

Cellular cryobiology: thermodynamic and mechanical effects

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Abstract

Several physical stresses kill cells at low temperatures. Intracellular ice is usually fatal, so survival of freezing temperatures involves combinations of dehydration, freezing point depression, supercooling and intracellular vitrification. Artificial cryopreservation achieves intracellular vitrification with rapid cooling, modest osmotic contraction and, often, added cryoprotectants. High warming rates are required to avoid crystallization during warming. Environmental cooling is much slower and temperatures less cold, but environmental freezing damage is important ecologically and agronomically. For modest sub-freezing temperatures, supercooling sometimes allows survival. At lower temperatures, extracellular water usually freezes and cells may suffer large osmotic contractions. This contraction concentrates solutes and thus assists vitrification, but is not necessarily reversible: the rapid osmotic expansion during thawing may rupture membranes. Further, membranes and other ultrastructural elements may be damaged by the large, anisotropic mechanical stresses produced when their surfaces interact via hydration forces. Solute reduce these stresses by osmotic, volumetric and other effects. © 2001 Elsevier Science Ltd and IIR. All rights reserved.

Keywords: Cryopreservation; Freezing; Cryoprotection; Vitrification; Parameter; Ice; Supercooling; Dehydration; Thawing; Heat transfer; Rate – survival

Cryobiologie cellulaire : effets thermodynamiques et mécaniques

Résumé

Plusieurs contraintes physiques tuent les cellules à basse température. La formation de glace intracellulaire est presque toujours mortelle, et donc la survie aux températures congélantes implique l'association de la déshydratation, l'abaissement du point de congélation, la surfusion, et la vitrification intracellulaire. La cryoconservation artificielle nécessite une vitrification grâce à un refroidissement rapide, une contraction osmotique modérée et souvent l'ajout de solutés cryoprotecteurs. Des vitesses élevées de réchauffement sont indispensables afin d'éviter la cristallisation en chauffant. Dans la nature, le refroidissement et la température sont plus modérés, mais les dégâts produits par le gel sont importants sur les plans écologique et agronomique. Aux températures congélantes modérées, la surfusion permet parfois la survie. Aux températures plus basses, l'eau extracellulaire gèle en général et les cellules peuvent subir de grandes contractions osmotiques. Cette concentration des solutés favorise la vitrification, mais elle n'est pas forcément réversible. Une expansion osmotique rapide pendant la fusion peut rompre des membranes. En outre, les membranes et d'autres éléments ultrastructuraux peuvent être

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endommagés par de grandes contraintes anisotropes mécaniques produites lorsque leurs surfaces interagissent par les forces d'hydratation. Les solutés réduisent ces contraintes par des effets osmotiques, volumétriques et autres. © 2001 Elsevier Science Ltd and IIR. All rights reserved.

Mots clés : Cryoconservation ; Cryocongélation ; Cryoprotection ; Vitricification ; Paramètre ; Glace ; Surfusion ; Déshydratation ; Décongélation ; Transfert de chaleur ; Vitesse – survie

1. Introduction

1.1. Cold, freezing and cryobiology

For the purposes of cryobiology, it is convenient to divide 'cold' into three ranges of temperatures. Chilling temperatures are cool, but above 0°C. Sustained exposure to temperatures in this range is damaging to many of the cells of homeotherms (including us) and to chilling sensitive plants, but is survived by a very wide variety of plants and animals. Freezing occurs in the range from 0 to about -40°C, and it is over this range that freezing damage occurs. The range of life that can withstand sustained exposures to temperatures in this range is much smaller. At very low temperatures, however, cryopreservation is possible. Sustained exposure to cryogenic temperatures is not in itself dangerous: at these temperatures, biochemistry and physiology occur at rates that are effectively zero. If cells or organs are undamaged when they achieve the temperature of boiling nitrogen, little is likely to change provided that the temperature is maintained (and that the possibly fragile samples do not suffer mechanical damage). One of the aims of cryopreservation is, therefore, to achieve these low temperatures without incurring chilling damage (if relevant) or freezing damage.

Freezing is damaging for a range of reasons. In particular, cells rarely¹ survive intracellular ice formation (IIF): indeed IIF is very widely used as an indicator of cell death. Why is the solid form of this vital substance so damaging? A major problem with ice is that it is a poor solvent. When an aqueous solution freezes, the ice is nearly pure, and the remaining unfrozen water is the solvent for all of the solutes, at concentrations that become very large as temperature falls. These concentrations may be toxic. High concentrations of electrolytes affect ionic interactions, including those that help stabilize the native state of enzymes. Unfolding and denaturation of enzymes is often irreversible. Further, ice and water interact differently with hydrophilic surfaces. This is potentially important because the surface tension of water (the hydrophobic effect) is also involved in maintaining the native state of enzymes. Ice

crystals may also be involved directly in mechanical damage to the cellular ultrastructure. On a supra-cellular scale, we should note that damage may be done to organs by ice crystals or by the volumetric expansion that accompanies freezing, but we shall not deal with these in this paper.

Before looking at the physical causes of freezing damage, let us briefly address the ecological, physiological and medical question: How to avoid IIF? Remaining above freezing temperatures is one strategy, and one which we warmly recommend, although it is unavailable to poikilotherms ("cold-blooded" animals) and plants in many environments. Four others are supercooling, freezing point depression, dehydration and vitrification, which 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 widely used as an indicator of cell death.

2. Avoiding intracellular ice formation

2.1. Supercooling

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 with respect to coexisting phases of ice and a more concentrated solution. While a very small volume of a pure solution may supercool tens of °C, biological solutions usually contain ice nucleators which may initiate crystallization either inside or outside the cell. Supercooling is a vital strategy for some Antarctic fish that live in seawater whose high concentration of salt gives it an equilibrium freezing point lower than those of their own tissue solutions. Their blood carries a potent protein "antifreeze". This component, present only in small molar concentrations, does not depress the equilibrium

¹ We draw attention, however, to recent reports of innocuous intracellular ice formation by [40] in a monolayer of canine kidney cells attached to glass coverslips.

freezing point, but works by impeding the growth of ice crystals [1]. The environmental temperature for such fish has a robust lower bound, determined by the equilibrium freezing point of the sea-water. 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 [2]. The importance of supercooling in cryopreservation is that it allows vitrification (discussed below).

2.2. Depression of equilibrium freezing point

Many plants and animals in cold environments accumulate solutes in the intracellular and often extracellular solutions [3–5]. One result is the depression of the equilibrium freezing temperature. Another is the osmotic resistance to dehydration, discussed below. Freezing point depression of more than a few °C requires rather highly concentrated solutions (see Fig. 1). Substantial increases in the concentration of ions are rare in biological

systems: the effects of electrolytes on electrical interactions are important in maintaining the active states of enzymes (mentioned above) and other aspects of cellular ultrastructure. 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 crystallization under conditions of relatively warm sub-zero temperature and low viscosity.

2.3. Dehydration

Freezing is a probabilistic process and, in most cases, the extra-cellular solution has a larger volume than the intracellular solution. For this and other reasons, extra-cellular freezing usually occurs first. When it does, the extra-cellular solutes are concentrated in a small quantity

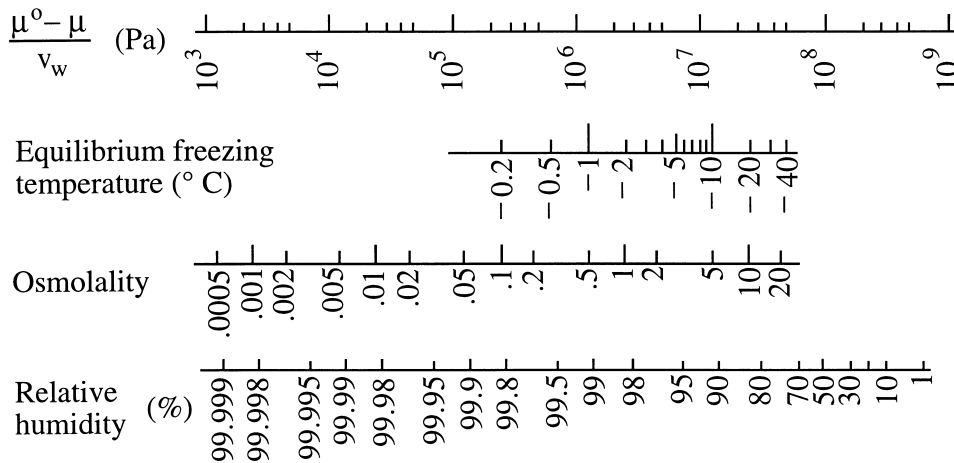


Fig. 1. Several variables related to aqueous solutions are plotted for comparison. The first scale shows, on a log scale, the reduction in the chemical potential with respect to pure water at atmospheric pressure. Dividing by the molecular/molar volume of liquid water v_w gives it the dimensions of pressure. To the extent that water is incompressible, this quantity equals $\Pi - P$ where Π is the osmotic pressure in the solution and P is its hydraulic pressure. The negative of this quantity, i.e. $P - \Pi$ is called the water potential [6]. 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 (for an ideal solution, the osmolality equals the number of moles of solute per kg of solvent). 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. Most human tissues have about 300 mosmol kg^{-1} , which gives a freezing point depression of about 0.5°C.

Fig. 1. Comparaison de plusieurs variables relatives aux solutions aqueuses. La première échelle logarithmique montre la diminution du potentiel chimique, comparée à celle de l'eau à la pression atmosphérique. En divisant par le volume molaire de l'eau liquide v_w , on obtient la dimension d'une pression. Etant donné l'incompressibilité de l'eau, cette pression est équivalente à $\Pi - P$ où Π est la pression osmotique de la solution et P est sa pression hydraulique. $P - \Pi$ égale le potentiel de l'eau [6]. La seconde échelle montre la température de congélation en équilibre pour une solution dont le potentiel de l'eau est donné par la première échelle. La troisième échelle montre la composition (en osmolal) d'une solution qui produirait une pression osmotique donnée par la première échelle (pour une solution idéale, l'osmolalité égale le nombre de moles de soluté par kg de solvant). La dernière échelle montre l'humidité relative d'une atmosphère en équilibre avec de l'eau possédant le potentiel d'eau donné par la première échelle, ou avec de la glace à une température donnée par la deuxième échelle. La plupart des tissus humains ont environ 300 mosmol kg^{-1} , ce qui donne lieu à un abaissement du point de congélation d'environ 0,5°C.

of unfrozen water, which necessarily has a higher osmotic pressure. This causes water to leave the cell. The characteristic time τ for hydraulic equilibration is $d/(3L_P\Pi_0)$ where d is a characteristic cellular dimension, L_P is the hydraulic permeability of the membrane and Π_0 is the osmotic pressure. For typical values ($d \sim 10 \mu\text{m}$, $L_P \sim 0.1 \text{ pm s}^{-1} \text{ Pa}^{-1}$, $\Pi_0 \sim 1 \text{ MPa}$, order of magnitude estimates only), τ is tens of seconds [7]. We refer to cooling as fast or slow according to whether substantial temperature changes are possible in times of this order. Cryopreservation often uses fast cooling and so, when extracellular ice occurs, cells do not have time to dehydrate severely, although there is usually some dehydration of cryobiological importance. In nature, cooling is slow and consequently water is usually close to equilibrium, except when vitrification occurs.

Many species of plants and animals, and especially their seeds and spores, survive freezing temperatures by achieving very low intracellular water contents. 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 (see Fig. 1). 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 (see Fig. 1). 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.

2.4. Nucleation and crystal growth

All molecules in a liquid undergo 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 which has a transient 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 (crystallizing, or freezing). This process is called nucleation and crystal growth. Nucleation can be either homogeneous (as described above) or heterogeneous, where an impurity (or the container wall) forms a substrate upon which nuclei can grow.

The probability of nucleation occurring in a supercooled liquid depends on a number of factors: the probability 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 which 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 without freezing.

2.5. Intracellular vitrification

In cryopreservation, the primary goal is to achieve intracellular vitrification while avoiding intracellular ice formation and membrane damage. One of the critical factors in determining success is the cooling rate. If a liquid is cooled sufficiently quickly, it will vitrify (form an amorphous, glass phase). The necessary cooling rates are extremely high for pure liquids (e.g. $10^7^\circ\text{C s}^{-1}$ for pure water), but much more realistic for solutions. For aqueous solutions of typical cryoprotectants, cooling rates of the order of $0.1\text{--}10^\circ\text{C s}^{-1}$ (of the order of $10\text{--}10^3^\circ\text{C min}^{-1}$) are sufficient to achieve vitrification.

If a solution is cooled to very low temperatures, the viscosity rises sharply, and molecular diffusion is reduced. If cooling is fast, then the viscosity rises rapidly, hindering nucleation. If cooling occurs 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 has the properties that it is amorphous (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 [8]. A glass is by definition in a state of stable (i.e. very long-lived) non-equilibrium.

The maximum possible cooling rate is limited by the size of the sample to be cooled. Cell suspensions (i.e. populations of cells suspended in a medium with an osmotic pressure equal to that of the cytoplasm) can be placed in containers with at least one small dimension (e.g. straws of small diameter or flat bags). These may be vitrified by very rapid cooling without the addition of further solutes. For macroscopic tissues, the concentration is increased by the addition of extra solutes called cryoprotectants.

3. Fast cooling and warming

Fig. 2 shows some of the pathways of fast cooling and warming of cells and some of the dangers associated with them. In fast cooling, the degree of volumetric

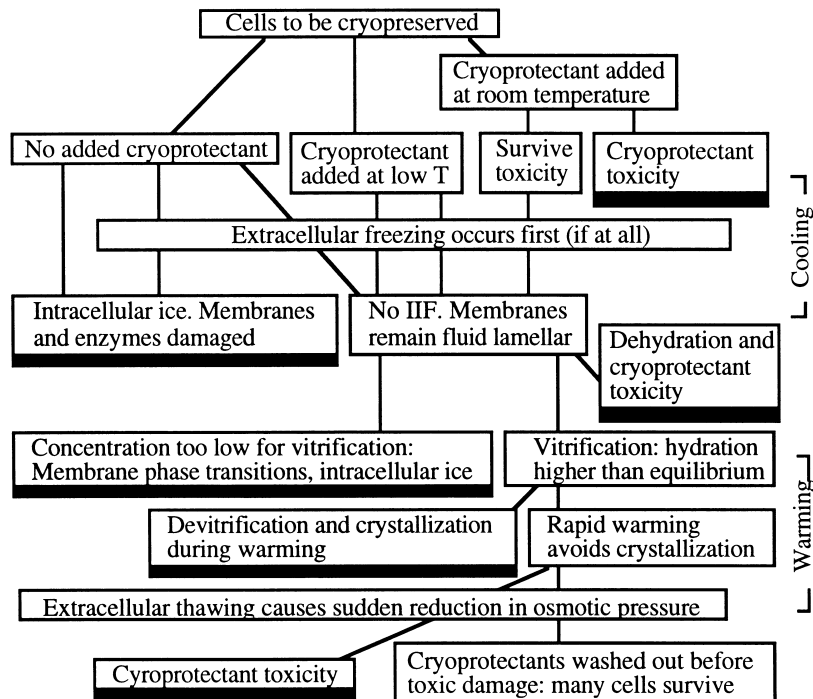


Fig. 2. A simplified and incomplete flow chart for cryopreservation showing some of the steps and some of the dangers. The black rectangles represent cell death. Not shown on this chart is the possibility of vitrification of small volumes in the absence of cryoprotectants, using extremely rapid cooling. [Injury at temperatures above 0°C may also be important in cryopreservation but is not shown here. Arav et al. [41] discuss the effect of chilling temperatures on phase transitions in the membranes and other ultrastructural elements of bovine egg cells. Baust et al. [42] briefly report “cryopreservation-induced apoptosis (programmed cell death)” in engineered tissue.]

Fig. 2. Schéma simplifié et incomplet de la cryoconservation, montrant certains stades et certains risques. Les cases soulignées en noir montrent la mort cellulaire. Ce schéma ne montre pas la possibilité de vitrifier des petits volumes en l'absence de cryoprotecteurs, utilisant un refroidissement très rapide. [Les altérations aux températures au dessus de 0°C peuvent être importantes en cryoconservation mais ne sont pas montrées ici. Arav et Coll. [41] ont étudié l'effet des températures de refroidissement sur les transitions de phase dans les membranes ou d'autres éléments structuraux des cellules d'ovocytes de boeuf. Baust et al [42] ont rapporté « de l'apoptose (more programmée des cellules) induite par la cryoconservation » dans les tissus en cours de manipulation.]

contraction due to osmotic effects is usually modest (discussed above). The dependence of this contraction on cooling rate [9] is in part responsible for an optimum in cooling rate. At very slow cooling rates, substantial volumetric contraction occurs, and this may be fatal in itself (discussed later). At very high rates, there is little volumetric contraction, so the solute concentration in the cytoplasm does not increase substantially. This makes vitrification less likely, and intracellular freezing more likely. A moderate rate (its value depending on the osmotic equilibration time of the cell and the propensity of its cytoplasm to nucleate ice) allows 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 option is to cool the cells to a relatively high sub-zero 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 [such as hydroxyethyl starch (HES) or dextran]

which decreases the chemical potential of the extracellular water. This has two important effects: the moderately contracted cells avoid IIF during the initial cooling; and the extracellular solution vitrifies at relatively high temperatures. The exact mechanisms of polymer cryoprotection are still unclear (e.g. [10] and references therein).

High intracellular concentration inhibits intracellular ice formation. If it is achieved by osmotic contraction, then the dehydration may be severe enough to cause damage, or the elevated concentration of ions may be dangerous. An alternative way of increasing the intracellular concentration is by using a permeating cryoprotectant, of which the two most common are glycerol and dimethylsulphoxide (DMSO). DMSO is especially widely used in cryopreservation because it permeates membranes rapidly. To permeate quickly, a solute needs to be membrane-soluble as well as water-soluble. The problem with this is that solutes which partition into the membrane change its structure and may thus damage it

and kill the cell. Permeating cryoprotectants are generally toxic. Thus cryopreservers must make a compromise among values of the parameters: concentration, duration of exposure to cryoprotectants at warm temperatures and the temperature of that exposure. A high concentration of cryoprotectants added to the supporting medium at warm temperatures with a relatively long exposure time would prevent IIF, but would be toxic. The optimum combination of concentration, time and temperature is usually determined empirically. There are two advantages in reducing the time of exposure to a permeating cryoprotectant at warm temperatures: first toxicity is reduced; second, a short exposure time leads to moderate dehydration, which helps avoid IIF. Cell membranes are more permeable to water than to all cryoprotectants, so when cryoprotectants are added to the extra-cellular solution, the initial osmotic response of a cell is contraction. As the cryoprotectant enters, dehydration slows and, if the exposure time is long, ultimately reverses.

The higher concentration of all solutes in the cytoplasm (achieved by a combination of dehydration and the addition of permeating cryoprotectants) and a sufficiently rapid cooling rate may together allow vitrification as discussed above (see also Fig. 2). Higher cooling rates would allow lower doses of the toxic cryoprotectants, but cooling rates are often limited 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 by a very large factor. Provided that the now brittle sample avoids mechanical shocks, the next threat it faces is crystallization 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 [11]. As for cooling, warming rates are usually limited by conduction. Microwave heating has been proposed, but the rapidly changing absorption spectrum makes this more difficult [12]. There is the further problem that rapid but inhomogeneous heating can produce dangerous mechanical stresses in macroscopic tissues.

4. Slow cooling and warming

Even at sunrise and sunset, cooling and warming rates in nature are usually relatively slow compared to

the characteristic time for osmotic equilibrium. Consequently, 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. Fig. 3 shows a flow chart for slow cooling and warming.

The required equilibrium dehydration can be substantial. Fig. 1 shows that freezing to -10°C requires osmotic pressures of more than 10 MPa and solutions of several osmolal. (The solutes are not the only cellular components to lower the chemical potential of water: the membranes, macromolecules and other ultra-structural components have colligative effects, as we shall see shortly.) For typical cells with osmolalities less than one, this requires a several-fold reduction in water content. This paper will be mainly concerned with the physical stresses produced by this freezing-induced dehydration, but we shall briefly describe two other damage mechanisms first.

4.1. Rupture during freezing and contraction

Some cells rupture during the process of freezing and osmotic contraction. One of the causes 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 [13]. (Electrical rupture of a cell usually requires an electrical potential difference of order 1 V. To readers accustomed to the potential differences (of order $kT/e \sim 20$ mV) which appear in equilibrium electro-chemistry, this may seem surprising. The key observation here is that a moving ice-water interface implies a large heat flux normal to the interface and therefore a system that is far from equilibrium. Ice dissolves some ions better than others and as a result the moving interface can generate a potential difference of tens or even hundreds of volts [13,38].)

4.2. Irreversible osmotic contraction

One important cause of damage is the irreversibility of the process of osmotic contraction. Freezing damage in some frost sensitive plant species is correlated with the observed inability of protoplasts (isolated plant cells) from leaves to contract and to re-expand osmotically. 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 are capable of much larger osmotic excursions [14]. Protoplasts have the property that they become spherical when suspended in media having a large range

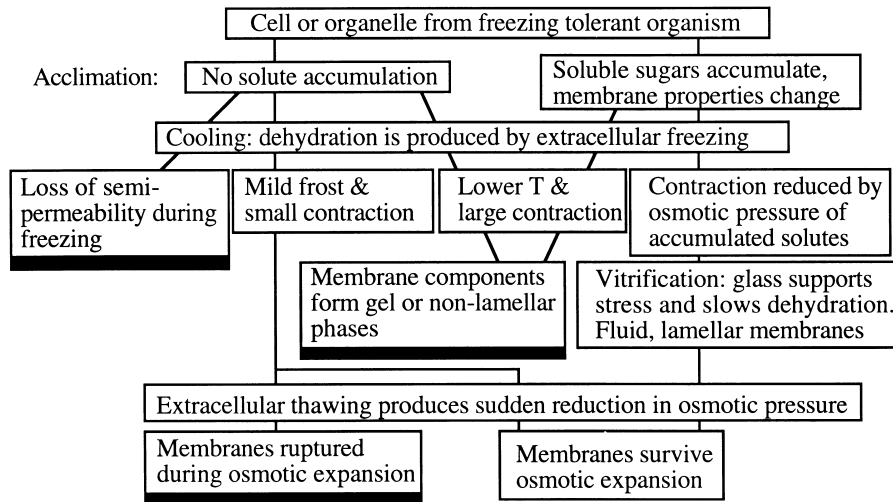


Fig. 3. A simplified flow chart for freeze–thaw damage in some species that are capable of acclimation including solute accumulation. The black rectangles represent cell death.

Fig. 3. Schéma simplifié des dégâts dus la congélation/décongélation pour certaines espèces capables d'acclimation lors de l'accumulation du soluté. Les cases soulignées en noir montrent la mort cellulaire.

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 protoplast plasma membrane has a small resting tension — a few tenths of a mN m^{-1} . 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 hence the surface area increases almost equally rapidly. The plasma membrane can support a tension of only several mN m^{-1} without rupture. Substituting this and a typical radius ($10 \mu\text{m}$) the Young–Laplace relation gives about 1 kPa as the maximum hydrostatic pressure that can be supported. This is about a thousand fold 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 in the order of 50% is required. The area elastic modulus for such a membrane is about 200 mN m^{-1} , 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 [15]. 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 [16–18]). 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.

4.3. 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 [3].

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. [19–21] 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 [22–25]. (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.

A simple model has been proposed [26–28] which can explain the above phenomena and, so far as we are aware, all the related data (e.g. [29]). 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 (P_0) is typically 10–100 MN m⁻². 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 (Fig. 4). Mechanical equilibrium in the normal direction requires that the negative² pressure 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

² At first the idea of a negative pressure of several MPa may seem odd. It should be noted that these are hydrophilic surfaces separated by a distance much less than the critical radius for cavitation [30] and so very large suctions can be supported without cavitation.

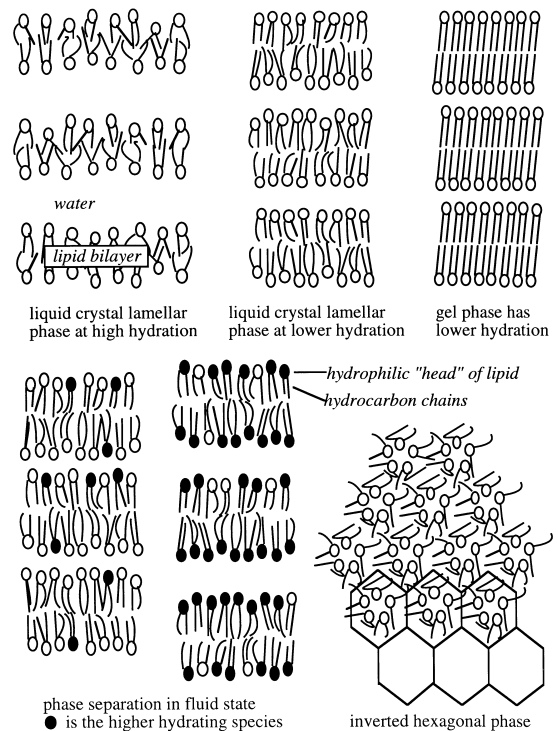


Fig. 4. A cartoon of the lipid water phases of model membranes exhibiting dehydration strains.

Fig. 4. Dessins montrant des phases d'eau liquide des modèles de membrane subissant des contraintes de déshydratation

in the normal direction. In regions of the cell rich in macromolecules, the macromolecules will also be pushed into close proximity and will suffer anisotropic internal stresses which compress them along their longer axes, although in this case the geometry is less simple [28]. We will concentrate only on membranes here. Coligative properties of membranes and solutes are compared in Fig. 5 and analysed in the Appendix.

Consider a single component lipid membrane. The gel-liquid crystal phase transition involves a reduction in area a per molecule and an increase in thickness t . 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 elevation ΔT of the transition temperature is given by a two dimensional version of the Clausius-Clapeyron equation:

$$\Delta T = \frac{T_o(a_l - a_g)\pi}{2L}, \tag{1}$$

where the subscripts l and g stand for liquid crystal and gel, T_o is the transition temperature at full hydration and L is the latent heat of the transition. The factor 2 arises

because each molecule contributes area to only one side of the membrane, while the compressive stress is given for the whole membrane. Using typical values this yields an elevation of order 0.5°C for each extra mN m^{-1} in lateral stress. At a separation of 0.5 m and with a repulsion of 20 mN m^{-2} , the lateral stress is about 10 mN m^{-1} (more detail in [26,28]).

The formation of the non-planar geometries is a discontinuous way in which the mechanical stress in the plane of the membrane can be relaxed and the volume

of water reduced. One such phase is the inverted hexagonal (H_{II}) phase (Fig. 4) in which approximately cylindrical volumes of aqueous solution are found in a hexagonal array. This transition cannot be analysed with such a simple model because it involves energies associated with the curvature of the interface. However, for weakly hydrating species, this geometry allows a large ratio of lipid volume to water volume (see Fig. 4).

In a membrane composed of mixtures of lipids having different phase transition temperatures, a number of the

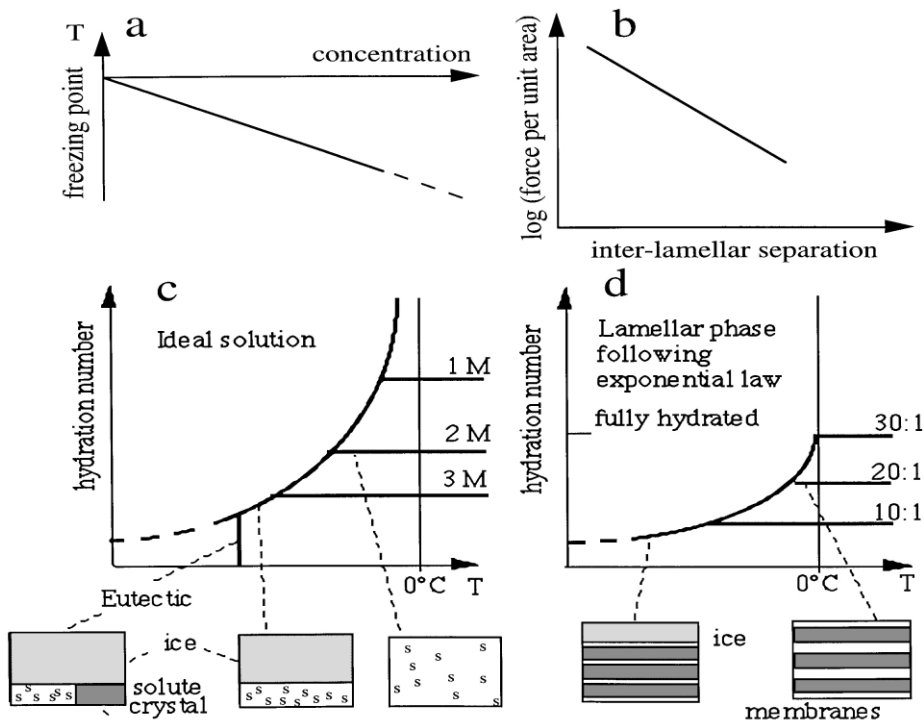


Fig. 5. Comparing the freezing point depression due to solutes and to membranes: (a) and (c) show the behaviour of an ideal solution. In (a) it is shown as equilibrium freezing point depression as a function of concentration (for sugars, the experimental curve is lower than the line at high concentration). 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 [28, 30, 39].

Fig. 5. Comparaison de l'abaissement du point de congélation du aux solutés et aux membranes : (a) et (c) montrent le comportement d'une solution idéale. (a) montre l'abaissement du point de congélation d'équilibre en fonction de la concentration (pour les sucres, la courbe expérimentale est plus basse que la courbe de la concentration supérieure). (c) montre la même relation que celle de l'eau en fonction du soluté, en fonction de la température. Les lignes horizontales montrent que, pour un échantillon avec une concentration connue, la composition reste constante au-dessus de la température de congélation en équilibre. Les effets colligatifs des membranes sont en général décrits en termes de répulsion entre membranes et de séparation entre membranes. Pour un large éventail de membranes lipidiques, on peut représenter cette relation par une répulsion qui diminue de façon exponentielle avec séparation, tel qu'on voit en (b). (d) est obtenu par la courbe de la relation eau/lipide en fonction de la température. On peut voir la similitude qualitative avec (c). Dans le cas des membranes, la relation eau/lipide a un plafond : vers une relation de 30 à 1, l'énergie entre les membranes est minimale (la force égale zéro), et si on ajoute de l'eau, on crée simplement une phase eau en excédent (aux températures au-dessus de celle de la congélation), ou davantage de glace (aux températures de congélation). Pour les données expérimentales, voir [28], [30] et [39].

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. If two or more components have very different hydration properties (e.g. different P_o), an homogeneous mix of the components has an internal energy that is rather higher than that of the separated phases. One phase has a higher concentration of the highly hydrating species and a higher inter-membrane separation than the other. In many cases, this difference in internal energy is large enough to overcome the entropy of demixing (Fig. 4).

This effect was first predicted theoretically [31], and then observed experimentally using small angle X-ray diffraction and solid state NMR [32] 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 42°C 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 [25].

The existence of fluid–fluid phase separations is not in itself 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. 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.

4.4. The effect of solutes

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 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 or trehalose (e.g. [3,19] and references in these papers).

4.5. Osmotic effects

At a given chemical potential of water, the presence of more solutes requires the presence of more water (see Fig. 1). 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 sub-zero 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 [33].

4.6. 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. At equilibrium and for small concentrations, the chemical potential depression is approximately proportional to the Celsius temperature, so we can write:

$$P - \Pi = \Psi \equiv \frac{\mu - \mu_o}{v_w} \cong \alpha(T - 0^\circ\text{C}) \quad (2)$$

Where Π is the inter-membrane osmotic pressure and for water α is 1.2 MPa °C⁻¹ (see the Appendix for further details).

An approximate equation, related to Eq. (2), is helpful to give an idea of the relative size of solute and pressure effects. For small freezing point depressions (making the approximations that the number fraction of solutes X_s is $\ll 1$ and neglecting the temperature dependence of the entropy of freezing), one obtains an expression for the change in the freezing point ΔT (Appendix)

$$\frac{\Delta T}{^\circ\text{C}} \cong -1.87 X_s + \frac{P}{1.2 \text{ MPa}} \quad (3)$$

The approximations used are increasingly poor at lower temperatures, but give approximate estimates for freezing point depressions of several degrees, and order of magnitude estimates for lower temperatures. Pure water has a concentration of 55 molar, so a concentration of 1 molar of a small non-dissociating solute gives $X_s \sim 0.02$ and a freezing point depression of about 2°C.

To achieve the same freezing point depression via hydration forces, a hydration force of order 2.4 MPa is required. For example, this is achieved between bilayer membranes of dioleoylphosphatidylcholine at water:lipid ratios of about 14:1 or an intermembrane separation of 1.2 nm [30].

The mechanical stress in the membrane π is $-Py$ where y is the inter-membrane separation, so:

$$\pi \cong \frac{\alpha (0^\circ\text{C} - T) - \Pi}{y} \quad (4)$$

Note that increasing Π also increases y so the effect on π is stronger than linear at high volume fraction of solute. Thus at similar concentrations, solutes with large partial molecular volumes should be more effective at reducing stress, all else equal.

Of course, the solutes only reduce the stress if they partition into the inter-membrane space. Polymeric solutes are often excluded from dehydrated membrane phases and so would be expected to contribute little reduction in stress. Solute 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.

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} [34].

Yoon et al. [30] 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 crystallization.

4.7. 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 [Eqs. (1) and (4)] is dramatically reduced, and the transition temperature can be reduced below the fully hydrated value T_o [21,29]. 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 Fig. 4). 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 [35].

In practice, the measured transition temperature in the presence of a glass is in the range $10\text{--}60^\circ\text{C}$ below the fully hydrated transition temperature T_o . Koster et al. [36] have measured the mechanical properties of a relevant sugar glass, and found Young's modulus to be about 15 GPa. Using parameters for DPPC [37], and Eq. (1), the compressive stress generated if the lipid remains fluid 20°C below T_o is about 45 mN m^{-1} , which corresponds to a strain of about 0.6% 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 crystallization 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.

Appendix. Combined colloidal and osmotic effects on freezing point depression

How big are the suctions produced in aqueous solutions equilibrated with ice? Because ice is a nearly pure phase, its chemical potential μ_{ice} is equal to the standard value at any temperature, whence the first equation in the series below. Equilibrium is not always achieved during cooling, especially at high cooling rates, as discussed in the text. This appendix, however, deals with equilibrium. μ_{ice} is therefore equal to the chemical potential of water μ_w whose value is given by the standard expression involving the number fraction X_w of water molecules, the hydraulic pressure P , the specific molar volume of water v_w and the gas constant R .

$$\mu_{ice}^o = \mu_{ice} = \mu_w = \mu_w^o + RT \ln X_w + P v_w \quad (A1)$$

In the absence of solutes ($X_w = 1$) or macroscopic objects (so that $P = 0$), of course $\mu_{ice}^o = \mu_w^o$. At temperatures below freezing, $\mu_{ice}^o < \mu_w^o$ which requires $\mu_w < \mu_w^o$. Solutes lower T_c via the translational entropy $-R \ln X_w$. Hydrophilic surfaces lower the chemical potential of water very near those surfaces. In principle this could be treated as a hydration energy that varied with distance from the surface, but in practice it is almost always treated as a repulsive hydration force between such surfaces, leading to a (negative) pressure in the water. (These treatments can be shown to be equivalent for suitable values of the parameters. See Appendix in [30].) Thus the presence of hydrophilic ultrastructural elements (membranes and macromolecules) allows a negative pressure in the intracellular solution, provided that the separation between them is within the range of the hydration force. We return to consider the size of these effects when we discuss solutes.

How big are these effects? The equation for solution-ice equilibrium can be cast in a very simple form making approximations, of which the most serious are neglecting the temperature dependence of the entropy of fusion and the pressure dependence of v_w , and using $X_s = (1 - X_w) \ll 1$, where X_s is the total number fraction of solutes. For equilibrium freezing,

$$\mu_w^o = \mu_{ice}^o + L - T \Delta S,$$

where L is the latent heat of fusion. At the melting temperature of pure water T_c , the melting entropy $\Delta S = L/T_c$. Neglecting the temperature dependence of L , we write

$$\mu_w^o = \mu_{ice}^o + L(1 - T/T_c),$$

so, substituting into (A1) gives

$$\mu_{ice}^o = \mu_{ice}^o + L(1 - T/T_c) + RT \ln X_w + P v_w \quad (A2)$$

Multiplying (A2) by T_c/L and writing the change in freezing point ΔT as $T - T_c$ gives

$$\Delta T = \frac{RT T_c}{L} \ln X_w + \frac{T_c v_w}{L} P$$

For $X_s \ll 1$, $\ln X_w = \ln(1 - X_s) \cong -X_s$. Further, set $T \cong T_c$. Subject to these approximations, and using standard values of the constants:

$$\frac{\Delta T}{^\circ C} \cong -1.87 X_s + \frac{P}{1.2 \text{ MPa}} \quad (A3)$$

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