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

The mass of the macromolecule in the cell can be defined either from its partial specific volume, $\overline{v}_m$, 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:

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

Now (5.2.4.3) may be rewritten as

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

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

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

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

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

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

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

For crystals in which the rules-of-thumb $w = 0.25$ and $\overline{v}_m = 0.74$ 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}):

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

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 (1999) 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

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