MINI-REVIEW

Freeze/Thaw-Induced Destabilization of the Plasma Membrane and the Effects of Cold Acclimation

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Abstract

Disruption of the plasma membrane is a primary cause of freezing injury. In this review, the mechanisms of injury resulting from freeze-induced cell dehydration are presented, including destabilization of the plasma membrane resulting from (a) freeze/thaw-induced osmotic excursions and (b) lyotropic phase transitions in the plasma membrane lipids. Cold acclimation dramatically alters the behavior of the plasma membrane during a freeze/thaw cycle—increasing the tolerance to osmotic excursions and decreasing the propensity for dehydration-induced lamellar to hexagonal-II phase transitions. Evidence for a casual relationship between the increased cryostability of the plasma membrane and alterations in the lipid composition is reviewed.

Key Words: Plasma membrane; freezing injury; cold acclimation; lipids; mesomorphic phase behavior; dehydration; liposomes; isolated protoplasts.

Introduction

As early as 1912, Maximov suggested that disruption of the plasma membrane was the primary cause of freezing injury. However, with the exception of the classical studies of Scarth, Levitt, and Siminovitch over the period of 1936 to 1940 (Scarth and Levitt, 1937; Scarth *et al.*, 1940; Levitt and Scarth 1936a, b; Levitt and Siminovitch, 1940; Siminovitch and Scarth, 1938), only recently have experimental studies been directed to the cellular and molecular aspects of freeze/thaw-induced lesions (see Steponkus, 1984). Although it is likely that all cellular membranes are vulnerable to freeze/thaw-induced destabilization, the stability of the plasma membrane is of

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foremost importance because of its central role in cellular behavior *during* a freeze/thaw cycle. The plasma membrane is the principal interface between the extracellular medium and the cytosol, and acts as a semipermeable barrier allowing for the efflux/influx of water during the freeze/thaw cycle while also serving as a barrier to preclude seeding of the intracellular solution by extracellular ice. In some cold hardy plants, it can maintain these structural and functional characteristics over a range of temperatures that can be as great as 200°C, in solutions on the order of 10–20 Osm and that result in removal of > 95% of the osmotically active water from the cell—while encased in a matrix of ice crystals. Clearly, cold acclimation transforms the plasma membrane into a rather durable entity, considering that most epoxy resins are not capable of withstanding such abuse.

Our approach to the study of freeze/thaw-induced lesions in the plasma membrane has been to determine the behavior and intrinsic properties of the plasma membrane *in situ* through studies of the cryobehavior of isolated protoplasts. This approach is yielding a comprehensive analysis of the manifestations of freezing injury, the mechanisms responsible, and the effects of cold acclimation on the cryobehavior of the plasma membrane that are responsible for its increased cryostability (see Steponkus, 1984). These studies provide a foundation for mechanistic studies to establish a causal relationship between alterations in the lipid composition and the altered cryobehavior of the plasma membrane following cold acclimation—a long-standing and controversial issue.

Freeze-Induced Cell Dehydration

During a freeze/thaw cycle, the plasma membrane is subjected to a multitude of stresses including thermal, mechanical, chemical, osmotic, and possibly even electrical perturbations [Steponkus (1984); see Franks (1985) and Taylor (1987) for comprehensive reviews of the physical/chemical events during the freezing of aqueous solutions]. These stresses do not occur independently; instead they are a concatenation of events ensuing with ice formation. Nevertheless, evidence to date suggests that, in the absence of intracellular ice formation, destabilization of the plasma membrane in isolated protoplasts is primarily a consequence of osmotic stresses and cell dehydration (Steponkus, 1984).

During cooling of a cell suspension, ice typically forms first in the suspending medium—either as a consequence of heterogeneous nucleation or seeding. During the subsequent growth of ice crystals, solutes and gases are largely excluded from the ice matrix and increase in concentration in the unfrozen portion of the solution. Substantial increases in the solute and gas

concentrations can occur within the diffusion layer that extends a distance of the order $X = D/R$ from the ice interface, where D is the solute diffusion coefficient and R is the freezing rate (Gross *et al.*, 1975a, b). Cells that are suspended in the medium impede diffusion of solute from the advancing ice front, and ice will propagate faster in the less-concentrated regions. As a result, at slow to moderate rates of cooling, cells tend to be encapsulated in channels or pockets of unfrozen solution. Ice will continue to form 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 will be equal to $(273 - T)/1.86$ and is independent of the initial solute concentration. The proportion of the original solution that is unfrozen following equilibration at a given subzero temperature depends on the initial solute concentration and can be approximated as the ratio of the initial to final osmolality, but is more accurately determined from the liquidus curve of the phase diagram for the particular solution. For a 0.5 Osm solution frozen to $-10°C$, $\sim 9\%$ of the solution will be unfrozen and will have an osmolality of 5.4 (an osmotic potential > 12 MPa).

Following ice formation in the suspending medium, a gradient in the chemical potential of the intra- and extracellular solutions will be established, and the cells or protoplasts will respond osmotically to the increased solute concentration in the partially frozen suspending medium. During cooling, the intracellular solution will be transiently supercooled—the extent being dependent on the rate of water efflux from the cell relative to the rate of cooling. For isolated cells or protoplasts, the rate of water efflux will be a function of the magnitude of the gradient in the chemical potential of the intra- and extracellular solutions, the water permeability of the plasma membrane, and the area/volume ratio of the protoplast. For excised tissues and organs, there will be additional resistance to water efflux that will increase the extent of supercooling at a given cooling rate. Although it has been long assumed that cold acclimation increases the water permeability of the plasma membrane (Levitt and Scarth, 1936a, b), determinations of volumetric behavior of isolated rye protoplasts indicate that there is no difference in the hydraulic conductivity of protoplasts isolated from non-acclimated or cold-acclimated leaves (Dowgert and Steponkus, 1983). In fact, for a given cooling rate, the rate of water efflux is somewhat less in acclimated protoplasts because of the higher internal solute concentration resulting from osmotic adjustment during cold acclimation.

Following osmotic equilibration, the extent of cell dehydration can be estimated from the Boyle van't Hoff relationship $V = V_b + x\,(\text{Osm}^{-1})$, with the osmolality being a function of the subzero temperature. For protoplasts isolated from nonacclimated rye leaves (NA protoplasts), the fractional

volume $V = 0.084 + 0.485$ (Osm^{-1}); for protoplasts isolated from cold-acclimated rye leaves (ACC protoplasts), $V = 0.146 + 0.879$ (Osm^{-1}).

Manifestations of Cryoinjury to the Plasma Membrane

In NA protoplasts, freeze-induced dehydration results in two different forms of injury—the incidence of which depends on the extent of cell dehydration (i.e., the minimum temperature imposed) (Steponkus *et al.*, 1982, 1984). When cooled to temperatures over the range of 0 to $-5°C$, the protoplasts respond osmotically and attain a minimum volume predicted by the Boyle van't Hoff relationship. For example, during cooling to $-5°C$, the osmolality of the partially frozen suspending medium increases from 0.5 to 2.69 Osm and $\sim 80\%$ of the osmotically active water is removed from the protoplasts. This extent of dehydration *per se* is not injurious because the protoplasts are osmotically responsive following warming and thawing of the suspending medium. However, lysis occurs during osmotic expansion before the protoplasts regain their initial volume. Hence, this form of injury is referred to as expansion-induced lysis. In contrast, when cooled to lower temperatures and subjected to a greater extent of cell dehydration (e.g., at $-10°C$, the osmolality will be 5.37 and $\sim 90\%$ of the osmotically active water is removed), the protoplasts are osmotically unresponsive during thawing of the suspending medium. This form of injury is referred to as loss of osmotic responsiveness. Although these two forms of injury occur as a result of cell dehydration, the mechanisms of injury are vastly different.

Expansion-Induced Lysis

Protoplasts behave as ideal osmometers (i.e., volume varies linearly with Osm^{-1}) over a wide range of osmolalities (Wiest and Steponkus, 1978). Following either osmotic contraction or expansion, the protoplasts are spherical, and the surface of the plasma membrane is smooth without any folding or pleating (Gordon-Kamm and Steponkus, 1984a), indicating that volumetric changes result in changes in the area of the plasma membrane. The changes in area can be quite large, ranging from 0.50 to 1.50 (Dowgert and Steponkus, 1984). Studies of the stress–strain relationship of the plasma membrane of isolated protoplasts indicate that the area elastic modulus k_A is ≈ 200 $mN \cdot m^{-1}$ and lysis occurs at tensions on the order of 4–6 $mN \cdot m^{-1}$ (Wolfe and Steponkus, 1981, 1983a, b). Thus, intrinsic or elastic expansion/contraction of the plasma membrane is limited to changes in area of ~ 2–3%. For larger changes in area, membrane material is either deleted from or incorporated into the membrane with the transfer of material dependent on

the surface tension (γ) of the plasma membrane (Wolfe and Steponkus, 1981). In isotonic solutions, the resting tension (γ_r) of the plasma membrane is $\sim 100 \, \mu\text{N} \cdot \text{m}^{-1}$. During osmotic expansion, the tension is increased and material is transferred into the membrane at a rate that is dependent on γ. During osmotic contraction, γ_r is relaxed to zero and material is deleted from the membrane until γ_r is reestablished.

High-resolution, computer-enhanced video microscopy (Dowgert and Steponkus, 1984) and electron microscopy (Gordon-Kamm and Steponkus, 1984a) have been used to document the deletion of membrane material. Osmotic contraction of NA protoplasts results in endocytotic vesiculation of the plasma membrane, and numerous clusters of vesicles that are 0.1–$1.5 \, \mu\text{m}$ in diameter are observed in the cytoplasm. Freeze-fracture studies reveal that the intramembrane particle density on both fracture faces of the plasma membrane remains unchanged following osmotic contraction. This suggests that endocytotic vesiculation involves a unit membrane deletion.

The expansion potential of isolated protoplasts is limited by a constant increment (tolerable surface area increment) rather than a constant maximum (Wiest and Steponkus, 1978). Thus, the expansion potential of protoplasts expanded from either isotonic or hypertonic solutions is similar, but lysis occurs at different critical areas. This suggests that the membrane material that is deleted during osmotic contraction does not enter the membrane reservoir that supplies material to the plasma membrane during osmotic expansion. As a result, sufficiently large area reductions (greater than the tolerable surface area increment) are irreversible. The tolerable surface area increment (TSAI) value varies among species (Steponkus and Wiest, 1979) and is influenced by the composition of the suspending medium (Steponkus et al., 1983). For rye protoplasts, the TSAI value is 0.25. However, this is only a phenomenological parameter used to characterize the expansion potential of a population of protoplasts (i.e., the TSAI value is the surface area increment at which lysis of 50% of the population occurs). For an individual protoplast, lysis or rupture of the plasma membrane is probabilistic. The probability of lysis during osmotic expansion increases with tension (Wolfe et al., 1985, 1986a, b) and the expected lifetime is $\sim 43 \, \text{sec}$ at $\gamma = 5.0 \, \text{mN} \cdot \text{m}^{-1}$ and is halved by each increase of $0.5 \, \text{mN} \cdot \text{m}^{-1}$.

Thus, expansion-induced lysis, which is the predominant form of freezing injury observed in NA protoplasts cooled to temperatures over the range of 0 to -5°C, is a consequence of mechanical strains incurred by the plasma membrane during osmotic expansion. However, these strains arise because of the deletion of membrane material during freeze-induced osmotic contraction, i.e., dehydration-induced endocytotic vesiculation of the plasma membrane.

Expansion-induced lysis is not a predominant form of injury in protoplasts isolated from cold-acclimated leaves (Dowgert and Steponkus, 1984).

This is because the behavior of the plasma membrane during osmotic contraction is dramatically altered following cold acclimation. Whereas the plasma membrane of NA protoplasts undergoes endocytotic vesiculation during osmotic contraction, the plasma membrane of ACC protoplasts forms exocytotic extrusions (Gordon-Kamm and Steponkus, 1984b). The extrusions, which resemble microvilli and tethered spheres, have a densely osmiophilic interior, suggesting a high lipid content. Freeze-fracture studies suggest that the lipidic material results from a preferential deletion of lipids from the plasma membrane. Following osmotic contraction, there is a substantial increase in the frequency of paracrystalline arrays in the plasma membrane and an increase in the intramembrane particle density of the noncrystalline regions. Nevertheless, osmotic contraction is readily reversible in ACC protoplasts and this lipidic material apparently is reincorporated into the plasma membrane during subsequent osmotic expansion when the extrusions are drawn back into the plasma membrane. Studies of the mechanics of osmotic contraction and expansion have established a causal relationship between the formation of exocytotic extrusions and the reversibility of osmotic contraction in ACC protoplasts (Dowgert et al., 1987). That is, measurements of membrane tension during osmotic expansion from hypertonic solutions demonstrate that the tension in the plasma membrane does not increase appreciably until the protoplasts are expanded beyond the isotonic volume.

Although differences in the behavior of the plasma membrane during osmotic contraction account for the differential sensitivity of NA and ACC protoplasts to osmotic excursions and hence the preclusion of expansion-induced lysis in ACC protoplasts, the manner by which this differential behavior is effected has been enigmatic. While we speculated that the differential behavior was a consequence of alterations in the composition of the plasma membrane, as opposed to control by the cytoskeleton (Dowgert and Steponkus, 1984), the nature of the compositional change was obscure. However, recent studies (Steponkus et al., 1988) provide compelling evidence that the differential cryobehavior of the plasma membrane of NA and ACC protoplasts is a consequence of differences in the lipid composition of the plasma membrane. These studies are discussed in detail in a following section.

Loss of Osmotic Responsiveness

Loss of osmotic responsiveness occurs in NA protoplasts cooled to temperatures below $-5°C$, which results in severe freeze-induced dehydration. For example, at $-10°C$, the protoplasts are subjected to a solution of 5.4 Osm with an osmotic potential > 12 MPa and $> 90\%$ of the osmotically

active water is removed. Under these conditions, injury is associated with several changes in the ultrastructure of the plasma membrane, including the appearance of lateral phase separations, aparticulate lamellae subtending the plasma membrane, and lamellar to hexagonal-II phase transitions in the plasma membrane and subtending lamellae (Gordon-Kamm and Steponkus, 1984c). These ultrastructural changes and the loss of osmotic responsiveness are consequences of freeze-induced dehydration rather than exposure to subzero temperatures *per se* and can be effected by osmotic dehydration (5.4 Osm sorbitol) in the absence of ice formation. In contrast, when the protoplast suspensions are supercooled to temperatures over the range of − 5 to − 15°C, survival remains at 100% and only small areas of lateral phase separations are observed. Studies of the covariance of survival and the incidence of hexagonal-II formation under a wide variety of conditions that alter the survival curve (% survival *vs.* freezing temperature) provide strong evidence, albeit correlative, for a causal relationship between lamellar to hexagonal-II phase transitions and loss of osmotic responsiveness (Gordon-Kamm and Steponkus, 1985; Steponkus and Gordon-Kamm, 1985; Pihakaski and Steponkus, 1987). Most important, dehydration-induced lateral phase separations and hexagonal-II phase structures are not observed in ACC protoplasts (Gordon-Kamm and Steponkus, 1984c).

Although our ultimate goal is to provide a molecular explanation for the differential propensity for dehydration-induced lamellar to hexagonal-II phase transitions in the plasma membrane of NA *vs.* ACC protoplasts, it is necessary first to provide a mechanistic understanding of the occurrence of hexagonal-II phase during freeze-induced dehydration of NA protoplasts. From studies of the phase behavior of phospholipids, it is well established that dehydration increases the liquid-crystalline to gel (L_α-to-L_β) phase transition temperature (Chapman *et al.*, 1967) and decreases the lamellar to hexagonal-II phase transition temperature (Luzzati, 1968). Thus, it has been suggested (Crowe and Crowe, 1984, 1986a, b) that severe dehydration of biological membranes compromises their structural and functional integrity because dehydration-induced L_α-to-L_β phase transitions in lipid species such as phosphatidylcholine result in demixing of the lipid mixture and the localized enrichment of nonbilayer-forming lipids such as phosphatidyl-ethanolamine which, upon further dehydration, would undergo a lamellar to hexagonal-II phase transition. This possibility is supported by the fact that, in mixtures of phosphatidylcholine–phosphatidylethanolamine, lowering the proportion of phosphatidylcholine increases the probability of a lamellar to hexagonal-II phase transition (Cullis and de Kruijff, 1979). However, lamellar to hexagonal-II phase transitions are *inter*bilayer events (see Cullis *et al.*, 1985) and involve more than just the removal of water from an isolated bilayer. During severe cell dehydration, spatial distances between the plasma

membrane and other endomembrane systems will be decreased and inter-bilayer events become a major concern.

When two membranes approach each other, they are subject to both long- and short-range forces. These include long-range van der Waals forces of attraction and electrostatic repulsive forces. However, at distances < 2 to 3 nm, the interbilayer forces are dominated by strongly repulsive hydration forces (LeNeveu et al., 1976, 1977; Cowley et al., 1978) that increase expo-nentially with a characteristic length of typically 0.25–0.35 nm (Parsegian et al., 1979). Hydration repulsion is the result of the affinity of the hydro-philic surfaces for water and presents a large energy barrier to the close approach of membranes. It is these forces that confer stability on the mem-branes by preventing their contact and fusion. From the perspective of biological cells at full hydration, the pressures required to overcome the hydration forces—on the order of several tens of MPa—appear enormous. However, from the perspective of freezing injury, these osmotic pressures are physiologically relevant: protoplast suspensions frozen to $-10°C$ are subject to an osmotic potential of 12 MPa, which increases to 25 MPa at $-20°C$. To put this in perspective of dehydration-induced mesomorphic phase tran-sitions, an osmotic pressure of 10 MPa raises the L_α-to-L_β phase transition temperature of dilauroylphosphatidylcholine from -2 to $+25°C$ (Parsegian et al., 1986).

Removal of water from adjacent bilayers has two major structural consequences: the bilayers are drawn closer together and the components within each bilayer pack more closely, creating a compressive stress and decreasing the molecular area (see Wolfe et al., 1986b; Wolfe, 1987). Several changes in bilayer structure have been predicted to occur when bilayers are forced together (see Rand, 1981; Rand and Parsegian, 1984; 1986; Rand et al., 1980, 1985). These include (a) L_α-to-L_β phase transitions, (b) demixing of lipid mixtures and segregation into separate coexisting lamellar phases, and (c) bilayer-to-nonbilayer transitions. Our observations of lateral phase separations, aparticulate lamellae, and hexagonal-II phase in the plasma membrane of NA protoplasts cooled to $-10°C$ are consistent with these predictions. Although aparticulate regions of the plasma membrane may be interpreted as representing gel phase regions from which proteins have been excluded, lateral segregation of membrane components (lipids and proteins) may also occur in the absence of a L_α-to-L_β phase transition. Because different lipids species exhibit distinct equilibrium separation distances (i.e., bilayer separations for a given force, Lis et al., 1982), lateral diffusion of lipids into or away from regions of closest approach is expected in multicom-ponent bilayers (Rand and Parsegian, 1986). Further experimental work is needed to characterize the physical state of the bilayer in these regions in order to distinguish between these two possibilities. The fact that these ultrastructural changes are not observed in ACC protoplasts indicates that

cold acclimation decreases the propensity to undergo demixing and lateral segregation of membrane components. The decreased incidence could be a consequence of alterations in the membrane composition (proteins and/or lipids) or even as a result of increased cytoplasmic concentrations of solutes such as sucrose or proline. Given the extensive documentation that a diverse array of carbohydrates preserve the functional and structural integrity of biological membranes during freezing and dehydration [see Crowe and Crowe (1984, 1986a, b) for reviews], it is likely that osmotic adjustment and the accumulation of solutes such as sucrose and proline contribute to the decreased propensity for lamellar to hexagonal-II phase transitions in the plasma membrane of ACC protoplasts. Nevertheless, the differential propensity could also be influenced by alterations in the membrane *per se*. In a subsequent section, evidence will be presented that alterations in the lipid composition of the plasma membrane following cold acclimation play a major role.

Effect of Cold Acclimation on the Plasma Membrane Lipid Composition

Although it is well established that changes in lipid composition occur during exposure to the low, nonfreezing temperatures required for cold acclimation, the majority of the studies have been restricted to either correlative studies of changes in lipid composition and cold hardiness or comparative studies of the lipid composition of species or varieties of differing hardiness (see Steponkus, 1978). The majority of these studies have been restricted to lipid analyses of whole tissue extracts or crude membrane fractions rather than the plasma membrane *per se*. Early studies did not have the advantage of an understanding of the molecular aspects of freezing injury to the plasma membrane and, as a result, the role of any changes in lipid composition was largely conjectural. In many instances, changes in lipid composition were viewed solely from the perspective of membrane "fluidity" without consideration of more contemporary views of lipid mesomorphism.

The improvement of techniques for isolating purified plasma membrane fractions (see Larsson, 1985) has provided the opportunity for studies of alterations in the lipid composition of the plasma membrane during cold acclimation. Analyses of plasma membrane isolated from a variety of tissues using two-phase partition techniques have demonstrated that the lipid composition is unique relative to other plant cell membranes (Uemura and Yoshida, 1984; Yoshida and Uemura, 1984, 1986; Lynch and Steponkus, 1987a, b; Rochester *et al.*, 1987). Typically, plant plasma membranes are enriched in free sterols and steryl derivatives such as steryl glucosides and acylated steryl glucosides. β-Sitosterol and campesterol are the predominant

sterols. Phospholipids, primarily phosphatidylcholine and phosphatidyl-
ethanolamine, are present in amounts comparable to those of free sterols.
Although their presence was overlooked in several early reports, recent
studies indicate that glucocerebrosides are a major component of plant
plasma membranes (Yoshida and Uemura, 1986; Lynch and Steponkus,
1987a, b; Rochester *et al.*, 1987). Glucocerebrosides from rye plasma mem-
brane are composed of hydroxynervonic, hydroxylignoceric, hydroxyerucic,
and hydroxybehenic acids and trihydroxy as well as dihydroxy long-chain
bases, including hydroxysphingenine, hydroxysphinganine, sphingadienine
isomers and sphingenine.

Although several studies have suggested that cold acclimation results in
only modest changes in the lipid composition of the plasma membrane
(Uemura and Yoshida, 1984; Yoshida and Uemura, 1984, 1986), the analyses
were limited in scope. A comprehensive lipid analysis of plasma membrane
from NA and ACC rye leaves has been published (Lynch and Steponkus,
1987b). To date, more than 100 lipid molecular species have been identified
as constituents of the plasma membrane. Although there are no lipid molecu-
lar species that are unique to either NA or ACC plasma membranes, cold
acclimation alters the proportion of virtually every lipid component.
Cumulatively, free sterols, steryl glucosides, and acylated steryl glucosides
constitute > 50 mol % of the total lipids in both NA and ACC plasma
membranes. Substantial changes in the proportions of the individual sterol
components are observed following cold acclimation. Free sterols increase
from 33 to 44 mol %, while steryl glucosides and acylated steryl glucosides
decrease from 15 to 6 mol % and 4 to 1 mol %, respectively. Following cold
acclimation, steryl glucosides and acylated steryl glucosides containing
β-sitosterol and campesterol decrease in amounts equivalent to the increases
in the respective free-sterol species, suggesting interconversion among these
three lipid classes during cold acclimation.

Following cold acclimation, the content of glucocerebrosides in rye
plasma membrane decreases from 16 mol % to < 7 mol %. Proportional
differences in the acyl chain composition, primarily an increase in hydroxy-
nervonic acid and decreases in hydroxybehenic acid and hydroxylignoceric
acid, reflect changes in the molecular species composition following accli-
mation (Cahoon and Lynch, 1988). Nevertheless, in both NA and ACC
samples, the predominant glucocerebroside species consists of hydroxy-
nervonic acid and hydroxysphingenine.

The phospholipid content of the plasma membrane increases from 32 to
42 mol % total lipid following cold acclimation. Phosphatidylcholine and
phosphatidylethanolamine increase from 15 to 20 mol % and 11 to 16 mol %,
respectively; and there are substantial differences in the individual molecular
species following acclimation. The principal mixed-chain molecular species

of phosphatidylcholine (1-palmitoyl-2-oleoylphosphatidylcholine, 1-palm-itoyl-2-linoleoylphosphatidylcholine, and 1-palmitoyl-2-linolenoylphos-phatidylcholine) increase only slightly, whereas the principal di-unsaturated molecular species (1-oleoyl-2-linoleoylphosphatidylcholine, dilinoleoylphos-phatidylcholine, 1-linoleoyl-2-linolenoylphosphatidylcholine, and dilin-olenoylphosphatidylcholine) more than double. The same trends are observed for phosphatidylethanolamine molecular species.

These results are in contrast to other studies which suggest that cold acclimation results in relatively few changes in the lipid composition of the plasma membrane. When the analysis is carried out at the molecular species level and the results expressed as mol % total lipid, it is evident that the proportion of virtually every lipid component is altered following accli-mation. The relevance of these changes is discussed below.

Increased Plasma Membrane Cryostability and Lipid Alterations Following Cold Acclimation: A Causal Relationship

As previously stated, most attempts to establish the role of lipid alter-ations in the cold-acclimation process have relied on correlative studies. However, the complexity of the lipid composition of the plasma membrane, the numerous changes following acclimation, and the fact that no single lipid molecular species is unique to either NA or ACC plasma membranes pre-clude the possibility that any simple correlative analysis of the changes will establish their role in the cold-acclimation process. Instead, mechanistic approaches must be taken to establish a causal relationship between alter-ations in the lipid composition and the increased cryostability of the plasma membrane. One approach is to determine whether liposomes derived from plasma membrane lipids exhibit the differential behavior observed in the plasma membrane of NA and ACC protoplasts (i.e., formation of endo-cytotic vesicles *vs.* exocytotic extrusions during osmotic contraction and the differential propensity for dehydration-induced lamellar to hexagonal-II phase transitions). A second, "membrane engineering," approach is to establish structure and function relationships by manipulation of the plasma membrane lipid composition via fusion of protoplasts with liposomes com-posed of specific lipids.

Cryobehavior of Liposomes

Membrane Flow During Freeze/Thaw-Induced Osmotic Contraction/ Expansion of Liposomes. During freeze/thaw-induced osmotic contraction/ expansion, the plasma membrane of NA protoplasts undergoes endocytotic vesiculation whereas that of ACC protoplasts forms exocytotic extrusions.

Using computer-enhanced, high-resolution video-cryomicroscopic tech-
niques, we have observed marked differences in the cryobehavior of large
(> 20-μm diameter) unilamellar vesicles (LUVs) prepared from plasma mem-
brane lipid extracts from nonacclimated and cold-acclimated rye leaves
during freeze-induced osmotic contraction (Steponkus and Lynch, 1989).
During osmotic contraction of NA LUVs, the liposome bilayer begins to
flutter and numerous vesicles are subduced from the liposome bilayer and
sequestered in the liposome interior. During subsequent osmotic expansion,
lysis occurs either as a series of transient lytic events during which time there
is partial release of intraliposomal solution allowing for a temporary estab-
lishment of osmotic equilibrium, or as a single lytic event without resealing
of the bilayer. Under similar conditions, ACC LUVs do not exhibit such
behavior. Instead, osmotic contraction results in the formation of either
exocytotic extrusions or vesicles that remain contiguous with the parent
bilayer. During subsequent osmotic expansion, the extrusions/vesicles are
observed to be drawn into the plane of the parent liposome bilayer.

*Propensity for Dehydration-Induced Lamellar to Hexagonal-II Phase
Transitions.* Freeze-fracture electron microscopy was used to determine
whether liposomes prepared from the total lipid extract of plasma membrane
fractions isolated from nonacclimated or acclimated rye leaves exhibited a
differential propensity for dehydration-induced lamellar to hexagonal—II
phase transitions (Cudd and Steponkus, 1988). When NA liposomes
prepared in 1.3 wt. % sorbitol were dehydrated at 4°C in 73 wt. % sorbitol
for 15 h (overnight), hexagonal-II phase was observed in aggregates of
apparently fused liposomes also having lamellar phase regions and loosely
ordered cylinders. This suggests that the formation of lipid domains leads to
the onset of the hexagonal-II phase transition. In ACC liposomes prepared
in a similar manner, there was no evidence of hexagonal-II phase formation
after dehydration under similar conditions (i.e., 73 wt. % sorbitol for 15 h at
4°C). Also, it is significant to note that little aggregation or apparent fusion
was observed in ACC liposomes exposed to these conditions.

These results demonstrate that NA and ACC liposomes respond dif-
ferently with respect to the propensity for dehydration-induced lamellar to
hexagonal-II phase transitions. This is of considerable significance consider-
ing that the propensity for hexagonal-II formation can also be influenced by
the presence of solutes such as sucrose or proline (Crowe and Crowe, 1984).
Thus, alterations in the lipid composition *per se* play a significant role
in determining the incidence of lyotropic phase transitions in the plasma
membrane. Because the lipid composition of NA and ACC liposomes
differs only in the proportion of the various lipid species, this differential
behavior is a consequence of composition-dependent alterations in lipid–
lipid interactions.

Membrane Engineering of the Plasma Membrane

Although the above studies of the cryobehavior of liposomes have established that the differential cryobehavior of the plasma membrane of NA and ACC protoplasts is a consequence of differences in the lipid composition, these studies do not reveal what specific changes are responsible. For this, we have begun studies of structure and function relationships by selective modification of the lipid composition of the plasma membrane. Using a protoplast × liposome fusion technique (Arvinte and Steponkus, 1988) to alter the lipid composition of the plasma membrane, the freezing tolerance of NA protoplasts can be significantly altered by enrichment of the plasma membrane with various lipid species (Steponkus *et al.*, 1988). Fusion of NA protoplasts with liposomes formed from the total phospholipid fraction of the plasma membrane of cold-acclimated rye leaves significantly increased the freezing tolerance of the treated protoplasts relative to that of the controls. For protoplasts fused with phospholipids isolated from the plasma membrane of cold-acclimated leaves, survival remained at 100% over the range of 0 to $-5°C$ and declined only after a freeze/thaw cycle to $-7.5°C$ or lower. In contrast, NA protoplasts exhibited a 50% decrease in survival over the range of 0 to $-5°C$. The increase in freezing tolerance of NA protoplasts was also elicited by fusion with liposomes composed of either 1-palmitoyl-2-oleoylphosphatidylcholine, 1-palmitoyl-2-linoleoylphosphatidylcholine, dioleoylphosphatidylcholine, dilinoleoylphosphatidylcholine, or dilinolenoylphosphatidylcholine, with dilinoleoylphosphatidylcholine or dilinolenoylphosphatidylcholine resulting in the maximum increase (equivalent to that of NA protoplasts fused with liposomes of ACC phospholipids) and 1-palmitoyl-2-oleoylphosphatidylcholine, 1-palmitoyl-2-linoleoylphosphatidylcholine, and dioleoylphosphatidylcholine somewhat less effective. In contrast, fusion with dipalmitoylphosphatidylcholine or dimyristoylphosphatidylcholine liposomes did not affect survival, but fusion with either dipalmitoylphosphatidylethanolamine or dioleoylphosphatidylethanolamine decreased survival at all temperatures.

At temperatures over the range of 0 to $-5°C$, the principal form of injury in NA protoplasts is expansion-induced lysis. Survival of NA protoplasts fused with mono- or di-unsaturated species of phosphatidylcholine did not decline appreciably until exposure to temperatures below $-5°C$, suggesting that enrichment of the plasma membrane with these lipid species greatly diminishes the incidence of expansion-induced lysis. Cryomicroscopic studies revealed that the plasma membrane of NA protoplasts fused with mono- or di-unsaturated species of phosphatidylcholine formed exocytotic extrusions during freeze-induced osmotic contraction. In contrast, osmotic contraction of control protoplasts resulted in endocytotic vesiculation of the plasma

membrane. Scanning electron-microscopic studies revealed that the mor-
phology of the extrusions is identical to that observed in ACC protoplasts.
Thus, enrichment of the plasma membrane of NA protoplasts with mono- or
di-unsaturated species of phosphatidylcholine alters the behavior of the
plasma membrane so that, during freeze-induced osmotic contraction, it
is both functionally and morphologically equivalent to that of ACC
protoplasts.

 This "membrane engineering" approach offers considerable promise for
studies of membrane structure and function relationships in relation to the
various forms of cryoinjury to the plasma membrane. Currently, such
studies in relation to the loss of osmotic responsiveness are in progress. We
are proceeding on the assumption that the differential cryobehavior of the
plasma membrane associated with the different forms of injury need not
be the result of common changes in the lipid composition of the plasma
membrane.

Mechanisms of Increased Cryostability Resulting from Lipid Alterations: Working Hypotheses

 Having demonstrated that alterations in membrane cryobehavior and
cryostability following acclimation are, in part, a consequence of changes in
lipid composition, our next goal is to develop a mechanistic understanding
of *how* lipid composition influences membrane cryostability—including both
the sensitivity of the plasma membrane of NA protoplasts and the increased
stability of ACC protoplasts. For expansion-induced lysis and loss of
osmotic responsiveness, this requires an understanding of the molecular
mechanisms of membrane dehydration-induced destabilization.

Membrane Flow During Osmotic Contraction

 Whereas the differential propensity for dehydration-induced L_α-to-L_β
and lamellar to hexagonal-II phase transition in NA and ACC protoplasts is
a consequence of lyotropic phase transitions, the differential behavior of
the plasma membrane during freeze-induced osmotic contraction is not
obviously related to lipid phase behavior—either thermotropic or lyotropic.
The formation of endocytotic vesicles or exocytotic extrusions is independent
of temperature over the range of $+25$ to $-5°C$, and the extent of dehy-
dration required to elicit either transformation is not sufficient to induce
lyotropic phase transitions. Instead, the formation of exocytotic vesicles in
NA protoplasts and liposomes *vs.* exocytotic extrusions in ACC protoplasts
and liposomes is triggered by osmotic contraction that results in relaxation

of the resting tension of the bilayer. Thus, the differential behavior of the plasma membrane of NA *vs.* ACC protoplasts is a consequence of a qualitatively different shape transformation of the *flaccid* membrane: that of NA protoplasts spontaneously forms endocytotic vesicles, whereas that of ACC protoplasts forms tubular extrusions. Although the transformation in the behavior of the plasma membrane can be effected by enrichment with mono- or di-unsaturated species of phosphatidylcholine, the transformation may be the result of interactions of phosphatidylcholine with other lipid species, rather than increased proportions of phosphatidylcholine *per se*. One possibility is that membrane morphology and shape transformations are influenced by phospholipid–cerebroside interactions.

Theoretical studies of self-assembly in model cerebroside–phosphatidylcholine systems (Maggio, 1985) have led to the prediction that the thermodynamic stability and overall geometry of the system are determined by the proportion and type of cerebrosides present. Consistent with these predictions, Curatolo and Neuringer (1986) have observed large variations in the morphology of phosphatidylcholine liposomes depending on the proportion and type of cerebroside added to the mixture and the molecular species of phosphatidylcholine. Although aqueous dispersions of 1-palmitoyl-2-oleoyl-phosphatidylcholine form large multilamellar vesicles, addition of 20% cerebrosides results in the formation of long, thin multilamellar tubules. Alternatively, dispersions of dipalmitoylphosphatidylcholine also form large multilamellar vesicles; however, dispersions containing 20% bovine cerebroside form small unilamellar vesicles. Addition of cholesterol reversed the apparent effect of cerebroside.

That lipid compositional changes accompanying cold acclimation may manifest alterations in cryobehavior via lipid–lipid interactions is conceivable, given that the ratio of cerebroside to di-unsaturated phosphatidylcholine decreases from 4:1 to 0.9:1 and the ratios of total phospholipid or free sterol to cerebroside increase from 2:1 to 6:1 following acclimation.

Dehydration-Induced Lamellar to Hexagonal-II Phase Transitions

Numerous studies have employed the concept of molecular shape or molecular packing to explain the polymorphic phase behavior of lipids and their respective propensities to form nonbilayer structures (see Cullis *et al.*, 1985; Gruner *et al.*, 1985). Paradoxically, examination of changes in lipid composition from the perspective of the molecular packing and polymorphic phase behavior of individual plasma membrane lipid components and their respective propensities to form nonbilayer phases tend to suggest that changes following cold acclimation would actually promote lamellar to hexagonal-II phase transitions. For example, di-unsaturated species of

phosphatidylethanolamine, potent "nonbilayer-forming" lipids, increase following acclimation. Concomitantly, free sterols, which facilitate thermotropic lamellar to hexagonal-II phase transitions in phosphatidylcholine-phosphatidylethanolamine mixtures (Cullis and de Druijff, 1978; Tilcock and Cullis, 1982; Tilcock et al., 1982), increase. Cerebrosides are "bilayer-forming" lipids (Abrahamsson et al., 1972; Ruocco et al., 1981; Curatolo 1982, 1987; Lynch and Steponkus, 1987c; Lynch, Caffrey, and Steponkus, in preparation), but decrease following acclimation.

Studies of lipid mixtures have provided insights that serve to explain this apparent paradox. For selected lipid mixtures containing both "bilayer-forming" and "nonbilayer-forming" lipids, the formation of hexagonal-II phase structures is preceded by lipid demixing, usually as a consequence of gel phase immiscibility and phase separation of the "bilayer-forming" lipid and the "nonbilayer-forming" lipid (Tilcock and Cullis, 1982; Papahadjopoulos et al., 1978) or as a result of hydration–repulsion forces (see above). If demixing occurs, the "nonbilayer-forming" lipids become enriched in localized regions and undergo a lamellar to hexagonal-II phase transition. However, in mixtures in which the lipid species exhibit miscibility, bilayer structure is preserved. Our working hypothesis proposes that (a) lipid demixing of NA plasma membrane lipid constituents occurs as a consequence of cellular dehydration and leads to localized domains enriched in "nonbilayer-forming" lipids and the formation of nonbilayer structures; and (b) changes in lipid composition following acclimation are such that dehydration-induced lipid demixing is precluded during freezing. With this working hypothesis, emphasis is away from lamellar to hexagonal-II phase transitions per se and toward aspects of freeze-induced demixing and lateral phase separations. As such, studies of the phase behavior of single lipids and simple lipid mixtures in progress in our laboratories center on elucidating the involvement of plasma membrane lipid components in modulating lipid miscibility and promoting/preventing demixing.

Decreasing water content leads to an increase in the gel to liquid-crystalline phase transition temperature (T_m) of phospholipids. For phosphatidylcholine species common to the plasma membrane, drying over P_2O_5 in vacuo for > 5 h at elevated temperatures increases the respective T_m's by $\sim 70°C$ over those of fully hydrated lipid samples. Thus, hydrated samples of 1-palmitoyl-2-oleoylphosphatidylcholine, dioleoylphosphatidylcholine, 1-palmitoyl-2-linoleoylphosphatidylcholine, dilinoleoylphosphatidylcholine, and dilinolenoylphosphatidylcholine have T_m's of -3, -18, -20, -53, and $-63°C$, respectively, whereas dry samples have T_m's of 68, 48, 37, 27, and 7°C, respectively (Lynch and Steponkus, 1988b). Relevant to this discussion, at intermediate levels of hydration, the T_m's of monounsaturated phosphatidylcholine species are near 0°C, whereas those of

di-unsaturated phosphatidylcholine species are below $-30°C$. Mixtures of 1-palmitoyl-2-linoleoylphosphatidylcholine, the major monounsaturated speices of the plasma membrane and dilinoleoylphosphatidylcholine, the predominant di-unsaturated species, exhibit a single endotherm (by differential scanning calorimetry) at all concentrations, indicative of ideal mixing. The respective T_m's increase nearly linearly with increasing proportions of 1-palmitoyl-2-linoleoylphosphatidylcholine such that a mixture having proportions of the two phospholipids corresponding to the present in NA plasma membrane exhibits a T_m 8°C greater than that of a mixture corresponding to ACC plasma membrane (Lynch and Steponkus, 1988b).

Phospholipids constitute $\sim 30\%$ of the lipid complement of the plasma membrane from nonacclimated rye leaves. Thus, any mechanistic hypothesis must also take into account the physical properties and lipid–lipid interactions of the other membrane constituents. For example, rye cerebrosides exhibit a high T_m (56°C). Mixtures containing 1-palmitoyl-2-oleoylphosphatidylcholine and $\geqslant 30$ mol % cerebroside exhibit nonideal mixing and gel phase immiscibility (Lynch and Steponkus, 1987c). Similar observations have been made using mixtures of brain cerebrosides and phospholipids (Curatolo, 1986; Johnston and Chapman, 1988) and synthetic cerebrosides, N-palmitoylgalactosyl-sphingosine (Ruocco and Shipley, 1984). Mixtures containing 25% brain cerebrosides, 25% brain phosphatidylcholine, and 50% cholesterol also exhibit gel phase immiscibility as a result of cerebrosides forming a separate phase.

Studies of phosphatidylcholine–sterylglucoside mixtures (Lynch and Steponkus, 1988a) suggest that steryl glucosides influence phospholipid phase behavior differently than do free sterols. Addition of 33 mol % free sterol to dipalmitoylphosphatidylcholine results in the absence of any detectable endotherm (McKersie and Thompson, 1979), whereas 33 mol % steryl glucosides from rye (Lynch and Steponkus, 1988a) or soybean (Mudd and McManus, 1980) shifts the T_m slightly and diminishes—but does not abolish—the endotherm of dipalmitoylphosphatidylcholine. This suggests that sterylglucosides do not interact in a stoichiometric fashion with phosphatidylcholine.

Although limited, the results of these studies support the contention that the plasma membrane of nonacclimated rye has a greater propensity to undergo phase separation and lipid demixing during freeze-induced cell dehydration. The high content of cerebrosides (especially if they are localized in the outer leaflet as is the case with animal plasma membranes) and sterylglucosides may manifest a limited degree of demixing and domain formation under nonstressed conditions that catalyzes pronounced demixing during cellular dehydration associated with freezing. This condition is exacerbated

by the phase behavior of the constituent phosphatidylcholine molecular species.

Extrapolating from the model studies of Lis, Rand, and Parsegian to the plasma membrane, close approach of bilayers as a consequence of cellular dehydration would lead to a lateral segregation of lipids (i.e., demixing) dependent upon their respective equilibrium separation distances. Lipid species such as free sterols or phosphatidylcholine (which exhibit relatively large equilibrium separation distances) would segregate away from regions of closest approach wheres phosphatidylethanolamine, which has a small equilibrium separation distance, would become enriched in the regions of closest approach. As stated above, phosphatidylethanolamine exhibits a propensity to undergo bilayer-to-nonbilayer phase transitions forming inverted cylindrical micelles (hexagonal-II phase). Thus, an enrichment of phosphatidylethanolamine in regions where adjacent bilayers are in close approach would facilitate lamellar to hexagonal-II phase transitions as observed for NA rye protoplast plasma membrane (Gordon-Kamm and Steponkus, 1984c).

Freeze-induced dehydration of ACC protoplasts does not lead to the formation of lateral phase separations, aparticulate lamellae, or hexagonal-II phase structures. This suggests that alterations in the lipid composition of the plasma membrane following cold acclimation increase membrane cryostability by maintaining miscibility during dehydration and preventing demixing. It is conceivable that increasing the free-sterol content and possibly the proportion of di-unsaturated species of phospholipid in conjunction with decreases in cerebrosides improve miscibility. In support of this contention, mixtures containing < 20 mol % N-palmitoylgalactosylphingosine in cholesterol-dipalmitoylphosphatidylcholine (1 : 1) exhibit complete lipid miscibility, and bovine brain cerebrosides (Curatolo, 1986) and rye cerebrosides (Lynch and Steponkus, 1987c) exhibit miscibility with phosphatidylcholine at cerebroside fractions < 20–30 mol %.

Conclusions

A mechanistic understanding of the cellular and molecular aspects of freeze/thaw-induced destabilization of the plasma membrane is emerging. Although freeze-induced dehydration is the primary stress involved, the mechanism of injury depends on the extent of dehydration. Cognizance of multiple forms of injury and their different causes allows for studies of the mechanisms by which cold acclimation increases the cryostability of the plasma membrane to be more acutely focused. Although cold acclimation is an amalgam of many changes, alterations in the lipid composition of the plasma membrane are a major facet of this process. In that subtle

changes in the proportions of the individual components dramatically alter the cryobehavior of the plasma membrane, further advances in understanding both freezing injury and cold acclimation require elucidation of lipid–lipid interactions in the complex mixture of lipids that comprise the plasma membrane.

Acknowledgments

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