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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.

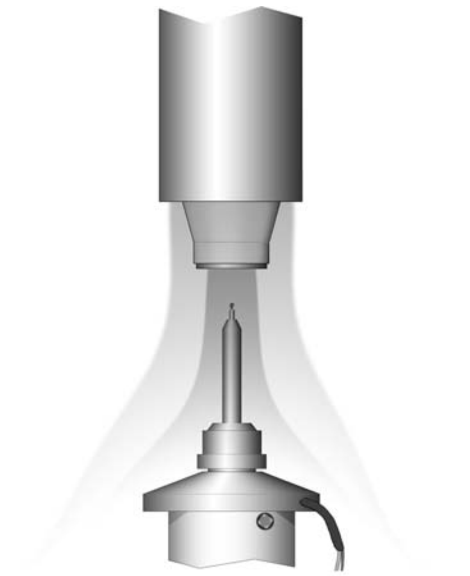

Figure 10.1.4.1
Schematic drawing of a dual-stream setup with the streams parallel to the diffractometer φ axis. The top part represents the outlet end of the stream delivery device. The outer stream (lighter grey) is dry, warm air. The goniometer head is protected by a shield.

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

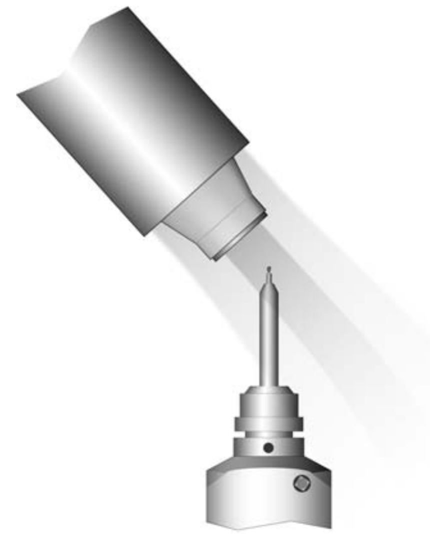

Figure 10.1.4.2
Schematic drawing of a dual-stream setup with the streams angled relative to the diffractometer φ axis. Stream representations are the same as in Fig. 10.1.4.1. The cold stream misses the goniometer head, so no shield is required.

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

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