

10. CRYOCRYSTALLOGRAPHY

10.1. Introduction to cryocrystallography

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10.1.1. Utility of low-temperature data collection

10.1.1.1. Prevention of radiation damage

Since about 1985, low-temperature methods in biocrystallography have moved from stumbling experimentation to mainstay production techniques. This would not have happened without good reason. A brief discussion of the advantages of data collection at cryogenic temperatures is given.

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 almost always 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 some loss of definition. Crystals cooled to near liquid-N₂ temperature typically show a greatly reduced rate of radiation damage, often to the extent that it is no longer an issue of concern. The protection from radiation damage was noted early on. Petsko (1975) observed numerous cases of this effect. A noteworthy example is the successful prevention of radiation damage to crystals of ribosome particles (Hope *et al.*, 1989).

Radiation damage appears to be related to the formation of free radicals. At sufficiently low temperature, two effects can influence the rate of damage: movement of the radicals is hampered, and the activation energy for reaction is not available. A revealing observation has been described by Hope (1990). A crystal that had been exposed to synchrotron radiation for many hours at 85 K showed no overt signs of radiation damage. However, while 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 affected little by temperature, so that when sufficient mobility and activation energy become available, the stored radicals will react.

10.1.1.2. Mechanical stability of the crystal mount

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

10.1.1.3. 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. If low resolution is the result of rapid radiation damage, lowering the temperature can lead to much improved resolution. However, if low resolution is mainly caused 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. The underlying assumption is that for any given temperature, 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 L_p 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. Cooling of biocrystals

10.1.2.1. 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 which 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 of water will freeze 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 $\text{m}^{-3} \text{s}^{-1}$ 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

10. CRYOCRYSTALLOGRAPHY

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 $\mu(\text{H}_2\text{O}, \text{solution})$ decreases, so $\mu(\text{H}_2\text{O}, \text{crystal}) > \mu(\text{H}_2\text{O}, \text{solution})$ 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 $\mu(\text{H}^+)$ 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 time. Typical room-temperature values for D for antifreeze molecules in water are around $10^{-9} \text{ m}^2 \text{ s}^{-1}$. 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 $\mu(\text{H}_2\text{O}, \text{solution})$, 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

10.1. INTRODUCTION TO CRYOCRYSTALLOGRAPHY

$1.5 \times 10^{-5} \text{ W m}^{-1} \text{ K}^{-1}$. N_2 boils at 77 K; propane remains liquid between 83 and 228 K. It is often thought that a gas bubble that can form around an object dipped in liquid N_2 makes it less effective as a coolant than liquid propane, which is much less likely to form bubbles. However, from model calculations, Bald (1984) suggested that the gas 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 K 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.

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. Cold gas supply

Two methods are 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.

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 $0.2 \text{ mol N}_2 \text{ min}^{-1}$; 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 also tend to cause undesirable cooling of diffractometer parts. No commercially offered device should be accepted if it does not meet the criterion given above.

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; unsatisfactory insulation is quite common. A major advantage is the availability of flexible transfer lines that greatly simplify the positioning of the cold stream relative to the diffractometer.

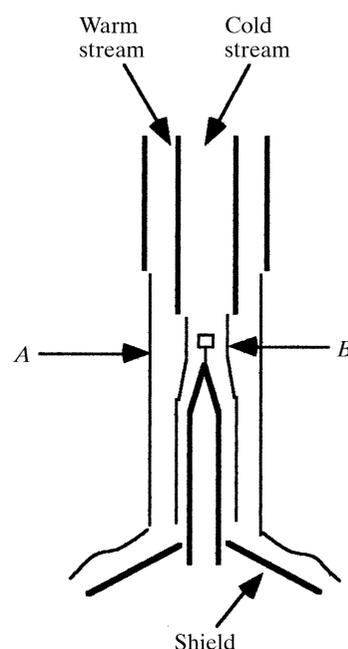


Fig. 10.1.4.1. Schematic drawing of a dual-stream setup with the streams parallel to the diffractometer φ axis. The top part represents the outlet end of the stream delivery device. *A* represents the outline of the warm shield stream and *B* represents the interface between the cold stream and the warm stream. The goniometer head (not shown) is protected by a shield.

10.1.3.2. 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 tube carrying the cold stream 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 unusual precautions are needed. The region of constant, minimum temperature will typically have a diameter of about 3 mm. Turbulent flow will result in no constant-temperature region, so it is 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

10. CRYOCRYSTALLOGRAPHY

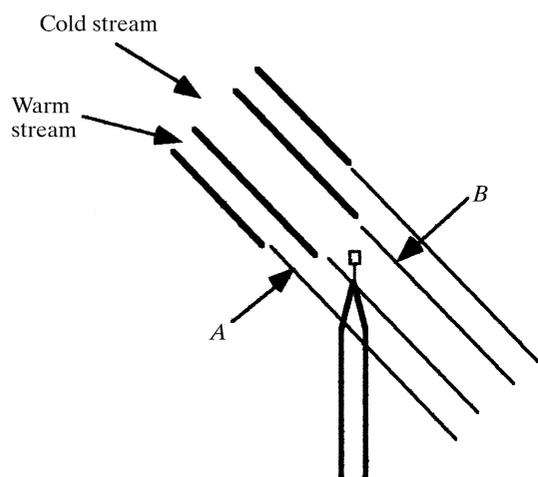


Fig. 10.1.4.2. Schematic drawing of a dual-stream setup with the streams angled relative to the diffractometer φ axis. *A* and *B* are the same as in Fig. 10.1.4.1. The cold stream misses the goniometer head, so no shield is required.

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 goniometer head will not be touched by the cold stream, 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

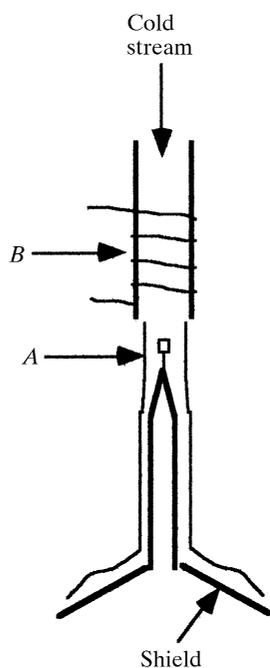


Fig. 10.1.4.3. Schematic drawing of a single-stream setup with the stream parallel to the diffractometer φ axis. *A* represents the outline of the cold stream. The tip of the outlet end is heated above the dew point with a heating coil *B*. The goniometer head (not shown) is protected by a shield.

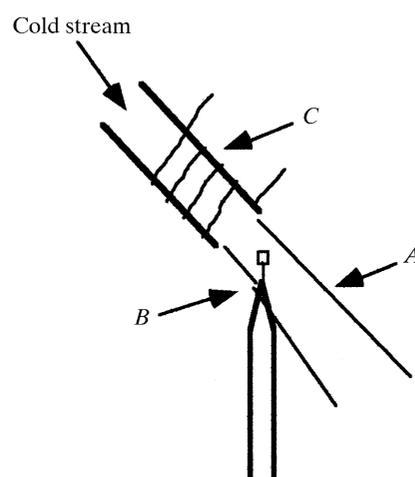


Fig. 10.1.4.4. Schematic drawing of a single-stream setup with the stream angled relative to the diffractometer φ axis. *A* represents the outline of the cold stream. At *B* the crystal mounting pin protrudes 1–2 mm into the cold stream. This prevents frost from forming on the mounting fibre. The tip of the outlet end is heated above the dew point with a heating coil *C*. 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.

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 enough to prevent frost or condensation. The head-on direction results in reliable frost protection for the crystal, but the goniometer head must be 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, though some precautions are needed. The mounting pin itself supplies the heat needed to prevent ice formation on the pin. The cone-shaped tip of the pin (full cone angle about 20°) should be smooth in order to prevent turbulence. About 1–2 mm (but not more) of the tip must protrude into the cold stream (see region *A* in Fig. 10.1.4.4). 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 it will also fail, because glass or other insulating mounting materials will invariably collect frost at the cold/warm interface. As frost prevention depends on heat conducted from the rest of the pin, it must be made from copper. 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.

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. 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. 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

10.1. INTRODUCTION TO CRYOCRYSTALLOGRAPHY

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.

10.1.4.3. *Temperature calibration*

Measurement of the temperature at the crystal site with thermocouples or other devices that require attached leads is very difficult, mainly because of heat conduction along the leads. The preferred method of calibration makes use of the known temperature of a phase transition of a crystal in the normal data-collection position. 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. Two methods are in use. One depends on a side-entry 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 other method relies on a magnetic platform on the goniometer head and a corresponding magnetic base on the mounting pin.

The use of liquid- N_2 cooling and side entry, and the requirement of reproducible knowledge of crystal temperature, 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, since there is no liquid to contain.

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.

10.2. CRYOCRYSTALLOGRAPHY TECHNIQUES AND DEVICES

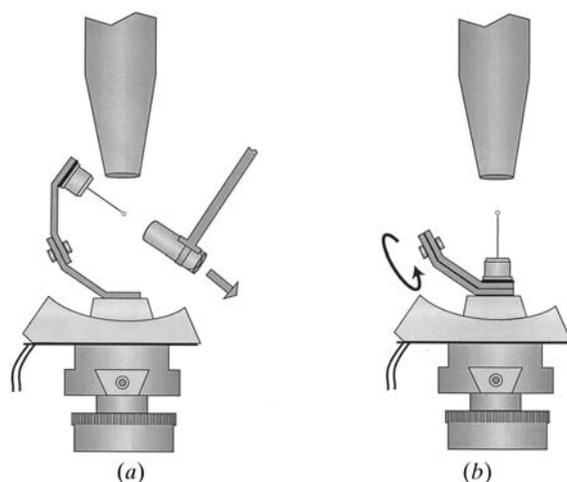


Fig. 10.2.5.2. Transfer using an alternative device for achieving the correct magnetic mount orientation. (a) A hinged mechanism is extended to orient the magnetic mount downward, and the loop assembly is attached. (b) The mechanism is rotated about the hinge to place the mount in the normal orientation for data collection.

nitrogen. The thermal mass of the tongs prevents warming as the crystal is then placed on the goniometer. The tongs are opened and removed to expose the crystal to the gas stream.

Any of these transfer procedures can be reversed in order to return the loop assembly to liquid nitrogen without thawing the crystal. The assembly and cryovial can then be placed in a Dewar designed for long-term storage. Some opening should be present in the loop-assembly bases, or the cryovials should be notched, to allow free movement of liquid nitrogen. The vials are conveniently held and organized using aluminium canes, which take up to five samples and have tabs that hold the loop assemblies in place. For

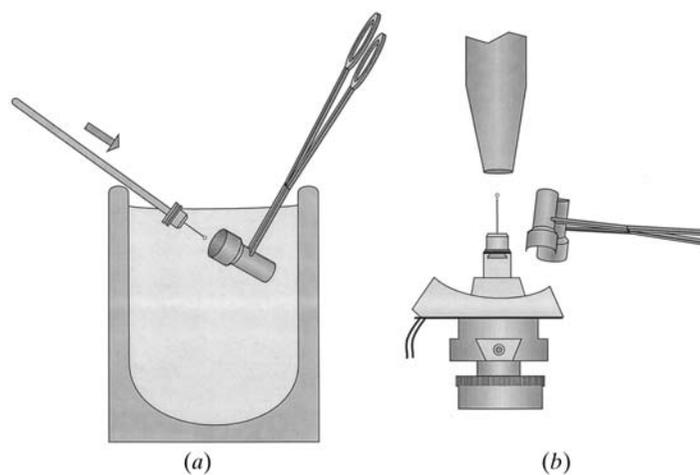


Fig. 10.2.5.3. Transfer using tongs. (a) The crystal is inserted into a split metal cup that allows the base to be held securely. (b) The tongs are inverted and used to place the loop assembly on the magnetic mount. The jaws of the tongs are then opened to separate the halves of the cup, and the tongs are withdrawn.

even more secure long-term storage, loop assemblies with threaded bases that screw into the cryovials are available.

The ability to store samples for long periods of time permits a number of crystals to be flash cooled under consistent conditions, which can be important for maintaining isomorphism, and crystals can also be stockpiled for later data collection at a synchrotron X-ray facility. In fact, crystals should be prescreened for quality in the laboratory before synchrotron data collection to make efficient use of time on the beam line. Finally, crystals that degrade in growth or harvest solutions, or that contain macromolecules in unstable or transient states, can be conveniently preserved by flash cooling and storage in liquid nitrogen.

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- 10.1**
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