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

life will have decay errors. Individual samples of biocrystals frequently have measurable differences in structure; merging of data will result in an average of the structures encountered, with concomitant loss of definition. Crystals cooled to near liquid-N_2 temperature typically show a greatly reduced rate of radiation damage, often to the extent that it is no longer an issue of major concern. The protection from radiation damage was noted early on (Haas & Rossman, 1970) and numerous cases were observed by Petsko (1975). A noteworthy example is the successful prevention of radiation damage to crystals of ribosome particles (Hope *et al.*, 1989).

Radiation damage appears to be most commonly initiated by photoelectrons and propagated by their inelastic scattering from nearby atoms, creating positively charged species and a cascade of secondary electrons (Garman & Nave, 2009). These can further interact with either protein or with water to form reactive, charged species and free radicals. At sufficiently low temperature, two effects can influence the rate of damage: movement of the reactive species is impeded and the activation energy for reaction is not available. A revealing observation has been described by Hope (1990), where a crystal that had been exposed to synchrotron radiation for many hours at 85 K showed no overt signs of radiation damage, but as the crystal was being warmed toward room temperature, it suddenly turned black and curled up like a drying leaf. More commonly, crystals turn yellow under X-ray irradiation, and bubbles and cracks appear on warming. The rate of free-radical formation would be little affected by temperature, so that when sufficient mobility and activation energy become available, the stored radicals will react.

There is a general consensus that radiation damage, even in high-flux synchrotron beams, can be slowed by cooling to liquid-helium temperatures. The extent of radiation damage is expected to depend on the nature of the macromolecule, and literature examples clearly illustrate this. Meents *et al.* (2007) have reported an extensive study of insulin and holoferritin at 15 and 90 K. They observed statistically significant but relatively small reductions in radiation damage; for holoferritin, 23% less damage at the lower temperature, and for insulin about 6%. In a study of *Streptomyces rubiginosus* D-xylose isomerase, Chinte *et al.* (2007) found a lifetime extension of 25% at 8 K compared to data collection at 100 K. Corbett *et al.* (2007) compared results from data collected at ~40 and at 110 K in a study of the metalloprotein putidaredoxin. They report that radiation-induced photoreduction at 110 K resulted in misleading structure interpretation. At 40 K the photoreduction did not occur. The damage mechanism in this case is related to a change in oxidation state of a central metal atom. The authors made a strong argument for measuring data for metalloproteins at the lowest possible temperature. Chinte *et al.* (2007) pointed out that the additional cost of liquid-helium-temperature measurements compared to 90–100 K measurements is small, and that the advantages can significantly outweigh the increased cost.

In recent years, the ability to calibrate X-ray beam intensity, to calculate reasonable approximations to accumulated radiation dose and to assess crystal degradation by means of various spectroscopies (*e.g.* UV–visible, IR, Raman, XAFS, XANES) in concert with diffraction has brought dramatic advances in understanding radiation damage by X-rays. A concise account has been given by Garman & Nave (2009). Useful suggestions for avoidance of radiation damage, including cases where multiple crystals are required, have been given by Holton (2009). Briefly, knowledge of beamline photon flux density (photons $\mu m^{-2}\,s^{-1}$), the dose ratio (a function of crystal composition and X-ray wavelength), the crystal shape and the cross section intensity profile of a given X-ray beam allows an estimate of the maximum tolerable exposure time for a particular crystal.

10.1.2.2. Mechanical stability of the crystal mount

The mechanical stability of samples is also of concern. Crystals mounted in capillaries and kept wet are prone to movement, giving rise to difficulties with intensity measurements. A crystal at cryotemperature is rigidly attached to its mount; slippage is impossible.

10.1.2.3. Effect on resolution

The effects on radiation damage and mechanical stability are clear-cut, and provide the main reasons for using cryotechniques. Resolution can also be affected, but the connection between temperature and resolution is neither simple nor obvious.

In any crystal, the Boltzmann distribution law is an important factor in determining the accuracy of the replication of structure from one unit cell to another. For many small-molecule crystals, just one arrangement corresponds to a distinct energy minimum. The result is a well ordered structure. With macromolecules, the typical situation is one where a number of arrangements correspond to similar energies. Accordingly, a number of atomic arrangements will be expressed in the crystal. Although the relative values of local minima depend on the temperature, one cannot count on a significant change in ordering by cooling the crystal. Instead, some distribution will be frozen in.

If poor resolution is the result of rapid radiation damage, data collection at cryotemperature can lead to much improved resolution. However, if poor resolution is caused mainly by inexact replication from one unit cell to another, lowering the temperature may have little effect on resolution. If the mosaic spread in the crystal increases upon cooling, resolution may even deteriorate.

In a model proposed by Hope (1988), a relationship between resolution r and temperature T is given by

$$r_2 = r_1[(B_0 + bT_2)/(B_0 + bT_1)]^{1/2}.$$

Here r_1 is the resolution at T_1, r_2 is the resolution at T_2, B_0 is the value of B at $T = 0$ and b is a proportionality constant. There are two underlying assumptions: (1) the overall atomic distribution does not change significantly with temperature and (2) for any given T, the temperature factor [*i.e.* $\exp(-B\sin^2\theta/\lambda^2)$] at the resolution limit has the same value; thus the effects of scattering factors and Lorentz–polarization factors are ignored. We see that if B_0 is the predominant term, lowering T will not have much effect, whereas for small B_0 (a relatively well ordered structure) the effect of T on r can be large. For example, if the room-temperature resolution is 1.5 Å, the resolution at 100 K can be around 1 Å, but if the room-temperature resolution is around 3 or 4 Å, little change can be expected. A qualitative assessment of these effects was clearly stated by Petsko (1975).

10.1.2.4. Annealing of biocrystals

Prior to about 1996, it was thought that thawing of a flash-cooled crystal would inevitably lead to its demise. In spite of anecdotal evidence that some biocrystals could survive warming and re-cooling, this notion persisted until work by Harp *et al.* (1998) and by Yeh & Hol (1998) showed that some biocrystals could be annealed under certain well defined conditions. The method of Harp *et al.* (1998) involved transfer of a flash-cooled crystal from the cold stream to a cryoprotective solution at room

temperature for 3 min followed by a second flash cooling. The Yeh & Hol (1998) technique, on the other hand, is performed *in situ* by simply blocking the cold stream for 1–2 s (*i.e.* until melting is observed), after which time the blockage is removed to re-cool the sample. Both these annealing protocols were shown to be capable of dramatic improvements in diffraction quality, both in terms of reduced mosaicity and improved resolution. A plausible mechanism involving the release of cooling-induced lattice stress by defect migration and solvent transport was suggested by Kriminski *et al.* (2002). Other work (Parkin & Hope, 2003; Juers & Matthews, 2004; Weik *et al.*, 2005) supports the notion of solvent transport, possibly as a result of solvent crystallization (Weik *et al.*, 2001) or other phase transition (Parkin & Hope, 2003) in the aqueous regions within biocrystals.

10.1.2.5. Additional benefits from sub-77 K cooling with helium

As is the case with nitrogen cooling, it is unlikely that thermodynamic equilibrium will be reached by cooling to liquid-helium temperatures. The change that will certainly take place on cooling to liquid-helium temperature is that true thermal motion will be greatly reduced. One result of this is that individual atom peaks will become much sharper. For example, electron-density maxima for well ordered atoms will increase by a factor of about three on cooling from 90 to 10 K. Potentially, this can allow a more detailed interpretation of a structure with a resolution limit better than about 1.5 Å, and also for the better ordered regions of a structure with poorer overall resolution. In general, however, it is not realistic to expect a significant resolution improvement in low-resolution structures based on the effects of temperature alone. Improvements related to diminished radiation damage, on the other hand, can be significant. Two studies illustrate the effects discussed here.

The effects of helium cooling on a high-resolution structure are well illustrated in a study by Petrova *et al.* (2006). They studied a complex of human aldose reductase at 15, 60 and 100 K. The complex has yielded data to 0.66 Å resolution, and thus represents a generally highly ordered structure. The emphasis of the study was on the behaviour of the atomic displacement parameters (ADPs). It was found that the major ADP component for well ordered atoms is temperature driven, as it would be in normal small-molecule structures. A large proportion of the atoms at 15 K have B values of 2 Å^2 or less (about 0.025 Å^2 or less in terms of U values). At 100 K, the corresponding cutoff is about 5 Å^2. Cooling to 15 K allows large portions of the structure to be determined with a precision that would be considered excellent for small molecules. However, the average isotropic B value for the 'best' Cα atoms at 15 K is still 3.9 Å^2 (a U value of about 0.05 Å^2). This indicates that the positional parameters for many of these atoms in reality are composites of closely spaced positions. The best average protein ADPs at 15 K are about the magnitude of small-molecule ADPs at room temperature. This sets unfortunate limits to the attainable accuracy of structural and electron-density parameters.

Hexagonal hen egg-white lysozyme has a relatively well ordered structure, but there are significant regions with multiple conformations. Brinkmann *et al.* (2006) measured diffraction data at 10 K to a resolution limit of 1.46 Å. The results indicate that major areas of disorder are present, illustrating that structural disorder persists at the lowest temperatures.

Although helium is more expensive than nitrogen as a coolant, the added cost for a helium-temperature data set is usually trivial. Equipment design and operating methods have developed to a stage where there is no significant operational difference between nitrogen and helium cooling when manual crystal handling is used.

10.1.3. Principles of cooling equipment

There are many ways to construct a low-temperature apparatus based on the cold-stream principle that functions well, but they are all made according to a small number of basic principles.

All gas-stream crystal-cooling devices must have three essential components: (*a*) a cold gas supply, (*b*) a system of cold gas delivery to the crystal, and (*c*) a system for frost prevention at the crystal site.

10.1.3.1. Liquid-nitrogen-based cold gas supply

Historically, two methods were commonly used: generation of gas by boiling liquid N_2 with an electrical heater, and cooling of a gas stream in a liquid-N_2 heat exchanger. The currently common methods are boiling, and cooling of the gas by means of a refrigerator.

Because precise voltage and current control are easily realized, the boiler method has the advantage of providing very accurate control of the flow rate with minimal effort. Precise control of the flow rate is typically not attained when the rate is controlled with standard gas-flow regulators, because they control volume, not mass.

In addition to control of the flow rate, precise control of the temperature requires exceptional insulation for the cold stream. The longer the stream path, the higher the requirements for insulation. As a rule, temperature rise during transfer should not exceed 15 K at a flow rate of 0.2 mol N_2 min^{-1}; preferably, it should be significantly lower. Higher cooling loss leads to excessive coolant consumption and to instability caused by changes in ambient temperature. High flow rates may also tend to cause undesirable cooling of diffractometer parts.

Appropriate insulation can be readily attained either with silvered-glass Dewar tubing or with stainless-steel vacuum tubing. Glass has the advantage of being available from local glassblowing shops; it generally provides excellent insulation. The main disadvantages are fragility and a rigid form that makes accurate positioning of the cold stream difficult. Stainless steel can provide superb insulation, given an experienced manufacturer. A major advantage is the availability of flexible transfer lines that greatly simplify the positioning of the cold stream relative to the diffractometer.

10.1.3.2. Liquid-helium-based cold gas supply

Open-stream cooling devices that can reach temperatures around 5 K are now commercially available. In principle, the design is simpler than that for liquid-nitrogen-based devices. A basic cooling apparatus consists of a liquid-helium transfer line from a pressurized delivery tank, an evaporation chamber and a cold gas delivery tube. The transfer line is an insulated capillary tube. The delivery tube is an insulated stretch of vacuum tubing with an electrically heated nozzle at the exit. The helium flow is controlled with a needle valve. Because of thermal loss, the flow rate also largely determines the temperature in the range below 20 K. Above about 20 K, an in-stream heating element is used for additional temperature control. This is necessary, because if the flow rate is too low, the cooling stream becomes unstable and will not reliably cover the sample.