

What is 'unfreezable water'? Is it the same as bound water or water of hydration?

Water can remain unfrozen at low temperatures and ordinary pressures because of

- freezing point depression due to hydration,
- freezing point depression due to osmotic effects (these are both equilibrium effects),
- supercooling: the delay or absence of formation of an ice crystal in the absence of efficient ice nucleators and/or
- the high viscosity produced by high concentrations of solutes (these are non-equilibrium effects).

Water that doesn't freeze under certain conditions is sometimes called 'unfreezable water' by various workers in the fields of cryobiology, anhydrobiology, food science and elsewhere. Here we give a brief explanation of the phenomenon in terms of the effects that slow or prevent freezing under various experimental conditions.

Some of the material in this short, informal essay is from the scientific paper "What is 'unfreezable water', how unfreezable is it and how much is there?" (Wolfe et al, 2002). Below we give references to this and other more formal and quantitative explanations in scientific journals. We also give very brief answers to two FAQs that have been raised by this page:

- Which solutes are best at preventing freezing?, and
- Which solutes are best at preserving ultrstructure? and

Conclusions and references.

Equilibrium effects

The freezing point of water may be lowered by the osmotic effects of dissolved solutes or by the hydration effects of macromolecules or biological ultrastructures such as membranes.

The **osmotic depression of freezing point** has been known for a long time. For each mole of dissolved molecules or ions one adds to a kilogram of water, one lowers the equilibrium freezing point by (approximately) two degrees.

How does osmotic depression of freezing work? Like many effects at equilibrium, freezing involves a compromise between minimising energy and maximising entropy. Molecules in ice have lower energy than they do in liquid water, because they are more strongly attracted to their neighbours, but they also have lower entropy because they

cannot move about. Entropy is more important at high temperatures, so fluids form at high temperatures and solids at low. Solutes scarcely dissolve in ice, so the entropy of ice is unaffected by their presence. However, dissolved solutes in water increase the entropy of the water molecules, and so the compromise between lowering energy and maximising entropy occurs at a lower temperature. (What do I mean by "entropy is more important at high temperatures"? Entropy is defined as the heat transferred in a reversible process divided by the temperature at which it is transferred. It has the dimensions of energy per temperature. In thermodynamics, the entropy therefore appears multiplied by temperature in such quantities as the chemical potential and the free energies. See below for formal references. A non-technical explanation is given on Boiling and freezing: the effects of solutes and of pressure.)

The **depression of freezing point by hydration** has only been studied quantitatively more recently. Water within about one nanometre (0.000001 mm) of a hydrophilic surface (one that attracts water) freezes at a lower temperature than water unaffected by surfaces. The closer to the surface, the lower the freezing temperature. The 'last' few layers of water, the water molecules closest to the surface, are very difficult to freeze or to remove. This is partly due to the equilibrium hydration effect that we are currently discussing, and partly due to non-equilibrium effects discussed later. But the important points are that the equilibrium freezing point of water falls as the water content decreases and that this effect can be quantified.

How do hydration effects cause depression of freezing point? The water near a surface is affected by that surface. The surface of biomolecules usually affects the energy and orientation of nearby water molecules. Some surfaces are charged or dipolar, and their electric field affects the orientation and energy of nearby water molecules. Some of them can form hydrogen bonds with water, and that disturbs the nearby water. Most surfaces don't hydrogen bond to water, but that disturbs water too: water hydrogen bonds to itself, so a surface that doesn't hydrogen bond the way water does causes a large disruption. In fact, even the water-air interface causes considerable local polarisation of water.

This disturbance of the attracted water extends several molecular diameters from a hydrophilic surface. It gives rise to hydration forces: when you try to bring two hydrophilic surfaces close together, you have to remove water molecules from between them. This gets harder and harder to do as the surfaces get closer, and the activity of the water gets lower and lower. This effect creates a hydration repulsion that has been characterised by many researchers, including Adrian Parsegian, Jacob Israelachvili and their teams. The force required increases exponentially as the surfaces approach, with a characteristic distance of typically 0.2 nm.

The increasing energy required to remove water molecules that are closer to the hydrophilic surface similarly gives rise to the greater freezing point depression. Undisturbed, bulk water freezes at its normal temperature (which depends on the solute concentration). The more strongly the water near a surface is held (ie the closer it is to the surface), the lower the temperature at which it freezes. See Wolfe et al, 2002 (reference below) for further explanation and quantitative detail.

Non-equilibrium effects

Supercooling is possible. Small volumes of water, or large volumes of very pure water, can be cooled considerably below 0°C before they freeze. The water in biological systems is found in very small volumes, such as the interiors of cells and organelles, and so can supercool substantially.

How does supercooling work? Ice crystals are only stable if they have a certain minimum size: one cannot, for instance, have one molecule of ice in a volume of liquid water. The minimum size of an ice crystal decreases with sub-freezing temperature, but just below freezing it is large. (See Bryant and Wolfe, 1999, for a more detailed explanation.) Now the chance that a large number of molecules will spontaneously form themselves into such a crystal is small. Consequently, pure water can be cooled well below zero (sometimes to -40°C) before ice forms. When you heat it up, it normally will melt at the equilibrium temperature.

Usually water will freeze at only a few degrees below zero. This is because a range of substances act as ice nucleators: they act as surfaces on which an ice crystal can grow. The more efficient the nucleator, and the more of them there are, the easier it is to freeze. Conversely, small volumes of very pure water can be supercooled most easily.

By the way, supercooling is a bit like superheating, but superheating can be much more spectacular!

High viscosity hinders equilibration. In biological systems, solutes are present. When some of the water freezes, the solutes are concentrated in the remaining unfrozen water, producing highly concentrated solutions. These are viscous, and become more viscous at lower temperatures. This has a few consequences. One is that the molecules of water are slower to diffuse and to rotate, so that ice nuclei are less likely to form and supercooling is more likely. Another is that water cannot quickly diffuse from the region near a hydrophilic surface to a region where ice has already started to form. Thus the amount of unfrozen water is higher than one would expect at equilibrium.

High viscosity and low temperature can produce a glass. A glass is a non-crystalline solid. Window glass is an example. Glasses are formed when the viscosity of a liquid becomes so high that it can resist shear stresses (resist changes in shape) for extremely long periods. When the water in cells forms a glass, diffusion, freezing and biochemistry are virtually stopped.

How hard is 'water of hydration' bound? The term 'bound water' is often misunderstood and often used in a misleading way. Unless vitrification has occurred or unless the aqueous medium has a very large viscosity, water of hydration can exchange rather rapidly with any other nearby water molecules. However, even moderately elevated viscosity in the aqueous phase of a low hydration system can make equilibration very slow. Just because an experimentalist has difficulty removing water from a sample doesn't mean that it is bound. For further discussion, download this paper.

Which solutes are best at preventing freezing?

The answer to this FAQ usually involves several specific details, so I'll only make a few general comments. These are dealt with in more detail by Wolfe and Bryant (2001) (see references below).

First, the solute should be **soluble** and, to achieve high concentrations, you may need high solubility. Supersaturation often occurs at freezing temperatures, but it helps if you start with high concentration. For osmotic freezing point depression, the direct effect of small solutes are rather similar at low concentration. Provided that one counts dissociating solutes (MgCl_2 in solution is three solute ions), the freezing point depression is approximately proportional to concentration. At high concentration, the osmotic effects of salts may be less than proportional to concentration. Conversely, the osmotic effect of many solutes such as sugars increases at high concentration by more than simple proportionality.

If the solute crystallises, this limits the concentration you can achieve, and hence the viscosity. So, to achieve large nonequilibrium effects, you need solutes that **do not crystallise easily**. One way to do this is to look through the handbooks for solutes that do not *individually* crystallise until very high concentration. Easier and often much cheaper is to use a **mixture of two or more solutes**.

High **viscosity** helps, for reasons discussed above. Some solutes have a greater effect on viscosity than others. Large solute molecules often have a greater effect on viscosity than small.

If you wish to prevent freezing in biological samples, you may also be interested in whether or not the solute is **toxic**. Also, you may want it to **permeate** the membrane. (If it doesn't the cell will contract osmotically, with potentially damaging physical and biochemical effects.) Solutes that permeate the membrane are usually toxic in high concentration, so some compromises are usually necessary: one may use a cocktail of permeating solutes and, after thawing, wash them out as quickly as possible.

Which solutes are best at preserving ultrastructure?

This is another subtle FAQ and it is complicated by the fact that some people in the cryopreservation industry have a pecuniary interest in certain solutes. Further, the cytoplasm is a rather special environment, and ultrastructural elements (membranes and macromolecules) are often stabilised by effects that, in a sense, balance each other. Nevertheless, some general observations may be made.

- Much ultrastructure is stabilised by the surface tension of water: it holds membranes together and is important in the secondary, tertiary and quaternary configuration of many proteins. So solutes that reduce substantially the surface tension of water (such as strong detergents) tend to disrupt ultrastructure.
- Ions (particularly polyvalent ions) shield the electrostatic interaction, and also to some

extent the dipolar interaction. Increasing the concentration of dissolved ions can reduce electrostatic repulsions and allow precipitation. So it's easy to imagine ultrastructural configurations that are sensitive to ion concentration.

- Membrane soluble molecules are potentially dangerous. The stability of a lipid bilayer depends on the geometry of its hydrophobic region, so hydrophobic molecules can disrupt the semipermeability and even the bilayer structure. This is a serious problem in cryobiology, because often one wishes to have the solute permeate the membrane, yet one doesn't wish to disrupt it.
- Some solutes interact with membranes, particularly at extreme dehydration. This statement is obvious: when one achieves a water content of say 20% or less, nearly all solute molecules are very near to a water-membrane or water-macromolecule interface.
- Some molecules appear to affect the hydration forces between membranes (and may therefore affect the hydration forces between macromolecules). This is potentially important, because the stresses produced by hydration forces at low water content can disrupt ultrastructure. Yoon et al (1998) studied the effects of dimethylsulphoxide (DMSO), sorbitol, sucrose and trehalose in this regard. The effects of DMSO and sorbitol were consistent with what one would expect from their osmotic properties alone. Sucrose and trehalose had a greater effect in reducing the stress due to hydration forces.

Conclusions

For all of the reasons listed above, biological samples, or other samples in which water occurs in finely divided volumes, can exhibit freezing over a wide range of temperatures. A substantial amount of water may never freeze under given experimental conditions. This water may be very difficult to remove, but still not 'bound'. So what is 'unfreezable water'? The practical answer is that it is water that did not freeze when the experimenter expected that it would. This answer is unsatisfactory because it is not objective. So we prefer not to use the term at all. Instead, it is better to recognize that water can freeze over a wide range of temperatures, and that, if the temperature is cold enough, non-equilibrium effects will slow or prevent any further freezing. **The 'water of hydration' that remains unfrozen may be considerably more than just a single layer of water molecules covering all hydrophilic surfaces.** For quantitative estimates, and for a more formal discussion, see "What is 'unfreezable water', how unfreezable is it and how much is there?".

Some relevant references

- Wolfe, J., Bryant, G. and Koster, K. (2002) "What is 'unfreezable water', how unfreezable is it and how much is there?" *Cryoletters*, 23, 157-166.
- Wolfe, J. (2002) "Cellular Thermodynamics" Encyclopedia of Life Sciences (<http://www.els.net>). London: Nature Publishing Group.
- Wolfe, J. and Bryant, G. (2001) "Cellular cryobiology: thermodynamic and mechanical effects" *International Journal of Refrigeration*, 24, 438-450.

- Bryant, G., K.L. Koster and J. Wolfe. (2001) "Membrane behavior in seeds and other systems at low water content: the various effects of solutes" *Seed Science Research*, 11, 17-25.
- Wolfe, J. and Bryant, G. (1999) "Freezing, drying and/or vitrification of membrane-solute-water systems" *Cryobiology*, 39, 103-129.
- Yoon, Y. H., Pope, J. and Wolfe, J. (1998) "The Effects of Solutes on the Freezing Properties of and Hydration Forces in Lipid Lamellar Phases" *Biophys. J.*, 74, 1949-1965.

More about water

- Despite its ubiquity, water is a remarkable and unusual chemical. See Martin Chaplin's page: Water structure and behaviour

Cryobiology links

- Introduction to cryobiology and anhydrobiology.
- Boiling and freezing: the effects of solutes and of pressure (a non-technical explanation).
- Society for Cryobiology. The scientific society in this field.
- CryoLetters, the rapid communication journal in the field.
- Membranes: homeostasis and regulation of area and tension, and how this relates to survival of freeze-thaw cycles.
- What the Hydration Forces Explanation *doesn't* say. A warning about some prevalent misquotations in cellular cryobiology.

Source:<http://www.phys.unsw.edu.au/~jw/unfreezable.html>