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REVIEW

Freezing, Drying, and/or Vitrification of Membrane–Solute–Water Systems

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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 behavior 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 behavior. 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 crystallization, vitrification, volumetric, partitioning, and permeability properties of the solutes.

Key Words: cryobiology; anhydrobiology; hydration forces; dehydration; vitrification; membranes; phase behavior; gel–fluid transition; lipids; macromolecules.

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 (70, 41, 42, 1, 22, 66, 75, 11, 62, 64) and these solutes are accumulated by some freezing-tolerant and desiccation-tolerant species (38, 61, 63, 82, 35). In this paper we analyze 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 (5), in the light of research in the past 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 simple 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

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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 on the order of 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 of 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. Macromolecule–solute–water systems are discussed in an appendix. Nonequilibrium 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 nonaqueous 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 (17, 71, 73, 80). (It is also likely that a cell with 10% water content contains regions of closely packed macromolecules, although this would be harder to recognize by electron microscopy.) Further, electron microscopy of freeze-dehydrated cells shows some interesting topological features that are correlated with damage (72, 16). These features, which we discuss in more detail later, are found in the membrane-rich regions or where pairs of membranes are close together. In some cases the regions appear to lack intramembrane particles and so may be plausibly modeled by stacks of bilayers in a lamellar phase. Macromolecules are often excluded from dehydrated lamellar phases (44, 6) so it is not surprising to find that dehydrated cells contain membrane-rich domains nor unreasonable to expect that they contain 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 mean that, while quantitative estimates may be difficult, the qualitative behavior should be similar. The laws of thermal physics and mechanics may be difficult to apply quantitatively to cells, but there is no reason to expect that they are violated.

PHASE EQUILIBRIA OF WATER, SOLUTE, AND 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 (83). The solid line in Fig. 1b shows the behavior 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 interlamellar force per unit area as a function of hydration or interlamellar 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 (Fig. 1d). At high hydrations (e.g., more than about 30 waters per lipid in the case of phosphatidylcholines) and above freezing temperatures, lipid–water suspensions separate into two different phases: a lamellar phase with about 30 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 (89). Figure 1d shows the equilibrium hydration of lamellar phases of dioleoylphosphatidylcholine (DOPC) at freezing temperatures. The data in Fig. 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 lamellae and is in a condition similar to that of water in lamellar

[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 $U - TS$.

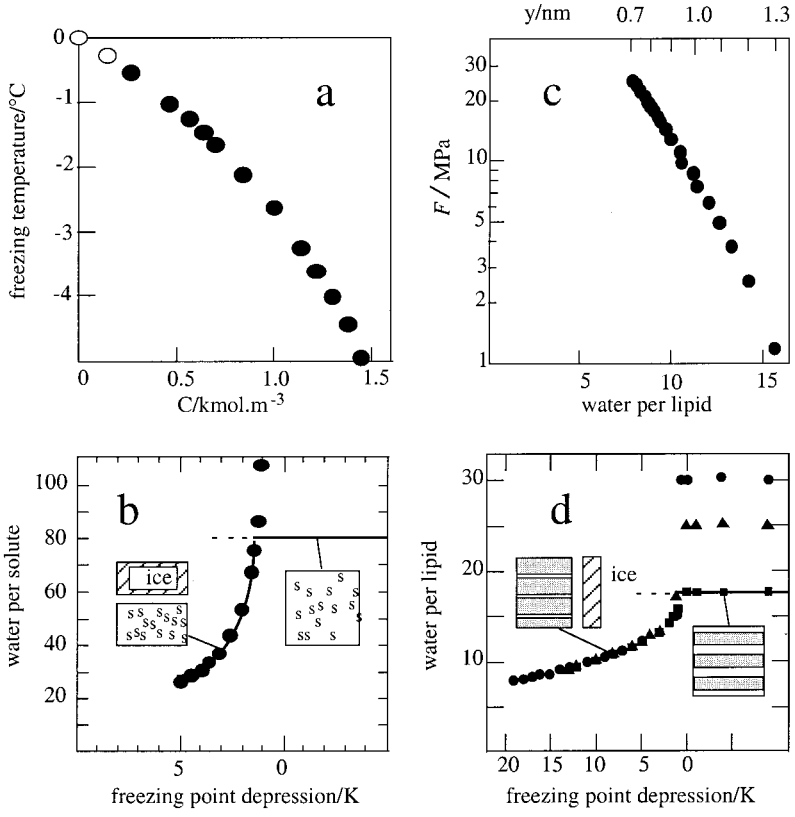

FIG. 1. (a) The equilibrium freezing temperature as a function of concentration for a solution of sucrose (83). In (b), the same data are plotted to show the composition of an unfrozen sucrose solution (expressed as the mole ratio solvent:solute) as a function of temperature. (The two unshaded points 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 interlamellar force per unit area as a function of mole ratio water:lipid or interlamellar separation *y* (90). On this semilog plot, the data are approximately linear, suggesting an exponential force law at small interlamellar separations. The hydration behaviors 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 behavior is shown by the solid lines in (b) and (d), supercooling is represented by the dashed horizontal lines to the left of the equilibrium curves.

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 lamellae. 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 lamellae 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 these lamellar phases have the same composition at the same freezing temperatures. Finally, the curves in Fig. 1d closely resemble the hydration behavior of lamellar phases in the absence of ice, as measured in diverse ways, as we shall see next. This behavior is usually represented in a rather different form, as shown in Fig. 1c, in which a repulsive force per unit area is measured as a function of the hydration or the interlamellar separation.

HYDRATION AND HYDRATION FORCES

When surfaces in water are brought to close separations (about one nanometer—Fig. 1c), 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 (25). 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 (29, 30). For the purposes of this discussion, the nature of the force is not of fundamental concern (but see the discussion by Bryant and Wolfe (5)).

Force-separation curves between bilayers and other surfaces may be measured directly using a technique developed by Israelachvili and coworkers (27, 23, 21). 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 lamellae are deposited (Fig. 2a).

In the osmotic stress technique (OST) of Rand, Parsegian, and colleagues (40, 44, 59), 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

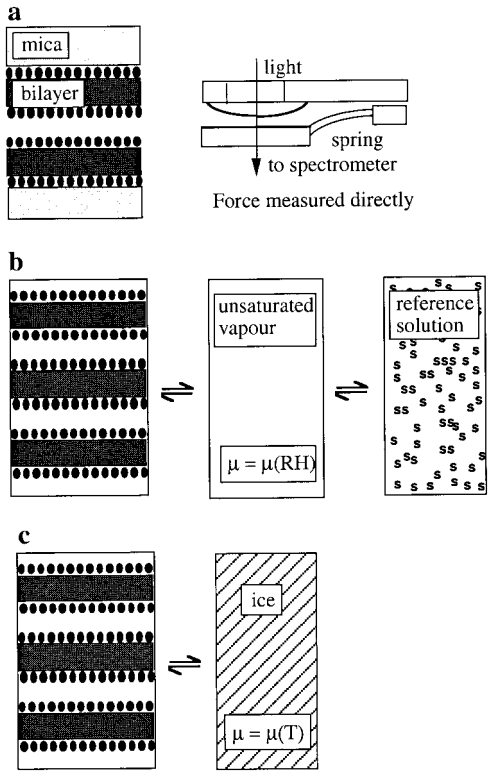

FIG. 2. Measuring lipid hydration and interlamellar forces. In the surface forces apparatus (a), the interlamellar force is measured directly and changes in the separation of the supporting surfaces are measured optically. In the osmotic stress technique (b), the force is determined from the equilibrium of the interlamellar water, a vapor phase, and a saturated solution. The interlamellar 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 intermembrane water with ice at known temperature. The intermembrane water content is determined from its NMR signal.

control the chemical potential of the reference phase. For modest dehydrations, high-molecular-weight polymers are introduced into the lipid/water mixture. As these do not permeate the membranes, they remain in a separate water–polymer phase, thus dehydrating the membranes. For moderate dehydrations, pressure is applied hydraulically through a membrane. For most moderate to severe dehydrations, a series of saturated solutions is used to control the vapor pressure, which is used to control the

hydration of lipid–water samples. Each lamellar phase sample is equilibrated with an unsaturated water vapor, 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 lamellae 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 interlamellar water. For mechanical equilibrium, the magnitude of the suction equals the repulsive force per unit area between the lamellae. As the lamellae 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 interlamellar 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 (24). In a variant on this method, the hydration, rather than the separation, is measured by weighing the sample (50) 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 behavior can also be studied using freezing, as is shown in Fig. 2c (henceforth freezing stress technique or FST). Consider first the case in which 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 interlamellar 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 lamellae 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. Figure 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 (90).

We note in passing that the OST has also been applied to determine force–separation relations for other geometries. Parsegian *et al.* (56) have measured the hydration repulsion and other forces in 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.

In the absence of solutes, the interlamellar 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 lamellae and produce a compressive stress[3] in them[4] (88). This is illustrated in Fig. 3. Note

[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).

[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 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.

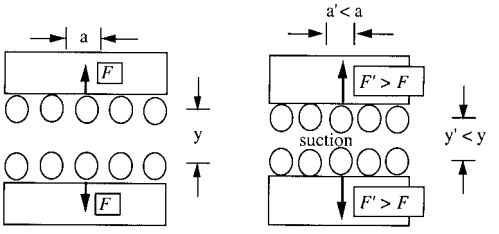

FIG. 3. *y* is the separation between the density weighted lipid–water interfaces and *a* is the area per lipid in (one side of) the lamella. The volume of water per lipid is *ay*/2. Removal of water from the interlamellar layer could produce reductions in either *a* or *y*. If the lamellae were infinitely 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 lamellae. Reductions in *a* are associated with increasingly large lateral compressive stress in the lamellae.

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 intramembrane 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.

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 (52, 87, 14). Because the lamellae 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 (44).

The most noticeable effect of lateral stress is on the gel–fluid (also known as gel–liquid crystal) transition in a planar bilayer (Fig. 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., 13, 78, 32, and references contained in these papers). The effect is readily explained in terms of a two-dimensional version of the Clausius–Clapeyron effect (5). 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_m is increased in proportion to the lateral pressure applied, at least for small applied stresses. Taking values (for DPPC) of $L \sim 5 \times 10^{-20}$ J · molecule^{-1} and $\Delta a \sim 0.15$ 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, Eq. [1] gives the depression of the transition temperature. Tensile stresses are possible when a vitrified interlamellar solution supports a 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 in-

[5] Phase diagrams in terms of T, π, and composition are given by (20) and (48).

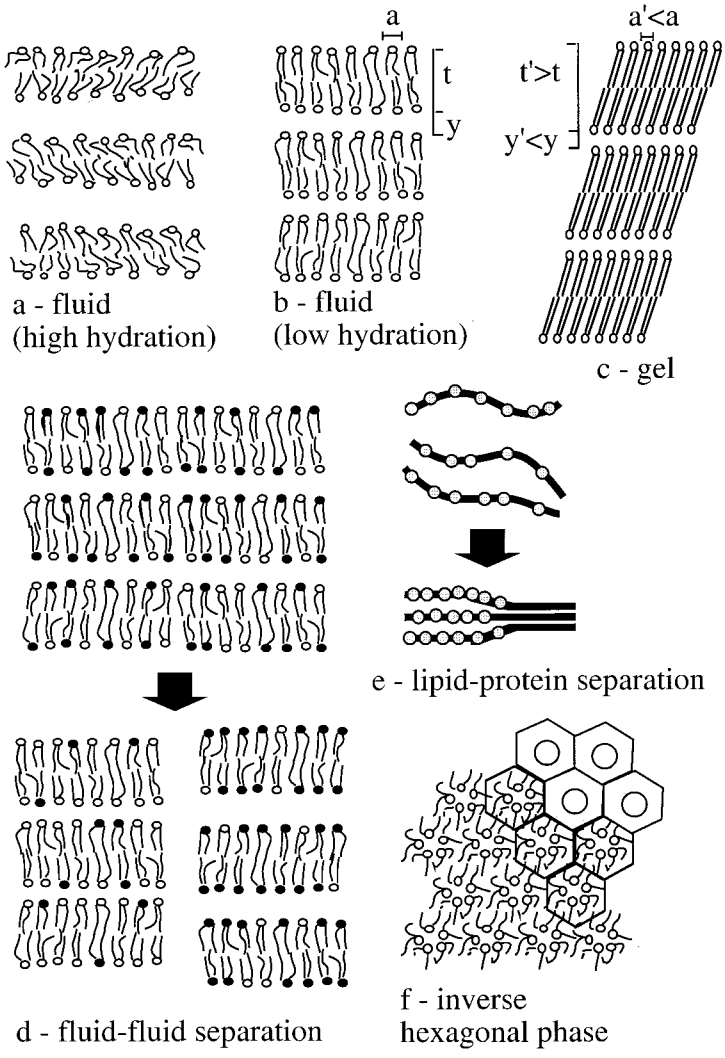

FIG. 4. The strains produced by dehydration-induced stresses. (a) A lamellar fluid phase (L_α) at high hydration. (b) The geometric strain produced at lower water content. The average area per lipid a and the interlamellar separation y are decreased, while the lamellar thickness t of the bilayers is increased. (c) At lower water contents, increased lateral stress (see text) can produce the transition to the gel phase ($L_{\beta'}$, 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 (Eq. [1]). In (d) 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 (d, top), 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 (d, bottom right), while the less strongly hydrating lipids are concentrated in domains with lower hydration (d, 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 (e, bottom). (f) A topological response to stress. At very low hydrations the lipids may undergo a transition to the hexagonal II phase, 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.

cludes 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 (6). This has been observed in two component lipid bilayers (4, 84) (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 Figs. 4b, 4c, and 4d, 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 (17, 71, 73, 80). 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 (72, 16).

In biological membranes, most of the lipids are strongly hydrating lipids that do not readily undergo transitions to nonbilayer 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 Fig. 4d) produces domains rich in the low hydrating component. These domains may then undergo a transition to a hexagonal II phase (Fig. 4f). The demixing may thus be an intermediate stage prior to formation of damaging inverse phases (6, 4, 84, 85).

[6] In some cases, membrane surfaces may have a spontaneous curvature and this transition may also lower the mechanical energy. This is analyzed by (28) and (19).

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 multilamellar[7] vesicles in pure water, in the presence of excess water. Water permeates easily and so the lamellae approach full hydration. Now add to the sample a nonpermeating nonionic solute. It is distributed (at least initially) in the bulk water phase. The osmotic pressure of the bulk solution now dehydrates the multilamellar vesicles. The extent of the dehydration is determined by the repulsive forces between the lamellae. The greater the solute concentration in the bulk, the greater the intermembrane repulsion and so the greater the intramembrane 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 the interlamellar 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. Nonspecific solute effects would produce little or no change in T_m at temperatures above the freezing temperature of the solution.

[7] A somewhat similar result may occur with unilamellar vesicles. If the vesicle radius is much greater than the membrane thickness, adding a nonpermeating 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.

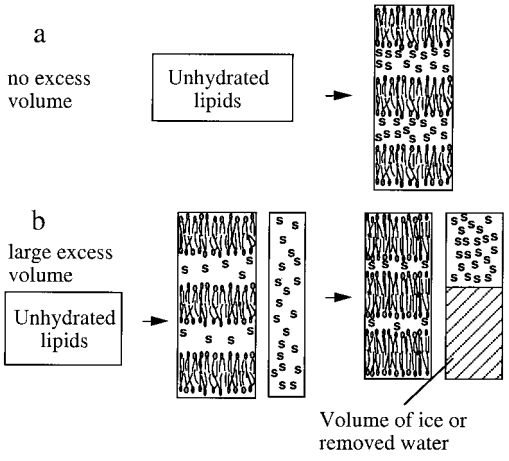

FIG. 5. The effect of different initial hydrations on solute redistribution. (a) A sample which is hydrated from the dry state with a small volume of a concentrated solution of solutes. There is no excess water, and so the solutes and water are all or nearly all in the interlamellar region (assuming no solute crystallization). (b) 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 interlamellar separation *y* are lower in case (b) than in (a). NMR of the solute or the water can be used to determine the distribution (90).

Freezing the sample further complicates the picture because it changes the solution compositions and can do so quite rapidly. Yoon *et al.* (90) 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.

Figure 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 interlamellar spaces. It is possible that there is also a macroscopic solution phase whose concentration is not necessarily the same as that of the interlamellar 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 interlamellar 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 interlamellar solution: repeated centrifugation with regular alternation of the orientation of the sample, repeated cycles of freezing and thawing, and the passage of time (weeks or more—P. Rand, personal communication). Nevertheless, it would be unwise to assume that, even after this treatment, the compositions of the interlamellar 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 sequestered in 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.

Specific vs nonspecific effects. Solutes affect the hydration of membranes, hydration forces, membrane–membrane interactions, and intramembrane 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 nonspecific, 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 interlamellar water is −1.2 MPa and so the interlamellar repulsion is 1.2 $MN \cdot m^{-2}$ (Appendix 1). Typically this gives rise to a lateral stress in the lamellae on the order of 1 $mN \cdot m^{-1}$, 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 $mol \cdot m^{-3}$ (for a nondissociating solute). One might expect that a solute concentration of this order in the interlamellar water would reduce the suction, the intermembrane repulsion, and the lateral stress to zero and increase the interlamellar separation. In practice, the osmotic effect of interlamellar solutes is a little more complicated for several reasons (90). The size of solute molecules is not negligible in comparison with the interlamellar 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 larger solutes. There may also be variations in solute distribution within the interlamellar layer due to interactions between the solute and the lamellae. In short, the purely osmotic effect of the presence of solutes in a fluid interlamellar layer is to increase the hydration, to decrease the intramembrane 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 freezing. Consider, for example, a suspension of unilamellar vesicles in pure water, to which is added a nonpermeating 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 intramembrane force and dehydration-induced intramembrane stresses at temperatures above freezing. In the presence of ice, the behavior of the lamellar phase is largely unaffected by the presence of these solutes, which are sequestered in a coexisting 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 nonrandom orientation of water propagating from the interface (29, 30). Solutes do not hydrogen bond in the same geometry as water, and the solute has a different (usually lower) polarizability. One might therefore expect all solutes, at sufficiently high volume fraction, to reduce the hydration repulsion and thus the intralamellar 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 interlamellar 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 (86). The molecular volume also affects the

steric interaction with membranes, discussed above. Finally, one of the most important effects of molecular size is that larger molecules are more likely to be excluded from the interlamellar layer.

Interactions among solutes, membranes, and water. After estimating and allowing for the osmotic and volumetric effects, Yoon *et al.* (90) reported that the disaccharides sucrose and trehalose, at concentrations of several kmol · m^{-3}, reduced the hydration force between dioleoylphosphatidylcholine bilayers to a greater extent than did the smaller solutes sorbitol and dimethyl sulfoxide. The effects of sorbitol and dimethyl sulfoxide on the interlamellar 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. 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 intermembrane sugar concentrations of much less than 1 kmol · m^{-3}, the effect on the hydration properties and intermembrane forces for most freezing temperatures is just that predicted from the osmotic effects (90). 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 polarize water and an altered surface mobility, or if they were excluded from the interface and thus created a very high concentration midway between the lamellae.

The results of Yoon *et al.* (90) were consistent with the exclusion, to a small extent, of sugars from the region closest to the bilayer surface. Crowe and co-workers (e.g., 12), on the other hand, observe alterations in the infrared spectra in the presence of trehalose and from this deduce that trehalose hydrogen bonds with the lipid head groups. Yu and Quinn (91) observe that DMSO reduces the lamellar repeat spacings and infer that the bilayer thickness decreases. From this they conclude that DMSO is preferentially located at the lipid–solvent interface.

The degree to which inter- and intramembrane stresses are modified by specific interactions between membranes and solutes is usually complicated by the osmotic effects of the solutes. The osmotic effects are often quite large and therefore may obscure specific effects. The SFA is quite different from and complementary to the OST and the 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.* (57) measured the effect of dimethyl sulfoxide, sorbitol, and trehalose on the force between 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 has been most extensively studied in colloid science, and much of the theory of the interaction between colloidal surfaces (81) has been carried over to analyze forces between membranes (see also 9, 26).

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 (49). 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 (81, 2, 47). The divalent ion Ca^{2+} may change the sign of electrostatic forces between charged surfaces (46). Electrical forces are potentially very important in determining the intermembrane 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 (60, 77). 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 nonionic, lower the chemical potential of water. Thus the purely osmotic effect of interlamellar solutes is to increase hydration and to reduce intramembrane 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 (3). 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 (51). To have such an effect directly, the compatible solutes must partition into, or be produced in, the solution in which the enzyme is found. It is also possible for nonpermeating solutes to have an effect by vitrification, which hinders osmotic equilibrium (this is discussed below). The effects of permeating and nonpermeating nonionic solutes can therefore be rather different. Shakir and Santarius studied the effect of complex media including both salts and nonionic solutes on photosynthetic reactions in thylakoid membranes (66). 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., 15). 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 nonequilibrium 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 favorable for more water molecules to grow on this nucleus, and the ice will

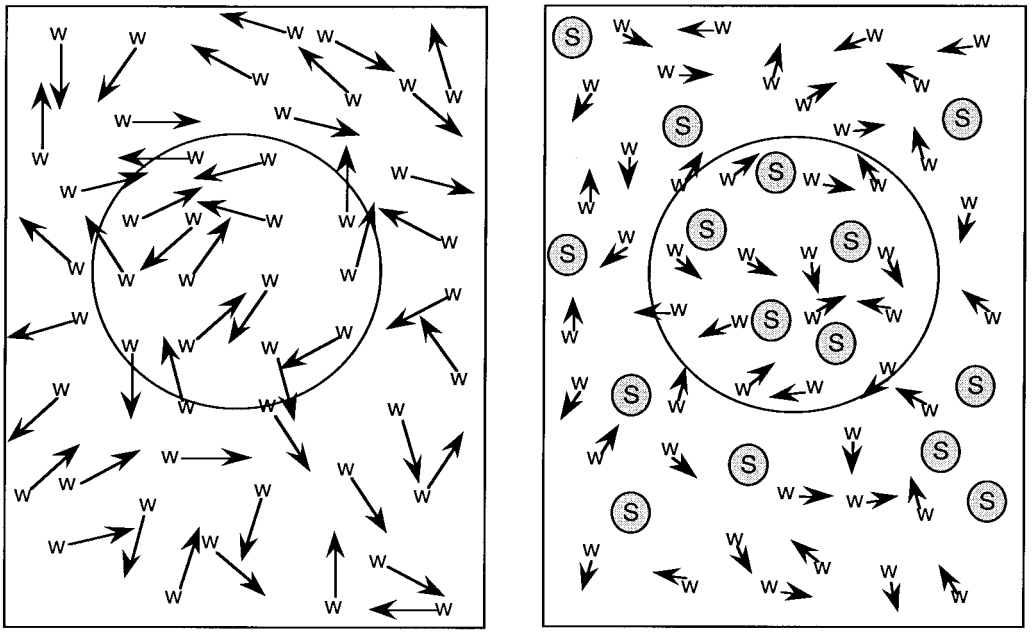

FIG. 6. A cartoon of the effects of solutes on the nucleation process. Water molecules and solutes are represented by the symbols w and s, respectively. The arrows represent diffusion, and the length of the arrows indicates the speed of diffusion. The large circles represent the critical nucleation radius. (a) The situation in which 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) The same situation in the presence of some hypothetical solutes. First, the solutes increase the viscosity, so diffusion is reduced (indicated by the smaller arrows in (b) than in (a)). Second, in order for a critical nucleus to form, a volume equal to or greater than a sphere with 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.

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 which 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 crystallize. Nucleation can also proceed via heterogeneous nucleation, in which a surface (such as the container walls), or a large particle in the solution (such as dust or protein), acts as a catalyst for the formation of ice nuclei (e.g., 15).

The probability of nucleation (i.e., the formation of nuclei larger than the critical volume) is related 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 molec-

[8] This is the primary reason 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.

ular rearrangements in the liquid become extremely slow or stop. Nucleation and crystal growth will be hindered, and the liquid will be in a stable nonequilibrium 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 (15). 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 Fig. 6b in which the arrows are shorter than in Fig. 6a) is usually larger with solutes than without, implying that the motion and reorientation of the water molecules into the ice structure take 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 Fig. 6b in which the solutes within the circle mean that an ice nucleus cannot form there at that instant). 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]

[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 (51).

At sufficiently high concentrations T_g may become larger than T_f, and ice cannot form. In the case of disaccharides, concentrations of greater than about 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 subzero temperatures (cooling) and has been suggested as a mechanism for membrane protection during dehydration (e.g., 7, 18). 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 extralamellar 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 interlamellar 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 interlamellar separation y will remain unchanged.

How does the presence of a glass protect membranes? It does at least three things: (i)

Once a glass has formed, further dehydration will be limited (i.e., lowering the subzero 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 crystallize, 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 necessarily take place. (iii) 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 (36, 32) 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 and 60°C, depending on the lipid species (34; Koster *et al.*, in preparation).

Zhang (92) and Zhang and Steponkus (93–95) 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 minimize this increase by vitrification only if T_g is below the fully hydrated transition temperature T_o (rather than T_m). When a 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 interlamellar 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 Eq. [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 (Koster *et al.*, in preparation). 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 $L \sim 5 \times 10^{-20}$ J · molecule^{-1}, $\Delta a \sim 0.15$ nm^2, and $T_o = 42$°C (8, 55), $\pi/T \sim 2$ mN · m^{-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 interlamellar 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 among 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 to first order be independent of the type of sugar, as long as T_g is higher than T_o. The studies of Koster and her colleagues and those of Zhang 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 nonspecific effects of solutes on the gel–fluid transition temperature as a function of hydra-

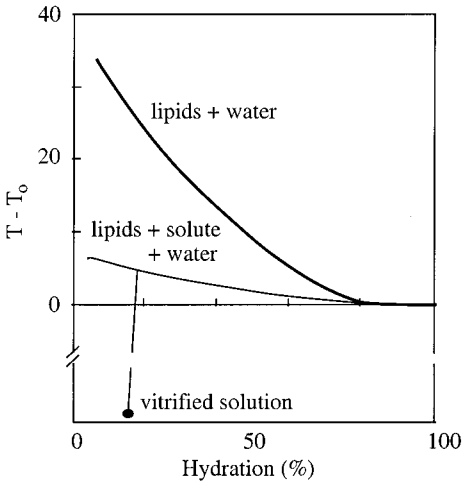

FIG. 7. A schematic of the nonspecific effects of *small* solutes on the gel–fluid transition temperature as a function of hydration. The y axis is $T - T_o$, where T_o is the transition temperature in excess water, indicated by the horizontal line. Values are approximate. The bold line shows $T - T_o$ 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 interlamellar 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 (95).

tion. The bold line is for a lipid–water system, in which dehydration causes the transition temperature to rise up to 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 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 at which the gel transition would occur in the absence of a glass.

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 probably 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 interlamellar 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 (92) 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.* (74, 75). In a review of this work (10), it is concluded 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,

[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 (94 and P. L. Steponkus, personal communication).

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 extralamellar volume. If the lamellar phase has time to dehydrate, the presence of the polymer will therefore have little direct effect on the freezing behavior 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 interlamellar space at high hydrations. Whether 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 interlamellar 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 (Eq. [1]). If they partition into the interlamellar solution and if they vitrify, then they could support lateral stresses of either sign and might thus change the membrane-transition temperature in either direction: depression if vitrification occurs between membranes in the fluid state or elevation if the membranes are in the gel state when the intervening fluid becomes glassy.

Two recent papers (10, 79) have examined the role of vitrification in protecting membranes and proteins. The experimental work presented and reviewed shows that, although dehydrated polymers such as dextran and hydroxyethyl starch vitrify at temperatures well above ambient, their ability to protect membranes and proteins (at moderate cooling rates) is limited. The authors 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 direct protection of the membranes if it occurs in the solution near them. 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 the layer between the membranes and limit their osmotic effect (on an equal weight basis). This is what is observed (see 79, 10, 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 worth noting 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 crystallization than many other sugars. Sucrose, on the other hand, crystallizes readily at high concentrations, although small amounts of raffinose reduce the tendency of sucrose to crystallize (e.g., 67, 33), so sucrose:raffinose mixtures avoid crystallization and can vitrify. The accumulation of small quantities of raffinose in some tolerant species allows sucrose (rather than tre-

[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., 69, 31, 76, 45).

halose) to play the role of vitrifier. It is possible that the most important reason trehalose is considered a better protectant at low hydrations is it does not crystallize readily and it has a high glass transition temperature. Koster and coworkers (68, 37) 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 (62). Levine and Slade (43) have written extensively on the nonspecific 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 macromolecules 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 appear to influence their freezing behavior (54), 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., 56, 39). The nonspecific effects of solutes on membrane–water interactions would therefore be expected to apply to macromolecule water systems that are dehydrated by freezing or desiccation. We know of no detailed analysis of the nonspecific effect of solutes on such stresses but we present a simple introduction in Appendix 4.

The effect of diverse solutes in minimizing 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 stabilization 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 in 53, 65). In Appendix 4, we derive relations among the hydration interaction of macromolecules, their contribution to the freezing point depression, and the intramolecular stress.

A recent review (10) discusses the effects of vitrified solutions on the stability of proteins. It points out the vast differences between the stabilizing effects of small solutes and polymers in the vitrified state. It also notes that proteins themselves vitrify in the dry state, but are not preserved, and concludes that vitrification is therefore insufficient to protect proteins. This argument appears not to recognize the different behaviors 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 usually occur in the bulk, providing little direct protection to the membrane structure.[12]

(3) When proteins are dried, they lose their structure as soon 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, in which the latter supports the anisotropic stress and thus limits strains in the whole phase.

[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.

SUMMARY OF THE NONSPECIFIC EFFECTS OF SOLUTES ON MEMBRANES AT LOW HYDRATION

At full or high hydration, the hydration force is negligible and intramembrane stresses are relatively small. At low hydration, intermembrane forces are dominated by the hydration repulsion. According to the analysis presented here, the nonspecific effects of solutes on membranes at low hydration can be summarized thus:

(1) At low or intermediate hydrations, the osmotic effect of the intermembrane 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 membrane-rich phases and elevate the gel–fluid transition temperature via Eq. [1].

(3) If the solutes are moderately 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 inverted 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 solutes do not crystallize. Some solutes can be concentrated to very high levels without crystallization (e.g., trehalose). Having mixtures of solutes also inhibits crystallization (e.g., sucrose/raffinose mixtures).

(6) At very low hydrations, vitrification often occurs. Where the solution between fluid membranes is vitrified, it lowers the intramembrane 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 lipid membranes and most solutes, with no specific interactions required. Indeed many of these effects would be expected in any hydrophilic nanostructures in aqueous solution, including much cellular ultrastructure. 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 (crystallization), and different glass transition temperatures. This does not rule out the possibility of specific effects, but much of the observed behavior of lipid–solute–water systems at low hydration can be explained without them.

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

$$\Delta s = \frac{L_{iw}}{T_c}, \qquad [A1]$$

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 Eq. [A1],

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

whence

$$P = -\frac{L_{iw}}{v_w}\left(1 - \frac{T}{T_c}\right) = \frac{L_{iw}}{v_w T_c}\Delta T, \quad [A2]$$

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,

$$P \cong (1.2\ \text{MPa} \cdot \text{K}^{-1}) \cdot \Delta T. \quad [A3]$$

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

$$F \cong -(1.2\ \text{MPa} \cdot \text{K}^{-1}) \cdot \Delta T. \quad [A4]$$

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 behavior are known to only 1 or 2 significant figures. If greater precision is required, linear or polynomial expressions for $L_{iw}(T)$ may be used. See (51) and (58) 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 atm) is unstable with respect to a volume of water vapor that can expand under the applied negative pressure. One cannot easily produce a suction of more than 1 atm—it is usually impossible to syphon water to a height exceeding 10 m. How then can the interlamellar water support a suction of several or tens of atmospheres without cavitating? The short answer is that, if the volume of water vapor is initially very small, the surface tension or surface free energy of water around it can support the suction and close up the bubble of vapor.

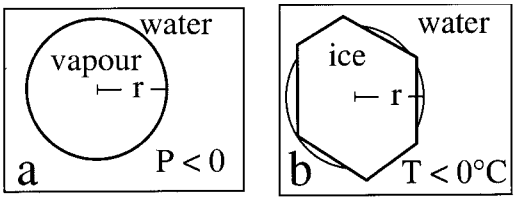

FIG. A1. See text.

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 analyze them together.

Figure A1a shows a small bubble of vapor formed in a liquid at negative pressure. The vapor 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,

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

Figure A1b 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 is 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

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

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,

$$dW = (P \cdot 4\pi r^2 + \gamma_w \cdot 8\pi r)dr,$$

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

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 subcritical 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

$$0 = P \cdot 4\pi r_c^2 + \gamma_w \cdot 8\pi r_c$$

$$0 = \frac{\rho L_{iw} \Delta T}{T_c} \cdot 4\pi r_f^2 + h \cdot \gamma_{iw} \cdot 8\pi r_f,$$

whence

$$r_c = -\frac{2\gamma_w}{P} \quad r_f = -\frac{2hT_c\gamma_{iw}}{\rho L_{iw}\Delta T}.$$

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

Despite the above argument, freezing of interlamellar water would be possible if the interfacial energy between ice and membranes were very low, i.e., if membranes were more icephilic than hydrophilic. If that were the case, however, one would expect lamellae 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 INTERLAMELLAR 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 $\pi = -Py$. For small deformations a can be written as

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

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

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

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 analyze the stresses for a hexagonal array of long cylindrical molecules (Fig. A2). This choice is sug-

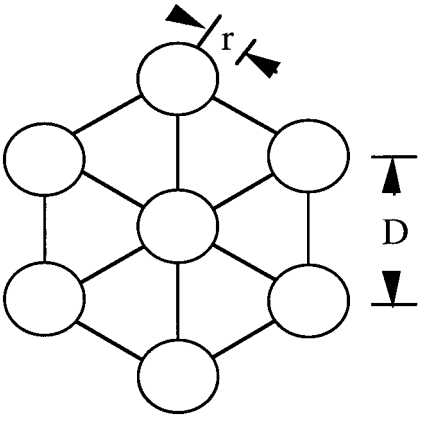

FIG. A2. Part of a hexagonal array, with interaxial spacing D, made up of long cylindrical molecules of radius r.

gested by its simplicity and also by the fact that such a system has been studied by Parsegian *et al.* (56). For other geometries, geometrical factors of order 1 will enter the analysis.

The pressure P is

$$P = \frac{dG}{dV} \cong \frac{dG'}{da},$$

where G is the free energy, G' is 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 neighbors interact, six pairwise interactions $f(D)$ give rise to G', whence

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

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 Eq. [A3] in Appendix 1, for the case in which there are no solutes present, P is given approximately as $(1.2\ \mathrm{MPa}\cdot\mathrm{K}^{-1})\cdot\Delta T$, 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

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

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

Intramolecular force and hydration properties. In this case of long molecules in a parallel array, the intramolecular 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

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

$$= -\left(\frac{3D}{2} - \sqrt{3}\,\frac{\pi r^2}{D}\right)f(D).$$

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

Intramolecular stress and freezing point. It is also possible to relate the intramolecular 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, $P \cong (1.2\ \text{MPa} \cdot \text{K}^{-1}) \cdot \Delta T$. For most biological macromolecules, $\rho_m/\rho_w \cong 1$. Thus, when no solutes are present in the macromolecular phase,

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

When solutes are present in the macromolecular phase and have an osmotic pressure Π, this becomes $P - \Pi \cong (1.2\ \text{MPa} \cdot \text{K}^{-1}) \cdot \Delta T$.

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

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

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