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3.2. Determination of the density of solids

By F. M. Richards and P. F. Lindley

3.2.1. Introduction (By P. F. Lindley)

The measurement of the density of a crystal has become a neglected art, and yet, in combination with an accurate knowledge of the unit-cell dimensions, it can provide vital information regarding the total molecular weight of the unit-cell contents. From this quantity, it is usually possible to determine the number of molecules in the unit cell and their individual molecular weights. The equation relating the crystal density (ρ), unit-cell volume (V), and the overall molecular weight is

$$\rho = Mm_a/V,$$

where m_a is the atomic mass unit (1.66057×10^{-24} g) and V is expressed in mm^3. Alternatively,

$$M = 0.602206V\rho,$$

where V is in units of Å^3. The mass per asymmetric unit can be determined by dividing M by the number of asymmetric units, Z (dependent on the space group), and this will normally correspond to the molecular weight. However, the quotient can either be a fraction of the molecular weight (normally 1/2) when the molecular symmetry permits the molecule to lie on a special position such as a centre of symmetry or a symmetry axis, or a multiple if the asymmetric unit contains more than one molecule. In either case, a special examination of the choice of unit cell and space group should be undertaken to ensure that the *correct* ones have been chosen. Normally, the measured and calculated densities should agree within at least 1.5%; discrepancies greater than this may indicate an incorrect molecular formula (not unknown in preparative chemistry) or the presence of solvent molecules or other additives. Incorrect choice of space group, inappropriate choice of unit cell, and incorrect asymmetric unit contents can all have profound effects on the success of a structure analysis and on the refinement of the resulting structure.

The classical techniques of density measurement are described by Tutton (1922) and by Reilly & Rae (1954). An excellent and detailed review of both the standard and the less common methods is given by Mason (1944), but, because this work can be difficult to obtain, some of the references compiled by this author are cited herein.

3.2.1.1. *General precautions*

Meticulous temperature control is essential for the highest precision. The allowable temperature fluctuation will depend on the thermal coefficient of expansion of the material and on the required accuracy of the measurement. The utmost care must be taken to avoid air bubbles and inclusions. In those techniques that require immersion of the solid in a liquid, it is assumed that no chemical or physical interaction occurs between the liquid and the solid, and that the volume of the liquid displaced represents the true volume of the solid. For most hard crystalline materials, liquids can easily be found for which these assumptions are valid. However, for amorphous powders, porous structures such as zeolites, crystalline proteins, and natural and synthetic fibres, the measured 'density' may depend markedly on the particular liquid chosen and on the details of the method applied. In these cases, *penetration* or *swelling* of the solid will depend on a variety of factors such as interfacial tension, the relation of pore size to molecular dimensions, adsorption, and electrostrictive forces. The structural unit to which the measured density applies may be very difficult to specify. Even with materials not subject to these difficulties, variability in the measured density is frequently found. Such variations may arise from differences in trace impurities or in the previous history of the sample (Johnston & Adams, 1912).

3.2.2. Description and discussion of techniques (By F. M. Richards)

The discussion here will be limited to six general methods, of which at least one may be adapted to the requirements of almost any problem. The method of choice will depend to a large extent on the nature of the material under study. The merits and disadvantages of each method will be discussed.

3.2.2.1. *Gradient tube*

This technique is simple, versatile, and capable of the greatest sensitivity. It is the method of choice except in those cases where immersion liquids with an appropriate density and chemical inertness cannot be found.

Originally devised by Linderstrom-Lang (Linderstrom-Lang, 1937; Linderstrom-Lang & Lanz, 1938) for the determination of the density of aqueous solutions, the procedure has been adapted for the measurement of crystal densities by Low & Richards (1952*a*). For the original solution measurements, a precision of 0.000001 g ml^{-1} was obtained, although no attempt has been made to attain that precision with solids. This technique was apparently developed and used quite independently in the sugar-cane industry [see, for example, Guo & White (1983) and earlier references contained therein].

3.2.2.1.1. *Technique*

When one liquid is layered over another of greater specific gravity, with which it is miscible, a linear gradient of density develops near the interface. Manipulation of a plunger-type stirrer in a vertical tube can extend the gradient over the greater part of the column. In the absence of convection, the process of diffusion in a column of this type is so slow that the gradient will be maintained virtually unchanged for many months.

A crystal introduced into the tube falls until it reaches a level corresponding to its own density, where it will remain stationary. The density gradient may be calibrated either by introducing immiscible liquid drops of known density, or by the use of a micro-Westphal balance designed for the purpose (Richards & Thompson, 1952).

With an adequate thermostat, measurements may be made at any temperature between the freezing and boiling points of the mixtures involved.

Powders and crystals with cavities or inclusions may be ground to a slurry with the lighter column liquid, subjected to reduced pressure to remove trapped air bubbles, and then introduced into the gradient tube. With hygroscopic materials, these operations are carried out in a dry atmosphere. Finely divided material settles rapidly if the tube is centrifuged. Although centrifugation does not markedly affect the gradient, the column should be calibrated after this step.

If such samples are homogeneous, they will form a thin layer after centrifuging. If, on the other hand, some air bubbles or

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Table 3.2.2.1. *Possible substances for use as gradient-column components*

Hydrophobic components			Hydrophilic components	
Liquid	Approximate density at 298 K (g ml^{-1})	α	Solute	Approximate maximum density of concentrated aqueous solution at 298 K (g ml^{-1})
Isooctane (2-methylheptane)	0.69	−0.8	Sodium chloride	1.20
Kerosene	0.79	−0.5 −0.8	Potassium chloride	1.40
m-Xylene	0.86	−0.85	Potassium iodide	1.63
Chlorobenzene	1.10	−1.1	Iron(III) sulfate	1.80
Bromobenzene	1.49	−1.3	Zinc bromide	2.00
Carbon tetrachloride	1.60	−1.9	Zinc iodide	2.39
Methyl iodide	2.28	−2.7	Thallium(I) formate	3.5
Bromoform	2.89	−0.9	Thallium(I) formate–malonate	4.3
s-Tetrabromoethane	2.96	−2.2		
Methylene iodide	3.32	−2.6	Ficoll* (60% *w*/*w* in water)	1.25

The density at temperature T K can be computed by substituting the values of the density at 298 K and α in the formula $d_T = d_{298} + 10^{-3}\alpha(T - 298)$. * Trade name for a synthetic high-molecular-weight polysaccharide derivative.

inclusions still remain, or if the sample is truly a mixture, a stable distribution of material will be observed. The density of the material of interest can then usually be obtained by measurement of the appropriate layer, generally the most dense, without further treatment of the sample. This is the only technique by which the homogeneity of the sample can be tested simply. All other methods provide an average density value. A satisfactory technique for removing crystalline powders from the gradient column has not been devised. If a precision of $\pm 0.002\,\mathrm{g\,ml^{-1}}$ is adequate, it is simplest to prepare a new wide-range column for each determination in a 10 ml test tube.

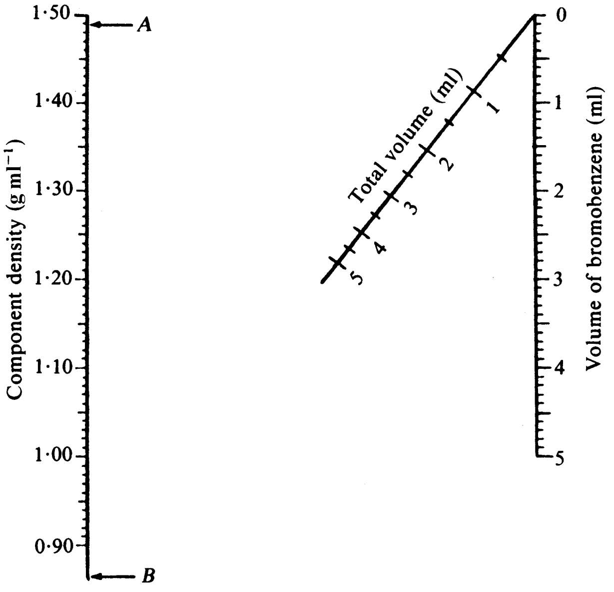

Fig. 3.2.2.1. Nomogram for the preparation of bromobenzene–xylene gradient column components at room temperature. From the desired component density and total volume, the required amount of bromobenzene is read from the chart, the volume difference being made up with xylene. To adapt this chart to any other pair of liquids, it is only necessary to change the component density scale. A uniform scale is drawn up such that the density of the heavy liquid lies at the point *A* while that of the light liquid is at *B*. The volume scales may be multiplied by any constant factor in order to change their range.

Detailed specifications for the preparation of large density-gradient columns are contained in the records of the British Standards Institution (1964). In the experience of the author, for ordinary laboratory use, the procedures described are unnecessarily complicated as is the large scale of the system. The large columns are not suitable for centrifuging and the settling times tend to be many hours. However, if extreme sensitivity (*i.e.* use of a shallow gradient) is required, the large column may be useful, as it was in the original studies of Linderstrom-Lang (Linderstrom-Lang, 1937; Linderstrom-Lang & Lanz, 1938).

In the specific application of this technique to protein crystals, where a gradient of organic liquids is used, it is necessary to have available crystals sufficiently large that they can individually be quickly wiped free of adhering mother liquor with dampened filter paper before insertion. The uncertainty of successful cleaning combined with rapid evaporation of liquid from the pores within the crystal always affect the estimated accuracy of the measurement. An important improvement in the technique has been made by Westbrook (1976, 1985) through the use of concentrated aqueous solutions of the water-soluble polymer Ficoll. This very high molecular weight polysaccharide can be dissolved in water to concentrations of at least 60% by weight. The solutions are very viscous but do provide satisfactory water-based gradient columns. The polymer is both too large to enter the solvent-filled pores of the protein crystals and too high in molecular weight to develop a significant osmotic pressure. An aqueous suspension of crystals can be added directly to the column. This procedure has been adapted for measurements of protein-crystal density under hydrostatic pressures from 1 to 2000 atm (1 atm ≡ 101 325 Pa) (Kundrot & Richards, 1988). The general principle of using high-polymer-based gradients can presumably be extended to other porous materials.

3.2.2.1.2. *Suitable substances for columns*

Some representative liquids are listed in Table 3.2.2.1; all are readily available. For further information, see Meyrowitz, Cuttitta & Hickling (1959), and for very heavy liquids Sullivan

(1927). Standardized solutions or mixtures from one list in Table 3.2.2.1 may be used as calibrating drops in gradients made from those of the other.

For rapid preparation of mixtures from stock solutions of the basic compounds, a nomogram is very useful, such as is given in Fig. 3.2.2.1 for the system bromobenzene–xylene at room temperature. In the construction of the nomogram, it has been assumed that the volumes of the liquids are additive. In general, this assumption is not valid, but it is a sufficiently good approximation for the purpose.

3.2.2.1.3. *Sensitivity*

The range of density covered by a column, and thus the accuracy of the determination, is controlled by the liquids or liquid mixtures chosen for the top and bottom components. A precision of about $\pm 0.002\,\mathrm{g\,ml^{-1}}$ can easily be obtained without any special precautions. If narrow-range columns are carefully protected from temperature changes and vibration, the accuracy of the measurement may be increased 10- to 100-fold.

3.2.2.2. *Flotation method*

Although historically used much earlier, this technique is essentially an approximation to the gradient-tube method. The specimen is immersed in a liquid, and a denser or less dense liquid miscible with the first is added until the sample neither rises nor sinks in the solution (Wulff & Heigl, 1931). The density of the immersion medium is then determined immediately by standard techniques such as pycnometry, by the Westphal balance, or by refractive index (Midgley, 1951). The method is reported as capable of a probable accuracy as great as 0.02%.

The compounds listed in Table 3.2.2.1 are also useful in this method. With slurries or with specimens smaller than 1 mm^3, a centrifuge must be used to achieve a reasonable rate of settling. As little as 0.05 mg of material has been used with good results (Bernal & Crowfoot, 1934). A modification of this method has been described in which the density of the immersion medium is varied by altering the temperature (Reilly & Rae, 1954; Wunderlich, 1957).

3.2.2.3. *Pycnometry*

This is one of the most demanding of the available techniques. A previously calibrated pycnometer containing the sample is weighed. A liquid of known density is then introduced, air bubbles are removed by reducing the pressure, and the filled bottle is reweighed. The volume of the sample and its mass may thus be determined. With care, a probable accuracy of 0.02% may be achieved (Johnston & Adams, 1912). Contrary to many published statements, the accuracy of this technique is not dependent to any significant extent on the use of immersion media of high density.

Liquids with low surface tension will facilitate the removal of air bubbles. In some cases, it is advantageous to fill the bottle with the mother liquor from which the crystal grew. Powders or many small crystals may be used as well as large single specimens. There is no restriction on the density of the materials for which this technique is suitable.

A micropycnometer for use with samples of total volume as small as 0.01 ml has been described (Syromyatnikov, 1935). An accuracy of better than 1% has been achieved with this instrument.

3.2.2.4. *Method of Archimedes*

The specimen is weighed in air and again in a liquid of accurately known density. From the apparent loss of weight the volume is computed, and thence the density (Reilly & Rae, 1954). The technique requires little special equipment and is capable of great accuracy when used with large, well formed crystals. The accuracy is maximized by using immersion liquids of density as close to that of the crystals as possible. For precise work, correction must be made for the interfacial tension between the supporting wire and the upper surface of the suspending medium.

A torsion microbalance has been adapted to the determination of crystals as small as 25 mg (Berman, 1939). A probable accuracy of better than 1% may be achieved with this micromethod.

A densitometer based on Archimedes principle with control of the composition of the gas phase and a wide temperature range has been described by Graubner (1986). The method is not suitable for finely divided materials.

3.2.2.5. *Immersion microbalance*

Some crystals, such as those of globular proteins grown from alcohol–water mixtures, rapidly change their composition, and thus their density, when removed from the mother liquor in which they were grown. The density may then be computed from the weight of the crystal immersed in its mother liquor, the density of the latter, and the volume of the crystal (Low & Richards, 1952*b*, 1954; Richards, 1954).

A horizontal quartz fibre, free at one end, is mounted in a glass case that can be filled with liquid. After calibration, the deflection of the fibre gives the weight of an immersed crystal suspended on the free end. The volume is computed from the crystal dimensions as determined from two photomicrographs of the immersed crystal taken at right angles to each other. The density of the mother liquor is measured by one of the standard techniques for liquids.

The method is suitable for single, well formed crystals having a volume of about 0.1 mm^3 or greater. The accuracy is related inversely to the difference in density between the crystal and its mother liquor.

3.2.2.6. *Volumenometry*

This is the only technique not requiring immersion of the sample in a liquid medium. The technique is therefore used in instances where the specimen would be attacked by the customary immersion media, or where one wishes to work over a temperature range where liquid media would be inappropriate.

The gas-pressure change caused by altering the volume of a calibrated vessel by a given amount is determined when the vessel is empty, and again after the weighed specimen has been introduced (Reilly & Rae, 1954).

Any gas inert to the crystal may be used. Powders and crystal fragments may be employed. A probable accuracy as great as 0.1% may be attained. Samples with an aggregate volume as low as 0.01 ml have been measured with a probable accuracy of 1% (Hauptmann & Schulze, 1934).

3.2.2.7. *Other procedures*

A novel procedure that may be useful in special circumstances is based on measuring the frequency of a vibrating string of the material in question. If the length of the string is fixed and the transverse deformation is small, the various harmonic frequen-

Table 3.2.3.1. *Typical calculations of the values of* V_M *and* V_{solv} *for proteins*

Protein	γB-Crystallin	γD-Crystallin	Ceruloplasmin
Space group	$P4_12_12$	$P2_12_12_1$	$P3_221$
Cell parameters (Å)	57.5 × 98.0	57.8 × 70.0 × 117.3	213.9 × 85.6
Molecular weight (kDa)	21	21	132
Z	8	4	6
V_M (Å^3 Da^{-1})	1.93	5.65*	4.95
$V_{\rm solv}$ (%)	36	78*	75
Resolution (Å)	1.5	2.0	3.1

* In the case of γD-crystallin, the values for V_M and $V_{\rm solv}$ are abnormally high. A recalculation assuming two molecules per asymmetric unit, $Z_a = 2$, gives more reasonable values of $V_M = 2.83$ Å^3 Da^{-1} and $V_{\rm solv} = 56\,\%$.

cies of vibration will be related inversely to the square root of the density for small oscillations. The potential accuracy of frequency measurements makes this useful for following density changes in the sample while altering the temperature or pressure; see Rabukhin (1982).

3.2.3. Biological macromolecules (By P. F. Lindley)

Biological macromolecules usually present particular difficulties with respect to density measurements and the determination of Z_a, the number of molecules per asymmetric unit, because of the presence in the crystals of variable amounts of solvent. However, it is often crucially important with respect to a structure determination, particularly using molecular-replacement techniques, to know Z_a. In many cases, $Z_a = 1$, although, as in the case of small molecules, crystals are also found with fractional values of Z_a when molecular symmetry axes coincide with crystal symmetry axes, or values greater than 1 if there are multiple copies of the molecule in the asymmetric unit. In practice, it is usually necessary only to know the solvent content of the crystals between rather coarse limits in order to distinguish possible values of Z_a, and this problem has been addressed for globular proteins by Matthews (1968). A rather more precise knowledge of the solvent may be essential for the use of density-modification techniques in phase refinement. Matthews defines a quantity, V_M, the crystal volume per unit of protein molecular weight (*i.e.* the ratio of the volume of the asymmetric unit determined from X-ray diffraction measurements to the molecular weight of the protein in the asymmetric unit) and shows that V_M bears a simple relationship to the fractional volume of solvent in the crystal. The range of observed values of V_M (1.68 to 3.53 Å^3 Da^{-1} for the 116 distinct crystal forms considered by Matthews with median and most common values of 2.61 and 2.15 Å^3 Da^{-1}, respectively) is essentially independent of the volume of the asymmetric unit. Matthews further defines the quantity $V_{\rm prot}$, the fraction of the crystal volume occupied by the protein:

$$V_{\rm prot} = 1.66\nu/V_M,$$

where ν is the partial specific volume of the protein in the crystal and for most proteins approximates to 0.74 ml g^{-1}. With this approximation,

$$V_{\rm prot} = 1.23/V_M$$

and, by difference, the fractional volume occupied by the solvent is therefore

$$V_{\rm solv} = 1 - 1.66\nu/V_M \approx 1 - 1.23/V_M.$$

On this basis, the range of V_M cited above converts to a solvent content ranging from 27 to 65%, with values near 43% occurring most frequently. For cases where the solvent content appears abnormally low or high in respect of the physical properties of the crystal and the resolution of the diffraction pattern, then some alteration to the value of Z_a may well be indicated. Some typical examples are given in Table 3.2.3.1. It should be noted that, although the method described above appears to obviate the need to measure the density of crystals, a precise experimental measurement of the crystal density, wherever practical, is always a useful investment.

In a recent development, Kwong, Pound & Hendrickson (1994) have devised an experimental method for the determination of Z_a using a volume-specific amino acid analysis. The crystal volume is determined from optical measurements of crystals mounted in glass capillaries, and the number of molecules in that volume is determined by amino acid analysis. From the unit-cell volume determined from X-ray measurements and the space-group symmetry, Z_a can be calculated from the number of molecules per crystal volume. The method requires extreme care to obtain precise measurements of the crystal volume and access to high-performance liquid chromatography and associated equipment for the amino acid analysis.

references