Determination of partial specific volume and absolute concentration by densimetry

Philip H. STOTHART

National Institute for Research in Dairying, Shinfield, Reading RG2 9AT, U.K.

(Received 13 January 1984/Accepted 23 February 1984)

The apparent partial specific volume, ϕ , of a protein (or other macromolecule) and the absolute concentration, c , can be determined from the densimetry of solutions and diffusates in $^1\text{H}_2\text{O}$, $^2\text{H}_2\text{O}$ and $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ mixtures if the ratios of concentrations (relative concentrations) are known. The densimetry method allows a non-destructive check on total macromolecule concentrations without prior knowledge of specific u.v.-absorption coefficient. The method may be especially useful in small-angle neutron-scattering studies.

The apparent partial specific volume, ϕ ,* of a protein (or other macromolecule) is needed for a number of physico-chemical techniques such as analytical ultracentrifugation, photon correlation spectroscopy, small-angle scattering of X-rays and neutrons, etc. It can be determined by densimetry of solutions (Kratky *et al.*, 1973) if the protein concentration is known. However, concentration determination by dry mass can be difficult with labile proteins (McKenzie & Murphy, 1970), and u.v. spectrophotometry is uncertain with systems (e.g. casein micelles) containing interacting mixtures of proteins with different specific absorption coefficients. The densimetry method (described below) permits simultaneous determination of apparent partial specific volume, absolute macromolecule concentration, and hence specific u.v.-absorption coefficient. The densimetry method and the method of obtaining ϕ by analytical ultracentrifugation in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ solvents (Edelstein & Schachman, 1967, 1973) both depend on the principle of varying the buoyant density of the macromolecule by varying the density of the solvent. The densimetry method is applicable to polydisperse association–dissociation systems (e.g. casein micelles) that are difficult to treat by the ultracentrifuge method (Edelstein & Schachman, 1973).

* The symbol ϕ is used, rather than $\bar{v}$, because reference is made to Casassa & Eisenberg (1964), a paper in which several definitions of partial specific volume are given.

Theory

The apparent partial specific volume, ϕ , of a solute is given by (Casassa & Eisenberg, 1964):

$$\phi = \frac{1}{\rho} \left(1 - \frac{\rho' - \rho}{c} \right) \quad (1)$$

where c is the solute concentration and ρ and ρ' are the solvent and solution densities respectively. Consider a non-diffusible solute (e.g. a protein), at concentration c , in a solution at dialysis equilibrium with a solvent containing only diffusible species. The density difference ($\rho' - \rho$) is attributed to the protein (neglecting any redistribution of ions across the dialysis membrane), and so the partial specific volume obtained is that termed ϕ' by Casassa & Eisenberg (1964). Here we neglect differences between ϕ and ϕ' , and use the symbol ϕ .

We require solvents of different densities in which the apparent molar volume of the protein is unchanged. This condition is best fulfilled by solvents that are chemically identical but differ isotopically. Solvents 1, 2 and 3 have approximate $^2\text{H}_2\text{O}/^1\text{H}_2\text{O}$ ratios of 0, 1.0 and ≥ 9 respectively. Their other diffusible components are identical. The M_r of the protein increases from M_r' in $^1\text{H}_2\text{O}$ solvent to M_r'' in $^2\text{H}_2\text{O}$ solvent because labile protons are exchanged for ^2H . If M_r increases linearly with x , the molar fraction of $^2\text{H}_2\text{O}$ in the water phase of the solvent, then:

$$M_r = M_r' (1 + Hx)$$

where H is a constant for a given protein.

Assuming the molar volume is the same in solvents 1, 2 and 3:

$$\phi_i = \phi_1 / (1 + x_i H) = \frac{1}{\rho_i} \left(1 - \frac{\rho'_i - \rho_i}{c_i} \right) \quad (2a, 2b \text{ and } 2c)$$

where $i = 1, 2$ or 3 , and $x_1 = 0$.

Put

$$c_i = m A_i \quad (3)$$

where A_i is a measurement proportional to concentration [e.g. u.v. absorbance, refractive-index difference, turbidity (if Beer's Law holds)] with m a constant, and substitute into eqns. (2a), (2b) and (2c) to obtain eqns. (4), (5) and (6):

$$m(1 - \phi_1 \rho_1) = (\rho'_1 - \rho_1) / A_1 = F_1 \quad (4)$$

$$m(1 - \phi_2 \rho_2) = m \{ 1 - [\phi_1 \rho_2 / (1 + x_2 H)] \} \\ = (\rho'_2 - \rho_2) / A_2 = F_2 \quad (5)$$

$$m(1 - \phi_3 \rho_3) = m \{ 1 - [\phi_1 \rho_3 / (1 + x_3 H)] \} \\ = (\rho'_3 - \rho_3) / A_3 = F_3 \quad (6)$$

For brevity, use ϕ in place of ϕ_1 .

If H is already known, either from using an assumed average value (Edelstein & Schachman, 1967, 1973), or by calculation from the amino acid sequence, then m and ϕ can be determined by simultaneously solving any two of eqns. (4)–(6). Hence, from eqns. (4) and (6):

where $G = A_1/A_3$. For a 10 mg/ml protein solution, an error of $\pm 10 \mu\text{g/ml}$ in $(\rho'_i - \rho_i)$ gives an error of 0.003 ml/g in ϕ . The required precision of density measurement varies inversely as concentration.

If $\rho_3 \approx 1/\phi$ (e.g. for 2M-CsCl in $^2\text{H}_2\text{O}$) then $(\rho'_3 - \rho_3) \approx 0$. Hence the terms in A_1/A_3 in eqns. (7) and (8) are minimized and so much reduce the effect on c_i and ϕ of errors in A_1/A_3 .

If H is unknown, then m , ϕ and H can be determined by solving eqns. (4), (5) and (6) simultaneously. The resultant quadratic equation has solution:

$$\phi = (-b - \sqrt{b^2 - 4hf}) / 2h \quad (9)$$

where:

$$h = \rho_1 [(x_2/x_3) F_2 (F_1 \rho_3 - F_3 \rho_1) - F_3 (F_1 \rho_2 - F_2 \rho_1)]$$

$$b = \rho_1 [(x_2/x_3) - 1] [2F_2 F_3 - F_1 (F_2 + F_3)] \\ + F_1 [\rho_3 (x_2/x_3) (F_1 - F_2) - \rho_2 (F_1 - F_3)]$$

$$f = [F_2 - F_1] [F_1 - F_3] [(x_2/x_3) - 1]$$

To minimize the effect of errors in densimetry, $(\rho_3 - \rho_1)$ should be as large as possible and $\rho_2 \approx \sqrt{\rho_1 \rho_3}$. m (and hence c_i) is found by substitution in eqn. (4), and H by substitution for m and ϕ in eqns. (5) or (6). In practice it is difficult to get accurate values of ϕ , as ϕ is extremely sensitive to errors in the F_i term. (see below).

$$\phi = (F_3 - F_1) / \left(F_3 \rho_1 - \frac{F_1 \rho_3}{1 + x_3 H} \right) \\ = [(\rho'_3 - \rho_3) \frac{A_1}{A_3} - (\rho'_1 - \rho_1)] / \left[(\rho'_3 - \rho_3) \rho_1 \frac{A_1}{A_3} - \frac{(\rho'_1 - \rho_1) \rho_3}{1 + x_3 H} \right] \quad (7)$$

$$c_1 = m A_1 = (\rho'_1 - \rho_1) \rho_3 - \frac{[(\rho'_3 - \rho_3) \frac{A_1}{A_3} \rho_1 (1 + x_3 H)]}{\rho_3 - [\rho_1 (1 + x_3 H)]} \quad (8)$$

Now $A_1/A_3 = c_1/c_3$, so only the ratio of the concentrations need be known in order to determine ϕ , c_1 and c_3 . Concentrations determined are absolute in that they are not referred to a secondary standard. The values of c_i and ϕ obtained are not sensitive to systematic errors in calibration of wavelength scale or absorbance scale of the spectrophotometer (as concentration ratios close to unity are used), in contrast with values of c_i obtained in the usual way, using literature values of absorptivity. An H -value of 0.0155 is often used for proteins in $^2\text{H}_2\text{O}$ (Edelstein & Schachman, 1967, 1973). c_i and ϕ are insensitive to H and not very sensitive to A_1/A_3 (or F_1/F_3); e.g. for $\rho_1 = \rho_{^1\text{H}_2\text{O}}$ and $\rho_3 \approx \rho_{^2\text{H}_2\text{O}}$:

$$\Delta c_i / c_i \approx 0.1 \Delta H / H \quad \text{and} \quad \Delta \phi / \phi \approx 0.03 \Delta H / H$$

$$\Delta c_i / c_i \approx 2 \Delta G / G \quad \text{and} \quad \Delta \phi / \phi \approx 0.7 \Delta G / G$$

Experimental test of the densimetry method for β -casein and α_{s1} -casein

(a) Materials and methods

α_{s1} - and β -Caseins (kindly donated by Dr. A. T. Andrews) had been obtained by ion-exchange chromatography (Andrews & Alichanidis, 1983). Solutions of α_{s1} - and β -casein were made by dissolving them in 0.07M-NaCl with 0.02% (w/v) NaN_3 and dialysing against this solvent. Soya-bean trypsin inhibitor (Trypsin Inhibitor type 1-S; Sigma) was added to the solution in the dialysis sac to a concentration of $1 \mu\text{g/ml}$. Two 4 ml samples of the solution were dialysed against this solvent made with $^1\text{H}_2\text{O}/^2\text{H}_2\text{O}$ mixture and with $^2\text{H}_2\text{O}$ (99.8 atom% ^2H ; Aldrich Chemical Co.). Final $^2\text{H}_2\text{O}$ molar fractions in the water phase (obtained from densities of diffusates) were

0.469 and 0.988 respectively. pH (or p^2H) of all solvents was 6.7 (Covington *et al.*, 1968). Densities of all solutions and diffusates were measured at 20.08°C with a Paar digital density meter (Kratky *et al.*, 1973), type DMA 60 (Anton Paar KG, Graz, Austria) by using a pair of matched cells, type DMA 602, in the PLL mode. These cells require 0.7 ml of sample, but smaller cells are available. Solutions and diffusates were exposed to the air for the minimum possible time between the end of dialysis and injection into the density meter. Densities were reproducible to $\pm 2 \mu\text{g/ml}$. U.V. absorbance of solutions was measured (with appropriate corrections) at 280 nm on a Perkin-Elmer type 552 double-beam spectrophotometer. Protein concentrations were all in the range 8–10 mg/ml. Samples for spectrophotometry were diluted with 10 mM-NaOH/10 mM-EDTA in $^1\text{H}_2\text{O}$, with volumes measured by micropipette or by weighing as shown in Table 1. High dilution factors minimized the effect of small differences in u.v. absorbance between proteins in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$.

(b) Results (see Table 1)

For H unknown (eqn. 9) the calculated ϕ values do not agree well with literature values, particularly for P-type data. Dilution by micropipette is clearly not sufficiently accurate, and weighing is necessary. ϕ is very sensitive to the F_i term, e.g. from this test we find $\Delta\phi/\phi \approx -40\Delta F_2/F_2$. It is possible to measure ($\rho_i - \rho_s$) to 0.1% for a protein solution of 7 mg/ml or more, but difficult to obtain the ratios of A_i values to this precision. The main source of error in F_i (and hence ϕ) is then in A_i .

For H assumed known (eqn. 7) the ϕ -values are far less sensitive to experimental error, e.g. for this test $\Delta\phi/\phi \approx 0.7\Delta F_1/F_1$. For W-type data, ϕ values in good agreement with the literature (McMeekin *et al.*, 1949) are obtained for $H = 0.0100$, whereas H -values calculated from the amino acid composition (Whitney *et al.*, 1976) for 100% exchange of

labile protons (Jacrot, 1976) are 0.0131 for β -casein and 0.0143 for α_{s1} -casein. This may indicate that the ^1H - ^2H exchange is only 60–80% complete, despite lengthy dialysis. The $A_{1\text{cm}}^{1\%}$ value obtained for β -casein agrees quite well with the literature value (Thompson & Pepper, 1964). The $A_{1\text{cm}}^{1\%}$ value obtained for α_{s1} -casein is lower than the literature value (Thompson & Kiddy, 1964), which may be a result of the pH at which u.v. absorbance was measured. The method has clearly given an accurate concentration, otherwise ϕ would have been in error. This illustrates the point that the method is not invalidated by conditions of pH, spectrophotometer calibration etc. that differ from those used to obtain specific absorption coefficients in the literature.

Discussion

It is clear that a rapid method of measuring density to high precision is required, e.g. the mechanical-oscillator method (Kratky *et al.*, 1973).

If H is unknown, then c , ϕ and H can in principle be determined, but concentration ratios must in general be known to very high accuracy ($\sim 0.1\%$) unless $\rho_2 \approx 1/\phi$, when somewhat lower accuracy will suffice.

If H is known or can be assumed, then the determination of ϕ and c from densimetry and concentration ratios is a practical possibility. In the special case that ϕ and c are required for a protein in a solvent (e.g. 2–3 M-CsCl) for which $\rho \approx 1/\phi$, then the method should give very good results. In small-angle neutron-scattering (s.a.n.s.) studies, accurate values of c and ϕ are often required (Jacrot, 1976). Solutions (of the necessary concentration) and diffusates in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$ are routinely prepared for s.a.n.s., so the method provides a useful check on concentration. It could be applied to samples with uncertain specific u.v.-absorption coefficient, such as mixed protein systems (e.g. casein micelles) or proteins with a

Table 1. Test of the densimetry method for β -casein and α_{s1} -casein

$A_{1\text{cm}}^{1\%}$ is the absorbance of a 10 mg/ml solution in a 1 cm-pathlength cell at 280 nm. For the 'W' data, the sample and the diluent were weighed into the spectrophotometer cell; for 'P' data they were pipetted. References: *McMeekin *et al.* (1949); **Thompson & Pepper (1964); ***Thompson & Kiddy (1964).

Casein	Data type	H assumed (eqn. 7)									
		Literature values		$H = 0.0131$				$H = 0.0100$			
		$\phi \uparrow$ (ml/g)	$A_{1\text{cm}}^{1\%}$	$\phi \uparrow$ (ml/g)	$A_{1\text{cm}}^{1\%}$	$\phi \uparrow$ (ml/g)	$A_{1\text{cm}}^{1\%}$	$\phi \uparrow$ (ml/g)	$A_{1\text{cm}}^{1\%}$	$\phi \uparrow$ (ml/g)	$A_{1\text{cm}}^{1\%}$
β -Casein	W	0.739*	4.6**	0.750	4.8	0.743	4.9	0.784	4.1	0.0286	
	P			0.749	4.8	0.743	4.9	0.798	3.8	0.0352	
α_{s1} -Casein	W	0.726*	10.0***	0.730	8.4	0.723	8.6	0.639			
	P			0.712	9.1	0.705	9.4	0.324			

† Adjusted to 20°C.

very small amount of nucleic acid contamination (Jacrot & Zaccai, 1981), systems in which the concentration of non-diffusible phase cannot be found accurately by drying (McKenzie & Murphy, 1970) because of lability etc.

The densimetry method gives the true concentration, c , and apparent partial specific volume, ϕ , of the non-diffusible species. It is not affected by the presence of diffusible species (salts, hydration water, peptides etc.) present in the 'dry' protein or in solution, provided that the final concentration of diffusible solute in the solution is low (Casassa & Eisenberg, 1964). If the concentration of diffusible solute is high and the non-diffusible species binds it preferentially, then the c and ϕ values obtained will be those of the complex of the two species. If the non-diffusible phase is a mixture of species (e.g. minerals and different caseins within a casein-micelle system), then the total dry concentration and a weight-average value of ϕ are obtained.

Relatively concentrated (≥ 7 mg/ml) solutions are required for the combined determination of c and ϕ . However, after the concentration proportionality constant m (eqn. 3) has been determined, ϕ can be determined with more dilute solutions by conventional densimetry (Kratky *et al.*, 1973).

The minimum volume of $^2\text{H}_2\text{O}$ required in total is less than 50 ml. Solutions and diffusates in $^2\text{H}_2\text{O}$ can contain up to approx. 10% $^1\text{H}_2\text{O}$ and so diffusates from previous dialysis may be used as solvents to reduce cost. The discussion has been confined to proteins in aqueous solution, but the principle might be applied to other systems of membrane, non-diffusible solute and solvent, whose density can be considerably changed by

isotopic substitution. The concentration ratios for a colloid could be obtained from turbidity measurements, provided that Beer's Law holds and that the colloid size distribution is the same in $^1\text{H}_2\text{O}$ and $^2\text{H}_2\text{O}$. This would extend the densimetry method to colloidal suspensions with no u.v. absorbance.

This work was supported by the Agriculture and Food Research Council of Great Britain.

References

- Andrews, A. T. & Alichanidis, E. (1983) *J. Dairy Res.* **50**, 275–290
- Casassa, E. F. & Eisenberg, H. (1964) *Adv. Protein Chem.* **19**, 287–395
- Covington, A. K., Paabo, M., Robinson, R. A. & Bates, R. G. (1968) *Anal. Chem.* **40**, 700–706
- Edelstein, S. J. & Schachman, H. K. (1967) *J. Biol. Chem.* **242**, 306–311
- Edelstein, S. J. & Schachman, H. K. (1973) *Methods Enzymol.* **27D**, 82–98
- Jacrot, B. (1976) *Rep. Prog. Phys.* **39**, 911–953
- Jacrot, B. & Zaccai, G. (1981) *Biopolymers* **20**, 2413–2426
- Kratky, O., Leopold, H. & Stabinger, H. (1973) *Methods Enzymol.* **27D**, 98–110
- McKenzie, H. A. & Murphy, W. H. (1970) in *Milk Proteins: Chemistry and Molecular Biology*, vol. 1 (McKenzie, H. A., ed.), p. 138, Academic Press, New York
- McMeekin, T. L., Groves, M. L. & Hipp, N. J. (1949) *J. Am. Chem. Soc.* **71**, 3298–3300
- Thompson, M. P. & Kiddy, C. A. (1964) *J. Dairy Sci.* **47**, 626–632
- Thompson, M. P. & Pepper, L. (1964) *J. Dairy Sci.* **47**, 633–637
- Whitney, R. McL., Brunner, J. R., Ebner, K. E., Farrell, H. M., Jr., Josephson, R. V., Morr, C. V. & Swaisgood, H. E. (1976) *J. Dairy Sci.* **59**, 795–815