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PART 10. CRYOCRYSTALLOGRAPHY

Chapter 10.1. Introduction to cryocrystallography

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Since about 1985, low-temperature methods in biocrystallography have evolved from stumbling experimentation to mainstay production techniques. This would not have happened without good reason. Most of the early development was done with nitrogen cooling. More recently, temperatures as low as 5 K have become accessible by means of liquid-helium-based techniques. A brief discussion of the methods, equipment and advantages of data collection at cryogenic temperatures is given.

10.1.1. Cooling of biocrystals

10.1.1.1. Physical chemistry of biocrystals

Crystals are normally brought from room temperature to the working, low temperature by relatively rapid cooling, either in a cold gas stream, or by immersion in a cryogen such as liquid nitrogen or liquid propane. One goal of the procedure is to avoid crystallization of any water present in the system, whether internal or external to the crystal. Ice formation depends on the formation of nuclei. Nuclei are formed either by homogenous nucleation, *i.e.* in bulk liquid, or by heterogeneous nucleation, *i.e.* at the surface of a phase other than the liquid. Although data pertaining to biocrystals are scarce, indications are that internal nucleation, whether homogenous or heterogeneous, is not common. Proteins that induce nucleation at mild supercooling are known, so presumably there exist regions in these proteins that help to prearrange water molecules so that they readily form ice nuclei. There are also proteins that hinder nucleation. At present there is no basis for predicting the outcome of cooling for any given protein crystal. Only in a statistical sense can one be reasonably confident that a given macromolecule will not promote the freezing of water.

Vali and coworkers (Götz *et al.*, 1991; Vali, 1995) have provided a quantitative treatment of ice nucleation that can serve as a guideline. They observe that the absolute rate of formation of nuclei increases with the volume of water and with decreasing temperature. The probability p that a volume V of water will begin freezing during a time span t is given by

$$p = J(T)Vt,$$

where $J(T)$ is the nucleation rate at temperature T. Based on empirical data, $J(T)$ is given by

$$J(T) = 6.8 \times 10^{-50} \exp[3.9(273 - T)],$$

where J is in $m^{-3}\ s^{-1}$ and T is in K. Note that $J(T)$ increases by a factor of 50 per K. As a practical limit, bulk water cannot be cooled 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 region 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 to prevent 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 include glycerol, ethylene glycols and MPD (2-methylpentane-2,4-diol). These compounds are added to reach a concentration sufficient 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 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 $\pm 10\%$). 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.

Figure 10.1.4.1
Schematic drawing of a dual-stream setup with the streams parallel to the diffractometer φ axis. The top part represents the outlet end of the stream delivery device. The outer stream (lighter grey) is dry, warm air. The goniometer head is protected by a shield.

Figure 10.1.4.2
Schematic drawing of a dual-stream setup with the streams angled relative to the diffractometer φ axis. Stream representations are the same as in Fig. 10.1.4.1. The cold stream misses the goniometer head, so no shield is required.

For a given setting of the flow control valve, the flow rate depends on the tank pressure. For a constant temperature, a constant pressure is required. The pressure is controlled with a pressure regulator that can reduce the tank pressure by releasing helium gas or raise it by adding helium gas from an external helium supply source. A typical delivery tank pressure is around 20 kPa. It is very important that the pressure is constant. The precise value attained is less important.

At atmospheric pressure, helium at around 40 K has the same density as room-temperature air. This means that very cold helium will rise in air, and has the capacity to seriously cool instrument parts in its way. Goniometer heads, beam stops and beam collimators are particularly vulnerable. A simple remedy is to use a small fan to mix the cold helium with room air.

10.1.3.3. Frost prevention

Three areas must be kept frost-free: the crystal, the crystal mount and the delivery end of the transfer tube. The first successful solution to this problem was the dual-stream design of Post *et al.* (1951). It provides for a cold stream surrounded by a concentric warm stream. If the warm stream is sufficiently dry, this will prevent frost around the outlet. The crystal will remain frost-free only if mixing of the two flows occurs downstream from the crystal. For a stream aligned with the axis of the goniometer head, an additional shield is needed to keep the goniometer head frost-free.

10.1.4. Operational considerations

10.1.4.1. Dual-stream instruments

Fig. 10.1.4.1 shows a schematic drawing of the region around the crystal in a traditional dual-stream apparatus, first described by Post *et al.* (1951). The device provides for a cold stream surrounded by a concentric warm stream. The diameter of the cold stream is typically around 7 mm with a shield stream of 2–3 mm. The two streams flow parallel to the axis of the crystal mount. In a properly functioning apparatus, the warm stream supplies enough heat to keep the tip of the cold-stream tube above the dew point. It is important that the streams do not mix, or the crystal temperature will not be stable. This is achieved by careful balancing of flow rates to minimize turbulence. (Absence of turbulence can be judged by the shape of the shadow of the cold stream in a parallel beam of bright light.) In a laminar cold stream, the crystal is well protected and no special precautions are needed. The region of constant, minimum temperature will typically have a diameter of about 3 mm. Turbulent flow will result in the absence of any constant-temperature region, so it is vitally important to verify the stream quality.

The cold stream has sufficient heat capacity to cool down the goniometer head, and sometimes other adjacent equipment parts as well. A simple solution consists of an aluminium cone equipped with a heating coil on the back. A shield that functions well has been described by Bellamy *et al.* (1994).

Fig. 10.1.4.2 illustrates a situation where the stream direction deviates substantially from the head-on direction in Fig. 10.1.4.1. An angle of 35–55° will give good results. An advantage of an angled delivery is that the cold stream will not touch the goniometer head, and therefore the heated stream deflector is not needed, resulting in simplified installation and operation.

Analysis of the dual-stream apparatus reveals a twofold function of the outer stream: it keeps the nozzle frost-free and it supplies heat to the mounting pin. Protection of the crystal is, in reality, already provided by the laminar cold stream. The nozzle can be kept frost-free simply with an electric heater. Ice formation on the crystal mount can be easily suppressed by appropriate design of the mounting pin and mounting fibre, and attention to their interaction with the cold stream. A successful solution is sketched in Figs. 10.1.4.3 and 10.1.4.4.

10.1.4.2. Electrically heated nozzle

Fig. 10.1.4.3 shows the functional equivalent of Fig. 10.1.4.1. Instead of the warm stream, an electrical heating element is used to keep the tip of the delivery tube ice free. An actual construction will usually consist of a nozzle that can be attached to the delivery tube. The heating element is made from standard resistance wire (*e.g.* Nichrome). About 5 W will usually be

Figure 10.1.4.3
Schematic drawing of a single-stream setup with the stream parallel to the diffractometer φ axis. The cold stream is represented by a grey region. The nozzle is heated above the dew point with a heating coil. The goniometer head is protected by a shield.

Figure 10.1.4.4
Schematic drawing of a single-stream setup with the stream angled relative to the diffractometer φ axis. The cold stream is represented by a grey region. The crystal mounting pin protrudes 1–2 mm into the cold stream. This prevents frost from forming on the mounting fibre. The nozzle is heated above the dew point with a heating coil. The cold stream misses the goniometer head, so no shield is required. In general, the simplest operation is attained with a setup similar to that shown here.

enough to prevent frost or condensation. The head-on direction results in reliable frost protection for the crystal, but requires that the goniometer head is equipped with a heated stream deflector.

Fig. 10.1.4.4 shows the functional equivalent of Fig. 10.1.4.2. This arrangement leads to the simplest design, although some precautions are needed. The mounting pin itself supplies the heat needed to prevent ice formation on the pin. The tip of the pin should be smooth in order to prevent turbulence. About 1–2 mm (but not more) of the tip must protrude into the cold stream. If too much of the pin is in the cold stream, the rest of the pin can become too cold and ice up. If the pin is too far out of the stream frost prevention will also fail, because glass or other insulating mounting materials will invariably collect ice at the cold/warm interface. As frost prevention depends on heat conducted from the rest of the pin, it must be made from copper (a requirement not strictly necessary for the dual-stream design). This design has been extensively tested and has been used for many years for the collection of a large number of data sets. Results are uniformly good, with simple operation and reliable frost prevention even in high humidity.

Fig. 10.1.4.5 is a photograph of a laboratory implementation of the setup of Fig. 10.1.4.4, with helium as the coolant. Operation is as simple as with nitrogen cooling.

Omission of the warm stream results in significant design simplification. The entire apparatus for production of the outer stream is left out, resulting in real savings in manufacture, operation and maintenance. There is no obvious disadvantage, as ice protection is as good as with the dual stream. The main cost to the user is in the requirement that the mounting system be constructed within somewhat narrower limits, including the requirement of a copper-shafted mounting pin. The fact that operator errors tend to become apparent through frost formation can actually be an advantage. With a dual-stream device, an improperly positioned cold stream or an improperly prepared crystal mount may not produce overt signs, even though the crystal temperature is ill-defined.

In all configurations shown, correct positioning of the cold stream is essential. The centre of the stream should not miss the centre of the diffractometer (and hence the crystal) by more than 0.5 mm.

With helium, the single-stream technique represents the best solution and reliable frost prevention at temperatures down to around 5 K is easily attained. Crystal mounting is simple and the sample is always visible, simplifying centring. In the authors' experience, liquid-helium cooling is as simple as liquid-nitrogen cooling.

10.1.4.3. Temperature calibration

Measurement of the temperature at the crystal site with sensing devices that require attached leads is very difficult, mainly because of heat conduction along the leads. It is usually necessary to loop the leads into the delivery nozzle.

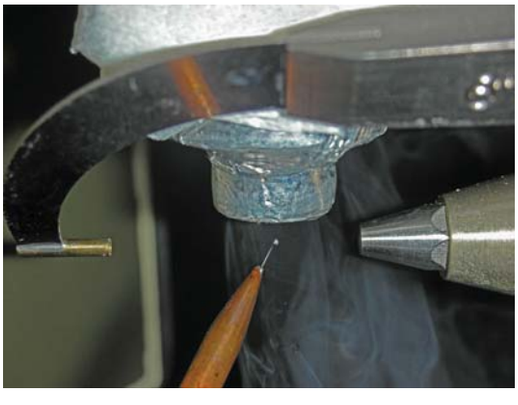

Figure 10.1.4.5
A crystal in a helium stream at 8 K in a setup corresponding to Fig. 10.1.4.4. A thin layer of fog forms at the helium–air interface. The cold stream breaks up well below the crystal position. Rising cold gas (visible as fog to the right of the cold stream) has been mixed with air to prevent cooling of diffractometer parts. The nozzle temperature is 295 K. The crystal is attached to a tapered glass fibre. The light-coloured region at top is not ice; it is part of the insulation. This photograph was taken after the end of data collection.

Both Si diodes and Pt resistance sensors have become sufficiently miniaturized to make them preferred choices at the lowest temperatures. Thermocouples are acceptable above about 80 K. A reliable method of calibration makes use of the known temperature of a phase transition of a crystal in the normal data-collection position. For example, KH_2PO_4 (often referred to as KDP) has a sharp transition at 123 K from tetragonal to orthorhombic, and is commonly used. Another possibility is KH_2AsO_4, which has a corresponding phase transition at 95 K.

Two readout temperatures suffice, one at room temperature and one at the phase transition. The difference between readout temperature and crystal-site temperature can be assumed to vary linearly with T, so interpolation or extrapolation is simple.

10.1.4.4. Transfer of the crystal to the diffractometer

Inspection of Figs. 10.1.4.1–10.1.4.4 reveals that the mounting of a crystal on a mounting pin *via* the traditional placement of the pin in the hole of a standard goniometer head is not simple, because the cooling nozzle is in the way. The solution to the problem is a design that allows side entry. This is most commonly achieved with a magnetic platform on the goniometer head and a corresponding magnetic base on the mounting pin, but an alternative means of side entry employs a slot on a modified goniometer head; the slot is equipped with a spring-loaded catch that allows a very smooth, but stable, catch of the pin.

The use of liquid-N_2 cooling and side entry, and the requirement of reproducible knowledge of crystal temperature at all times, led to the development of a set of tools for crystal mounting as described by Parkin & Hope (1998). The tools include special transfer tongs used for moving crystals from liquid N_2 to the goniometer head. The temperature of the crystal is maintained by the heat capacity and low heat conductance of the tongs. The operation is independent of the orientation of the goniometer head because there is no liquid to contain.

10.1.4.5. Automated robotic crystal handling

The era of structural genomics has necessitated a move to high throughput at high-intensity synchrotron sources. To meet this goal, a number of robotic crystal-handling devices have been developed. These in turn have had the beneficial effect of increased standardization in mounting-pin geometry.

10.1.5. Concluding note

With correctly functioning low-temperature equipment and appropriate techniques, a crystal can be maintained frost-free for the duration of a data-collection run. Formation of frost on the crystal indicates malfunction of the equipment, or operator error. The most likely cause is operator error, but faulty equipment cannot be ruled out. The techniques described here have been used for collecting thousands of data sets from ice-free crystals and crystal mounts. There is no reason to accept frost problems as an unavoidable part of cryocrystallography.

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