

## WHAT IS 'UNFREEZABLE WATER', HOW UNFREEZABLE IS IT AND HOW MUCH IS THERE?

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### Summary

Water that remains unfrozen at temperatures below the equilibrium bulk freezing temperature, in the presence of ice, is sometimes called 'unfreezable' or 'bound'. This paper analyses the phenomenon in terms of quantitative measurements of the hydration interaction among membranes or macromolecules at freezing temperatures. These results are related to analogous measurements in which osmotic stress or mechanical compression are used to equilibrate water of hydration with a bulk phase. The analysis provides formulæ to estimate, at a given sub-freezing temperature, the amount of unfrozen water due to equilibrium hydration effects. Even at tens of degrees below freezing, this hydration effect alone can explain an unfrozen water volume that considerably exceeds that of a single 'hydration shell' surrounding the hydrophilic surfaces. The formulæ provided give a lower bound to the amount of unfrozen water for two reasons. First, the well known freezing point depression due to small solutes is, to zeroth order, independent of the membrane or macromolecular hydration effect. Further, the unfrozen solution found between membranes or macromolecules at freezing temperatures has high viscosity and small dimensions. This means that dehydration of such systems, especially at freezing temperatures, takes so long that equilibrium is rarely achieved over normal experimental time scales. So, in many cases, the amount of unfrozen water exceeds that expected at equilibrium, which in turn usually exceeds that calculated for a single 'hydration shell'.

**Keywords:** unfreezable water, water of hydration, bound water, dehydration, freezing, freezing point depression

### INTRODUCTION

The term 'unfreezable water' is often applied to water that did not freeze under conditions where the experimenter expected it to freeze. This is an unsatisfactory definition because it depends upon the expectations of the experimenter: one person's 'unfreezable' water is another's viscous vicinal solvent. Franks (1) implicitly recommends calling it simply 'unfrozen water'. This recommendation does not seem to have been noted and/or accepted by a number of practitioners in cryobiology, food science, etc. who observe unfrozen water at tens of °C below the equilibrium freezing temperature, in the presence of ice crystals. If this water is not unfreezable, such an investigator might reasonably ask, why has it not frozen? As Mazur (13) points out, the water of hydration of ultrastructural components not only

remains unfrozen in cells, but does not freeze in the presence of ice crystals *in vitro*. Why not? The purpose of this paper is to answer these questions with reference to quantitative measurements of the relevant parameters in some simple systems.

The existence of unfrozen water at freezing temperatures, in the presence of ice or other nucleators, can be attributed to (at least) three effects: (i) The well known freezing point depression due to small solutes, (ii) The less well known freezing point depression due to macromolecules, membranes and other hydrophilic ultrastructure, (iii) The effects of viscosity. (i) and (ii) are equilibrium effects, (iii) is non-equilibrium. We consider these in turn.

*i) Freezing point depression due to small solutes.* The first contributor to the stability of unfrozen water is the osmotic freezing point depression due to solutes. Because of the entropy of mixing, the equilibrium freezing point of an aqueous solution is lower than that of pure water, and the amount of the freezing point depression is approximately proportional to the osmotic pressure of the solution, which depends directly on the osmolality.

Because solute concentrations *in vivo* are usually not much greater than a few hundred mOsm, osmotic pressures *in vivo* are usually not much more than 1 MPa or so, a value that lowers the freezing point by only about one degree. Once ice is present, however, the effect is enhanced because solutes are excluded from the ice structure and become more concentrated in the remaining unfrozen fraction. As the temperature falls, successively greater osmotic pressure is required to keep a solution at equilibrium with the pure ice. Thus, the concentration rises until, in principle, solutes could begin to crystallise when the saturated concentration is reached. In practice, this is unlikely *in vivo*, first because many different solutes are present, and second because equilibration is slowed at very low temperatures, as we discuss below. Freezing point depression due to small solutes and freezing induced concentration has been described elsewhere (*e.g.* 14), so we do not discuss it further here.

*ii) Freezing point depression due to macromolecules and membranes.* Macromolecules, membranes, and indeed any hydrophilic components in a solution also depress the freezing point at sufficiently low hydration. Like osmotic freezing point depression, this is an equilibrium effect. However, the mechanism is different. In the case of solutes, the freezing point depression is due to the entropy of mixing, and is approximately proportional to the number of solutes. Although macromolecules and other structures have an analogous effect, it is relatively small because the numbers are small. The main contributor to freezing point depression for such mesoscopic objects is due to the low energy of water molecules near a hydrophilic surface. This is the main topic discussed in this paper.

*iii) Non-equilibrium effects.* Under some conditions, aqueous solutions, or even pure liquid water, may not freeze, even when they are unstable with respect to ice. Bulk volumes of supercooled solution can exist virtually indefinitely in the absence of ice nucleators. In solutions below their equilibrium freezing temperature, if ice crystals are already present, they grow. As Franks (2) points out, the viscosity of very concentrated solutions (elevated by cooling and solute concentration) slows the growth of the ice interface to millimetres per century. In such circumstances, some regions of a solution may remain unfrozen, below their freezing temperature, even if there is an ice crystal only microns away. When the viscosity is sufficiently high, the phase is called a glass or a vitreous phase.

#### *Hydration and freezing point depression.*

It is possible for modest volumes of an aqueous solution to remain unfrozen, in the presence of ice crystals, at tens of °C below the equilibrium freezing temperature of a bulk solution with the same concentration. The unfrozen water in question is affected by the presence of mesoscopic objects (such as membranes, macromolecules and other ultrastructural

elements in cells, tissues, food products etc.). This is possible even in the absence of solutes. The quantity of unfrozen water may exceed the expected amount of 'water of hydration' or 'hydration shell'.

In one usage, 'water of hydration' refers to the population of water molecules found near to solutes and macromolecular surfaces, whose physical properties (*e.g.* relaxation times, polarisation, partial molecular volume) differ from those of pure water. In another usage, it refers to a (first) 'hydration shell' of molecules, one layer deep, covering the water facing surfaces of non-aqueous components. In this paper, we are concerned with the water that hydrates membranes and macromolecules, and we follow the former usage.

In the vicinity of a polar or dipolar region of a membrane or macromolecule, the dipolar water molecule has a non-random average orientation and thus a lower potential energy than it does in bulk (7-9). This energy may be further lowered by hydrogen bonding to the surface, if it occurs. Because of its non-random average orientation, the specific entropy of this vicinal water is less than that of bulk water, but greater than that of ice. One would expect the specific potential energy and specific entropy to be strongly dependent on the distance of the water from the surface which attracts it: the first 'hydration shell' would be more strongly attracted than the second, and so forth. This is observed experimentally: it becomes more difficult to remove the water closer to the surface and, in some geometries, the work to remove water decreases exponentially with distance (3,5,15,16). Depending upon the freezing temperature and the hydrophilicity of the surface, the amount of water whose properties are sufficiently affected so as to prevent their freezing usually exceeds that of the first 'hydration shell'.

This result, obtained with a variety of techniques and on a variety of different biochemical systems, is important to our understanding of hydration and freezing point depression. If we regard the unfrozen water near membrane or macromolecular surfaces as water of hydration, then *water of hydration becomes a quantitative rather than a qualitative distinction*: all the water molecules within a nanometre or so of a hydrophilic surface are affected to some extent by the hydration interaction. The work required to remove the water decreases exponentially as distance from the surface increases. The strength of the hydration interaction also depends on the surface: its charge, its polarisation and the area density of hydrogen bonds, if present. More work is required to remove water from a strongly hydrophilic surface, so, if equilibrium is obtained at a given chemical potential of water, a strongly hydrophilic surface (*e.g.* a phosphatidylcholine membrane) will have higher hydration per unit area than will a weakly hydrophilic one (*e.g.* a phosphatidylethanolamine membrane).

How unfreezable is this water of hydration, and how much is there? We turn to these questions next.

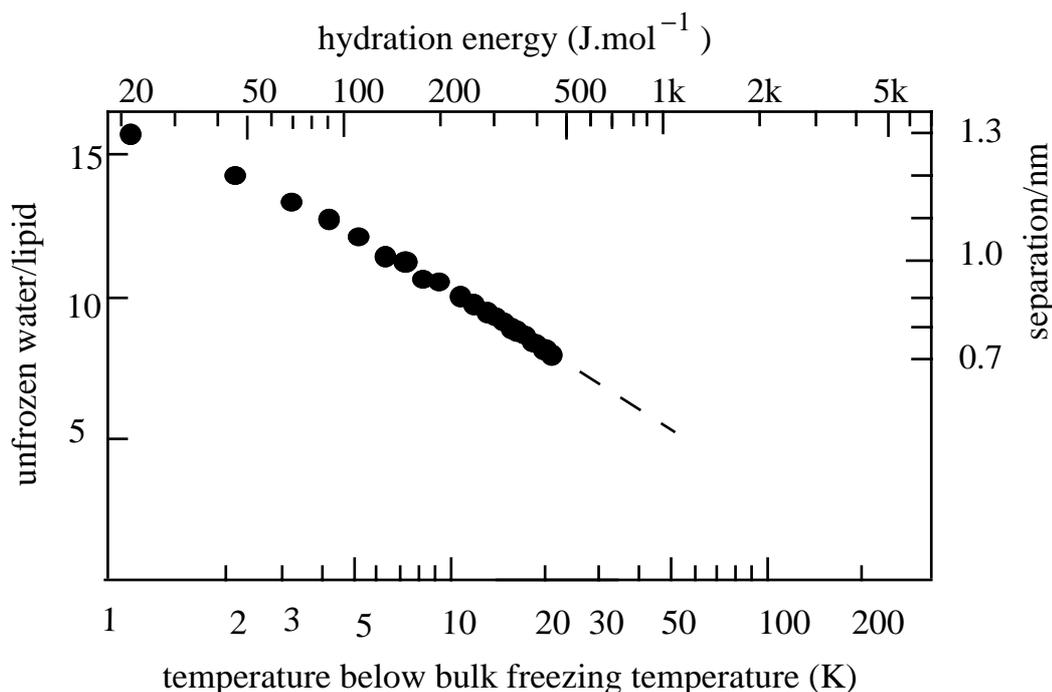
### ***Unfrozen water near membranes.***

*Hydration vs freezing.* A multi-lamellar phase provides a system with a simple geometry that can be measured with and without solutes present, to isolate the two effects. The freezing behaviour of such a system has been measured previously (18,19), and some representative data for DOPC are shown in Figure 1 as number of unfrozen water molecules per lipid molecule *vs.* temperature below the bulk freezing temperature (on a log scale). From the graph it can be seen that, at ten degrees below freezing, there are about 10 unfrozen water molecules per lipid. This number decreases logarithmically with temperature. (This logarithmic dependence results from, and is usually expressed as, the exponential dependence of the hydration interaction with distance from the hydrophilic surface.)

It is also important to note the freezing point depression at which the data cease: beyond a freezing point depression of 20 K, the investigators could not investigate the equilibrium properties, because the technique prohibited equilibration periods longer than several hours.

Thus in this instance, the non-equilibrium effects are important even for membranes in pure water, at a temperature only twenty degrees below freezing. This is a result of the confined geometry: with an interlamellar spacing of 0.7 nm, the viscosity of the thin intermembrane layer, although still in the fluid phase, slows the equilibration time to many hours.

The upper axis in Figure 1 gives the hydration energy<sup>1</sup>, determined from the freezing point depression, as described below. If one extrapolates the exponential dependence of hydration energy with hydration number to even lower hydrations (an extrapolation supported to some extent by data discussed later), one predicts very large freezing point depressions for the lowest levels of hydration, and correspondingly higher viscosities and greater difficulties in reaching equilibrium.



**Figure 1.** The hydration number for a multi-lamellar phase of dioleoylphosphatidylcholine (DOPC), in the absence of solutes, plotted as a function of temperature (data from (19)). The horizontal axis is also plotted approximately as the energy of hydration<sup>1</sup>. The vertical axis on the right is the inferred inter-membrane separation. The hydration energy decreases by a factor of  $e$  when the distance from the hydrophilic surface is increased by a characteristic distance  $\lambda = 0.17$  nm.

The exponential dependence of energy of hydration with distance from a hydrophilic surface has been observed in a number of different contexts and described in terms of different parameters, because there are a number of different ways of removing water from hydrophilic surfaces. During dehydration, the chemical potential  $\mu_w$  of the water of hydration is less than that of pure bulk water at atmospheric pressure,  $\mu_w^0$ . At the equilibrium freezing temperature, the chemical potential water equals that of ice:  $\mu_w = \mu_i^0$ . At lower temperatures,  $\mu_i^0 < \mu_w$ . In the example we have just seen, as the temperature falls, some of the water that initially hydrates the lipid membranes gradually leaves the vicinity of the membrane and joins a growing ice crystal, until either the remaining unfrozen water is in equilibrium with ice ( $\mu_w = \mu_i^0$ ), or until the viscosity is so large that the freezing process becomes so slow that equilibrium is not achieved. Making the approximation that neither the latent heat nor entropy of fusion depends on temperature, one can use thermodynamics to relate the energy of hydration to the

<sup>1</sup> The hydration energy in this geometry is due to the two hydrophilic surfaces on either side of the water layer. For a single hydrophilic surface in bulk solution, the hydration energy is half this value (see Appendix in (19)).

temperature below freezing at which that hydration is achieved (18). This approximation is insufficient for precise calculations, but suffices for the general argument presented here, and it has been used to calculate the hydration energy in the approximate axis shown in Figure 1. The cartoons in Figure 2 depict freezing of the bulk solution, the resultant close approach of membranes and macromolecules, and the hydration force between the hydrophilic surfaces. The effects of solutes, also depicted in figure 2, are discussed further below.

*Hydration vs. evaporation.* In an unsaturated atmosphere, the chemical potential of water is lower than that of pure, bulk, liquid water. Consequently, membranes can also be dehydrated by equilibration or partial equilibration with an atmosphere with relative humidity RH less than one. The hydration can be determined gravimetrically or from the geometry, which can be measured by *e.g.* X-ray diffraction. Again making approximations, the energy of hydration can be determined from the relative humidity. Before the freezing dehydration studies described above, several different investigators had used such techniques to determine the hydration energy as a function of hydration of lipid membranes (*e.g.* 16,12), and had measured an exponential decrease in hydration energy with number of waters of hydration or inter-membrane separation. Because such experiments are conducted at temperatures of typically 20°C, the viscosity is less than in freezing experiments. Further, rather longer equilibration times are conveniently available. As a result, such studies had observed that the exponential dependence extends to lower hydrations than those reported in the freezing-hydration studies, which lends some support to the extrapolation in Figure 1.

Since Langmuir's report (9), the hydration of hydrophilic surfaces has been reported as a short-range repulsive force between such surfaces. The force per unit area that must be applied to bring two surfaces together at small separation is equal to the suction required to remove the water of hydration, which is equal to the work of hydration per unit volume. The forces per unit area are usually no more than a few percent of the bulk modulus of water. Consequently, researchers have usually made the assumption that the molecular volume of water was approximately constant<sup>2</sup> and reported the equivalent force per unit area rather than the energy of hydration per unit volume. Thus the figures in the papers cited in the preceding paragraph show a repulsive force per unit area that decreases exponentially with increasing membrane separation. (The close relationship between such a force per unit area and a hydration energy per unit volume is described explicitly in (19).)

*Hydration vs mechanical expulsion.* Repulsive forces due to the hydration of membranes and other hydrophilic surfaces separated by only a few molecular layers of water have also been measured directly (3,5,6). In this technique, the water close to the hydrophilic surfaces is expelled into a reservoir of bulk pure water while the force per unit area and the separation between surfaces are measured. As well as being conducted at ambient temperatures, this technique has a relatively small area of close approach, to which large forces per unit area may be applied. Consequently, this technique has reported hydrations reduced so low that membranes fuse together.

*Unfrozen, unevaporated and unexpelled water.* Removal of water of hydration by freezing, evaporation and mechanical expulsion all give qualitatively similar results: the energy of hydration decreases exponentially with separation, having a characteristic decay length  $\lambda$  of typically 0.2 nm (a length comparable with the linear dimensions of a water molecule) and an extrapolated intercept that varies considerably depending on the hydrophilic surface in question.

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<sup>2</sup> This approximation is usually appropriate over the experimental range of hydration, but may be violated at the very large suctions extrapolated at or near zero hydration. The bulk modulus for water is 2 GPa.

There are some relatively small differences among the parameters as measured by the three techniques (4,19), which may be attributed to the different determinations of the position of the surface and to the different mechanical constraints and different temperatures under which they operate. The different techniques are compared in more detail in (4,17).

It should be noted that the energy-hydration dependence derived thus is, of course, a measure of all of the inter-surface forces, including van der Waals and electrostatic, if present. At sufficiently short range (less than about one or two nanometres), the hydration interaction is much greater than the others, which can therefore be neglected. At larger separations, the other longer-range forces become important and the exponential dependence no longer obtains.

#### *Water of hydration near macromolecules.*

To date, we know of no studies of the equilibrium unfrozen hydration of macromolecules as a function of freezing temperature. One of the problems in such studies is the difficulty of obtaining homogeneity and regular geometry, and of obtaining the amount of unfrozen water reliably. Parsegian *et al.* (15) have measured the hydration of arrays of DNA in equilibrium with solutions of different osmotic pressures. Again, they obtained an exponential decrease in hydration energy with distance. The extrapolated value at zero hydration depends on the suspending solution but is of the order of 100 MPa. The characteristic decay length is about 0.15 nm. Li *et al.* (10) report hydration isotherms for starch. When these are plotted as hydration energy *vs.* average inter-polymer spacing, they also yield an exponential decay with an extrapolated intercept of 270 MPa and a characteristic length of 0.12 nm. In these cases, the shorter decay length may be related to the different geometry<sup>3</sup>.

#### *Effect of solutes.*

Solutes lower the chemical potential of the water in solution, and so the water of hydration in a solution has its chemical potential lowered by both the solute and the presence of the hydrophilic surface. This means that a lower sub-freezing temperature is required to remove it, or equivalently that more water of hydration is retained at a given temperature. To first order, the effect of solutes on the hydration of membranes at freezing temperatures is explained by their osmotic effect (19). However, very large concentrations of some solutes, including sucrose, can weaken the hydration interaction: at very large solute concentration, the water structure may become disrupted (8,19).

Figure 2 is a cartoon representing the equilibrium effects of freezing on a system containing membranes, macromolecules and solutes in water.

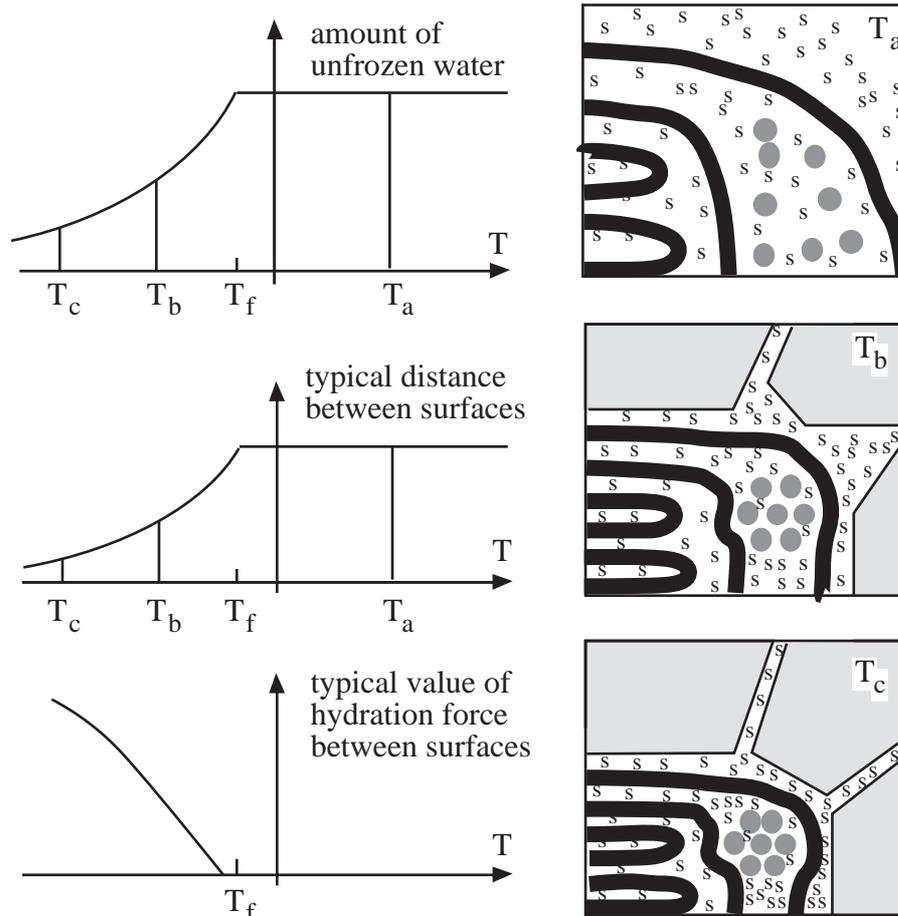
#### *Non-equilibrium effects.*

Solutes and low temperatures both increase the viscosity of the solution. This effect can be very great at freezing temperatures because of the concentration of the solutes in the small remaining amount of unfrozen water. At low temperatures and high solute concentrations, the viscosity may become so large as to make equilibration impossible over ordinary time scales. Thus, the amount of hydration retained at such a temperature may considerably exceed the amount calculated for equilibrium.

The temperature dependence of the viscosity is one of the causes of hysteresis in hydration. Dehydration at a temperature above freezing, followed by cooling to some sub-zero temperature T, may result in a lower overall hydration than freeze-dehydration at the same temperature T: in the first case the dehydration occurs at a higher temperature where the viscosity is lower, and therefore equilibration times are shorter. For other causes, see (1,17).

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<sup>3</sup> In an array of long macromolecules, closely pushed together by dehydration, there are several nearest neighbours, and fields have an extra distance dependence of approximately 1/(axial separation). Details in (15,17).



**Figure 2.** A cartoon representing unfrozen water as a function of temperature due to osmotic and hydration effects in a system containing membranes (dark lines), macromolecules (shaded circles), solutes (s), water (white) and ice (shaded). The presence of solutes and hydrophilic surfaces depresses the equilibrium freezing temperature to  $T_f < 0$  °C. When  $T < T_f$ , the chemical potential of the unfrozen water may be lowered to equal that of ice by one, or usually both, of two effects: the concentration of solutes in the remaining unfrozen water, and the suction that may be supported by the hydration force between hydrophilic surfaces<sup>4</sup>.

*Is the water of hydration bound?*

The water of hydration nearest a hydrophilic surface is sometimes called bound water. Certainly such water is attracted to the surface and therefore has a lower potential energy than does bulk water. In a crude approximation, the work required to remove the last 'layer' of hydration can be estimated by extrapolating the data from the different techniques to low hydration. Hydration forces are usually summarised by the exponential force law:

$$P = P_0 \exp(-d/\lambda) \tag{1},$$

where  $d$  is the separation,  $P$  is the suction or negative pressure required to achieve equilibrium,  $P_0$  is its extrapolated value at  $d = 0$  and  $\lambda$  is the decay length.

<sup>4</sup> The chemical potential of water is given by  $\mu_w = \mu_w^0 + kT \ln X_w + Pv_w$ , where  $\mu_w^0$  is the standard chemical potential,  $X$  is the molecular fraction of water, and  $v_w$  the partial molecular volume of water. Respectively, the osmotic and hydration effects make the second and third terms on the right hand side more negative, and so allow equilibration with ice when, at sub-freezing temperatures,  $\mu_w^0 > \mu_i^0$ .

Using the freezing stress technique (18,19), it is difficult to achieve equilibration with suction below a few tens of MPa. At higher temperatures and using the osmotic stress technique, forces per unit area of up to 100 MPa are inferred (1,11,15). For molecules of modest hydrophilicity, the values extrapolated at zero separation between surfaces are hundreds of MPa to several GPa. For strongly hydrophilic species, considerably higher values are extrapolated, although it should be noted that these are the cases in which the extrapolation is most extended and in which the approximations made are least accurate, and in which the irregular geometry of the surface makes calculation difficult.

Making the approximation (see footnote 2) that water has a negligible compressibility, one may equate a difference in chemical potential per unit volume (in J per m<sup>3</sup>) to the suction or negative pressure required to remove water molecules (in Pa) to the work of hydration (in J per mol or per molecule), for which purpose it is useful to note that

$$1 \text{ GPa} = 1 \text{ GJ.m}^{-3} = 18 \text{ kJ.mol}^{-1} = 3 \times 10^{-20} \text{ J.molecule}^{-1} \cong \text{several } kT.\text{molecule}^{-1},$$

where  $k$  is Boltzmann's constant.  $kT$  is the thermal energy at modest freezing temperatures.

For lipids with a  $P_0$  of 1 GPa, the implied maximum work of hydration due to one surface<sup>5</sup> is of order a few kJ.mol<sup>-1</sup> or a couple of times the thermal energy. This represents a weak 'binding' in the molecular kinetic sense: the water molecules hydrating such a surface could exchange relatively rapidly with their neighbours. (One might put this informally by saying that, although it is hard to remove a water of hydration and replace it with nothing, it is easy to replace it with another water molecule.) For the more strongly hydrophilic species, and subject to the caveats above, considerably stronger attractions may, however, apply. In such cases, molecular exchange may be slow.

The work of hydration is only one obstacle to dehydrating a surface. For water between two surfaces with a  $P_0$  of say 1 GPa, the interaction between the surface and the nearest water molecules is only several times the thermal energy, so one might not anticipate difficulties in dehydrating it completely. In some cases, the difficulty observed by experimentalists is a kinetic problem. Water in a very narrow, viscous layer is hard to remove even if the attraction to the hydrophilic surface is only modest. This is particularly the case at low temperatures because of the strong dependence of viscosity on temperature. Such water would fail to freeze not because it is in equilibrium with ice, but rather because it can remain in disequilibrium for a time exceeding the patience (or even the lifetime) of the experimentalist. In such cases, 'bound' water may be not so much tied up as unavoidably detained.

*How much unfrozen water remains near hydrophilic surfaces at a given freezing temperature?*

This question is hard to answer exactly because it depends on the geometry of the hydrophilic surfaces and how strongly they attract water. An order of magnitude estimate may however be made, using the experimentally determined, exponential hydration law (eqn 1) for the hydration energy per unit volume. If  $v_w$  is the molecular or molar specific volume of water, and assuming that water occupies most of the volume of the hydration shell, then the number of molecules or moles per unit hydrophilic area is

$$n \cong \frac{A d}{2 v_w} \cong - \frac{A \lambda}{2 v_w} \ln \frac{P}{P_0} ,$$

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<sup>5</sup> To estimate the work of hydration for the most intimate layer of water molecules, one should extrapolate not to zero separation, but to the linear dimensions of one water molecule. Remembering that most of the published data are for the interactions with two interfaces, the value of interest is less than  $P_0$  by about  $2e \cong 5$ . Further, *one should treat such extrapolations with caution*. The separation is usually defined between the volume weighted average surfaces. At very close approach, the complicated surface geometry makes this approximation rather crude.

where the geometrical factor 2 applies to membranes (and A is the area of one side of the membrane). Making the same severe approximations mentioned above, the equilibrium value of P is related (17) to the latent heat  $L_{iw}$  and the equilibrium freezing temperature  $T_c$  of the solution in question by

$$P \cong \frac{L_{iw}}{v_w} \left(1 - \frac{T}{T_c}\right) = \frac{L_{iw}}{v_w T_c} \Delta T .$$

$$\text{So } \frac{n}{A} \cong -\frac{\lambda}{2v_w} \ln \frac{P}{P_0} \cong -\frac{\lambda}{2v_w} \ln \frac{L_{iw} \Delta T}{P_0 v_w T_c} ,$$

where  $\Delta T$  (in K or °C) is the freezing point depression due to the hydration effect alone.

Fortunately for our estimate, the most variable parameter,  $P_0$ , is in the argument of the log function and so has a relatively small effect on the estimates. Readers are invited to insert values most relevant to their own cases. For example, let us take 1 GPa as an order of magnitude estimate of  $P_0$  for the membrane geometry, a characteristic length  $\lambda$  of 0.2 nm,  $1 \times 10^{-20}$  J.molecule<sup>-1</sup> for the latent heat of fusion of ice and  $3 \times 10^{-29}$  m<sup>3</sup>.molecule<sup>-1</sup> as the specific volume of water. This gives an estimate of the number of waters of hydration per unit of hydrophilic area as a function of temperature:

$$\frac{\text{water of hydration}}{\text{unit area}} \sim 3 \times 10^{18} \ln \frac{10^3}{\Delta T} \text{ molecules.m}^{-2} \sim 5 \times 10^{-6} \ln \frac{10^3}{\Delta T} \text{ moles.m}^{-2}.$$

Note that  $\Delta T$  does not include the effects of solutes: it is the difference between T and the equilibrium freezing temperature of a bulk solution having the composition of the hydration shell. For lipid molecules having an area per molecule of 0.6 nm<sup>2</sup> and in the absence of solutes, this rough calculation predicts that at 20 K below freezing the amount of unfrozen water at equilibrium would be of the order of 7 waters/lipid. When small solutes are present, the equilibrium water content is higher and depends on the ratio of solutes to lipids, on the volume of the solutes and on non-equilibrium effects (1,17,19).

## CONCLUSION

At temperatures about 20 °C or K below freezing and when equilibrated with ice, membranes and macromolecules retain sufficient water of hydration to provide a layer up to a few molecules deep. In the presence of solutes, such a layer may be retained to even lower temperatures. The equilibrium of this water of hydration is well understood in terms of the hydration interaction and is consistent with hydration interactions measured in systems dehydrated by osmotic stress, unsaturated atmosphere or by direct mechanical stress.

In the case of dehydration by freezing, the concentrated solutions at low temperatures have viscosities that slow both the equilibration of water in confined spaces and the growth of the ice interface to an extent that makes freezing effectively stop.

We return to the questions of the title. What is 'unfreezable' water? It is water that did not freeze, for various reasons including those discussed. How much is there? An estimate of the unfrozen water of hydration at equilibrium is given above. (Non-equilibrium hydrations will usually be higher.) How 'unfreezable' is it? Its ease of freezing decreases with increasing solute concentration, proximity to a hydrophilic surface and viscosity. It may also depend upon hysteresis and thus on sample preparation. Its 'unfreezability' may therefore depend upon the ingenuity of someone intent on freezing it. Better then simply call it unfrozen.

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