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 much higher than those needed to suppress ice formation (Mitchell & Garman, 1994) can preserve low mosaic spread. Work by Kriminski *et al.* (2002) suggests 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 should 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 achieved by immersion in liquid N_2.

10.1.1.2. 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.

10.1.1.3. Removal of the solvent layer

Because of their tendency toward rapid loss of internal solvent, biocrystals rarely survive prolonged exposure to the atmosphere. A solution to this problem was described by Hope (1988), where 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 of loop mounts to carry along too much oil (Teng, 1990), which can cause excessive background scattering. An advantage is that absorption can become nearly isotropic. The most commonly used oil is the polyisobutene Infineum V8512, formerly known as Infineum Parabar 10312, Exxon Paratone-8277 or Paratone-N. Contrary to popular myth, there is nothing magical or mysterious about this particular oil. Important properties are that it is inert, has a useful viscosity, forms a glass on cooling and has a coefficient of thermal expansion which appears to match that of many biocrystals.

10.1.1.4. Cooling rates

The time dependence of nucleation probability suggests that faster is safer. Although few 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 1.5×10^{-5} W m^{-1} K^{-1}. N_2 boils at 77 K; propane remains liquid between 83 and 228 K. It is often thought that the gas layer that can form around an object dipped in liquid N_2 as a result of the Leidenfrost effect (Leidenfrost, 1756) makes liquid N_2 less effective as a coolant than liquid propane, which is much less likely to form bubbles. However, from model calculations, Bald (1984) suggested that this Leidenfrost insulation problem in liquid N_2 would not be significant in the cooling of small objects of low thermal conductivity, because there is not enough heat transport to the surface to maintain the gas layer. He also concluded that liquid N_2 could potentially yield the highest cooling rate among commonly used coolants, but in a review of plunge-cooling methods, Ryan (1992) gives preference to liquid ethane. Walker *et al.* (1998) measured the cooling rates in N_2 gas (100 K), liquid N_2 (77 K) and liquid propane (100 K) of a bare thermocouple and of a thermocouple coated with RTV silicone cement. The thermocouples were made from 0.125-mm wire and the coating was about 0.20–0.25 mm thick. With the gas stream, cooling of the centres of the samples from 295 to 140 K took 0.8 and 2 s, respectively; with liquid N_2 the times were 0.15 and 0.6 s, and with liquid propane they were 0.15–0.18 and 1.2 s (time reproducibility is to within ±10%). Given the simplicity of liquid-N_2 immersion, there seems little reason to choose the more complicated and more hazardous liquid-propane technique. As the field of low-temperature biocrystallography has matured, liquid-propane methods have all but died out, and liquid-N_2 immersion is now by far the most commonly employed method.

10.1.2. Beneficial effects of low temperature

10.1.2.1. Suppression of radiation damage

Biocrystals near room temperature are sensitive to X-rays and generally suffer radiation damage during data measurement. Often this damage is so rapid and severe that a number of different crystals are needed for a full data set. On occasion, damage is so rapid that data collection is impossible. Crystal decay is typically accompanied by changes in reflection profiles and cell dimensions, which alter the positions of diffraction maxima, exacerbating the problem of changing diffraction intensities. The use of more than one crystal invariably introduces inaccuracies. Intensities from a crystal near the end of its usable