccid, isotropic tension is zero, and curvature energies are negligible (5–7) and precludes osmotic expansion during thawing of the suspending medium. Various possibilities by which alterations in the semipermeable characteristics of the plasma membrane are effected have been suggested. These include (*i*) concentration of toxic solutes such as electrolytes (11–13), (*ii*) thermally-induced liquid crystal-to-gel phase transitions in the plasma membrane, which are proposed to be either injurious (14, 15) or to predispose the membrane to dehydration injury (16, 17), and (*iii*) the effects of dehydration *per se* on membranes (18).

Dehydration-induced phase transitions in biological membranes, in particular the lamellar-to-hexagonal$_{II}$ (H_{II}) phase transition, have previously been suggested as possible causes of injury during extreme cellular desiccation (18–20). The transition from lamellar to H_{II} phase has been looked for but not found in plant cells that undergo extensive dehydration such as pollen (21) or in lipid extracts from seeds (22). However, based on freeze-fracture evidence, it is suggested that H_{II} lipid phase occurs in dehydrated lettuce seeds (23).

Recently, Crowe *et al.* (18) and Steponkus (1) suggested that lamellar-to-H_{II} phase transitions may be involved in freezing injury. In order to investigate this possibility, changes in the morphology of the plasma membrane of nonacclimated protoplasts were determined by freeze-fracture following freeze-induced dehydration. To further determine whether these changes were the result of dehydration or low temperature, comparable osmotic manipulation and supercooling studies were also done.

MATERIALS AND METHODS

Protoplast Isolation. Seeds of *Secale cereale* L. cultivar Puma were sown in vermiculite and germinated at 20°C/15°C (day/night) temperatures (16-hr photoperiod). Nonacclimated plants were grown in this environment for 2 weeks. Plants were acclimated to cold by exposing 1-week-old plants to 13°C/7°C (11.5-hr photoperiod) for an additional week and then to 2°C (10-hr photoperiod) for 4 weeks. Protoplasts were enzymically isolated from the leaves (24) and resuspended in isotonic sorbitol [0.53 and 1.0 osmolal (osm) for nonacclimated and acclimated, respectively].

Cooling Protocol and Freeze-Fracture. Small drops of either a nonacclimated or acclimated protoplast suspension were placed in gold cups (Balzers BB133 142-2) on a copper tube connected to a circulating ethanol bath (Neslab, Endocal ULT-80). Temperature was monitored with a copper/constantan thermocouple in one droplet. The suspensions were cooled to −2°C and seeded with a small ice crystal. After ice formation, the samples were cooled to −5°C (nonacclimated), −10°C (nonacclimated and acclimated), or −35°C (acclimated) at a rate of 1°C/min. After an isothermal period of 15 min (all temperatures), 1 hr (nonacclimated, −10°C), or 2 hr (acclimated, −35°C), the copper bar was inverted, and the frozen samples fell into a small cup

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Abbreviations: osm, osmolal; H_{II}, hexagonal$_{II}$.

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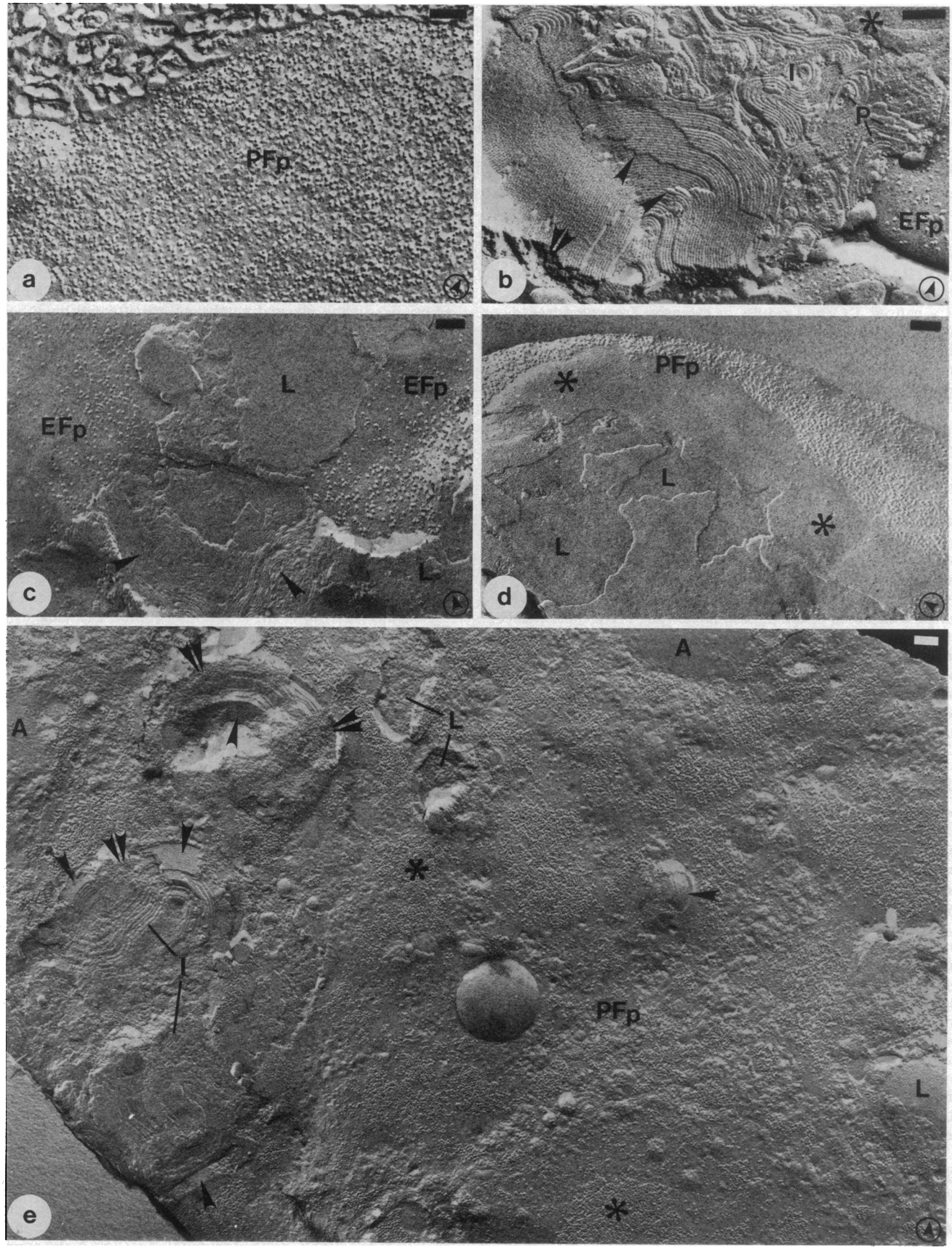

FIG. 1. (*a*) Plasma membrane protoplasmic face (PFp) of a nonacclimated protoplast suspended in isotonic sorbitol (0.53 osm). The particle distribution appears even, with no particle aggregation or bare patches on the membrane. (×65,000.) (*b–e*) Nonacclimated protoplasts following freeze-induced dehydration by cooling to −10°C. (*b*) Hexagonal$_{II}$ lipid phase. The individual cylinders (arrowheads) within each layer of the well-ordered H_{II} region are apparent. Intramembrane particles (P) can be discerned in areas of intermediate (I) H_{II} morphology,

of nitrogen slush positioned under the copper bar. After quenching of the samples, they were fractured on a Balzers 360M freeze-fracture apparatus, coated with platinum and carbon, and the replicas were cleaned for viewing. (For further details of sample preparation see ref. 8.)

Osmotic Manipulation. The osmolality of a partially frozen solution varies as a function of the subzero temperature (osm $\approx$ 273-T/1.86°C). Thus, the osmolality of the unfrozen solution at −10°C is ≈5.37 osm. By osmotically contracting nonacclimated protoplasts in 5.37 osm sorbitol at 0°C, a similar extent of dehydration was imposed in the absence of low temperatures or extracellular ice. Protoplasts remained in the hypertonic sorbitol (5.37 osm) for 15 min before being quenched for freeze-fracture as described above.

Supercooling. Gold cups were loaded with a small drop of nonacclimated protoplast suspension and were placed on the cooling stage at 0°C. The samples then were cooled at a rate of 1°C/min to −10°C. After 15 min at −10°C, the samples were quenched in nitrogen slush. Supercooled drops distorted as they fell into the coolant and, thus, were easily recognized. The shape of samples that froze on the stage prior to being quenched remained a perfect dome. These were discarded.

RESULTS

In nonacclimated protoplasts suspended in 0.53 osm sorbitol at 0°C, the intramembrane particles were evenly distributed on the plasma membrane fracture faces (Fig. 1*a*). After the samples were cooled to −5°C, the particle distribution and plasma membrane morphology appeared unchanged (micrograph not shown). However, when cooled to −10°C and held for 15 min, several changes in membrane morphology were observed. These included lamellar-to-H_{II} phase transitions (Fig. 1*b*), the appearance of aparticulate lamellae underneath the plasma membrane (Fig. 1 *c* and *d*), and lateral phase separations in the plasma membrane (Fig. 1*d*).

The spatial relationship of these changes is shown in Fig. 1*e*. Regions of H_{II} phase ranged in area from 0.1 μm^2 to well over 1 μm^2 and were interspersed over the membrane (Fig. 1*e*). Longitudinally fractured H_{II} appeared as cylinders lying parallel to the membrane, while cross-fracturing resulted in patches of an uneven, bumpy texture (Fig. 1*e*). Within regions of H_{II}, both tightly spaced cylinders and more loosely ordered cylinders were observed. In Fig. 1*e*, lamellae lying underneath the plasma membrane are also apparent. Lateral phase separations, which varied in extent (cf. Figs. 1*d* vs. 1*e*), resulted in clusters of intramembrane particles over the surface of the protoplasmic face.

The surface area of the plasma membrane fracture faces that were examined ranged between 5 and 10 μm^2 (e.g., the protoplasmic face in Fig. 1*e* is slightly over 10 μm^2). Even though this represents only a small proportion of the total surface area in these contracted nonacclimated protoplasts (<1%), regions of H_{II}, aparticulate lamellae, and lateral phase separations were observed in 50% of the fracture faces examined in the 15-min treatment. After 1 hr at −10°C, this increased to >80%.

The morphology within individual H_{II} regions ranged from highly ordered stacks composed of several layers of parallel cylinders (average spacing of cylinders, 7.5 nm ± 0.5 nm) to areas of loosely organized, undulating furrows or striations (Fig. 1*b*). The intermediate morphology (i.e., more loosely ordered) appeared to intergrade between the well-ordered H_{II} and smooth bilayer. Usually the H_{II} configurations were aparticulate, although some intramembrane particles were observed in the intermediate regions.

The aparticulate or nearly aparticulate lamellae observed beneath the protoplasmic face (Fig. 1 *d* and *e*) or lying on top of the exoplasmic face (Fig. 1*c*) were closely appressed to the plasma membrane with little or no space observed between them. When more than one lamellae was observed (Fig. 1*c*), these also appeared to be closely appressed. In Fig. 1*c*, regions of the lamellae are present in which loosely ordered intermediate H_{II} morphology can be seen.

H_{II}, aparticulate lamellae, and lateral phase separations in the plasma membrane were observed after hypertonic contraction in 5.37 osm sorbitol at 0°C. The degree of lamellar-to-H_{II} transition after osmotic dehydration was comparable to that seen after freeze-induced dehydration and ranged from loosely ordered undulating furrows in the membrane to areas of highly ordered H_{II} (Fig. 2*a*). In contrast, when nonacclimated protoplasts were supercooled to −10°C, only small areas of lateral phase separation (Fig. 2*b*) were observed in some (<20%) plasma membrane protoplasmic faces. The high degree of lateral phase separation observed after freeze-induced dehydration was not observed at −10°C in the absence of extracellular ice.

In contrast to nonacclimated protoplasts, when protoplasts isolated from acclimated leaves were cooled to −10°C, no lateral phase separations, aparticulate lamellae, or H_{II} configurations were observed. Even after 2 hr at −35°C, no H_{II} and a low incidence (<20%) of lamellae and phase separation was observed (micrographs not shown).

DISCUSSION

Under conditions that result in severe freeze-induced dehydration and the loss of osmotic responsiveness of nonacclimated protoplasts (slow cooling to −10°C), we observed lateral phase separations in the plasma membrane, aparticulate lamellae associated with the plasma membrane, and lamellar-to-H_{II} phase transitions involving both the plasma membrane and the underlying lamellae. These changes also occurred when dehydration was effected by direct osmotic manipulation at 0°C. In contrast, only a small degree of lateral phase separation was observed in the plasma membrane after supercooling to −10°C. Thus, we conclude that the membrane alterations observed in isolated protoplasts are largely a consequence of freeze-induced dehydration and the volume of water frozen rather than direct effects of temperature *per se*. This is contrary to the proposal by Rajashekar *et al.* (14) that low temperature and not dehydration is responsible for membrane changes during freezing. However, Rajashekar *et al.* (14) did not separate low-temperature effects from those due to dehydration but instead assumed that changes in dehydration were negligible at the subzero temperatures used in their study.

More recently, Yoshida (16, 17) proposed that phase transitions in the plasma membrane are induced by subzero temperatures and predispose the membrane to dehydration-induced injury. However, we have found that for nonacclimated

which appears to intergrade between the well-ordered H_{II} and the bilayer (asterisk). Double arrowheads indicate cross-fractured H_{II}. EFp, plasma membrane exoplasmic face. (×78,000.) (*c*) Lamellae (L) lying next to the EFp with portions undergoing lamellar-to-hexagonal$_{II}$ phase transition (arrowheads). (×50,500.) (*d*) Large aparticulate domain observed on the PFp (asterisks) indicative of lateral phase separation and an aparticulate lamella lying beneath the plasma membrane (L). (×54,400.) (*e*) Large expanse of PFp showing regions of H_{II} (arrowheads), lamellae lying underneath the plasma membrane (L), and lateral phase separation in the plasma membrane seen as aggregations of intramembrane particles (asterisks) and aparticulate domains (A). The regions of hexagonal$_{II}$ phase in the plasma membrane can be seen in longitudinal fracture (single arrowheads) and in cross-fracture (double arrowheads). Also within an individual H_{II} region, both well-ordered cylinders (arrowheads) and more loosely-ordered cylinders (I) can be observed. (×40,300.) Circled arrowheads indicate shadowing direction. (Bars = 0.1 μm).

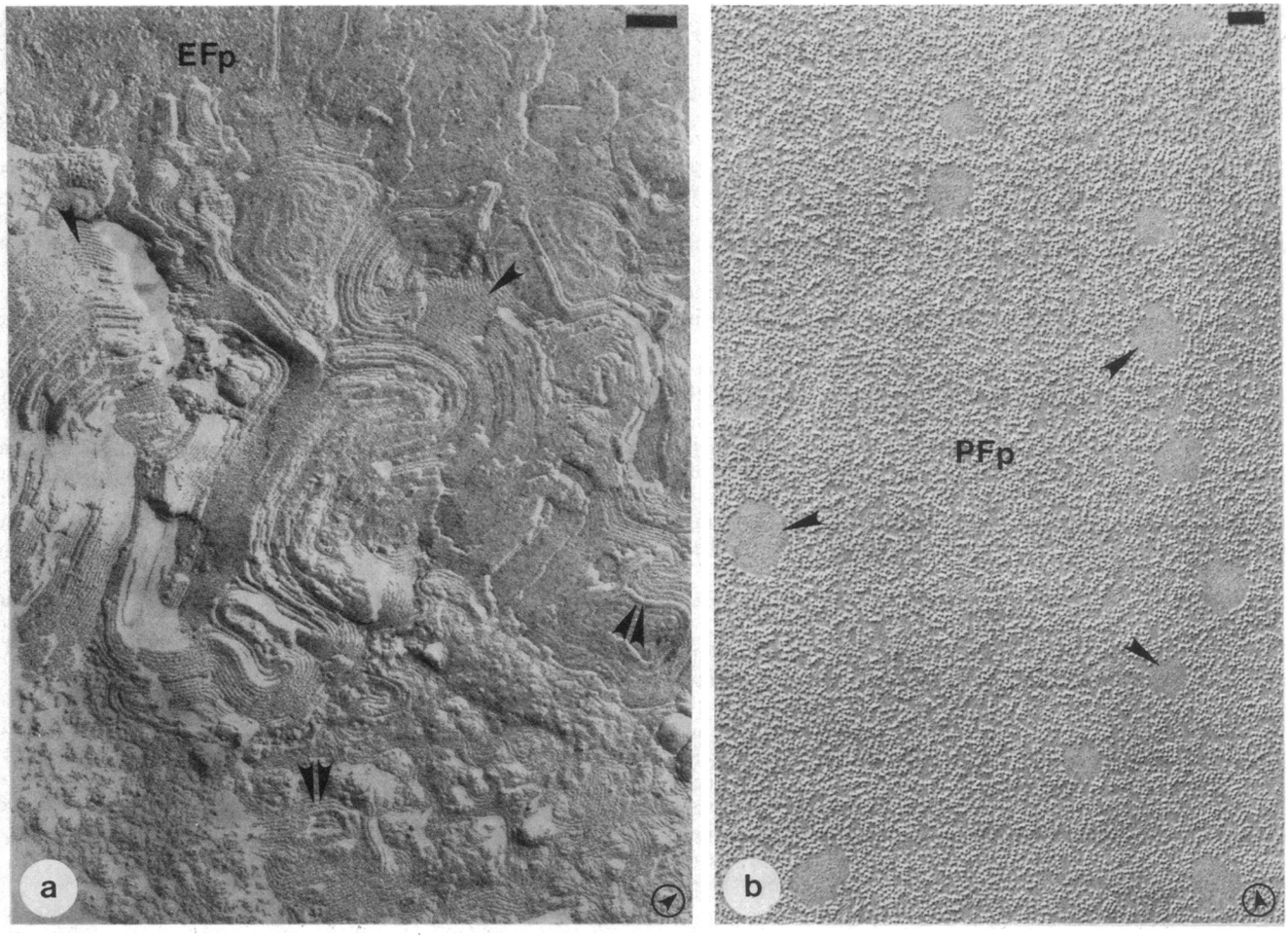

FIG. 2. (*a*) Various stages of lamellar-to-hexagonal$_{II}$ phase transition in the plasma membrane exoplasmic face (EFp) of a nonacclimated protoplast hypertonically contracted in 5.37 osm sorbitol (at 0°C). Highly ordered hexagonal$_{II}$ regions are present (arrowhead) as well as regions where the striations or cylinders are less ordered (double arrowheads). (×67,600.) (*b*) Plasma membrane protoplasmic face (PFp) of a nonacclimated protoplast supercooled to −10°C. Small areas of phase separation (arrowheads) that are clear of intramembrane particles are present. (×46,100.) Circled arrowheads indicate shadowing direction. (Bars = 0.1 μm.)

mated protoplasts, osmotic manipulation in the absence of subzero temperatures resulted in ultrastructural alterations similar to those caused by freezing to −10°C. Thus, it is unlikely that the changes we observed with isolated protoplasts are due to either temperature *per se* or temperature-induced phase changes that render the membrane more susceptible to dehydrative stresses.

Both lateral phase separations (25, 26) and the appearance of lamellae next to the plasma membrane (25–27) have been observed in previous studies of plasma membrane morphology following freeze-induced cellular dehydration. Although no indication of H_{II} lipid phase was reported in these studies, this would be extremely difficult to visualize in thin sections of freeze-substituted material (27).

The various changes in plasma membrane morphology induced by dehydration of nonacclimated protoplasts are probably closely interrelated. The presence of multilamellar structures appears to be a prerequisite to the formation of the H_{II} lipid phase (see refs. 28 and 29). In dehydrated nonacclimated protoplasts, the H_{II} phase occurs in multilamellar or stacked bilayer regions, generally involving the plasma membrane and underlying lamellae. It is possible that various organelle membranes and/or endomembrane components could participate in the lamellar-to-H_{II} transition, and occasionally H_{II} or intermediate configurations were observed in the membranes of the chloroplast envelope (micrographs not shown). However, in nonacclimated protoplasts, a likely source of membrane material during contraction is the plasma membrane itself. During hypertonic contraction of nonacclimated protoplasts, endocytotic vesiculation of the plasma membrane occurs (4, 8). The majority of this vesiculation occurs at warm subzero temperatures (i.e., above −5°C) and results in large clusters of vesicles in the cytoplasm, often lying just beneath the plasma membrane. These vesicles would become increasingly appressed into multilamellar stacks, which may fuse forming the appressed sheets observed underneath the plasma membrane as dehydration progressed. In contrast, neither endocytotic vesiculation at warm subzero temperatures (30) or H_{II} phase after severe freeze-induced dehydration occurs in acclimated protoplasts.

As in other biological membranes (20, 31, 32), the H_{II} configurations in nonacclimated protoplasts are oriented parallel to the membrane. These regions consist of layers of parallel, inverted cylindrical micelles. The spacing of these parallel cylinders (≈7.5 nm) in the well-ordered regions is in good agreement with studies of lipid mixtures in model systems (29, 33). However, the spacing varied considerably once outside the tightly ordered regions. Loosely ordered, undulating striations or furrows were often observed, appearing to intergrade between the smooth bilayer and the tightly ordered H_{II} regions. The morphology in these regions is similar in appearance to intermediate stages of the lamel-

lar-to-H_{II} transition observed in freeze-fracture of model systems (33).

The areas of intermediate morphology may represent regions that have only undergone a partial transition. In model systems, the diameter of the cylinders varies as a function of the molar ratios of the participating lipid species and the degree of dehydration (see ref. 29 for a review). The variation in H_{II} morphology and the fact that H_{II} phase only appeared to occur in localized regions may be due to spatial variation in membrane composition. Nonbilayer phases such as H_{II} are either inhibited or promoted by the relative concentration of "nonbilayer" to "bilayer" lipids in the mixture (28, 29, 33). Therefore, in a biological membrane it is reasonable to expect that lateral segregation of lipids might result in areas where H_{II} transitions were favored.

Although the changes we observed in plasma membrane morphology (lateral phase separations, apposition of lamellae, and H_{II} formation) occurred under the same conditions that result in loss of osmotic responsiveness in nonacclimated protoplasts (slow cooling to -10°C or hypertonic contraction at 0°C), further work is required to establish a causal relationship. There are, however, several possible ways in which these membrane alterations could result in loss of osmotic responsiveness. Transition from the lamellar to H_{II} phase should dramatically alter the semipermeable characteristics of the plasma membrane, since the bilayer is the only lipid structure compatible with a nonleaky membrane (34). Protoplasts that are osmotically unresponsive after freeze-induced dehydration or direct exposure to hypertonic solutions are readily permeated by fluorescein or Evans blue (10).

Also, even though the transition from lamellar to H_{II} has been shown to be reversible in both model systems (see ref. 29 for a review) and biological membranes such as sarcoplasmic reticulum (20), considerable reorganization of all the membrane components in these regions is possible (i.e., both lipid and protein). For example, protein displacement from the bilayer and/or denaturation of integral proteins could occur as possible consequences of the rearrangement of lipids during the lamellar-to-H_{II} transition (19, 20). The H_{II} transition dramatically inhibits Ca^{2+}-ATPase activity in sarcoplasmic reticulum vesicles, even after the bilayer is reestablished upon full hydration (20).

The changes in membrane morphology that occur at -10°C in nonacclimated (coincident with the loss of osmotic responsiveness) are not observed in acclimated protoplasts cooled to the same temperature. In addition, even when acclimated protoplasts were cooled below the minimal temperature for 50% survival (i.e., to -35°C), no H_{II} was observed. This suggests that membrane alterations occur during cold acclimation that preclude H_{II} formation during freeze-induced dehydration at the temperatures used in this study. These and other (4, 8, 30) differences in plasma membrane behavior between nonacclimated and acclimated protoplasts during freezing most likely reflect compositional differences in the plasma membrane.

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