International Tables for Crystallography (2006). Vol. F, Section 10.1.2.3, p. 198.

below 233 K without freezing. However, given a sufficiently small volume and high cooling rate, it is possible to supercool water to form a glassy state that is at least kinetically stable. Stability requires a temperature below 140 K; at higher temperatures crystallization eventually takes place. For the cooling rates typically attained with small crystals (up to a few hundred $K\,s^{-1}$) it seems impossible to avoid crystallization of water in the mother liquor adhering to a crystal, unless it is modified in some way. Once ice forms at the crystal surface, freezing may propagate through the entire crystal, effectively destroying it. Even if the crystal remains intact, diffraction from polycrystalline ice will render parts of any data set from that crystal useless. Because the probability of a nucleation event increases with time, it seems prudent to use a rapid cooling process. However, we note that the expression for $J(T)$ is formulated for pure water and cannot be valid for all conditions; it is well established that a majority of biocrystals can be cooled below 140 K.

A consequence of the foregoing is that for prevention of ice growth one should first focus attention on the situation immediately outside the crystal, rather than on its interior. Two approaches have been shown to have merit: (*a*) modification of the solvent layer, and (*b*) removal of the solvent layer.

The goal of solvent modification is the prevention of ice formation in that layer. Commonly used modifiers (referred to as antifreezes or cryoprotectants) are water-soluble organic compounds of low molecular weight with good hydrogen-bonding properties; examples are glycerol, monomeric ethylene glycol and MPD (2-methyl-2,4-pentanediol). These compounds are used in sufficient concentration to suppress nucleation and thereby prevent ice formation. Typical concentrations are in the 15–30% range, depending on the compound and the original composition of the mother liquor. The required concentration must be determined by experiment. Some suitable starting points are given by Garman & Mitchell (1996). The modified solution is tested by cooling a small drop to the working temperature. If the drop remains clear, there is no ice formation.

It is important to keep in mind that any change in the properties of the medium surrounding the crystal will have consequences for its crystallographic stability. In order to protect the crystal, two fields should be considered: thermodynamics and kinetics.

For a crystal in equilibrium with its mother liquor, the chemical potential of each species will be the same inside the crystal and in the mother liquor. If the solution surrounding the crystal is altered by the addition of an antifreeze, the chemical potential μ of water (and other species) will change and the crystal will no longer be in equilibrium with its surrounding solution. The typical result is that $\mu(H_2O,\ \text{solution})$ decreases, so $\mu(H_2O,\ \text{crystal}) > \mu(H_2O,\ \text{solution})$ and there will be a thermodynamic drive to remove water from the crystal. The activation energy for water diffusion is low, so if the process is allowed to proceed, the end result is loss of water with likely deterioration in crystal quality (but see below). Considerations of this kind led Schreuder *et al.* (1988) to develop procedures for solvent modification that would prevent destruction of the crystal. Although some success was reported, sufficient problems were encountered that the approach cannot be considered to be a general solution.

It is important to note that loss of water does not always lead to loss of crystal integrity. For example, Esnouf *et al.* (1998) and Fu *et al.* (1999) have shown that controlled dehydration can result in substantially improved resolution. In addition, antifreeze concentrations substantially higher than those needed to suppress ice formation (Mitchell & Garman, 1994) can preserve low mosaic spread. These phenomena may be connected.

In earlier work, Travers & Douzou (1970) emphasized the importance of keeping the dielectric constant unchanged when modifying the mother liquor. Petsko (1975) made observations that support the significance of this approach and, based on systematic studies, also showed that keeping $\mu(H^+)$ constant is of great importance. Hui Bon Hoa & Douzou (1973) and Douzou *et al.* (1975) have presented tables of solvent compositions that facilitate the preparation of successful cryoprotective solutions. It should be noted that a significant aim in Petsko's work was to keep the solvent liquid, so as to permit manipulation of enzyme substrates. Studies of enzyme kinetics are much more demanding than the rapid cooling to about 100 K that is of primary interest here.

In most cases it is only necessary to consider kinetic effects, *i.e.*, how long it takes before the crystal itself begins to change. When a crystal in a drop of its original mother liquor is dipped into a drop of modified mother liquor, diffusion begins immediately. The speed of propagation in the liquid phase can be estimated from a standard equation for the mean-square travel distance of a diffusing species,

$$\overline{x^2} = 2Dt,$$

where D is the diffusion coefficient and t is the the time. Typical room-temperature values for D for antifreeze molecules in water are around $10^{-9}\ m^2\ s^{-1}$. Thus, a root-mean-square travel distance of 0.1 mm requires about 5 s. For a solvent layer about 0.1–0.2 mm thick, a contact time of 5–20 s will then provide a sufficient level of modification to prevent freezing, while the risk of crystal damage is small. It is often important to stop any ongoing process as soon as protection from freezing has been attained. This can conveniently be done by immersion in liquid N_2.

10.1.2.2. *Internal ice or phase transition*

If there are good indications that ice formation does start internally, or that a destructive phase transition takes place, an attempt can be made to modify the internal water structure. An important consideration of Petsko (1975) was never to allow large deviations from equilibrium. This can be accomplished by a slow, gradual change in $\mu(H_2O,\ \text{solution})$, allowing enough time for the crystal to re-establish equilibrium. A number of successful experiments were reported.

10.1.2.3. *Removal of the solvent layer*

Because of their tendency for rapid loss of internal solvent, dried biocrystals rarely survive exposure to the atmosphere. A solution to this problem was described by Hope (1988). The solvent is removed while the crystal is submerged in a hydrocarbon oil. After the liquid has been removed, a small drop of oil is allowed to encapsulate the crystal, allowing it to tolerate brief exposure to air. Even under such mild conditions, some crystals still lose water and suffer damage. A remedy for this is to keep the oil saturated with water. One disadvantage of the oil technique is the tendency to carry along too much oil, which can cause excessive background scattering. One advantage is that absorption can become nearly isotropic. The most commonly used oil is Infineum Parabar 10312, formerly known as Exxon Paratone-8277 or Paratone-N.

10.1.2.4. *Cooling rates*

The time dependence of nucleation probability suggests that faster is safer. Although no systematic data are available, it is commonly assumed that crystal cooling should be as rapid as possible. Studies related to cryopreservation of biological samples for electron microscopy provide a number of measurements of cooling rates in various coolants, but it is difficult to extract information directly relevant to cryocrystallography. From a practical point of view, the coolants to be considered are liquid N_2 and liquid propane (and, to a lesser extent, liquid ethane). Thermal conductivities for small-molecule compounds in liquid form tend to be of similar magnitude – around

Copyright © 2006 International Union of Crystallography

references