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The Influence of Cold Acclimation on the Lipid Composition and Cryobehaviour of the Plasma Membrane of Isolated Rye Protoplasts [and Discussion]

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Phil. Trans. R. Soc. Lond. B 1990 **326**, 571-583
doi: 10.1098/rstb.1990.0032

Phil. Trans. R. Soc. Lond. B **326**, 571–583 (1990)

Printed in Great Britain

The influence of cold acclimation on the lipid composition and cryobehaviour of the plasma membrane of isolated rye protoplasts

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Destabilization of the plasma membrane, which is a primary cause of freezing injury, is a consequence of freeze-induced osmotic stresses and cell dehydration. However, the mechanism of injury depends on the magnitude of the osmotic stress and the extent of cell dehydration. Over the range of 0 to −5 °C, destabilization of the plasma membrane in protoplasts isolated from non-acclimated rye leaves is a result of osmotic excursions, because freeze-induced osmotic contraction results in endocytotic vesiculation of the plasma membrane and sufficiently large area reductions are irreversible. At lower temperatures, the protoplasts are subjected to extremely large osmotic pressures (−12 MPa at −10 °C), and there are several changes in the ultrastructure of the plasma membrane, including the formation of aparticulate domains and lamellar- to hexagonal$_{II}$-phase transitions. These changes, which are manifestations of demixing of the membrane components, are predicted by a theory of bilayer interactions at low levels of hydration.

During cold acclimation, the cryobehaviour of the plasma membrane is altered; osmotic contraction results in the reversible formation of exocytotic extrusions and the propensity for dehydration-induced demixing and lamellar- to hexagonal$_{II}$-phase transitions is decreased. In both cases, the differential behaviour is also observed in liposomes prepared from plasma membrane lipids isolated from non-acclimated and cold-acclimated leaves. However, as no lipid species are unique to the plasma membrane of either non-acclimated or cold-acclimated leaves, the differential behaviour is caused by altered lipid–lipid interactions because of different proportions of the lipid species. Hence the behaviour of the plasma membrane can be altered by using a protoplast–liposome fusion procedure to selectively modify the lipid composition of the plasma membrane. These studies provide direct evidence that the increased cryostability of the plasma membrane is a consequence of alterations in its lipid composition.

Introduction

Cold acclimation of higher plants is a complex developmental process that increases the tolerance of winter annuals, biennials, and perennials to freezing temperatures. The increase in freezing tolerance may range from a few degrees in herbaceous species to tens of degrees in winter cereals to over 100 degrees in some deciduous species. The cold acclimation process, which typically requires four to six weeks to achieve the maximum increase in freezing tolerance, is triggered by environmental cues, most notably temperatures in the range of 0 to 5 °C and, in some species, decreasing photoperiod. During the cold acclimation process there are numerous biochemical changes including hormonal changes, altered gene activity and new gene products and alterations in metabolism that result in the accumulation of carbohydrates, and alterations in lipid metabolism. Often, these changes are viewed as disparate events.

However, given that freezing injury is a consequence of membrane destabilization, it is more appropriate to consider the integration of these changes as ultimately contributing to the increased cryostability of cellular membranes.

Although there are many examples of freeze-induced destabilization of the various cellular membranes, the plasma membrane is of primary importance because of its central role in cellular behaviour during a freeze–thaw cycle. To minimize the extent of supercooling of the cytosol and to preclude intracellular ice formation during exposure to subzero temperatures, the cells must behave as osmometers and dehydrate following extracellular ice formation. For this to occur, it is necessary to maintain the semipermeable characteristics of the plasma membrane, including its function to serve as a barrier to the extracellular ice. Lysis or alterations in the semipermeable characteristics of the plasma membrane are a primary cause of freezing injury (Steponkus 1984; Steponkus & Lynch 1989*a*). Under conditions that preclude intracellular ice formation, disruption of the plasma membrane is a consequence of freeze-induced cell dehydration. However, destabilization can be effected by different mechanisms depending on the extent of cell dehydration (see below).

The role of lipid alterations in the cold acclimation process has been controversial. One major concern is whether the changes in lipid composition observed after an exposure to the low temperatures required for cold acclimation are causally related to the cold acclimation process or merely low temperature responses. Because the majority of the early studies were restricted to correlative studies of changes in lipid composition and increases in cold hardiness, the mechanistic significance of the changes could not be discerned. Also, many of the studies involved lipid analyses of whole tissues or crude membrane fractions with relatively few reports of the effect of cold acclimation on the lipid composition of the plasma membrane. Although several reports have suggested that there are only modest changes in the plasma membrane lipid composition following cold acclimation, these studies were restricted to analyses of the various lipid classes (see, for example, Hellergren *et al.* 1984; Uemura & Yoshida 1984; Yoshida & Uemura 1984). However, analyses of the individual lipid molecular species of the plasma membrane reveal that there are substantial differences following cold acclimation (Lynch & Steponkus 1987*a*).

This paper reviews the evidence for a causal relation of the changes in the lipid composition based on a mechanistic analysis of freeze–thaw-induced destabilization of the plasma membrane. This analysis derives from studies of the intrinsic properties and cryobehaviour of the plasma membrane of protoplasts and liposomes prepared from plasma membrane lipids isolated from winter rye (*Secale cereale* L. cv. Puma).

Lipid composition of the plasma membrane

The lipid composition of the plasma membrane of rye leaves is unique in comparison with other plant cell membranes (Lynch & Steponkus 1987*a*). The most notable features are the relatively high content of sterols and steryl derivatives and the presence of glucocerebrosides. Cumulatively, sterols (33 mol %), sterylglucosides (15 mol %), and acylated sterylglucosides (4 mol %) compose approximately 52 mol % of the total lipids of the plasma membrane; glucocerebrosides, 16 mol % and phospholipids the remaining 32 mol %.

After cold acclimation, free sterols increase from 33 to 44 mol %, sterylglucosides decrease from 15 to 6 mol % and acylated sterylglucosides decrease from 4 to 1 mol %; glucocerebrosides

decrease from 16 to 7 mol % and phospholipids increase from 32 to 42 mol %. Within these lipid classes, more than 100 different lipid molecular species have been identified to date (Lynch & Steponkus 1987*a*; Cahoon & Lynch 1988). However, there is no molecular species that is unique to either the plasma membrane fraction isolated from non-acclimated or cold-acclimated rye leaves. Instead, cold acclimation alters the proportion of virtually every molecular species.

The major free sterols are β-sitosterol and campesterol with smaller amounts of stigmasterol and cholesterol. These sterols are also the principal steryl moieties in the sterylglucosides and acylated sterylglucosides. For both the sterylglucosides and acylated sterylglucosides, glucose is the only sugar constituent. The major acyl chain of the acylated sterylglucosides (esterified at the 6–0 position of glucose) is palmitate, with lesser amounts of oleate, linoleate, linolenate and behenate. After cold acclimation, the increases in β-sitosterol and campesterol are proportional to the decreases in the sterylglucoside and acylated sterylglucoside species containing these sterol moieties, possibly because of interconversions by deacylation or deglucosylation, or both.

The plasma membrane contains more than 26 different molecular species of glucocerebrosides (Cahoon & Lynch 1988). Glucocerebrosides comprise glucose as the sugar moiety, a long chain base (hydroxysphingenine and sphingadienine are the major bases), and primarily C_{22} and C_{24} saturated and unsaturated hydroxy fatty acids. Although there is a proportional increase in the hydroxynervonic acid-containing species, the mol % of glucocerebrosides is decreased following cold acclimation.

The predominant phospholipids are phosphatidylcholine and phosphatidylethanolamine with lesser amounts of phosphatidylglycerol, phosphatidylserine, and phosphatidylinositol. For phosphatidylcholine and phosphatidylethanolamine, the major molecular species comprise mono-unsaturated (1-palmitoyl-2-linoleoyl and 1-palmitoyl-2-linolenoyl) and di-unsaturated (dilinoleoyl and 1-linoleoyl-2-linolenoyl) species. Following cold acclimation, there is no significant change in the relative proportions of the individual phospholipid classes. However, there are numerous changes in the individual molecular species, with a twofold increase in the principal di-unsaturated species of both phosphatidylcholine and phosphatidylethanolamine.

To determine the mechanistic significance of the changes in the plasma membrane lipid composition during cold acclimation, it is necessary first to establish the mechanisms by which dehydration results in destabilization of the plasma membrane.

Osmotic stresses during a freeze–thaw cycle

During freezing of a protoplast suspension, the protoplasts are subjected to large osmotic stresses, the magnitudes of which depend on the subzero temperature. Large osmotic pressures occur during the freezing of an aqueous solution because solutes are largely excluded from the ice matrix and accumulate in the unfrozen portion of the mixture. Ice formation continues until the chemical potential of the unfrozen solution is in equilibrium with that of the ice, which is a direct function of the subzero temperature. At equilibrium, the osmolality of the unfrozen solution is equal to $(273-T)/1.86$ and is independent of the initial osmolality of the solution. For example, the osmolality of the unfrozen portion is 2.69 at −5 °C and 5.37 at −10 °C, regardless of the solute or its initial concentration. The amount of water that must be frozen to achieve the given osmolality is dependent on both the species of solute and its initial

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concentration. See Franks (1985) and Taylor (1987) for a more comprehensive description of the physical and chemical aspects of ice formation in aqueous solutions.

Following ice formation in the suspending medium and the concentration of the extracellular solutes, a gradient in the chemical potential of the intra- and extracellular solutions will result in cell dehydration, the extent of which can be estimated from the Boyle van't Hoff relation for the particular cell type. Because the osmolality of the suspending medium increases linearly as a function of the subzero temperature and cell volume varies linearly with the reciprocal of the osmolality of the suspending medium, the osmotic volume of the cell decreases greatly at relatively high subzero temperatures.

In protoplasts isolated from non-acclimated rye leaves (hereafter referred to as NA protoplasts), the freeze-induced osmotic stresses result in two different forms of injury to the plasma membrane, depending on the magnitude of the osmotic stress. Over the range of 0 to −5 °C, destabilization of the plasma membrane is largely a consequence of the osmotic excursions incurred during the freeze–thaw cycle, whereas at lower temperatures, injury is a consequence of the large osmotic potentials that result in more severe cell dehydration *per se* (see Steponkus (1984); Steponkus & Lynch (1989*a*)). In both cases, cold acclimation increases the cryostability of the plasma membrane.

Destabilization of the plasma membrane during osmotic excursions

When suspensions of protoplasts isolated from non-acclimated rye leaves are frozen to temperatures over the range of −2 to −5 °C, the protoplasts respond osmotically and attain a minimum volume predicted by the Boyle van't Hoff relation (Dowgert & Steponkus 1983, 1984). Although approximately 80% of the osmotically active water is removed at −5 °C, this extent of dehydration *per se* is not injurious as the protoplasts are osmotically responsive during warming and thawing of the suspending medium. However, lysis occurs during osmotic expansion before the protoplasts regain their initial volume. This form of injury is referred to as expansion-induced lysis (see Steponkus (1984); Steponkus & Lynch (1989*a*)).

Expansion-induced lysis is a consequence of the behaviour of the plasma membrane during freeze–thaw-induced osmotic excursions. During freeze-induced osmotic contraction of NA protoplasts, the plasma membrane undergoes endocytotic vesiculation and the surface area of the plasma membrane is not conserved (Dowgert & Steponkus 1984; Gordon-Kamm & Steponkus 1984*a*). Although endocytotic vesiculation *per se* is not injurious, sufficiently large area reductions are irreversible. Because intrinsic elastic expansion of the plasma membrane is limited to 2–3% (Wolfe & Steponkus 1981, 1983) the larger increases in area that occur during osmotic expansion following thawing of the suspending medium require that new membrane material be incorporated into the plane of the plasma membrane. However, the area expansion potential is limited by a constant increment (approximately 0.25 in the case of protoplasts isolated from non-acclimated rye leaves). This constant is independent of the extent of previous osmotic contraction (Wiest & Steponkus 1978), which suggests that the material deleted in the form of endocytotic vesicles is not readily reincorporated into the plasma membrane during subsequent osmotic expansion. As a result, sufficiently large area reductions (greater than 0.25) are irreversible. (See Dowgert *et al.* (1987); Wolfe *et al.* (1985, 1986) for a detailed analysis of membrane exchange during osmotic excursions).

Expansion-induced lysis is not observed in protoplasts isolated from leaves of cold-acclimated

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rye seedlings (hereafter referred to as ACC protoplasts). This is because cold acclimation alters the behaviour of the plasma membrane during osmotic contraction such that the plasma membrane forms exocytotic extrusions rather than endocytotic vesicles (Dowgert & Steponkus 1984; Gordon-Kamm & Steponkus 1984*b*). As a result, the surface area of the plasma membrane is conserved during osmotic contraction. See Steponkus & Uemura (1989) for a more detailed review of the mechanism of expansion-induced lysis.

Studies of the cryobehaviour of large unilamellar vesicles (LUVS) prepared from plasma membrane lipid extracts indicate that the differential behaviour of the plasma membrane during osmotic contraction of NA and ACC protoplasts is also observed in LUVS prepared from the respective plasma membrane lipid extracts (Steponkus & Lynch 1989*b*). During osmotic contraction of LUVS prepared from the plasma membrane lipid extracts of non-acclimated rye leaves, numerous daughter vesicles are subduced from the parent bilayer into the interior of the LUV. In contrast, LUVS prepared from plasma membrane lipid extracts of cold-acclimated rye leaves form tubular or vesicular extrusions during osmotic contraction. Thus the differential behaviour of the plasma membrane observed during osmotic contraction of NA and ACC protoplasts is a consequence of alterations in the lipid composition of the plasma membrane. Further, because there is no lipid species that is unique to the plasma membrane fraction isolated from either non-acclimated or cold-acclimated rye leaves (Lynch & Steponkus 1987*a*), the differential cryobehaviour is a consequence of altered lipid–lipid interactions resulting from the changes in the proportions of the individual lipid species.

Subsequent studies in which the lipid composition of the plasma membrane was selectively modified have established that the differential behaviour is associated with the increase in the unsaturated species of phosphatidylcholine (Steponkus *et al.* 1988). By using a protoplast–liposome fusion technique (see Arvinte & Steponkus (1988)) to enrich the plasma membrane of isolated protoplasts with specific lipids, the cryobehaviour of the plasma membrane and the freezing tolerance of the protoplasts were significantly altered. For example, fusion of NA protoplasts with liposomes composed of the total phospholipid fraction isolated from the plasma membrane of cold-acclimated leaves resulted in 100% survival over the range of 0 to −5 °C. In untreated NA protoplasts survival decreases by 50% over this range because of expansion-induced lysis. The increase in freezing tolerance was also elicited by fusion of NA protoplasts with liposomes composed of either mono- or di-unsaturated species of phosphatidylcholine, with dilinoleoyl- and dilinolenoylphosphatidylcholine the most effective. However, fusion with disaturated species neither increased nor decreased the survival over the range of 0 to −5 °C.

The increased survival of NA protoplasts over the range of 0 to −5 °C following enrichment of the plasma membrane with mono- or di-unsaturated species of phosphatidylcholine was the result of a transformation in the cryobehaviour of the plasma membrane. Enrichment of the plasma membrane of NA protoplasts with dilinoleoylphosphatidylcholine resulted in the formation of the exocytotic extrusions during freeze-induced osmotic contraction. The morphology and frequency of the extrusions are indistinguishable from those observed in ACC protoplasts. When subjected to an isotonic–hypertonic–hypotonic osmotic excursion, survival does not decrease until after the fused protoplasts are expanded beyond the isotonic volume, as occurs in ACC protoplast. In NA protoplasts fused with liposomes of dilinoleoylphosphatidylcholine, the osmotic expansion potential during an isotonic–hypotonic osmotic excursion is identical to that of untreated NA protoplasts, indicating that the increased

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tolerance to isotonic–hypertonic–hypotonic excursions is not a consequence of excess membrane material following fusion.

During cold acclimation, the transformation in the cryobehaviour of the plasma membrane is completed within the first seven to ten days of the acclimation period (Uemura & Steponkus 1989). At that time the incidence of expansion-induced lysis is less than 10% at any subzero temperature and exocytotic extrusions are formed during osmotic contraction. During this time, the major changes in the phospholipid molecular species of the plasma membrane are also completed (Cahoon *et al.* 1989) and enrichment with dilinoleoylphosphatidylcholine has no effect on the freezing tolerance. Therefore, we conclude that the alteration in the cryobehaviour of the plasma membrane that results in the formation of exocytotic extrusions rather than endocytotic vesicles during osmotic contraction is an initial step in the cold acclimation process and is a consequence of alterations in the phospholipid composition of the plasma membrane.

Destabilization of the plasma membrane following severe dehydration

At temperatures below −5 °C, destabilization of the plasma membrane of isolated rye protoplasts is a consequence of the large osmotic pressures and severe cell dehydration. For example, at −10 °C, the osmolality of the suspending medium is 5.4 with an osmotic potential of approximately −12 MPa and more than 90% of the osmotically active water is removed from the protoplasts. Under these conditions, injury in NA protoplasts is manifested as a loss of the semipermeable characteristics of the plasma membrane such that the protoplasts are osmotically unresponsive. This form of injury is associated with several changes in the ultrastructure of the plasma membrane including the formation of large aparticulate domains in the plasma membrane, aparticulate lamellae subtending the plasma membrane, and lamellar- to hexagonal$_{II}$-phase transitions in the plasma membrane and subtending lamellae (Gordon-Kamm & Steponkus 1984*c*). These ultrastructural alterations are a consequence of freeze-induced dehydration rather than exposure to subzero temperatures and can also be induced by dehydration in a 5.4 osmolal sorbitol solution at 0 °C. These ultrastructural changes are not observed in ACC protoplasts. This differential propensity for dehydration-induced lamellar- to hexagonal$_{II}$-phase transitions is also observed in multilamellar lipid vesicles prepared from the plasma membrane lipids of non-acclimated and cold-acclimated rye leaves (Cudd & Steponkus 1988). Liposomes prepared from the plasma membrane lipid fraction of non-acclimated leaves and subjected to osmotic dehydration undergo aggregation, fusion, and the formation of inverted cylindrical micelles (hexagonal$_{II}$-phase structures), whereas liposomes prepared from the plasma membrane lipids of cold-acclimated leaves do not.

To explain the differential propensity for dehydration-induced lamellar- to hexagonal$_{II}$-phase transitions in the plasma membrane of NA versus ACC protoplasts and liposomes prepared from the plasma membrane lipids isolated from non-acclimated versus cold-acclimated rye leaves, it is necessary to develop an understanding of lamellar- to hexagonal$_{II}$-phase transitions as a consequence of freeze-induced dehydration that is consistent with the alterations in plasma membrane lipid composition following cold acclimation. The concept of molecular shape or molecular packing characteristics of individual lipid species (Cullis *et al.* 1985; Gruner *et al.* 1985), appropriate when considering the polymorphic phase behaviour of individual lipids or binary mixtures, does not adequately explain the differential propensities of plasma

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membrane lipids isolated from non-acclimated and cold-acclimated rye leaves to form non-bilayer structures. If the various lipid components are considered individually, the changes following acclimation would seem to actually promote lamellar- to hexagonal$_{II}$-phase transitions. For example, di-unsaturated species of phosphatidylethanolamine, which are 'non-bilayer-forming' lipids, increase following acclimation. Free sterols, which facilitate thermotropic lamellar- to hexagonal$_{II}$-phase transitions in mixtures of phosphatidylcholine and phosphatidylethanolamine (Cullis & de Kruijff 1978; Tilcock & Cullis 1982; Tilcock *et al.* 1982) also increase following acclimation. Concomitantly, the content of cerebrosides, strong 'bilayer-forming' lipids (Abrahamson *et al.* 1972; Curatolo 1987), decreases following acclimation.

Studies of lipid mixtures provide insight to explain this apparent paradox. For lipid mixtures containing both 'bilayer-forming' and 'non-bilayer-forming' lipids, as is the case with the plasma membrane, the formation of hexagonal$_{II}$-phase structures is preceded by lipid demixing. That is, mixtures in which the constituent lipid species exhibit miscibility exist in a bilayer form. If demixing occurs (as a consequence of temperature or dehydration, or both), the 'non-bilayer-forming' lipids become enriched in localized domains and, free from the influence of the 'bilayer-forming' species, undergo a lamellar- to hexagonal$_{II}$-phase transition. Our working hypothesis proposes that (i) lipid demixing of NA plasma membrane lipid constituents occurs as a consequence of dehydration and leads to localized domains and the formation of non-bilayer structures and (ii) changes in lipid composition following acclimation are such that dehydration-induced lipid demixing is precluded. With this working hypothesis, emphasis is away from lamellar- to hexagonal$_{II}$-phase transitions *per se* and towards establishing the mechanism of dehydration-induced demixing and the involvement of plasma membrane lipid components in modulating lipid miscibility.

The ultrastructural alterations of the plasma membrane of NA protoplasts effected by freezing or osmotic dehydration (see above) reflect demixing of protein and lipid components of the bilayer. The aparticulate regions of the plasma membrane may be interpreted as representing gel-phase regions of lipid. As such, lipid demixing is accomplished by a dehydration-induced liquid-crystalline- to gel-phase transition that precedes the observed formation of non-bilayer structures. This interpretation appears consistent with studies of the phase behaviour of phospholipids, which demonstrate that dehydration increases the liquid-crystalline- to gel-phase transition temperature (Chapman *et al.* 1967) and decreases the lamellar-to hexagonal$_{II}$-phase transition temperature T_m (Luzzati 1968). Thus it has been proposed that the deleterious effects of dehydration of biological membranes are the result of lyotropic, liquid-crystalline- to gel-phase transitions in lipid species such as phosphatidylcholine that lead to demixing of the lipid mixture and the localized enrichment of 'non-bilayer-forming' lipids such as phosphatidylethanolamine, which undergo a lamellar-to hexagonal$_{II}$-phase transition upon further dehydration (Crowe & Crowe 1984, 1986*a*, *b*). However, for this model to be relevant to the changes observed in rye protoplasts, lyotropic, liquid-crystalline- to gel-phase transitions of phosphatidylcholine must be induced at a water potential of *ca.* −12 MPa, which occurs during freeze-induced dehydration at −10 °C when the protoplasts are subjected to a 5.4 osm solution. Recent studies of the phase behaviour and hydration characteristics of phosphatidylcholine species indicate that under conditions of temperature and dehydration encountered during freezing and that result in membrane destabilization, unsaturated species common to the plasma membrane would be expected to remain in the

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liquid crystalline phase (Lynch & Steponkus 1988*a*, 1989*a*). Although other plasma membrane lipid constituents may be involved in gel-phase formation and phase separation, no apparent transition is observed over the range of −40 to −70 °C with plasma membrane lipid dispersions in excess water or equilibrated at a water potential of −16 MPa. Taken together, these results suggest that the dehydration-induced ultrastructural alterations in the plasma membrane of NA protoplasts frozen to −10 °C are not the result of liquid-crystalline- to gel-phase transitions *per se*. An alternate mechanism of demixing, which is also consistent with the observed changes in plasma membrane ultrastructure during freeze-induced cell dehydration, is based on the studies of Parsegian, Rand and co-workers (Lis *et al.* 1982; Rand 1981) involving repulsive hydration forces during the close approach of bilayers. When two bilayers approach to within 2–3 nm of each other, interbilayer forces are dominated by strongly repulsive hydration forces that increase exponentially with a characteristic decay length of typically 0.25–0.35 nm (LeNeveu *et al.* 1976, 1977; Cowley *et al.* 1978; Parsegian *et al.* 1979). Hydration repulsion is the result of the affinity of the hydrophilic surfaces for water and presents a large energy barrier to the close approach of membranes. Removal of water from between adjacent bilayers results in the bilayers being drawn closer together and the components within each bilayer being packed more closely (see Wolfe *et al.* (1986); Wolfe (1987)). Several changes in bilayer structure have been predicted to occur when bilayers are forced together (see Rand (1981); Rand & Parsegian (1984, 1986); Rand *et al.* (1980, 1985)). These include (i) liquid-crystalline- to gel-phase transitions; (ii) demixing of liquid mixtures and segregation into separate co-existing lamellar phases; (iii) bilayer-to-non-bilayer transitions. Although liquid-crystalline- to gel-phase transitions (increases in T_m) are observed at very short distances (i.e., when the bilayers are subjected to very high forces) demixing has been predicted to occur readily. This is because different lipids (and proteins) have different hydration characteristics and exhibit different equilibrium separation distances. Differences in the hydration repulsion and equilibrium separation distances of phosphatidylcholine and phosphatidylethanolamine have been documented. Lis *et al.* (1982) calculated the equilibrium separations of egg phosphatidylethanolamine and dioleoylphosphatidylcholine to be 1.7 nm and 2.2 nm, respectively. Marra and Israelachvili (1985) have shown that the hydration repulsion of dipalmitoylphosphatidylcholine is several orders of magnitude greater than that of dipalmitoylphosphatidylethanolamine at separations of $\leqslant 2$ nm. Thus lateral diffusion of lipids into or out of regions of close approach is expected in multicomponent bilayers that are being forced together as a consequence of dehydration (Lis *et al.* 1982; Rand 1981; Bryant & Wolfe 1989). Forces of the order of 10 MPa are sufficient to effect the close approach of bilayers and consequently after bilayer structure (Lis *et al.* 1982). Based on these measurements, it is suggested that the water potential (−12 MPa) and the resultant freeze-induced dehydration that occurs at −10 °C may be sufficient to induce demixing in certain lipid mixtures in the absence of a liquid-crystalline- to gel-phase transition. Similar calculations and predictions have been made for protein–lipid systems (Bryant & Wolfe 1989). Thus aparticulate regions of the plasma membrane and demixing of plasma membrane lipids (and proteins) as well as bilayer-to-non-bilayer phase transitions may occur in the absence of a liquid-crystalline- to gel-phase transition in a subpopulation of plasma membrane lipid species.

To test this hypothesis and to provide a molecular explanation for the differential propensity for lamellar- to hexagonal$_{II}$-phase transitions in the plasma membrane of NA versus ACC protoplasts (Gordon-Kamm & Steponkus 1984*c*) and plasma membrane-derived liposomes

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(Cudd & Steponkus 1988), we have examined the hydration characteristics and phase behaviour of individual lipids, simple lipid mixtures, and plasma membrane lipid extracts. Although many previous studies of dehydration and hydration repulsion in lipid systems have focused on the properties and behaviour of phosphatidylcholine and phosphatidylethanolamine, these two phospholipids together compose only 25% and 35% of the lipid complement of the plasma membrane from non-acclimated and cold-acclimated rye leaves, respectively. Thus any mechanistic hypothesis of the differential behaviour of the plasma membrane observed in NA versus ACC protoplasts must also take into account the physical properties (phase behaviour and hydration characteristics) and lipid–lipid interactions of the other membrane constituents including free sterols, sterylglucosides, acylated sterylglucosides, and glucocerebrosides.

At high water contents, dispersions of plasma membrane lipids isolated from non-acclimated and cold-acclimated rye leaves do not exhibit an obvious phase transition as detected by differential scanning calorimetry because the plasma membrane contains free sterols in proportions sufficient to preclude any detectable phase transition in a homogeneous mixture with phospholipids. However, when equilibrated at water potentials between −16 and −160 MPa, phase transitions occur between 26 and 54 °C in plasma-membrane lipids of both non-acclimated and cold-acclimated rye leaves, suggesting that demixing occurred during equilibration at these water potentials. When equilibrated at similar water potentials above −70 MPa, the respective T_m values of dispersions of plasma membrane lipids of non-acclimated rye leaves are higher than those of cold-acclimated leaves. This is attributable to the fact that, at equivalent water potentials, the water content of plasma-membrane lipids of cold-acclimated leaves is greater than that of plasma membrane lipids isolated from non-acclimated leaves. Because the values for T_m of both samples are similar when expressed as a function of water content, the differences in the hydration characteristics must account for differences in the calorimetric behaviour. Moreover, the fact that plasma membrane lipid preparations from cold-acclimated leaves have a higher water content (and so presumably exhibit greater interbilayer distances or water layer thickness) for a given water potential may serve to decrease the likelihood of lamellar- to hexagonal$_{II}$-phase transition, which is an interbilayer phenomenon.

To understand how changes in lipid composition following acclimation may account for the different hydration characteristics of the plasma membrane lipid extracts, desorption isotherms have been determined for individual lipid components (Lynch & Steponkus 1989*a*, *b*). Of the plasma membrane lipid components investigated, phosphatidylcholine has the greatest water content for a given water potential (19.5 wt% water or 10–11 mol H_2O per mol lipid at −10 MPa) followed by phosphatidylethanolamine (13 wt% or 6 mol H_2O per mol lipid at −10 MPa). At a water potential of −10 MPa, free sterols, sterylglucosides, and glucocerebrosides had water contents of 6 wt%, 5 wt%, and 2 wt% water, respectively, corresponding to 1–2 mol H_2O per mol lipid. Mixtures of phosphatidylcholine and phosphatidylethanolamine containing 33–66 mol% of phosphatidylcholine exhibited hydration properties similar to that of pure phosphatidylcholine. The addition of equimolar amounts of sterol or sterylglucoside to phosphatidylcholine or phosphatidylcholine–phosphatidylethanolamine mixtures (to yield a 1:1:1 molar ratio) resulted in only a minor decrease in the level of hydration at any given water potential. In contrast, phosphatidylcholine–glucocerebroside mixtures had water contents of approximately 14 wt% at −10 MPa. These results suggest that increases in

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phospholipids and free sterols and concomitant decreases in glucocerebrosides after acclimation alter the hydration characteristics of the plasma membrane.

Two lipid classes common to the plasma membrane, glucocerebrosides and sterylglucosides, have distinct physical properties and limited miscibilities with phospholipids. For example, mixtures containing 1-palmitoyl-2-oleoyl-phosphatidylcholine and $\geqslant 30$ mol % rye cerebrosides exhibit non-ideal mixing and gel phase immiscibility (Lynch & Steponkus 1987*b*). Similar observations have been made by using mixtures of brain cerebrosides and phospholipids (Curatolo 1987; Johnson & Chapman 1988). Mixtures containing 25% brain cerebrosides, 25% phosphatidylcholine and 50% cholesterol also exhibit a separate phase (Johnston & Chapman 1988).

Studies of phosphatidylcholine–sterylglucoside mixtures (Lynch & Steponkus 1988*b*) suggest that sterylglucosides influence phospholipid phase behaviour differently than do free sterols. The addition of 33 mol % sterylglucosides to dipalmitoylphosphatidylcholine, 1-palmitoyl-2-oleoylphosphatidylcholine, or dioleoylphosphatidylcholine slightly shifts the T_m values and diminishes, but does not eliminate, as do free sterols, the respective endotherms. This suggests that sterylglucosides are less miscible than free sterols in mixtures with phosphatidylcholine.

Even when fully hydrated, the high content of glucocerebrosides and sterylglucosides present in the plasma membrane of non-acclimated rye leaves may result in a limited degree of demixing and domain formation that catalyses further demixing during freeze-induced dehydration. This may be exacerbated by the hydration characteristics of the lipid components that contribute to the differential hydration characteristics of the plasma membrane of non-acclimated and cold-acclimated rye leaves. Thus the changes in lipid composition following cold acclimation may serve to increase membrane cryostability by increasing the extent of hydration and decreasing the likelihood of demixing during freeze-induced dehydration.

Portions of this work were supported by the United States Department of Energy (Grant No. DE-FG02-84ER13214) and the United States Department of Agriculture Competitive Research Grant Program (Grant No. 5-CRCR-1-1651 and Grant No. 88-37264-3988).

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Discussion

U. Heber (*Institute of Botany and Pharmaceutical Biology, University of Würzburg, F.R.G.*). When a protoplast is frozen down to the temperature of the lamellar- to hexagonal$_{II}$-phase transition, does Professor Steponkus know what happens to intracellular solutes? What are the permeability properties of the altered plasmalemma during and after the alteration?

P. L. Steponkus. Lamellar- to hexagonal$_{II}$-phase transitions are observed in protoplasts frozen to -10 °C. Following osmotic equilibration at this temperature, the osmolality of the cytosol is 5.37 as a result of concentration of the intracellular solutes.

Following exposure to -10 °C, the protoplasts (isolated from non-acclimated leaves) are osmotically unresponsive. Presumably, this is because of an irreversible loss of the

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semipermeable characteristics of the plasma membrane. However, it should be emphasized that the lamellar- to hexagonal$_{II}$-phase transition is presumed to be reversible following thawing of the suspending medium.

R. S. Pearce (*Department of Agricultural and Environmental Science, University of Newcastle-upon-Tyne, U.K.*). Professor Steponkus ascribes hex$_{II}$ formation to demixing of the membrane lipids in the stressed material. Hex$_{II}$ is not found in the hardy material. What other potentially damaging consequences of demixing could occur that might explain damage when the hardy material is sufficiently stressed?

P. L. Steponkus. At present we do not have any evidence that demixing occurs in the plasma membrane of protoplasts isolated from cold-acclimated leaves. Apparently, cold acclimation alters the lipid composition such that demixing is precluded, hence lamellar- to hexagonal$_{II}$-phase transitions do not occur. As a result, injury to protoplasts isolated from cold acclimated leaves is a consequence of another lesion.

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