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Chapter 5.2. Crystal-density measurements

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5.2.1. Introduction

Crystal-density measurements have traditionally been a valuable and accurate (±4%) method for determining molecular weights of proteins (Crick, 1957; Coleman & Matthews, 1971; Matthews, 1985). But since exact chemical compositions of proteins being crystallized today are usually known from DNA sequences, crystal densities are rarely used for this purpose. Rather, crystal-density measurements may be necessary to define a crystal's molecular-packing arrangement, particularly when a crystal has an unusual packing density (very dense or very open); when there are a large number of subunits in the crystallographic asymmetric unit; when the structure consists of heterogeneous subunits, so the molecular symmetry or packing is uncertain; and for crystals of nucleic acids, nucleic acid/protein complexes and viruses.

5.2.2. Solvent in macromolecular crystals

Crystals of biological macromolecules differ from crystals of smaller molecules in that a significant fraction of their volume is occupied by solvent (Adair & Adair, 1936; Perutz, 1946; Crick, 1957). This solvent is not homogeneous: a part binds tightly to the macromolecule as a hydration shell, and the remainder remains free, indistinguishable from the solvent surrounding the crystal.

Hydration is essential for macromolecular stability: bound solvent is part of the complete macromolecule's structure (Tanford, 1961). Diffraction-based studies of macromolecular crystals verify the presence of well defined bound solvent. Typically, 8–10% of the atomic coordinates in each Protein Data Bank file are those of bound water molecules. The consensus observation of protein hydration (Adair & Adair, 1936; Perutz, 1946; Edsall, 1953; Coleman & Matthews, 1971; Kuntz & Kaufmann, 1974; Scanlon & Eisenberg, 1975) is that every gram of dry protein is hydrated by 0.2–0.3 g of water: this is consistent both with the presence of a shell of hydration, the thickness of which is about one water molecule (2.5–3 Å), and with the rule-of-thumb that approximately one water molecule is found for every amino-acid residue in the protein's crystal structure. Matthews (1974) suggests setting this hydration ratio, w, to 0.25 g water per gram of protein as a reasonable estimate for typical protein crystals.

Crystallographic structures also exhibit empty regions of 'free' solvent. Such voids are to be expected: closely packed spheres occlude just 74% of the space they occupy, so to the extent that proteins are spherical, tight packing in their crystals would leave 26% of the crystal volume for free solvent. Although the distinction between free and bound solvent is not sharp (solvent-binding-site occupancies vary, as do their refined B factors), it is a useful convention and is consistent with many observed physical properties of these crystals.

5.2.3. Matthews number

In an initial survey of 116 crystals of globular proteins (Matthews, 1968) and in a subsequent survey of 226 protein crystals (Matthews, 1977), Matthews observed that proteins typically occupy between 22 and 70% of their crystal volumes, with a mean value of 51%, although extreme cases exist, such as tropomyosin, whose crystals are 95% solvent (Phillips *et al.*, 1979). The volume fraction occupied by a macromolecule, φ_m, is reciprocally related to V_M, the Matthews number, according to

$$V_M = \overline{\upsilon}_m / N_o \varphi_m = V/nM, \qquad (5.2.3.1)$$

where $\overline{\upsilon}_m$ is the partial specific volume of the macromolecule (Tanford, 1961), N_o is Avogadro's number, V is the volume of the crystal's unit cell, n is the number of copies of the molecule within the unit cell and M is the molar weight of the macromolecule (grams per mole). V_M is the ratio between the unit-cell volume and the molecular weight of protein contained in that cell. The distribution of V_M (2.4 ± 0.5 Å^3 Da^{-1}) is asymmetric, being sharply bounded at 1.7 Å^3 Da^{-1}, a density limit consistent with spherical close packing. The upper limits to V_M are much less distinct, particularly for larger proteins. Matthews observed a slight tendency for V_M to increase as the molecular weight of proteins increases. V_M values below 1.9, or above 2.9, can occur but are relatively rare (beyond a 1σ cutoff).

The unit-cell volume, V, is determined from crystal diffraction. The partial specific volume of a macromolecule, $\overline{\upsilon}_m$, is the rate of change in the volume of a solution as the (unhydrated) macromolecule is added. It can be measured in several ways, including by ultracentrifugation (Edelstein & Schachman, 1973) and by measuring the vibrational frequency of a capillary containing a solution of the macromolecule (Kratky *et al.*, 1973). $\overline{\upsilon}_m$ typically has a value around 0.74 cm^3 g^{-1} for proteins and around 0.50 cm^3 g^{-1} for nucleic acids (Cantor & Schimmel, 1980). Values of $\overline{\upsilon}_m$ are tabulated for all amino acids and nucleotides, and $\overline{\upsilon}_m$ of a macromolecule can be estimated with reasonable accuracy as the mean value of its monomers. Commercial density-measuring instruments are available to determine $\overline{\upsilon}_m$ by the Kratky method. Because M is usually well known from sequence studies, n – the number of copies of the macromolecule in the unit cell – can be calculated thus:

$$n = V/V_M M = V N_o \varphi_m / \overline{\upsilon}_m M. \qquad (5.2.3.2)$$

For proteins, evaluating this expression with $V_M = 2.4$ usually provides an unambiguous integer value for n – which must be a multiple of the number of general positions in the crystal's space group! Setting n to its integer value then provides the actual value for V_M. If the calculated V_M value lies beyond the usual distribution limits, if n has an unexpected value or a large value, or if the crystal contains unusual components or several different kinds of molecular subunits, the crystal density may need to be measured accurately.

5.2.4. Algebraic concepts

Let V be the volume of one unit cell of the crystal. Let m_c be the total mass within one unit cell, and m_m, m_{bs} and m_{fs} be the masses, within one unit cell, of the macromolecule, bound solvent and free solvent, respectively. Let ρ_c, ρ_m, ρ_{bs} and ρ_{fs}, respectively, be the densities of a complete macromolecular crystal, its unsolvated macromolecule, its bound-solvent compartment and its free-solvent compartment. Let φ_m, φ_{bs} and φ_{fs}, respectively, be the

fractions of the crystal volume occupied by the unsolvated macromolecule, the bound solvent and the free solvent. By conservation of mass,

$$m_c = m_m + m_{bs} + m_{fs}. \tag{5.2.4.1}$$

The volume fractions must all add to unity:

$$\varphi_m + \varphi_{bs} + \varphi_{fs} = 1. \tag{5.2.4.2}$$

The density of the crystal is the total mass divided by the unit-cell volume:

$$\rho_c = \frac{m_c}{V} = \frac{m_m}{V} + \frac{m_{bs}}{V} + \frac{m_{fs}}{V}. \tag{5.2.4.3}$$

The mass in each solvent compartment is the product of its density and the volume it occupies:

$$m_{bs} = \rho_{bs} V \varphi_{bs}, \qquad m_{fs} = \rho_{fs} V \varphi_{fs}. \tag{5.2.4.4}$$

The mass of the macromolecule in the cell can be defined either from its partial specific volume, $\overline{\upsilon}_m$, the unit-cell volume, V, and the molecules' volume fraction, φ_m, or from the molar weight, M, the number of molecular copies in the unit cell, n, and Avogadro's number, N_o:

$$m_m = V\varphi_m/\overline{\upsilon}_m = nM/N_o. \tag{5.2.4.5}$$

Now (5.2.4.3) may be rewritten as

$$\rho_c = \varphi_m/\overline{\upsilon}_m + \rho_{bs}\varphi_{bs} + \rho_{fs}\varphi_{fs}. \tag{5.2.4.6}$$

Define a mean solvent density, ρ_s:

$$\rho_s = \frac{\rho_{bs}\varphi_{bs} + \rho_{fs}\varphi_{fs}}{\varphi_{bs} + \varphi_{fs}}. \tag{5.2.4.7}$$

This allows (5.2.4.6) to be rewritten as

$$\rho_c = \varphi_m/\overline{\upsilon}_m + (1 - \varphi_m)\rho_s. \tag{5.2.4.8}$$

Upon rearrangement, this gives expressions for the volume fraction of a macromolecule and for the molecular-packing number:

$$\varphi_m = \frac{\rho_c - \rho_s}{(\overline{\upsilon}_m)^{-1} - \rho_s} = \frac{n\overline{\upsilon}_m M}{VN_o},$$
$$n = \frac{VN_o}{\overline{\upsilon}_m M}\frac{\rho_c - \rho_s}{(\overline{\upsilon}_m)^{-1} - \rho_s}. \tag{5.2.4.9}$$

In (5.2.4.9), all terms can be measured directly, except ρ_s. The treatment of ρ_s will be discussed in Section 5.2.7. (5.2.4.9) defines the total macromolecular mass in the unit cell, $m_m = nM/N_o$, from a measurement of the crystal density ρ_c. If M were known from the primary sequence of the molecule, this measurement determines the molecular-packing number, n, with considerable certainty. If the molar weight were not accurately known, it could be determined by measuring the crystal density.

5.2.5. Experimental estimation of hydration

During refinement of crystal structures, crystallographers must decide how many solvent molecules are actually bound to the macromolecule and for which refined coordinates are meaningful. The weight fraction of bound solvent to macromolecule in the crystal, w, is estimated for most protein crystals to be about 0.25 (Matthews, 1974, 1985). However, its true value can be derived experimentally in the following manner. Since all relevant studies identify the bound solvent as water, it is reasonable to set the density of bound solvent as $\rho_{bs} = 1.0$ g ml^{-1}. Therefore, w can be expressed algebraically as

$$w = \frac{m_{bs}}{m_m} = \frac{\varphi_{bs}\rho_{bs}\overline{\upsilon}_m}{\varphi_m} = \frac{\varphi_{bs}\overline{\upsilon}_m}{\varphi_m}. \tag{5.2.5.1}$$

For crystals in which the rules-of-thumb $w = 0.25$ and $\overline{\upsilon}_m = 0.74$ cm^3 g^{-1} are valid, (5.2.5.1) implies that bound solvent occupies about one-third of the volume occupied by protein.

The crystal density, ρ_c, changes linearly with the density of free solvent surrounding the crystal. Let ρ_o be defined as the density the crystal would have if all its solvent were pure water ($\rho_s = 1.0$ g ml^{-1}):

$$\rho_o = 1 + \varphi_m(1/\overline{\upsilon}_m - 1). \tag{5.2.5.2}$$

A plot of crystal density against density of the supernatant (free solvent) solution should be a straight line with an intercept (at $\rho_{fs} = 1.0$ g ml^{-1}) of ρ_o and a slope of φ_{fs}. Therefore, by making a few crystal-density measurements, each with the crystal first equilibrated in solutions of varying densities, experimental values for ρ_o and φ_{fs} can be derived. If the partial specific volume is known for this molecule, φ_m, φ_{bs} and w can be derived from the expressions above. This approach was used by Coleman & Matthews (1971) and Matthews (1974) to measure molecular weights of six crystalline proteins, assuming $w = 0.25$, but their measurements could alternatively have assigned more accurate values to w, had the molecular weights been previously known. Scanlon & Eisenberg (1975) measured w for four protein crystals by this method (values betwen 0.13 and 0.27 were observed) and also confirmed that bound solvent exhibited a density of 1.0 g ml^{-1}.

5.2.6. Methods for measuring crystal density

Density measurements of macromolecular crystals are complicated by their delicate constitution. These crystals tolerate neither dehydration nor thermal or physical shock or stress. Furthermore, since macromolecular crystals contain free solvent, their densities will change as the density of the solvent in which they are suspended is changed. They cannot be picked up with tweezers, nor rinsed with arbitrary solvents, nor placed out to dry on the table.

The other experimental problem with these crystals is that they are very small. Typically, their linear dimensions are 0.1–0.2 mm, volumes are 1–10 nl and weights are 1–10 µg. Molecular structures can now be determined from even smaller crystals (linear dimensions as small as 20 µm) using synchrotron radiation, so density-measurement methods compatible with very small crystals are required. With such small samples, it is far easier and more accurate to measure *densities* than to measure directly *volumes* and *weights*.

The physical properties of macromolecular crystals constrain the methods by which their densities can be measured accurately. In all circumstances, great care must be taken to avoid artifacts such as air bubbles or particulate matter which often adhere to these crystals. All measurements should be made at one tightly controlled temperature, since thermal expansion can change densities and thermal convection can corrupt density gradients. Because crystals contain solvent, it is bad to dry them, since this process usually disrupts them, changing all parameters in unpredictable ways. Yet many density-measurement methods require that all external solvent first be removed from the crystals, since the measured densities will be some average of crystal and any remaining solvent. This can be an almost insurmountable problem for crystals containing cavities and voids. Unfortunately,

many crystallization mother liquors are viscous and difficult to remove, for example if they contain polyethylene glycol (PEG).

Richards & Lindley in Chapter 3.2 of *IT* C (2004) list six methods for measuring crystal densities: pycnometry, the method of Archimedes, volumenometry, the immersion microbalance, flotation and the gradient tube. The first three methods require direct weighing of the crystal and are therefore of limited value for crystals as small as those used in macromolecular diffraction, although these methods are used in various applications, such as mineralogy and the sugar industry. The latter three methods measure densities and density differences, and can therefore be used in macromolecular crystallography. A new method specifically for protein crystals has recently been described (Kiefersauer *et al.*, 1996), involving direct tomographic measurement of crystal volumes coupled with quantitative amino-acid analysis. Because the gradient-tube method remains the method of choice for most crystal-density measurements, it will be discussed last and most thoroughly here.

5.2.6.1. Pycnometry

Pycnometry measures the density of a liquid by weighing a calibrated volumetric flask before and after it is filled with the liquid. To measure a crystal's density, the pycnometer is first calibrated and weighed, and then the crystal sample, from which all external liquid has been removed, is introduced. The pycnometer is now reweighed, thus determining the crystal's weight. Next, liquid of known density is added, and the pycnometer is reweighed. The crystal volume is derived from the difference in volumes of the pycnometer with and without the crystal present. The method requires direct measurement of the crystal's weight, yet it is difficult to make microbalances with sensitivity and accuracy limits better than about 0.01 mg. Micropycnometry methods have been developed to determine mineral densities with as little as 5 mg of material (Syromyatnikov, 1935), but typical macromolecular crystals are 1000 times smaller than that.

5.2.6.2. Volumenometry

This technique measures the increase in gas pressure due to changes in the volume of a calibrated container into which the crystal, having previously been weighed, is introduced (Reilly & Rae, 1954). Almost any gas can be used for this technique if it is compatible with the apparatus and does not interact with the crystal. The empty, calibrated chamber is pressurized by adding a measured gas bolus, and this pressure is measured. After it is weighed, the crystal sample is placed in the container, which is repressurized by adding the same volume of gas. The difference in pressures between the two measurements is due to the change in volume of the container due to the crystal's presence. In principle, this method is compatible with powdered crystal samples, multiple crystals, or irregularly shaped crystals. As with micropycnometry, this technique is not appropriate for macromolecular crystals. The crystal must be free of external solvent, yet not dried, and the microbalance must be able to measure the crystal's weight precisely and accurately. The useful lower limit of crystal size for this technique, reported by Richards & Lindley in Chapter 3.2 of *IT* C (2004), is 0.01 ml.

5.2.6.3. The method of Archimedes

Known for thousands of years, this method measures the difference in weight of an object in air and in a liquid of known density. The difference divided by the liquid density gives the object's volume. The crystal is suspended by a vertical fibre or wire from a microbalance as it is dipped into the liquid. The surface tension of the liquid acting on the supporting fibre must be accounted for and corrected. The accuracy of this method improves as the density of the liquid used approaches that of the crystal. This method has been used with crystals as small as 25 mg (Berman, 1939; Graubner, 1986), but it does not lend itself well to density measurements of objects as small as macromolecular crystals.

5.2.6.4. Immersion microbalance

Barbara Low and Fred Richards developed this ingenious method, which permits the crystal to be weighed in a liquid environment. A microbalance (consisting of a thin horizontal quartz fibre, free at one end) is kept entirely within the liquid bath. Its vertical deflection, observed with a microscope, is initially calibrated as a function of weight (Low & Richards, 1952*b*; Richards, 1954). The density of the liquid can be determined with high precision by standard techniques. Each crystal's volume (in the 1952–1954 studies) was calculated from two orthogonal photomicrographs (this required that the crystal morphology be regular). The crystal density can then be calculated:

$$\rho_c = \rho_{\text{liquid}} + \frac{\text{apparent crystal weight}}{\text{crystal volume}}. \qquad (5.2.6.1)$$

The easiest and most accurate part of the method is measuring the liquid density. Therefore, experimental error in determining crystal weight and volume can be minimized by using a liquid with a density close to that of the crystal. In the limit where the crystal and liquid densities are the same, this method is equivalent to the flotation method – the fibre deflection is zero and the accuracy of the crystal-density measurement should be high. As originally implemented, the method is useful only for crystals with simple shapes, for which orthogonal photomicrographs can yield good estimates for the volume. Perhaps the method might be generalized if the tomographic volume-measuring method were adopted, as described by Kiefersauer *et al.* (1996). Richards & Lindley in Chapter 3.2 of *IT* C (2004) state that the method is only suitable for large crystals (volumes of 0.1 mm^3 or greater).

5.2.6.5. Flotation

The crystal must first be wiped completely free of external liquid and then immersed in a mixture of organic solvents, the density of which is adjusted (by addition of denser or lighter solvents) until the crystal neither rises nor sinks. Note that if the liquid used were aqueous, the crystal density would change as the surrounding liquid density is changed (*e.g.* by adding salt), since the crystal's free-solvent compartment would exchange with the external liquid. In this case, the equilibrium density, ρ_e, is a function only of the hydration number, w, and the macromolecule's partial specific volume, $\overline{\upsilon}_m$:

$$\rho_e = (w + 1)/(w + \overline{\upsilon}_m). \qquad (5.2.6.2)$$

ρ_e is about 1.25 g ml^{-1} for all protein crystals, regardless of packing arrangements or molecular weights, since $w \simeq 0.25$ and $\overline{\upsilon}_m \simeq 0.74$ cm^3 g^{-1}.

When the crystal just floats, the liquid's density (which now equals the crystal density) can be measured by standard techniques with high accuracy. Flotation measurements can be made with small samples (Bernal & Crowfoot, 1934) and with slurries of microcrystals. Centrifugation should be used to accelerate the crystal settling rate each time the liquid density is altered. The

Table 5.2.6.1
Organic liquids for density determinations

Name	Density (g ml^{-1})
Carbon tetrachloride (tetrachloromethane), CCl_4	1.5940
Bromobenzene, CH_5Br	1.4950
Chloroform (trichloromethane), $CHCl_3$	1.4832
Methylene chloride (dichloromethane), CH_2Cl_2	1.3266
Chlorobenzene, CH_5Cl	1.1058
Benzene, CH_6	0.8765
m-Xylene, 1,3-$(CH_3)_2C_6H_4$	0.8642
Iso-octane (2-methylheptane), $C_5H_{11}CH(CH_3)_2$	0.6980

Table 5.2.6.2
Inorganic salts for density determinations

The densities are approximate values for aqueous solutions at 20 °C.

Solute	Density (g ml^{-1})
Sodium chloride	1.20
Potassium tartrate	1.40
Potassium iodide	1.63
Iron(III) sulfate	1.80
Zinc bromide	2.00
Zinc iodide	2.39

method can be tedious, so its practitioners rarely achieve an accuracy better than 0.2–1.0% (Low & Richards, 1952*a*).

5.2.6.6. Tomographic crystal-volume measurement

Recently, a new method for density measurement which is specific for protein crystals has been reported (Kiefersauer *et al.*, 1996). The crystal volume is calculated tomographically from a set of optical-shadow back projections of the crystal, with the crystal in many (>30) orientations. This measurement is analogous to methods used in electron microscopy (Russ, 1990). The crystal is mounted on a thin fibre which is in turn mounted on a goniostat capable of positioning it in many angular orientations. The crystal must remain bathed in a humidity-regulated air stream to avoid drying. The uncertainty of the volume measurement improves asymptotically as the number of orientations increases (estimated to be 10–15%). The images are captured by a digital charge-coupled device camera, transferred to a computer and processed with the program package *EM* (Hegerl & Altbauer, 1982). This same crystal must then be recovered and subjected to quantitative amino-acid analysis (the authors used a Beckman 6300 amino-acid analyser). With a lower limit of 100 pmol for each amino acid, the uncertainty of this measurement was estimated to be 10–20% for typical protein crystals. The method appears to work for crystals with volumes ranging between 4–50 nl. Errors in the determined values of n ranged from 4–30%.

Implementation of the method requires complex equipment and considerable commitment (in terms of hardware and software) by the research laboratory. The accuracy of the method is sufficient to determine n unambiguously in many cases, but it is not as high as can be obtained with gradient-tube or flotation methods if care is taken. The method has the virtue that once established in a research laboratory, it might lend itself to a considerable degree of automation, thereby reducing the activation barrier to measuring crystal densities for members of the research group.

5.2.6.7. Gradient-tube method

This is the most commonly used method for measuring densities of macromolecular crystals. It is simple and inexpensive to implement. It can be used to measure densities of very small crystals and crystalline powders. Practised with care, the gradient-tube method is capable of measuring crystal densities with a precision and accuracy of ± 0.002 g ml^{-1}.

Although density gradients were used earlier for other purposes, the application of the gradient-tube method for crystal-density measurement was first described by Low & Richards (1952*a*). The gradient can be formed in a long glass column (preferably with volume markings, such as a graduated cylinder), in which case the crystal sample will settle by gravity in the tube; or in a transparent centrifuge tube, in which case the crystal's approach to its equilibrium density may be accelerated by centrifugation. The gradient may be made by two organic liquids (Table 5.2.6.1) with different densities, or it may be made by a salt concentration gradient in water (Table 5.2.6.2). In either case, formation of the gradient is simplified with a standard double-chamber 'gradient maker' – however, a *glass* gradient maker should be used if the gradient is made of organic solvents! Be aware that all these substances are toxic, particularly to the liver, and some are listed as carcinogens, so avoid prolonged exposure.

Desired upper and lower density limits for the gradient can be made by mixing two of these liquids in appropriate ratios. The sensitivity and resolution of the measurement can be enhanced by using a shallow gradient covering the expected density. These organic liquids have a nontrivial capacity to dessicate the crystal sample, so it is important that they be water-saturated before use. Also, when an alcohol is the precipitant of a crystal, organic solutions may be inappropriate for density measurements.

For aqueous gradients, the salts listed in Table 5.2.6.2 may be added to water to create a dense liquid.

A widely used variant of the method has been to form aqueous gradients with Ficoll, a sucrose polymer cross-linked with epichlorhydrin (Westbrook, 1976, 1985; Bode & Schirmer, 1985). Manufactured by the Pharmacia Corporation specifically for making density gradients used in the separation of intracellular organelles or intact cells, Ficoll is a large polymer ($M_r = 400\ 000$) which is very hydrophilic and soluble, and has chemical properties similar to sucrose. Since it is highly cross-linked, each Ficoll molecule tends to be globular and is so large that it is effectively excluded from the crystal. Ficoll precipitates protein from solution on a per-weight basis as effectively as polyethylene glycol and can prevent protein crystals from dissolving, even in the absence of other solutes. A 60% (w/w) solution of Ficoll has a density of about 1.26 g ml^{-1}, sufficiently dense that almost all protein crystals will float in this solution (nucleic acid crystals are usually too dense for Ficoll). Used with care (see below), Ficoll gradients seem to yield the most reproducible crystal-density measurements. Concentrated Ficoll solutions are quite viscous, so these gradients are usually made by manually overlaying small volumes (0.5 ml each) of decreasing density, rather than with a gradient maker. In a standard cellulose nitrate centrifuge tube of about 5 ml capacity, this procedure makes an almost continuous gradient which works satisfactorily.

The density column must be calibrated once it has been formed. This is performed by introducing small items of known density into the column and noting their vertical positions. The density of the gradient as a function of vertical position can then be defined by interpolating between adjacent calibrated points. Usually, the calibrating points are made from small drops of immiscible liquid. Thus, in an organic solvent gradient, the drops are made of salt water; in an aqueous gradient, the drops are

made of mixed organics (previously saturated with water). To make each calibration drop, a solution is made up with approximately the desired density, and its exact density (± 0.002 g ml^{-1}) is measured pycnometrically or by refractive index (Midgley, 1951). The drops can be inserted into the gradient with a flame-narrowed Pasteur pipette (this takes practice). Once calibrated, these gradients tend to be extremely stable over many months.

With an organic liquid gradient, two methods have been used to introduce the crystal sample to be measured. It can be extracted from its mother liquor with a pipette and extruded onto filter paper, which wicks away all exterior aqueous liquid. When free of moisture, but before it dessicates, the crystal must be shaken, flipped, or scraped onto the gradient top surface and allowed to sink to its equilibrium position. The second method involves injection of the crystal sample, in an aqueous droplet, into the gradient solution with a Pasteur pipette. A very thin syringe (home-made or commercial) is then used to draw off all extraneous liquid, while the crystal remains submerged in the organic liquid. Either method requires considerable manual dexterity and practice, especially with very small crystals. A significant advantage of Ficoll and aqueous salt gradients is that the crystal does not need to be manipulated at all: any liquid surrounding the crystal, which was introduced into the gradient at the start, rapidly dilutes into the aqueous solution and does not appear to interfere with further measurements.

With very small crystals, the approach to equilibrium is so slow that it is wise to use centrifugation, especially if it is suspected that the density is changing with time (see below). Nitrocellulose centrifuge tubes compatible with swinging-bucket rotors are typically 1 cm diameter, 5 cm long cylinders and are suitably transparent for this work. Centrifugation at 2500–5000 r.p.m. for as little as five minutes is sufficient for most crystals to reach a stable position in the gradient. It can be difficult to find the crystal after centrifugation, so the one or two most likely density values should 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

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

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 $\overline{\upsilon}_m = 0.74$ 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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