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Cold-Induced Lipid Phase Transitions [and Discussion]

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Cold-induced lipid phase transitions

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The structural organization of biological membranes is largely determined by the weak interactions existing between their components and between these components and their aqueous environment. These interactions are particularly sensitive to changes in temperature and hydration. The factors influencing membrane lipid phase behaviour are briefly reviewed and used to develop a phase-separation model describing the response of biological membranes to stress. The factors affecting the interaction of cryoprotectants with membrane lipids are explored and their role in the stabilization of membrane organization at low temperatures discussed. It is suggested that the basis of their protective action lies in an ability to preserve the balance of interactions between membrane components at low temperatures at a level similar to that existing under physiological conditions.

1. Introduction

The organization and overall stability of biological membranes is determined by the relative strengths of the interactions between their components. These involve electrostatic interactions in the form of Van der Waals forces, salt linkages and hydrogen bonds and entropic and steric factors such as those involved in hydrophobic interactions. The fact that these interactions have different dependencies on factors such as temperature, pH, ionic strength and extent of membrane hydration means that their relative strengths, and hence the organization of membrane components, change under different conditions.

A great deal of effort has gone into attempting to correlate membrane lipid changes with changes in growth temperature and the ability of different organisms to resist temperature stresses. Unfortunately, very few general principles relating to membrane stability have emerged. An alternative approach is to attempt to identify the rules determining lipid interactions in model systems and to examine the extent to which they give an insight into the problem. The strategy adopted here is to examine the phase properties, first of single lipids, and then of lipid mixtures, with the aim of devising a model that can be used to explain the phase-separation characteristics of biological membranes. Particular attention will be paid to factors of specific interest to low temperature biology including the possible importance of sub-gel phases, osmotic dehydration and the mechanism of action of cryoprotective agents.

2. Phase behaviour of single lipids

2.1. *Bilayer-forming and non-bilayer-forming lipids*

In terms of lipid phase behaviour, it is useful to classify membrane lipids in terms of the different structural arrangements that they take up when dispersed in water. On this basis, they can be divided into micelle-, bilayer- and non-bilayer-forming lipids. Micelle-forming lipids,

such as lysolecithin, are rarely found in biological membranes and can be ignored in the present discussion. Most membrane lipids fall into the bilayer-forming and non-bilayer-forming categories. Of these, the bilayer-forming group is by far the larger. Most biological membranes, however, contain significant amounts of non-bilayer-forming lipids; typically 15–25 % rising to 40–50 % in some extreme cases. Phosphatidylethanolamine, monogalactosyldiacylglycerol and cardiolipin are all common examples of non-bilayer-forming lipids.

The structural properties of bilayer and non-bilayer lipids are conveniently discussed in terms of phase assignments derived from low-angle X-ray diffraction (Luzzati *et al.* 1968). Bilayer-forming lipids are characterized by the fact that they form lamellar phases in both the liquid-crystal (L_α) and the gel state (L_β). The non-bilayer-forming lipids, however, tend to form inverted lipid micelles in the liquid-crystal state giving rise, in the condensed state, to the inverted hexagonal phase (Hex_{II}). Some non-bilayer-forming lipids, as discussed below, can also form L phases depending on the conditions. In the gel state, non-bilayer-forming lipids all form lamellar phases of the type found for bilayer-forming lipids.

The structures formed by bilayer- and non-bilayer-forming lipids are determined by the competing tendencies of the lipids to maximize contact between water and their polar head groups while at the same time minimizing contact between water and their hydrocarbon chains. The balance between these two requirements is governed partly by the relative strengths of the different interactions between neighbouring lipid molecules, and between these molecules and water, and partly by steric constraints imposed by the geometry of the molecules. Several theoretical treatments of the interaction between these factors are available. Of these, the analysis by Israelachvili *et al.* (1980) giving rise to the 'shape' model of lipid interactions has proved particularly useful.

The shape model is based on the calculation of a packing factor, p, defined by:

$$p = V/a_0 \times l, \tag{1}$$

where V is the volume swept out by the hydrocarbon chains of the lipid, a_0, the optimal surface area per lipid molecule and l, the length of the molecule. The calculation of the individual contributions to p is complex but the relation between the value of the packing factor and the resulting structure, as illustrated in figure 1, is very simple.

The packing factor concept has proved extremely helpful in providing a simple qualitative framework for understanding the potential effects of such factors as changes in temperature, the length of the lipid chain and chain saturation on lipid phase behaviour. Rises in temperature, for example, would be expected to increase the kinetic motion of the hydrocarbon chains and hence increase the value of V. This, in turn, would increase the value of p and hence be expected to stabilize non-bilayer structures with respect to bilayer structures. It is important to remember, however, that the value of p is ultimately determined by the overall thermodynamic balance of the system and limitations of molecular geometry and that accurate predictions of lipid behaviour, as opposed to simple rationalizations of the type cited above, require considerable detailed analysis.

The shape model with its concept of a variable packing factor emphasizes the fact that lipids assume different structures under different conditions. Non-bilayer-forming lipids, such as phosphatidylethanolamines, can form lamellar (L_α) or inverted hexagonal (Hex_{II}) phases in the liquid-crystalline state. The formation of non-bilayer structures is favoured at higher temperatures and low degrees of hydration. A typical phase diagram for such a lipid is shown

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packing factor	critical packing shape	structures formed
<1/3	cone	spherical micelles
1/2 - 1	truncated cone	vesicles
>1	inverted truncated cone	inverted micelles

FIGURE 1. Classification of membrane lipids in terms of packing factors (adapted from Israelachvili *et al.* (1980)).

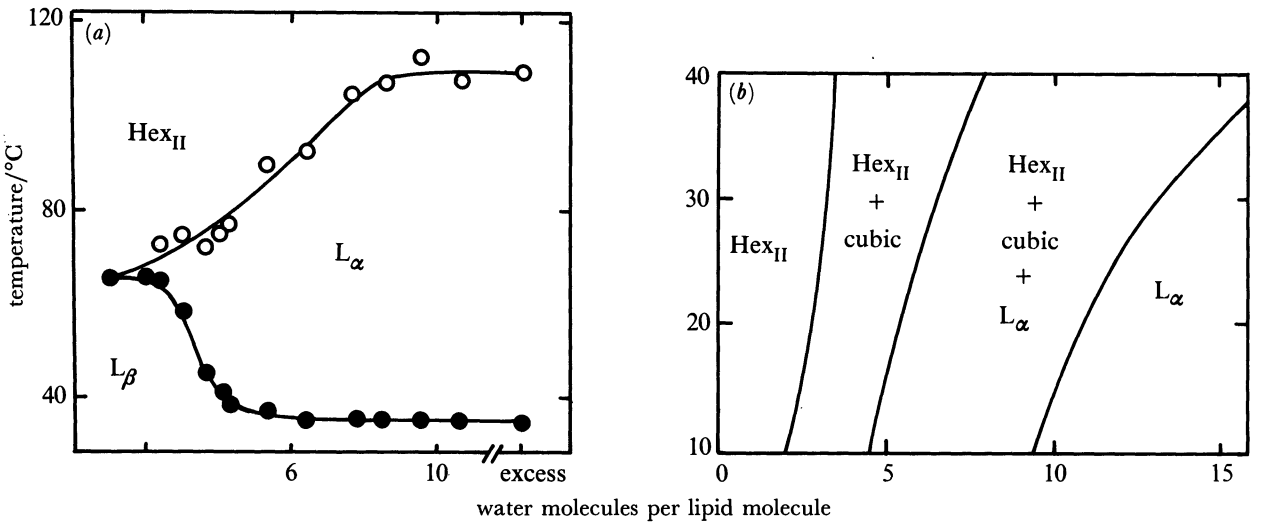

FIGURE 2. Phase diagrams of (*a*) didodecyl phosphatidylethanolamine and (*b*) a dispersion of monogalactosyl- and digalactosyl diacylglycerol (2:1) molar ratio. (Data adapted from the work of Seddon *et al.* (1982) and Brentel *et al.* (1985) respectively.)

in figure 2*a*. The energy barrier associated with L_α-to-Hex_{II} transitions is surprisingly low. This is reflected in the low enthalpy value associated with L_α-to-Hex_{II} phase transitions as opposed to L_β-to-L_α transitions. The molar enthalpy for the L_β-to-L_α transition for dipalmitoyl-phosphatidylethanolamine, for example, is 33.1 kJ mol^{-1} whereas the corresponding value for the L_α-to-Hex_{II} transition is only 1.3 kJ mol^{-1} (Seddon *et al.* 1983).

2.2. *Low temperature phases*

In addition to the L_β-to-L_α and L_α-to-Hex_{II} phase transitions involving the liquid-crystal state, it is now widely recognized that many, if not all, lipids undergo transitions between

different gel-phase forms. The most widely studied system is that involving the $L_{\beta'} \rightarrow P_{\beta'} \rightarrow L_{\alpha}$ transitions occurring in phosphatidylcholines. This is the series of transitions that gives rise to the characteristic pre-transition–transition behaviour seen in these lipids (Janiak *et al.* 1976). The $L_{\beta'}$ phase corresponds to a phase in which the hydrocarbon chains are packed on a hexagonal lattice (as in the conventional L_{β} phase) but are inclined at an angle to the bilayer plane. The chains are packed on a similar lattice in the $P_{\beta'}$ phase but with an altered chain orientation. Under these conditions, packing constraints distort the bilayer into a ripple phase, giving rise to the characteristic ripple appearance seen in freeze-fracture electron micrographs of phosphatidylcholines thermally quenched from temperatures between their pre-transition and main transition temperatures (Luna & McConnell 1978).

Much interest has been generated in recent years regarding the existence of other low-temperature phases. These have been variously referred to as 'crystalline' or 'sub-gel' phases. The existence of such phases was first reported by Chen *et al.* (1980) working with phosphatidylcholines equilibrated for several days at about 0 °C. X-ray diffraction studies indicate that the formation of these crystalline (L_c) phases involves a repacking of the hydrocarbon chains on an orthorhombic as opposed to hexagonal lattice. This rearrangement appears to involve a dehydration of the lipid headgroups (Sen *et al.* 1983). The existence of L_c phases in phosphatidylethanolamines, phosphatidylglycerols and galactolipids is now well established. There is also clear evidence, from measurements of bilayer thicknesses, that some lipids form chain interdigitated gel phases (L_{β_i}) under low hydration conditions. These are normally only seen at extreme levels of dehydration. A detailed discussion of these phases can be found in the recent review by Quinn (1989).

3. Phase behaviour of simple lipid mixtures

To apply the knowledge gained from studies on single lipids to the study of biological membranes, it is necessary to understand the way that lipids behave in mixed systems. It is particularly important to know how the presence of additional lipids is likely to affect the gel–liquid crystal ($L_{\beta} \rightarrow L_{\alpha}$) and bilayer–non-bilayer ($L_{\alpha} \rightarrow Hex_{II}$) transitions of individual components of lipid mixtures. In the context of cryobiology, information on their effects on the sub-gel transitions ($L_c \rightarrow L_{\alpha}$) and ($L_{\beta_i} \rightarrow L_{\alpha}$) is also of considerable potential interest.

3.1. *Liquid-crystal–gel-phase separations*

The behaviour of mixed lipids is determined by the extent of ideality of their mixing. If ideal mixing were to occur in the gel and liquid-crystal states, the phase transition temperature would be a linear function of mole fraction and the composition of the liquid and solid phases identical. In practice, ideal behaviour is never seen. In cases where the deviations from the ideal are small as, for example, for two lipids with identical head-groups and similar length fatty acyl chains, the lipids show complete miscibility in the liquid and the solid states and the composition of the two phases is not too dissimilar (Shimshick & McConnell 1973). In contrast, mixtures of lipids having the same headgroup but very different acyl chains, or mixtures of lipids with different headgroups often show solid-state, and sometimes even limited liquid-state, immiscibility. The phase diagrams of such systems can be extremely complex involving multiple liquid-crystal and gel phases.

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3.2. *Bilayer–non-bilayer transitions*

Relatively little work has been carried out on the systematic analysis of the behaviour of bilayer–non-bilayer transitions in mixed lipid systems. Hui *et al.* (1981) have reported a partial phase diagram for phosphatidylethanolamine:phosphatidylcholine mixtures and showed that the proportion of lipid in non-bilayer structures decreased rapidly with increasing additions of bilayer lipid. Brentel *et al.* (1985) have examined the phase behaviour of 1:2, 1.2:1 and 2:1 mole ratio mixtures of mono- and digalactosyl diacylglycerol over the temperature range 10–40 °C and for water contents up to 14 mol water per mole lipid by using deuterium nuclear magnetic resonance and X-ray diffraction techniques. A simplified version of their phase diagram for the 2:1 mole ratio mixture is shown in figure 2*b*.

The formation of inverted structures such as Hex_{II} is driven by the tendency of the non-bilayer forming lipids to minimize their exposure to the aqueous phase by forming structures with a high radius of curvature. This process is opposed by packing constraints that increase the free energy of such structures compared to that of the bilayer configuration. Addition of molecules such as dolichol (Gruner 1985) or long-chain lipids (Tate & Gruner 1987) that can relax these constraints by filling potential gaps in non-bilayer structures, leads to a stabilization of the non-bilayer configuration resulting in a marked lowering of $L_\alpha \rightarrow Hex_{II}$ transition temperatures. Conversely, the addition of bilayer-forming lipids, which tend to interact more strongly with water and hence have higher optimal surface areas per molecule, appear to stabilize the bilayer arrangement at the expense of non-bilayer configurations.

3.3. *Sub-gel transitions*

Little attention has been paid to the possible occurrence of sub-gel transitions in mixed lipid systems. Boyanov *et al.* (1986) has shown that the occurrence of the L phase of L-1,2,dipalmitoylphosphatidylcholine is suppressed by the presence of more than eight percent of the D-enantomer. The same authors reported that the presence of 15% of L-1,2,dipalmitoyl-phosphatidylethanolamine has a similar effect. Measurements of the effects of the added lipids on the pre-transition endotherm suggest, however, that the mechanisms involved are rather different.

It seems likely that sub-gel phases are of little importance in systems showing solid-state miscibility. However, it remains to be determined whether or not they can be formed in mixed lipid dispersions showing solid-state immiscibility.

4. Phase behaviour of total membrane lipid extracts

The phase behaviour of total membrane lipid extracts is extremely complex. Studies on lipid extracts of chloroplast membranes have, however, provided several useful insights into the part played by different membrane components. The most striking observation is that total polar lipid extracts of this type do not necessarily form bilayer structures on dispersion in aqueous media. Chloroplast lipids can be induced to form small unilamellar liposomes if dispersed in distilled water but the addition of low concentrations of monovalent or divalent cations leads to the formation of inverted lipid micelles, followed by liposome fusion and precipitation (Gounaris *et al.* 1983). Similar changes were triggered by changes in pH. The formation of non-bilayer structures can also be induced in mixtures of bilayer and non-bilayer forming lipids by

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heat or by the addition of membrane dehydrating agents such as ethylene glycol and dimethylsulphoxide (Sen *et al.* 1981, 1982). Studies of this type highlight the fact that changes in lipid organization can be triggered by a wide range of factors other than changes in temperature. This is particularly important in the context of changes occurring during freezing, as discussed in §5.

Dispersion of total lipid extracts of chloroplast membranes in media of composition similar to that used in chloroplast isolation leads to the formation of lipid aggregates dominated by non-bilayer structures (Gounaris *et al.* 1983). The absence of analogous structures in native chloroplast membranes indicates that other membrane components, not present in the extracts, must play a role in stabilizing the lipids into a bilayer conformation. The obvious candidates for such a role are membrane proteins. The ability of certain proteins and oligopeptides to influence the phase behaviour of membrane lipids is well documented. Taraschi *et al.* (1982), for example, have demonstrated the ability of glycophorin to stabilize the non-bilayer forming lipid dioleoylphosphatidylethanolamine into a bilayer configuration. Cytochrome *c*, and apocytochome *c*, in contrast, have been shown to have the ability to destabilize the bilayer structure of mitochondrial membranes and to induce fusion between the inner and outer membranes (Van Venetie & Verkleij 1982). Studies on the incorporation of gramicidin into model membrane systems indicate that quite subtle changes in polypeptide chain length and organization can lead to major changes in lipid organization (Killian *et al.* 1989).

Clearly, if membrane proteins play a major role in stabilizing non-bilayer lipids within the membrane bilayer, the presence of such lipids would be expected to confer some advantages in terms of membrane structure or function, or both. Several possible roles have been suggested. One possibility is that non-bilayer lipids play a role in the efficient incorporation, or sealing, of proteins into the membrane bilayer. Some evidence in support of this view has come from studies of the effects of thermal stress on isolated chloroplasts (Gounaris *et al.* 1984; Williams *et al.* 1984). Relatively minor increases in temperature lead to the breakdown of the light-harvesting apparatus of photosystem II and a major re-organization of the thylakoid membrane that is accompanied by an irreversible phase-separation of non-bilayer forming lipids. Cullis, de Kruijff and Verkleij and their co-workers have suggested that non-bilayer forming lipids may play a functional as well as a structural role in membranes and have implicated them in the transport of polar molecules across membranes and as intermediates in membrane fusion (see Verkleij (1984)).

Studies on the phase behaviour of total polar lipid extracts of chloroplast membranes and the comparison of the behaviour of such extracts with that of native membranes have led to the suggestion by Williams (1988) that the phase behaviour of biological membranes, to a first approximation at least, is explicable in terms of the type of diagram presented in figure 3. This diagram represents a first attempt to define the general phase behaviour of membranes. The different regions in the diagram, it must be stressed, refer to regions of phase-separation rather than uniform phases and the boundaries indicate the onset of such phase separations rather than the formation of discrete phases.

The diagram is divided into three main regions corresponding to the conventional non-bilayer, lamellar liquid-crystal and gel phases seen in phase diagrams of non-bilayer forming lipids and bilayer–non-bilayer mixtures (cf. figure 2). The non-bilayer region is sub-divided into two parts. A lower temperature region in which non-bilayer lipids are constrained within the lipid bilayer by the presence of membrane proteins and a higher temperature region in

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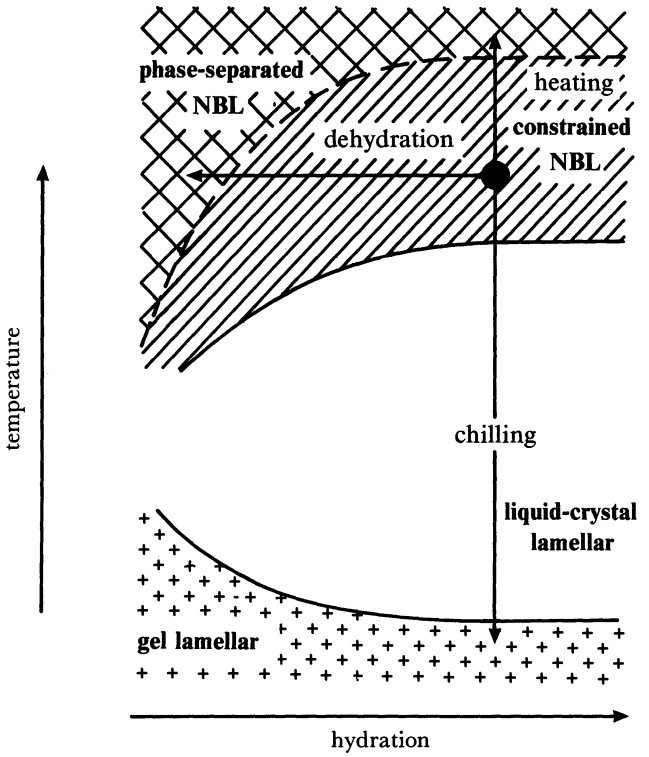

FIGURE 3. Diagram illustrating the types of phase separations that might be anticipated on subjecting a natural membrane to environmental stress. See text for details.

which they are able to phase-separate. The liquid-crystal lamellar region corresponds to the region in which the non-bilayer lipids can be accommodated in the lipid bilayer even in the absence of proteins and the low temperature gel lamellar region to a region in which the higher melting-point lipids tend to separate out as gel-phase patches.

Under normal conditions, the stable range of the membrane corresponds to the constrained non-bilayer region. Rises in temperature or membrane dehydration, or both, would thus be expected to result in the phase-separation of non-bilayer components. There is good evidence for such effects in chloroplasts (Gounaris *et al.* 1984) and sarcoplasmic reticulum (Crowe & Crowe 1984). Decreases in temperature, as discussed below, would be expected to lead to the destabilization of lipid–protein interactions and the phase separation of gel-phase lipid.

5. APPLICATIONS TO CRYOBIOLOGY

Discussion of membrane changes associated with low temperatures are conveniently divided into those involving chilling and freezing temperatures. This allows a distinction between those phenomena driven predominantly by temperature changes and those that contain significant contributions from other stresses.

5.1. *Cold shock and chilling injury*

The term cold-shock is often used to describe the sort of damage, associated with extensive phase separation of higher melting-point lipids, seen on cooling thermophilic microorganisms.

This type of chilling injury is readily explained in terms of the phase-separation diagram described above. The boundaries between liquid-crystal and gel-phase lipid are known to be particularly leaky and to act as loci for cell leakage. The formation of such gel-phase regions has been particularly well characterized in the cyanobacterium *Anacystis nidulans* where abundant evidence for phase separation is available from freeze-fracture (Furtado *et al.* 1979; Ono & Murata 1982), differential scanning calorimetry (Furtado *et al.* 1979; Ono *et al.* 1983; Mannock *et al.* 1985) and wide angle X-ray diffraction studies (Tsukamoto *et al.* 1980). Ono & Murata (1981) have also demonstrated a close correlation between the appearance of such gel-phase regions in the cytoplasmic membranes of *Anacystis* and the onset of extensive K^+ leakage.

Although the type of chilling injury seen in *Anacystis* is readily explicable in terms of lipid phase behaviour, there are many other systems that exhibit chilling injury at temperatures well above those associated with detectable phase separations of gel-phase lipid. This is particularly true of 'chilling sensitive' plants, which are characterized by the occurrence of chilling injury on exposure to temperatures below 5–10 °C despite the fact that the bulk of their membrane lipids form gel phases at temperatures well below 0 °C (Williams & Quinn 1987). Two possible explanations of chilling sensitivity have been proposed to explain this type of injury. The first is that the presence of minor lipid components with relatively high gel-to-liquid crystal transitions allows a limited phase separation at temperatures well above the corresponding transition temperature for bulk lipid components. This view is based on the existence of a strong correlation between the presence of highly saturated molecular species of phosphatidylglycerol in the chloroplasts of a wide range of plants and their susceptibility to chilling damage (Murata *et al.* 1983). The gel-to-liquid crystal phase transition temperature of such lipids, which typically represent 5–8% of the total membrane lipid, is about 35 °C (Murata & Yamaya 1984) but this would be expected to be much lower in the presence of the more unsaturated lipids found in the native membrane. An alternative explanation, favoured by Quinn & Williams (1985) is that lower temperatures lead to a reduced ability of the non-bilayer forming lipids to seal key intrinsic membrane proteins into cell membranes. This might be expected to lead to leakage at protein–lipid interfaces akin to that occurring at gel–liquid-crystal interfaces in membranes exhibiting gel-phase separation.

In either case, chilling injury is likely to be the result of secondary changes associated with a limited breakdown of membrane permeability properties. It could well involve the leakage of specific ions such as Ca^{2+} as proposed by Minorsky (1985), or it may reflect a more general inability to preserve metabolite balances.

5.2. *Damage due to freezing*

One of the major features of freezing damage, as opposed to chilling injury, is the phenomenon of freeze-dehydration. When ice forms in a suspension of cells this tends to occur preferentially in the extracellular medium. The osmotic strength of the unfrozen fraction is thus raised imposing a gradient of water potential on the unfrozen cells. Loss of intracellular water in response to this gradient results in cell shrinkage, cellular dehydration and a consequent increase in concentration of cellular solutes. Exposure to high electrolyte levels, and changes in cellular pH associated with freeze dehydration are likely to have marked effects on the weak interactions between membrane components. In particular, the strength of intramolecular

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hydrogen bonds between neighbouring lipids, between lipid head-groups and proteins and between water and both of these components may be altered. Hydrophobic interactions tend to be strengthened and electrostatic interactions between lipid head-groups to be weakened by ion-shielding.

Seddon *et al.* (1983) have shown that high concentrations of salts such as NaCl lead to a marked stabilization of the non-bilayer phase of phosphatidylethanolamines with respect to the liquid-crystal lamellar phase and a consequent lowering of the ($L_\alpha \rightarrow Hex_{II}$) phase transition temperature. Smaller, but nevertheless significant changes are seen in the ($L_\beta \rightarrow L_\alpha$) transition temperature. In this case, the lower temperature L_β phase is stabilized with respect to the L_α phase and the transition temperature is raised. Gounaris *et al.* (1983) working with aqueous dispersions of total membrane lipid extracts of chloroplasts, as mentioned above, have demonstrated that changes in ionic strength or pH can lead to major changes in the extent of non-bilayer lipid phase separation.

Direct demonstrations of freeze-induced phase separations of non-bilayer forming lipids in the plasma membranes of protoplasts isolated from non-acclimated rye leaves have been reported by Gordon-Kamm & Steponkus (1984) and Pihakaski & Steponkus (1987). Cudd & Steponkus (1988) have reported similar effects in liposomes prepared from the lipids of such membranes. Caffrey (1987) has also reported the occurrence of freeze-induced changes in the temperature of the $L_\beta \rightarrow L_\alpha$ phase transition in dioleoylphosphatidylserine liposomes.

5.3. *Role of cryoprotectants*

The role of cryoprotectants in the preservation of biological activity is a subject of great interest to cryobiologists. Many cells have the ability to synthesize compatible solutes such as proline and betaine that act as natural cryoprotectants. Other cryoprotectants, most commonly glycerol and dimethylsulphoxide, are widely used in the cryopreservation of both plant and animal tissues. All these compounds share an ability to interact strongly with water altering its structure and its interactions with solute molecules (Taylor 1987). A great deal of information is available regarding their mode of action in terms of their abilities to reduce losses of intracellular water and to stabilize protein structure. Until recently, however, relatively little attention had been paid to their effects on membrane stability.

Interest in the interaction of cryoprotectants with membranes in general and membrane lipids in particular was greatly spurred by work showing the ability of disaccharides, such as trehalose, to stabilize biological organisms against low temperature damage (Crowe & Clegg (1973). Trehalose, and to a lesser extent other disaccharides, appear to have the ability to lower the temperature of the gel to liquid-crystal transition of dry lipid dispersions from those typical of the anhydrous, or near anhydrous, state to values similar to, or lower than, the transition temperature of fully hydrated lipid.

Some confusion has arisen regarding the interpretation of these observations. It is becoming increasingly clear that the results obtained in such systems are strongly influenced by the method of sample preparation adopted (Quinn *et al.* 1988). Considerable differences are seen between samples in which the lipids are dried down from organic solvents and those that are prepared by freeze-drying from an initially hydrated state. In the former case, the lipids tend to be in a largely disordered state (Lee *et al.* 1986) whereas in the latter they tend to retain their original bilayer configuration (Caffrey *et al.* 1988; Crowe & Crowe 1988; Quinn *et al.* 1988).

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It is now generally accepted that disaccharides, sugar alcohols and polyols such as glycerol can act as 'water replacement' agents preserving bilayer structures under conditions of extreme dehydration.

A rather different aspect of the interaction of cryoprotectants and membranes is the effect of cryoprotectant–water mixtures on the phase behaviour of non-bilayer lipids. This work stems from observations in our laboratory that the addition of cryoprotectants tends to induce the formation of non-bilayer structures in aqueous dispersions prepared from mixtures of bilayer and non-bilayer forming lipids (Sen *et al.* 1981, 1982). These observations led to the suggestion that cryoprotectants might play an important role in preserving the appropriate balance between the bilayer and non-bilayer forming tendencies of membrane lipids at low temperatures (Quinn 1985). Strong support for this view comes from recent studies demonstrating that additions of sugars, sugar alcohols and polyols can bring about a marked lowering of the temperature of the L_α-to-Hex_{II} phase transition of hydrated phosphatidylethanolamines (Bryszewska & Epand 1988; Koynova *et al.* 1989).

We have recently investigated the phase behaviour of the non-bilayer forming lipid distearoylphosphatidylethanolamine dispersed in glycerol–water, dimethylsulphoxide–water and sucrose–water mixtures. A preliminary phase diagram for the glycerol–water system is shown in figure 4. As the concentration of glycerol is increased, there is a dramatic reduction in the temperature of the $L_\alpha \rightarrow Hex_{II}$ transition and a small increase in that of the $L_\alpha \rightarrow L_\beta$

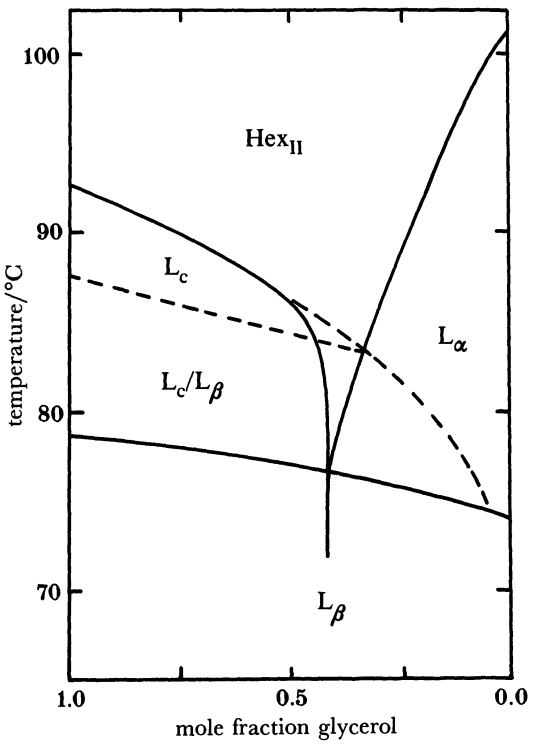

FIGURE 4. Phase diagram for distearoyl phosphatidylethanolamine in the presence of excess glycerol–water mixtures constructed on the basis of differential scanning calorimetry and X-ray diffraction data. Dashed lines indicate phase boundaries of equilibrated dispersions and solid lines the boundaries observed for samples reheated immediately after cooling from the hexagonal$_{II}$ phase. (Unpublished data of W. P. Williams, P. J. Quinn, R. D. Koynova and L. Tsonev).

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transition. The stabilization of the Hex_{II} phase, as pointed out by Koynova *et al.* (1989), can be explained in terms of the Hofmeister effect. Molecules such as sugars and the common cryoprotectants tend to stabilize the structure of bulk water. The effect of such agents is to reduce the extent of interaction of water with lipid head-groups. In terms of the shape hypothesis, this is equivalent to reducing the value of a_0 and thus increasing that of the packing factor p. Observations by Yeagle & Sen (1986) that the chaotropic agents such as guanidine hydrochloride, urea and sodium thiocyanate, which tend to destabilize the structure of bulk water, bring about a stabilization of the L_α phase at the expense of the non-bilayer Hex_{II} phase add strong support to this argument.

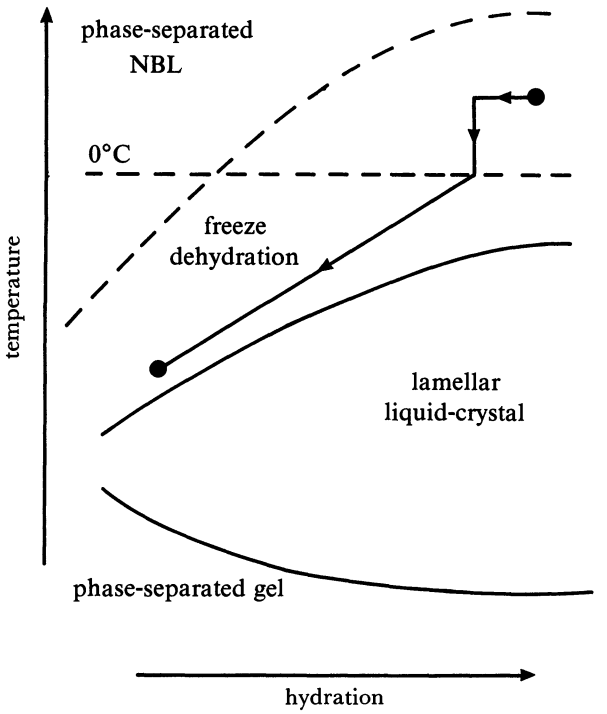

FIGURE 5. Diagram illustrating the effect of cooling biological membranes in the presence of cryoprotectant. The cryoprotectant ensures that the balance of forces between membrane components remains relatively constant and that the membrane stays within its stable range. See text for details.

The implications of these results in terms of the effects of cryoprotectants on membrane stability are clear. Addition of cryoprotectants, especially under freezing conditions where their effective concentration is likely to be increased by freeze-dehydration, would be expected to combat the normal tendency for the distinction between bilayer and non-bilayer forming lipids to be reduced at low temperatures. The effect of this, as indicated in figure 5, should be to maintain the balance of interactions between membrane components at a level similar to that existing under physiological conditions and hence reduce membrane damage on cooling.

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Discussion

D. Chapman (*Department of Protein and Molecular Biology, Royal Free Hospital School of Medicine, London, U.K.*). Dr Williams has suggested that non-lamellar lipids present in a natural biomembrane may interact in some way with the intrinsic proteins. Can he provide some model of this type of interaction?

W. P. Williams. There is convincing evidence from studies on model systems that certain proteins, such as glycophorin, can stabilize non-bilayer forming lipids in a bilayer configuration (Taraschi *et al.* 1982). Unfortunately, no clear idea of the molecular interactions involved has, as yet, emerged. In general, non-bilayer structures are commonly observed in total lipid extracts of membranes but not in the native membrane. Clearly, some factor associated with the membrane is involved in constraining the non-bilayer forming lipids within the membrane bilayer. This could be associated with lipid–protein interactions. The possibility that the

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formation of non-bilayer structures involves interactions between neighbouring lipid layers, which is possible in the extracts but may be suppressed in the native membrane, cannot however be completely excluded.

D. Chapman. Dr Williams has said that demixing of bilayer forming lipids from non-bilayer forming lipids can occur under certain conditions by lateral phase separation. A change in lipid asymmetry of a membrane is another method by which (at least in principle) demixing might occur causing a potential instability to be produced in the biomembrane structure.

W. P. Williams. I would certainly agree that changes in lipid asymmetry could trigger demixing of lipids. This process may well play a role in some aspects of membrane damage but there is no evidence to suggest that it is a major factor in low-temperature injury.

U. Heber (*Institute of Botany and Pharmaceutical Biology, University of Würzburg, F.R.G.*). If I understood Dr Williams correctly, in his system cryoprotectants stabilize non-bilayer structures, whereas chaotropic agents stabilize bilayers. It is well-known that the functionability of biomembranes is preserved during freezing by cryoprotectants and destroyed by chaotropic solutes. How does Dr Williams explain what appears to be a contradictory situation?

W. P. Williams. Low temperatures tend to stabilize bilayer structures at the expense of non-bilayer structures. As the temperature is lowered, non-bilayer forming lipids will tend to be more easily accommodated in the membrane bilayer. If these lipids play a specific role in stabilizing membranes, for example by helping to seal intrinsic membrane proteins into a bilayer, this ability will be progressively lost on cooling. Addition of cryoprotectants tends to stabilize non-bilayer structures and thus to compensate for such changes; chaotropic agents tend to aggravate them. The presence of cryoprotectants would help preserve the balance of forces between membrane components at low temperatures at a level similar to that normally existing at physiological temperatures.

Reference

Russell, N. J. 1989 Adaptive modifications in membranes of halotolerant and halophilic microorganisms. *J. Bioenerg. Biomemb.* **21**, 93–114.

F. Franks (*Pafra Ltd, 150 Cambridge Science Park, Cambridge, U.K.*). Timasheff and his colleagues have shown that in solution, carbohydrates raise the chemical potential of protein, i.e. carbohydrates repel proteins (Gekko & Morikawa (1981)). A similar effect might be expected to operate between lipids and carbohydrates, yet, in the almost dry state, carbohydrates *protect* proteins and lipids against physiologically damaged phase changes, so that carbohydrates are said to substitute for water. What, if anything is known about the change-over in the interactions during the gradual desiccation of solutions which contain 'protectant' carbohydrates?

Reference

Gekko, K. & Morikawa, T. 1981 Thermodynamics of polyol-induced thermal stabilization of chymotrypsinogen. *J. Biochem.* **90**, 51–60.

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W. P. Williams. The idea that carbohydrates substitute for water and prevent the occurrence of phase transitions is based on studies involving the bilayer forming lipid phosphatidylcholine where the presence of carbohydrate leads to a marked decrease in the temperature of the gel to liquid-crystal phase transition of the dry tri-hydrate to a value close to that of the fully hydrated lipid. Given the very limited amount of water present, the carbohydrate is necessarily in contact with the lipid head-groups. The similarity of the transition temperatures suggests that the balance of forces existing at the bilayer surface in the two systems is very similar, hence the idea that carbohydrate can substitute for water. It is not clear, however, whether or not a direct binding of carbohydrate is involved.

Carbohydrates have a much more marked effect on the properties of dispersions containing appreciable amounts of water. As illustrated in my figure 5, the addition of relatively low proportions of glycerol lead to marked decreases in the temperature of $L \rightarrow Hex_{II}$ transitions and rather smaller increases in $L_\beta \rightarrow L_\alpha$ transitions. Both these changes involve the stabilization of phases in which the extent of the lipid–solvent interface is minimized. The system thus shows interesting parallels with the work of Timasheff and his colleagues that are certainly worthy of closer examination.

To the best of my knowledge, no information exists regarding the changes at molecular level that occur in these systems during dessication. It is, however, known for sugars at least, that reproducible results are best obtained by drying hydrated samples rather than by partially hydrating lipids dried down from organic solvents. This probably reflects the importance of starting from a system with pre-formed bilayers.

R. Jaenicke (*Institute for Biophysics, University of Regensburg, F.R.G.*). 1. Referring to the effects of glycerol on the lipid phase transitions, what does happen to membrane proteins or soluble proteins at the extremes of glycerol concentrations that Dr Williams was discussing?

2. An alternative cause of low water activity, apart from drying, would be high salt. What is the effect of salts on lipid-phase transitions in halophilic organisms? Low temperature is assumed to weaken hydrophobic interactions; how do salts interfere with this tendency?

W. P. Williams. I can only refer Professor Jaenicke to the work of Timasheff and his colleagues mentioned by Professor Franks. They found that high glycerol concentrations stabilize globular proteins against thermal denaturation. Their results indicate that this is caused by entropic factors possibly associated with solvent ordering effects.

High concentrations of sodium chloride lead to similar changes in the phase behaviour of non-bilayer lipids such as phosphatidylethanolamine to those seen on dehydration (Seddon *et al.* 1982). This stabilization is usually attributed to the reduction of electrostatic interactions between neighbouring head-groups by electrostatic shielding. It would, however, be interesting to extend these studies to cover the Hofmeister series to see whether lipids show a similar pattern of behaviour to that reported for proteins. Halophilic organisms accommodate themselves to high salt concentrations in several different ways. The effect of changing salt concentration on the membrane lipids of such organisms have been extensively studied (see the recent review of Russell (1989)). Moderate halophiles adapt to high salt by increasing their proportion of anionic lipids. The net result of this, as discussed by Russell, appears to be to keep the balance of bilayer–non-bilayer behaviour of their lipids at a constant level.

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F. Franks. I know of no evidence that compatible (osmoregulating) solutes or other chemical cryoprotectants interact strongly with water. Hydrogen bonds are the only possible interaction and all available theoretical treatments show that, by whatever criterion binding is determined, the interactions between water molecules are stronger than those between water and polar groups on organic molecules.

W. P. Williams. I would certainly agree with Professor Franks' statement but this is not really the question at issue. The presence of cryoprotectants appears to reduce the extent of interaction between the lipid head-groups and the solvent whereas chaotropic agents tend to increase interaction. This suggests a possible role of solvent ordering. It is the relative ability of these agents to promote, or disrupt, such ordering that is of potential interest.

Membrane lipids do not normally show undercooling. Nucleation appears to be very rapid and efficient as evidenced by the extremely rapid cooling rates required to avoid phase separations in freeze-fracture studies (see Sternberg *et al.* (1989)).

Reference

Sternberg, B., Galle, P. & Watts, A. 1989 The effect of temperature and protein content on the dispersive properties of bacteriorhodopsin from *H. bacterium* in reconstituted DMPC complexes free from endogenous lipids: a freeze-fracture electron microscopy study. *Biochim. biophys. Acta* **980**, 117–126.

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