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.

This work was supported by the US Department of Energy, Office of Biological and Environmental Research, under contract W31-109-ENG-38.

References

Adair, G. S. & Adair, M. E. (1936). *The densities of protein crystals and the hydration of proteins. Proc. R. Soc. London Ser. B*, **120**, 422–446.

Berman, H. (1939). *A torsion microbalance for the determination of specific gravities of minerals. Am. Mineral.* **24**, 434–440.

Bernal, J. D. & Crowfoot, D. (1934). *Use of the centrifuge in determining the density of small crystals. Nature (London)*, **134**, 809–810.

Bode, W. & Schirmer, T. (1985). *Determination of the protein content of crystals formed by Mastigocladus laminosus C-phycocyanin, Chroomonas spec. phycocyanin-645, and modified human fibrinogen using an*

improved Ficoll density gradient method. Biol. Chem. Hoppe–Seyler, **366**, 287–295.
Cantor, C. R. & Schimmel, P. R. (1980). *Biophysical Chemistry*. San Francisco: W. H. Freeman & Co.
Coleman, P. M. & Matthews, B. W. (1971). *Symmetry, molecular weight, and crystallographic data for sweet potato β-amylase. J. Mol. Biol.* **60**, 163–168.
Cornick, G., Sigler, P. B. & Ginsberg, H. S. (1973). *Characterization of crystals of type 5 adenovirus hexon. J. Mol. Biol.* **73**, 533–538.
Crick, F. (1957). *X-ray diffraction of protein crystals. Methods Enzymol.* **4**, 127–146.
Edelstein, S. J. & Schachman, H. (1973). *Measurement of partial specific volumes by sedimentation equilibrium in H_2O − D_2O solutions. Methods Enzymol.* **27**, 83–98.
Edsall, J. T. (1953). *Solvation of proteins.* In *The Proteins*, edited by H. Neurath & K. Bailey, Vol. 1, part B, pp. 549–726. New York: Academic Press.
Graubner, H. (1986). *Densitometer for absolute measurements of the temperature dependence of density, partial volumes, and thermal expansivity of solids and liquids. Rev. Sci. Instrum.* **57**, 2817–2826.
Hegerl, R. & Altbauer, A. (1982). *The EM program system. Ultramicroscopy*, **9**, 109–116.
Kiefersauer, R., Stetefeld, J., Gomis-Rüth, F. X., Romão, M. J., Lottspeich, F. & Huber, R. (1996). *Protein-crystal density by volume measurement and amino-acid analysis. J. Appl. Cryst.* **29**, 311–317.
Kratky, O., Leopold, H. & Stabinger, H. (1973). *The determination of the partial specific volume of proteins by the mechanical oscillator technique. Methods Enzymol.* **27**, 98–110.
Kuntz, I. D. & Kaufmann, W. (1974). *Hydration of proteins and polypeptides. Adv. Protein Chem.* **28**, 239–345.
Low, B. W. & Richards, F. M. (1952*a*). *The use of the gradient tube for the determination of crystal densities. J. Am. Chem. Soc.* **74**, 1660–1666.
Low, B. W. & Richards, F. M. (1952*b*). *Determination of protein crystal densities. Nature (London)*, **170**, 412–415.
Matthews, B. W. (1968). *Solvent content of protein crystals. J. Mol. Biol.* **33**, 491–497.
Matthews, B. W. (1974). *Determination of molecular weight from protein crystals. J. Mol. Biol.* **82**, 513–526.
Matthews, B. W. (1977). *Protein crystallography.* In *The Proteins*, edited by H. Neurath & R. L. Hill, pp. 403–590. New York: Academic Press.
Matthews, B. W. (1985). *Determination of protein molecular weight, hydration, and packing from crystal density. Methods Enzymol.* **114**, 176–187.
Midgley, H. G. (1951). *A quick method of determining the density of liquid mixtures. Acta Cryst.* **4**, 565.
Perutz, M. F. (1946). *The solvent content of protein crystals. Trans. Faraday Soc.* **42B**, 187–195.
Phillips, G. N., Lattman, E. E., Cummins, P., Lee, K. Y. & Cohen, C. (1979). *Crystal structure and molecular interactions of tropomyosin. Nature (London)*, **278**, 413–417.
Quiocho, F. A. & Richards, F. M. (1964). *Intermolecular cross linking of a protein in the crystalline state: carboxypeptidase-A. Proc. Natl Acad. Sci. USA*, **52**, 833–839.
Reilly, J. & Rae, W. N. (1954). *Determination of the densities of solids by voluminometry.* In *Physico-Chemical Methods*, Vol. 1, pp. 577–608. New York: van Nostrand.
Richards, F. M. (1954). *A microbalance for the determination of protein crystal densities. Rev. Sci. Instrum.* **24**, 1029–1034.
Richards, F. M. & Lindley, P. F. (2004). *Determination of the density of solids.* In *International Tables for Crystallography*, Vol. C. *Mathematical, Physical and Chemical Tables*, edited by E. Prince, ch. 3.2. Dordrecht: Kluwer Academic Publishers.
Russ, J. C. (1990). *Computer-Assisted Microscopy: the Measurement and Analysis of Images.* New York: Plenum Press.
Scanlon, W. J. & Eisenberg, D. (1975). *Solvation of crystalline proteins: theory and its application to available data. J. Mol. Biol.* **98**, 485–502.
Syromyatnikov, F. V. (1935). *The micropycnometric method for the determination of specific gravities of minerals. Am. Mineral.* **20**, 364–370.
Tanford, C. (1961). *Physical Chemistry of Macromolecules.* New York: John Wiley & Sons.
Westbrook, E. M. (1976). *Characterization of a hexagonal crystal form of an enzyme of steroid metabolism, D5–3-ketosteroid isomerase: a new method of crystal density measurement. J. Mol. Biol.* **103**, 659–664.
Westbrook, E. M. (1985). *Crystal density measurements using aqueous Ficoll solutions. Methods Enzymol.* **114**, 187–196.