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

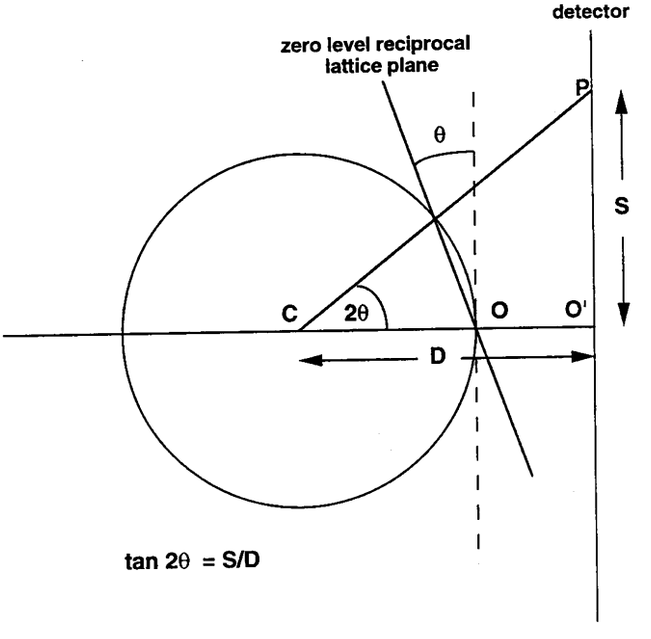

Fig. 3.4.2.1. A zero-layer reciprocal-lattice plane will record on a flat-plate detector placed at a distance D from the crystal C as an ellipsoid of maximum dimension S from the direct-beam position O'.

& 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).

references