The Significance of the "Partial Specific Volume" Obtained from Sedimentation Data*

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ABSTRACT: A parameter designated the *effective* or *hydrodynamic* partial specific volume, and defined by the equation $\bar{V}_h = 1/\rho^0$ (where ρ^0 is the solvent density at which sedimentation no longer occurs), can be experimentally determined by ultracentrifugation. This parameter, $\bar{V}_h$, has been determined, in various solvent systems, for ovalbumin, conalbumin, lysozyme, β-lactoglobulin, bovine serum albumin, edestin, human hemoglobin, and human fibrinogen.

A comparison of such data with the partial specific volumes, $\bar{V}$, determined by conventional density measurements, indicates that $\bar{V}_h$ is numerically equal to $\bar{V}$ *only* in solvent systems where selective solvation effects are nonexistent.

The ultracentrifugal determination of the molecular weight of a macromolecule requires, among other things, a knowledge of both the partial specific volume of the macromolecule and the density of the medium. The classical technique for determining partial specific volume based upon the pycnometric measurement of solution densities at various solute concentrations requires large volumes of solution and is quite time consuming. The magnetic float technique described by MacInnes *et al.* (1951) and variously modified by others is inherently more rapid and accurate than pycnometric methods but, nevertheless, still requires a relatively large volume of solution. Recently, Beams and Clarke (1962) have described two somewhat elaborate techniques utilizing the magnetic float principle which require very small volumes of solution.

Svedberg and Pedersen (1940) have drawn attention to the hypothesis, supported by the early work of McBain (1936) and Adair and Adair (1936), that the partial specific volume of a hydrated macromolecule can be estimated by adjusting the specific volume of the solution until sedimentation no longer occurs. It is assumed that under these conditions the partial specific volume of the sedimenting complex is equal to the specific volume of the solution. This technique has been utilized in studies on the hydration of virus characteristic particles (Lauffer and Bendet, 1954) and proteins (Martin *et al.*, 1956; Cox and Schumaker, 1961a,b); indeed, it has been used for many years to determine the "density" of such particles. Schachman and Lauffer (1950) have noted the ambiguities in interpretation which can arise in such studies and have treated, as have others (Katz and Schachman, 1955; Katz, 1956; and Schumaker and Cox, 1961), the theoretical aspects of such measurements.

Ambiguities of this nature have been noted in our laboratory. Indeed, in a recent publication from this laboratory on the urea denaturation of ovalbumin (Gagen and Holme, 1964), a substantially different (and inconsistent) interpretation of the sedimentation data would have resulted had not the effects of selective solvation been considered. These limited observations concerning selective solvation effects quite naturally led to the investigations reported in this communication, where it is hoped that some of the ambiguities encountered in such studies will be clarified. In addition it seems desirable to emphasize the dangers inherent in the indiscriminant use of ultracentrifugal data for the determination of "partial specific volumes."

Experimental Section

Proteins. The proteins used were ovalbumin (Worthington Biochemical Corp., Freehold, N. J.), bovine serum albumin (Armour and Co., Chicago, Ill.), β-lactoglobulin, conalbumin, lysozyme, edestin, human hemoglobin, and human fibrinogen (all from Nutritional Biochemical Corp., Cleveland, Ohio).

Reagents. The deuterium oxide, 99.7% minimum purity, was purchased from General Dynamics Corp., San Carlos, Calif. The salts (sodium chloride, urea, ammonium nitrate, etc.) were all reagent grade and used without further purification. In those instances where deuterium oxide was used as a solvent, reagents having exchangeable hydrogens were deuterated prior to use.

Density Measurements. Solution densities were determined using a scaled-down modification of the magnetic density balance apparatus of L. Benjamin (1965, in preparation). The dimensions of the reservoir and float are such that densities can be determined using approximately 10–15 ml of solution; small projections on the floats minimize the effects of sidewall adhesion. Floats were calibrated using solutions of known density. Several comparisons with pycno-

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TABLE I: Comparison of Density Determinations by Magnetic Float and Pycnometric Methods.

	ρ^{30} (g/ml)	
Soln	Magnetic Float	Pycnometry
NaCl(0.1 M)	0.99986	0.9998
NaCl(0.2 M)	1.00395	1.0038
Acetate buffer (μ = 0.1, pH 3.8)	1.00340	1.0035
Phosphate buffer (μ = 0.1, pH 7.0)	1.00256	1.0024
Phosphate buffer (μ = 0.2, pH 7.0)	1.00902	1.0088
Veronal buffer (μ = 0.1, pH 8.5)	1.00410	1.0040

metrically determined densities, indicating the accuracy of the apparatus, are shown in Table I.

Partial Specific Volume Measurements. Partial specific volumes were determined, using the magnetic float technique for determining solution densities, ρ, from plots of ϕ (the apparent partial specific volume) *vs.* concentration, φ being defined (Casassa and Eisenberg, 1961) as

$$\phi = \frac{1}{\rho_s}\left[1 - \frac{\Delta\rho}{c}\right] \quad (1)$$

where ρ_s is the density of the solvent, $\Delta\rho$ the increment in density (*i.e.*, $\rho - \rho_s$), and c the solute concentration in grams per milliliter. Solutions were prepared in the usual manner, by direct dissolution of lyophilized protein in appropriate buffer without subsequent dialysis. Protein concentrations were determined by spectrophotometric measurements and/or semimicro-Kjeldahl nitrogen analyses.

The ultracentrifugal technique for determining the *effective* or *hydrodynamic* partial specific volume (Gagen and Holme, 1964), $\bar{V}_h$, utilized the sedimentation of the protein in H_2O solution and D_2O solution as described by Martin *et al.* (1956), to yield

$$\bar{V}_h = \left[\frac{\eta_2}{\eta_1} - k\frac{s_1}{s_2}\right]\left[\rho_1\frac{\eta_2}{\eta_1} - \rho_2\frac{s_1}{s_2}\right]^{-1} \quad (2)$$

where η_1, ρ_1, and s_1 are the viscosity, density, and sedimentation coefficient, respectively, in H_2O solution, and η_2, ρ_2, and s_2 are the comparable values in D_2O solution. The value for k, the ratio of molecular weights in the two media, was calculated from the amino acid composition assuming complete deuteration of exchangeable hydrogens.

An alternative technique employed was the determination of $\bar{V}_h = 1/\rho^0$ (ρ^0 = the solvent density at $\eta s = 0$) from linear plots of $\eta s/k'$ *vs.* ρ/k' for the protein in solvents containing deuterium oxide as the density-increasing additive. (Here k' is the ratio of molecular weights, calculated as above, assuming that the degree of deuteration is proportional to the mole fraction of D_2O.) For several of the proteins a similar parameter, designated $\bar{V}_h'$, has been calculated from ηs *vs.* ρ plots for solutions in which sucrose or urea was used as the density-increasing component.

Ultracentrifugation. A Spinco Model E ultracentrifuge equipped with temperature control and a phase-plate schlieren optical system was employed for the sedimentation analyses. Most of the sedimentation studies were made at protein concentrations of 0.25% or less using a synthetic boundary cell of the capillary-fill type. Sedimentation coefficients were determined in the usual manner (Schachman, 1959).

Results

Comparison of the Ultracentrifugal Technique with Conventional Techniques for Determining Partial Specific Volumes. The partial specific volumes of several proteins in various solvent media have been determined by both the magnetic float density technique and the ultracentrifugal technique described by Martin *et al.* (1956). A comparison of these results with the values reported by other workers is shown in Table II.

In agreement with the conclusion of Martin *et al.* (1956), the results shown in Table II indicate that the sedimentation method can yield data comparable to those obtained by pycnometric and magnetic float density measurements. This, indeed, has been assumed by numerous workers who have made use of this technique.

Effect of Solvent Media upon the "Partial Specific Volume" Determined by Ultracentrifugation. The results shown in Table II, particularly those for edestin in 1.0 M NaCl and in 6 M urea–0.1 M NaCl, seemingly indicate that the ultracentrifugally determined *effective* partial specific volume, $\bar{V}_h$, is not influenced by the composition of the solvent media. However, data for human fibrinogen, conalbumin, and human hemoglobin, together with previously reported data on ovalbumin (Gagen and Holme, 1964), given in Table III, show that this is not the case. Hence, one cannot indiscriminantly employ the ultracentrifugal technique to determine the thermodynamically defined partial specific volume, $\bar{V}$, since in some solvent systems $\bar{V}_h \neq \bar{V}$.

Values for $\bar{V}_h'$ were obtained on five of the proteins in this study (ovalbumin, conalbumin, bovine serum albumin, human hemoglobin, and lysozyme) using sucrose as the density-increasing component. For each of the five proteins the $\bar{V}_h'$ value was greater than the corresponding partial specific volume, $\bar{V}$; the increases ranging from 0.04–0.05 ml/g for ovalbumin and bovine serum albumin to 0.07–0.08 ml/g for conalbumin, human hemoglobin, and lysozyme. These studies, conducted in aqueous 5–30% sucrose–Veronal buffer (ionic strength = 0.1), indicated a linear relationship between ηs and solvent density in all instances, as shown in Figures 1 and 2. In similar studies Hill and

TABLE II: Comparison of the Ultracentrifugal Technique with Conventional Techniques for Determining Partial Specific Volumes.

Protein	Solvent Medium	$\bar{V}_h$ (ml/g)	$\bar{V}$ (ml/g)
β-Lactoglobulin	0.1 M NaCl, pH 7.0	0.749	0.751[a] 0.751[b]
Bovine serum albumin	Water, pH 3.0	0.728	0.734[a]
	Water, pH 7.0	0.724	0.734[c]
Lysozyme	0.1 M NaCl, pH 7.0	0.689	0.688[d]
Ovalbumin	Water, pH 7.0	0.750	0.752[e] 0.748[c]
	Phosphate buffer, pH 7.0	0.747	0.747[a] 0.749[b]
	Veronal buffer, pH 8.5	0.746	0.747[a]
Edestin	1.0 M NaCl, pH 7.0	0.730	0.727[e]
	6 M urea + 0.1 M NaCl	0.730	0.734[a]
Human fibrinogen	0.1 M NaCl, pH 7.0	0.729	0.725[f]

[a] This work. [b] Svedberg and Pedersen (1940). [c] Dayhoff *et al.* (1952). [d] Colvin (1952). [e] Charlwood (1957) (measured in 0.75 M NaCl + 0.25 μ phosphate). [f] Armstrong *et al.* (1947).

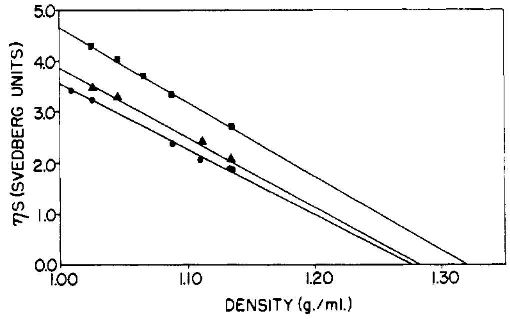

FIGURE 1: Plots of ηs (the viscosity-corrected sedimentation coefficient) *vs.* ρ (the solvent density) for ovalbumin (-●-●-), human hemoglobin (-▲-▲-), and bovine serum albumin (-■-■-) in sucrose–Veronal buffer solutions.

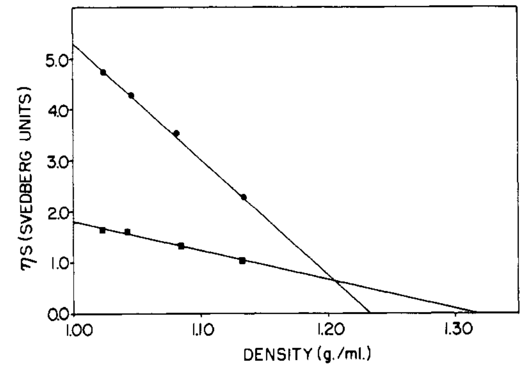

FIGURE 2: Plots of ηs *vs.* ρ for conalbumin (-●-●-) and lysozyme (-■-■-) in sucrose–Veronal buffer solutions.

TABLE III: Additional Comparisons of the Ultracentrifugal Technique with Conventional Techniques for Determining Partial Specific Volumes.

Protein	Solvent Medium	$\bar{V}_h$ (ml/g)	$\bar{V}$ (ml/g)
Ovalbumin	6 M urea, pH 7.0	0.796	0.730
Conalbumin	0.1 M NaCl, pH 7.0	0.693	0.736
	6 M urea + 0.1 M NaCl, pH 7.0	0.685	0.731
Human hemoglobin	0.1 M NaCl, pH 7.0	0.694	0.752
Human fibrinogen	6 M urea + 0.1 M NaCl, pH 7.0	0.639	0.725[a]

[a] Armstrong *et al.* (1947); all other values, this work.

Cox (1965) have reported curvature in the ηs *vs.* ρ plots for ovalbumin in *sucrose–phosphate buffer* solutions.

Similar studies with ovalbumin in which urea was used as the density-increasing component yielded a value for $\bar{V}_h'$ (0.80 ± 0.01) which was not significantly different than the $\bar{V}_h'$ obtained using sucrose. Interestingly, if one determines $\bar{V}_h$ at a given urea concentration between 3 and 6 M, employing deuterium oxide as the density-increasing component, one obtains similar results (Gagen and Holme, 1964).

Some additional $\bar{V}_h$ data concerning the effects of various solvents upon the protein ovalbumin are shown in Table IV. One can reasonably assume that the partial specific volume, $\bar{V}$, of ovalbumin in each of these solvents will be in the range 0.73–0.75, the values found, respectively, in 6 M urea and in water (Gagen and Holme, 1964).

TABLE IV: The *Effective* or Hydrodynamic Partial Specific Volume, $\bar{V}_h$, of Ovalbumin in Various Solvent Media.[a]

Solvent Medium (M)	$\bar{V}_h$ (ml/g)	Solvent Medium (M)	$\bar{V}_h$ (ml/g)
Water	0.750	NH_4F (0.01)	0.724
		NH_4F (0.02)	0.722
NaCl (0.01)	0.754	NH_4F (~2.0) (7% soln)	0.736
NaCl (0.05)	0.736		
NaCl (0.10)	0.745	Urea (3)	0.817
NaCl (0.20)	0.747	Urea (4)	0.804
		Urea (6)	0.796
NH_4NO_3 (~1.25) (10% soln)	0.700	0.5% sodium dodecyl sulfate	0.824

[a] Note (for comparative purposes): $\bar{V}$ (determined pycnometrically) = 0.748–0.752 ml/g in water. $\bar{V}$ (determined pycnometrically) = 0.730 ml/g in 6 M urea.

Effect of Temperature upon the Effective Partial Specific Volume. The effect of temperature, over the range 10–35°, upon the effective partial specific volume, $\bar{V}_h$, of ovalbumin at pH 8.5 has been examined. Although the observed sedimentation coefficients in both H_2O and D_2O were temperature dependent, the effect was associated simply with the concomitant changes in solvent densities and viscosities. Hence, the values for $\bar{V}_h$ determined at various temperatures within this range were not significantly different. Values for $\bar{V}_h'$ determined using sucrose as the density-promoting component were likewise unaltered by temperature over this limited range. Further studies in the region where ovalbumin loses its heat stability (*i.e.*, 65–75°) are in progress.

Effect of Centrifugal Field Strength upon the Effective Partial Specific Volume. The effective partial specific volumes of ovalbumin in water and in 0.1 M NaCl were determined, by the ultracentrifugal method, as a function of the angular velocity. The values obtained at angular velocities of 2.277, 4.402, and 6.260 × 10^3 radians/sec were 0.745, 0.751, and 0.747, respectively. As anticipated, the angular velocity does not influence the value $\bar{V}_h$ obtained for an essentially globular protein, ovalbumin, although this would not preclude a possible effect in systems exhibiting shear-dependent sedimentation behavior.

Discussion

The partial molar volume of a solute in a two-component system, $\bar{V}_2$, is a thermodynamically defined parameter

$$\bar{V}_2 = \left[\frac{\partial V}{\partial n_2}\right]_{n_1,T,P} \tag{3}$$

where V is the volume of the system, T the temperature, P the pressure, and n_1 and n_2 the number of moles of components 1 and 2, respectively. Obviously, as Scheraga (1961) has pointed out, this parameter cannot be associated with the actual volume occupied by the solute; indeed, it need not be positive since negative or zero values are not theoretically precluded and, in fact, have been found (*e.g.*, magnesium sulfate in H_2O; Lewis and Randall, 1923). The experimental determination of partial molar volumes (or, more generally, partial specific volumes) by conventional techniques or by the ultracentrifugal technique utilizes somewhat different approaches which warrant investigation.

The conventional technique for determining $\bar{V}_2$ uses the relationship

$$V = n_2\phi + n_1v_1^0 \tag{4}$$

where V is the total volume of the system, n_1 and n_2 are the number of moles of solvent and solute, respectively, v_1^0 is the molar volume of the solvent, and ϕ is the *apparent* molar volume of the solute. When differentiated with respect to n_2 (at constant n_1, T, and P), eq 4 yields

$$\left[\frac{\partial V}{\partial n_2}\right]_{n_1,T,P} = \phi + n_2\frac{\partial \phi}{\partial n_2} \tag{5}$$

which, of course, defines $\bar{V}_2$. From measurements of solution densities at various solute concentrations one obtains both terms on the right-hand side of eq 5 and thus determines $\bar{V}_2$ (see Experimental Section, eq 1).

The general equation describing the sedimentation behavior of a macromolecule can be written (Schachman, 1959)

$$f\eta s_2 = M_2(1 - \bar{V}_2\rho) + \alpha M_1(1 - \bar{V}_1\rho) \tag{6}$$

where f is the frictional coefficient, η the solvent viscosity, ρ the solvent density, α the preferential absorption coefficient for component 1, M the molecular weight, and $\bar{V}$ the partial specific volume (subscript 2 denoting the macromolecule and subscript 1 the component present in greatest amount, usually water). This equation is applicable to macromolecules in either simple solvents (those not exhibiting preferential solvation effects, $\alpha = 0$) or complex solvents (those exhibiting preferential solvation effects, $\alpha \neq 0$). Obviously, if the term $f\eta s_2$ can be reduced to zero, *i.e.*, if sedimentation no longer occurs,

$$\frac{1}{\rho^0} = \frac{M_2\bar{V}_2 + \alpha M_1\bar{V}_1}{M_2 + \alpha M_1} \tag{7}$$

where ρ^0 is the solvent density at which $f\eta s_2 = 0$. Although this parameter is dimensionally similar to a partial specific volume, it is hydrodynamic in nature; therefore, the term *effective* (or *hydrodynamic*) *partial specific volume*, $\bar{V}_h$, defined as

$$\bar{V}_h = \frac{1}{\rho^0} \qquad (8)$$

has been introduced (Gagen and Holme, 1964). Any of the variants of the ultracentrifugal technique, *if performed in such a manner that M, $\bar{V}$, f, and α remain constant*, would be expected to yield the parameter, $\bar{V}_h = 1/\rho^0$, in any solvent system. However, examination of eq 7 shows that only in simple solvents ($\alpha = 0$) would this value, $\bar{V}_h$, be equivalent to $\bar{V}_2$; in complex solvents ($\alpha \neq 0$), this equivalence would not be observed, *i.e.*, in such solvents $\bar{V}_h \neq \bar{V}_2$.

In the present study it has been shown that when deuterium oxide is used as the density-increasing additive the ultracentrifugal technique does indeed provide acceptable values for the partial specific volumes of macromolecules in simple solvents. However, the data emphasize the inapplicability of this technique for such measurements in complex solvents. In addition, these data show that it cannot be *a priori* established whether a solvent be considered as simple or complex (*e.g.*, 6 M urea + 0.1 M NaCl acts as a simple solvent for edestin, while 0.1 M NaCl acts as a complex solvent for conalbumin). One cannot, therefore, indiscriminantly employ the ultracentrifugal technique to determine the thermodynamically defined partial specific volume, $\bar{V}$.

In complex solvents the significance of the sign and magnitude of the numerical difference between $\bar{V}_h$ and the thermodynamically defined $\bar{V}$ has not been fully explored. If $\bar{V}_h$ does indeed reflect solute–solvent interactions which do not influence $\bar{V}$, it would seem that the difference between the two parameters could be correlated with such phenomena as extent of solvation and/or exposure of hydrophobic regions of the macromolecule.

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