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5. CRYSTAL PROPERTIES AND HANDLING

5.1. Crystal morphology, optical properties of crystals and crystal mounting

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5.1.1. Crystal morphology and optical properties

When crystals of a biological macromolecule are grown, they are first examined under the microscope. This can show the crystal quality and may reveal crystal symmetry. Some external properties of macromolecular crystals are described here, including information on shape, habit, polymorphism, twinning and the indexing of crystal faces. Optical properties are also described. Every crystal form, however, has to be treated individually; only by working with it can the crystallographer discover its physical properties. The diffractionist aims to be able to mount a macromolecular crystal in a totally stable manner so that it does not deteriorate or slip in position during the data collection. Some methods for mounting such macromolecular crystals for X-ray diffraction studies are described here together with the necessary tools. For general information on purchasing supplies to do this see the list at http://www.hamptonresearch.com.

5.1.1.1. *Crystal growth habits*

5.1.1.1.1. *The shape of a crystal – growth habits*

Morphology is the general study of the overall shape of a crystal, that is, the arrangement of faces of a crystal. It can often provide useful information about the internal symmetry of the arrangement of atoms within the crystal (Mighell *et al.*, 1993). The periodicity of the arrangement of molecules or ions in a crystal can be represented by three non-collinear vectors, **a**, **b** and **c**, which give a unit cell in the form of a parallelepiped with axial edges a, b and c, and interaxial angles α, β and γ (α between **b** and **c**, *etc.*). The vectors **a**, **b** and **c** from the chosen origin of the unit cell are, by convention, selected in a right-handed system. Since there may be several possible choices of unit cell, the simplest, with the smallest possible repeats and with interaxial angles nearest to 90°, is the best choice. One method used to highlight the periodicity of the atomic arrangement within a crystal is to replace each unit cell by a point; this mathematical construction gives the crystal lattice. The entire crystal structure is the convolution of the unit-cell contents with the crystal lattice.

Biological macromolecules are, in general, chiral and can only crystallize in those space groups that do not contain symmetry operations that would convert a left-handed molecule into a right-handed one (improper symmetry operations). Proper symmetry operations involve translations, rotation axes and screw axes. These maintain the chirality of the molecule and hence are appropriate for crystals of biological macromolecules. The number of possible space groups is therefore reduced by this constraint on the types of symmetry operations allowed from the usual 230 for molecules in general down to 65 for chiral molecules.

The appearance of a crystal that has grown under a particular set of experimental conditions is called its habit. It is a result of the different relative growth rates of various crystal faces, and these rates, in turn, depend on the nature of the interactions between the molecules in the crystal, the degree of supersaturation of the solution and the presence of any impurities which may affect the growth rates of specific crystal faces. The term 'habit' is only used to describe the various appearances of crystals that are composed of identical material and maintain the same unit-cell dimensions and space group. The faces that have developed on these crystals are various subsets of those implied in the overall morphological description of the crystal. Any change in the experimental conditions under which a crystal is grown may alter its habit; a judicious selection of experimental conditions may permit formation of crystals with a chunky habit that are more suitable for X-ray diffraction analysis than thin plates or needle-like crystals. Examples of the crystalline forms of haemoglobins are provided by Reichert & Brown (1909).

Various descriptions of crystal habits appear in the literature. These include terms such as 'tabular', 'platy' or 'acicular' crystals, 'hexagonal rods' and 'truncated tetragonal bipyramids', among others. Some crystal habits are not very appropriate for X-ray diffraction analyses; these include spherulites, which are polycrystalline aggregates of fine needles with an approximately radial symmetry, and dendrites, which have a tree-like structure. The habit of a crystal can sometimes give information on the molecular arrangement within it. For example, flat molecules that stack readily upon each other produce long crystalline needles, because interactions in the stacking direction are stronger than those in other directions.

A crystal is bounded by those faces that have grown most slowly. Fast-growing faces quickly disappear as more and more molecules are deposited on them, constrained by surrounding faces that are growing more slowly. Any factor that changes the relative rates of growth of crystal faces, such as impurities in the crystallizing solution, will affect the overall habit. Different faces of protein crystals have different arrangements of side chains on their surfaces; thus, an impurity may bind to certain faces rather than others. Adsorption of an impurity on a particular face of a crystal may retard the growth of that face, causing it to become more prominent than normal in the growing crystal.

5.1.1.1.2. *Quality of protein crystals*

Protein and nucleic acid crystals contain a high proportion of water in each unit cell and are therefore fragile. The proportion of solvent to macromolecule in the crystal can be expressed, as described by Matthews (1968), as V_m in $\text{Å}^3\ \text{Da}^{-1}$ for the asymmetric unit. Values in the range 1.7 to 4.0 are usual for proteins, but nucleic acid crystals generally have a higher water content. Crystal fragility due to water content may be used to determine whether or not a crystal contains protein or buffer salt. Pressure with a fine probe will settle this question because a protein crystal will shatter, while a salt crystal, which is much sturdier, will generally withstand such treatment. If crystals have grown into one another, or appear as clumps, it is sometimes possible to split off a single crystal by prodding the clump gently at the junction point between the crystals with a scalpel or a glass fibre.

5.1.1.1.3. *Polymorphism*

Intermolecular contacts between protein molecules in the crystalline state determine the mechanical stability of the crystal. If the conditions used for crystallization vary, the number and identity of these contacts may be changed, and polymorphs will result. Polymorphism is the existence of two or more crystalline forms of a given material. Polymorphs have different unit-cell dimensions and hence different molecular arrangements within

them. This property is common for biological macromolecules and can be used to select the best crystalline form for X-ray diffraction studies. Different polymorphs of a particular material are often prepared by varying the crystallization conditions. They may also develop in the same crystallizing drop because supersaturation conditions may change while a crystal is growing. Examples of polymorphs are provided by hen egg-white lysozyme, which can form tetragonal, triclinic, monoclinic or orthorhombic crystals, depending on the pH, temperature and nature of added salts in the crystallization setup (Steinrauf, 1959; Ducruix & Geigé, 1992: Oki *et al.*, 1999).

A regular surface that offers a charge distribution pattern that is complementary to a possible protein layer in a crystal can sometimes be useful in producing a starting point for the nucleation of new protein crystals. Epitaxy is the oriented growth of one material on a crystal of an entirely different material. Regularities on the surface of the first crystal can act as a nucleus for the oriented growth of the second material. Generally, there should be similar, but not necessarily exactly matching, repeat distances in the two crystals. Epitaxy has been used with considerable success for the growth of protein crystals on selected mineral surfaces (McPherson & Shlichta, 1988). For example, lysozyme crystals grow well on the surface of the mineral apophyllite. Crystals of related macromolecules can also be used as nucleation sources for protein crystallization. Epitaxy can, however, sometimes be a nuisance rather than a benefit if the crystallization setup contains surfaces with unwanted regularity.

A change in the environment around a protein crystal may also cause a change in unit-cell dimensions, and possibly even in space group. For example, the transference of a RuBisCO crystal from a high-salt, low-pH mother liquor to a low-salt, high-pH synthetic mother liquor produced a more densely packed polymorph. The overall unit-cell dimensions were smaller in the latter (V_m changed from 3.16 to 2.74 Å^3 Da^{-1}) (Zhang & Eisenberg, 1994).

5.1.1.1.4. *Twinning*

Twinning is a phenomenon that can cause much grief in X-ray diffraction data measurements. It has been described as 'a crystal growth anomaly in which the specimen is composed of separate crystal domains whose orientations differ in a specific way . . . some or all of the lattice directions in the separate domains are parallel' (Yeates, 1997). Thus, a twin consists of two (or more) distinct but coalescent crystals. This effect has been described in terms of their diffraction patterns as follows: 'Some crystals show splitting of diffraction spots owing to the different tilts of the two lattices. Others pretend to be single crystals with no split spots, and their symmetries of intensity distribution vary with every data set. These latter have been called hemihedral, and in them the unique axes of the two crystals are exactly reversed parallel with each other.' (Igarashi *et al.*, 1997). Perfect hemihedral twinning (when there are equal proportions of each twin member) can be detected from the value of $\langle I^2\rangle/\langle I\rangle^2$ for the acentric data; it is near 2 for untwinned data and 1.5 for twinned data (Yeates, 1997).

Twinning may sometimes be prevented by introducing variations into the crystallization setup. Such changes could involve the pH or the nature of the buffer. Variations in the seeding technique used, or the introduction of additional agents, such as metal ions or salts, detergents, or certain amino acids, can also be tried. One method for estimating the degree of twinning is based on the fact that each measured X-ray diffraction intensity is the sum of the intensities from the two (or more) crystal lattices (suitably weighted according to the proportion in which each lattice alignment occurs in the crystal). The relative proportion of each component can therefore be estimated. Detwinned intensities obtained by this method should only be positive or zero (not negative) within experimental error (Stanley, 1972; Britton, 1972; Rees, 1980). Crystal structures of hemihedrally twinned crystals are now being determined (Gomis-Rüth *et al.*, 1995; Breyer *et al.*, 1999).

5.1.1.2. *Properties of crystal faces*

5.1.1.2.1. *Indexing crystal faces*

Crystal faces are described by three numbers, the Miller indices, that define the relative positions at which the three axes of the unit cell are intercepted by the crystal face (Blundell & Johnson, 1976; Phillips, 1957). The crystal face that is designated *hkl* makes intercepts $x = a/h$, $y = b/k$ and $z = c/l$ with the three axes (a, b and c) of the unit cell. When the Miller indices are negative, because the crystal face intercepts an axis in a negative direction, they are designated $-h-k-l$ or $\bar{h}\bar{k}\bar{l}$. Most faces of a crystal are, as required by the law of rational indices, represented by small integers. For symmetry reasons, planes in hexagonal crystals are conveniently described by four axes, three in a plane at 120° to each other. This leads to four indices, *hkil*, where $i = -(h+k)$. Crystal faces are designated by round brackets, *e.g.* (100), as shown in two simple examples in Fig. 5.1.1.1. A set of faces then defines a crystal form. All sets of planes *hkl* related by symmetry, such as the main faces of an octahedron (111), (11$\bar{1}$), (1$\bar{1}$1), ($\bar{1}$11), (1$\bar{1}\bar{1}$), ($\bar{1}$1$\bar{1}$), ($\bar{1}\bar{1}$1) and ($\bar{1}\bar{1}\bar{1}$), may be represented by the use of curly brackets, *e.g.* $\{111\}$. Square brackets, *e.g.* [111], are used to indicate a direction

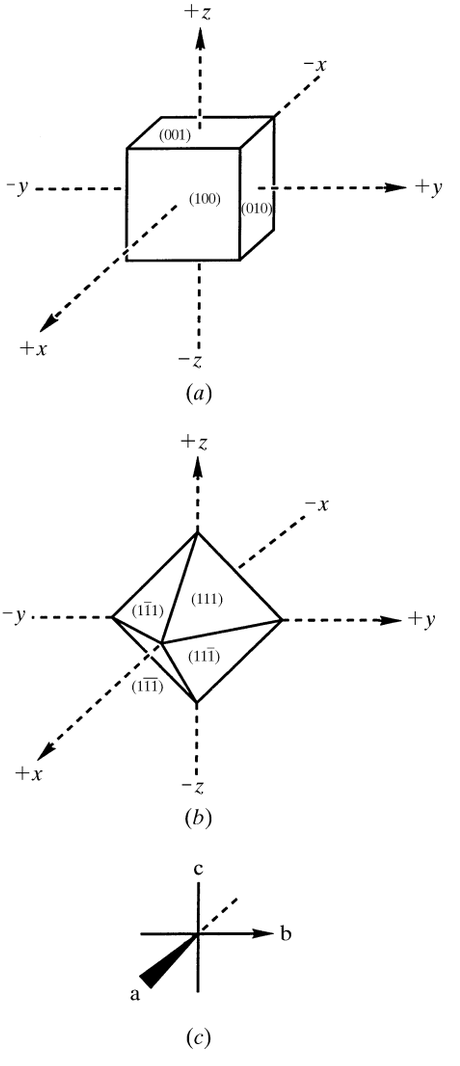


Fig. 5.1.1.1. Crystal faces. (*a*) Cube and (*b*) octahedron. (*c*) Unit-cell axes.

in a crystal, [001] being the *c* axial direction. Thus, information about several faces of a crystal is contained in information about one face if the relevant symmetry of the crystal is known. If the atomic structure of the crystal is known, it is possible to determine which aspects of the macromolecule lie on the various crystal faces.

5.1.1.2.2. *Measurement of crystal habit*

The habit of a crystal may be described in detail by measurements, particularly of the angles between adjacent faces. This can be done with an optical goniometer, in which a collimated visible beam of light is reflected from different faces as the crystal is rotated, and the angles between positions of high light intensity are noted. Such studies can be combined with X-ray diffraction measurements of the crystal orientation of the unit cell with respect to the various crystal faces (Oki *et al.*, 1999).

5.1.1.3. *Optical properties*

Crystals interact with light in a manner which depends on the arrangement of atoms in the crystal structure, any symmetry in this arrangement and the chemical nature of the atoms involved. Refraction is seen as a change in the direction of a beam of light when it passes from one medium to another (such as the apparent bend in a pencil placed in a beaker of water). The refractive index is the ratio of the velocity of light in a vacuum to that in the material under investigation and is greater than unity. It is measured by the extent to which the direction of a beam of light changes on entering a medium.

If a protein crystal has grown in the cubic system, its refractive index will be the same in all directions and the crystal is described as optically isotropic. Most protein crystals, however, form crystals with anisotropic properties, that is, their properties vary with the direction of measurement of the crystal. For example, some crystals appear differently coloured when viewed in different directions, and are described as pleochroic. The absorption of light is greatest when light is vibrating along bonds of chromophoric groups in the molecules in the crystals rather than perpendicular to them, and therefore they show interesting effects in plane-polarized light (in which the electric vectors lie in one plane only) (Bunn, 1945; Wahlstrom, 1979).

Anisotropy of the refractive index of a protein crystal, that is, its birefringence, can be used by biochemists to determine whether or not a protein has been crystallized. If the protein preparation in a test tube is held up to the light and shaken, birefringent protein microcrystals are revealed by light streaks, a schlieren effect. This occurs because the crystals have a different refractive index from the bulk of the liquid. Birefringence implies that there is double refraction as light passes through a crystal, and the light is split into two components (the ordinary and extraordinary rays) that travel with different velocities and have different properties (those of the ordinary ray being normal). Iceland spar (calcite) provides the ideal example of double refraction. Birefringence is measured as the difference between the refractive indices for the ordinary and extraordinary rays, and a crystal is described as positively birefringent if the refractive index is greater for the extraordinary ray. If a crystal is positively birefringent $(n_E > n_O)$, it can be assumed to contain rod-like bodies lying parallel to the single vibration direction of greatest refractive index (n_E). If a crystal is negatively birefringent $(n_O > n_E)$, it can be assumed to contain plate-like bodies lying perpendicular to the single vibration direction of least refractive index (n_E). For example, in crystalline naphthalene, the highest refractive index is in the direction of the highest density of atoms, along the long axis of the molecule. Similar arguments can be applied to crystalline macromolecules, such as haemoglobin, which is strongly pleochroic, appearing dark red and opaque in two extinction directions, and light red and transparent in the third (Perutz, 1939). Thus, the coefficient of absorption is high when the electric vibration of plane-polarized light is parallel to the haem groups, but is low in other directions. Similarly, a specific carotenoid protein has been found to appear orange or clear depending on the orientation of the crystal relative to the direction of polarization of the light hitting it (Kerfeld *et al.*, 1997). The darkest orange colour, corresponding to a maximum absorbance of the carotenoid cofactor, is found when the polarizer is aligned along the *a* axis (the long axis of the crystals). This suggests that all the carotenoid cofactor molecules in this crystal structure lie nearly parallel to the *a* axis.

The directions along which double refraction is observed can be used to give some information on the crystal class. Some crystals are found to have one, and only one, direction (the optic axis) along which there is no double refraction. Crystals with this property are called uniaxial. They have two principal refractive axes and are tetragonal, hexagonal or rhombohedral. Other crystals are found to have two directions along which there is no double refraction (two optic axes), and these are called biaxial. Such crystals are either orthorhombic, monoclinic or triclinic and have three principal refractive indices.

5.1.1.3.1. *Crystals between crossed polarizers*

Most protein crystals are birefringent and are brightly coloured in polarized light. In order to view these effects, crystals (in their mother liquor) are set on a microscope stage with a Nicol prism (polarizing material) between the light source and the microscope slide (the polarizer). Another Nicol prism is set between the crystal and the eyepiece (the analyser). The crystal should not be in a plastic container, since this would produce too many colours. If the vibration plane of the analyser is set perpendicular to that of the polarizer (to give 'crossed Nicols'), no light will pass through in the absence of crystals, and the background will be dark. If the crystal is isotropic, the image will remain dark as the crossed Nicol prisms are rotated. If, however, the crystal is birefringent (with two refractive indices), the crystal will appear coloured except at four rotation positions (90° apart) of the crossed Nicol prisms, where the crystal and background will be dark (extinguished). At these positions, the vibration directions of the Nicol prisms coincide with those of the crystal. If one is looking exactly down a symmetry axis of a crystal that is centrosymmetric in projection (such as a tetragonal or hexagonal crystal), the crystal will not appear birefringent, but dark. By noting the external morphology of the crystal with respect to its angle of rotation, one can often deduce the directions of the unit-cell axes in the crystal (Hartshorne & Stuart, 1960). Examination of a crystal under crossed Nicol prisms can also provide information on crystal quality. For example, sometimes the components of a twinned crystal extinguish plane-polarized light independently. Other methods of examining crystals include Raman spectroscopy (Kudryavtsev *et al.*, 1998).

5.1.1.3.2. *Refractive indices and what they tell us about structure*

The refractive index of a crystal can be measured by immersing it in a mixture of liquids of a known refractive index in which the crystal is insoluble. The liquid composition is then varied until the crystal appears invisible. At this point, the refractive indices of the crystal and the liquid are the same. If the refractive index is the same in all directions, the crystal is optically isotropic, but most protein crystals are optically anisotropic and have more than one refractive index. For example, tetragonal crystals have different refractive indices for light vibrating parallel to the fourfold axis and for light vibrating perpendicular to it. These refractive indices are measured by the use of plane-polarized light.

5.1.1.4. *Packing of molecules in crystals*

Growth kinetics of the different faces should be correlated with the structural anisotropy of the intermolecular contacts. It has been found that a judicious mutation of a single surface residue of a protein can markedly affect its solubility and hence crystallizability. This method has been used with great success for crystallizing a retroviral integrase (Dyda *et al.*, 1994).

The relationship between crystal morphology and internal crystal structure was examined in the mid-1950s (Hartman & Perdok, 1955*a*,*b*,*c*). It was shown that the morphology of a crystal is determined by 'chains' of strong intermolecular interactions (hydrogen bonding, van der Waals contacts, molecular stacking) running through the entire crystal. For a crystal to grow in the direction of a strong interaction ('bond'), these bonds must form an uninterrupted chain through the structure, giving rise to the periodic bond chain theory. The stronger the interaction between molecules, the more likely the crystal is to be elongated in that direction. If a bond chain contains interactions of different kinds, its influence on the shape of the crystal is determined by the weakest interaction present in a particular chain. Prominent faces are parallel to at least two high-energy bond chains. This enables a correlation to be made between the crystal lattice and the crystal morphology, based on the fact that direct protein–protein contacts, reinforced by well ordered solvent molecules, are important in determining crystal packing (Frey *et al.*, 1988). Studies of the morphology of tetragonal lysozyme (Nadarajah & Pusey, 1996; Nadarajah *et al.*, 1997) showed that the crystallizing unit is a helical tetramer (centred on the 4_3 crystallographic axes).

5.1.2. Crystal mounting

5.1.2.1. *Introduction to crystal mounting*

Once crystals have been obtained and visually characterized, the next procedure involves the transfer of a selected crystal to an appropriate mounting device so that the crystal may be characterized using X-rays. Macromolecular crystals are generally obtained from and stored in a solution containing the precipitant or precipitants and other substances such as uncrystallized protein or other macromolecules. The object is to mount the crystal in such a way that it is undamaged by cracking, drying out, dissolving *etc.* during this operation. In some cases, the crystal may have been stored in a solution containing volatile solvents. Alternatively, the crystals may have been grown at a temperature lower than room temperature and therefore may require special handling in order to avoid crystal deterioration. In other cases, it may be desirable to prepare the crystal for study at cryogenic temperatures. This section deals with the mounting of crystals for all these conditions and concentrates on the mounting of crystals for diffraction experiments at or just below room temperature. Procedures such as 'flash cooling' are used to reduce radiation damage. Crystal-mounting techniques for cryogenic experiments are covered in detail in Part 10 and are only mentioned briefly here. In general, the most difficult part of mounting macromolecular crystals is the transfer of the crystal from a holding solution to a suitable mount. A capillary or, if cryogenic experiments are to be carried out, a cryoloop should be used.

5.1.2.2. *Tools for crystal mounting*

In order to facilitate the process of mounting macromolecular crystals for X-ray diffraction experiments, it is necessary to have the appropriate tools for the task. Fig. 5.1.2.1 shows a collection of some useful tools for the mounting of crystals. These include a binocular microscope, tweezers (two types), thin glass capillaries, Pasteur pipettes, a heater, paper wicks, and a thumb pump. Other

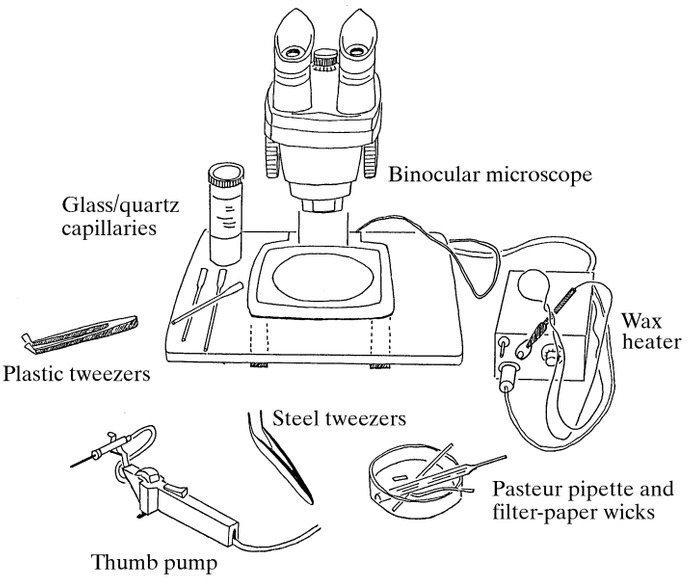


Fig. 5.1.2.1. Tools commonly used for mounting crystals.

useful tools and supplies include surgical scissors, dental wax, latex tubing, light vacuum oil, a cryogenic mounting loop, Plasticine, mounting platforms, mounting pins, absorbent dental points and micropipettes with plastic tips. There are many other items that might be useful, and several variations are found in different laboratories. An important factor in the transfer of crystals from a holding solution to a capillary is that the experimenter needs to feel at ease with the process. The method that will be detailed here has evolved over time and has proved to be a relatively anxiety-free process. Other methods for crystal mounting may be found in the literature (Rayment, 1985; Sawyer & Turner, 1992; McRee, 1993). All of the methods outlined here and in the literature have the same goal, namely, the successful transfer of a macromolecular single crystal to a suitable mount for X-ray data collection.

5.1.2.2.1. *Microscope*

Perhaps the single most important piece of equipment for examining and mounting crystals is a binocular dissection microscope. This should have variable zoom capabilities, and there should be sufficient distance (*e.g.* 5–10 cm) between the objective lens of the microscope and the microscope stage to accommodate the necessary equipment and allow manipulation of the crystals and solutions. A magnification of between 10 and 40 times is probably best in practice. It is also important to ensure that the light source of the microscope is not so intense that it heats the microscope stage, thereby damaging the macromolecular crystals. If the microscope is fitted with crossed polarizers, the quality of the crystals can be assessed.

5.1.2.2.2. *Capillaries*

The capillaries used for crystal mounting are made of thin-walled glass. These capillaries range from 0.1 to 2.0 mm in diameter and have a stated wall thickness of 0.01 mm. In practice, however, the larger the diameter of the capillary, the thinner the glass wall. Therefore, handling of the larger-diameter capillaries is generally very difficult because they are so fragile. Capillaries made of fused quartz are also available, but are not recommended for general use because they produce a higher background with X-rays. Quartz capillaries are not as fragile as the thin-walled glass capillaries, however, and may be useful in experiments where the tensile strength of the capillary is important, for example, when a

diffraction flow-cell experiment is planned (Petsko, 1985). In addition, small-diameter capillaries (produced in the laboratory by drawing out glass tubing or Pasteur pipettes) will be needed to aid in the removal of excess liquid around the crystal after the transfer from the crystallization dish to a capillary has been completed.

5.1.2.2.3. *Thumb pump*

The thumb pump is a simple micropipetting device for transferring very small amounts of liquid in a highly controlled manner, making it an extremely useful tool for directly transferring protein crystals from solution to capillary, thus minimizing the chance of crystal damage. This simple device allows the experimenter to have much more control over volume transfers than any other device we have tried. The mechanism is simple and easy to operate. The device shown in the illustration can be held and manipulated with one hand. The capillary is held firmly in place and the pipetting action is controlled by a thumb wheel (part of the thumb pump), which affords a great deal of control over the volume of liquid being transferred with the crystal.

5.1.2.2.4. *Heater*

The heater illustrated here consists of a variable rheostat and a heating element. The latter is a short piece of Nichrome wire which has been coiled and attached to the rheostat *via* wires that run through a ball-point pen barrel. This permits a fine temperature control for melting dental wax and for controlling where the heat is applied.

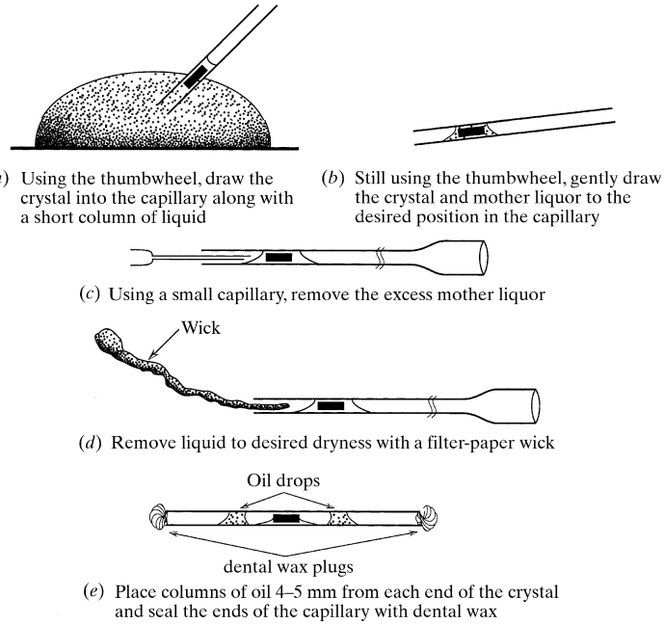
5.1.2.3. *Capillary mounting*

One must first select a capillary for mounting the crystal. A general rule to follow is to select a capillary that has a diameter that is approximately twice the size of the crystal dimension to be placed along the breadth of the capillary. Thus, to mount an elongated parallelepiped with the longest crystal dimension parallel to the capillary, it is necessary to take into account the cross section of the crystal perpendicular to the longest dimension. This rule is only a guide and is probably broken most of the time. Indeed, for 'chunky' crystals, it may be advantageous to use a capillary only slightly larger than the crystal so that the crystal may be in contact with the capillary wall in more than one place, thereby making the mount more stable. The object is to have enough of the crystal in contact with the capillary wall to allow the crystal to be held in place with a small amount of mother liquor. One possible problem that occurs with very thin crystals is that the crystal may bend to conform to the shape of the capillary wall. In this case, the crystal is rendered unsuitable for X-ray diffraction experiments.

The capillary is prepared by first removing the flame-sealed tip. This is done with surgical scissors or by pinching with surgical tweezers and then gently tapping the capillary tip against a smooth hard surface to remove the jagged edges which may have resulted from this cutting. The removal of the jagged edges at the broken end of the capillary will simplify the transfer of the crystal from the holding solution to the capillary. The large flared end of the capillary is left intact, and, at this time, a rubber transfer tube with a mouthpiece can be fitted over the flared end of the capillary. The capillary can then be rinsed with distilled deionized water, or it may even be desirable to treat the capillary with some other solution, such as EDTA, or perhaps with a solution identical to that surrounding the crystal. The rinse solution is gently drawn up into the capillary, and the solution is then blown out into a waste container. This can be accomplished fairly rapidly, and then the capillary can be dried by gently drawing air in through the capillary tip. Only the excess liquid should be removed at this point if the rinse solution contains salts.

The crystal must now be transferred to a capillary from the storage location, which may be a shallow well in a depression slide, a droplet on a cover slip, or perhaps a vial containing crystals. Direct transfer from a droplet on a cover slip or from a shallow well of a depression slide to the final capillary is possible, but can be complicated if several crystals are present in the drop. The easiest way to set up the final crystal transfer is to first remove it from the original drop or vial using a micropipette with a tip that has been enlarged so that it will accommodate the desired crystal. The crystal is then transferred together with a few microlitres of solution to a siliconized cover slip or depression well using the micropipette. It may even be easier to place 5–10 µl of solution on a siliconized cover slip or depression well and then use a cryoloop to capture the crystal and deposit it in the solution. The crystal can now be easily drawn up into the capillary with the aid of the thumb pump. It will be accompanied by a small column of mother liquor, and the thumb pump can be used to position the crystal with its liquid at the desired location in the capillary. The excess mother liquor can now be removed by using a capillary that is much smaller than the data-collection capillary, as well as smaller than the crystal. A final drying can be accomplished using appropriately sized filter-paper wicks or absorbent dental points. A very small amount of liquid should be left behind to keep the crystal moist and to 'glue' the crystal to the capillary wall *via* surface tension. A crystal that is too dry will probably deteriorate and be useless for diffraction experiments, while a crystal that has too much liquid can slip during data collection. On the other hand, moderate drying has been found, in certain cases, to give a crystal with improved diffraction.

After the crystal is safely in position in the capillary, the capillary must be sealed in order to maintain the moisture necessary to prevent crystal deterioration. If desired, a short column of mother liquor may be placed in the capillary a few millimetres away from the crystal. This is usually necessary if capillaries larger than 1 mm in diameter are used. A small strip of filter paper may also be placed in the capillary and then dampened with mother liquor. Both methods allow the moisture level in the crystal to be maintained. A reasonably good first seal may consist of a short column of light vacuum oil on both sides of the crystal, again, a few millimetres away from it. At this time, a ring of molten dental wax is placed along the capillary beyond the oil drop nearest the flared end of the



(*a*) Using the thumbwheel, draw the crystal into the capillary along with a short column of liquid

(*b*) Still using the thumbwheel, gently draw the crystal and mother liquor to the desired position in the capillary

(*c*) Using a small capillary, remove the excess mother liquor

(*d*) Remove liquid to desired dryness with a filter-paper wick

(*e*) Place columns of oil 4–5 mm from each end of the crystal and seal the ends of the capillary with dental wax

Fig. 5.1.2.2. Mounting a crystal in a capillary.

capillary, and the capillary is then cut or broken just beyond the wax. The final seal may then be accomplished using molten dental wax or perhaps even epoxy at each end of the capillary. The diffraction equipment and arrangement will dictate the position of the crystal in the capillary, and this should be accommodated before the final seals are put in place. The geometry of the capillary could aid in preventing slippage of the wedged crystal during data collection (Åkervall & Strandberg, 1971). Alternatively, a specific crystal coating which effectively glues the crystal to the interior of the capillary can be used (Rayment *et al.*, 1977). The capillary with its crystal is now ready to be placed on the platform of choice for placement on the goniometer head in final preparation for diffraction experiments. Fig. 5.1.2.2 illustrates the steps in mounting a crystal in a capillary in preparation for the X-ray experiment.

The above method deals only with crystals which are to be mounted at or near room temperature for experiments at or near room temperature. An alternative approach is to grow a crystal in a capillary (Åkervall & Strandberg, 1971), which could eliminate the need to manipulate the crystal manually. When crystals have been grown in the presence of detergents or gels, specific methods may be required for mounting (McRee, 1993). The appropriate procedure for flash cooling of crystals is detailed in Part 10.

Acknowledgements

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be calculated in advance, and looked for first. The positions of calibration drops and of crystals in these centrifuge tubes can be measured with a hand-held ruler to a resolution of about 0.5 mm.

Particularly for crystals with high values of V_M (*i.e.*, loosely packed) or for crystals of large molecular weight proteins, the apparent crystal density may increase with time: the crystal continues to sink and there is no apparent equilibrium spot. This behaviour is seen in both organic solvent gradients and Ficoll gradients, and the reasons for it are unclear. It may be that, in organic solvent gradients, some of the solvent can dissolve into the crystal; or the crystal may condense from slow dessication. In Ficoll gradients, it may be that sucrose monomers or dimers are present, which diffuse into the crystal over time. A careful study of this behaviour (Bode & Schirmer, 1985) in Ficoll gradients suggested that useful density values can still be obtained for these crystals by fitting the apparent density to an exponential curve:

$$\rho_c(t) = a + b\exp(-\lambda t). \qquad (5.2.6.3)$$

In this expression, parameters a, b and λ must be derived from the fitted curve. The crystals were inserted into the gradient with flame-narrowed Pasteur pipettes. Each crystal was initially surrounded by a small amount of mother liquor, which rapidly diffused into the Ficoll solution. Time zero was assigned as the time when centrifugation first began. It was necessary to observe crystal positions within the first minute, and at two- to five-minute intervals thereafter, to obtain a reasonable time curve for the density function. The experimental goal in the Bode & Schirmer experiment was to obtain a good estimate for the density value at time zero, $\rho_c(0) = a + b$. This was realized in all six of the crystal forms that manifested time-dependent density drift in the study.

5.2.7. How to handle the solvent density

It is necessary to have an accurate estimate of the mean solvent density, ρ_s, in (5.2.4.9). The Ficoll gradient-tube method is particularly convenient for this reason: the gradient can be made without any significant solute other than Ficoll. Since the free-solvent compartment of the crystal is entirely water, $\rho_s = \rho_{bs} = \rho_{fs} = 1.0$ g ml^{-1}. Therefore, in Ficoll density gradients, the crystal density becomes ρ_o, as defined in (5.2.5.2), and the packing number n can be calculated from

$$n = \frac{VN_o}{M}\frac{(\rho_c - 1)}{(1 - \overline{v}_m)}. \qquad (5.2.7.1)$$

Another way to set $\rho_s = 1.0$ g ml^{-1} is to cross-link the crystals with glutaraldehyde (Quiocho & Richards, 1964; Cornick *et al.*, 1973; Matthews, 1985), making the crystals insoluble even in the absence of stabilizing solutes. Once cross-linked, crystals can be transferred to a water solution prior to the density measurement, thereby substituting water for its free solvent. Care must be taken with cross-linking, however. Overnight soaking in 2% glutaraldehyde solutions can substantially increase the crystal density, while destroying its crystalline order (Matthews, 1985). Even 0.5% glutaraldehyde concentrations may change the observed density of some crystals if the exposure is for many hours – which may be necessary to render the crystal completely insoluble. Therefore, the densities observed from cross-linked crystals should be regarded with caution.

If it is necessary to carry out density measurements in an organic solvent gradient, then it is necessary in general to measure the crystal density at more than one free solvent density, since the relative volume fractions of the crystal's components are not known *a priori*. However, if this is a well behaved protein crystal, by setting $\overline{v}_m = 0.74$ ml g^{-1}, $V_M = 2.4$ Å^3 Da^{-1} and $w = 0.25$ g bound water per g protein, one can guess the crystal's volume compartments to be:

$$\varphi_m = 0.51, \qquad \varphi_{fs} = 0.32, \qquad \varphi_{bs} = 0.17,$$

and the mean solvent density to use in (5.2.4.9) would be

$$\rho_s \simeq 0.35 + 0.65\rho_{fs}. \qquad (5.2.7.2)$$

This may give reasonably reliable derivations for n in (5.2.4.9), with just one crystal-density measurement. Over-reliance on parameter estimates, however, can lead to bogus results, and (5.2.7.2) should be used with caution.

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