Thermodynamic Analysis of the Effects of Small Inert Cosolutes in the Ultracentrifugation of Noninteracting Proteins†

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ABSTRACT: Considerations of the effect of a small cosolute on the sedimentation equilibrium distribution for a noninteracting protein have led to the development of a simple procedure for evaluating both the molecular weight of the protein and the second virial coefficient describing the excluded volume interaction between protein and cosolute. Its application is illustrated by analysis of sedimentation equilibrium distributions for bovine thyroglobulin and horse liver alcohol dehydrogenase in the presence of a range of sucrose concentrations, and also of those for aldolase in the presence of urea to obtain the subunit molecular weight of this tetrameric enzyme. The effects of sucrose concentration on the sedimentation coefficients of thyroglobulin, catalase, and horse liver alcohol dehydrogenase are also examined to demonstrate that the second virial coefficients for protein−cosolute excluded volume interaction may be determined, albeit with less precision, from the cosolute concentration required to render the sedimentation coefficient zero by virtue of its effect on the buoyancy term. These findings serve to reinforce the fact that the effects of small cosolutes usually ascribed to changes in "protein solvation" are envisaged more realistically in terms of excluded volume.

Interest in the effects of added inert solutes on the centrifugal behavior of macromolecules stems from early attempts to evaluate the partial specific volume of the macromolecule as the reciprocal of the solvent density required to decrease the sedimentation coefficient to zero (Sharp et al., 1944; Schachman & Lauffer, 1949; Katz & Schachman, 1955; Martin et al., 1959). The fact that the magnitude of this density depended upon the inert solute chosen to effect the increase in solvent density has clearly precluded its use as a measure of the partial specific volume. In keeping with the concept of a protein as a solvated hydrodynamic particle (Oncley, 1941), the range of such densities for a given macromolecular solute was attributed to different extents of solvation (hydration) of the macromolecule in the presence of the various inert cosolutes (Sharp et al., 1944; Schachman & Lauffer, 1949; Katz & Schachman, 1955).

On the grounds that the extent of such preferential solvation increased with increasing size of the inert solute, the phenomenon was attributed tentatively but correctly by Kauzman to an excluded volume effect [see footnote cited in Schachman and Lauffer (1949)]. In other words, because the distance of closest approach between the centers of protein and cosolute molecules (both assumed spherical for simplicity) is the sum of their radii, the protein molecule is surrounded by a volume shell that is necessarily occupied by solvent due to the exclusion of cosolute molecules from this region. Consideration of this solvent shell as part of the protein gives rise to the concept of differences in the extent of preferential protein solvation by inert cosolutes. However, the extent of this preferential solvation reflects mainly the difference in sizes of the solvent and cosolute molecules, rather than any cosolute-dependent change in the dimensions of an actual solvated protein molecule. Nevertheless, preferential solvation has remained the favored description of the effect, an unfortunate consequence of which is its possible misinterpretation as a change in the dimensions of the solvated protein in the presence of cosolute.

The formal equivalence of the protein solvation and excluded volume treatments has recently been established in relation to the stabilization of protein structure by inert solutes (Wills & Winzor, 1993; Winzor & Wills, 1995). Such stabilization of protein structure by high concentrations of small nonelectrolytes had previously been interpreted in terms of preferential occupancy of the protein domain by solvent at the expense of small solute (Cohen & Eisenberg, 1968; Reisler & Eisenberg, 1969). However, the partial specific volume measurements (Lee & Timasheff, 1981; Timasheff, 1995) that had been used to define a thermodynamic parameter for interaction of small cosolute (M) with protein (A), ξ_M, could also be interpreted in terms of molecular space-filling (Winzor & Wills, 1986; Wills et al., 1993). Indeed, by deriving the relationship between ξ_M and the excluded volume for protein and cosolute (B_{AM}), it was shown unequivocally that the statistical mechanical (excluded volume) and preferential solvation treatments merely afford alternative ways of accounting for the magnitude of the second virial coefficient that describes thermodynamic nonideality of the protein arising from the presence of small

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cosolute (Wills & Winzor, 1993; Winzor & Wills, 1995). For the reasons outlined above, the statistical mechanical analysis leaves less room for misinterpretation of the quantitative statement of that thermodynamic nonideality.

In the present investigation, we illustrate the manner in which the excluded volume treatment can allow quantitative interpretation of the effects of inert solutes on the sedimentation behavior of macromolecules. For macromolecules which do not self-interact, it is shown that sedimentation equilibrium distributions obtained for the macromolecular component in the presence of a small (nonsedimenting) inert solute can be used to evaluate the second virial coefficient describing the excluded volume interaction between macromolecule and small solute; and hence to provide information analogous to that derived from densitometric measurements of partial specific volumes (Cohen & Eisenberg, 1968; Reisler & Eisenberg, 1969; Lee & Timasheff, 1981; Timasheff, 1995). In addition, the same logic is used to determine approximate values of the excluded volume parameter from the apparent partial specific volume deduced from sedimentation velocity studies of the macromolecule in the presence of different concentrations of an inert solute.

THEORY

Consider a situation in which a macromolecular solute, A, which does not self-associate or isomerize, is dialyzed exhaustively against buffer supplemented with a small inert solute, M, before being subjected to ultracentrifugation at angular velocity ω and temperature T. At sedimentation equilibrium, the distributions of the two solutes, A and M, are given by the expression (i = A or M):

$$z_i(r) = z_i(r_F) \exp[M_i(1 - \bar{v}_A\rho_s)\omega^2(r^2 - r_F^2)/2RT] \quad (1)$$

which relates the molar thermodynamic activities, defined at constant chemical potential of solvent, of the two solutes at radial distance r, $z_i(r)$, to their values at some fixed radial distance, $z_i(r_F)$. M_i and $\bar{v}_i$ are the molecular weight and partial specific volume, respectively, of solute i, and R is the universal gas constant. That ρ_s is the density of pure unsupplemented solvent was first shown for a two-component system (Wills et al., 1993), and subsequently for multicomponent systems (Wills et al., 1996). Provided that the magnitude of $M_M(1 - \bar{v}_M\rho_s)$ is sufficiently small for only minor redistribution of M to occur at sedimentation equilibrium, it can be helpful to regard the small solute as part of the solvent. In this situation, the distribution of a dilute macromolecular solute can be described by the expressions [see, *e.g.*, eq 5.27 of Eisenberg (1976)]

$$C_A(r) \approx C_A(r_F) \exp[M_A(1 - \phi_A'\rho_d)\omega^2(r^2 - r_F^2)/2RT] \quad (2)$$

$$\rho_d = \rho_s + (1 - \bar{v}_M\rho_s)M_M C_M \quad (3)$$

where C_A denotes molar concentration and ρ_d is the density of the diffusate with which the macromolecular solute has been brought to dialysis equilibrium prior to centrifugation (Casassa & Eisenberg, 1964). Comparison of eqs 1 and 2 shows the thermodynamic activity of A to contain a buoyancy factor described in terms of the density of unsupplemented solvent (ρ_s) and the partial specific volume of macromolecular solute ($\bar{v}_A$), whereas the corresponding factor in the expression for concentration contains the density of the supplemented diffusate (ρ_d) and the apparent partial specific volume (ϕ_A') defined appropriately under conditions of constant chemical potentials of solvent (μ_s) and small solute (μ_M) (Cohen & Eisenberg, 1968; Reisler & Eisenberg, 1969; Wills et al., 1993). We now demonstrate how consideration of thermodynamic nonideality leads to the derivation of eq 2 from eq 1.

The thermodynamic activity of macromolecular solute, $z_A(r)$, can be expressed as a function of molar concentration, $C_A(r)$, and the molar activity coefficient, $\gamma_A(r)$ (Wills et al., 1993; Wills & Winzor, 1993):

$$z_A(r) = \gamma_A(r)C_A(r) \quad (4a)$$

$$\ln \gamma_A(r) = 2B_{AA}C_A(r) + B_{AM}C_M(r) + ... \quad (4b)$$

where B_{AA} and B_{AM} are the respective second virial coefficients reflecting nonideality due to self-interaction of A and its excluded volume interaction with small solute: this expression is correct to first order in solute concentrations [$C_i(r)$]. On the basis that the experiments are conducted under conditions of low concentration of macromolecular solute ($B_{AM}C_M \gg 2B_{AA}C_A$), eq 4b simplifies to

$$\ln \gamma_A(r) \approx B_{AM}C_M(r) \quad (5)$$

whereupon substitution of eq 5 into eq 1 (written for A) gives

$$C_A(r) \approx C_A(r_F) \exp\{M_A(1 - \bar{v}_A\rho_s)\omega^2(r^2 - r_F^2)/2RT + B_{AM}[C_M(r_F) - C_M(r)]\} \quad (6)$$

In order to obtain results consistent to first order in C_M (as in eq 5), the dependence of $C_M(r)$ upon radial distance may be written as

$$C_M(r) \approx C_M(r_F) \exp\{[M_M(1 - \bar{v}_M\rho_s)\omega^2(r^2 - r_F^2)/2RT] + ...\} \quad (7a)$$

or, on expression of the exponential term as a truncated power series:

$$C_M(r) = C_M(r_F)[1 + M_M(1 - \bar{v}_M\rho_s)\omega^2(r^2 - r_F^2/2RT + ...] \quad (7b)$$

Substitution of eq 7b into eq 6 then gives

$$C_A(r) \approx C_A(r_F) \exp[M_A(1 - \bar{v}_A\rho_s)\omega^2(r^2 - r_F^2)/2RT] \times \exp[-B_{AM}C_M(r_F)M_M(1 - \bar{v}_M\rho_s)\omega^2(r^2 - r_F^2)/2RT] \quad (8a)$$

$$\approx C_A(r_F) \exp\{[M_A(1 - v_A\rho_s) - B_{AM}C_M(r_F)M_M(1 - \bar{v}_M\rho_s)]\omega^2(r^2 - r_F^2)/2RT + ...\} \quad (8b)$$

This expression is formally identical to eq 2 with the buoyant molecular weight, $M_A(1 - \phi_A'\rho_d)$, defined correct to first order in C_M as

$$M_A(1 - \phi_A'\rho_d) = M_A(1 - \bar{v}_A\rho_s) - (1 - \bar{v}_M\rho_s)B_{AM}M_M C_M(r_F) + ... \quad (9)$$

in which the first two terms are the buoyant molecular weights, $(d \ln A_{280}/dr^2)(2RT/\omega^2)$, that emanate directly from

the sedimentation equilibrium distributions of limitingly dilute macromolecular solute in the presence and absence, respectively, of inert solute: in view of the relatively minor redistribution of small solute, $C_M(r_F)$ may be approximated by C_M, the concentration of small solute in the diffusate. B_{AM} may thus be determined as the sole parameter of unknown magnitude in eq 9.

It is noted that eq 9, divided throughout by M_A, has been derived previously [eq 17 of Wills et al. (1993)] in the context of interpreting densitometric studies of thermodynamic nonideality arising from the presence of a dialyzable inert solute. We have now shown that this expression may also be used to interpret the linear variation of the buoyant molecular weight of the macromolecule with the molar concentration of small solute included in the diffusate.

A similar approach may be adopted, in principle, to determine B_{AM} from the effect of small inert solutes upon the sedimentation coefficient of the macromolecular species. Such results are plotted (Schachman & Lauffer, 1949; Katz & Schachman, 1955; Cheng & Schachman, 1955) as a dependence of the product of the measured sedimentation coefficient and the relative viscosity of the supplemented buffer ($s_A\eta_{rel}$) upon solvent density (ρ_d). Extrapolation of the plot to the abscissa ($s_A\eta_{rel} = 0$) yields the density at which the sedimentation coefficient is zero, $(\rho_d)_{s_A=0}$. Because (i) the buoyant molecular weight is zero under these conditions and (ii) the absence of any net sedimentational or diffusional flow allows the system to be treated in thermodynamic equilibrium rather than hydrodynamic terms, $(\phi_A')_{s_A=0}$ may be equated with $1/(\rho_d)_{s_A=0}$. Furthermore, because aqueous solutions are essentially incompressible, the concentration of inert solute required to effect that solvent density, $(C_M)_{s_A=0}$, may be obtained from eq 3.

Substituting these magnitudes of $(\phi_A')_{s_A=0}$ and $(C_M)_{s_A=0}$ into eq 9 should thus provide an estimate of B_{AM}. Even though such an estimate is subject to considerable uncertainty because of the extrapolation to obtain $(\rho_d)_{s_A=0}$ and the consequent values of $(\phi_A')_{s_A=0}$ and $(C_M)_{s_A=0}$, such an endeavor serves to stress that the solvation effects inferred from sedimentation velocity studies of macromolecules in the presence of small solutes are also amenable to interpretation in terms of thermodynamic nonideality emanating from molecular crowding by the inert solute.

EXPERIMENTAL PROCEDURES

General Procedural Details. Bovine thyroglobulin, horse liver alcohol dehydrogenase, bovine liver catalase, and rabbit muscle aldolase were crystalline preparations obtained from Sigma Chemical Co., St. Louis, MO. These protein preparations were dissolved in the appropriate buffer (supplemented with small cosolute, where appropriate), after which aliquots (50−100 μL) were subjected to zonal chromatography at 0.2 mL/min on a 600 × 7.8 mm Biosep-SEC-S3000 column (Phenomenex, Torrance, CA), preequilibrated with the same buffer. This exclusion chromatography step served not only to remove any contaminating material with markedly different size characteristics but also to provide a purified solution in dialysis equilibrium with the buffer to be used in the ultracentrifuge studies. Protein solutions were then subjected to sedimentation at 20 °C in a Beckman XL-A analytical ultracentrifuge, and the resulting distributions were recorded spectrophotometrically at 280 nm. Buffer (diffusate) densities were determined routinely by the standard procedure in an Anton Paar density meter; and their relative viscosities were determined by conventional Ostwald viscometry.

Studies of Thyroglobulin. Solutions of thyroglobulin (0.2 − 0.3 mg/mL) in either Tris−chloride buffer (0.01 M Tris/HCl−0.1 M KCl), pH 7.5, or the same buffer supplemented with sucrose (0.1 − 0.5 M) were centrifuged at 3000 and 25 000 rpm for measurement of equilibrium distributions and sedimentation coefficients, respectively. An absorption coefficient ($A^{1\%}_{1cm}$) of 10.0 for thyroglobulin at 280 nm (Van der Walt et al., 1983) was used for converting absorbance measurements to protein concentrations.

Studies of Alcohol Dehydrogenase. Corresponding studies of horse liver alcohol dehydrogenase employed an imidazole−chloride buffer (0.04 M imidazole/hydrochloric acid−0.144 M NaCl), pH 7.5, supplemented with 0 − 0.5 M sucrose. Loading concentrations of approximately 0.4 mg/mL enzyme were used in equilibrium as well as velocity experiments, which entailed rotor speeds of 10 000 and 55 000 rpm, respectively. Concentrations were calculated on the basis of the absorption coefficient ($A^{1\%}_{1cm}$) of 4.2 at 280 nm reported by Ehrenberg and Dalziel (1958).

Studies of Aldolase. Solutions of aldolase (0.25−0.35 mg/mL) in acetate−chloride buffer (0.035 M sodium acetate/acetic acid−0.2 M NaCl), pH 5.0, and in the same buffer supplemented with urea (1−8 M), were centrifuged at 12 000−24 000 rpm to obtain equilibrium distributions for evaluating the effects of this denaturing solute on the buoyant molecular weight of the enzyme. Absorbance measurements on enzyme solutions in unsupplemented acetate buffer were converted to protein concentrations on the basis of an absorption coefficient ($A^{1\%}_{1cm}$) of 9.1 at 280 nm for aldolase (Baranowski & Niederland, 1949). Protein concentrations could not be measured precisely from corresponding absorbance measurements in the presence of urea because of changes in the absorption coefficient accompanying the dissociation and unfolding of aldolase. However, the loading concentrations (0.25−0.35 mg/mL) would have been sufficiently small for the buoyant molecular weight determined from the dependence of ln A_{280} upon the square of radial distance to reflect effectively the value extrapolated to zero protein concentration.

Studies of Catalase. Solutions of bovine liver catalase (0.5 mg/mL) in phosphate buffer (0.1 M KH_2PO_4), pH 7.0, and in the same buffer supplemented with 0.05−0.20 M sucrose, were centrifuged at 45 000 rpm to quantify the effect of the small cosolute on the sedimentation coefficient of the enzyme. An absorption coefficient ($A^{1\%}_{1cm}$) of 15.8 at 280 nm (Hiraga et al., 1964) was used for the determination of protein concentration.

RESULTS

As noted in the introduction, the aim of this investigation has been to rationalize the dependence of the buoyant molecular weight of a protein upon concentration of a small cosolute in terms of thermodynamic nonideality arising from excluded volume interactions between protein and cosolute molecules. We consider first the effects of such nonideality upon the analysis of sedimentation equilibrium distributions reflecting protein concentrations that are sufficiently small for self-interaction of the macromolecule to be negligible:

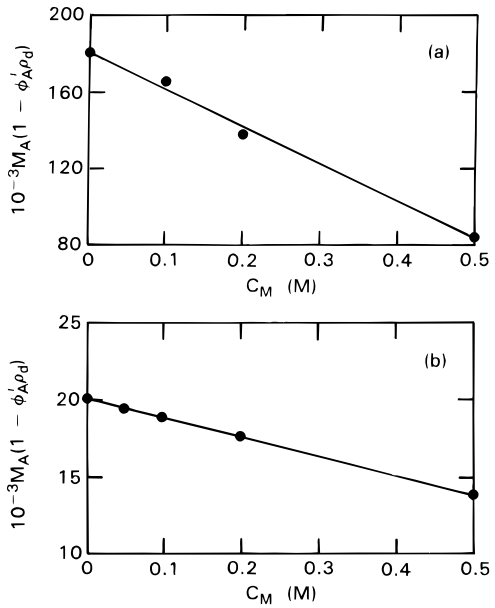

FIGURE 1: Effect of sucrose concentration (C_M) on the buoyant molecular weight of (a) bovine thyroglobulin in Tris−chloride buffer, pH 7.5, and (b) horse liver alcohol dehydrogenase in imidazole−chloride buffer, pH 7.5. The lines denote the best-fit linear relationships (see eq 9).

the buoyant molecular weight is thus regarded as the value in the limit of zero protein concentration.

Effect of Sucrose on the Sedimentation Equilibrium Behavior of Proteins. The dependences of the buoyant molecular weight, $(\mathrm{d} \ln A_{280})/\mathrm{d}r^2)(2RT/\omega^2)$, upon sucrose concentration for thyroglobulin and horse liver alcohol dehydrogenase are summarized in Figure 1a,b, respectively. Analysis of these results for thyroglobulin in terms of eq 9 yields values of 180 000 (±9000) for the buoyant molecular weight and 1500 (±300) L/mol for B_{AM}: in both instances, the uncertainty is expressed as twice the standard error. For horse liver alcohol dehydrogenase, the corresponding values are 20 200 (±110) and 97 (±4) L/mol. These findings now need to be rationalized in terms of parameters for the two protein-cosolute systems.

In the limit of zero cosolute concentration, the apparent partial specific volume (ϕ_A') becomes indistinguishable from $\bar{v}_A$, whereupon the buoyant molecular weights deduced from the ordinate intercepts of Figure 1 should yield the protein molecular weights (eq 9). Combination of the estimate of buoyant molecular weight for thyroglobulin with the reported partial specific volume of 0.723 mL/g (Derrien et al., 1949) yields a molecular weight of 660 000 (±33 000), an estimate consistent with the accepted value of 670 000 (Steiner & Edelhoch, 1961). In a similar vein, substitution of a partial specific volume of 0.750 mL/g for alcohol dehydrogenase into the expression for buoyant molecular weight yields a molecular weight of 82 000 (±450), which is again in good agreement with the reported value of 83 000 (Ehrenberg & Dalziel, 1958).

Having demonstrated consistency of the ordinate intercepts in Figure 1 with the molecular weights of the two proteins, it now remains to rationalize the slopes in terms of the excluded volume. For a small uncharged cosolute such as sucrose, the excluded volume is synonymous with the covolume, $4\pi N(R_A + R_M)^3/3$, where R_A and R_M denote the effective thermodynamic radii of protein and cosolute, respectively. For sufficiently small cosolutes, $R_A + R_M \approx R_A$, and hence, to a good approximation, the covolume may be equated with the hydrodynamic volume of the protein (Winzor & Wills, 1986; Wills et al., 1993). For thyroglobulin, a radius of 8.4 nm is inferred from the sedimentation coefficient of 19.4 S (Derrien et al., 1949; Steiner & Edelhoch, 1961), in which case a value of 1490 L/mol is calculated for the effective hydrodynamic volume: this value duplicates the experimentally determined estimate of B_{AM}. Likewise, combination of the molecular weight of 83 000 for horse liver alcohol dehydrogenase with the sedimentation coefficient of 5.11 S (Ehrenberg & Dalziel, 1958) yields an effective hydrodynamic volume of 116 L/mol. The agreement between this value and the estimate of 96 L/mol for B_{AM} is reasonable, considering the approximation involved in modeling the molecules as spheres.

The above two examples have served to illustrate the plausibility of evaluating protein−cosolute excluded volumes from the thermodynamic nonideality incorporated into the dependence of buoyant molecular weight upon C_M. A corollary of this finding is that an approximate value of the protein−cosolute covolume may be calculated from the effective hydrodynamic volume, whereupon ϕ_A' may be calculated from eq 9 divided throughout by M_A. An example of this application of the present treatment is to be found in a study of the effect of sucrose on the molecular weight of lactate dehydrogenase at pH 5 (Hall et al., 1995).

Subunit Molecular Weight of Aldolase. We now consider a situation in which the inert cosolute is replaced by a small denaturant such as urea. Clearly, the assumed inertness of cosolute that is implicit in eq 9 precludes its application to results in the denaturant concentration range conducive to progressive disruption of quaternary structure in the protein. However, for denaturant concentrations commensurate with complete dissociation of the protein into its constituent subunits, the dependence of buoyant molecular weight upon denaturant concentration should be described by eq 9 with M_A reidentified as the subunit molecular weight. Linear extrapolation of this region of the dependence of buoyant molecular weight to the ordinate ($C_M = 0$) thus has the potential to yield the buoyant molecular weight of the subunits in the absence of denaturant, $M_A(1 - \bar{v}_A\rho_s)$, and hence the subunit molecular weight because $\bar{v}_A$ may reasonably be equated with the partial specific volume of the native protein. Sedimentation equilibrium studies of aldolase in the presence of urea are used to illustrate this proposition.

The dependence of the buoyant molecular weight of aldolase upon the concentration of urea is presented in Figure 2. At low concentrations of denaturant, the steeper slope can be rationalized in terms of progressive dissociation of the tetrameric enzyme into subunits, whereas the lesser slope thereafter (solid line) presumably reflects the nonideality arising from excluded volume interactions between denatured subunits and cosolute. Linear regression analysis of the results in the latter region signifies an ordinate intercept of 10 900 (±1400) and a slope of 460 (±280) L/mol. Combination of this estimate of the ordinate intercept with the reported partial specific volume of 0.742 mL/g (Taylor & Lowry, 1956) and the measured buffer density (ρ_s) of 1.0095 g/mL leads to a subunit molecular weight of 43 000 (± 5000). The molecular weight of native enzyme deduced from experiments in the absence of denaturant is 165 000 (±10 000), which agrees well with earlier estimates (Kawa-

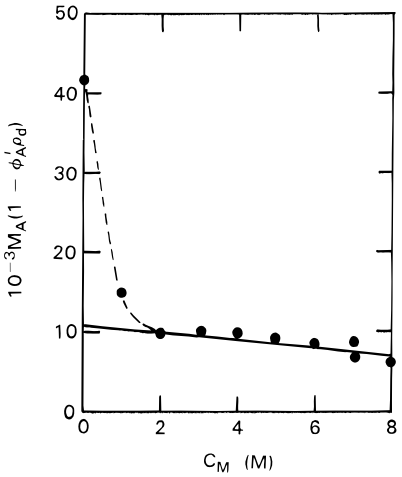

FIGURE 2: Effect of the concentration of urea on the buoyant molecular weight of aldolase in acetate−chloride buffer, pH 5.0. The ordinate intercept of the limiting slope at high denaturant concentration defines the buoyant molecular weight of aldolase subunits in unsupplemented buffer (see text).

hara & Tanford, 1966; Masters & Winzor, 1981) for this tetrameric enzyme.

Although the slope of the solid line drawn in Figure 2 describes the second virial coefficient due to nonideality arising from interactions between denatured enzyme subunits and cosolute, its magnitude cannot easily be interpreted in terms of excluded volume for two reasons. First, the concept of the completely unfolded subunit as a rigid impenetrable sphere is clearly inappropriate. Second, the isodesmic self-association of urea (Schellman, 1955) precludes its consideration as a single cosolute species: on the basis of the equilibrium constant of 0.04 m^{-1} obtained for urea self-association, only 74% of the cosolute would be monomeric in 8 M urea. Consequently, there is no theoretical concentration dependence to guide the extrapolation to obtain $M_A(1 - \bar{v}_A\rho_s)$ as the ordinate intercept in Figure 2.

By measuring the buoyant molecular weight of aldolase as a function of denaturant concentration, we have avoided the problem of interpreting results at a single denaturant concentration in terms of $M_A(1 - \phi_A'\rho_d)$, where ρ_d is the supplemented diffusate density and ϕ_A' the apparent partial specific volume obtained from the difference between the densities of dialyzed protein solution and supplemented diffusate (Casassa & Eisenberg, 1964). Such measurements of ϕ_A' require not only relatively large amounts of protein to obtain sufficiently accurate measurement of the density difference but also recalibration of the measurement of protein concentration if spectrophotometry is used for that purpose: the prediction of magnitudes of ϕ_A' by measurements of preferential solvation (Lee & Timasheff, 1974, 1979; Prakash & Timasheff, 1981) is fraught with the same problems. Although the same material requirements apply to the experimental determination of $\bar{v}_A$ in unsupplemented solvent, that restriction is frequently avoided by considering the partial specific volume of native protein to be obtainable from its amino acid composition (Cohn & Edsall, 1943; Laue et al., 1992). Combination of $\bar{v}_A$, however determined, with the density (ρ_s) of unsupplemented solvent (diffusate) suffices to obtain the subunit molecular weight from the extrapolated ordinate intercept in Figure 2. In that regard, Stellwagen and Schachman (1962) also interpreted their measurements of the buoyant molecular weight of aldolase in 6 M urea as

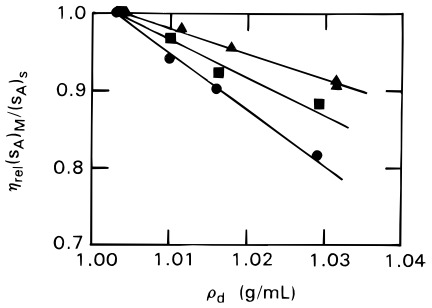

FIGURE 3: Dependence of the sedimentation coefficient, corrected for supplemented buffer viscosity, upon the density of the diffusate (ρ_d) for solutions of horse liver alcohol dehydrogenase (▲), bovine liver catalase (■), and bovine thyroglobulin (●) dialyzed against sucrose-supplemented buffers: results are expressed relative to the the sedimentation coefficient of aldolase in unsupplemented buffer.

signifying a subunit molecular weight of 40 000. However, that estimate was based on a mismatch of partial specific volume and density terms in that the buoyant molecular weight was incorrectly taken as $M_A(1 - \bar{v}_A\rho_d)$.

Protein−Cosolute Excluded Volumes from Sedimentation Velocity Experiments. We now return to consideration of the effects of small inert cosolutes with a view to identifying the so-called preferential protein solvation effect in sedimentation velocity studies as an excluded volume phenomenon. The effect of sucrose supplementation of protein solutions upon their sedimentation velocity behavior is summarized in Figure 3, which presents the dependence of the normalized sedimentation coefficient, $\eta_{rel}(s_A)_M/(s_A)_s$, upon buffer density for horse liver alcohol dehydrogenase (▲), catalase (■), and thyroglobulin (●). Inasmuch as a small protein concentration (0.5 mg/mL or lower) was used in these experiments, the results essentially describe the density dependence of the corresponding ratio of sedimentation coefficients extrapolated to infinite dilution, $\eta_{rel}(s_A^0)_M/(s_A^0)_s$. Whereas such changes in the normalized sedimentation coefficient have traditionally been interpreted in terms of protein solvation, we shall adopt the viewpoint (see Theory) that the effect can be considered in terms of excluded volume. In that regard, extrapolation of the results (Figure 3) to $\eta_{rel}(s_A)_M/(s_A)_s = 0$ yields the buffer densities, $(\rho_d)_{s_A=0}$, required to decrease the sedimentation coefficients of the three proteins to zero. These buffer (diffusate) densities are summarized in Table 1, together with the sucrose concentrations, $(C_M)_{s_A=0}$, obtained from eq 3. Substitution of these values of $(\rho_d)_{s_A=0}$ and $(C_M)_{s_A=0}$ for their respective counterparts in eq 9 yields, on taking into account the fact that the buoyant molecular weight is zero, the magnitudes of B_{AM} listed in the penultimate column of Table 1. Such estimates of the excluded volume (B_{AM}) clearly cannot exhibit the precision of their counterparts evaluated by sedimentation equilibrium (Figure 1) because of the large experimental uncertainty inherent in the extrapolation (Figure 3) to obtain $(\rho_d)_{s_A=0}$ and the correlated parameter, $(C_M)_{s_A=0}$. Nevertheless, there is reasonable agreement between excluded volumes so determined and the effective hydrodynamic volumes of the proteins (final column of Table 1), which should provide a reasonable approximation of B_{AM} for the excluded volume of a protein and a small uncharged cosolute such as sucrose (Winzor & Wills, 1986, 1995; Wills et al., 1993). Indeed, the fact that B_{AM} often underestimates the hydrody-

Table 1: Evaluation of Protein−Sucrose Excluded Volumes by Sedimentation Velocity

protein	$(\rho_A)_{s_A=0}$[a] (g/mL)	$(C_M)_{s_A=0}$[b] (M)	B_{AM}[c] (L/mol)	V_h[d] (L/mol)
thyroglobulin	1.142 (1.138−1.145)	1.064 (1.010−1.018)	1300 (1230−1360)	1490
catalase	1.205 (1.182−1.233)	1.544 (1.368−1.758)	330 (285−367)	330
HLADH[e]	1.232 (1.218−1.249)	1.744 (1.636−1.874)	90 (84−97)	116

[a] Estimate of buffer density associated with zero sedimentation of protein. [b] Corresponding concentration of sucrose to effect that density (eq 10). [c] Excluded volume calculated from eq 9. [d] Stokes volume based on the following parameters: M_A= 670 000, $\bar{v}_A$= 0.723 mL/g, and $s_{20,w}$ = 19.4 S for thyroglobulin (Derrien et al., 1949; Steiner & Edelhoch, 1961); M_A = 240 000, $\bar{v}_A$ = 0.730 mL/g, and $s_{20,w}$ = 11.4 S for catalase (Samejima & Yang, 1963); M_A = 83 000, $\bar{v}_A$ = 0.750 mL/g, and $s_{20,w}$ = 3.58 S for alcohol dehydrogenase (Ehrenberg & Dalziel, 1958). [e] Horse liver alcohol dehydrogenase.

namic volume can be attributed to asymmetry/irregularity of the protein shape.

DISCUSSION

From the experimental viewpoint, the major outcome of this investigation is the demonstration of a simple and economic means to overcome the evaluation of ϕ_A', the apparent partial specific volume of a protein, in the presence of a large concentration of small cosolute. Casassa and Eisenberg (1964) had provided the initial lead by showing that analysis of the sedimentation equilibrium distributions could be simplified to that for the standard single-solute system by predialysis of the protein solution against buffer supplemented with small cosolute. However, this action introduced the problem of evaluating the consequent experimental parameter ϕ_A'. Although this apparent partial specific volume may, in principle, be determined by densitometry, the amount of protein required for such characterization has rendered the problem either intractable or unattractive. What we have shown is that performance of sedimentation equilibrium studies at a series of cosolute concentrations obviates the necessity to evaluate ϕ_A', thereby rendering more feasible the possibility of characterizing both the molecular weight and the thermodynamic nonideality of nonassociating proteins by sedimentation equilibrium. This finding is of particular significance to the determination of subunit molecular weights of proteins by sedimentation equilibrium in the presence of high concentrations of a denaturant (Figure 2).

Although the estimation of second virial coefficients for excluded volume interactions between protein and cosolute (B_{AM}) by sedimentation equilibrium is clearly inferior to their determination by sedimentation equilibrium, the results presented in Figure 3 and Table 1 serve to reinforce the identification of the so-called protein solvation effects in sedimentation velocity studies as manifestations of thermodynamic nonideality arising from excluded volume interactions between protein and cosolute. That different cosolutes give rise to different effects on the sedimentation velocity characteristics of a protein (Schachman & Lauffer, 1949) is not a consequence of changes in the radius (molecular dimensions) of the protein in the presence of the cosolutes. Rather, it is a consequence of the different sizes of the cosolutes. In the belief that use of the term "protein solvation" to describe the effect may be responsible for frequent misinterpretation of its meaning, we emphasize the Kauzman explanation of the phenomenon by depicting schematically (Figure 4) the situation for preferential solvation of a protein (A) by two cosolutes (M_1, M_2) with different effective radii (R_1, R_2). Because the centers of the two molecules, A and either M_1 or M_2, can approach no closer than the sums of their radii, there is a volume shell (shaded area) which can only be occupied by solvent. There may, of course, also be regions within the protein volume that are accessible to solvent but not cosolute: such solvation is taken into account in the effective protein radius R_A. Use of a larger cosolute increases the volume of the shell occupied solely by solvent, and hence the protein is said to be more highly solvated relative to the cosolute. Inasmuch as it is not the protein volume but the protein−cosolute excluded volume that has been changed, description of the protein as being more highly solvated in the presence of the larger cosolute is readily misconstrued. It is hoped that this reiteration of the Kauzman protein solvation concept may clarify the situation for any researchers who may have adopted the literal interpretation of the term.

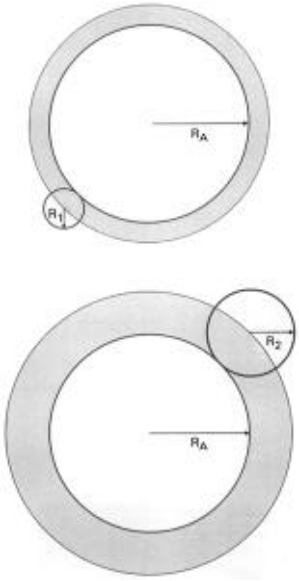

FIGURE 4: Schematic representation of the origin of the greater extent of protein solvation (hatched areas) that occurs in the presence of a larger cosolute (M_2): the difference reflects a change in protein−cosolute excluded volume, and not a change in protein dimensions (the literal interpretation of a change in extent of protein solvation).

REFERENCES

Baranowski, T., & Niederland, T. R. (1949) *J. Biol. Chem. 180*, 543−551.
Casassa, E. F., & Eisenberg, H. (1964) *Adv. Protein Chem. 19*, 287−395.
Cheng, P. Y., & Schachman, H. K. (1955) *J. Polym. Sci. 16*, 19−30.
Cohen, G., & Eisenberg, H. (1968) *Biopolymers 6*, 1077−1100.
Cohn, E. J., & Edsall, J. T. (1943) in *Proteins, Amino Acids and Peptides as Ions and Dipolar Ions*, pp 370−381, Reinhold, New York.
Derrien, Y., Michel, R., Pedersen, K. O., & Roche, J. (1949) *Biochim. Biophys. Acta 3*, 436−441.
Ehrenberg, A., & Dalziel, K. (1958) *Acta Chem. Scand. 12*, 465−469.
Eisenberg, H. (1976) *Biological Macromolecules and Polyelectrolytes in Solution*, Oxford University Press (Clarendon), London.

Hall, D. R., Jacobsen, M. P., & Winzor, D. J. (1995) *Biophys. Chem. 57*, 47−54.
Hiraga, M., Anan, F. K., & Abe, K. (1964) *J. Biochem. (Tokyo) 56*, 416−423.
Katz, S., & Schachman, H. K. (1955) *Biochim. Biophys. Acta 18*, 28−35.
Kawahara, K., & Tanford, C. (1966) *Biochemistry 5*, 1578−1584.
Laue, T. M., Shah, B. D., Ridgeway, T. M., & Pelletier, S. L. (1992) in *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S. E., Rowe, A. J., & Horton, J. C., Eds.) pp 90−125, Royal Society of Chemistry, Cambridge, U.K.
Lee, J. C., & Timasheff, S. N. (1974) *Arch. Biochem. Biophys. 165*, 268−273.
Lee, J. C., & Timasheff, S. N. (1979) *Methods Enzymol. 61*, 49−57.
Lee, J. C., & Timasheff, S. N. (1981) *J. Biol. Chem. 256*, 7193−7201.
Martin, W. G., Winkler, C. A., & Cook, W. H. (1959) *Can. J. Chem. 37*, 1662−1670.
Masters, C. J., & Winzor, D. J. (1981) *Arch. Biochem. Biophys. 209*, 185−190.
Oncley, J. L. (1941) *Ann. N. Y. Acad. Sci. 41*, 121−150.
Prakash, V., & Timasherff, S. N. (1981) *Anal. Biochem. 117*, 330−335.
Reisler, E., & Eisenberg, H. (1969) *Biochemistry 8*, 4572−4578.
Samejima, T., & Yang, J. T. (1963) *J. Biol. Chem. 238*, 3256−3261.
Schachman, H. K., & Lauffer, M. A. (1949) *J. Am. Chem. Soc. 71*, 536−541.
Schellman, J. A. (1955) *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim. 29*, 223−229.
Sharp, D. G., Taylor, A. R., McLean, I. W., Jr., Beard, D., & Beard, J. W. (1944) *J. Biol. Chem. 159*, 29−44.
Steiner, R. F., & Edelhoch, H. (1961) *J. Am. Chem. Soc. 83*, 1435−1444.
Stellwagen, E., & Schachman, H. K. (1962) *Biochemistry 1*, 1056−1069.
Taylor, J. F., & Lowry, C. (1956) *Biochim. Biophys. Acta 20*, 109−117
Timasheff, S. N. (1995) in *Protein−Solvent Interactions* (Gregory, R. B., Ed.) pp 445−482, Marcel Dekker, New York.
Van der Walt, B., Kotze, B., Edelhoch, H., & Van Jaersveld, P. P. (1983) *Biochim. Biophys. Acta 744*, 90−98.
Wills, P. R., & Winzor, D. J. (1992) in *Analytical Ultracentrifugation in Biochemistry and Polymer Science* (Harding, S. E., Rowe, A. J., & Horton, J. C., Eds.) pp 311−330, Royal Society of Chemistry, Cambridge, U.K.
Wills, P. R., & Winzor, D. J. (1993) *Biopolymers 33*, 1627−1629.
Wills, P. R., Comper, W. D., & Winzor, D. J. (1993) *Arch. Biochem. Biophys. 300*, 206−212.
Wills, P. R., Jacobsen, M. P., & Winzor, D. J. (1996) *Biopolymers 38*, 119−130.
Winzor, D. J., & Wills, P. R. (1986) *Biophys. Chem. 25*, 243−251.
Winzor, D. J., & Wills, P. R. (1995) in *Protein−Solvent Interactions* (Gregory, R. B., Ed.) pp 483−520, Marcel Dekker, New York.

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