Cryobiology and anhydrobiology of cells

Cryobiology and anhydrobiology share some features at the cellular level. This page analyses thermal and physical stresses at low temperature and/or low hydration

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Low temperature damage in cells can be divided into damage produced by three effects: (i) low temperature *per se*; (ii) direct effects of freezing and (iii) indirect effects of freezing. Cryobiology (the study of life at low temperatures) and anhydrobiology (the study of life at low water contents) have some features in common. This is because, in environmental freezing, one of the major causes of damage is freezing induced dehydration. This essay gives an introduction to several types of cryobiological and anhydrobiological damage at the cellular level. It concentrates however on freezing- or desiccation-induced cellular dehydration, and on our biophysical research in this area and on related systems. At the end we list references to the scientific papers summarised here.

Contents

Introduction

The study of damage produced by freezing and/or low temperatures and by low water contents is important in a variety of fields, of which here are some examples: In medicine, surgeons would like to be able to cryopreserve organs for transplants. To date, however, the cryopreservation of large organs (except blood) has a poor success rate. Blood and sex cells are routinely frozen and thawed for later use but even then, in many cases, the cellular survival rates are unacceptably low. Cryopreservation is also important in maintaining germplasm for important or endangered species. Frost damage is an important agronomic concern: if farmers can get a crop into the ground before the last frost, then they have a longer growing season and a greater yield. Damage in seeds during drying and rehydration may also be agronomically and ecologically important. Drying and freeze drying are also important in the food industry.

Freezing is worse than cold

Low temperatures and not really so bad, once we get over some homeothermic prejudices. *Homeotherms* (warm blooded animals like us) may be seriously damaged if our core temperature falls even several degrees for a sustained period. But this is rare in the animal world, and almost unknown in the plant world: most animals and almost all plants are *poikilotherms* – their temperature is not very different from that of the surroundings – so they survive the environmental temperature variations. With some exceptions (parasites of homeotherms, cave dwellers etc), environmental temperatures do vary substantially. Some large animals can reduce temperature variation by moving, by sweating, by metabolism etc, and some large plants control their temperatures by controlling radiation exchange, metabolism, and transpiration. But the overwhelming majority of living species can survive changes in temperature of tens of °C – many survive freezing. Small ranges of hydration are survived by most organisms. Many plants and lower animals (especially seeds and spores) can survive desiccation in air, but most cannot.

Further, **cryogenic temperatures** (those near the boiling point of nitrogen) are not dangerous *per se*. At liquid nitrogen temperatures, almost all biochemistry and physiology is so slow that nothing (including damage!) happens at those temperatures. Cooling to cryogenic temperatures may kill, and warming up may kill---we'll discuss these later---but little damage occurs in the cryopreserved state.

Freezing, however, is often deadly. On the scale of organs, the formation of ice can cause mechanical damage by expansion, or rupture as pointed ice crystals grow through the tissue. Further, if the ice forms inside the cell, the cell almost always dies. There have been a few recent reports of cells surviving **intracellular ice formation**

(IIF) *in vitro*, but IIF is usually taken by working cryobiologists as indicating cell death.

Ice is a poor solvent. When ice forms in an aqueous solution, most solutes are excluded from the ice, and remain in a concentrated unfrozen solution. So concentrations become very large and may be toxic. High concentrations of salts affect electrical or ionic interactions, including those that help stabilise the native state of enzymes. The unfolding and denaturation of enzymes is often irreversible. Further, ice and water interact differently with the surfaces of membranes and macromolecules, which is important because these interactions (via the surface tension of water or the hydrophobic effect) are also involved in maintaining the healthy state of the cell's internal anatomy or *ultrastructure*.

Before we go on to look at freezing damage, consider the question: **How to avoid intracellular ice formation?** It may be asked and answered at the physiological, ecological and medical levels. **Keeping warm** is the simplest answer, although this strategy is unavailable to many living things in most environments. Four other strategies are **supercooling**, **freezing point depression**, **dehydration** and **vitrification**,. These occur to varying extents in both the artificial process of cryopreservation and in environmental freezing. In all cases, the integrity of the cell's plasma membrane is critical. An intact membrane is necessary to prevent extracellular ice from nucleating intracellular ice. But an intact membrane is vital for other reasons too (e.g. maintaining the different compositions of intra- and extracellular solutions) and rupture of the membrane is also widely used as an indicator of cell death.

Avoiding intracellular ice formation

Supercooling

Supercooling refers to taking a liquid below its equilibrium freezing point, without freezing. Biological solutions *in situ* can usually supercool a couple of °C or more. From the point of view of the organism, this freezing avoidance mechanism has the advantage that the solutions remain liquid and allow relatively normal, though slower, metabolism. It has the disadvantage that a supercooled solution is unstable: if an ice crystal is introduced, it tends to freeze immediately, forming ice and a more concentrated solution. A very small volume of a pure solution may supercool as much tens of °C, but biological solutions are threatened by ice nucleators which may initiate freezing either inside or outside the cell. Supercooling is a vital strategy for some Antarctic fish that live in a solution with a higher concentration and greater freezing point depression than those of their own tissues. Their blood carries a potent protein "antifreeze". This substance, present only in tiny molar concentrations, does not

depress the equilibrium freezing temperature, but works by impeding the growth of ice crystals (DeVries, 1984). The environmental temperature for such fish has a robust lower bound: they will not encounter temperatures colder than the freezing temperature of the ocean. Without such a lower bound, the supercooling strategy is a dangerous one. Plant leaves often supercool a few degrees and may thus survive mild frosts without freezing damage, whereas slightly colder temperatures can cause extensive damage (Lutze et al., 1998). The importance of supercooling in cryopreservation is that it allows vitrification, which we look at later.

Freezing point depression

Many plants and animals found in cold environments accumulate soluble molecules (*solutes*) in their intracellular and often extracellular solutions (Leopold, 1986, Lee, 1989, Koster and Lynch, 1992). These solutes lower the equilibrium freezing temperature. Another result is that the organism is more resistant to dehydration, for osmotic reasons discussed below. Freezing point depression of more than a few °C requires rather highly concentrated solutions (see the conversion chart below). Large increases in the concentration of salts are rare in biological systems for the electrical reasons mentioned above. Solutes which can be tolerated in high concentrations (**compatible solutes**) include a number of sugars. High concentrations of such molecules increase the viscosity and thus reduce diffusion in solutions. This slows metabolism, but has advantages for slowing further dehydration and for vitrification, which we discuss later. The main importance of freezing point depression in cryopreservation is in the avoidance of crystallisation in the high-temperature, low viscosity régime.

Dehydration

Freezing usually occurs outside the cell first, because that's where there are more freezing nuclei, and because the extra-cellular solution has a larger volume than the intracellular solution. When this happens, the extra-cellular solutes are concentrated in a small quantity of unfrozen water, which necessarily has a higher osmotic pressure. This causes water to leave the cell. The characteristic time for water to flow out of cells under these conditions is tens of seconds (Wolfe and Bryant, 1992), so we refer to cooling as fast or slow according to whether substantial temperature changes are possible over this time scale. Cryopreservation usually uses fast cooling and so, when and if extracellular ice occurs, cells do not have time to dehydrate severely, although there is usually some dehydration of cryobiological importance. Environmental cooling, on the other hand, is slow so water is usually close to equilibrium, except under extreme dehydration.

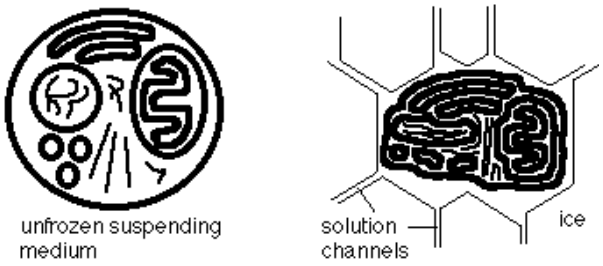

Figure: A very simplified sketch shows a cell before and after the freezing of its tissue or supporting medium. The elevated osmotic pressure causes a large reduction in the aqueous volume of the cell. Consequently, all of the non-aqueous components are brought into close proximity. In this state, stacks of membranes begin to resemble lamellar phases.

Many species of plants and animals, and especially their seeds and spores, survive freezing temperatures by cellular dehydration. Dehydration increases the osmotic pressure of the intracellular solution (the cytoplasm) which depresses its freezing temperature and promotes vitrification---both inhibit intracellular ice formation. Equilibration with ice at about -20 °C or with an atmosphere of about 80% relative humidity requires that a cellular interior have a composition of about 10 *osmolal*. Osmolal means roughly 'osmotically effective moles of solute per kg of water'. The amount of dehydration required to achieve this depends on the initial composition. If all solutes were ideal, and if the initial composition were 1 osmolal (a typical value for a plant cell, and a few times higher than that of most animal cells), then only 10% of the initial intracellular water would remain. If the initial composition were 2 osmolal, then 20% would remain. In practice, these water contents are underestimates because the osmotic pressure of many solutions increases more than linearly with concentration, and because of colligative properties of the cellular ultrastructure. Dehydration itself can cause damage, which we discuss later in some detail. The next figure shows the relationship among several variables that depend upon the chemical potential of water, which is a function of the freezing temperature or the atmospheric humidity.

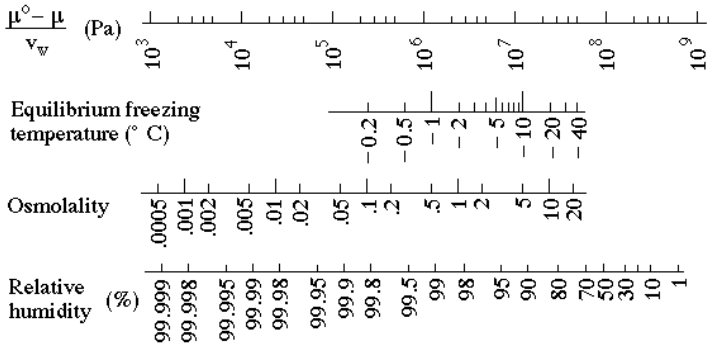

Figure: This figure allows conversion among several variables related to aqueous solutions. The first scale shows, on a log scale, the reduction in the chemical potential with respect to pure water at atmospheric pressure. Dividing

this by the molecular volume of liquid water gives it the dimensions of pressure. To the extent that water is incompressible, this quantity equals the osmotic pressure (Π) minus the hydrostatic pressure (P). The negative of this quantity is called the **water potential** (Slayter, 1967). The second scale shows the equilibrium freezing temperature of a solution whose water potential is given in the first scale. The next scale shows the composition (in osmolal) of a solution which would produce an osmotic pressure given by the first scale. The scale at the bottom shows the relative humidity of an atmosphere equilibrated with water having the water potential given in the first scale, or with ice at a temperature given by the second.

Intracellular vitrification

In cryopreservation, the usual goal is to achieve intracellular vitrification while avoiding intracellular ice formation and membrane damage. One of the deciding factors is the cooling rate. If a liquid is cooled sufficiently quickly, it can avoid freezing and vitrify (form an amorphous, glass phase). The necessary cooling rates are extremely high for pure liquids (e.g. 10 million degrees per second for pure water), but much more realistic for solutions. For aqueous solutions of typical cryoprotectants, cooling rates of about 0.1-10 °C/s (roughly 10-1,000 °C/min) are sufficient to achieve vitrification.

Molecules in a liquid undergo random, Brownian motion. For freezing to occur in a supercooled liquid, the diffusing molecules must spontaneously form a small cluster (called a nucleus or embryo) of molecules that temporarily has a structure similar to that of ice. In a supercooled liquid such clusters form and dissipate rapidly. If however the cluster is larger than some critical size, it becomes energetically favourable for other diffusing molecules to join the structure, and it grows through the sample (crystallising, or freezing). This process is called nucleation and crystal growth. Nucleation can be either homogeneous (as described above, and in the figure below) or heterogeneous, where an impurity (or the container wall) forms a substrate upon which nuclei can grow.

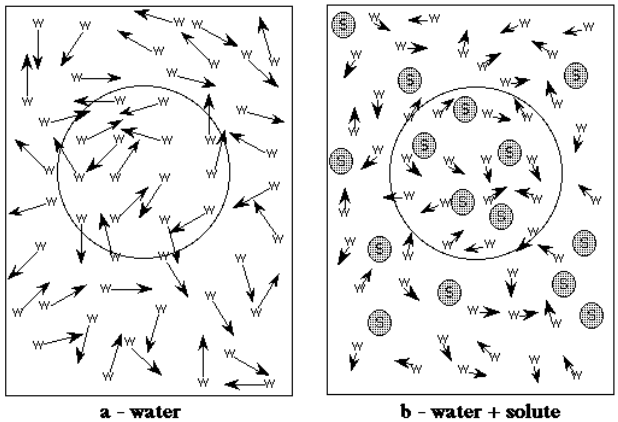

Figure: A simplified sketch of the effects of solutes on the nucleation of freezing. Water molecules and solutes are represented by the symbols w and s respectively. The arrows represent diffusion, and the length of the arrows

indicates the speed of diffusion. The large circles represent the critical nucleation radius. In (a), only water is present. For a critical nucleus to form, the water molecules in the volume represented by the circle must spontaneously arrange themselves into a regular ice-like structure. If this regular lattice is larger than critical size, then the crystal will grow. (b) shows the situation in the presence of solutes (the solute:water molar ratio is 1:4). First, solutes increase the viscosity, so diffusion is reduced (hence smaller arrows in (b) than (a)). Second, in order to form a critical nucleus, a volume equal to or greater that the critical radius must be completely free of solute molecules. This is not the case here. As the concentration of solutes increases, this effect becomes stronger, further reducing the chance of nucleation.

The probability of nucleation in a supercooled liquid depends on several factors: it increases with the volume of the sample and the degree of supercooling; it decreases with increasing solution concentration; and it also increases in the presence of impurities that can act as heterogeneous nuclei. A pure liquid in a small volume with no impurities can be supercooled a long way below its equilibrium freezing point. Small volumes (microlitres) of pure water, for example, can be cooled to about -40 °C.

At very low temperatures, the viscosity of a solution rises sharply, and molecular diffusion is reduced. If cooling is fast, then the viscosity rises rapidly, hindering nucleation. If cooling is sufficiently fast, the viscosity can become so large that molecular diffusion is effectively halted, and the probability of nuclei formation becomes negligible. The sample is then said to be a **glass** or **vitreous solid**, and the process is called **vitrification**. A glass is *amorphous* (unlike a crystal, it has no long range order) but has the mechanical properties of a solid. A material is said to be a glass if its viscosity reaches 10^{14} Pa.s (Franks, 1982). A glass is by definition in a state of very long-lived non- equilibrium.

Fast cooling and warming

So, how to get cells past the dangers that await them in freezing and warming? The next figure shows how for the case of fast cooling and warming---i.e. in artificial cryopreservation. In this case, the degree of osmotic contraction is usually small, as discussed above. Osmotic contraction depends on cooling rate (Mazur, 1963), which is in part responsible for there being an optimum in cooling rate. At very slow cooling rates, substantial osmotic contraction occurs, and this may be fatal in itself (discussed later). At very high rates, there is little osmotic contraction, so the solute concentration in the cytoplasm remains low. This makes vitrification less likely, and intracellular freezing more likely. At moderate rates (the value depends on the osmotic equilibration time of the cell and the propensity of its cytoplasm to nucleate ice), some non-fatal contraction which causes the concentration to increase sufficiently that vitrification can occur. A moderate osmotic contraction can be controlled by means other than cooling rate: one method is to cool the cells to a relatively high freezing temperature and allow them to contract to equilibrium prior to fast cooling. Another is to add a **non-penetrating cryoprotectant**, i.e. an extracellular solute which increases

the extracellular osmotic pressure. Hydroxyethyl starch (HES) or dextran are used. This has two effects: the moderately contracted cells avoid IIF during the initial cooling; and the extracellular solution vitrifies at relatively high temperatures. The detailed mechanisms of polymer cryoprotection are still unclear (e.g. Bryant et al., 94).

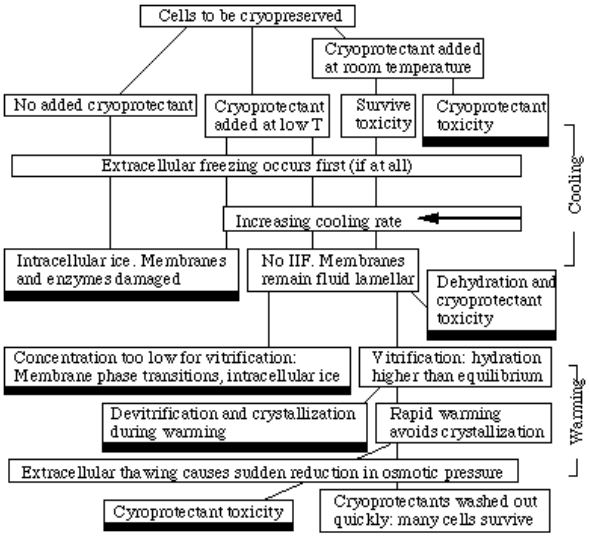

Figure: A simplified and incomplete flow chart for cryopreservation showing some of the steps and some of the dangers. The black rectangles represent cell death. Not shown on this chart is the possibility of vitrification of small volumes in the absence of cryoprotectants, using extremely rapid cooling.

The cytoplasm has a higher solute concentration, because of a combination of dehydration and the addition of cryoprotectants that can permeate through the membrane. This, together with the rapid cooling rate, may together allow vitrification as discussed above. Higher cooling rates allow lower doses of the toxic cryoprotectants, but cooling rates are often limited in practice by heat conduction in the samples being cooled, particularly for macroscopic organs.

Once the cell is vitrified, "**suspended animation**" is nearly achieved: all processes of metabolism and injury are slowed to almost zero. Provided that the sample avoids mechanical shocks (glasses are brittle!), the next threat it faces is crystallisation during warming. The vitreous state is unstable with respect to ice plus concentrated solution, but it is prevented from achieving the stable state by its very high viscosity. As the temperature rises, the viscosity falls, molecular motion becomes less slow, and water molecules may diffuse and rotate into the configurations required to nucleate ice, or to add to existing nuclei. The chance of large scale ice nucleation and/or growth occurring depends on the time the sample is exposed to lower viscosities while it is below the equilibrium freezing temperature. Thus successful warming should cross this temperature range quickly (Rall et al., 1984). As is the case with cooling,

warming rates are usually limited by conduction. Microwave heating has been proposed, but the rapidly changing absorption spectrum makes this more difficult (Baudot, 1997). There is the further problem that rapid but inhomogeneous heating can produce dangerous mechanical stresses in macroscopic tissues.

Slow cooling and warming

Even at sunrise and sunset, cooling and warming rates in nature are usually low compared to the characteristic time for osmotic equilibration. As a result, extracellular freezing often causes dehydration which approaches hydraulic equilibrium. An exception occurs when an intracellular solution vitrifies and thereafter undergoes no or very little further loss of water. In some cases of dehydration without vitrification, the viscosity may still be large enough to prevent or to slow further dehydration. The next Figure shows a flow chart for slow cooling and warming.

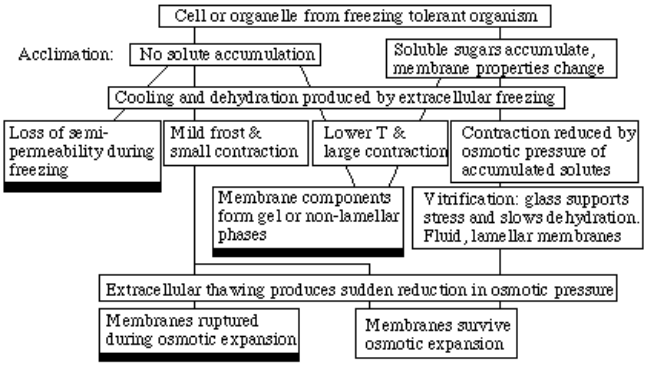

Figure: A simplified flow chart for freeze-thaw damage in some species that are capable of acclimation including solute accumulation. The black rectangles represent cell death.

The required equilibrium dehydration can be substantial. In an earlier figure we saw that freezing to -10° C requires osmotic pressures of more than 10 MPa and solutions of several osmolal. For typical cells with osmolalities less than one, this requires a several-fold reduction in water content. We are mainly concerned with the physical stresses produced by this freezing-induced dehydration, but we shall briefly describe two other damage mechanisms first.

Rupture during freezing and contraction

During this process of freezing and osmotic contraction, some cells rupture. Simple mechanical rupture by the advancing ice crystals is one likely cause. Another cause of rupture may be electrical: the large transient electric field associated with an advancing ice front in a weak electrolyte solution causes a potential difference across the cells, which is large enough to rupture membranes, and which is correlated with rupture (Steponkus et al., 1985).

Irreversible osmotic contraction

Osmotic contraction is often irreversible. Cells isolated from the leaves of some frost sensitive plant species have a limited ability to contract and to re- expand osmotically, and this is correlated with the occurrence of freezing damage. Plants which have been acclimated to low (but not freezing) temperatures are much less susceptible to frost damage at modest freezing temperatures, and their *protoplasts* (isolated cells) are capable of much larger osmotic excursions (Wiest and Steponkus, 1978). Protoplasts have the property that they become spherical when suspended in media having a large range of osmotic pressures. When the concentration of the suspending medium is increased, (the osmotic equivalent of extracellular freezing) the protoplasts initially become flaccid, but over several minutes they become spherical with a smaller area. The plasma membrane has a small resting tension---a few tenths of a mN/m. This is the process which is not always reversible. When the external medium is abruptly diluted back to its original composition (the osmotic equivalent of thawing), the volume increases rapidly and the area increases almost equally rapidly. The plasma membrane can support a tension of only several mN/m without rupture. Substituting this and a typical radius (10 microns) the Young-Laplace relation gives about 1 kPa as the maximum hydrostatic pressure that can be supported. This is about a thousand times less than the osmotic pressures involved, and so may be neglected for such cells (though not necessarily for small robust cells in less concentrated media, such as red blood cells). It follows that the area of the cell is determined by the flux of water into the cell. For a halving of the concentration of the suspending medium, an area increase of order 50% is required. The area elastic modulus for such a membrane is about 200 mN/m, so it is capable of an elastic expansion of only a few percent without rupture. Once the membrane stretches and its tension increases, membrane material is incorporated into the membrane at a rate which is a strong function of the applied tension (Wolfe and Steponkus, 1983). The biophysics of this problem is attractive in that all of the relevant parameters and functions can be measured: the osmotic properties of the solution, the hydraulic conductivity of the membrane, its relevant elastic modulus, the rate of membrane material incorporation and the probability of rupture as functions of tension. Further, the differential equations for tension and probability of lysis have analytic, though awkward, solutions (for details, see Wolfe et al., 1985, 1986, Dowgert et al., 1987). One result is unexpected: membranes from acclimated plants are slightly less robust than those from non-acclimated. However, they more than compensate for this by having a much greater rate of incorporation of new material.

Damage produced by severe dehydration

Freezing damage to frost tolerant plants is correlated with a different cellular malfunction: loss of membrane semipermeability in the freezing-induced dehydrated

state. Like the irreversible osmotic contraction described above, this symptom can be reproduced by osmotic manipulation at room temperature: protoplasts dehydrated in high concentration suspending media dehydrate but, when the medium is diluted, they fail to expand osmotically. A range of desiccation tolerant species of plants and animals also support substantial dehydrations but, in some cases, dehydration below a critical value (of order 10% water content) can be fatal. In this case, freezing damage and desiccation damage are similar and cryobiology and anhydrobiology overlap. In both cases, accumulation of solutes can reduce or prevent damage in model systems and species that are freezing or desiccation tolerant are observed to accumulate solutes, especially sucrose and trehalose (Leopold, 1986).

A range of symptoms of damage have been reported in the severely dehydrated state for both model membrane systems and the membranes of living cells. (i) In the lipids that are the major molecular component of cell membranes, the gel-liquid crystal phase transition occurs at higher temperatures (e.g. Crowe et al., 1988, Tsvetkov et al., 1989, Koster et al., 1994 and references in these papers). This is important because coexistence of the phases has been associated with reduced semipermeability. (ii) Membranes may undergo topological changes. Electron micrographs show membranes associated with arrays of long cylinders which resemble the inverted hexagonal phase formed by some lipid-water dispersions at very low hydration. In this phase, the water is found in long narrow cylinders on a hexagonal array, each cylinder surrounded by the hydrophilic moiety of the lipids. This geometry renders them unsuitable for forming a semipermeable barrier. Other topological changes have also been reported (Fujikawa 1995, Gordon-Kamm and Steponkus 1984, Uemura et al., 1995, Webb et al., 1993). (iii) Lateral phase separations may occur in the fluid state. At low hydrations, large areas of protein free membrane are observed in electron micrographs. Further, phase separation may occur to produce one phase rich in highly hydrating lipid species, and another rich in weakly hydrating species. The significance here is that the inverted hexagonal phases can most easily be formed by weakly hydrating lipids in the absence of protein.

We have proposed a simple model (Bryant and Wolfe, 1992, Wolfe and Bryant, 1999, 2001), which explains the above phenomena and, so far as we are aware, all the related data. Consider a cell whose water content has been reduced to (say) 10% by volume. Let us suppose that a membrane rich region of the cell has this same water content, and that its membranes are 5 nm thick. The membranes are therefore on average 0.5 nm apart. At this separation, all hydrophilic surfaces in water (including membranes) experience a strong repulsion called the hydration force which decreases approximately exponentially with separation, has a characteristic length of about 0.2 nm and whose extrapolated value at zero repulsion (Po) is typically 10-100 MPa. Removal of inter-membrane water in this regime either reduces the

thickness of the inter-membrane layer, and thus does considerable work against this strong repulsion, or else reduces the area of the inter-membrane layers and thus compresses the membranes in their plane. In practice it does both.

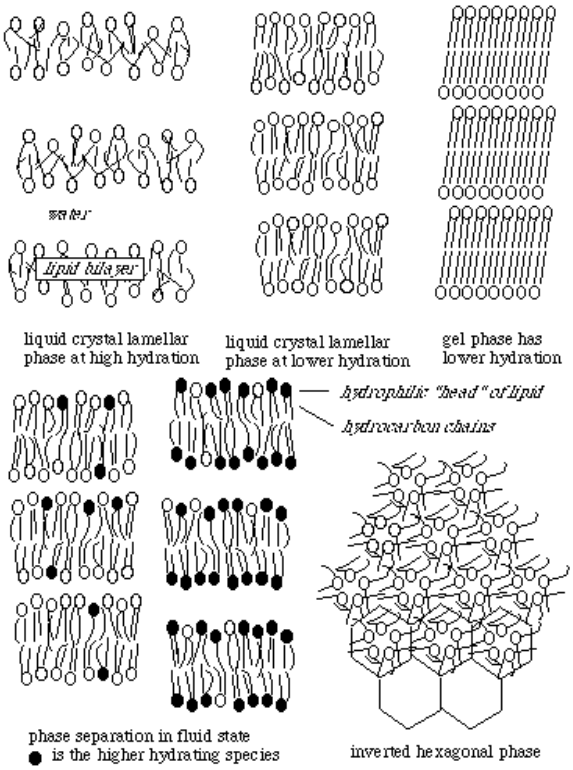

Figure: A cartoon of the lipid water phases of model membranes exhibiting dehydration strains

Mechanical equilibrium in the normal direction requires that the suction in the inter-membrane phase have the same magnitude as the repulsive force. In the lateral direction it requires that the membranes support a compressive stress equal in magnitude to the inter-membrane suction times the inter- membrane separation. Severe dehydration of membrane rich regions thus causes stacks of membranes which resemble a lamellar phase, while compressing them laterally to make them thicker in the normal direction. In regions of the cell rich in macromolecules, the macromolecules will also be pushed into close separation and will suffer anisotropic internal stresses which compress them along their longer axes, although in this case the geometry is less simple (Wolfe and Bryant, 1999). We will concentrate only on membranes here. Colligative properties of membranes and solutes are compared in the figure below.

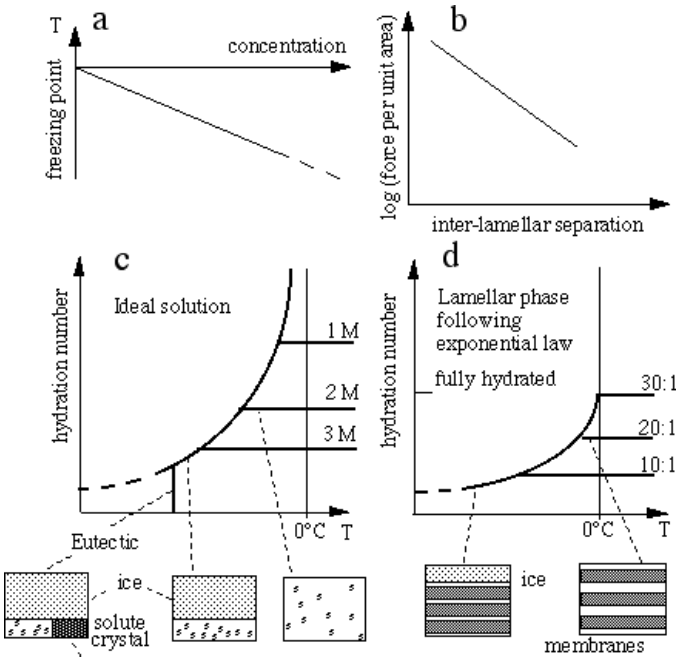

Figure: Comparing the freezing point depression due to solutes and to membranes. (a) and (c) show the behaviour of an ideal solution. In (a) it is shown as equilibrium freezing point depression as a function of concentration (for sugars, the experimental curve is lower than the line at high concentration). In (c) the same relation is represented as the molecular ratio water:solute, as a function of temperature. The horizontal lines show the simple fact that, for a sample with a given composition, the composition is constant above the equilibrium freezing temperature. The colligative effects of membranes are usually described in terms of the inter- membrane repulsion and the inter-membrane separation. For a large range of lipid membranes, this relation is well approximated by a repulsion that decreases exponentially with separation, as shown in (b). Converting this to a plot of water:lipid ratio as a function of temperature gives (d). Note the qualitative similarity to (c). In the membrane case, the water:lipid ratio has an upper limit: at about 30:1 the inter-membrane energy is a minimum (the force is zero) and so adding further water to such a sample simply creates an excess water phase (at temperatures above freezing), or more ice (at freezing temperatures). For experimental data, see Yoon et al. (1998) or Wolfe and Bryant (1999).

How large are these effects? Consider a single component lipid membrane. The gel-liquid crystal phase transition involves a reduction in area per molecule and an increase in its thickness. When these quasi two dimensional objects are compressed in the plane by a lateral stress π, the gel phase, which has a lower area per molecule, is favoured and the temperature of the transition is therefore elevated due to the Clausius-Clapeyron effect. (This is the effect which, in three dimensions, causes the boiling point of water to depend upon atmospheric pressure.) Using typical values this yields an elevation of order 0.5 °C for each extra mN/m in lateral stress. At a separation of 0.5 nm and with a repulsion of 20 MPa, the lateral stress is about 10 mN/m (more detail in Wolfe and Bryant, 1992; 1999).

One spectacular way in which membranes can respond to this lateral stress is by forming non-planar geometries, including the inverted hexagonal (H_{II}) phase, as shown in a previous figure. For weakly hydrating species, this geometry allows a large ratio of lipid volume to water volume, however this transition cannot be

analysed with such a simple model because it involves energies associated with the curvature of the interface.

In a membrane composed of mixtures of lipids having different phase transition temperatures, a number of the phases described above can coexist (e.g. gel-fluid coexistence) over a range of temperature. This is analogous to solid-liquid phase coexistence between the melting points of the components of mixtures of three dimensional materials.

In membranes at low hydration, a different mechanism can give rise to separation into two coexisting liquid crystal phases. Consider the case where two or more components have very different hydration properties (e.g. different P_o) - ie you have a mixture of a highly hydrating species and a weakly hydrating species. In this case an homogeneous mix of the components has an internal energy that is rather higher than that of the separated phases. In many cases, this difference in internal energy is large enough to overcome the entropy of demixing, and the mixture will phase separate. previous figure. Here one phase has a higher concentration of the highly hydrating species and a higher inter-membrane separation than the other.

This effect was first predicted theoretically (Bryant and Wolfe, 89), and then observed experimentally using small angle X-ray diffraction and solid state NMR (Bryant et al., 1992) for mixtures of POPC and POPE (two mixed chain unsaturated phospholipids typical of those found in plant membranes). In excess water, the two species are completely miscible, forming a single lamellar phase. At 10% water content and 315 K however, the mixture separates into two separate lamellar phases with different water separations. Dehydration induced fluid-fluid phase separations have since been observed for other systems (Webb et al. 1993).

The existence of fluid-fluid phase separations is not, in itself, necessarily a danger to biological materials. However, it could be a necessary intermediate stage in the formation of damaging inverted phases (such as H_{II}). Cell membranes are composed mostly of strongly hydrating lipids that tend to form lamellar phases at all hydrations. Weakly hydrating species (that tend to form inverted phases at low hydrations) are normally in the minority: if they were not, then the bilayer membranes would not be stable. Even under severe dehydrations, inverted phases are unlikely to occur in these membranes if they remain homogeneous. However, if fluid-fluid phase separation occurs, the weakly hydrating lipids are concentrated into low hydration fluid phases, and are then free to undergo the transition to an inverted phase if the hydration is low enough. Thus fluid-fluid phase separations may be a precursor to the formation of the inverted phases which have been correlated with membrane damage during dehydration and freezing.

The effect of solutes (cryoprotectants)

The presence of high concentrations of low molecular weight solutes in model membrane systems reduces the incidence of the effects associated with dehydration damage: they reduce the elevation of the membrane transition temperature and they reduce the occurrence of non-lamellar phases. This may be one reason why freeze- and desiccation- adapted species have evolved to accumulate solutes, often sucrose, trehalose or other sugars (eg Leopold, 1986, Crowe et al., 1988).

Osmotic effects

At a given chemical potential of water, the presence of more solutes requires the presence of more water (see first figure above). A cell or a vesicle that has a higher internal concentration at temperatures above freezing will contract less in equilibrium with ice at any given freezing temperature. Further, the addition of any new solute requires a reduction in the concentration of others already present. The presence of a high concentration of sugars reduces the concentration of ions that is required to produce a given osmotic pressure. So the presence of sugars reduces the dangerous high ion concentrations mentioned earlier. Solutes which can be accumulated in large concentrations without producing toxic effects of their own are called compatible solutes (Brown, 1976).

Reduction in mechanical stress

Provided that the solutes partition into the inter-membrane layers, their presence contributes osmotically to the lowering of the chemical potential of water. The larger the osmotic term, the smaller the suction and so the lower the stress imposed on the membrane. This effect can be considerable (Yoon et al, 1998; Wolfe and Bryant, 1999).

The proviso about partitioning is very important: many solutes, especially polymers, are excluded from the inter-membrane regions. As a result, their osmotic effect may have no direct effect on reducing the membrane stress, and it may even increase it (Koster et al, 2000). Solutes which remain external to the cell or to the vesicles in model systems will also give no direct reduction to membrane stress. At temperatures above freezing, the addition of the solute to the external solution can, in sufficiently high concentration, dehydrate the cell or vesicle sufficiently to increase the stress. Permeating cryoprotectants (such as DMSO) partition readily into the inter-membrane space. In model systems, it is not simple to produce high concentrations of non-permeating solutes in the inter-membrane space, so comparisons among different experiments should be made carefully, unless the inter-membrane concentration, rather than the total sample concentration, is measured.

Yoon et al. (1998) studied lipid-solute-water systems at freezing temperatures and used the nuclear magnetic resonance signal of the water to determine the distribution of solute and solvent between lamellar phases and a concentrated bulk solution phase in equilibrium with ice (they used either D_2O or deuterated solutes). For the small solute molecules (DMSO and sorbitol) they found that the phase behaviour was close to that expected using the effects discussed above and assuming no specific effects. They found that the disaccharides sucrose and trehalose (which have about twice the volume of the others) increased the hydration less than would be expected from their osmotic effects alone. This effect was consistent with a model in which these molecules were excluded from a very thin layer of water closest to the lipids. Yoon et al. found that all of the solutes studied decreased the intra-membrane stress, but that the disaccharides decreased it more than the smaller solutes. This is only one reason why sucrose and trehalose may occur so widely as natural cryoprotectants, however. Other reasons concern vitrification and crystallisation.

In principle, solutes could affect the membrane stress in specific ways. If the solutes bound to the membrane surface, for example, one would expect a modification in the hydration force. One study of inter-membrane forces using the Surface Forces Apparatus found no specific effects on the inter- membrane force due to DMSO, sorbitol or trehalose, however this study was limited for technical reasons to concentrations of about 1 $kmol.m^{-3}$(Pincet et al., 1994).

Vitrification and ultrastructure

An important extra consideration arises when and if the inter-membrane layer vitrifies. It has been found that under these conditions the dehydration- induced increase in the transition temperature is dramatically reduced, and the transition temperature can be reduced below the fully hydrated value T_o (Koster et al., 1994, Crowe et al., 1998). Consider a membrane at a fixed hydration at a temperature a few degrees above its transition temperature T_m. As the temperature is lowered to T_m the transition takes place, accompanied by a reduction in area per lipid (see previous figure). If however the inter-membrane aqueous solution is vitrified, the transition can not take place. The vitrified layer is a solid, so is capable of supporting considerable mechanical stress. If the temperature is lowered through T_m, the glass will impede the reduction in area necessary for the gel phase to form. As the temperature is lowered below T_m, the glass will support an increasing compressive stress in the membrane. At some point below T_o, the stress will become large enough to overcome the presence of the glassy matrix, and the gel transition will occur. During warming, the transition temperature will remain the same. Conversely, if the membrane is in the gel phase when vitrification occurs, the transition temperature will be raised above the fully hydrated transition temperature T_o, during both cooling and warming (Zhang and Steponkus, 1996).

In practice, the measured transition temperature in the presence of a glass is in the range 10-60°C below the fully hydrated transition temperature T_o. Koster et al. (2000) have measured the mechanical properties of a relevant sugar glass, and found Young's modulus to be about 20 GPa. Using parameters for DPPC (Guldbrand et al., 1982), and eq. (1), the compressive stress generated if the lipid remains fluid 20°C below T_o is about 40 mN/m, which corresponds to a strain of about 0.4% in the glass. That level of strain is easily supportable by a solid.

The importance of this effect is clear: if the solution is vitrified while the lipids are in the liquid crystal phase, then the transition temperature will be lowered dramatically, and the membranes will remain in the fluid state. In addition, the formation of the glass has two other important effects. First, if the solution vitrifies then further dehydration will be extremely limited; and second, if the sample is vitrified, solute crystallisation will be restricted. This is beneficial as the protective effects of solutes mentioned in the previous sections can only occur if the solutes remain in solution.

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Much of the above essay comes from the paper

- Wolfe, J. Bryant, G., (1999) Freezing, drying and/or vitrification of membrane-solute-water systems. *Cryobiology*, **39**, 103-129.

Other parts come from some of our other publications, also available on line:

- Yoon, Y. H., Pope, J. and Wolfe, J. (2003) "Freezing stresses and hydration of isolated cell walls" *Cryobiology*, **46**, 271-276.
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A list of publications by Joe Wolfe.

Related links

- What is 'unfreezable water'?. A FAQ in cryobiology and anhydrobiology.
- Membranes: homeostasis and regulation of area and tension, and how this relates to survival of freeze-thaw cycles.
- What the Hydration Forces Explanation *doesn't* say. A warning about some misquotations in this work.
- Freezing in alpine forests. A collaborative study with Marilyn Ball of ANU.