PHYSICAL PRINCIPLES OF MEMBRANE DAMAGE DUE TO DEHYDRATION AND FREEZING

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Water is the solvent for the ions, the organic solutes and many of the biochemicals necessary for active life, and the substrate for some of its reactions including photosynthesis. Water also allows relatively rapid diffusion of the reagents and products of vital reactions. Water is necessary, and it must be liquid water: ice is a much poorer solvent, and the diffusion in ice of solutes other than the hydroxyl ion is very slow.

Therefore there is almost no active life in organisms or tissues from which most of the water is removed, or in which most of the water is frozen. Any remaining unfrozen water forms highly concentrated solutions with very high viscosities, or is bound tightly to other molecules. The rates of diffusion and of biochemical reactions are very slow or zero.

As a result, the nearly dry or frozen state is, for some tissues and organisms, a state of suspended animation[1]: the processes of biochemistry and physiology slow almost to a halt, and recommence only when liquid water reappears. Seeds, for instance, survive with only a small fraction of water (5-15% by weight); so do the spores of bacteria. The tissues of resurrection plants are capable of rehydration to active life. The animals and micro-organisms of the soil such as nematodes, rotifers and tardigrades survive drying and rehydration. Similarly, some organisms, tissues and cells survive temperatures which freeze the environmental water (eg. woody tissues of plants, some insects, suspensions of some cells).

[1] The observation that seeds are a state of suspended animation must be almost as old as agriculture. Several millenia later, van Leeuwenhoek (1702) observed that some lower animals too could be resurrected to vigorous life from the almost inert, desiccated state. Three centuries after van Leeuwenhoek, anhydrobiology is now a recognized inter-disciplinary research area (Leopold, 1986) which incudes physiology, cell and molecular biology, biochemistry, colloid and surface science, thermodynamics and statistical mechanics.

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Such cases, however, are the exception rather than the rule: removal of most of the liquid water is usually fatal to cells, tissues and organisms. The explanation for this involves other properties of water: its surface tension and its capacity to form hydrogen bonds. These properties are of central importance in cellular ultrastructure.

In a textbook illustration of a cell, it appears that the cell membranes hold together the largely aqueous cytoplasm and the organelles. The reverse is also true: water holds cell membranes together. The surface tension of water - or more explicitly the high free energy associated with putting CH_2 and other non-polar groups into water - provides the attractive term in the free energy of the lipid matrix of biological membranes. The surface tension of water is also a dominant effect contributing to the tertiary structure of proteins. Proteins in their native state are folded so that their hydrophobic regions (such as the α helices) are in contact with other hydrophobic groups rather than water. Hydrogen bonding both within the protein and with water may also be involved.

For this ultrastructural role, the water must be liquid. The surface tension of ice is only about half that of liquid water, and the geometry with which it forms hydrogen bonds is different. As a result, proteins denature in ice. This denaturation may be irreversible, because there are often many possible tertiary structures in water. For an enzyme, the exact molecular geometry is important for its biochemical function, and only one (or a small number) of the possible foldings performs that function. Intracellular ice formation appears to be universally fatal (the authors know of no reported case of a cell surviving intracellular freezing) and it is likely that one of the causes is this irreversible denaturation of some vital enzymes.

In those cases where cells survive sub-freezing temperatures, the plasma membrane of the cell plays the crucial role in preventing the entry of ice. In nature, and in many of the clinical situations where cell suspensions are frozen, this entails allowing most of the intracellular water to leave the cell. Therefore cells which withstand freezing temperatures must also withstand dehydration, and in many cases the damage caused to cells by freezing temperatures is the damage caused by dehydration rather than by low temperature *per se*. For this reason we shall consider dehydration and freezing together in this paper.

The hydration of biological cells

In their active state, nearly all biological cells are surrounded by a water-permeable membrane. Most are also surrounded by an external medium which is or includes a relatively dilute aqueous solution. The hydraulic permeability of a membrane L_P is defined by $J_V = L_P(\Delta P - \sigma\Delta\Pi)$ where J_V is the volume flux of water, P the pressure, Π the osmotic pressure and σ the reflection coefficient for solutes. Consider a cell (initial volume V_o, area A_o), with an ideal semipermeable membrane ($\sigma = 0$), with $P = 0$ and with an osmotic pressure Π_o. Suppose that exposure to a change in osmotic pressure $\Delta\Pi$ results in a final volume change of ΔV. In the approximation $\Delta V \cong -V_o\Delta\Pi/\Pi_o$,

$$\frac{dV}{dt} = -AL_P\Delta\Pi \cong \frac{A_oL_P\Pi_o}{V_o}\Delta V = \frac{3L_P\Pi_o}{d}\Delta V = \frac{\Delta V}{\tau} \qquad (1)$$

where $d = 3V_o/A_o$ is a characteristic length of the cell (the radius in the case of a sphere) and where $\tau = d/3L_P\Pi_o$ is the characteristic time for equilibration of water through the membrane. For an order of magnitude, take $d = 10\ \mu m$, $L_P = 0.1\ pms^{-1}Pa^{-1}$ and $\Pi_o = 1$ MPa. Thus the characteristic time for osmotic equilibration is typically tens of seconds[2]. Because changes in humidity and temperature and other relevant variables are usually slower than this, the intracellular and extracellular water are usually at or near equilibrium, at least for relatively high hydration[3].

Equilibrium between intracellular and extracellular water (subscripts i and e) requires that the chemical potential of water μ be constant:

$$\mu = \mu^o + kT \ln a_i + P_iv_w = \mu^o + kT \ln a_e + P_ev_w \qquad (2)$$

where a is the activity of water, P its pressure, v_w its molecular volume and where μ^o, the standard chemical potential, is the value of μ for pure water at atmospheric pressure. The plasma membrane of animal cells cannot resist a substantial pressure difference, so at moderate or high hydration $a_i = a_e$ (in many cases this implies that the extracellular and intracellular solutions have similar (total) solute concentrations). In contrast, plant cell plasma membranes are supported by a cell wall which resists stretching beyond several percent, and so limits the cell volume. Under normal conditions the extracellular

[2] It is assumed that diffusion is much more rapid than permeation (as is the case for small cells) or that there is rapid mixing of the internal and external solutions.

[3] At sufficiently low hydration the elevated concentration of solutes gives the intracellular solution a very high viscosity and thus reduces the rate of water diffusion, particularly at low temperature. More on this anon.

solution is much more dilute (a_e approaches 1) than the intracellular solution, so $P_i > P_e$. If P_e is not too negative, P_i is positive and this pressure excess (called turgor pressure) distends the cell wall and contributes to the rigidity of leaves. The cell wall of most plant cells resists only small negative (internal) pressures, however, so cells collapse rather than suffer substantially negative P_i (Tyree and Hammel, 1972).

There is therefore a large range of hydration over which (intact) plant and animal cells behave approximately osmotically: changes in μ change a_i but $P_i \approx 0$. The water content (and thus the volume) of the cell is determined by μ and the number of solutes in the cell. Non-osmotic behaviour becomes pronounced at low levels of hydration.

Under most conditions, the water content of cells is typically about 90% by weight, where the 10% non-aqueous content comprises varying amounts of membranes, macromolecules and a range of solutes which give an osmotic pressure of between a few hundred kPa (for animal cells) and several MPa (for plants adapted to relatively dry environments). The water content of seeds and "dried" tissues is typically several percent. The water content of intact cells can fall to several percent if the chemical potential of the external water is lowered by about kT/3. All terms in μ may be varied: the pressure and temperature may be changed; μ^o may be varied by a phase change[4]; and the activity of water depends on the solute concentration. So a reduction in μ of kT/3 can be caused by:

i) equilibration with an atmosphere of relative humidity about 80%,
ii) freezing the external water to about -25 °C,
iii) increasing the external solute concentration to several kmol.m^{-3},
iv) applying a suction in the external water of about 30 MPa.

All of the above occur in nature - not to mention the laboratory[5] - with a range of severity. Equilibration with an unsaturated atmosphere confronts seeds, spores and many organisms separated from a source of water. Freezing of the external medium befalls not only the habitants of shallow ponds in

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[4] ($\mu^o_{water} - \mu^o_{ice}$) and ($\mu^o_{vapour} - \mu^o_{water}$) are the latent heats of fusion and evaporation respectively.

[5] where scientific ingenuity has produced other methods for dehydration, such as the application of large pressures in a "pressure bomb" (Scholander *et al.*, 1964).

intemperate climes, but also the cells in any tissue exposed to sufficiently low temperatures. Hyperosmosis (low a_c) is another insult imposed by extracellular

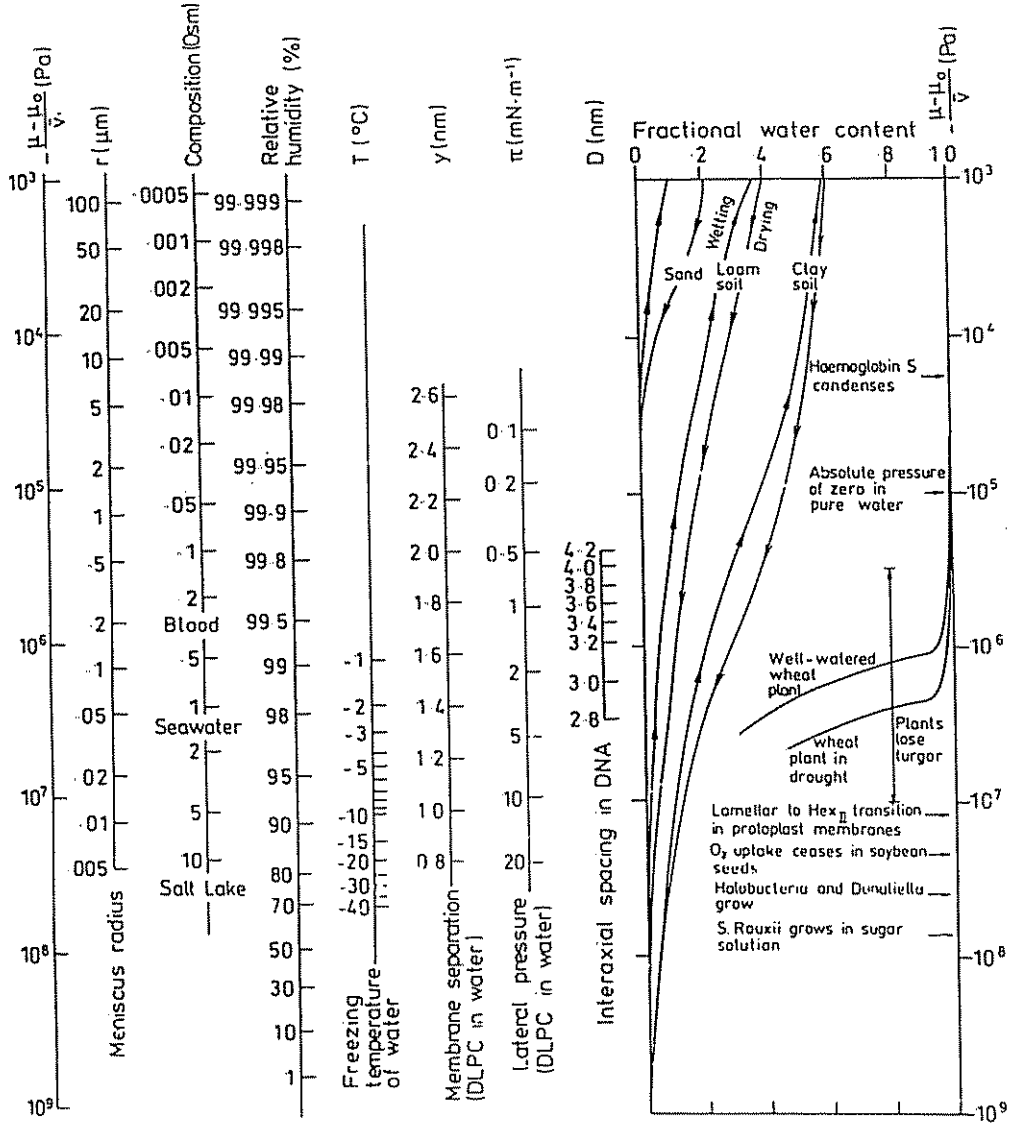

Figure 1. The equilibrium relations among several quantities relevant to low hydration. The data for dilaurylphosphatidylcholine are from Lis *et al.*, (1982) and those for DNA are from Parsegian *et al.*, (1986).

freezing, and it also strikes the residents of small, evaporating volumes of water evaporating ponds. Suctions are produced in the microscopic water volumes in soil or in plant cell walls - these suctions are applied by evaporation and are supported by highly curved menisci.

Relations among the relevant physical parameters are shown in Figure 1. Biologists use the quantity $(\mu-\mu^o)/v_w$ (Slatyer, 1967) which has dimensions of pressure and which they call the water potential Ψ. In the approximation that v_w is constant, (eqn 2) gives[6]: $\Psi = P - \Pi$. The logarithmic scale on the left is $-\Psi$, and it can be considered as the suction (the negative gauge pressure) in Pa. The next scale is the radius of a spherical meniscus of pure water which would support this suction in air at atmospheric pressure. The third scale is the solute concentration (in osmolal or moles per kg of water for an ideal solution) of a solution at atmospheric pressure in equilibrium which has the same μ. The fourth scale is the relative humidity of an atmosphere which has the same μ; the fifth is the temperature of (pure) ice which has the same μ. Thus the table shows for example that seawater can be in equilibrium with ice at about -3 °C (as is the nearly the case in arctic oceans), with an atmosphere of about 98% relative humidity, or pure water with a suction about 3 MPa, such as could be supported by a meniscus[7] with radius about 50 nm. Hydration relations for a lipid-water lamellar phase, DNA, a wheat leaf and for several soils are also shown.

Freezing of cells in aqueous suspension

Figure 2 shows several of the possible responses of cells in suspension to the freezing and thawing of the suspending medium. The extracellular medium freezes first for two reasons. First, the nucleation of freezing is a probabilistic phenomenon and, as the external solution has a much greater volume than any intracellular solution, nucleation is much more likely to occur outside. Second, it seems that many cells have a low concentration of ice nucleators.

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[6] Another component of Ψ called the Matric Potential is sometimes identified. This quantity can always be identified with components of the pressure or osmotic pressure. Passioura (1980) explains its use, and recommends against it.

[7] The law of Young and Laplace is applicable for menisci whose radii of curvature is as small as a few nm (Fisher and Israelachvili, 1980).

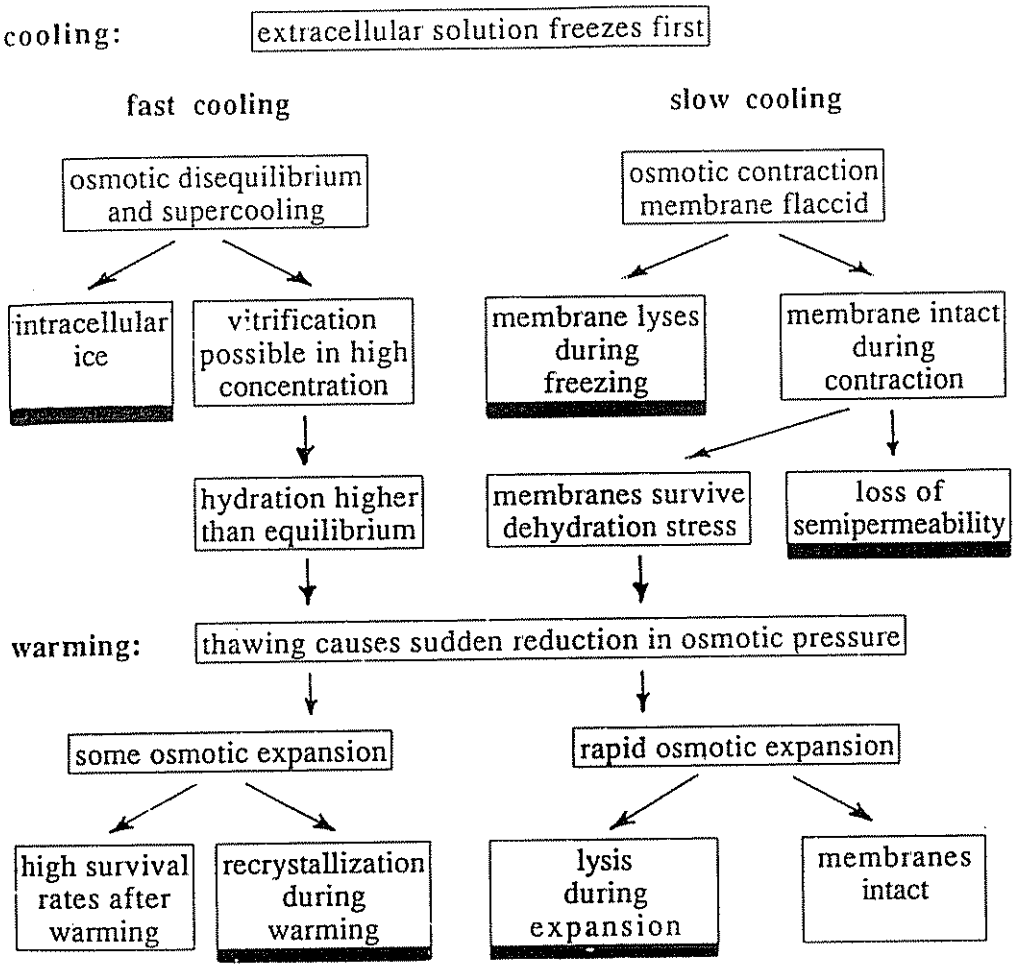

Figure 2. Pathways in a freeze-thaw cycle

Whatever the composition of the external medium (low concentration for plants, higher for animals), the solutes are much less soluble in ice than water, so external freezing produces a large fraction of nearly pure ice and a small fraction of concentrated solution whose chemical potential is determined by the temperature and pressure (see figure 1). Most of the external water freezes within a few degrees of zero so there can be a very rapid reduction in μ_c and a sudden increase in Π_c, especially if the solution supercools by a few degrees. Water diffuses out of the cell i.e. the cell contracts osmotically. In the figure "slow cooling" means that the temperature falls sufficiently slowly that water

can leave to maintain $\mu_i \equiv \mu_e$. "fast cooling" means that heat leaves the cell faster than water does, there is relatively little osmotic contraction, and so the internal solution becomes supercooled. Sufficient supercooling will usually cause ice nucleation in the cell at some temperature above about -40 °C, and thus cell death. (In figure 2 the horizontal lines signify cell rupture or death.)

A supercooled intracellular solution may however remain unfrozen indefinitely if it vitrifies[8], i.e. if it becomes exceedingly viscous (10^{12} to 10^{14} Pa s), due to the effects of high solute concentration and low temperature (Franks *et al.*, 1990). This is the route pursued in the cryopreservation of cell suspensions *in vitro*, where the internal concentration is often increased by adding a membrane-permeating solute called a cryoprotectant (eg. dimethylsulphoxide). Another method of provoking vitrification[9] is to allow some osmotic contraction (eg. by cooling first to a relatively warm sub-zero temperature), and then rapidly cooling. In many cases, vitrified cells may be stored in liquid nitrogen for years. The Scylla and Charibdis that face the cells during warming are recrystallization and osmotic rupture. Recrystallization, a paradoxical freezing during warming, occurs if the temperature rise causes the viscosity to fall sufficiently to allow the supercooled water molecules to orient and to form crystals. On the other hand there will be some osmotic expansion as the external medium thaws, and this may rupture the membrane. If these straits be safely passed, high rates of cellular survival are possible. Among the problems which limit the wider application of cryopreservation are these: the required concentrations of cryoprotectants may be toxic; and the cooling rates required for vitrification may not be achievable. The latter is particularly a problem for macroscopic organs[10] as distinct from cell suspensions.

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[8] Icecream is a common example of a metastable, supercooled solution whose viscosity at very low temperatures prevents it from separating into ice and a concentrated solution of sugar with suspended fats. If the mixture is warmed and cooled without stirring, it separates into its thermodynamically stable configuration.

[9] In some dehydration tolerant species, vitrification occurs at warm temperatures due to high concentrations of internal solutes. Some solutes such as trehalose and sucrose are reputed to be effective in this role (Crowe *et al.*, 1988).

[10] To any reader who may be contemplating paying a large sum of money for the right to have his/her body or parts thereof frozen in the hope of being reanimated by a more competent future civilization, the authors suggest that the vendors of this service be asked whether the cytoplasm of the cells of vital organs will be vitrified or crystallized. If the latter, then reanimation would require rapid reconstruction of denatured proteins *in situ*. If the former, then there are many serious cryobiolgists who would like to know the details, so as to be able to preserve human organs.

The right hand side of figure 2, the response to slow cooling, is the régime that is relevant to freezing and thawing in nature. Again the external medium contains some almost pure ice and some highly concentrated solution. The osmotic contraction that follows freezing to even several degrees below freezing usually requires a severalfold increase in osmotic pressure (see figure 1). This usually produces a severalfold decrease in the volume of the cell. Even for a cell that was spherical initially, the result is an irregular shape with a flaccid membrane. Thawing will produce a sudden decrease in osmotic pressure and allow a rapid osmotic expansion. The survival of a cycle of freezing and thawing has much in common[11] with the survival of osmotic dehydration and rehydration.

The big question is: how can a cell retain its ultrastructure during such a cycle? Maintaining the conformation of the proteins is obviously important, because denaturation can be irreversible. In the case of freezing, the maintenance of the cell membrane is vital, because its rupture allows ice to nucleate into the cytoplasm, and this, so far as the authors know, is always fatal. The integrity of membranes is almost equally important in the survival of any cycle of dehydration and rehydration because of their important topological function in separating different solutions - in the case of the plasma membrane, the quick from the dead.

On the right hand side of figure 2 (slow cooling and warming) there are three dead ends for the cell:

i) lysis (i.e. rupture of the cell membrane) during freezing,
ii) lysis during osmotic expansion, and
iii) loss of osmotic response at low hydration.

Our research on the first two was conducted in collaboration with Steponkus and co-workers at Cornell University. These studies used protoplasts (single cells from which the wall has been removed) isolated from rye seedlings that

[11] There are also many differences. First the low temperature itself (rather than its osmotic effects) may be injurious: biochemical reaction rates are differentially sensitive to temperature and so metabolic imbalances and the buildup of potentially toxic intermediary products is one possibility. The direct temperature effect is however less important in the study of freezing than it is in the study of chilling (damage inflicted by cold non-freezing temperatures). This may be because at freezing temperatures reaction and diffusion rates are so slow that metabolism is nearly stopped. Other differences are that environmental freezing usually lowers the chemical potential less than does air drying (see figure 1), and that the macroscopic mechanical effects in tissues are rather different for the two cases.

had either been exposed to cool but not freezing temperatures (acclimated) or not (nonacclimated). Currently we are concentrating on (iii).

One dead end in figure 2 is lysis during freezing. This is surprising, as one would not expect large mechanical stresses to appear in the membrane during the osmotic shrinkage that accompanies freezing: the membrane in cells of ordinary size bends rather than support compressive stress. For protoplast suspensions at least, this injury is unique to freezing and cannot be simulated by osmotic manipulation. This rupture occurs in the vicinity of the ice front and we accuse the very strong local electric field near the freezing interface of rupturing the membrane electrically. The evidence is circumstantial but strong: the rupture occurs in those conditions which give rise to a large transient potential difference and does not occur when the measured transient is small; and rupture of this type occurs with higher frequency in cells which are also damaged easily by electric fields (Steponkus *et al.*, 1984, 1985).

Another dead end for cells is rupture during osmotic re-expansion. The damage caused to protoplast suspensions by freezing to warm sub-zero temperatures and then thawing can be simulated at room temperature by equivalent osmotic manipulation. This damage has been correlated with frost tissue damage in non-hardy tissues. When protoplasts from non-acclimated seedlings are osmotically contracted, they become spherical at the new volume

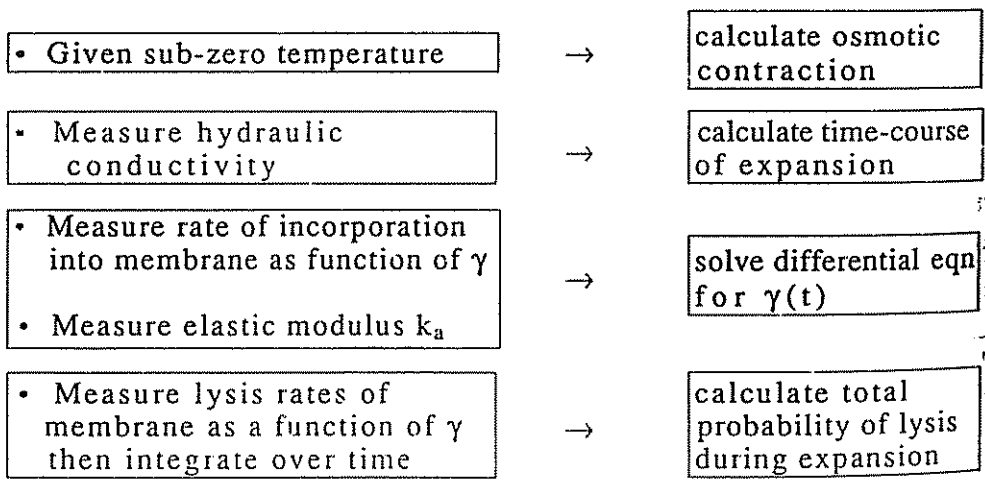

Figure 3. Analysis of cell lysis during thawing

by removing membrane material from the cell surface. When they are re-expanded (by dilution or thawing of the external medium), the surface tension in the membrane rises, and membrane material is re-incorporated. The chance of cell survival depends on the dynamics of this process, and on the dependence of membrane rupture on membrane tension. The relevant parameters may all be measured independently using micropipette aspiration, and the analysis of the process is set out in Figure 3. In this analysis, all parameters are directly measured, and there is good agreement with experiment (Wolfe and Steponkus, 1982; Wolfe *et al.*, 1985; Dowgert *et al.*, 1987).

Membrane damage at low hydration

Cells without walls behave osmotically in aqueous suspension over a large range of hydration: down to about 10 or 20% water. Protoplasts isolated from cold acclimated plants usually survive dehydration-rehydration or freeze-thaw cycles in the osmotic range, but suffer membrane damage while exposed to very low hydration, whether imposed osmotically or by freezing. They do not expand osmotically when the external solution is thawed or diluted.

The phenomenon is more general: many types or symptoms of damage occur in the range of water contents below about 20% by weight. This is also the range over which the mechanical stresses in membranes cease to be negligible in comparison with the relevant elastic moduli[12].

Removal of water at low hydration involves close approach of everything else in the cell, including the many internal membranes which are folded and forced together. When the thickness of solution separating the non-aqueous components of the cell is reduced to a few nanometres or less, large, repulsive hydration forces are encountered[13]. This repulsion can support a negative solution pressure or suction. Thus, at very low hydration, reductions in μ must

[12] At a final, summary session of a Rockefeller conference on anhydrobiology, C. Vertucci observed that many speakers had reported damage or unusual effects in the hydration range several-20%. (The conference was held in Bellagio, Italy. A.C. Leopold was the convenor and editor of the proceedings, 1986). Vertucci enquired whether there might be a general phenomenon involved, and Wolfe made the above observation about the magnitude of the stresses in membranes (elaborated by Wolfe, 1987).

[13] Aspects of hydration and other interfacial forces are treated by Israelachvili, Marcelja, Parsegian, Simon and Rand in other chapters in this volume.

produce reductions in pressure P as well as reductions in a_i, and this gives rise to non-osmotic behaviour[14].

Nearly all of our knowledge of hydration forces comes from simple systems. The model of biological membranes is the lipid bilayer or lamellar phase. These have been investigated by Israelachvilli, Parsegian, Rand and co-workers. Applying the same arguments to cells is a leap of faith, but a leap which is made less vertiginous by the observation of hydration repulsions among other cellular constituents including proteins and DNA (eg Parsegian *et al.*, 1986); and by the geometric similarities between various lipid water phases and the ultrastructure of dehydrated cells inferred from electron microscopy.

For membranes at very close approach, reduction of the water volume causes both reduction of intermembrane separation and contraction in the plane of the surface. Very close approach is opposed by the strong repulsion and the lateral contraction produces compressive stresses in the membrane. The stresses in the membrane interior are highly anisotropic - large and compressive in the plane of the membrane, much smaller in the normal direction. Several different strains are possible - of which the simplest is contraction in the plane of the membrane and thickening in the normal direction (Lis *et al.*, 1982). Others involve phase changes and phase separations.

In a quasi-two dimensional system such as a membrane, the lateral stress π (the compressive force per unit length in the plane of the membrane) is analogous to the bulk pressure in a three dimensional system, and it has a similar effect on the phase properties (Evans and Needham, 1987). Lipid bilayers contract in area (and increase in thickness) when they "freeze" from the fluid to the gel phase, so the temperature of this transition is lowered by lateral pressure (and raised by applied surface tension). Thus dehydration to the range which increases lateral pressure raises the transition temperature (Ulmius *et al.*, 1977; Kodama *et al.*, 1982; Seddon *et al.* 1984; Lynch and Steponkus, 1989).The area change is typically 0.2 nm^2, the latent heat of order 5×10^{-20} J per molecule, and so the change is of order 1 K for each mNm^{-1} of applied stress.

[14] Hydration forces between membranes may be measured directly (the Israelachvili method) or else by applying a suction hydrostatically or osmotically (the method of Rand, Parsegian and co-workers).

Hydration forces and the lateral stresses they cause may produce another effect in membranes at low hydration: fluid-fluid phase separations or demixing. This is possible because of the large range of magnitudes of the hydration force among different molecular species - among lipids this range is two orders of magnitude (Rand and Parsegian, 1989). Consider a lamellar phase with water and only two lipid components: one strongly hydrating which (on its own) gives rise to a large hydration repulsion, and another which hydrates weakly and has a small repulsion. If an homogeneous mixture of the lipids separates into one phase rich in the weakly hydrating species, and another rich in the strongly repelling species, then the former will have a smaller inter-membrane separation than the homogeneous mixture, and the latter a larger separation (see figure 4). Now because the hydration repulsion is strongly nonlinear, this configuration has a lower potential energy than does the homogeneous phase. If the difference in energy is greater than the entropy of demixing associated with the separation, then the separation is stable. An analysis of this effect is reported by Bryant and Wolfe (1989).

Such a separation has been observed in a model system. Palmitoyloleoyl-phosphatidylcholine (POPC) and palmitoyloleoylphosphatidylethanolamine (POPE) were chosen for the study because they are mixed chain unsaturated phospholipids (and thus are typical of plant lipids), and because the hydration repulsion of POPC is much greater than that of POPE. Above the thermotropic phase transition temperatures of both, these two lipids are

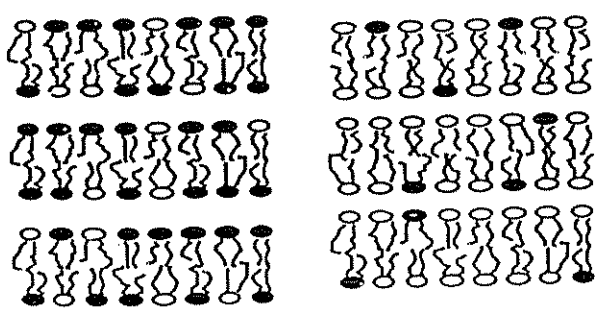

Figure 4. Lateral demixing in a lipid mixture. The lipids whose head groups are shaded in this sketch have a larger repulsion.

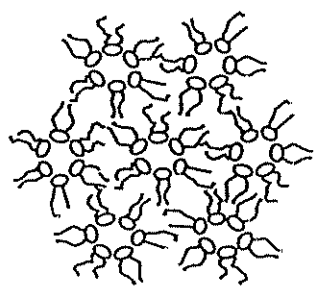

Figure 5. A section through an inverted hexagonal phase, perpendicular to the axes of the cylinders.

completely miscible in excess water. At 315 K and 10% water content, however, they separate into two fluid phases with different repeat spacings (Bryant, 1991). X-ray diffraction patterns of the mixture show two different repeat spacings consistent with fluid phases of POPE-rich and POPC-rich compositions. Further, the D_2O-NMR spectra of the sample shows two superimposed Pake patterns indicating that the water is distributed between two regions with different degrees of ordering. The ratio of hydration repulsions expected for these two lipid is within the range of the ordinary components of biological membranes, and so it is likely that similar separations occur *in vivo* in living systems[15] similarly dehydrated. The importance of such separations is that such demixing concentrates the lipids which can form the inverted hexagonal phase (discussed below), and thus may be a necessary precursor to its formation.

The transition of a lipid water mixture from the lamellar phase to the inverted hexagonal (H_{II}) phase is topologically spectacular. In the former, thin extended planes of water separate lipid bilayers; in the latter extended cylinders of water (whose axes lie on an hexagonal array, whence the name) are surrounded by the hydrophilic parts of the lipids while the hydrophobic parts fill the remaining space (Luzzatti and Husson, 1962; Luzzatti, 1968; Tardieu *et al.*, 1973; Luzzatti and Tardieu, 1974). This transition relaxes the compressive stress in the plane of the lipid-water interface and, in some cases, may also lower energy associated with the curvature moduli (see analyses by Kirk *et al*, 1984; Gruner *et al*, 1986). At a given hydration, the H_{II} phase is stable at higher temperatures than the lamellar, but at a given temperature it is stable at lower water contents. Thus the H_{II} phase may be induced by freezing cell suspensions: it is of course the osmotic dehydration and not the temperature reduction which is responsible. Gordon-Kamm and Steponkus (1984) studied electron micrographs of protoplasts that had been contracted either by freezing or by equivalent osmotic contraction at temperatures above freezing, and observed arrays of cylinders resembling the H_{II} phase. They correlated the

[15] In fact another example of such a separation is regularly reported in studies of dehydrated cells. When electron micrographs show regions of membranes rich in particles, these are interpreted as being rich in proteins, and conversely. Two effects (at least) may account for this separation: First, the proteins may be differentially soluble in two lipid phases which have demixed either due to the thermotropic transition, or due to the effect discussed above. Alternatively, the proteins may demix in a fluid membrane due to differential hydration repulsion. Membrane proteins are large and often protrude from the membrane surface, so they are expected to have a large hydration effect compared to lipids. The thermodynamics of this separation is discussed by Bryant and Wolfe (1989).

frequency of observation of these arrays with lack of osmotic response damage to protoplasts. The ultrastructural implications of this are clear: were a stack of membranes (say a folded region of the plasma membrane of a dehydrated cell, or a dehydrated mitochondrion or chloroplast) to form regions of hexagonal phase, the lumens would lose their erstwhile independence, and the osmotic response of the cell and its organelles would be lost.

The effect of cytoplasmic solutes

Plants exposed to cold but not freezing temperatures, or to low but not fatal μ_{water} may acclimate, i.e. become less susceptible to freezing or dehydration damage. Acclimated plants usually have higher cytoplasmic osmotic pressures. Dissolved sugars (trehelose in particular) are reported to contribute to the stability of membranes at low hydration (Crowe and Crowe, 1986; Crowe *et al.*, 1988; Clegg, 1986).

Cytoplasmic solutes have several effects which mitigate the damage caused by environmental dehydration: (i) they can slow or prevent the approach to equilibrium of water; (ii) they cause that equilibrium to occur at higher water content, and (iii) they reduce the strains imposed at a given water content. Let us consider these in turn:

i) Solutes are concentrated by dehydration and this may increase the viscosity to the range 10^{12} to 10^{14} Pa s where supersaturated or supercooled solutions form glasses (Franks *et al.*, 1990). In this state the diffusion of water is so slow that hydration levels above equilibrium may be sustained.

ii) High initial solute concentration (and higher osmotic pressure) at full hydration requires a larger volume of solution at any given μ_{water} due to osmotic pressure.

iii) At any given μ_{water}, higher Π implies less negative P (eqn 2). This smaller suction requires less intermembrane repulsion and thus creates less intra-membrane stress. Burke (1986) gives a review of the work on (i). We have investigated the effects (ii) and (iii) using the intensity of the narrow resonance of a D_2O-NMR signal[16] as a measure of the fraction of unfrozen water.

[16] The broad resonance is due to the ice, but its bandwidth is so large that there is no danger of confusing the two. This technique also yields data related to the degree of ordering of that remaining unfrozen water.

Freezing of lipid-solution mixtures or of cell suspensions allows a very simple control of the chemical potential of water[17]: $\mu - \mu_o = L(T/T^* - 1)$ where L is the latent heat and T^* is the freezing temperature of pure water. Consider a lamellar phase of lipid and solution in equilibrium with ice. The pressure in the solution equals -1 times the hydration repulsion per unit area, so write

$P = - P_o e^{-y/\lambda}$, where λ is the characteristic length of the repulsion and P_o is its extrapolated value at separation $y = 0$, and assume that these parameters are independent[18] of solute concentration (for more detail see the chapter by Rand). Let there be a volume V_u of (unfrozen) water and n molecules of an improbably ideal solute with partial volume v. Suppose that the total (one-sided) membrane area is A so that the solution volume is $Ay = nv_s + V_u$. With the approximation that the molecular volume v_w of (liquid) water is constant, eqn 2 gives:

$$\frac{\mu - \mu_o}{v_w} = P - \Pi \cong - P_o e^{-y/\lambda} - \frac{nkT}{V_u} = - P_o e^{-(nv_s + V_u)/\lambda A} - \frac{nkT}{V_u} \quad (3)$$

Several simple conclusions can be read from these equations. First, higher solute concentration (larger n) requires larger intermembrane separation y. Higher osmotic pressure means less negative pressures, and so smaller repulsions, which occur at larger separations. Further, solutes with larger partial volumes cause larger separations and less negative pressures. Finally, all else equal, (3) shows that solutes with larger partial volumes v_s imply a *smaller* volume of unfrozen water (though not a smaller volume of unfrozen solution). From (3):

$$\frac{dV_u}{dn} = \frac{kTA\lambda}{-PV_u} - v_s \quad (4)$$

This function is positive for small suctions, but negative for large suctions. For typical numbers, consider membranes separated by a few nm, λ ~0.2 nm and v_s a few times 10^{-28} m^3. The characteristic pressure is of order MPa. The dehydration properties of dipalmitoylphosphatidylcholine (DPPC) bilayers in different D_2O solutions is shown in Figure 6. The vertical axis is the integral of the narrow resonance of the NMR spectrum (approximately proportional to the quantity of unfrozen water). For pure D_2O this falls abruptly to zero a little below the equilibrium freezing temperature (277 K). For DPPC lamellar phases, the fraction of unfrozen water decreases continuously with decreasing

[17] and thus may be useful in dehydration as well as in cryobiological studies.
[18] Increasing the solute concentration should lower the dipole concentration and the dielectric permittivity, which might lower P_o and increase λ.

temperature. At low temperatures (low chemical potentials) the quantity of unfrozen water is least for the lamellar phase with sucrose solution, greater for sorbitol solution, and greater again for lamellae plus pure D_2O. For sucrose the unhydrated partial volume is 0.52 nm^{-3} and for sorbitol it is -0.27 nm^{-3}, so these results are qualitatively in agreement with (3) and (4).

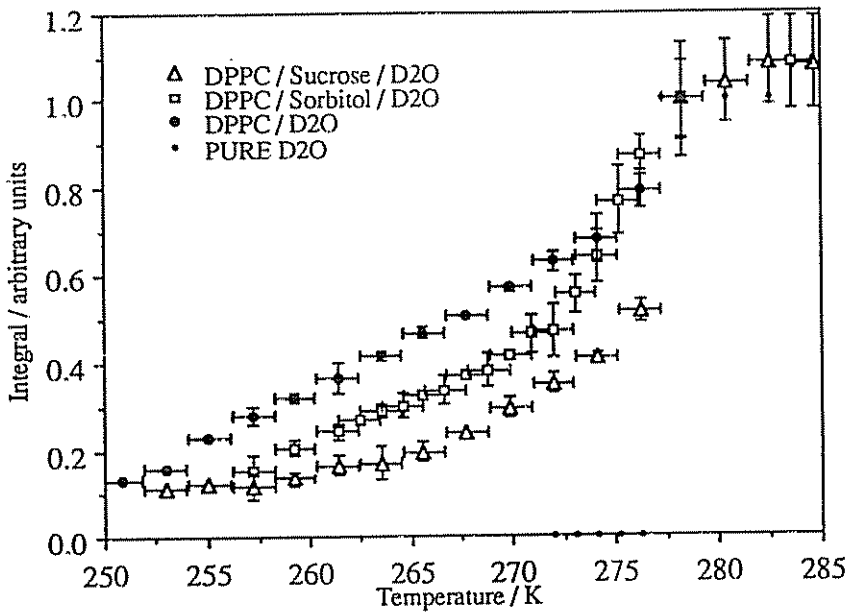

Figure 6. The intensity of the D_2O-NMR signal as a function of temperature.

The biological importance of this model is its implications for membrane damage. The lateral compressive stress π in the membrane (expressed as a lateral pressure or force per unit length) is just $\pi = |P_y|$, so the presence of solutes which can enter between the membranes lowers the intra-membrane stresses at any given chemical potential of water. This effect is greater for large solute molecules than for small. Figure 7 shows the calculated π as a function of temperature and water potential for membranes with pure D_2O and with three different solutions: a hypothetical ideal solute with zero partial volume, and ideal solutes with partial volumes corresponding to sorbitol and sucrose.

This model is still highly simplified: in particular no specific chemical effects of solutes have been included. Some solutes may have a large effect on P_o due

to interactions at the membrane interface, and this will influence membrane stress. Trehelose, for example, appears to have a specific interaction with the membrane and creates strains even in excess water where external stresses[19]

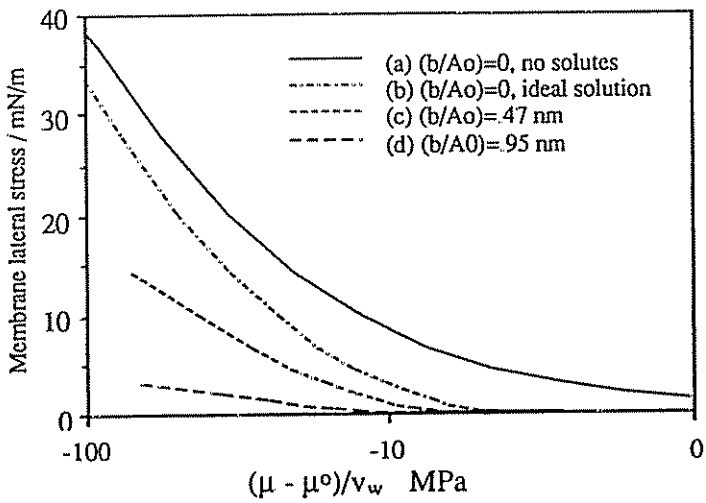

Figure 7. Calculated stresses in membranes as a function of the chemical potential of water in the presence of different solutions. The data for curves c and d are appropriate for sorbitol and sucrose respectively.

are negligible (Wistrom *et al.*, 1989). Nevertheless this simple model predicts substantial non-specific effects for large solutes on membrane freezing and dehydration behaviour. These predictions are in at least qualitative agreement with experimental results from model systems and, in preliminary experiments, from cell suspensions (Bryant, 1991). Analysis of specific solute effects on membrane stability will be a non-trivial exercise, and any such analysis must first consider the magnitude of these simple solute properties.

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