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RESEARCH PERSPECTIVE

# Membrane behaviour in seeds and other systems at low water content: the various effects of solutes

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## Abstract

A common feature of desiccation-tolerant organisms, such as orthodox seeds, is the presence of large quantities of sugars, especially di- and oligosaccharides. These sugars may be one component of the suite of adaptations that allow anhydrobiotes to survive the loss of most of their cellular water. This paper describes the physical effects of dehydration on cellular ultrastructure, with particular emphasis on membranes, and explains quantitatively how sugars and other solutes can influence these physical effects. As a result of dehydration, the surfaces of membranes are brought into close approach, which causes physical stresses that can lead to a variety of effects, including demixing of membrane components and fluid-to-gel phase transitions of membrane lipids. The presence of small solutes, such as sugars, between membranes can limit their close approach and, thereby, diminish the physical stresses that cause lipid fluid-to-gel phase transitions to occur during dehydration. Thus, in the presence of intermembrane sugars, the lipid fluid-to-gel phase transition temperature ($T_m$) does not increase as much as it does in the absence of sugars. Vitrification of the intermembrane sugar solution has the additional effect of adding a mechanical resistance to the lipid phase transition; therefore, when sugars vitrify between fluid phase bilayers, $T_m$ is depressed below its fully hydrated value ($T_0$). These effects occur only for solutes small enough to remain in very narrow spaces between membranes at low hydration. Large solutes, such as polymers, may be excluded from such regions and, therefore, do not diminish the physical forces that lead to membrane changes at low hydration.

**Keywords: dehydration, desiccation, membranes, phase transition, solutes, sugars, vitrification**

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## Introduction

Membrane phase behaviour during dehydration and rehydration is important to the survival of seeds and other anhydrobiotic tissues. To give just one example, Hoekstra *et al*. (1999) recently showed that rehydration of pollen at low temperatures, at which the membranes were thought to be in the gel phase, resulted in the visible disruption of plasma membranes and cellular death. Rehydration at a higher temperature, at which the membranes were thought to be in the fluid phase, did not cause disruption of the membranes, and the pollen survived. The phase of the membrane lipids – fluid, gel or non-lamellar – is determined by the composition of the membrane, the degree of hydration, the temperature, and the composition and state of nearby solutions. Soluble sugars have profound effects on the phase behaviour of membrane lipids in desiccated model systems (Caffrey *et al*., 1988; Crowe and Crowe, 1988; Koster *et al*., 1994, 1996, 2000; Crowe *et al*., 1996). Several mechanisms, including general effects and specific interactions, have been proposed to explain how sugars can help maintain membranes in the fluid phase as dehydration occurs. In order to understand the diverse ways that sugars and other solutes can influence membrane phase behaviour, one must first understand how dehydration physically affects membrane lipids. It is then possible to explain the various ways in which sugars and other solutes can affect this process.

The phase behaviour of membranes and solutions depends on such general physical conditions as temperature, mechanical constraints, osmotic pressures, distribution of components and rate or state of equilibration. The phase behaviour may also depend on specific interactions among chemical components. The first purpose of this paper is to explain briefly the general effects on membrane phase

behaviour that result from the loss of water and to show how these effects explain the observed membrane phase behaviours in the presence of solutes. The second is to resolve some apparent misunderstandings that have appeared in the literature. In this paper we shall not discuss specific solute–membrane interactions, such as those proposed by the water replacement hypothesis (Crowe *et al.*, 1996). This does not, of course, mean that such interactions are absent. Nevertheless, there are good reasons for omitting them here. First, because there are so many compounds with specific interactions that might be discussed, consideration of them would be a long process. More importantly, all or nearly all of the observed behaviours can be explained without recourse to such interactions.

## Why does dehydration deform membranes and raise transition temperatures?

Membranes are considered one of the primary sites of lethal damage to cells that are not desiccation tolerant, and protecting membrane components from the effects of desiccation is a likely feature of desiccation tolerance (e.g. Crowe and Clegg, 1978; Leopold, 1986). Use of electron microscopy has revealed structural changes in cellular membranes damaged by dehydration. Such structural changes include the formation of aparticulate regions, in which integral membrane proteins have been excluded from lipid-rich domains, and regions of the non-bilayer hexagonal II phase, which destroy the effectiveness of the membrane as a permeability barrier (Crowe and Crowe, 1982; Gordon-Kamm and Steponkus, 1984; Platt-Aloia, 1988). Another structural change that can occur in the phospholipid component of the membrane during dehydration is the transition from the fluid to the gel phase (Chapman *et al.*, 1967; Crowe *et al.*, 1984; Caffrey *et al.*, 1988). The temperature at which this transition occurs, $T_m$, depends on many factors in addition to water content, including the presence of impurities in the bilayer (e.g. McMullen *et al.*, 1994) and the presence of solutes in the suspending solution (reviewed by Wolfe and Bryant, 1999). For ease of discussion, we define the fluid–gel phase transition temperature of the pure phospholipid in excess water to be $T_o$, and we use this value as a basis for comparison of the effects of dehydration on the lipid phase transition temperature, $T_m$ (Wolfe and Bryant, 1999; Koster *et al.*, 2000).

As pure phospholipids are subjected to gradually decreasing water contents, there is a corresponding gradual increase in their phase transition temperature $T_m$ above $T_o$ (Chapman *et al.*, 1967; Lynch and Steponkus, 1989; Webb *et al.*, 1993; Koster *et al.*, 1994, 2000) (Fig. 1). This increase in $T_m$ generally becomes detectable at water contents less than approximately 0.2 g $H_2O$/g DW, which result from water potentials less than about –11 MPa (Lynch and Steponkus, 1989; Zhang and Steponkus, 1995; Koster *et al.*, 2000). As dehydration of the pure phospholipid grows more severe, $T_m$ rises further above $T_o$; $T_m$ for the anhydrous lipid may be 60 or more degrees above $T_o$ (Chapman *et al.*, 1967; Lynch and Steponkus, 1989). Similar increases in the apparent $T_m$ of membranes with drying have been reported in pollen (Hoekstra *et al.*, 1992) and seed embryos (Sun *et al.*, 1994). The increase in phospholipid $T_m$ during dehydration can be explained quantitatively through an understanding of hydration forces and the physical

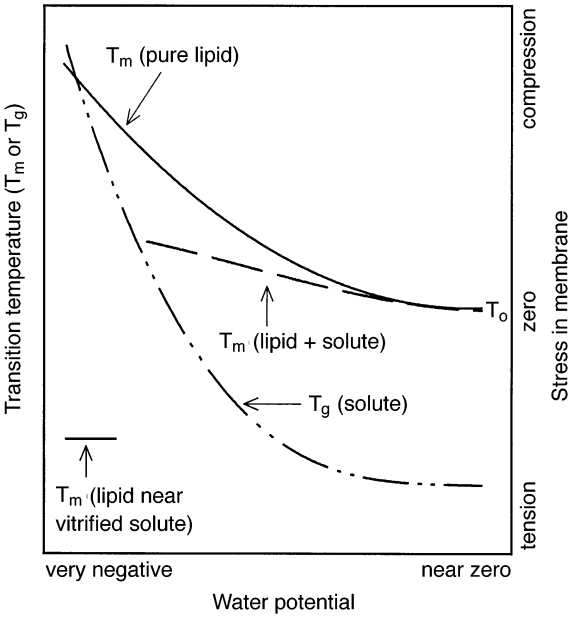


**Figure 1.** Phase diagram showing the various effects of small solutes on phosphatidylcholine phase behaviour. At high water potential, the intermembrane water content is high, and the lipid fluid-to-gel transition temperature is that of the lipid in excess water, $T_o$. As the water potential decreases, water leaves the intermembrane space, the compressive stress in the membrane increases, and the transition temperature $T_m$ of the pure lipid, represented by the solid line, rises above $T_o$. If the sample contains small solutes that remain between the membranes, the compressive stress does not increase to the same extent during drying as it does in the absence of such solutes. Hence, $T_m$ (dashed line) also does not rise much above $T_o$. If the small solutes should vitrify in the intermembrane space while the lipids are in the fluid phase (i.e. if $T_g$ is above $T_m$ for the lipid dried with solutes), then the mechanical resistance of the glass exerts a tension on the membrane that hinders the transition to the gel phase. Thus, $T_m$ for the lipid near the vitrified solute is depressed below $T_o$.

stresses that are imposed upon membranes and macromolecules at low water contents (Bryant and Wolfe, 1992; Wolfe and Bryant, 1999).

## The hydration forces explanation

The mechanisms for membrane damage discussed here are important only at rather severe dehydration, at water contents below approximately 0.2 g $H_2O$/g DW, depending upon the composition of the system being studied. In a cell at initially high water content, dehydration removes water from the bulk aqueous solutions in the cell. This changes the cell's geometry (i.e. it produces strains), but the physical stresses involved are small compared to those encountered at low hydration. The key point is that, at sufficiently low hydration, all of the ultrastructural elements, such as membranes and proteins, are very close together. Consider first a membrane-rich phase. At approximately 20% water, the average separation between membranes is about 1 nm (Fig. 2). This observation is independent of the initial geometry. A similar argument can be made for a cell: throughout the cell, the hydrophilic surfaces on membranes, macromolecules and ultrastructural elements are, on average, brought to within nanometres of each other by extreme dehydration.

What happens if more water is removed? Let's concentrate on the water between two membranes (Fig. 2), although it could equally well be the water between a membrane and another hydrophilic surface. The water volumes tend to contract in all directions. Bringing the membranes closer together is difficult at this range and is opposed by the very strong hydration force, described more fully below. Very large negative water potentials can produce the strong suctions needed to overcome this force and, thus, can reduce the intermembrane separation to less than a nanometre or so (Rand and Parsegian, 1989)

The hydration force is a large, repulsive force that can be measured between two hydrophilic surfaces at small separation (e.g. Rand and Parsegian, 1989). The most widely accepted explanation of the origin of the hydration force attributes it to a non-random polarization of water molecules, which decreases exponentially with distance from the surface (Kjellander and Marčelja, 1985a, b). The molecular explanation for these forces is not of central interest to our argument, however. What is important is that they are large, repulsive, decrease rapidly with separation, and begin to have important effects at a range of around 1 nm or so from the surface. The hydration force between membranes has been measured by many researchers, most notably Parsegian, Israelachvili and their colleagues (Marra and Israelachvili, 1985; Rand and Parsegian, 1989), and it has also been measured between DNA molecules in hexagonal arrays (Parsegian *et al*., 1986). Such forces have not yet been measured as a precise function of separation between protein molecules because the less regular geometries of proteins make it difficult to use the geometrical analysis and experimental techniques used by Parsegian *et al*. (1986). The fact that hydration forces have been measured in all hydrophilic systems thus far studied

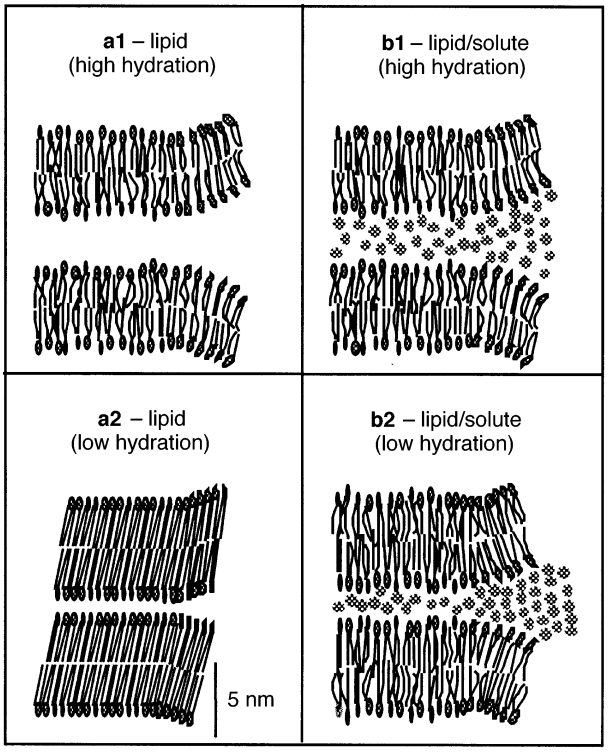


**Figure 2.** The simple thermodynamic effect of small solutes on membrane phase behaviour: (a) a membrane/water system, and (b) a membrane/water/small solute system. The solutes (small shapes between the membranes) represent disaccharides. The lipids and solutes are to scale in two dimensions, and the white space between membranes and around solutes represents water. The discussion assumes a constant temperature. At moderate to high hydration (water potential not much below zero), the membranes are fluid without (a1) or with (b1) solutes present. As the membranes without solutes are dehydrated, water is removed from between the membranes, which causes lateral compressive stress in the membrane plane and transition to a gel phase, as described in the text. This phase has a lower area per lipid molecule and a greater membrane thickness (a2). If, however, solutes are present, their osmotic and volumetric properties mean that at a particular water potential, the hydration will be higher, the separation between membranes will be larger, the lateral stress will be lower, and the membrane may remain in the fluid phase (b2).

strongly suggests that they act among soluble macromolecules and on the hydrophilic surfaces of intrinsic membrane proteins.

At very low water potentials, sufficiently large suctions can overcome the hydration force, but what do these large suctions do to membranes? As long as the solution is a liquid, the suction (negative pressure) is the same in all directions. The suction reduces the volume of intermembrane water. Acting perpendicular to the membranes, it reduces the separation; acting parallel to the membranes, it causes a contraction in the plane of the membrane (Lis *et al*., 1982). This contraction reduces the surface area of the aqueous interface and compresses the membrane components in the plane of the interface. Because the suctions are large, the compressive stresses in the membrane are considerable. Membrane lipids in the gel phase have a lower surface area per molecule than those in the fluid phase, so this compression favours the gel phase and causes the fluid–gel transition to occur at a higher temperature than it would in lipids at full hydration (Evans and Needham, 1987). In other words, if a fully hydrated membrane is in the fluid phase at a particular temperature, as it is dehydrated, the suction causes a compression in the membranes, and the transition to the gel phase may occur without changing the temperature (Evans and Needham, 1987).

The mechanics and thermodynamics of the dehydration-induced stresses and strains in membranes and macromolecules have been analysed and reviewed (Wolfe, 1987; Bryant and Wolfe, 1992; Wolfe and Bryant, 1999). These analyses have been applied effectively to explain the large body of data showing that the fluid–gel phase transition temperature of pure phospholipids, $T_m$, increases gradually during progressive dehydration (Fig. 1) (e.g. Chapman *et al*., 1967; Lynch and Steponkus, 1989; Webb *et al*., 1993; Koster *et al*., 1994, 2000). In brief, the effect of the stress in the plane of the bilayer on the $T_m$ of a phospholipid is determined to first order by a two-dimensional version of the Clausius–Clapeyron equation:

$$\Delta T_m = \frac{T_o \Delta a}{2L} \pi \quad (1)$$

which relates the change in the transition temperature, $\Delta T_m$, to the transition temperature at full hydration ($T_o$), the change in the lipid surface area between fluid and gel phases ($\Delta a$), the enthalpy of the transition ($L$), and the lateral stress in the bilayers ($\pi$) (Bryant and Wolfe, 1992; Wolfe and Bryant, 1999; Koster *et al*., 2000). Thus, increasing the compressive stress in the bilayers by bringing them into close approach causes an increase in the $T_m$ of the phospholipid, as shown graphically in Fig. 1.

## What are the effects of solutes?

When phospholipid bilayers are dehydrated in the presence of small solutes, such as the sugars glucose, sorbitol, sucrose and trehalose, there is less elevation of the fluid–gel transition temperature, $T_m$, than when the lipids are dried in the absence of such solutes (Fig. 1). This has been demonstrated experimentally in numerous studies, including those of Caffrey *et al*. (1988), Crowe and Crowe (1988), Koster *et al*. (1994, 1996, 2000), Zhang and Steponkus (1995) and Crowe *et al*. (1996). The observations that sugars can hinder the dehydration-induced increase in $T_m$ can be explained by understanding the effects of the sugars on the separation between the membranes.

The main effect of small cytoplasmic solutes on membrane phase behaviour is due to simple water relations. Increasing the solute concentration increases the osmotic pressure. A higher osmotic pressure results in a smaller suction (i.e. a less negative pressure) at any given negative water potential, which means that less water is removed from between the membranes. The result is a larger average separation between ultrastructural components and thus a smaller hydration force between the membranes (Fig. 2b). The relationship among interlamellar solute content, water potential and water content is shown qualitatively in Fig. 3A. Solutes that remain between the membrane bilayers during dehydration also have a volumetric effect: the molecular volume of the solutes themselves contributes to the separation of the bilayers at very low water contents and thereby reduces the hydration force between the membranes (Bryant and Wolfe, 1992; Wolfe and Bryant, 1999). Thus, the presence of high concentrations of interlamellar solutes diminishes the stresses and strains in the membranes at low hydrations (Fig. 3B), i.e. the effect that favours the transition to the gel phase is reduced.

Some have misinterpreted the hydration forces explanation proposed by Bryant and Wolfe as suggesting that solutes act to prevent massive shrinkage and shape changes in cells (or liposomes) during dehydration (Crowe *et al*., 1996; Oliver *et al*., 1998). In fact, Bryant and Wolfe (1992) explain that the hydration forces and related effects are encountered only after membranes and macromolecules are brought into close approach by the osmotic shrinkage achieved at very low water contents. Thus, shrinkage and shape changes brought about by dehydration are central to the hydration forces explanation (Bryant and Wolfe, 1992; Wolfe and Bryant, 1999).

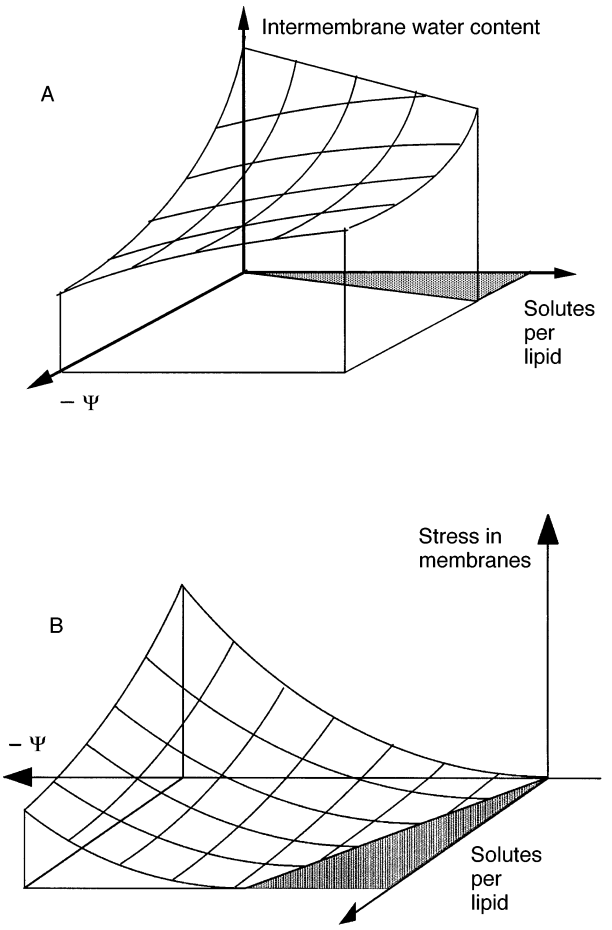


**Figure 3.** (A) The equilibrium composition of a simple model system containing only lipid membranes, small solutes and water. The water content is shown as a function of the water potential, $\Psi$, and the number of solute molecules in the intermembrane spaces. Increasing the amount of solute present increases the osmotic pressure, $\Pi$, of the solution and thus contributes a larger negative component to $\Psi$. If $\Pi + \Psi = 0$, the forces between the membranes are zero and bulk solution may exist in equilibrium with the membrane-solution phase. The shaded area, for which no values of water content are shown, represents $\Pi + \Psi > 0$. At increasingly negative $\Psi$, water is drawn out of the intermembrane spaces, reducing the separation. The presence of solutes small enough to partition into these spaces gives the interlamellar solution an osmotic pressure and thus maintains a higher water content and increased interlamellar spacing than in the absence of such solutes. (B) The membrane stress as a function of water potential and intermembrane solute content. The axes have been rearranged for convenience of viewing. The stress in the membranes is determined by the hydration force and is, therefore, a strong function of the intermembrane separation and, hence, of the water content. These graphs are only qualitative: for explicit calculations, see Wolfe (1987).

## Where does the water replacement hypothesis fit in?

Another suggested mechanism for the stabilization of membranes at low hydration is that certain sugars may replace water around the polar groups of macromolecules and, by hydrogen bonding to them, can stabilize membranes and macromolecules in the absence or near absence of water (Crowe *et al*., 1996; Oliver *et al*., 1998). According to this model, direct interactions between the sugars and polar groups are necessary to maintain the membranes in a physical state similar to their state in excess water (Crowe *et al*., 1996; Oliver *et al*., 1998). So far as we know, there is no controversy over the proposition that solutes interact with membranes or that this interaction increases with solute concentration. All solutes that approach close enough must interact with membranes via van der Waals forces. Ionic solutes will interact via various forms of electrical interaction. Other solutes may interact with membranes via hydrogen bonds. Water interacts with membranes via all of these interactions. In the present context, the interesting questions to ask about the replacement of water (and its own interactions with membranes) by solutes (and their interactions with membranes) are these: Are the solute–membrane interactions specific to special types of solutes, are they important in determining the phase behaviour, and can they explain the observed data quantitatively? We shall return to these questions below.

## What about glass formation?

As an aqueous solution is dehydrated, the concentration of solutes increases and the viscosity increases. If the viscosity rises to ~$10^{14}$ Pa s, then the solution is vitrified and the resulting solid is called a glass. A glass is by definition a non-equilibrium state, so it is possible to have both glassy and non-glassy regions within a heterogeneous sample (Slade and Levine, 1995). Within the glassy domains, the rate of processes that require translational diffusion, such as the glycation of proteins, can be slowed dramatically (e.g. Karmas *et al*., 1992). The diffusion of water from vitrified samples is also slowed but is not prevented. Numerous studies (reviewed by Slade and Levine, 1995) have demonstrated that water molecules, probably due to their low molecular weight and small molecular volume, have greater mobility in a glassy matrix than had been previously predicted. Thus, vitrified samples can still be dehydrated. The presence of a glassy matrix can stabilize dried macromolecules such as proteins (Sun *et al*., 1998). How much of an effect a glass will have on membranes depends strongly on where the glass is located.

Koster *et al*. (1994) noted that when sugar solutions vitrified between fluid phase bilayers during dehydration, $T_m$ of the phospholipid was depressed below $T_o$ (e.g. from $T_o$ at –3°C to a $T_m$ at –25°C for 1-palmitoyl-2-oleoyl-phosphatidylcholine, POPC). Zhang and Steponkus (1996) subsequently confirmed the observation of Koster *et al*. (1994) and proposed that the mechanical resistance of the glass is responsible for the depression of $T_m$ below $T_o$. Because glasses are metastable solids, they can support a considerable mechanical stress, especially over short periods of time. If a glass forms between two fluid phase membranes during dehydration, then the rigidity of the glass makes it more difficult for the membranes to contract in area, as they must do to form a gel phase. As the temperature falls, the glass resists the normal compression in the plane of the membrane, and the lipids remain in the fluid phase below $T_o$ (Zhang and Steponkus, 1996; Koster *et al*., 2000). If the temperature continues to drop, a point is reached where the tendency of the membrane to contract is sufficient to overcome the hindrance of the glass, and the fluid-to-gel transition will occur. Thus, $T_m$ is depressed below $T_o$ by the presence of the intermembrane glass (Fig. 1). In effect, the presence of the interlamellar glass exerts a tension in the plane of the bilayer that, in turn, depresses $T_m$ below $T_o$ (Zhang and Steponkus, 1996; Wolfe and Bryant, 1999; Koster *et al*., 2000). The Clausius–Clapeyron equation given in equation (1) can be used to quantify the lateral tension (–π) in the membrane brought about by the interlamellar glass (Koster *et al*., 2000). Zhang and Steponkus (1996) have also suggested that if the lipid is in the gel phase when the intermembrane layer becomes vitrified, then the rigidity of the glass makes it more difficult for the membrane to expand in area, as it must do to form a fluid phase. In this case, a rise in temperature would cause the membrane to expand in area, putting the glass under tension and the membrane under a lateral compressive stress. In this event, $T_m$ could be elevated above $T_o$.

To summarize, vitrification of the intermembrane layer causes the membranes to tend to remain in the phase they were in at the time of vitrification. Vitrification with the membranes in the fluid phase causes a decrease in $T_m$ below $T_o$, while vitrification with the membranes in the gel phase causes an increase in $T_m$ above $T_o$. This is explained in greater detail by Wolfe and Bryant (1999) and Koster *et al.* (2000).

The proposed role of glass formation by sugar solutions on phospholipid $T_m$ has led to some confusion and even controversy in the literature. One of the reasons for confusion is that different authors have used different transition temperature reference points, which has led to misunderstandings of some of the proposed mechanisms, and these misunderstandings have been propagated in the literature (e.g. Crowe *et al*., 1996, 1998; Oliver *et al*., 1998). For this reason, we now use the following nomenclature: $T_m$ is the fluid–gel transition temperature of the lipid under the measured conditions, and $T_o$, used as a reference, signifies the lipid phase transition temperature at full hydration without solutes present (Wolfe and Bryant, 1999; Koster *et al*., 2000). One misinterpretation about the proposed effects of sugars on phospholipid phase behaviour is that vitrification of sugar solutions was suggested to be responsible for preventing the increase in $T_m$ above $T_o$ during dehydration of membranes (Crowe *et al*., 1996, 1998; Oliver *et al*., 1998). A hypothesis derived from this misinterpretation is that there should be a direct relationship between the $T_g$ of the sugar and its ability to prevent the increase in $T_m$ (Crowe *et al*., 1996, 1998; Oliver *et al*., 1998). In fact, Koster *et al*. (1994) proposed that the limiting of the rise in $T_m$ during dehydration was due to the osmotic and volumetric effects of sugars, and they did not suggest that it was dependent upon vitrification. Thus, no direct relationship between the $T_g$ of a solute and its ability to maintain $T_m$ near $T_o$ was hypothesized (Koster *et al*., 1994). Instead, only the depression of $T_m$ below $T_o$ was suggested to be caused by vitrification of the sugar solution (Koster *et al*., 1994).

An alternative model to explain the depression of $T_m$ below $T_o$ has been suggested by Crowe and coworkers, who argue that the ability of disaccharides, such as trehalose and sucrose, to depress the $T_m$ of dry dipalmitoyl-phosphatidylcholine (DPPC) below $T_o$ does not result from vitrification of these solutes, but rather is a result of their insertion between adjacent lipids in the same bilayer (Crowe *et al*., 1998). According to this model, disaccharides have the proper size needed to spread the lipids apart within the bilayer, and thus, hinder their transition into the gel phase. Crowe *et al*. (1998) state that glucose, a monosaccharide, is unable to depress the $T_m$ of dry DPPC because the small size of the monosaccharide cannot spread the lipids to the extent that the disaccharides can. In contrast, the proposal by Koster *et al*. (1994) suggests that disaccharides depressed the $T_m$ of dry DPPC below $T_o$ because the dried sugar solutions vitrified between fluid bilayers of the phospholipid. Glucose, which does not vitrify at temperatures above $T_o$ for DPPC (42°C), cannot depress $T_m$ for this lipid. The model of Koster *et al*. (1994) predicts, however, that glucose can depress the $T_m$ of other phospholipids below $T_o$, provided that the glucose vitrifies between fluid phase bilayers, e.g. that $T_g$ is above $T_o$ for the lipid. Recent experimental data show that this is indeed the case: vitrified glucose solutions depressed the $T_m$ of dehydrated phospholipids below their respective $T_o$ values, but only when $T_g$ was above $T_o$ for that lipid (Koster *et al*.,

2000). For example, at water contents less than about 0.5 g $H_2O$/g DW, $T_g$ values for glucose solutions were above –5°C, the $T_o$ for OPPC (1-oleoyl-2-palmitoyl-phosphatidylcholine). In these samples, $T_m$ of the phospholipid was depressed to –30°C by the presence of the vitrified glucose solution (Koster *et al*., 2000). Thus, in contrast with the model proposed by Crowe *et al*. (1998), glucose, a monosaccharide, can have an effect qualitatively similar to that of trehalose and other disaccharides: both defer the phase transition of the lipid to a temperature below $T_o$, provided that the solutions vitrify between the lipid bilayers.

## Different solutes

It is important to note that the effects described in the previous section can only occur if vitrification occurs near the membrane surface. If vitrification occurs in the bulk solution, then it will have little effect on the membranes, although it might have effects on other processes in the dry system, such as slowing reaction rates of some chemical reactions (Karmas *et al*., 1992; Slade and Levine, 1995). (Note that the word 'bulk' here means any volume larger than the average distance between hydrophilic surfaces, so volumes larger than about 1–2 nm in all directions can be considered bulk in this context.) Large polymers, for example, are excluded from between membranes and other ultrastructural elements at low hydration (e.g. Rand and Parsegian, 1989). Because they are excluded from the intermembrane space, large polymers will neither prevent the increase in phospholipid $T_m$ above $T_o$ during dehydration, nor will they depress $T_m$ below $T_o$ if they vitrify (Wolfe and Bryant, 1999; Koster *et al*., 2000). The partitioning of large polymers into a bulk solution phase is one of the principles of operation of the osmotic stress technique for dehydrating membranes (Rand and Parsegian, 1989) and can actually result in the elevation of $T_m$ above $T_o$ (e.g. Crowe *et al*., 1996; Koster *et al*., 2000). If large polymers vitrify, they will usually do so in the bulk solution rather than in the interlamellar space, and the glass will have little effect on membrane phase behaviour (Wolfe and Bryant, 1999; Koster *et al*., 2000).

The size of the glass-forming solute is an important consideration that has led to further confusion and controversy about the proposed role of vitrification on membrane stabilization in desiccated systems. Some authors have suggested that the inability of large glass-forming polymers, such as dextran and hydroxyethyl starch, to depress $T_m$ of a dry phospholipid below $T_o$ means that vitrification, *per se*, is not responsible for the observed depression of $T_m$ below $T_o$ when phospholipids are dried in the presence of smaller sugars (Crowe *et al*., 1996, 1998; Oliver *et al*., 1998). We maintain that the size of the solute must be considered in making such assertions, and that vitrification of small solutes that are not excluded from the intermembrane space can have a profound effect on phospholipid $T_m$ that is different from the purely osmotic and volumetric effects of the solutes (Wolfe and Bryant, 1999; Koster *et al*., 2000). Vitrification will have a significant effect on membrane phase behaviour only if the glass forms between the membranes, or between a membrane and other hydrophilic ultrastructural elements. The difference between large and small solutes is illustrated in Fig. 4.

Along with their size, solubility of the solutes is also important. As a solution is dehydrated, it can

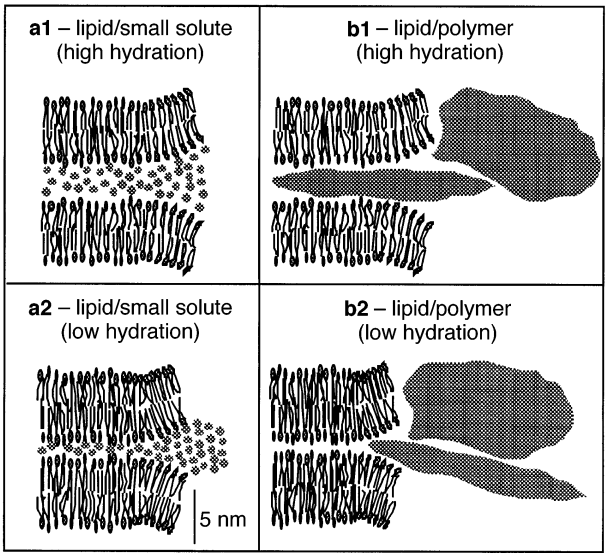


**Figure 4.** Schematic illustration of the partitioning of small and large solutes and the effects of vitrification in membrane-rich systems at low hydration: (a) shows small solutes (the size of disaccharides), and (b) shows large solutes (a polymer with $M_r$ about 40,000). The lipids and solutes are to scale in two dimensions, and the white space between membranes and around solutes represents water. The discussion assumes a constant temperature. At water potentials near zero (high hydrations), both small (a1) and large (b1) solutes can enter between membranes. As the water potential becomes more negative (and hydration therefore decreases), the average separation between the membranes is reduced, and the larger solutes (in this case polymers) are progressively excluded from between the membranes (b2), while the small solutes remain (a2). If the hydration and temperature are low enough, the solute–water mixture may vitrify. If vitrification occurs between fluid membranes (small solutes, a2), the presence of the glass will hinder the transition to the gel phase. In the case of the excluded large solutes, vitrification will have little direct effect on the membranes because vitrification takes place in bulk volumes of solution.

only undergo vitrification if the solutes remain in solution. High concentrations of some solutes may lead to their crystallization. Crystals have a minimum stable size and are not found in regions where the membranes are very close together. When crystallization occurs, vitrification is much less likely, and the osmotic effects of solutes on lipid phase transitions also will be substantially reduced because the crystalline solutes will not be present in the intermembrane space (Caffrey *et al*., 1988; Koster *et al*., 1996).

## Summary of solute effects on membrane phase behaviour

As described above, small solutes have been observed to have two effects on membrane lipid phase transitions in dehydrated systems. First, the presence of solutes between the bilayers hinders their close approach during dehydration and, thus, diminishes the compressive stress that would otherwise force the lipids into the gel phase. In this case, the presence of small solutes keeps $T_m$ from increasing much above $T_o$ during dehydration (e.g. for POPC, $T_m$ stays near –3°C instead of increasing to 61°C; Koster *et al*., 1994, 2000). Secondly, if the solutes should happen to vitrify while between the bilayers, the mechanical resistance of the glass will further hinder transitions between the fluid and gel phases. If the lipids are in the fluid phase when the glass forms, $T_m$ will be depressed below $T_o$ (e.g. for POPC, $T_m$ is depressed to –25°C instead of remaining near –3°C; Koster *et al*., 1994, 2000).

## Specific interactions between sugars and solutes

Are there specific interactions between sugars and lipids and, if so, how important are they in affecting the state of membranes at low hydration? Specific interactions are rather more difficult to discern than general ones. Furthermore, at low hydration, both specific and non-specific interactions are greater, simply because the concentration of solutes and membranes is higher. So the observation of an interaction that increases at low hydration is insufficient to show that it dominates the phase behaviour. Finally, the specific properties of solutes themselves, such as different molecular volumes, and different tendencies to vitrify or crystallize, may be more important than specific interactions with membranes.

That hydrogen bonds form between sugars and the polar groups of membranes and macromolecules in the absence or near absence of water is not surprising, and there is evidence for such interactions (Crowe *et al*., 1984, 1996). The data that support the existence of hydrogen bonds between sugars and membrane phospholipids have been obtained from systems at very low water contents (e.g. <0.02 g $H_2O$/g DW). For many pure phospholipids, desiccation to such low water contents would result in the elevation of $T_m$ by about 30°C above $T_o$, yet the presence of sugars during drying limits the increase in $T_m$ (Koster *et al*., 2000). The idea that hydrogen bonding between sugars and membranes is responsible for maintaining $T_m$ near $T_o$ in the nearly anhydrous system (water contents near 0 g $H_2O$/g DW) does not account for the observed effects of sugars on $T_m$ at intermediate water contents (between approximately 0.05 and 0.2 g $H_2O$/g DW), at which $T_m$ rises in the absence of sugars (Fig. 1). The hydration forces explanation, on the other hand, predicts this gradual increase in $T_m$ in the absence of intermembrane solutes, and effectively explains the mechanism by which the presence of intermembrane solutes limits the rise in $T_m$ (Bryant and Wolfe, 1992; Wolfe and Bryant, 1999).

Specific solute–membrane interactions may exist and may be important in extremely dry systems. However, for the moment, the effects of solutes on the phase behaviour of membranes as a function of hydration over the entire hydration range are consistent with the simple thermodynamic model described here and in Wolfe and Bryant (1999).

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