Table 3.4.1.3. *Cryoprotectants commonly used for biological macromolecules*

Protectant	Concentration (% by volume)
Glycerol	13–25
Ethylene glycol	11–30
Poly(ethylene glycol) 400	25–35
Xylitol	22
(2*R*,3*R*)-Butane-2,3-diol	8
Erythritol	11
Glucose	25
2,4-Methylpentanediol	28–45

Pressure cells. Tilton (1988) has described an attachment that can be used on conventional diffractometers for collecting X-ray data from biomolecular crystals under gas pressures up to 300 atm (30 MPa). The crystals are coated with mineral oil to minimize dehydration (see Subsection 3.4.1.5) and mounted in a quartz glass capillary between two layers of cotton fibres. These fibres give mechanical support to the specimen and protect it from shock during gas pressurization. No plugs of mother liquor or oil are used so that the gas flow is unimpeded. Kundrot & Richards (1986) describe an adaptation of the flow cell for hydrostatic pressure studies up to 0.2 GPa. More recently, Kroeger & Kundrot (1994) have described a gas cell that allows data sets at several partial pressures to be collected from the same crystal.

3.4.1.5. *Cryogenic studies of biological macromolecules*

Useful recent reviews on protein crystallography at low temperatures have been written by Hope (1990) and Watenpaugh (1991).

3.4.1.5.1. *Radiation damage*

Crystals of biological macromolecules are very susceptible to radiation damage, and this can severely limit the amount and quality of diffraction data that can be collected per crystal. There have been relatively few systematic studies of this phenomenon (Young, Dewan, Nave & Tilton, 1993; Gonzalez & Nave, 1994; Nave 1995), but one of the first effects of radiation damage is the deterioration of the high-resolution regions of the pattern, followed by increasing loss of crystallinity. Improvement of crystal lifetime in X-ray beams has been obtained by the addition of free-radical scavengers (Zaloga & Sarma, 1974) and the replacement of the mother liquor with solutions containing 10–20% polyethylene glycol 4000 or 20000 (Cascio, Williams & McPherson, 1984). The use of synchrotron radiation has also led to improved data-per-crystal ratios (Lindley, 1988). The high intensity allows fast collection of data, and the high collimation permits different sections of the same crystal to be used for data collection. This is particularly useful for prismatic crystals, which can be mounted along their largest morphological axis. An alternative method of surmounting this problem, however, is to freeze the protein crystal. As the temperature is decreased, the rate of diffusion of free radicals is reduced, with a corresponding reduction in radiation damage. Appreciable reduction in diffusion rate is achieved even at 250 K, and at 100 K diffusion essentially ceases. Cryogenic measurements not only minimize radiation damage but often lead to improved resolution owing to decrease in thermal motion in the crystal. Increasing the crystal lifetime may be particularly important with respect to multiwavelength anomalous-dispersion measurements in order to derive phase information. Since crystals of biological macromolecules contain substantial amounts of solvent, typically between 35 and 80% by volume, the technical problem is to force the solvent to cool in an amorphous glass-like state, rather than as crystalline ice. The latter normally degrades the crystallinity by expansion and gives rise to powder rings, which complicate data measurement.

3.4.1.5.2. *Cryoprotectants*

Cryoprotectants are normally required to avoid ice formation, and the choice of cryoprotectant will depend on the nature of the mother liquor from which the crystals have been grown. Crystals grown from high salt will usually require high salt concentration in the cryobuffer to avoid dissolution, although the addition of organic solvents may be a useful alternative. Table 3.4.1.3 lists commonly used cryoprotectants and their typical concentrations (Gamblin & Rogers, 1993).

The introduction of the cryoprotectant can be achieved through: (*a*) crystal growth in the cryoprotectant; (*b*) direct transfer of crystal from mother liquor into cryoprotectant buffer either in a single step or in steps of increasing cryoprotectant concentration; (*c*) dialysis, either direct or stepwise; or (*d*) exchange of liquor using a flow cell and a gradient maker.

3.4.1.5.3. *Crystal mounting and cooling*

Experience indicates that small crystals are better for cryogenic purposes, presumably because the rate of diffusion of small molecules and the rate of heat loss during rapid freezing is significantly faster than for large crystals. In most cases, there is an increase in the mosaicity (typically by a factor of 2–3), and in large specimens the increase may render the crystals useless for data collection. Successful freezing is often indicated by the crystal remaining transparent. Opacity usually indicates considerable breakdown in the crystallinity. Three commonly used methods for mounting crystals of biological macromolecules for cryogenic measurements are detailed below.

(i) *Coating methods*. Useful accounts of this method are given by Dewan & Tilton (1987) and Hope (1988). The crystal is first transferred to a hydrocarbon environment, mounted on a glass fibre attached to a brass pin on a goniometer head, and then fast cooled by introduction into a nitrogen-gas stream. The crystal adheres to the fibre by surface-tension effects, and the hydrocarbon also prevents loss of solvent during transfer into the gas stream. Paratone-N (Exxon) mixed with mineral oil (25–50% mineral oil) has a suitable viscosity, and excess oil should be removed by draining. This method has been successfully used for a number of biological macromolecules including crambin (Teeter, Roe & Heo, 1993) and the bovine eye lens protein, γB-crystallin (Lindley *et al.*, 1993). In the case of γB-crystallin, it was found that large crystals, $0.5 \times 0.5 \times 1.0$ mm, often became opaque after freezing, indicating gross damage to the crystallinity, or showed appreciable mosaic spread in the subsequent diffraction patterns, rendering them useless for data collection. Smaller crystals, $0.2 \times 0.2 \times 0.8$ mm, gave good diffraction patterns with an increase in the mosaic spread of only a factor of

about two, compared with room-temperature measurements, presumably because of smaller angular and size distributions of the mosaic blocks. For γB-crystallin, the effective resolution was extended from 1.5 Å to at least 1.2 Å. A coating and flash-freezing method has been employed to obtain data from physically fragile and very radiation sensitive crystals of 50S ribosomal particles (Hope *et al.*, 1989). The crystals were transferred to an inert hydrocarbon environment, or to solutions similar to the crystallization medium but with higher viscosities, and flash frozen on a thin glass spatula by immersion in liquid propane. They were then transferred to a cold-nitrogen-gas stream for data measurement. The immersion in a slurry of propane near its melting point gives good wetting of the crystal surface and a heat transfer rate appreciably faster than direct introduction into a cold-gas stream. Transfer from the propane to the gas stream has to be achieved rapidly to avoid ice formation on the surface of the protein owing to condensation of moist air.

(ii) *Loop techniques*. Loops (Teng, 1990; Gamblin & Rogers, 1993), made from fine wire, glass, and a range of thin fibres, can provide very useful mounts for cryocrystallography. Typically, the loops are folded and the two ends glued inside a glass capillary mounted on a goniometer head. Rayon and hair fibres give relatively low backgrounds in diffraction patterns and can readily be made into loops with diameters from 200 to 800 μm. Larger-diameter loops tend to fold over, and glass fibres are more appropriate. Wire loops have a distinct disadvantage in that a plane of diffraction data in which the X-rays are blocked by the wire loop is inaccessible. The diameter is chosen so that the crystal just fits inside the loop and is held in place by surface tension with a thin film of the crystallization/cryoprotectant buffer. The loop with the crystal can then be flash frozen by immersing in liquid propane or fast frozen by direct introduction into a cold-gas stream. Hope (1990) describes a device that can rapidly transfer crystals mounted in loops from a liquid-propane bath to the cooled-gas stream. Indeed, once crystals have been frozen in loops they can be transferred to liquid-nitrogen containers and kept almost indefinitely. A typical application of the loop technique is provided by the crystal structure determination of an extracellular fragment of the rat CD4 receptor (Lange *et al.*, 1994).

(iii) *Liquid-helium cryostat: neutron diffraction*. Slow freezing using a liquid-helium cryostat (Archer & Lehmann, 1986), over a period of hours, has been successfully used with crystals of the coenzyme of vitamin B_{12} to 15 K (Bouquiere, Finney, Lehmann, Lindley & Savage, 1993), where the solvent content is relatively low, 16–17 water molecules per asymmetric unit. Whether biological macromolecular crystals can be annealed to low temperatures with progressive sets of cooling, heating and cooling stages is not well researched.

3.4.1.5.4. *Cooling devices*

Several airstream devices have been described to cool protein crystals to around 250 K [Marsh & Petsko (1973), temperature range 253 to 303 K; Rossi (1989), temperature range 242 to 335 K; Machin, Begg & Isaacs (1984), 258 to 293 K; Fischer, Moras & Thierry (1985), temperature range 263 to 293 K; Fraase Storm & Tuinstra (1986), 250 to 350 K; Arndt & Stubbings (1987), 248 to 353 K]. The devices of Machin, Begg & Isaacs, Fraase Storm & Tuinstra and Arndt & Stubbings involve thermoelectric modules utilizing the Peltier effect. The space available to accommodate the sample is usually very limited and care has to be taken with the length of the capillary and other aspects of crystal mounting. Hovmöller (1981) has designed an extension to the cooling delivery tube that minimizes air turbulence at the sample. Various devices have been described that operate down to near liquid-nitrogen temperature and that can be fitted to a variety of data-collection systems. These include the rotation camera (Bartunik & Schubert, 1982), and a universal cooling device for precession cameras, rotation cameras and diffractometers (Hajdu, McLaughlin, Helliwell, Sheldon & Thompson, 1985). One of the more versatile devices is the cryostream described by Cosier & Glazer (1986), which uses a pump to effectively separate the liquid-nitrogen supply from the gas outflow; this arrangement eliminates instabilities in the cooling-gas stream; the device works in the range 77.4 to 323.0 K and is commercially available (Oxford Cryosystems, England).

3.4.1.5.5. *General*

Cryocrystallography not only minimizes the effects of radiation damage but also often allows the collection of high-quality, high-resolution data from a single specimen. In the case of very labile systems such as ribosomal particles, it is sometimes the only means of obtaining useful diffraction data. Further, cryocrystallography permits the study of temperature effects on the structure and dynamics of biological macromolecules. In this latter regard, examples include multiple-temperature crystallographic studies on sperm whale myoglobin (Frauenfelder, Petsko & Tsernoglou, 1979; Hartmann *et al.*, 1982; Frauenfelder *et al.*, 1987) and, more recently, ribonuclease-A (Tilton, Dewan & Petsko, 1992; Rasmussen, Stock, Ringe & Petsko, 1992). The future will no doubt see the routine emergence of cryogenic techniques for data collection, using both conventional and synchrotron X-ray sources, from biological macromolecules, with consequent improvement in structure quality and detail.

3.4.2. Setting of single crystals by X-rays

3.4.2.1. *Introduction*

With regard to X-ray structure analysis, the use of automated data-collection devices in conjunction with sophisticated software packages has, in the most part, eliminated the need for accurate crystal-setting techniques, although it should be remembered that the determination of the precise crystal orientation with respect to the instrument axes is a prerequisite for data processing. Furthermore, in the case of samples that are highly radiation sensitive (*e.g.* viruses), the lifetime of the sample in the X-ray beam does not permit accurate setting. However, the exercise of setting a crystal so that a certain morphological feature and/or unit-cell edge is perpendicular or parallel to the X-ray beam at the start of the experiment is often very useful, not only in establishing the quality of the crystal diffraction pattern (spot dimensions, mosaicity, twinning, limit of resolution, susceptibility to radiation damage, *etc.*), but also in ensuring that intensity data are collected in the most efficient manner and that the data set is as complete as possible (see also Subsection 3.4.2.8). Mounting a crystal specimen in a random orientation can often lead to inefficient data collection (some reflections measured several times and volumes of reciprocal space not measured at all), and in extreme cases can lead to inappropriate or incorrect choice of cell and space group. Optical examination, crystal density measurement, and careful analysis of diffraction data should still be regarded as important components of crystal structure analysis, even though data collection may be fully automated.

In most cases, the problem of crystal setting by X-rays is composed of two parts (Jeffery, 1971):

references