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

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