Freezing, drying and/or vitrification of membrane-solute-water systems.

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Abstract

Membranes are often damaged by freezing and/or dehydration, and this damage may be reduced by solutes. In many cases, these phenomena can be explained by the physical behaviour of membrane-solute-water systems. Both solutes and membranes reduce the freezing temperature of water, although their effects are not simply additive. The dehydration of membranes induces large mechanical stresses in the membranes. These stresses produce a range of physical deformations and changes in the phase behaviour. These membrane stresses and strains are in general reduced by osmotic effects, and possibly other effects of solutes—provided of course that the solutes can approach the membrane in question. Membrane stresses may also be affected by vitrification where this occurs between membranes. Many of the differences among the effects of different solutes can be explained by the differences in the cystallization, vitrification, volumetric, partitioning and permeability properties of the solutes.

Key words

Cryobiology, anhydrobiology, hydration forces, dehydration, vitrification, membranes, phase behaviour, gel-fluid transition, lipids.

Introduction

Membranes are often damaged during the process of freezing and thawing, or during desiccation and rehydration. Indeed rupture of the plasma membrane is one of the most commonly used indicators of cell death. Freezing may also impair activity in biological membranes. Various solutes limit this damage, both in living organisms and in model systems (Steponkus, 1984; Leopold, 1986; Anchodorguy et al, 1987; Hincha, 1989; Sun *et al*, 1996; Shakir and Santarius, 1995; Crowe *et al.*, 1997; Ring and Danks, 1998; Sampedro *et al*, 1998) and these solutes are accumulated by some freezing-tolerant and desiccation tolerant species (Lee, 1989; Ring, 1980; Rojas *et al*, 1986; Waslyk *et al*, 1988; Koster and Lynch, 1992). In this paper we analyse the interactions of membranes with water and solutes at freezing temperatures and/or low hydration. We also consider briefly the hydration of macromolecules in freezing solutions. This paper extends a previous analysis of this topic by the same authors (Bryant and Wolfe, 1992), in the light of research in the last several years, over which time considerable progress has been made in understanding the effects of solutes on membrane hydration and interactions, and on the effects of vitrification. The approach taken is to give physical explanations and illustrations in the text, with the mathematical and formal thermodynamical detail relegated to appendices. The first half of the paper concentrates mainly on the physical principles involved and the second half on the effects of solutes on membrane properties at freezing temperatures.

In discussing solutes, we can loosely divide them into three broad categories: salts (small, charged), sugars and other medium sized related molecules (usually uncharged), and macromolecules. Some of the effects we discuss apply to all solute types (e.g. they all occupy volume). Others may differ among groups (e.g. macromolecules are less likely to permeate membranes and to partition into lamellar phases). Most of our discussion concerns sugars and macromolecules, and the electrical effects of ionic solutes are discussed here only briefly.

The cooling of cells can be divided into slow and fast cooling by comparing the times for thermal and hydraulic equilibrium. The cooling rate that marks this division varies considerably among cells of different type and size. In the natural environment, cooling rates are slow and so the distribution of water often has the time to approach equilibrium. In this paper we consider processes and phenomena that are relevant mainly to slow cooling. Extracellular freezing usually occurs before intracellular freezing (discussed later). The formation of extracellular ice concentrates the extracellular solutes. This elevates the extracellular osmotic pressure and thus causes water to leave the cell osmotically. Water contents of order 10% or less are possible. Thus slow freezing and desiccation in an atmosphere with low humidity have many features in common.

In some cases, equilibrium thermodynamics allows the calculation of the mechanical stresses to which membranes are exposed and the way in which solutes affect these. We shall therefore begin by considering the equilibrium thermodynamics of solutions, of membrane-water phases, and membrane-solute-water phases. For simplicity we shall discuss lipid bilayer membranes, although we anticipate that much of the discussion will be applicable to other hydrophilic membranes as well. Non-equilibrium effects, especially vitrification, are discussed later.

From models and theories to living cells

Experimental investigations of these thermodynamic and mechanical effects have often been conducted on model systems comprising only several different chemical components, but in which the composition is both known and controlled. This also facilitates theoretical analysis. Much of this review concerns such simple systems. Caution should of course be exercised when comparing phenomena in a lamellar phase of lipid membranes or a regular hexagonal array of macromolecules with those occurring in biological cells. Nevertheless, in the case of freezing-induced dehydration, the analogy is relatively strong. When the water content of a cell falls to say 10 or 20%, all of the non-aqueous components are crushed very close together. Electron micrographs of such freeze dehydrated cells show stacks of membranes which closely resemble lamellar phases, and sometimes hexagonal II phases (Gordon-Kamm and Steponkus, 1984; Steponkus, 1993; Steponkus *et al.*, 1993, Uemura *et al.*, 1995). (It is also likely that a cell with 10% water content also contains regions of closely packed macromolecules, although this would be harder to recognize in electron microscopy.) Further, electron microscopy of freeze-dehydrated cells shows some interesting topological features that are correlated with damage (Steponkus and Webb, 1992; Fujikawa, 1995). These features, which we discuss in more detail later, are found in the membrane-rich regions or where membranes are close together. In some cases the regions appear to lack intra-membrane particles and so may be plausibly modelled by stacks of bilayers in a lamellar phase. Macromolecules are often excluded from dehydrated lamellar phases (Lis et al, 1982; Bryant and Wolfe, 1989) so it is reasonable to expect that a dehydrated cell contains membrane-rich domains and macromolecule-rich domains.

Moreover, many of the thermodynamic and mechanical effects discussed in this paper would be expected in any system comprising nanometer-sized hydrophilic objects in aqueous solution. As we shall show (Appendix 4), different geometries give similar equations, differing chiefly in numerical factors. The complicated geometries and usually unknown composition of cell components means that, while quantitative estimates may be difficult, the qualitative behaviour should be similar. The laws of thermal physics and those of Newton may be difficult to apply quantitatively to cells, but there is no reason to expect that they are violated.

Phase equilibria of water, solute, membrane combinations

It is worth reviewing freezing and the effect of solutes on freezing, so that we can compare this with the effect of membranes.

Freezing point depression due to solutes. Freezing represents a balance between the lower enthalpy of the solid phase and the higher entropy of the liquid phase[1]. An equilibrium phase transition occurs at a temperature T where

$$T\Delta S = L$$

where L is the latent heat of fusion and ΔS is the change in entropy at fusion. The presence of solutes in liquid water increases the entropy of the water molecules. When ice forms, its crystalline structure excludes almost all solutes, so that ice is an almost pure, single component phase. As a result, the entropy of the ice is almost unchanged by the presence of solutes in an ice-solution sample. On the other side of the equation, the presence of solutes makes little difference to the latent heat of fusion. In the presence of solutes, ΔS is larger so the equilibrium freezing temperature T is lower. (Some of the thermodynamics for this section is developed in Appendix 1.)

This familiar result—commonly called freezing point depression—is usually plotted as freezing temperature T *vs* solute concentration C (Fig 1a). For the purposes of comparison with membrane hydration and for cryobiology, however, it is helpful to consider T as the independent variable. It is also helpful for the comparison to represent the composition of the solution as the hydration of the solute, i.e. the mole ratio of water to solute, rather than the concentration. This is shown in Fig 1b. At low (solute) concentrations, the hydration of the solute is approximately proportional to the reciprocal of the concentration, so the nearly linear region in Fig 1a approximates a hyperbola in Fig 1b. The data in Fig 1a are standard data for sucrose (Weast, 1990). The solid line in 1b shows the behaviour of a hypothetical sample whose total composition has 80 water molecules for each sucrose molecule. This composition has a freezing point of about –1.5 °C so, above this temperature, the sample is a single, homogeneous solution phase. This is indicated by the horizontal line. At lower temperatures, the equilibrium condition for this sample comprises a pure ice phase in equilibrium with a solution whose composition is given by the curve. This solution phase contains all of the solute and a quantity of liquid water, which decreases as the temperature falls. Samples with different total composition would be represented by different horizontal lines above freezing, but all follow the same curve below freezing.

Freezing point depression due to membranes. A standard way of representing the colligative or hydration properties of a lamellar phase is a plot of the inter-lamellar force per unit area as a function of hydration or inter-lamellar spacing (Fig 1c). We return to this representation later but, for the purposes of this comparison, we shall first consider the hydration of a lipid lamellar phase as a function of temperature (1d). At high hydrations (more than about thirty waters per lipid) and above freezing temperatures, lipid-water suspensions separate into two different phases: a lamellar phase with about thirty waters per lipid, and a bulk phase of nearly pure water. At lower hydrations and/or freezing temperatures, however, there is no excess water phase: just a single lamellar phase. When a highly hydrated sample is frozen, the bulk water freezes and the lamellar phase begins to dehydrate, so it is sufficient here to consider low hydration phases. Nuclear magnetic resonance (NMR) can be used to measure the amount of liquid water present as a function of temperature (Yan et al, 1993). Fig 1d shows the equilibrium hydration of lamellar phases of DOPC at freezing temperatures. The data in 1d are for three samples having different total compositions: these compositions are each shown by points on a horizontal line. (These points are measured above the equilibrium freezing temperature for each sample.) Below these temperatures, ice and water coexist. The NMR signal from the liquid water gives information about the size and geometry of the contributing volumes. These signals indicate that the water lies between the lamellæ and is in a condition similar to that of water in lamellar phases at low hydration when no ice is present. There are several other reasons to believe that the ice forms a separate, macroscopic phase and that there is no ice in the narrow spaces between adjacent pairs of lamellæ. First, at any given freezing temperature, there is a minimum size for ice crystals below which they are unstable with respect to water and there is insufficient space for stable ice between lamellæ at the average separation (see Appendix 2). Second, the hydration curves for samples with different initial hydrations superpose very closely in Fig 1d, which suggests that the these lamellar phases have the same composition at the same freezing temperatures. Finally, the curves in Fig 1d closely resemble the hydration behaviour of lamellar phases in the absence of ice, as

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[1] Liquid water has a higher internal energy (U) than does ice: the latent heat of fusion (L) is just the difference in internal energy per unit mass. But liquid water also has higher entropy (S) than does ice, because its molecules can translate and rotate more freely. The entropy is more important at high temperatures: expressions for the Gibbs and Helmholtz free energies include the term U – TS.

measured in diverse ways, as we shall see next. This behaviour is usually represented in a rather different form, as shown in 1c, in which a repulsive force per unit area is measured as a function of the hydration or the inter-lamellar separation.

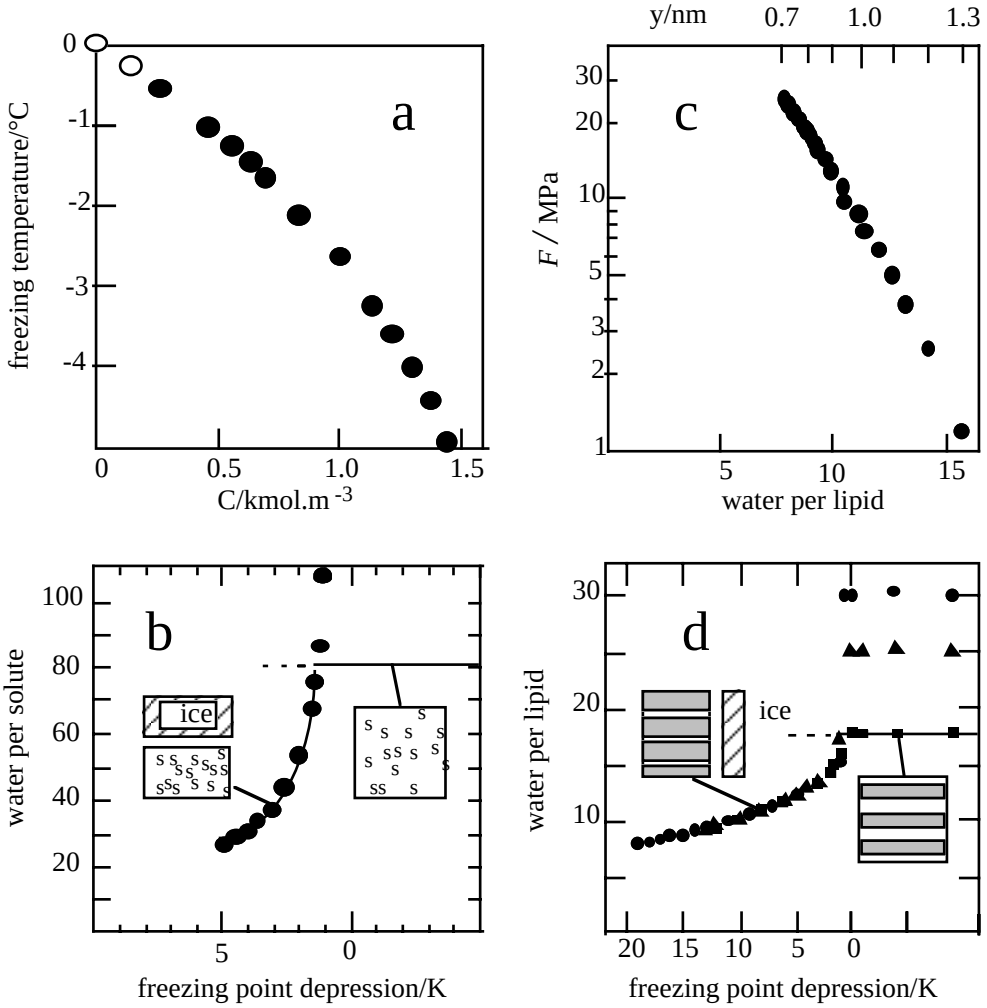

Figure 1. (a) shows the equilibrium freezing temperature as a function of concentration for a solution of sucrose (Weast, 1990). In (b), the same data are plotted to show the composition of an unfrozen sucrose solution (expressed as the mole ratio solvent:solute) as a function of temperature. (The two unshaded points in (a) are omitted in (b).) The solid line represents the water:sucrose ratio in a sample whose total composition has a mole ratio of 80:1. Such a sample is a single, homogeneous phase above about –1.5 °C. Below that temperature, ice and solution coexist, as shown by the cartoon insets, in which *s* represents a solute molecule and white represents water. In (c), the hydration properties of a DOPC lamellar phase are shown as the inter-lamellar force per unit area as a function of mole ratio water:lipid or inter-lamellar separation *y* (Yoon et al, 1998). On this semi-log plot, the data are approximately linear, suggesting an exponential force law at small inter-lamellar separations. The hydration behaviour of most lipids are qualitatively similar (though quantitatively different), so one would expect qualitatively similar results from other lipids that did not undergo a phase transition in the temperature range investigated. The data in (c) are the same as those also plotted in (d). Here they are plotted as the composition of a lamellar phase of DOPC:D_2O as a function of temperature. At sufficiently low temperatures, an ice phase coexists with the dehydrated lamellar phase, as shown in the cartoon inset, in which shaded bars represent the bilayers. At higher temperatures, there is no ice and, for any given sample, the hydration does not change with temperature. The different symbols represent samples with three different total compositions: mole ratios 30 (●), 25 (▲) and 17.7 (■). For both solutions and lamellar phases, supercooling is possible. For the samples whose equilibrium behaviour is shown by the solid line in (b) and (d), supercooling is represented by the dashed horizontal lines to the left of the equilibrium curves.

Hydration and hydration forces

When surfaces in water are brought to close separations (a few tenths of a nanometer), a very large repulsive force, called the hydration force, is measured. Hydration forces have been investigated using a number of different and complementary methods. The origin of the hydration force is still not unanimously accepted. Some researchers attribute it to normal motion of the surface, either individual

molecular motion or surface undulations (Israelachvili and Wennerström, 1996). A more widely held view is that it is due to ordering of water at the surface, which propagates out from the surface with decreasing strength (Kjellander and Marčelja, 1985a,b). For the purposes of this discussion, the nature of the force is not of fundamental concern (but see the discussion by Bryant and Wolfe, 1992).

Force-separation curves between bilayers and other surfaces may be measured directly using a technique developed by Israelachvili and co-workers (Israelachvili and Adams, 1978; Horn, 1984; Helm et al, 1989). In the Surface forces apparatus (SFA) the deflection of a calibrated spring measures the force and sophisticated optical interference methods are used to measure the changes in separation of the atomically smooth surfaces upon which the lamellæ are deposited (Fig 2a).

In the Osmotic Stress Technique (OST) of Rand, Parsegian and colleagues (LeNeveu et al, 1976; Rand and Parsegian, 1989), the force between bilayers is determined thermodynamically by equilibrating the water in the phase to be studied with a reference aqueous phase. Depending on the range of hydration to be studied one of three methods is used to control the chemical potential of the reference phase. For modest dehydrations, pressure is applied hydraulically through a membrane. For moderate dehydrations large molecular weight polymers are introduced into the lipid/water mixture. As these do not permeate the membranes, they remain in a separate water-polmer phase, thus dehydrating the membranes. For most moderate to severe dehydrations, a series of saturated solutions is used to control the vapour pressure, which is used to control the hydration of lipid-water samples. Each lamellar phase sample is equilibrated with an unsaturated water vapour, which in turn is equilibrated with one of a series of reference solutions (Fig 2b). The chemical potential of water (μ) is known for each of the reference solutions, and at equilibrium it equals the chemical potential of water in the lamellar phase. In a solution, μ is lower than it is in a pure water phase at zero pressure because of the osmotic effect of the solutes, which lower the entropy of the water. The water between the lamellæ contains no solutes, but its chemical potential can be lowered by lowering the hydrostatic pressure in this region, so a negative pressure or suction is developed in the inter-lamellar water. For mechanical equilibrium, the magnitude of the suction equals the repulsive force per unit area between the lamellæ. As the lamellæ approach closer, the repulsive force can be very large (tens of MPa) and it requires successively lower chemical potentials of water to draw water out of the inter-lamellar regions[2]. The repeat spacing and the separation may be measured by X-ray diffraction to give force-distance curves. At close approach, the hydration force dominates other forces (the attractive van der Waals interaction, electrostatic interactions) and the force depends approximately exponentially on separation, with a characteristic length of about 0.2 nm, as shown in Fig 1c. The two methods (Figs 2a and 2b) are quite different and the constraints upon the bilayers are different. Nevertheless, the force curves measured are qualitatively similar, and may be quantitatively reconciled (Horn et al, 1988). In a variant on this method, the hydration, rather than the separation, is measured by weighing the sample (Marsh, 1989) to give force-hydration relations. Knowledge of the bilayer geometry and mechanical properties allows comparison of force-distance and force-hydration curves (Appendix 3).

Hydration force behaviour can also be studied using freezing, as is shown in Fig 2c (henceforth Freezing Stress Technique or FST). Consider first the case when there are no solutes present. When a lamellar phase equilibrates with a macroscopic phase of pure ice, the chemical potential of the ice depends on its temperature—in fact it decreases approximately linearly with temperature. As the temperature falls, the chemical potential of the inter-lamellar water also falls, again by supporting an increasingly negative hydrostatic pressure. Again the magnitude of this suction must equal the repulsive force per unit area, and so the force between the lamellæ may be calculated directly from the temperature (Appendix 1). The hydration may be measured directly by NMR, as described above, to give force-hydration relations. Fig 1c shows the data from Fig 1d replotted in this way. These measurements also give an approximately exponential force law with parameters similar to those determined by the two other methods (Yoon et al, 1998).

We note in passing that the OST has also been applied to determine force-separation relations for other geometries. Parsegian et al (1986) have measured the hydration repulsion and other forces in

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[2] Despite these very large suctions, cavitation is highly improbable. This is because the surfaces are very hydrophilic and the separations are smaller than the critical diameter for cavitation (Appendix 2).

hexagonal arrays of DNA. In principle the OST and the FST may be used to determine force-hydration relations for a variety of ultrastructural elements, provided that their geometries are known.

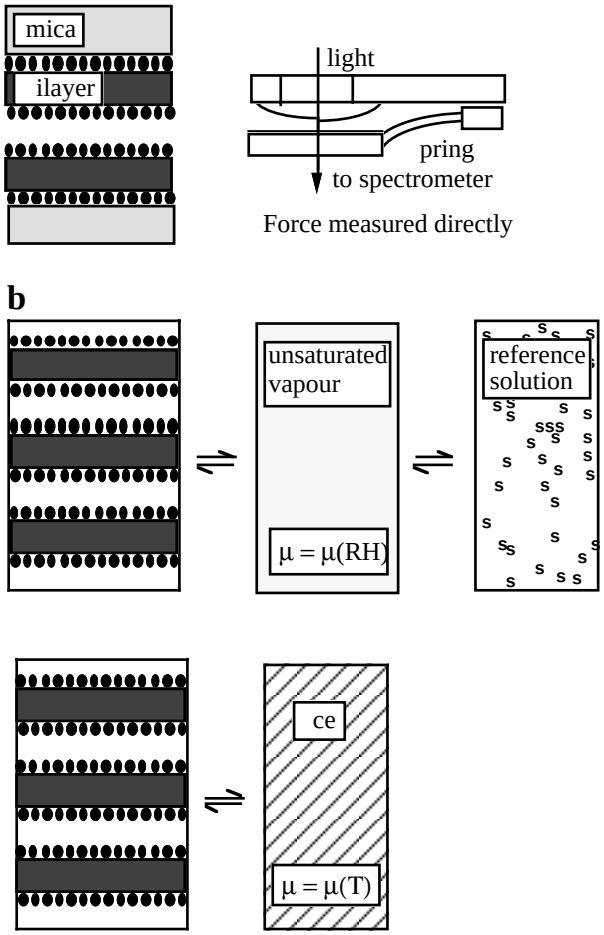

Fig 2. Measuring lipid hydration and inter-lamellar forces. In the Surface Forces Apparatus (a), the inter-lamellar force is measured directly and changes in the separation of the supporting surfaces are measured optically. In the Osmotic Stress Technique (b), the force is determined from the equilibrium of the inter-lamellar water, a vapour phase and a saturated solution. The inter-lamellar separation is determined from X-ray diffraction or the hydration is determined gravimetrically. In the Freezing Stress Technique (c), the force is determined from the equilibrium of the unfrozen inter-lamellar water with ice at known temperature. The inter-lamellar water content is determined from its NMR signal.

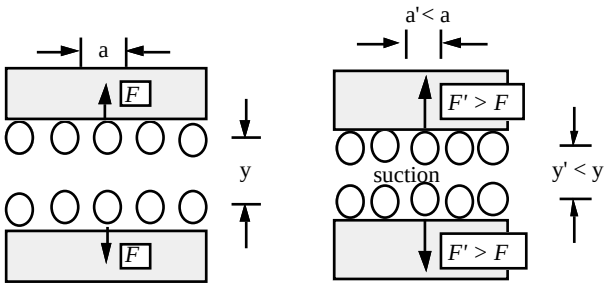

Fig 3. *y* is the separation between the density weighted lipid-water interfaces and *a* is the area per lipid in (one side of) the lamella. The volume of water per lipid is *ay*/2. Removal of water from the inter-lamellar layer could produce reductions in either a or y. If the lamellæ were infinitely rigid, only *y* would be reduced by a reduction in water volume. If the hydration repulsion were an infinite step function, then only *a* would be reduced. In practice, both are reduced (Appendix 3). Reductions in *y* are balanced by an increasingly large hydration repulsion between the lamellæ. Reductions in *a* produce increasingly large lateral compressive stress in the lamellæ.

In the absence of solutes, the inter-lamellar layer is expected to remain fluid at quite low temperatures and separations. Consider the forces acting in the lamellar phase. In the direction normal to the bilayers, the suction in this layer is balanced by the hydration repulsion. In the lateral direction, it acts to compress the lamellæ and produce a compressive stress[3] in them[4] (Wolfe, 1987). This is illustrated in Figure 3. Note that this lateral stress can be produced by desiccation in equilibrium with an unsaturated atmosphere (*cf* Fig 2b) or by freezing induced dehydration (*cf* Fig 2c). Consequently, much of the following discussion has relevance to both cryobiology and anhydrobiology.

Stresses and strains in membranes

These intra-membrane stresses produce several strains and other responses: geometrical strains, topological strains, thermotropic changes and spontaneous demixing. They are illustrated in Fig 4. Some of these have been associated with membrane damage in freezing or dehydration of living cells or model systems.

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[3] In this paper, "stress" is used in its strict physical sense: a force per unit area. "Strain" means a deformation produced by the stress. The words "stress" and "strain" are often used metaphorically in cryobiology.

[4] The compressive stress could be considered as a force per unit area acting at a point in any surface perpendicular to its plane. Integrating this three dimensional stress across the membrane thickness gives a lateral force per unit length which we call lateral pressure or lateral stress π.

The geometric strain of a membrane is the simplest. If a membrane at initially high hydration (Fig 4a) is dehydrated (Fig 4b), a compressive lateral stress is associated with a reduction in area per molecule. For small changes, the two are proportional and the constant of proportionality is called the area elastic modulus. This has been measured for lipid bilayers, and for animal and plant membranes using micropipette aspiration (Mitchison and Swann, 1954; Wolfe and Steponkus, 1983; Evans and Needham, 1987). Because the lamellæ have very low volumetric compressibilities, a fractional reduction in area is associated with a nearly equal fractional increase in thickness. This has been measured by X-ray diffraction (Lis et al, 1982).

The most noticeable effect of lateral stress is on the gel-fluid (also known as gel-liquid crystal) transition in a planar bilayer (4c). Dehydration elevates the transition temperatures for lipid-water phases as much as 40 °C above the excess water transition temperature T_O. This effect has been observed by many investigators for a wide range of lipids (e.g. Crowe et al. 1988; Tsvetkov et al., 1989; Koster et al. 1994; and references contained in these papers). The effect is readily explained in terms of a two dimensional version of the Clausius-Clapeyron effect (Bryant and Wolfe, 1992). When the bilayer goes from gel to fluid, its area in the plane increases by an amount Δa per molecule. In a dehydrated phase, this occurs against a lateral pressure π in the bilayer, or $\pi/2$ in each monolayer, so it incurs an extra energy cost of $\pi\Delta a/2$. This makes the gel phase more stable with respect to the fluid, and so the transition temperature is elevated. The two dimensional version of the Clausius-Clapeyron equation may be written:

$$\Delta T = \frac{T_o \Delta a}{2L} \pi \qquad (1)$$

where ΔT is the increase in the transition temperature due to a lateral stress π, L is the latent heat of the transition, and $\Delta a = (a_f - a_g)$ is the difference in molecular areas between the gel (g) and fluid (f) phases. Thus the transition temperature T_o is increased in proportion to the lateral pressure applied, at least for small applied stresses. Taking values (for DPPC) of L ~ 5 10^{-20} J.molecule^{-1} and Δa ~ 0.15 nm^2, the transition temperature is elevated by ~ 0.5 K for each mN.m^{-1} of applied lateral stress[5]. (For membranes under a tensile stress, Eqn (1) gives the depression of the transition temperature. Tensile stresses are possible when a vitrified inter-lamellar solution supports the compressive stress, as we shall discuss later.)

Another deformation produced by lateral stresses is lateral demixing in membranes of more than one component. If a membrane includes components that differ sufficiently greatly in their hydration interaction, then in some regions of the hydration-temperature phase diagram, they separate into two fluid phases with different compositions (Bryant and Wolfe, 1989). This has been observed in two component lipid bilayers (Bryant et al, 1992; Webb *et al*, 1993) (Fig 4d). It can also explain the exclusion of proteins from areas of fluid mosaic membranes under suitable conditions (Fig 4e), although other explanations are also possible. The possible significance of this demixing is discussed below.

Apart from the geometric deformation shown in Fig 4b,c and d, there is another way in which the aqueous volume can be reduced: via a discontinuous change in the shape of the interface. Hexagonal II phases (inverse hexagonal phases) have tubes of water surrounded by lipids, as shown in Fig 4f. For inverse cubic phases, approximately spherical volumes of water are surrounded by lipids[6]. The geometry of these inverse phases is ill suited to perform the role of semipermeable separation, which is an important function of membranes, and so it is not surprising that observation of these phases, or ultrastructural features resembling them, has been associated with damage at low hydration (Gordon-Kamm and Steponkus, 1984; Steponkus, 1993; Steponkus *et al.*, 1993, Uemura *et al.*, 1995). Several further topological changes have also been reported in plant cell membranes that are brought closely together during freezing, and these have been related to freezing damage (Steponkus and Webb, 1992; Fujikawa, 1995).

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5 Phase diagrams in terms of T, π, and composition are given by Guldbrand et al (1982) and Marcelja and Wolfe (1979).(46) and (18).

6 In some cases, membrane surfaces may have a spontaneous curvature and this transition may also lower the mechanical energy. This is analysed by Kirk et al, 1984 and Gruner et al, 1985.

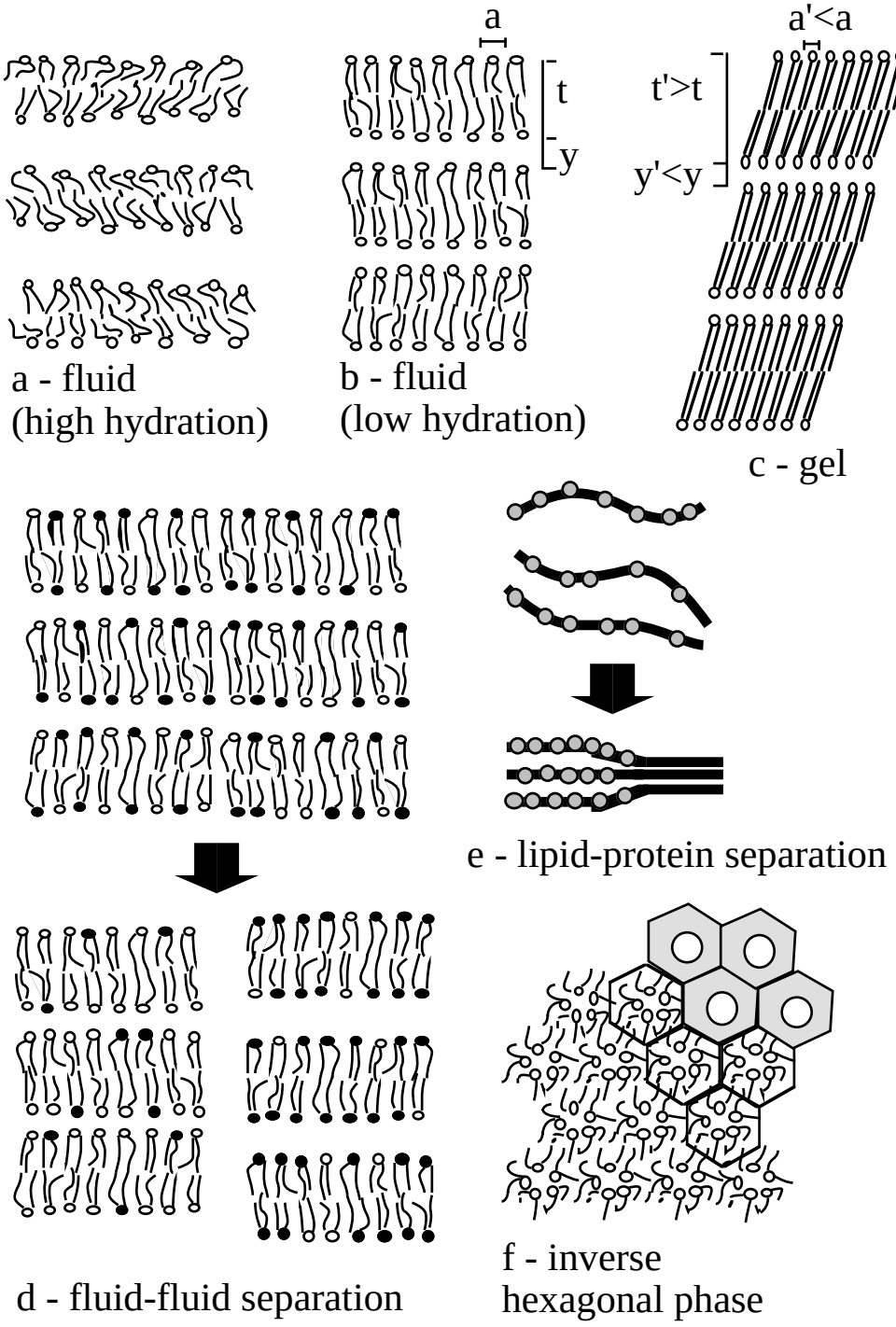

Fig 4. The strains produced by dehydration-induced stresses. (a) shows a lamellar fluid phase (L_α) at high hydration. (b) shows the geometric strain produced at lower water content. The average area per lipid *a* and the inter-lamellar separation *y* are decreased, while the lamellar thickness *t* of the bilayers is increased. At lower water contents, increased lateral stress (see text) can produce the transition to the gel phase (L_β (with straight chains) or $L_{\beta'}$ (with chains at a fixed angle, as shown)). In the gel phase a'<a, t'>t and y'<y. Dehydration stress produces this transition at elevated temperatures (equation 1). In figure 4d the shaded circles represent the lipid species with the greater hydration, and the unshaded circles represent the lipid with the lower hydration. At high hydrations the two lipids form a single mixed phase (4d, upper figure), but as hydration is reduced, they separate into two separate phases. The lipid with the greater hydration is preferentially sequestered in domains with relatively high hydration (4d, bottom right), while the less strongy hydrating lipids are concentrated in domains with lower hydration (4d bottom left)(,(e) large hydrophilic molecules, such as intrinsic membrane proteins (circles in this diagram), have a larger hydration interaction and can therefore be demixed by dehydration stresses (lower diagram). (f) shows a topological response to stress. At very low hydrations the lipids may undergo a transition to the hexagonal II phase (H_{II}), which consists of small cylinders of water surrounded by lipids. At the top of the diagram, lipids are represented by the shaded area and water by the unshaded. The hexagons are the repeat units of the structure. In the lower part of the diagram, individual lipid molecules are represented.

In biological membranes, most of the lipids are strongly hydrating lipids that do not readily undergo transitions to non-bilayer phases. However, some membrane components are less strongly hydrating. Even relatively small fractions of the weakly hydrating species may however be important, because the fluid-fluid demixing that results from dehydration stresses (discussed above—see figure 4d) produces domains rich in the low hydrating component. These domains may then undergo a transition to a hexagonal II phase (figure 4f). The demixing may thus be an intermediate stage prior to formation of damaging inverse phases (Bryant and Wolfe, 1989, Bryant et al 1992, Webb et al., 1993, 1995).

Effects of solutes

Solute partitioning. Membranes are poorly permeable to many solutes, especially when the solute molecules are large. It follows that solutes may not always equilibrate between phases, and that the composition of the phases of a sample with a particular overall composition may depend on the history of its preparation.

As a simple example, consider a suspension of multi-lamellar[7] vesicles in pure water, in the presence of excess water. Water permeates easily and so the lamellæ approach full hydration. Now add to the sample a non-permeating non-ionic solute. It is distributed (at least initially) in the bulk water phase. The osmotic pressure of the bulk solution now dehydrates the multi-lamellar vesicles. The extent of the dehydration is determined by the repulsive forces between the lamellæ. The greater the solute concentration in the bulk, the greater the inter-membrane repulsion and so the greater the intra-membrane stress. In this case we would expect lateral compression of the membrane, elevation of the membrane liquid crystal-gel transition temperature (T_m) and perhaps other strains if the bulk solution were sufficiently concentrated. Compare this sample with one of the same overall composition, but in which the solute partitions between the bulk and inter-lamellar water (by one of the means discussed below), until both solute and water reach equilibrium. Here there is no osmotic pressure difference, little or no dehydration of the lamellar phase and little or no lateral stress. Non-specific solute effects would produce little or no change in T_m at temperatures above the freezing temperature of the solution.

Freezing the sample further complicates the picture because it changes the solution compositions, and can do so quite rapidly. Yoon et al (1998) reported experiments that compared lipid:sugar:water samples having similar lipid:sugar ratios, but different levels of initial hydration. When these were frozen, the lamellar phase was dehydrated and the water equilibrated between that phase and a bulk solution phase. The number of solutes in the lamellar phase was higher in samples whose initial hydration was low, simply because there was no (or less) excess solution. As a result, the samples with low initial hydration produced lamellar phases with higher hydration at a given freezing temperature.

Fig 5 illustrates this point for a hypothetical solute to which the membrane is completely impermeable. In case a, anhydrous lipids are hydrated with a relatively small amount of a highly concentrated solution. This is likely to produce a lamellar phase with a highly concentrated solution in the inter-lamellar spaces. It is possible that there is also a macroscopic solution phase whose concentration is not necessarily the same as that of the the inter-lamellar solution. In case b, the same amount of anhydrous lipid is hydrated by adding a large volume of dilute solution, which contains (for the purposes of this illustration) the same amount of solute. This will form a lamellar phase with a dilute inter-lamellar solution and a large volume of bulk solution. The second sample is then either frozen or dehydrated in an unsaturated atmosphere until (let us suppose) it has the same total water content as the first sample. If the solute fails to permeate the membrane, then the final result may be that the second sample comprises a lamellar phase containing less solute (and also less water) than the first sample, and a larger bulk volume of concentrated solution. If the dehydration were sufficient, it might also contain crystals of the solute. A range of procedures may be applied to the second sample to increase the solute content of the inter-lamellar solution: repeated centrifugation with regular alternation of the orientation of the sample, repeated cycles of freezing and thawing, and

[7] A somewhat similar result may occur with unilamellar vesicles. If the vesicle radius is much greater than the membrane thickness, adding an impermeant solute to the suspending medium will cause the vesicles to collapse until the membrane separation is determined by the hydration force. At low hydration, regions of flattened vesicles may resemble–and respond like–lamellar phases.

the elapse of time (weeks or more—P. Rand, personal communication). Nevertheless, it would be unwise to assume that, even after this treatment, the composition of the inter-lamellar phase and the bulk phase were the same. First, the solute may still not have reached equilibrium between the two phases. Second, the equilibrium may not correspond to equal concentrations. Some solutes may be preferentially distributed into the lamellar phase, others excluded from it. The purpose of this illustration is to warn that sample preparation and history must be considered in any comparison of data, and that the composition of the lamellar phase component of a lipid-solute-water sample is not readily determined from the total composition of the sample.

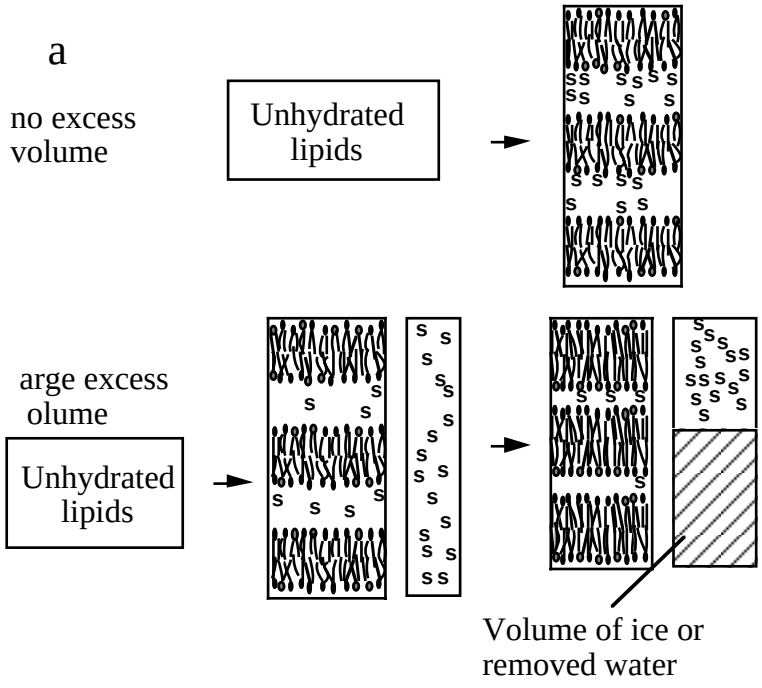

Fig 5. The effect of different initial hydrations on solute redistribution. (a) shows a sample, which is hydrated from the dry state with a small volume of a concentrated solution of solutes. There is no excess water, and so the solutes and water are all or nearly all in the inter-lamellar region (assuming no solute crystallization). (b) shows lipids being hydrated from the dry state with the same ratio of solute to lipid, but enough water to create a substantial excess volume. Upon removing some of the water, so that the total water volume is the same as in (a), many of the solutes concentrate outside the lamellar region. Both the number of solutes per lipid in between the membranes, and the inter-lamellar separation y, are lower in case (b) than (a). NMR of the solute or the water can be used to determine the distribution (Yoon et al, 1998).

Specific *vs* non-specific effects. Solutes affect the hydration of membranes, hydration forces, membrane-membrane interactions and intra-membrane stresses in a number of ways. The interactions of solutes with water and with membranes may be specific to particular solutes. There are also, however, some important effects that are non-specific, in the sense that any solute (or any solute of similar size) would have a similar effect.

Osmotic effects. All solutes have an osmotic effect: they increase the entropy and lower the chemical potential of the water in which they are dissolved. Consider first a lamellar phase containing no solutes, in equilibrium with ice at –1 °C. The ice and the lamellar phase compete for water, with the result that the pressure in the inter-lamellar water is – 1.2 MPa and so the inter-lamellar repulsion is 1.2 $MN.m^{-2}$ (Appendix 1). Typically this gives rise to a lateral stress in the lamellæ of order 1 $mN.m^{-1}$, although the value depends on the type of lipid. Now consider a system with solutes *in the interlamellar fluid*. Ice at –1 °C can equilibrate with a solution having zero hydrostatic pressure and a solute concentration of about 500 $mol.m^{-3}$ (for a non-dissociating solute). One might expect that a solute concentration of this order in the inter-lamellar water would reduce the suction, the intermembrane repulsion and the lateral stress to zero, and increase the inter-lamellar separation. In practice, the osmotic effect of inter-lamellar solutes is a little more complicated for several reasons (Yoon et al, 1998). The size of solute molecules is not negligible in comparison with the inter-lamellar separation, so the excluded volume near the interfaces must be considered. Due to this effect, a solute has a greater osmotic effect in a confined space than it would in bulk solution, and the effect increases somewhat for large solutes. There may also be variations in solute distribution within the inter-lamellar layer due to interactions between the solute and the lamellæ. In short, the purely osmotic effect of the presence of solutes in a fluid inter-lamellar layer is to increase the hydration, to decrease the intra-membrane stress and thus to reduce the dehydration induced increase in the gel-fluid transition temperature, but the effect is somewhat less than predicted by the simplest model.

The foregoing discussion concerns the osmotic effect of solutes which have partitioned into the interlamellar space. If solutes are too large to partition into the interlamellar layer, or if the sample preparation and their impermeability has kept the solutes out of that space, then their osmotic effects are indirect. They can have an effect on hydration because they affect the chemical potential of water, but this is most important at temperatures above freeing. Consider, for example, a suspension of unilamellar vesicles in pure water, to which is added a non-permeating solute. First consider temperatures above freezing: Water leaves the vesicles, which then shrink until further dehydration is prevented by the hydration force when membranes are pushed close together. When the water is at equilibrium, the osmotic pressure of the solution equals the suction in the interlamellar water. In this case, the excluded solute produces an intra-membrane force and dehydration-induced intra-membrane stresses at temperatures above freezing. In the presence of ice, the behaviour of the lamellar phase is largely unaffected by the presence of these solutes, which are sequestered in a co-existing concentrated solution phase.

Effects on the hydration force. The presence of the solute (in high concentration) may be expected to affect the hydration force. Many researchers believe the hydration force to be due to the non-random orientation of water propagating from the interface (Kjellander and Marčelja, 1985a,b). Solutes do not hydrogen bond in the same geometry as water, and the solute has a different (usually lower) polarisability. One might therefore expect all solutes, at sufficiently high volume fraction, to reduce the hydration repulsion and thus the intra-lamellar stress. At equal concentration, a solute with a larger volume would be expected to have a larger effect, all else equal.

Volumetric effects. The volume of solutes (when not negligible in comparison with the volume of water) itself increases the volume of the inter-lamellar solution. If this layer of solution has negative pressure, that suction now acts on a greater thickness of solution, and this increases the lateral stress. Except for large volume fractions, this effect is relatively small (Wolfe and Bryant, 1992). The molecular volume also affects the steric interaction with membranes, discussed below. Finally, one of the most important effects of molecular size is that larger molecules are more likely to be excluded from the inter-lamellar layer.

Interactions among solutes, membranes and water. After estimating and allowing for the osmotic and volumetric effects, Yoon et al (1998) reported that the disaccharides sucrose and trehalose, at concentrations of several $kmol.m^{-3}$, reduced the hydration force between dioleylphosphatidylcholine bilayers to a greater extent than did the smaller solutes sorbitol and dimethylsulphoxide. The effects of sorbitol and dimethylsulphoxide on the inter-lamellar repulsion were very similar to what these authors calculated from their osmotic and volumetric effects. It should be noted that, at high volume fractions of solute, the effect of solutes makes a bigger difference to a plot of force vs separation than it does to a plot of force vs hydration. Yoon et al made comparisons in terms of force-hydration curves. The larger effect of the larger solutes sucrose and trehalose might be because of their increased perturbation of water structure: the volume fractions reached as high as 50% and one would expect substantial disruption of the hydrogen bonding network at these concentrations. An alternative explanation is that they are due to specific effects of these solutes on the hydration force.

Solutes could affect hydration forces either if they were adsorbed onto the membrane-water interface, in which case they would produce an interface with an altered capacity to polarise water and an altered surface mobility, or if they were excluded from the interface and thus created a very high concentration midway between the lamellæ. The results of Yoon et al (1998) and Pincet et al (1994) were consistent with the exclusion, to a small extent, of sugars from the region closest to the bilayer surface.

For all of the effects of solutes discussed above, rather large concentrations (several $kmol.m^{-3}$ or more) are required to produce substantial effects. For inter membrane sugar concentrations of much less than 1 $kmol.m^{-3}$, the effect on the hydration properties and inter membrane forces for most freezing temperatures is just that predicted from the osmotic effects (Yoon et al, 1998).

The Surface Forces Apparatus (SFA) is quite different from and complementary to the Osmotic Stress Technique (OST) and the Freezing Stress Technique (FST) in that, in the SFA, measurements are conducted in the presence of a large volume of excess solution. Consequently the SFA is unaffected by osmotic forces, except at very close separations when exclusion effects may be

important. As a result, the SFA is well suited to examining the specific effects of different solutes. A limitation on the technique is that it is difficult to use very large concentrations of solutes because, in order to compare with controls, the aqueous medium must be replaced during an experiment. Pincet et al (1994) measured the effect of dimethylsulphoxide, sorbitol and trehalose on the force between dioleylphosphatidylcholine (DOPC) bilayers. For the saccharides, their study was limited to concentrations of only 1.5 to 2 kmol.m^{-3}. Their results showed little specific difference among the solutes. Their results also suggested that, when bilayers were brought very close together in the presence of a reservoir of solution, sorbitol and trehalose were, to some extent, excluded from the region very near the membrane surface.

Electrical interactions. The effect of ions on charged surfaces was first studied in colloid science, and much of the theory of the interaction between colloidal surfaces (Verwey and Overbeek, 1948) has been carried over to analyse forces between membranes (see also Cevc, 1990 and Israelachvili, 1991). Experimentally, the effect of ionic solutes on intermembrane forces has been studied in considerable detail using the SFA, and chiefly at concentrations that are modest in comparison with those found in freeze-dehydrated or desiccated cells. The most spectacular effects are on charged membranes and they have a large proportional effect on the electric double layer forces at moderate to high hydrations (Marra and Israelachvili, 1985). The effects of different ions upon the interactions between surfaces with various charges are varied and complicated, and are reviewed by others. The effects of monovalent ions on the repulsion between surfaces is usually to reduce it (Verwey and Overbeek, 1948; Attard, 1996; Marčelja, 1997). The divalent ion Ca^{++} may change the sign of electrostatic forces between charged surfaces (Marčelja, 1992). Electrical forces are potentially very important in determining the inter membrane spacing in highly hydrated systems, and have been invoked to explain such effects as the stacking and unstacking of thylakoid membranes in fully hydrated chloroplasts. Further, the interactions between ions and membranes are capable of producing responses that include demixing and changes in the phase transition temperature (Raudino et al, 1987; Tamura-Lis et al, 1986). In membranes at low hydration, however, close approach almost always produces a very large repulsive force, as discussed above, and this paper is concerned primarily with the effects of such forces, rather than a detailed discussion of their origins.

Compatible solutes. All solutes, whether ionic or non-ionic, lower the chemical potential of water. Thus the purely osmotic effect of inter-lamellar solutes is to increase hydration and to reduce intra-membrane stresses at any given freezing temperature. The effects of different solutes on the activity of enzymes may however be quite different and some solutes are toxic in high concentrations.

At equilibrium, the effect of any one solute, at a given freezing temperature or chemical potential of water, is to lower the concentration of the others by reducing the amount of ice present. Compatible solutes are those that can be accumulated in large concentrations with no deleterious effects (Brown, 1976). The interaction between ions and enzymes affects the state and activity of the enzyme, so one effect of compatible solutes is that they result in a reduction in the concentration of ionic solutes (Mazur, 1963). To have such an effect directly, the compatible solutes must partition into the solution in which the enzyme is found. It is also possible for non-permeating solutes to have an effect by vitrification, which hinders osmotic equilibrium (this is discussed below). The effects of permeating and non-permeating non-ionic solutes can therefore be rather different. Santarius and co-workers studied the effect of complex media including both salts and nonionic solutes on photosynthetic reactions in thylakoid membranes (Shakir and Santarius, 1995). They conclude that the colligative action of penetrating cryoprotectants is the primary mechanism for protection of the photosynthetic reactions in the thylakoid. We do not know of any study of the effect of freezing induced stresses in the thylakoid, and the extent to which permeating solutes reduce these stresses. Shakir and Santarius also discuss possible interactions between solutes and membranes.

Freezing and vitrification of water

The normal fluid to solid phase transition occurs by a process of nucleation and growth (e.g. Franks, 1982). This process is the same for any liquid, but here it will be explained by the example of the water-ice transition. Consider undercooled water at a temperature T, which is a few degrees below the equilibrium freezing point T_f. If the water is pure and the volume small, the water can remain in this non-equilibrium undercooled state almost indefinitely. In order for freezing to occur, the water molecules, which are undergoing Brownian motion, must spontaneously adopt a

configuration that is "ice-like". The probability of this happening to the entire sample at the same time is vanishingly small. Locally, however, small clusters of molecules with an ice-like structure (called homogeneous nuclei) are continuously forming and breaking up. If one of these nuclei reaches a critical size (see appendix 2), then it becomes energetically favourable for more water molecules to grow on this nucleus, and the ice will propagate rapidly through the entire sample. This two stage process is called nucleation and crystal growth.

A cartoon of the nucleation process is shown schematically in Fig. 6a. Each water molecule (indicated by a *w* with an arrow) undergoes Brownian motion with a characteristic diffusion coefficient (the magnitude of that is indicated by the arrows). The circle represents the critical radius for nucleation, and the water molecules inside the radius are, at this snapshot in time, arranged in a pseudo regular ("ice-like") manner. If the regularity grows to be larger than the critical radius, then the sample will crystalise. Nucleation can also proceed via heterogeneous nucleation, where the water molecules near a surface (such as the container walls), or large particles in the solution (such as dust, proteins etc), act as a catalyst for the formation of ice nuclei (e.g. Franks, 1982).

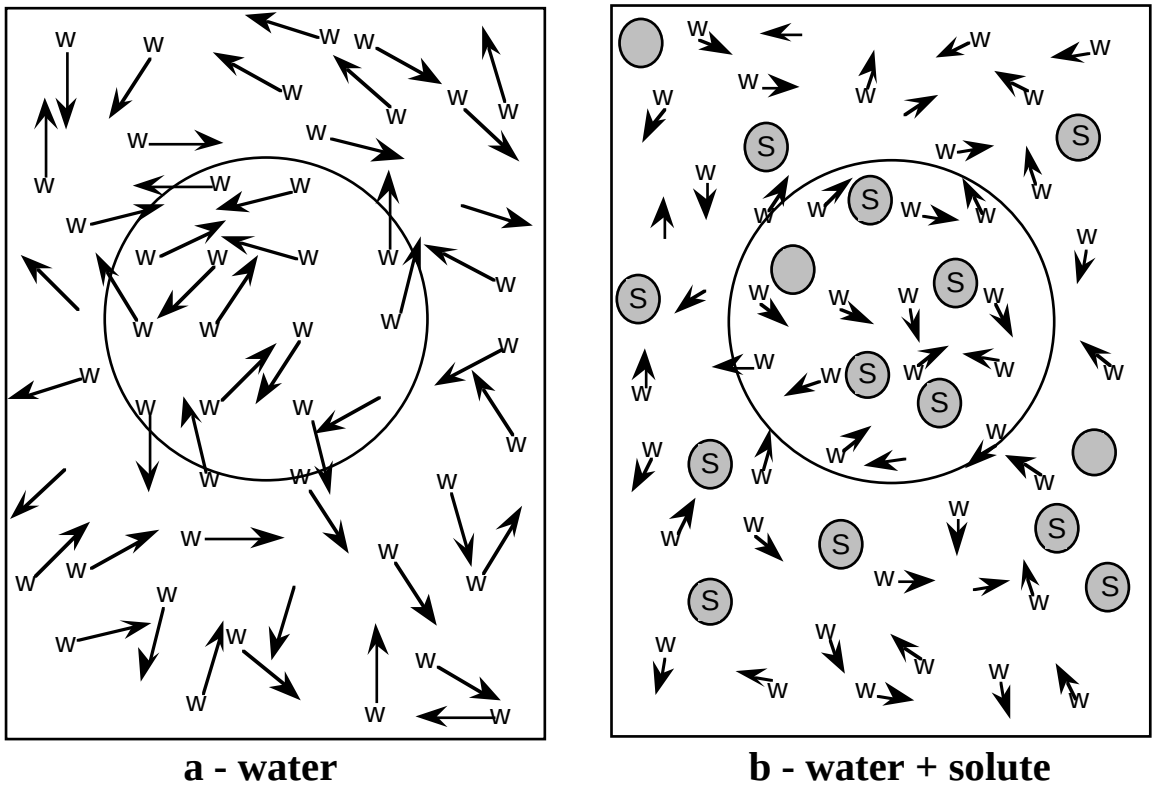

Fig. 6. A cartoon of the effects of solutes on the nucleation process. Water molecules and solutes are represented by the symbols *w* and *s* respectively. The arrows represent diffusion, and the length of the arrows indicates the speed of diffusion. The large circles represent the critical nucleation radius. (a) shows the situation where only water is present. For a critical nucleus to form, the water molecules in the volume represented by the circle must spontaneously arrange themselves (through Brownian motion) into a regular ice-like structure. If this regular lattice is larger than the critical radius, then the crystal will grow. (b) shows the same situation in the presence of some hypothetical solutes (for a solute:water molar ratio of 1:4). First, the solutes increase the viscosity, so diffusion is reduced (indicated by the smaller arrows in (b) than (a)). Second, in order for a critical nucleus to form, a volume equal to or greater that the critical radius must be completely free of solute molecules. In the situation shown in (b) this is not the case. As the concentration of solutes increases, this effect becomes even stronger, further reducing the chance of nucleation occurring. As the solutes of interest are much larger than water molecules, solute diffusion is much slower than water diffusion, so no arrows have been drawn on the solute molecules.

The probability of nucleation (i.e. the formation of nuclei larger than the critical volume) is proportional to sample volume[8], the amount of undercooling ($\Delta T = T_f - T$), and the viscosity of the liquid. As the liquid is cooled, the viscosity rises. If the liquid is cooled sufficiently quickly the viscosity may become so great that molecular rearrangements in the liquid become extremely slow or

8 This is the primary reason that freezing occurs in the extracellular solution before it occurs inside individual cells. A second reason is that the number of heterogeneous nucleation sites inside cells is exceedingly low.

stop. Nucleation and crystal growth will be hindered, and the liquid will be in a stable non-equilibrium phase, which is amorphous (i.e. it has no long range order, like a liquid), but which has mechanical properties like a solid. Such a phase is called a glass or vitrified solid, and the process by which it forms is called vitrification. A solution is said to be vitrified if its viscosity is greater than 10^{14} Pa.s (Franks, 1982). For comparison the viscosity of water is ~ 1 mPa.s at 20 °C.

Freezing and vitrification of aqueous solutions

In many single component systems such as water, the rate of cooling must be extremely high ($> 10^7$ K.s^{-1}) to achieve vitrification. However, in systems with two or more components, vitrification is easier to achieve. The addition of solutes decreases the probability of nucleation and growth for two reasons. The first effect is that the viscosity at any particular temperature (shown schematically in Figure 6b where the arrows are shorter than in figure 6a) is usually larger with solutes than without, implying that the motion and reorientation of the water molecules into the ice structure takes longer. The higher viscosity therefore hinders both nucleation and growth. Second, because the solutes are incompatible with the ice structure, the physical presence of the solutes hinders the formation of nuclei - an ice nucleus can form only if, at a particular time, a volume greater than or equal to the critical volume is free of solute molecules. At high concentrations this is unlikely (as shown in Figure 6b where the solutes within the circle mean that an ice nucleus cannot form there at that point in time). The probability of nucleation occurring at any particular temperature is reduced with increasing concentration. For both these reasons, as the concentration of solutes is increased, the temperature T_g at which vitrification will occur increases, and the cooling rate needed to achieve vitrification is reduced[9].

At sufficiently high concentrations T_g may become larger than T_f, and ice cannot form. In the case of disaccharides, concentrations of 90% (by weight) are sufficiently high to vitrify under ambient conditions. A familiar example of such a sugar glass is toffee, which we mention here because we shall soon discuss the mechanical properties of sugar glasses.

Vitrification in membrane models and biological materials

Vitrification can occur in biological systems at ambient temperatures (desiccation) or sub zero temperatures (cooling), and has been suggested as a mechanism for membrane protection during dehydration (e.g. Burke, 1986; Green and Angel, 1989). In both cases, if the viscosity rises to ~10^{14} Pa.s (caused by either higher concentrations or lower temperatures) then the solution is vitrified. In cells or lamellar phases at low hydration, the vitrification will occur where the sugars are located. If the sugars are between the membranes, then vitrification should occur there. If the sugars are excluded from the region between the membranes, then vitrification may occur in extra-lamellar volumes near the membranes, but not between them. The fact that membranes can be protected from dehydration by vitrification suggests that vitrification does occur in the inter-lamellar spaces, but the evidence is only circumstantial. It is possible that vitrification in volumes outside the lamellae may provide protection from further dehydration if the membranes are completely encased in the glass, though this seems unlikely to be the case in general (see below).

If vitrification does occur between the lamellae, there are a number of consequences. First, ordinary thermodynamic equilibrium cannot be assumed (though thermal equilibrium still applies). The force between the lamellae in a glass is unknown, but it is not needed - because the glass is solid it cannot be deformed to any substantial degree, so the inter-lamellar separation y will remain unchanged.

How does the presence of a glass protect membranes? It does three things: (i) once a glass has formed, further dehydration will be limited (i.e. lowering the sub-zero temperature or the humidity will have little effect on the intermembrane separation). The membranes will thus have an effective hydration higher than at equilibrium. (ii) Vitrification lowers the probability of crystallization. When solutes crystalise, they no longer lower the chemical potential of a solution and so further dehydration is possible. If however the solution starts to vitrify, this limits the increase in the concentration of the unvitrified solution. Crystallization is therefore less likely and further dehydration does not

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[9] At cooling rates within a couple of orders of magnitude of 1 K.s^{-1}, the intracellular concentration is itself a function of cooling rate, because cells dehydrate osmotically in the presence of extracellular ice (Mazur, 1963).

necessarily take place. (3) Finally, a glass may allow the membranes to remain in the fluid lamellar phase at hydrations and temperatures that normally would lead to deleterious phase transitions. This last point is discussed in the following section.

Koster and co-workers (Koster et al., 1993; Koster et al., 1994) reported that, for POPC and small solutes, if the glass transition temperature T_g of the concentrated solution exceeds the value of the gel-fluid transition temperature (T_m), then the gel-fluid transition at low hydration occurs about 20 °C below the fully hydrated transition temperature T_o. They found similar effects in other lipids, but the range of depression of the gel-fluid transition temperature varies between about 10 °C and 60 °C, depending on the lipid species (Koster and Anderson, 1995; Koster et al., unpublished).

Zhang (1998) and Zhang and Steponkus (1995; 1996; ms submitted) studied a range of lipids and small solutes chosen to give a wide range of T_o and T_g, and developed a model to understand the process. While they report that dehydration elevates the gel-fluid transition temperature T_m, they find that (small) solutes minimise this increase only if T_g is below the fully hydrated transition temperature T_o (rather than T_m). When the transition occurs in a glassy matrix ($T_g > T_o$), the effect depends on the thermal history of the sample. If the lipid was in the fluid state when the inter-lamellar layer vitrified, T_m is depressed (both for cooling and warming). If it was in the gel phase when the glass was formed, T_m is elevated above T_o. Zhang and Steponkus propose that the glassy matrix impedes the conformational change associated with the lipid phase transition. A glass can support a substantial anisotropic stress. For a lamellar phase that was gel at vitrification, heating would create compressive stress in the bilayers and tensile stress in the glass, and T_m would be elevated, according to Eqn (1). For a lamellar phase that was fluid at vitrification, cooling would create tensile stress in the bilayers and compressive stress in the glass, and T_m would be depressed.

Is the glass matrix sufficiently rigid for this model? The elastic properties of a relevant sugar glass (a solution of sucrose:raffinose 85:15 at a concentration of 90%) have recently been measured (Martin and Bryant, in prep). The Young's modulus, Y, is about 20 GPa (compared to 9 GPa for ice). Using the Clausius-Clapeyron equation, an estimate of the compressive stress for a membrane 20°C below its T_o can be made. Using typical values (for DPPC) of $L \sim 5\ 10^{-20}$ J.molecule^{-1}, $\Delta a \sim 0.15$ nm^2, and T_o = 42 °C (Caffrey, 19**????**, Nagle et al, 1996), $\partial\pi/\partial T \sim 2$ mNm^{-1}K^{-1}. If the glass were to support the stress of a membrane down to 20 °C below T_o, this would correspond to a stress of ~40 mN.m^{-1}. If this stress were supported over half the thickness of the inter-lamellar separation (say ~0.5 nm), this would lead to a stress of 80 MPa. For a glass with Y = 20 GPa, this corresponds to a strain in the glass of about 0.4%, which is easily supported.

It seems reasonable to assume that Young's modulus would not differ greatly between sugar glasses composed of different sugars, so the model would predict that the depression of the phase transition temperature due solely to this effect would be independent of the type of sugar, as long as T_g is higher than T_m. The studies of Koster and her colleagues and those of Zhange and Steponkus provide experimental confirmation of this prediction. The magnitude of the effect varies with lipid species, however, because of the variation in Δa and L among lipids.

Figure 7 schematically summarizes the main non-specific effects of solutes on the gel fluid transition temperature as a function of hydration. The bold line is for a lipid-water system, where dehydration causes the transition temperature to rise several tens of degrees above the excess water transition temperature T_o. The full line shows the effects of the osmotic and volumetric effects of small, uncharged solutes such as sugars, which reduce the membrane stress and hence the transition temperature at any hydration. If vitrification occurs at a particular hydration, then the transition temperature will fall by an amount in the range ~10 °C to 60 °C, depending on the lipid species (indicated by the filled circle in Fig 7), and then remain almost constant as any further dehydration will be limited in extent and rather slow[10]. This effect is most likely due to the mechanical properties of the glass, as it can support an anisotropic stress, and can thus support the membrane in the fluid state at temperatures where the gel transition would occur in the absence of a glass.

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[10] Diffusion is slowed but not stopped in vitrified materials. Further, highly viscous samples may be inhomogeneous and not all regions may vitrify at the same time or temperature. Thus some further dehydration may occur over periods of weeks (Steponkus, personal communication; Zhang and Steponkus, 1995).

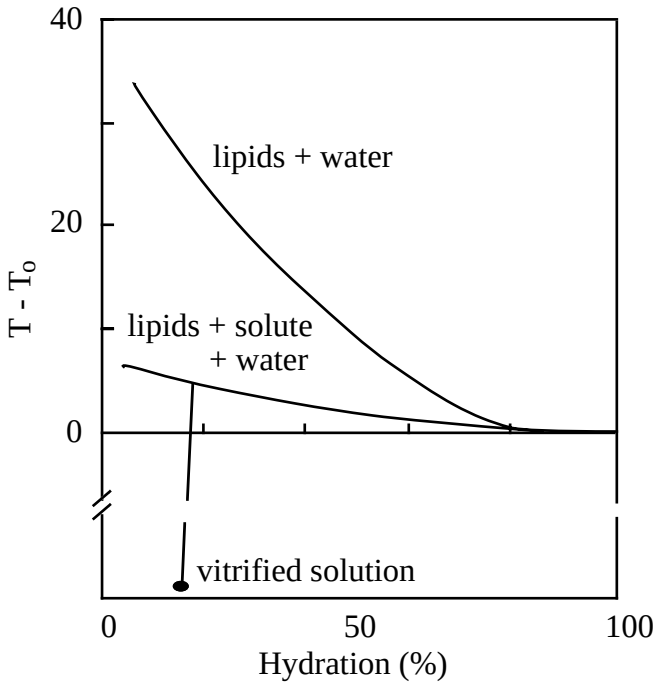

Fig. 7. A schematic of the non-specific effects of *small* solutes on the gel-fluid transition temperature as a function of hydration. The y axis is $T-T_o$, where T_o is the transition temperature in excess water, indicated by the horizontal line. Values are approximate. The bold line shows $T-T_o$ as a function of hydration for a lipid-water system. The full line is for a lipid-water-hypothetical solute system, and illustrates the effect of the inter-lamellar solutes reducing membrane stress and hence the transition temperature. The filled circle indicates the transition temperature if vitrification occurs while the lipid is in the fluid phase, as suggested by Zhang and Steponkus (1996).

Another effect of the presence of the glass phase, (and indeed highly viscous fluids that have not vitrified), is that the viscosity may hinder dynamic phase transitions. This is not important in the slow cooling rates in the natural environment, but it may have an important consequence in the laboratory. Rapid rates of temperature scanning could lead to increased hysteresis because of the effects inter-lamellar viscosity may have on the time taken for the lipids to rearrange themselves between configurations.

Glasses and very viscous fluids also reduce diffusion of solutes. Zhang (1998) has pointed out that this may reduce the leakage of solutes through membranes that otherwise would allow solute leakage at the phase transition. Leakage of electrolytes and markers from dry liposomes has been studied extensively by Sun et al. (1994, 1996). Crowe et al (1998), reviewing this work, conclude that the rate of leakage drops considerably below the glass transition, but does not stop completely until 10-20 °C below the T_g. Two complications should be mentioned. First, the glass transition is a poorly defined, second order transition and the T_g measured by DSC is only one measure of the glass transition temperature, and there is disagreement among researchers about how to define it. Second, as the sample is cooled towards T_g, diffusion slows dramatically, and local inhomogeneities in concentration do not come to equilibrium. Consequently, some areas of the sample will vitrify at lower temperature than others. It is therefore possible that the leakage measured at temperatures just below the sample average T_g, as measured by DSC, may occur in regions of the sample that are not vitrified.

Polymers *vs*. small solutes

The bulk of the discussion so far has concentrated on the effects of small solutes such as disaccharides. Solutions of larger molecules, such as polymers, also undergo vitrification during dehydration. In model systems containing lipids, water and polymers, large polymer molecules are often excluded from the lamellar phase at low hydrations and form separate bulk phases in regions outside the lamellar structure (see Fig 5). This partitioning is the basis of operation of one version of the osmotic stress technique (discussed above). Thus their *direct* osmotic and volumetric effects on the membranes will be small. Between the bilayers will be water with little or no macromolecular solute, and so the presence of large polymers will have little direct effect on membrane stress, and hence little effect on membrane protection. When vitrification occurs in a system of membrane-water-large polymer, it will occur in the extra-lamellar volume. If the lamellar phase has time to dehydrate, the presence of the polymer will therefore have little direct effect on the freezing behaviour of the

lamellar phase[11]. (Again, excluded solutes do have an osmotic effect at temperatures above freezing, as discussed above.)

Relatively small polymers may partition into the inter-lamellar space at high hydrations. Whether on not they are excluded from a dehydrated lamellar phase depends upon the preparation and history of the sample. If such molecules produce vitrification, the effect on membrane transitions will depend on whether they are in the inter-lamellar phase or in a separate bulk phase. The osmotic pressure of polymers at modest weight fractions is smaller than that of the same weight fraction of small solutes. Thus small polymers would be expected to have little effect on the membrane transition temperature via the Clausius-Clapeyron effect (equation 1). If they partition into the inter-lamellar solution and if they vitrify, then they could support lateral compressive stresses and might depress the membrane transition temperature.

In a recent review, Crowe et al. (1998) have examined the role of vitrification in protecting membranes and proteins. They review experimental work, which shows that, although dehydrated polymers such as Dextran and hydroxyethylstarch (HES) vitrify at temperatures well above ambient, their ability to protect membranes and proteins (at moderate cooling rates) is limited. They conclude from this that vitrification alone is not sufficient to provide membrane protection, and appeal to specific effects to solve the dilemma. The appeal to specific effects is unnecessary for the reasons explained above. The vitrified solution can only provide substantial protection to membranes if it occurs between the bilayers. In the absence of any specific effects, one would expect the protective effects of carbohydrates to decrease with increasing molecular mass above a certain size, which would limit their partitioning into membrane phases, and limit their osmotic effect (on an equal weight basis). This is what is observed (see Crowe et al 1998 and references therein).

Trehalose *vs*. other sugars

What is special about trehalose, that its protective effects seem to be significantly better than other similar sugars such as sucrose and raffinose? Why are disaccharides better than monosaccharides? It is important to note the different physical properties of the various sugars before appealing to specific solute-membrane interactions. First, at any particular concentration, trehalose has a higher glass transition temperature than most other sugars. Second, highly concentrated trehalose is less prone to crystallisation than many other sugars. Sucrose on the other hand crystalises readily at high concentrations, although small amounts of raffinose reduce the tendency of sucrose to crystalise (Koster, 1991), so sucrose:raffinose mixtures avoid crystallization and can vitrify. The accumulation of small quantities of raffinose in some tolerant species allows sucrose (rather than trehalose) to play the role of vitrifier. It is possible that the most important reasons trehalose is considered to be a better protectant at low hydrations is because it does not crystalise readily, and because it has a high glass transition temperature. Koster and co-workers (Sommervold et al., 1995; Koster et al., 1996) showed that the ability of samples to vitrify is important in reducing the incidence of solute crystallization during storage. Other biologically important properties of trehalose—its low reactivity and reducing power and its high stability—are cited by Ring and Danks (1998). Levine and Slade (1992) have written extensively on the non-specific effects of trehalose in dehydrated systems.

Macromolecule-solute-water interactions

This paper has concentrated on membrane-solute-water interactions. Some of the observations would be expected to apply to macromolecule-solute-water interactions. Most biological molecules are hydrophilic in their native state and so one would expect strong hydration repulsion at close approach. These generate internal stresses in the macromolecules (Appendix 4). The mechanical properties of cross-linked polymers appears to influence their freezing behaviour (Murase et al, 1997), which is consistent with the suggestion that unfrozen water under suction generates mechanical stresses in the polymers. In some geometries, such as long chains or flat sheets, these stresses are anisotropic and thus give rise to geometrical deformations and structural transitions (e.g. Parsegian et al, 1986; Leiken et al, 1994). The non-specific effects of solutes on membrane-water interactions would therefore be expected to apply to macromolecule water systems that are dehydrated

[11] Note that the cryoprotective properties of many polymers on samples frozen at very high cooling rates in the laboratory are due to different mechanisms, and higher hydrations are maintained when vitrification occurs (e.g. Sputtek et al., 1993; Körber et al., 1985; Takahashi et al.,1988; Macfarlane et al. 1990).

by freezing or desiccation. We know of no detailed analysis of the non-specific effect of solutes on such stresses but we present a simple introduction in Appendix 4.

The effect of diverse solutes in minimising damage to biological macromolecules has been widely reported. In the case of enzymes, electrical interactions with ions, and ion mediated interactions between macromolecules are obviously important in maintaining activity. The colligative action of solutes is also acknowledged to be of considerable importance. For this reason, we suggest that it may be constructive to examine the hydration interaction and mechanical stresses produced by freezing or drying of aqueous macromolecular phases. As is the case with lamellar phases, vitrification of the aqueous phase would reduce the extent of (further) mechanical stress in the macromolecules, and this may be an important part of the contribution of cryoprotectants to the stabilisation of biological macromolecules. The situation is however complicated by the different partitioning effects of different solutes, their different effects on vitrification and their different specific effects on enzyme activity (see discussions by Moreira et al, 1998; Santarius and Franks, 1998). In Appendix 4, we derive relations among the hydration interaction of macromolecules, their contribution to the freezing point depression, and the intra-molecular stress.

Crowe et al. (1998) discuss the effects of vitrified solutions on the stability of proteins. They point out the vast differences between the stabilizing effects of small solutes and polymers in the vitrified state, and they also suggest that because proteins themselves vitrify in the dry state, but are not preserved, that vitrification is not sufficient to protect proteins. This argument appears not to recognize the different behaviour found in three very different situations:

1) Small solutes can vitrify in the spaces inside the protein structure, and then maintain that structure against further dehydration.
2) Vitrification of polymers will occur in the bulk, providing no direct protection to the membrane structure[12].
3) When proteins are dried, they immediately lose their structure as the water is removed. The protein "glass" is therefore made of proteins that have already suffered substantial strains. This contrasts with ultrastructural elements in an aqueous glass, where the latter supports the anisotropic stress and thus limits strains in the whole phase.

Summary of the non-specific effects of solutes on membranes at low hydration

At full or high hydration, the hydration force is negligible and intra-membrane forces are small. At low hydration, intermembrane forces are dominated by the hydration repulsion. According to the analysis presented here, the non-specific effects of solutes on membranes at low hydration can be summarised thus:

1) At low or intermediate hydrations, the osmotic effect of the inter-lamellar solutes reduces the stress on the membranes. In sufficient concentration, it may keep the gel-fluid transition temperature near the value it has in fully hydrated membranes. This effect is expected with any solute (salts, sugars etc).
2) The solutes will have these effects only if they remain between membranes. If the solutes are excluded from the membrane region, then these effects will be significantly reduced. Solutes that are completely excluded can, in sufficient concentration, dehydrate lamellar phases and elevate the gel-fluid transition temperature via Eq. 1.
3) If the solutes are relatively large (e.g. disaccharides), they will have an additional volumetric effect, which affects the stress described in Eq. (1).
4) The reduction of lateral stress by solutes will, all else equal, reduce the tendency for freezing or dehydration to produce non-lamellar phases, such as the hexagonal II phase.
5) As the solutes are further concentrated by dehydration, further stress reducing effects will occur only if the solute does not crystalise. Some solutes can be concentrated to very high levels without crystallisation (e.g. trehalose). Having mixtures of solutes also inhibits crystallisation (e.g. sucrose/raffinose mixtures).

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[12] The presence of polymers can, however, affect the concentration of other smaller solutes, if present, and the different solute concentration may affect protein structure.

6) At very low hydrations, vitrification occurs. Where the solution between fluid membranes is vitrified, it lowers the intra-membrane stress and this further lowers the gel-fluid transition temperature. Such vitrification will usually maintain the membranes in the fluid phase, and stop or severely slow any further dehydration. Conversely, the vitrification of the solution between membranes in the gel phase will usually elevate the gel-fluid transition temperature.

None of the effects listed above are specific to any particular sugar or lipid—they occur to varying degrees for all lipids and most solutes, with no specific interactions required. In the case of sugars, much of the reported differences in efficacy at protecting membranes during dehydration are primarily a consequence of their different physical properties—different sizes (volumetric effects), different solubilities (crystallisation) and different glass transition temperatures. This does not rule out the possibility of specific effects, but much of the observed behaviour of lipid-solute-water systems at low hydration can be explained without them.

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Appendix 1: Freezing of lamellar phases

Consider reversible freezing of water at uniform pressure. From the definition of entropy, the specific entropy Δs of the transition is

$$\Delta s = \frac{L_{iw}}{T_c} \qquad \text{(A1)}$$

where L_{iw} is the latent heat of fusion of ice and T_c is the equilibrium freezing temperature for water at atmospheric pressure. Both L_{iw} and Δs are weak functions of temperature and pressure. Now consider ice at atmospheric pressure in equilibrium with water at pressure P. At equilibrium, the chemical potentials are equal, so

$$\mu_i^o = \mu_w^o + Pv_w$$

where the subscripts i and w refer to ice and water, and where v_w is the specific volume of water. The standard chemical potential of water $\mu_w^o = \mu_i^o + L_{iw} - T\Delta s$, so substituting from A1,

$$\mu_i^o = \mu_i^o + L_{iw} - T\frac{L_{iw}}{T_c} + Pv_w$$

whence $$P = -\frac{L_{iw}}{v_w}\left(1 - \frac{T}{T_c}\right) = \frac{L_{iw}}{v_w T_c}\Delta T \qquad \text{(A2)}$$

where $\Delta T = T - T_c$. When $T < T_c$, the pressure in the liquid phase is negative. For small temperature variations, L_{iw} and ΔS can be considered approximately constant so the suction is approximately proportional to ΔT. Substituting standard values for water,

$$P \cong (1.2\ \text{MPa.K}^{-1}) \,.\, \Delta T. \qquad \text{(A3)}$$

For mechanical equilibrium, the force per unit area *F* between the lamellæ equals the suction in the inter-lamellar layer, so, taking repulsion as positive, and remembering that $\Delta T < 0$ below freezing,

$$F \cong -(1.2\ \text{MPa.K}^{-1}) \,.\, \Delta T. \qquad \text{(A4)}$$

The approximation that the latent heat of fusion is independent of temperature is adequate for most applications in this discussion because several other parameters related to lipid geometry and hydration behaviour are known only to one or two significant figures. If greater precision is required, linear or polynomial expressions for $L_{iw}(T)$ may be used. See Mazur (1963) and Pitt (1990) for further details.

Appendix 2: Metastable phases: suctions and supercooling

A volume of water under a negative absolute pressure (a gauge pressure lower than –1 Atmosphere) is unstable with respect to a volume of water vapour that can expand under the applied negative pressure. One cannot easily produce a suction of more than one atmosphere—it is usually impossible to syphon water to a height exceeding 10 m. How then can the inter-lamellar water support a suction of several or tens of atmospheres without cavitating? The short answer is that, if the volume of water vapour is initially very small, the surface tension of surface free energy of water around it can support the suction and close up the bubble of vapour.

A volume of liquid water at a temperature below freezing is unstable with respect to ice. Why then can supercooled water exist? Again, the answer is related to the surface free energy of the water interface surrounding a small ice nucleus. The two cases are similar so we shall analyse them together.

Fig (a) shows a small bubble of vapour formed in a liquid at negative pressure. The vapour pressure inside is small so we shall neglect it. The work W required to create this bubble has two terms. The work done in displacing the liquid is PV, where V is the volume. This is negative because $P < 0$. The work done in creating an area A of interface is $\gamma_w A$ where γ_w is the interfacial free energy per unit area.

For the spherical bubble,

$$W = P \cdot \frac{4}{3}\pi r^3 + \gamma_w \cdot 4\pi r^2$$

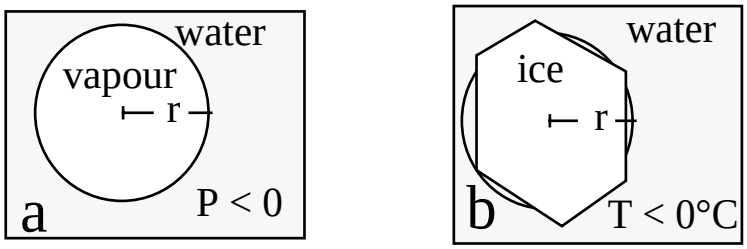

Fig (b) shows a small ice crystal formed in water at T < 0°C. The free energy required to form it has two terms. The free energy of freezing contributes a term $\rho V L_{iw}\Delta T/T_c$, where ρ is the density of water, L_{iw} is the (specific) latent heat of freezing, V is the volume of water frozen, T_c the equilibrium bulk freezing temperature and $\Delta T = T - T_c$. The work done in creating the ice-water interface of area A is $\gamma_{iw}A$ where γ_{iw} is the interfacial free energy per unit area of that interface. Let r be the radius of a sphere having volume V. The energy required to create this ice nucleus is

$$U = \frac{\rho L_{iw}\Delta T}{T_c} \cdot \frac{4}{3}\pi r^3 + h.\gamma_{iw} \cdot 4\pi r^2$$

where h is a dimensionless geometrical factor, which is larger than but of order 1.

The extra work dW required to expand the bubble and the extra energy dU required to expand the ice crystal are, respectively:

$$dW = \left(P.4\,\pi r^2 + \gamma_w.8\pi r\right)dr \qquad dU = \left(\frac{\rho L\Delta T}{T_c}.4\pi r^2 + h.\gamma_{iw}.8\pi r\right)dr$$

The bubble or crystal will expand indefinitely if dW/dr or dU/dr is negative. If it is positive, then the bubble or crystal will (usually) shrink. The value of r at which these derivatives are zero is the critical size for nucleation of cavitation and freezing respectively. (The word "usually" is included because thermal activation may take a sub-critical nucleus over the critical size, if it is within a few thermal energies of the critical energy.) So the critical radii for cavitation (c) and freezing (f) satisfy

$$0 = P.4\,\pi r_c^2 + \gamma_w.8\pi r_c \qquad 0 = \frac{\rho L_{iw}\Delta T}{T_c}.4\pi r_f^2 + h.\gamma_{iw}.8\pi r_f$$

whence $r_c = -\frac{2\gamma_w}{P}$ $\qquad$ $r_f = -\frac{2hT\gamma_{iw}}{\rho L_{iw}\Delta T_c}$

(Note that the result for r_c is just the equation of Young and Laplace.) For a suction of 10 MPa (100 Atmospheres), $r_c \sim 15$ nm. For a freezing point depression of 10 °C, $r_f \sim 7$ nm. Both of these are rather larger than the inter-lamellar separations encountered in lamellar phases exposed to desiccation or freezing temperatures.

Despite the above argument, freezing of inter-lamellar water would be possible if the interfacial energy between ice and membranes were very low, i.e. if membranes were more ice-philic than hydrophilic. If that were the case, however, one would expect lamellæ to be efficient nucleators for ice, which is inconsistent with the observation that the water in the lamellar phases can readily be supercooled by tens of K.

Appendix 3: Calculating inter-lamellar separation.

We assume that the water is incompressible, and that it has the same specific volume as in the bulk. One would expect the former to be a good approximation for pressures of magnitude much less than the bulk modulus, which is 2.0 GPa for water. It is unknown how good the latter approximation is for water near a strongly hydrophilic surface. Let *y* be the separation between the density-weighted

interfaces between lipid and water. *a* is the area per lipid molecule in one side of the bilayer. If there are *f* water molecules per lipid, and *g* solute molecules per lipid, then

$$fv_w + gv_s = \frac{1}{2} ya.$$

where v_s is the partial specific volume of the solute. The area *a* is a function both of the temperature and of the lateral stress in the bilayer. Express the lateral stress as π, the total force per unit length in the plane of the bilayer, where π = –Py. For small deformations *a* can be written as

$$a = a_o\left(1 + \alpha(T - T_r) + \frac{P\,y}{k_a}\right)$$

where α is the coefficient of area expansion, k_a is the area elastic modulus of the bilayer and T_r is a reference temperature. Solving these two equations for y gives

$$y = -\frac{k_a}{2P}\left(1 + \alpha(T - T_r) - \sqrt{\left(1 + \alpha(T - T_r)\right)^2 + \frac{8(fv_w + gv_s)P}{k_a a_o}}\right)$$

Appendix 4. Stresses in macromolecules

Hydration forces between hydrophilic macromolecules can produce freezing point depression and stresses within the macromolecules. Hydrated macromolecular phases can have a variety of different geometries. Here we analyse the stresses for a hexagonal array of long cylindrical molecules. This choice is suggested by its simplicity, and also by the fact that such a system has been studied by Parsegian et al (1986). For other geometries, geometrical factors of order one will enter the analysis.

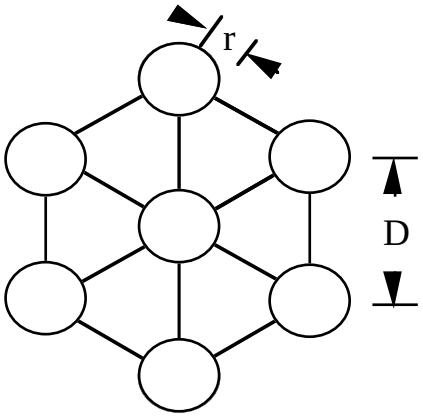

Figure. Part of a hexagonal array, with inter-axial spacing D, made up of long cylindrical molecules of radius r.

The pressure P is $P = \frac{dG}{dV} = \frac{dG'}{da}$ where G is the free energy, G' the free energy per unit length and a is the cross section of the array divided by the number of cylinders.

$$a = \frac{D^2\sqrt{3}}{2}$$

so $da = dD\sqrt{3}D$

whence $P = \frac{dG'}{dD}\frac{1}{\sqrt{3}D}$

The force per unit length f(D) contributes to the free energy of interaction. Assuming that only first neighbours interact, 6 pairwise interactions f(d) give rise to G' whence

$$dG' = -\frac{1}{2}\,6\,f(D)\,dD$$

where the half is included because two cylinders contribute to the mutual interaction energy.

Thus $P = \frac{dG'}{dD}\frac{1}{\sqrt{3}D} = -\sqrt{3}\,\frac{f(D)}{D}$

Freezing point depression by macromolecules. From (A3) in Appendix 1, for the case where there are no solutes present, P is given approximately as $(1.2\ MPa.K^{-1}).\Delta T$, where ΔT is the

change in freezing point. Thus, when no solutes are present in the macromolecular phase, the macromolecules produce a freezing point change of

$$\Delta T \cong -(1.4\ \mu K.Pa^{-1})\ \frac{f(D)}{D}$$

To first order, this is added to the freezing point variation due to the osmotic pressure of solutes, where present.

Intra-molecular force and hydration properties. In this case of long molecules in a parallel array, the intra-molecular stress is compressive along the axis of the macromolecule. If we integrate it over the cross section of the molecule we get a longitudinal force F, which, for mechanical equilibrium, balances the suction in the fluid. Consider one triangle of the array, which contains half a cylinder (three sixths of a cylinder) and a cross section of liquid with area $D^2\sqrt{3}/4 - \pi r^2/2$. The suction acting on this area balances the longitudinal force F, so

$$F = 2P\left(D^2\sqrt{3}/4 - \pi r^2/2\right) = -\left(\frac{3D}{2} - \sqrt{3}\ \frac{\pi r^2}{D}\right) f(D)$$

Thus the force along the axis of the macromolecule can be related to the hydration force for that molecule.

Intra-molecular stress and freezing point. It is also possible to relate the intra-molecular stress directly to the freezing point depression in a macromolecular phase. Let P_m be the compressive stress along the axis of the macromolecule. Mechanical equilibrium requires that

$$fP_m = (f - 1)P$$

The area ratio $f/(f - 1)$ is simply related to the ratio of densities of macromolecule (ρ_m) and water (ρ_w) and the composition expressed as the mass ratio h of water to macromolecule: $h = (f\rho_w)/((1–f)\rho_m)$. Thus the compressive stress P_m is

$$P_m = -(\rho_m/\rho_w)hP$$

Again, where no solutes are present, $P \cong (1.2\ MPa.K^{-1}).\Delta T$. For most biological macromolecules, $\rho_m/\rho_w \cong 1$. Thus, when no solutes are present in the macromolecular phase,

$$P_m \cong -(1.2\ MPa.K^{-1}).\ \Delta T.h.$$

When solutes are present in the macromolecular phase and have an osmotic pressure Π, this becomes

$$P - \Pi \cong (1.2\ MPa.K^{-1}).\ \Delta T.$$

$$P_m \cong (\Pi - 1.2\ MPa.K^{-1}.\ \Delta T)\ h$$

Substantial deformation of the macromolecule will occur when P_m becomes more than few percent of its Young's modulus.