Chilling injury in plants—
the role of membrane lipid fluidity

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Abstract. Many authors have associated chilling injury in
plants with changes in the mobility of membrane lipids
but have not proposed specific mechanisms for this
association.

This paper explains how the mobility of membrane
lipids can affect membrane thickness, membrane per-
meability, the electric field, cation concentration and
water ordering near a membrane and hence change the
conformation (and thus activity) of a membrane-bound
enzyme. The complications in such a model due to
protein-lipid interactions and lateral phase separations
are also discussed.

Introduction

Whole plants will die if the temperature is sufficiently
low. Damage at various temperatures below 0°C is called
freezing injury, and, as the name implies, the formation
of ice crystals in tissue has been proposed as a mechan-
ism for it (Tattar & Blanchard, 1976). Tissue damage or
death suffered at temperatures above freezing but below
about 15°C is called chilling injury and its explanation
poses a difficult problem. Such injury is important since
many commercial crops are chilling-sensitive, for ex-
ample: maize, sorghum, sugarcane, sweet potato, cap-
sicum, tomatoes and citrus fruit. The symptoms vary,
but some examples are: surface pitting, discolouration
and failure of fruit to ripen (Lyons, 1973); incomplete
chlorophyll development in leaves (Slack, Roughon &
Bassett, 1974); and loss of water (Wright & Simon,
1973).

At first glance, one might suppose that as temperature
decreased all reactions would continue, but more slowly,
and therefore the plant would survive and continue to
grow at a smaller rate. However, it is possible that below
a critical temperature an enzyme becomes completely
inactive and thus a critical reaction step may cease.
Further, when competitive reactions are involved—such
as production and photo-destruction of chlorophyll, a
competition important in chilling injury (McWilliam &
Naylor, 1967; Bagnall & Wolfe, 1978)—a dispropor-
tionate change in one reaction rate can cause the net
production of a vital component to fall to zero. Either

complete inactivation or a disproportionate change in
reaction rate can be explained by a change in configura-
tion of an enzyme molecule: a small change might change
the energy of its activated complex and thus alter its
activity; and a large change might render impossible the
formation of the activated complex.

A plant's survival is dependent not only on tem-
perature, but also on irradiance (McWilliam & Naylor,
1967), humidity (Wright and Simon, 1973), and the
amount of 'cold hardening' (i.e. exposure to low but not
chilling temperature) it has received (Guinn, 1971) and
there is therefore no single critical temperature below
which a plant always dies and above which it always
lives. However in any particular circumstances of irradi-
ance, humidity and other conditions a chilling-sensitive
plant suffers damage at low temperatures. The varied
symptoms lead one to look for a fundamental process
which might be responsible, and the change in the
fluidity of membrane lipids will be shown to be a likely
candidate. The circumstantial evidence is strong: all bio-
logically common lipids have their melting range roughly
in the range of growth temperatures (Williams & Chap-
man, 1970; Shimshick & McConnell, 1973), and there
seems to be a correlation between the lowest survival
temperature of a plant and the melting points of its
membrane lipids (Lyons & Asmundson, 1965). That is,
chilling-resistant plants generally have more unsaturated
and shorter chained lipids than chilling-sensitive plants.
Lyons & Raison (1970) were the first to use such an
explanation for chilling injury. In their theory (Lyons,
1973) there is some critical temperature at which the
membrane lipids of mitochondria in various parts of the
plant suddenly undergo a phase change. By an un-
explained mechanism this causes a change in configura-
tion of some membrane-bound enzymes, which changes
the energy of the activated complexes of some key rate-
limiting reactions.

However, it is also possible that the solidification of
membrane lipids is directly responsible for chilling in-
jury: various processes, particularly passive trans-
membrane diffusion are thought to rely on the lateral
and vertical mobility of lipid molecules. The gradual
termination of these processes by the reduction in lipid
mobility with decreasing temperature may also play a
role in chilling injury.

The Raison-Lyons hypothesis is flawed in its present form since it predicts for one species a critical temperature below which some vital process cannot be maintained. Experimentally it has been shown (Wright & Simon, 1973) that plants can survive at temperatures below their supposed critical temperature (depending on other conditions), and that some parameters of whole plant growth expected to change abruptly at the critical temperature in fact vary continuously with temperature (Bagnall & Wolfe, 1978). Further, the hypothesis includes no specific mechanism whereby the lipid fluidity affects the membrane proteins.

In this paper I argue that a change in conformation will not occur for all membrane proteins at the same temperature due to lateral/lateral phase separations of the lipids, and non-specific lipid–protein interactions which lead to preferential adsorption. This means that the enzyme activity is a continuous function of temperature and thus the difference in limiting survival temperatures with different conditions can be explained. I propose specific ways in which lipid fluidity can affect protein confirmation and some diffusion mechanisms.

Theory

It is useful here to ask the question: Why are many enzyme proteins embedded in, or attached to, the membrane instead of floating around in the cytoplasm? Obviously those molecules or complexes of molecules whose functions involve the different environments which may exist on opposite sides of a membrane must be sited protruding through that membrane. Further, the membrane is part of the architecture of the cell and embedding a protein in it keeps it more or less in one place. But apart from this the membrane has several peculiar properties, many of which vary with temperature, which must affect the structure and function of intrinsic and (in some cases) extrinsic proteins. Some of these are discussed below.

Fluidity

Some species have the ability to increase the fluidity of their membranes when exposed to low temperatures by increasing the proportion of lipids with lower melting points (Kuiper, 1970; Gerloff, Richardson & Stahmann, 1966). The implication that some metabolic processes are facilitated by the existence of regions of lipids in the fluid state has important consequences for the membrane-bound enzyme. First, such a molecule in a fluid bilayer can undergo slight changes in shape and thereby deform the adjacent lipids. The operation of some enzymes involves their changing from one conformation to another, slightly different, and back again (Koshland, 1973). Thus the activity of such an enzyme is dependent on the mobility of the surrounding lipids. Second, the lipids, while fluid, can distort to pack around an irregularly shaped protein (within limits). Once the lipids freeze, their tails will straighten out and thus tend to impose straight sides on the hydrophobic

region of adjacent proteins. A conformational change may be thus induced. Third, the collisions with fluid lipids provide the protein with an input of kinetic energy (Junge, 1972) which may be necessary for its function (Duppel & Dahl, 1976).

Amphiliphic properties

The bilayer has a hydrophobic zone sandwiched between two hydrophilic zones. A protein which when dissolved in water exposes its hydrophilic groups, must, to become an intrinsic protein, lessen its surface energy by reconfiguring to expose a region of hydrophobic groups to the tails in the bilayer. If the protein protrudes on both sides then it must be circled by a largely hydrophobic band, about 3–5 nm wide. In the frozen bilayer the lipids pack closer together and therefore, to conserve volume, the bilayer is thicker than when in the fluid state (Marsh, 1974). Thus, in addition to (but related to) the different 'straightening' effect discussed above, the frozen and fluid phases will have hydrophobic regions of different thickness. Where both phases exist at the same time (i.e. in a phase separation) a protruding protein molecule will preferentially dissolve in one phase or other (depending on the width of its hydrophobic band) (Chen & Hubbell, 1973; Overath, Thilo & Träuble, 1976). If forced to remain in the unfavourable phase the resultant stress may cause the protein to change shape.

Surface charges and dipoles

Many biological lipids dissociate, leaving the head group negatively charged. This surface charge causes an electric field in the solution near the membrane, and, if the membrane is asymmetric or if there is a trans-membrane potential, in the hydrophobic region also (McLaughlin, Szabo & Eisenman, 1971; Nelson, Colonomos & MacQuarrie, 1975).

These fields are very strong ($\sim 10^7 \mathrm{Vm^{-1}}$—the same order as the dielectric breakdown field) and therefore a small fractional change is still a large absolute change in field strength.

As a result of the negative surface charge, an excess of positive ions approaches the membrane, and the density of cations increases from its bulk value to a much higher value in the layer in contact with the membrane (e.g. Vervey & Overbeek, 1948). Further, any amphiliphic head group which is not charged must be dipolar, or zwitterionic—it is this that makes the head groups hydrophilic. So there will be a dipolar field near the membrane surface as well.

In the solid-like phase, the lipids pack more closely together and so the surface density of both charges and dipoles will be greater than in the fluid-like phase (Träuble et al., 1976). The larger negative surface charge density will cause a larger field in the aqueous solution and a larger cation concentration. [This relationship is reversible and the temperature at which the phase separation begins or ends can be altered by changing the pH or ion concentration (Haller & Freiser, 1976).] It will

also change the field inside the membrane and thus cause some counter-ions to traverse the membrane to maintain ⁻ ° same net membrane potential.

A protein molecule has many charged and dipolar groups and its conformation will depend upon the interactions between these groups with each other and with any externally applied fields. The change in surface charge associated with lipid freezing is expected to deform the protein so that its negative groups are further from the bilayer and any positive groups closer. The local increase in cation concentration will more effectively shield negative groups and will change the dissociation equilibria so that there are fewer negative charges.

The freezing of the membrane lipids can thus change both the shape and state of ionization of membrane-bound proteins and thus their activation energies.

Water ordering

The difference between a solid and a liquid is that the former is more 'ordered'—its molecules vary little in position and orientation compared with those of the latter. The order parameter is a convenient way of expressing the degree of molecular motion, and takes on values between one (for a rigid molecule) and zero (for rapid isotropic tumbling).

Water molecules are dipolar and thus in the presence of a strong electric field will experience different molecular ordering from those in the bulk state (Hasted, 1973). Thus an ordered state is observed near interfaces several ᵒns of degrees above the bulk freezing temperature ᴖlifford, 1975). In this quasi-crystalline state the water molecules have a different spacing and orientation, and a mobility, from those in bulk water (Chapman & McLaughlan, 1967), and this may extend a nanometer or more beyond the head groups (Drost-Hansen, 1973).

Many solute molecules in water find themselves trapped within what is known as a clathrate cage (Davidson, 1973)—a complicated framework of water molecules which leaves no hydrogen bonds exposed. The hydrophilic regions of an intrinsic protein will be surrounded by a similar quasi-static cage, hydrogen bonding and other interaction with which will be critical in determining its configuration (Hagler & Moult, 1978). Highly ordered water molecules also occur 'inside' the protein's quaternary structure and play an important part in holding it together (Hagler & Moult, 1978).

The structure of this 'cage' will be partly determined by the local electric field and ion concentration, and therefore may be altered by a change in the surface density of lipid head groups (Forslind & Kjellander, 1975; Drost-Hansen, 1973). Thus a protein may be stressed into a new configuration by the change in spacing and orientation of hydrogen bonds with water as a result of lipid freezing.

Thus, four of the physical properties of the environment of membrane proteins are highly temperature dependent, and since conformation is affected by these ᵖroperties, this will be temperature dependent too (see ᴖg. 1).
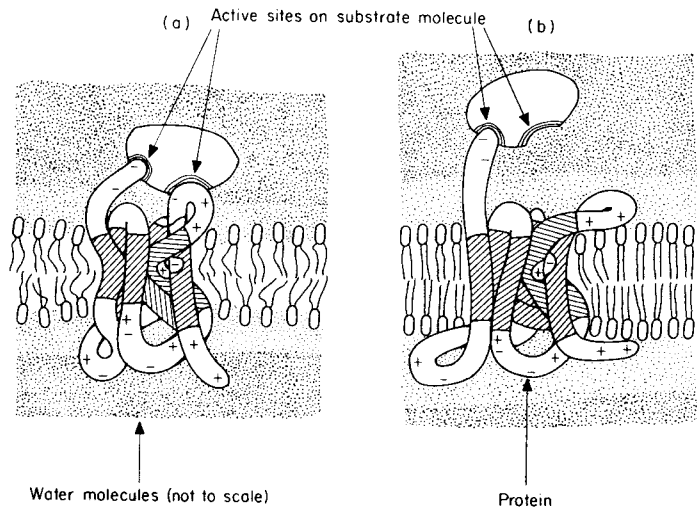

Figure 1. A highly idealized representation of an intrinsic protein in a lipid bilayer. In (a) the lipids are fluid and their deformability allows the hydrophobic regions of the protein (shaded) freedom to configure in any shape which leaves a narrow band exposed to the bilayer. This conformation is geometrically suitable for forming the activated complex as shown. In (b) the lipids are solid and the more rigid, thicker hydrophobic region is deforming the hydrophobic areas of the protein. The more ordered water phase extends further and the electric field is stronger, so the protein has deformed so that it can no longer easily form the same activated complex.

Phase transitions and separations

A bilayer made of one species of lipid only and containing no protein, will, over a small range of temperature, change its molecular mobility drastically from a two-dimensional fluid to a solid-like state—that is, it will closely approximate a first-order phase transition. The temperature at which this occurs will depend on the salt and hydrogen ion concentration of the aqueous phase (Jacobson & Papahadjopoulos, 1975; Trauble et al., 1976), the transverse and lateral pressure (Hui et al., 1975), and the curvature of the bilayer.

The bilayer in a membrane is, regrettably, rather more complex (Lyons, Wheaton & Pratt, 1964). When different miscible species of lipids with different transition temperatures are mixed, the bilayer does not suddenly freeze at a particular temperature (Reisman, 1970). Its behaviour is usually represented on a phase diagram (see Fig. 2). As the temperature of a mixture of some composition is gradually lowered, it separates into domains of different composition. Thus, small regions of the frozen state, with a high concentration of the higher melting point component appear in the otherwise fluid bilayer (see e.g. Rushbrooke, 1949; Shimshick & McConnell, 1973; Lee, 1977). These increase in size (and composition approaches that of the original mixture) as the temperature continues to fall until eventually, on the solidus curve, the whole bilayer is frozen. The situation with more than two components is more complicated (Lang & Widom, 1975) but qualitatively similar, i.e. there will be a range of temperatures over which the
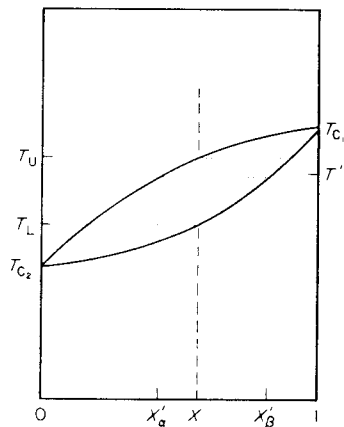
Figure 2. A simple, two-component phase diagram. Fractional composition of species 1 on the x axis, temperature on the y axis. A mixture of X of species 1 and $(1-X)$ of species 2 will separate into two phases between T_U and T_L. At T', one phase will have X'_α of species 1 and the other X'_β of species 1. T_{C_1} and T_{C_2} are the critical temperatures of (pure) species 1 and 2.

bilayer comprises domains of various compositions, some frozen, some fluid. Another interesting complication is that if a single species bilayer has a dissociation equilibrium such that there are substantial amounts of both charged and uncharged lipid, it too will phase separate (Forsyth et al., 1977).

If we were now able to insert proteins into our bilayer without perturbing it, we should expect to find that, as we lower the temperature, progressively more protein molecules find themselves in frozen domains, and so, over a range of temperatures, the fraction of enzymes surrounded by frozen lipids varies from zero to one. Over this range we would expect any membrane-bound reaction rate to be a weighted average of the rates of the reactions with the two different activation energies.

Boundary effects

To complicate the problem further, however, the interaction between lipid and protein is different from that between lipid and lipid, and thus the boundary layer of lipids round a protein is in a different environment from that of bulk liquids. The next layer is less affected and so on, but there are several layers of lipids round a protein molecule whose mobilities (and, it will be shown, composition) will be different from the bulk values. This boundary layer effect is evident in the experiments of Jost et al. (1973) and others.

The tails of a lipid in the bulk bilayer can bend and wriggle (and the end of the tail thrash about) because the 'next door' lipid can bend and wriggle to accommodate this. If the molecule next to the lipid is a large and relatively rigid protein molecule it will be considerably less mobile, and reduce the mobility of the next layer. Using this concept together with Molecular Field Theory, Marčelja (1976) has calculated the order parameter of

lipids as a function of temperature and distance from a protein. With his boundary conditions, the order increases (and therefore the mobility decreases) near the protein, and the order of the boundary layers is continuous function of temperature. Thus even if the protein molecules were embedded in a pure, one-species bilayer the lipids nearby would not undergo an abrupt phase change.

Now if the free energies of interaction between the protein molecule and each species of lipid molecule were equal, then, in order to maximize entropy, the lipid composition would be homogeneous up to and including the boundary layer. If, however, the free energy interaction is lowest with one species, then that species will be preferentially adsorbed onto the protein; i.e. that species will form a larger fraction of the boundary layer than it does of the bulk (Boggs et al., 1977). This might be brought about simply by geometrical packing considerations (Israelachvili, 1977) and is most easily shown in Fig. 3.

A protein with wide hydrophobic band would preferentially adsorb lipids with long chains; one which bulges in the hydrocarbon region would be surrounded mainly by lipids with large head-group areas and so on. Another pertinent selection criterion is mobility. In or near the phase-separation region the free energy change associated with the adsorption into the boundary layer region will vary with lipid species because of the large range of mobilities. Marčelja's boundary conditions (Marčelja, 1976)—a flat, rigid protein wall—will preferentially adsorb the highest melting point species, that is
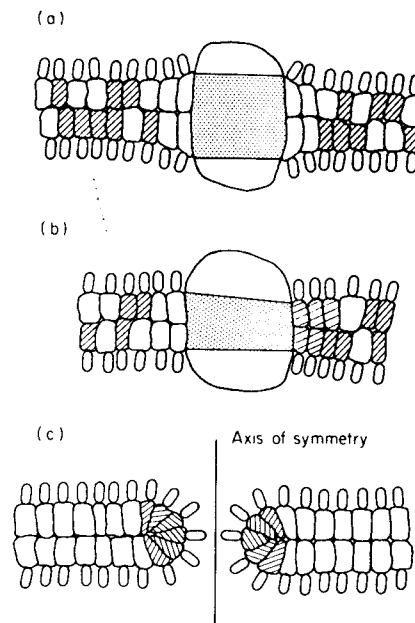
Figure 3. (a) and (b) show respectively how protein molecules with wide and narrow hydrophobic regions preferentially adsorb long chained (unshaded) and short chained (shaded) lipids in the boundary region. (c) Shows how the formation of a pore requires a domain of single-chained lipids (shaded) i.e. lipids with long chains but a large ratio of head group area to hydrocarbon volume.

the species predominating in the solidus domains. At temperatures well above the fluidus curve, the high ˙˙ ˙lting point lipids (those with long chains and/or most ˙˙urated) begin to congregate about such proteins and, as the temperature falls, this systems 'seeds' the formation of the freezing domains. Alternatively, if the geometry of the protein requires the boundary layer to remain fluid in order to pack around it, the more fluid (lower melting point) species will be over-represented in the boundary region. (This is a possible interpretation of the experiments of Chen & Hubbell, 1973). If a protein had a narrow hydrophobic region (selecting for short-tail lipids) and were shaped such that the boundary layer tails could not straighten out, then it would be surrounded by fluid lipids below the freezing temperature of even the most fluid species. A plant whose membrane-bound enzymes had narrow, curved hydrophobic regions would therefore be better equipped for withstanding chilling temperatures.

The perturbation of the local lipid environment by a protein molecule is a fundamental difficulty in any theory (such as that of Lyons (1973)) which attempts to relate quantitatively enzyme conformation change with pure lipid phase transition temperatures: the lipids which can affect the protein are those near to it, and these will have a different mobility and composition from the bulk lipids. A coincidence between 'critical' temperatures of bulk lipid mobility and enzyme condition is fortuitous.

rmeability mechanisms

Some researchers have speculated that the movement of ions and water molecules through bilayers involves purely mechanical use of lipid fluidity, though evidence for the existence of specific mechanisms must as yet be indirect. Two such theories are the lipid pore theory (Lee & Chan, 1977) and the bobbing-up-and-down hypothesis (Robertson & Thompson, 1977; Robertson & Boardman, 1975). The formation of a lipid pore in the form of the inner half of a torus fitted into a slab—see Fig. 3— requires the aggregation of a domain of lipid molecules having a large ratio of head group area to hydrocarbon tail volume, as well as some fluidity. Such pores then would be unlikely to form at low temperatures with the tails tending to straighten out, and they would also be unlikely at temperatures well above melting since the maximization of entropy is not consistent with separation into such distinct phases as are implied by this model. That is, the formation of these pores would only be likely over a range of some tens of degrees which would roughly coincide with that of biological processes. Since permeability changes have been accused of producing some effects of chilling injury (Levitt, 1972), this mechanism may also play a part.

The bobbing-up-and-down hypothesis proposes that Cl^- ions are transported through the membrane by attaching to a phosphate group which, its hydrophilicity ˙˙minished, 'bobs down' and dissolves HCl in the tail ˙gion of bilayer. It is then picked up by another head group and transferred to the other side. Obviously such a process as this requires the fluidity of the membrane lipids and is therefore a process which would be affected by chilling over a range of temperatures depending on the lipid composition.

Conclusion

An analysis of temperature-dependent changes in a membrane is distressingly complex. At say 30°C, the lipid bilayer is completely fluid, allowing intrinsic proteins to make small oscillations about their current configuration, and is homogeneous, except around proteins whose packing shapes require a preponderance of some species. There is some water structuring due to the electric field near the bilayer and this is influencing the 'clathrate cage' of the hydrophilic regions of the protein. Perhaps also there are diffusion processes (pores, bobbing up and down) which are facilitated by the easy lateral and vertical movement of the lipids. As the temperature falls, the rates of such diffusion processes fall dramatically. As the lipids gradually lose mobility, they provide the protein with a more rigid environment. Their tails extend more, thus causing stresses in the hydrophobic bonds, and they pack closer together thus increasing both the electric field and the cation concentration in the nearby water, which in turn change the electric forces on the charged or dipolar groups on the protein. The water-ordering extends further, is less fluid, and the hydrogen bonds shift position slightly. The composition of the lipid environment is changing and, depending on its shape, the protein may be preferentially caught in local regions of fluidity or solidity. The energy of its activated complex will at least be different—and thus its activity will change—or the forces may be great enough to cause a conformational change which precludes the possibility of the enzymes' functioning at all.

The relative importance of these mechanisms may be tested by experiment as follows. If the membrane proteins of chilling-sensitive species are distinguished from those of chilling-resistant species by having rigid flat sides onto which solid-like lipids can adsorb, then these proteins will be more prevalent in the solid-like domains of phase separations. Those of chilling-resistant species, hypothesized to have irregular hydrophobic regions, would be more prevalent in the fluid-like regions. This could be determined from freeze fracture electron micrographs frozen rapidly from phase separation temperatures (cf. the experiments of Chen & Hubbell, 1973). It is not necessary to postulate that the analogous proteins of chilling-sensitive and chilling-resistant species are very different—several minor changes in primary structure could cause a large change in quaternary structure.

If the activation energy of an enzyme is changed by changing electric field and/or changing cation concentration, then its activity should be affected by change in the concentration in the aqueous solution of divalent cations. I predict that the activity of enzymes from chilling-sensitive species will be more dependent on

divalent cation concentration than that of those from chilling-resistant species.

If the anomalously high Cl⁻ flux ('explained' by the up-and-down hypothesis) vanishes at temperatures below the lipid freezing regime, then it will imply that lipid mobility is necessary for the transport mechanism.

Such experiments are within the range of current techniques and, if undertaken, should yield useful information about the mechanisms of chilling injury.

Since changes in enzyme activity and membrane permeability occur gradually over a range of temperatures, one does not expect to find that there is one critical temperature above which a species always survives and below which it always dies. Rather, over a critical range of temperatures, survival will depend on other conditions, such as irradiance and humidity.

Acknowledgments

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