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 (1999), 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:

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

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 (1999) 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, $\rho_e$, is a function only of the hydration number, $w$, and the macromolecule's partial specific volume, $\overline{v}_m$:

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

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

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 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





references