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3.4. Mounting and setting of specimens for X-ray crystallographic studies

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3.4.1. Mounting of specimens

3.4.1.1. *Introduction*

This section deals with the mounting of two categories of specimens:

(1) polycrystalline;

(2) single crystal.

Category 2 is further divided into single crystals of small organic and inorganic molecules, and those of biological macromolecules at both ambient and cryogenic temperatures. Commonly used methods of mounting specimens for both camera and diffractometer, and most other detector systems are described.

The bibliography is necessarily selective and wherever possible has been restricted to journals and textbooks that are readily accessible to a crystallographic laboratory. It should also be noted that there exist, worldwide, various centres specializing in synchrotron-radiation and neutron diffraction techniques. Within these centres lies a wealth of experience in sample handling and preparation. For specialist purposes, communication with local contacts at such centres may provide invaluable assistance.

3.4.1.2. *Polycrystalline specimens*

3.4.1.2.1. *General*

Informative accounts of the powder method of recording diffraction patterns have been given by Klug & Alexander (1954), D'Eye & Wait (1960) and Dent Glasser (1977). There are three principal methods of preparing polycrystalline specimens for mounting in powder cameras:

(1) encased;

(2) bonded;

(3) fibre supported.

The most common method of preparing samples of polycrystalline materials is to *encase* them in thin-walled capillary tubes, for Debye–Scherrer camera work, or into sample holders, for Guinier camera and diffractometer measurements. This technique has the advantage that the sample can be readily protected from attack by oxygen, carbon dioxide and water vapour, and, if necessary, the sample preparation can be undertaken in an inert atmosphere (Lange & Haendler, 1972; D'Eye & Wait, 1960). The precise details of sample preparation and mounting will be dependent on the type of camera or diffractometer used, but the particle size should be generally less than 10 μm for stationary samples and diffractometer work. A slightly larger particle size, 45 μm, can be used for Debye–Scherrer camera work if the specimen is rotated. Foit (1982) has described a simple method of filling thin-walled capillaries using an ultrasonic vibrator. A frequent problem affecting intensity measurements from powder specimens is caused by preferred orientation when powder samples are packed or pressed. McMurdie, Morris, Evans, Paretzkin & Wong-Ng (1986) have described a method of sample preparation suitable for a diffractometer that minimizes this problem.

Capillaries made from lithium beryllium borate (Lindemann glass), borosilicate (*e.g.* Pyrex glass), or fused silica are commercially available in a variety of internal diameters. For very high temperatures, thin-walled ceramic or metal capillaries can be used. The diffraction pattern of the metal can be used as an internal standard. Capillaries that are suitable for materials that react with glass can be made from various organic polymers. Table 3.4.1.1 lists details of capillaries and other containers suitable for encasing powder specimens.

In the *bonded* method, the polycrystalline material is mixed with an adhesive such as gum tragacanth or ethyl cellulose, and the mixture is wetted with water or aqueous alcohol to form a viscous paste. The paste is then rolled between two glass slides or extruded through a glass capillary, using a glass or metal piston, to form a cylindrical sample. This can be cut to length and either glued, fixed with plasticine, or cemented (for high-temperature work) to the camera mounting pin. Alternatively, the sample can be compressed and compacted in a die to form a solid rod, or, for diffractometers, into a disc. In the case of very small quantities of material, the powder can be smeared with silicone vacuum grease over the surface of a disc-shaped silica crystal. The silica can then be used as an internal standard.

In the *fibre-supported* method, a silica, Lindemann, or borosilicate glass fibre moistened with adhesive (Canada balsam diluted with xylene, collodion, gum tragacanth and water, dilute fish glue) is dipped into the powder. Experience has shown that powder adhesion to the fibre is often improved if non-drying glues or viscous oils are employed. Hairs of fine organic filaments have been used in place of glass fibres, and for high temperature above 1270° C metal wires are useful. Once again, the metal diffraction patterns can act as internal standards. For extruded metal wires, the wire itself acts as the specimen, and the diameter can be reduced by etching if it is too large, or a glancing-angle diffraction technique can be employed. Various specialized holders for diffraction studies of polycrystalline samples can be found in annual conference proceedings such as EPDIC (*European Powder Diffraction Conference*, Switzerland: Trans Tech Publications) and Advances in X-ray Analysis (*Proceedings of the Annual Conference on the Applications of X-ray Diffraction*, New York/London: Plenum). The journals *Reviews of Scientific Instruments* (American Institute of Physics) and *Nuclear Instruments and Methods* (Elsevier, North-Holland) also provide useful sources of information.

3.4.1.2.2. *Non-ambient conditions*

A number of devices have recently been described to study polycrystalline specimens under non-ambient conditions. Rink, Mathias & Schlenoff (1994) have designed a portable sample housing for work at room temperature with samples that are air or moisture sensitive. A review of designs and desirable features for high-temperature furnaces suitable for X-ray diffractometers has been given by McKinstry (1970). More recently, Puxley, Squire & Bates (1994) have described an *in situ* cell fitted to a Siemens D-500 powder diffractometer that allows samples in flowing or static reactive gas environments at atmospheric pressure and at temperatures up to 1273 K. These authors also review other developments in the field of high-temperature furnaces for polycrystalline X-ray diffraction published since the McKinstry article in 1970. Brown, Swapp, Bennett & Navrotsky (1993) have devised methods to minimize the uncertainties in temperature at the sample and in the position of the sample itself. Tarling, Barnes & Mackay (1984) have adapted a Guinier–Lenné high-temperature powder camera to include a gas rinsing system and a specially designed mini-environment cell in which conditions of industrial furnacing can be simulated. In the neutron area, Lorenz, Neder, Marxreiter, Frey & Schneider

Table 3.4.1.1. *Single-crystal and powder mounting, capillary tubes and other containers*

Material	Temperature range (K)	Comments
(*A*) Capillary tubes		
Glass Lindemann glass Vitreous silica	< 773 < 773 < 1373	Lindemann glass scatters less, but is moisture sensitive Thinner walled tubes that are less sensitive to atmospheric influences can be obtained using other types of glass
Collodion Polyvinyl methylal resin (*e.g.* Formvar) Cellulose acetate	93 to 343 < 323 < 373	These capillaries can be made by coating a copper wire with a solution of the polymer in an appropriate organic solvent. When dry, the metal core may be removed by stretching, to reduce its diameter
Polyethylene	< 373	Tubes may be drawn from the molten polymer using a glass tube and a slow stream of air. The polymer gives a distinct diffraction pattern
(B) Other containers		
Gelatin capsules	< 303	Vessels with very thin, 20 μm, windows can be made
Methyl methacrylate resin (*e.g.* Perspex)	< 338	
Mica	< 1073	Mica windows useful in vessels for small-angle scattering, but the wall size is generally thicker, ~0.3 mm, and there are discrete lines at 10.00, 3.34 and 2.60 Å in the diffraction pattern
Regenerated cellulose film (*e.g.* cellophane)	Ambient	

For optimum results, tube diameters should be between 0.3 and 0.5 mm with wall thicknesses of 0.02 to 0.05 mm. The materials listed above, except where stated, give diffuse diffraction patterns. If necessary, control diffraction patterns, recorded only from the capillary or other container, should be taken.

(1993) have developed a mirror furnace working at up to 2300 K and suitable for polycrystalline or single-crystal samples.

A comprehensive account of cryogenic studies pertinent to both polycrystalline and single-crystal samples is given by Rudman (1976). Nieman, Evans, Heal & Powell (1984) have described a device for the preparation of low-temperature samples of noxious materials. The device is enclosed in a vanadium can and is therefore only suitable for neutron diffraction studies. Ihringer & Küster (1993) have described a cryostat for powder diffraction, temperature range 8–300 K, for use on a synchrotron-radiation beam line at HASYLAB, Germany (Arnold *et al.*, 1989).

3.4.1.3. *Single crystals (small molecules)*

3.4.1.3.1. *General*

Small single crystals of inorganic and organic materials, suitable for intensity data collection, are normally glued to the end of a glass or vitreous silica fibre, or capillary (Denne, 1971*b*; Stout & Jensen, 1968). A simple device that fits onto a conventional microscope stage to facilitate the procedure of cementing a single crystal to a glass fibre has been constructed by Bretherton & Kennard (1976). The support is in turn fixed to a metal pin that fits onto a goniometer head. For preliminary studies, plasticine or wax are useful fixatives, since it is then relatively easy to alter the orientation of the support, and hence the crystal, as required. For data-collection purposes, the support should be firmly fixed or glued to the goniometer head pin. The fibre should be sufficiently thin to minimize absorption effects but thick enough to form a rigid support. The length of the fibre is usually about 10 mm. Kennard (1994) has described a macroscope that allows specimens to be observed remotely during data collection and can also be used for measurement of crystal faces for absorption correction. Large specimens can be directly mounted onto a camera or onto a specially designed goniometer (Denne, 1971*a*; Shaham, 1982). A method using high-temperature diffusion to bond ductile single crystals to a metal backing, for strain-free mounting, has been described by Black, Burdette & Early (1986).

Prior to crystal mounting, it is always prudent to determine the nature of any spatial constraints that are applicable for the proposed experiment. Some diffractometers have relatively little translational flexibility, and the length of the fibre mount or capillary is critical. For some low-temperature devices where the cooling gas stream is coaxial with the specimen mount, the

Table 3.4.1.2. *Single-crystal mounting – adhesives*

Adhesive	Temperature range (K)	Comments
Durofix, Duco cement *etc.* (celluloid composition dissolved in organic solvent)	93 to 373	* Dries rapidly
Shellac dissolved in alcohol	<423	* Correct amount of solvent is critical
Fish glue (*e.g.* Seccotine)	<423	* Unsuitable for humid atmospheres
Dental cement	93 to 573	Adheres well to glass or asbestos, but not metals
Epoxy resin (epichlorohydrin, *e.g.* Araldite)	93 to 373	* Permanent fixing, fast (minutes) and slow (hours) available. 'Uncured' adhesive, *i.e.* minus hardener, useful for cryogenic mounting
Vacuum grease (*e.g.* Apiezon)	<473	Can protect crystal from moisture
Silicone high-vacuum grease	<373	Can protect crystal from moisture
Vaseline		Low temperatures down to liquid helium
Canada balsam	<333	† Dilute with xylene.
Mixture of wax and resin, ~1:1	93 to 303	†
Aluminium	<873	Large crystals set in molten metal, irradiate only protruding part of crystal
Aluminium cement	<1973	Irradiate only protruding part of crystal

* These glues tend to pull in setting and may require adjustment during the drying process. † Useful adhesives if the crystal requires grinding to shape after fixing.

orientation of the fibre (and crystal) on the goniometer head may also need careful alignment.

Many proprietary adhesives can be used (see Table 3.4.1.2), but it should be remembered that adhesives such as epoxy resins are often permanent, and attempts to dismount specimens lead to crystal damage. Some adhesives contain organic solvents that may react with the sample, and others may be X-ray sensitive and deteriorate with exposure. In low-temperature work, some adhesives shrink or become brittle. Ideally, the adhesive should have the same thermal characteristics as the crystal and its mount. An account of how strong stresses on adhesives, typically used to mount single crystals, induced by low and high temperatures is given by Argoud & Muller (1989*a*). The stresses appear to cause anisotropic modifications to secondary extinction, leading to discrepancies in the intensities of symmetry-related reflections. Beeswax and paraffin wax were found to be free from such stresses. Crystals that are sensitive to air can be mounted inside capillary tubes or other containers, as listed in Table 3.4.1.1. A useful summary of the methods available has been provided by Rao (1989). All adhesives and containers will give diffraction patterns, typically comprising diffuse bands, that contribute to the general background, and that may change with ageing. Minimal amounts of adhesive and thin-walled capillaries should be used. If the background diffraction is critical, it is highly recommended that diffraction patterns of the container and/or adhesive are recorded separately as controls.

The morphology of a given crystal will normally dictate the way that it is mounted, particularly for data-collection purposes. Thus, prismatic crystals and needle-shaped crystals are usually mounted with the longest dimension parallel to the fibre, in order to minimize systematic errors due to absorption. Jeffery (1971) and Wood, Tode & Welberry (1985) have described devices for shaping crystals into spheres and cylinders, respectively. A solvent lathe whereby a string moistened with solvent is used to shape the crystal is described by Stout & Jensen (1968).

3.4.1.3.2. *Non-ambient conditions*

As in the case of polycrystalline samples, a number of devices have been described to study single crystals at elevated pressures and at a range of temperatures. The mounting of the sample is very dependent on the device and radiation used. In recent years, the field of high-pressure crystallography has expanded significantly, and several sample holders based on the diamond-anvil cell have been reported for pressures up to 10 GPa. Recent papers include those by Alkire, Larson, Vergamini, Schirber & Morosin (1985) for neutron diffraction, and Malinowski (1987) and Leszczynski, Podlasin & Suski (1993) for X-ray diffraction.

Various types of furnace have been designed for high-temperature studies of single crystals. These are based either on radiative heat transfer mechanisms [*e.g.* Swanson & Prewitt (1986); temperatures up to 1400 K], electrically heated gas streams [*e.g.* Tsukimura, Sato-Sorensen & Ghose (1989); temperatures up to 1600 K], or flame heaters [*e.g.* Miyata, Ishizawa, Minato & Iwai (1979); temperatures up to 2600 K]. Furnaces specific to Weissenberg geometry (Adlhart, Tzafaras, Sueno, Jagodzinski & Huber, 1982) and Laue diffraction (Bhat, Clark, El Korashy & Roberts, 1990) have also been reported. There are many techniques available for mounting single crystals for high-temperature diffraction (Hazen & Finger, 1982), and a detailed account using an MgO-based ceramic cement is given by Swanson & Prewitt (1986). A recent paper by Peterson (1992) summarizes previous work in the design of high-temperature furnaces and describes a flame-heated gas-flow furnace operating in the range 373 to 1573 K. For this system, the crystals can be mounted either directly onto the thermocouple bead with a paste of fine platinum particles and oil, particularly useful if the crystal is to be exposed to a gas mixture that controls oxygen fugacity, or sealed under high vacuum in an ampoule made from 0.2 or 0.3 mm diameter silica capillary. In the latter case, the main support for the crystal is a 0.2 mm Pt wire threaded through a 0.3 mm diameter silica glass capillary. A 0.05 mm Pt/13Rh lead is welded to the end of this support to form the thermocouple bead. The wire is then wound around the outside of the capillary. A 0.05 mm Pt lead is welded to the other end of the 0.2 mm Pt wire and is threaded through a hole in the capillary near the base. The whole assembly can be mounted on a goniometer head that has only translational adjustments. For neutron diffraction, Lorenz, Neder, Marxreiter, Frey & Schneider (1993) have described a mirror furnace operating upto 2300 K. The sample support is normally a thin ceramic tube or rod of Al_2O_3 or ZrO_2 to which the sample may be glued with a ceramic cement. Neder, Frey & Schulz (1990) have described a versatile holder for high-temperature neutron studies. One part of the crystal is ground away to leave a stem, which is then fixed to an alumina rod with a ceramic glue based on zirconia. The ceramic glue is surrounded by a cylinder of BN to minimize spurious scattering.

A comprehensive account of low-temperature diffraction is given by Rudman (1976). Procedures for the selection and transfer of crystals to diffractometers have been described by Boese & Bläser (1989) and Kottke & Stalke (1993). These procedures are applicable down to temperatures of 213 and 193 K, respectively. The latter authors do not recommend the use of capillaries, but describe a device employing the oil-drop mounting technique pioneered by Hope (1987, 1988). Lippman & Rudman (1976) have used a mechanically refrigerated gas stream to achieve temperatures down to approximately 150 K, and the use of liquid nitrogen extends the range to 77 K. Devices such as the Oxford Cryostream can be readily fitted to diffractometers and other types of camera. Closed-cycle refrigerators, liquid-helium-based devices (*e.g.* Henriksen, Larsen & Rasmussen, 1986; Argoud & Muller, 1989*b*; Zobel & Luger, 1990; Graafsma, Sagerman & Coppens, 1991; Toyoshima, Hoya & Ohshima, 1991) further extend the low-temperature limit to 5 K, but often involve substantial blind regions and collision zones. For sample mounting in these devices, it is essential to have good heat conduction to the crystal. Zobel & Luger (1990) describe a taper-formed sample holder made of special copper screwed to the cold head. A steel injection needle with a Be wire inside (0.3 mm diameter and exactly 2 mm in length) is fitted into a 0.5 mm bore hole. The crystal is glued with Araldite to the Be needle, which has little X-ray absorption but good heat conduction. In addition to diffractometer-based devices, Moret & Dallé (1994) have described an adaptation of the closed-cycle refrigerator for a precession goniometer, and various authors have reported systems utilizing Weissenberg geometry for both X-rays and neutrons (*e.g.* Hohlwein & Wright, 1981; Aldhart & Huber, 1982; Allen *et al.*, 1982). Reference should be made to the individual papers for methods of mounting, including spatial and any other constraints.

3.4.1.4. *Single crystals of biological macromolecules at ambient temperatures*

Crystals of biological macromolecules are normally grown from an aqueous solution (see Subsection 3.1.1.2), and when growth is complete are in equilibrium with the mother liquor. Changes in this equilibrium may often result in crystal damage, so the most important aspect of crystal mounting in this case is to preserve the crystal in its state of hydration. This is most readily accomplished by sealing the crystal in a thin-walled quartz or glass capillary tube (King, 1954; Holmes & Blow, 1966). The crystal adheres to the inside of the tube by surface-tension effects through a small droplet of liquid, and a further pool of liquid at one end maintains the required degree of hydration. The general principles involved are well described by Rayment (1985). D'Aprile & Moretto (1975) have described two simple devices, a small electric heater for melting the wax used for sealing the capillary and a refrigerating microcell to prevent heat affecting the wet crystal, which are very useful for mounting wet single crystals in capillary tubes.

Alternatively, crystals can be grown directly within capillary tubes (Phillips, 1985) or microdialysis cells such as those described by Zeppezauer, Eklund & Zeppezauer (1968). A further mounting device particularly useful for enzymatic studies is the flow cell (Wyckoff *et al.*, 1967), in which the specimen is immobilized while mother liquor, or buffer with substrates or inhibitors, is allowed to flow over the crystal. A useful account of this device is given by Petsko (1985). More recently, Edwards (1993) has described a yokeless flow cell, which uses a plastic cone fixed to a brass mounting pin with a wire harness to support the quartz capillary. Although the device was originally designed for Laue studies, its simplicity and practicality should make it useful for a wide range of diffraction experiments. Pickford, Garman, Jones & Stuart (1993) have designed a mounting cell that allows the humidity around a protein crystal to be varied in a controlled manner. This may be particularly useful for crystals where the solvent content is high and the molecular packing, and hence the diffraction intensities, highly dependent on the precise amount of solvent present.

The relatively short crystal lifetimes and large volumes of intensity data often dictate that crystals of biological macromolecules be mounted so that data collection can be accomplished in the most efficient manner, for example, with a symmetry axis parallel to the rotation axis of the collection device. Samples crystallizing in the form of thin plates that have to be aligned perpendicular to the capillary axis can be wedged using cotton lint fibres (Narayana, Weininger, Huess & Argos, 1982), or mounted on a fibre plug (Przybylska, 1988).

One of the key problems in collecting diffraction data from wet crystals is movement of the specimen within the capillary, *i.e.* crystal slippage. Numerous ways have been suggested to surmount this problem, including flattening of the capillary surface, surrounding the crystal with a thin film of plastic (Rayment, Johnson & Suck, 1977) and supporting the crystal with fibre plugs in contact with the mother liquor.

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):

(1) equatorial setting, whereby a particular reciprocal-lattice plane is aligned perpendicular to a given direction. This setting is equivalent to bringing a direct-lattice vector (perpendicular to the reciprocal-lattice plane) parallel to the given direction;

(2) azimuthal setting, whereby a reciprocal-lattice vector, in the equatorial plane, is positioned to make a given angle with the plane containing the given direction and the X-ray beam.

In the rotation or oscillation methods, the given direction is the camera rotation axis, but precession geometry requires a direct-lattice vector to be aligned along the X-ray beam. This section will briefly discuss:

(1) equatorial setting using a rotation camera;

(2) setting and orientation using stationary-crystal methods;

(3) rotation geometry setting for crystals with large unit cells;

(4) diffractometer setting considerations.

Specialized methods for orientating and cutting large single crystals are not covered, but two-axis goniometers have been designed by Denne (1971*a*) and Shaham (1982), and methods for cutting single crystals along any desired direction have been reported by Campos, Cardoso & Caticha-Ellis (1983) and Desai & Bhatt (1984).

3.4.2.2. *Preliminary considerations*

Prior to the commencement of the setting process, it is useful to align the crystal optically so that prominent morphological features bear fixed geometrical relationships with the component parts of the goniometer head. Thus, a prismatic crystal could be aligned with its longest axis parallel to the mount, but in addition with a face perpendicular to the rotation axis of one of the goniometer arcs. Many modern goniometer heads have a rotatable component to which the crystal mount can be fixed, and judicious use of this facility can considerably simplify the setting process. This may be particularly important for crystals that are very sensitive to X-radiation. It is also useful if the arc readings on the goniometer head are equal or close to zero. Large deviations away from the zero positions can lead to mechanical collision with other parts of the camera (*e.g.* the layer-line screens of a Weissenberg camera), or in extreme cases to interference with the primary or diffracted X-ray beams. If the crystal mount is fixed to the goniometer head with wax or plasticine, this can often be achieved by manual manipulation of the mount and the wax. The use of less-pliable adhesives requires careful monitoring during the hardening process. Although the detailed alignment will depend on the geometry of the recording device, care taken at the mounting stage will always result in increased efficiency in setting. Sensible orientation of the goniometer head on the camera may also lead to increased efficiency, and it is often useful to start with the axes of the goniometer arcs perpendicular and parallel to the X-ray beam.

3.4.2.3. *Equatorial setting using a rotation camera*

Methods for equatorial setting are well described by Jeffery (1971). The aim is to identify reciprocal-lattice layer lines from X-ray oscillation photographs and, by measuring the degree and directions of curvature of the zero-layer line, to adjust the crystal setting until the layer-line patterns are made perpendicular to the rotation axis, *i.e.* the crystal lattice vector perpendicular to these reciprocal-lattice layers lies parallel to the rotation axis. For crystals of well defined morphology, initial alignment of a crystal lattice vector with the rotation axis can be achieved optically, often to within a degree. For setting errors of less than 5°, reciprocal-lattice layer lines should be readily identifiable on X-ray oscillation diffraction patterns. The use of unfiltered X-radiation often assists in this regard (as well as reducing exposure times), and a device described by Kulpe (Kulpe, 1963; Kulpe & Dornberger-Schiff, 1965) may prove useful in the identification of the zero-layer equatorial pattern on photographic films.

For accurate final setting, a general 'double-oscillation' method such as that of Weisz & Cole as modified by Davies (Jeffery, 1971) is preferred, although Suh, Suh, Ko, Aoki & Yamazaki (1988) have provided a rationale for adjustment of both goniometer arcs simultaneously from a 'single-oscillation' photograph. With the 'double-oscillation' technique, two single oscillations, separated by a φ reading of 180°, are recorded on the same image, but with significantly different exposure times, so that the patterns are related by a mirror plane and are readily distinguishable. The goniometer arcs are placed at 45° to the X-ray beam. Measurements of the relative displacements of the two patterns at the $2\theta = 90°$ position on the image readily yield corrections to both goniometer-head arcs. No translational movement of the film cassette is required, but the crystal must diffract to at least a θ angle of 45°. Hanson (1981) has devised a technique suitable for a Weissenberg camera that is a combination of double oscillation with displacements and measurements at low-2θ angles. This method is particularly suitable for crystals with large unit cells.

In the case where layer lines are not readily locatable, but the crystal unit-cell dimensions are known, Jeffery (1971) also describes an equatorial setting technique that relies on the indexing of at least three low-angle Laue streaks.

Okasaki & Soejima (1986) have described two simple goniometer attachments that may prove useful for crystals that have been mounted so that the angular movements required to achieve setting exceed the range commonly available on goniometer heads.

3.4.2.4. *Precession geometry setting with moving-crystal methods*

Methods of setting crystals so that a crystal lattice vector lies along the X-ray beam have been fully described by Buerger (1964). Optical alignment precedes small-angle (typically 2–5°) precession photographs taken with unfiltered radiation. The use of a screen with a central hole may assist the identification of the outer ends of the white-radiation streaks on the zero-layer pattern by preventing the recording of the upper-layer patterns. The deviation of the zero-layer pattern from cylindrical symmetry about the direct beam leads to the measurement of simultaneous corrections for the spindle angle and goniometer-head arcs. These adjustments are particularly easy if the goniometer-head arcs are perpendicular and parallel to the X-ray beam, and both arcs read zero. Reider (1975) has proposed an approximate stereographic method of making appropriate corrections when these ideal conditions are not fulfilled. Where optical alignment is not possible, or recognition of a zero-layer pattern is difficult, reciprocal space can be systematically explored by taking a series of small-angle precession photographs at regular intervals (*e.g.* 15°) around the spindle axis until a suitable zero-layer pattern is found. In such cases, and particularly for non-orthogonal crystal systems, the use of the complementary rotation technique is recommended (see Subsection 3.4.2.3).

In the final alignment when the crystal lattice vector is parallel to the X-ray beam, it is also desirable to have a reciprocal axis parallel to the spindle axis. With this combined setting, it is possible to survey the whole of reciprocal space (to a θ limit equal to the maximum precession angle mechanically available) with one mounting of the crystal.

3.4.2.5. *Setting and orientation with stationary-crystal methods*

3.4.2.5.1. *Laue images – white radiation*

The azimuthal and back-reflection Laue methods for setting crystals with relatively small unit cells have been described by Jeffery (1971). The former is capable of achieving an accuracy of setting of $\pm 0.05^\circ$, whereas the latter is important in metallurgy, where the Laue method is often the only possibility because of the large size of the specimens. Schiller (1985) has emphasized the importance of the back-reflection Laue technique for setting specimens with a precision of 0.1° needed in semiconductor surface preparation.

In recent years, there has been a resurgence of the Laue technique, in conjunction with synchrotron radiation, to record intensity data from biological macromolecules in very short time scales. The overall experimental strategies involved are described by Helliwell *et al.* (1989) and Clifton, Elder & Hajdu (1991). Crystals are not usually set in a precise orientation for these types of experiment prior to data acquisition because of radiation damage. The post-determination of the precise crystal orientation with respect to the instrument axes from the recorded Laue pattern therefore forms an essential part of the data processing. Most methods are based on the indexing procedure of Riquet & Bonnet (1979), and an interactive computer program for the interpretation and simulation of Laue patterns has been written by Laugier & Filhol (1983). An orientation-matrix approach has been reported by Jacobson (1986), and the work of Helliwell *et al.* (1989) has led to a comprehensive set of Laue processing programs. In addition to enabling trial-and-error visual matching of images, this program suite includes an auto-indexing procedure based on a known unit cell, and refinement of the orientational parameters. More recently, Carr, Cruickshank & Harding (1992) have developed a method whereby a gnomonic projection of the Laue diffraction pattern can be used to determine the cell dimensions and orientation of a crystal. The axial ratios and interaxial angles can be determined precisely, but the absolute scaling of the cell is dependent on the accuracy with which the minimum wavelength used in the experiment is known.

3.4.2.5.2. *'Still' images – monochromatic radiation*

More recently, the azimuthal method has proved of great value in the rapid alignment of crystals with large unit cells prior to data collection on devices using rotation geometry. After optical alignment, a 'still' photograph taken with monochromatic radiation (or a very small angle rotation photograph, typically 0.05–0.20°), is used to locate a zero-layer reciprocal-lattice plane (Fig. 3.4.2.1). Such a plane will record on a flat detector placed at a distance D mm from the crystal, C, as an ellipsoidal trace of maximum dimension S mm from the direct-beam position, O'. In order to make the plane perpendicular to the X-ray beam (*i.e.* the real axis parallel to the X-ray beam), it must be rotated through an angle θ such that $\tan 2\theta = S/D$.

If the vector $O'P$ makes an angle α with the rotation axis, the angle θ can be resolved into a vertical component, $\theta \sin\alpha$, corresponding to a rotation of the spindle axis, and a horizontal component, $\theta \cos\alpha$, corresponding to a rotation of the goniometer arc whose axis is perpendicular to the X-ray beam (assuming a perpendicular and parallel setting of the goniometer head). Rotation of the reciprocal-lattice plane within its own plane can then be achieved with the goniometer arc whose axis is parallel to the beam. This technique is also applicable to preliminary setting on a precession camera.

However, with very radiation sensitive crystals, it is inadvisable to waste time accurately setting the crystal prior to data collection, since the crystal is subject to continuous radiation damage from the beginning of the first exposure (Rossmann & Erickson, 1983). In this case, two 'still' images are collected, preferably separated by a 90° rotation, after data collection but before the crystal is irretrievably damaged. In principle, the orientation can be determined from a single still, but the precise crystal orientation is better determined by identifying and measuring the orientations of two real axes relative to the camera axes, from the sets of ellipses on two stills. The orientation of the reciprocal axis, perpendicular to these two real axes, can then be calculated, and, provided that the unit-cell dimensions are known, the orientation of the third real axis readily determined. Given the directions of the three real axes, the direction cosines of the reciprocal axes can be computed and a matrix determined that specifies the crystal orientation with respect to the camera axes. This method obviates the need to index the 'partial' reflections on still images (Jones, Bartels & Schwager, 1977).

3.4.2.6. *Setting and orientation for crystals with large unit cells using oscillation geometry*

The use of the screenless rotation technique is now routine as a method for large-molecule data collection (Arndt & Wonacott, 1977; Usha *et al.*, 1984). In general, the setting of the crystal for data-collection purposes does not need to be precise, although efficient data collection may dictate that a particular direct axis is set along the rotation axis (Munshi & Murthy, 1986), and subsequent data processing may be simpler. An accurate knowledge of the crystal orientation relative to the axial system of the camera is, however, absolutely essential for the final data processing.

Historically, determination of the crystal setting was normally undertaken using 'still' photographs (see Subsection 3.4.2.5) and the final orientation then determined from two such photographs taken orthogonally (Jones, Bartels & Schwager, 1977; Rossmann

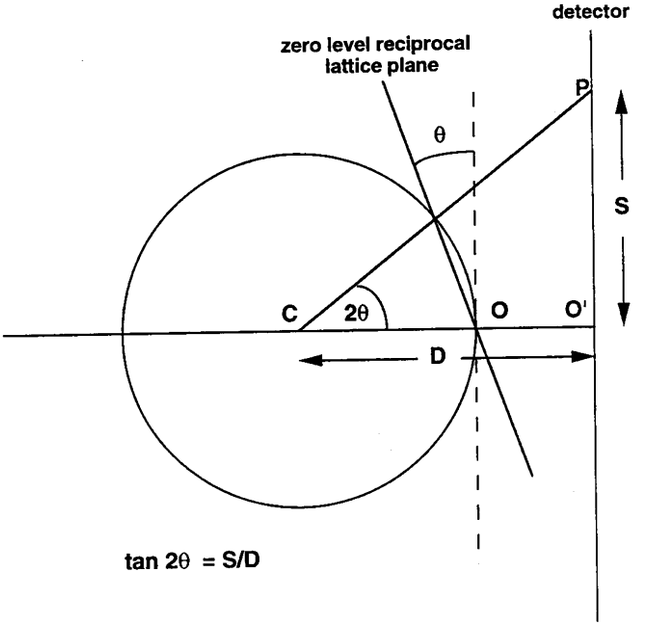

Fig. 3.4.2.1. A zero-layer reciprocal-lattice plane will record on a flat-plate detector placed at a distance D from the crystal C as an ellipsoid of maximum dimension S from the direct-beam position O'.

& Erickson, 1983). In order to minimize the problem of radiation damage, various graphical methods were then devised for determining the precise setting without the need to record 'still' images (Dumas & Ripp, 1986; Moews, Sakamaki & Knox, 1986; Sarma, McKeever, Gallo & Scuderi, 1986). Vriend, Rossmann, Arnold, Luo, Griffith & Moffat (1986) reported a 'post-refinement' technique in which the intensities of partially recorded reflections on oscillation images are compared with their full intensities observed elsewhere on the same or a different image. The degree of partiality is dependent on the crystal orientation so that this provides a very sensitive method of refining the setting parameters (cell parameters, crystal mosaicity and X-ray beam characteristics may also be refined). In a further development, Vriend & Rossmann (1987) described how to determine the orientation from a single oscillation photograph. The method was again devised for crystals that have short lifetimes in the X-ray beam and is based on correlating the unique set of calculated normals to reciprocal-lattice planes with the observed zone axes on the oscillation image.

Currently, auto-indexing procedures based on a single still/oscillation image or preferably several images well separated in reciprocal space are used to determine the precise crystal setting prior to data processing. Kim (1989) has devised an auto-indexing algorithm based on methods previously developed for four-circle diffractometers (*e.g.* Sparks, 1976). The algorithm includes *ab initio* cell-parameter and orientation-matrix determination, followed by reduced-cell calculation and transformation of the reduced cell to one of higher symmetry, where appropriate. Kim's method does require, however, that the diffraction image is large enough to display many lunes. Higashi (1990) has also developed an auto-indexing program for single and or multiple still/oscillation images. The very effective auto-indexing routine of Kabsch (1988*a*, 1993) has been incorporated into the *XDS* program suite (Kabsch, 1988*b*).

Several types of area-detector diffractometer have been developed for fast and accurate measurement of intensity data for macromolecular crystals. Crystal alignment and general strategies for typical devices are described by Xuong, Nielsen, Hamlin & Anderson (1985), Messerschmidt & Pflugrath, (1987), Higashi (1989), and Sato *et al.* (1992).

3.4.2.7. *Diffractometer-setting considerations*

General setting considerations for three- and four-circle diffractometers have been discussed by Busing & Levy (1967). In principle, crystals can be placed on a four-circle diffractometer in any general orientation, although it is often useful to have a setting such that the reciprocal-lattice axis lies parallel to the φ rotation axis. This setting is a prerequisite for effective use of the empirical absorption correction method of North, Phillips & Matthews (1968).

In the case where the crystal orientation is not precisely determined, setting is normally achieved using automatic procedures that involve finding a set of general reflections and generating a **UB** matrix from their angular positions. Generation of the **UB** matrix can be achieved by finding the shortest non-coplanar reciprocal-lattice vectors and assigning these as the reciprocal-cell axes (Hornstra & Vossers, 1974). The resulting unit cell is always primitive, and additional manipulations are required to determine the conventional cell and type of Bravais lattice. This reciprocal-space method is adopted by the Nonius CAD4 diffractometer software (CAD4 Manual, 1989). Alternatively, the 'auto-indexing' method originated by Sparks (1976, 1982) and Jacobson (1976) can be used whereby direct-lattice vectors are generated, again through an initial cell. Clegg (1984) has described an enhancement of the direct-lattice vector method so that the initial cell is used to produce direct-lattice vectors systematically. In order to confirm that a generated vector is a true direct vector, the condition is applied that the scalar multiplication of a true direct vector and any true reciprocal vector (*i.e.* the observed reflection vectors) results in an integer. If a great majority of the products of a putative direct vector and each of the measured observed reflection vectors are integers, the direct vector is accepted. The final cell can be obtained from the set of accepted direct vectors. Subsequently, Duisenberg (1992) developed a method of auto-indexing that is particularly applicable to difficult cases such as twin lattices, incommensurate structures, fragmented crystals, long axes, and even unreliable data. Finding the reciprocal lattice from a distribution of reciprocal-lattice points (*i.e.* observed reflections) is reduced to finding elementary periods in one-dimensional rows, obtained by projecting all observed points onto the normal to the plane formed by any three of these points. Row periodicity and offending reflections are readily recognized. Each row, by its direction and reciprocal spacing, defines one direct-axis vector, based upon all co-operating observations. A primitive cell can be obtained from the direct vectors and refined against the fitting reflections, resulting in one main lattice, or a main lattice and a set of alien reflections (see also Subsection 3.4.2.6).

3.4.2.8. *Crystal setting and data-collection efficiency*

Although it has become modern practice to determine the orientation of crystals after data collection using auto-indexing procedures, rather than to carry out accurate alignment prior to data collection, such a procedure, as indicated earlier in this section, can lead to inefficient data collection. In the case of anomalous-dispersion measurements, and particularly multiple-wavelength anomalous diffraction (MWAD) for phase determination (*e.g.* Kahn *et al.*, 1985), it is often very important to orientate the crystal so that Bijvoet pairs of reflections are recorded simultaneously. The use of synchrotron radiation, where access is usually very limited and crystals are highly radiation sensitive, often leads to insufficient care being taken in the data-collection procedure. An efficient data-collection strategy should aim to measure a set of data as complete as possible (preferably $> 90\%$) in the shortest possible time. Contiguous regions of reciprocal space, such as the 'cusp' region for oscillation geometry, and low-resolution shells should *not* be omitted. In addition, a reasonable number of reflections should be measured more than once to check for internal consistency in the data set. For biological macromolecules, in particular, the temptation to collect data beyond the practical resolution limit should be avoided. Two useful indicators from the outer resolution shell are (*a*) the proportion of significant data should not fall below 70%, and (*b*) the internal consistency index for data measured more than once should not rise above 20%. In general, rotation of crystals along the highest rotation symmetry axis (*i.e.* the fourfold axis for tetragonal systems) will require the least amount of data to be collected, and it is advisable to mount crystals so that this rotation axis is parallel to the fibre or capillary axis, provided that this is sensible in terms of the crystal morphology.

Munshi & Murthy (1986) have discussed strategies of data collection using the screenless oscillation method based on the Laue group and the nature of the crystal axis parallel to the rotation axis. More general strategies for area-detector systems have been reported by Xuong, Nielsen, Hamlin & Anderson (1985) and Zhang & Matthews (1993).

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