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Nonequivalence of second virial coefficients from sedimentation equilibrium and static light scattering studies of protein solutions

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Abstract

Experimental data for ovalbumin and lysozyme are presented to highlight the nonequivalence of second virial coefficients obtained for proteins by sedimentation equilibrium and light scattering. Theoretical considerations confirm that the quantity deduced from sedimentation equilibrium distributions is B_{22}, the osmotic second virial coefficient describing thermodynamic nonideality arising solely from protein self-interaction. On the other hand, the virial coefficient determined by light scattering is shown to reflect the combined contributions of protein–protein and protein–buffer interactions to thermodynamic nonideality of the protein solution. Misidentification of the light scattering parameter as B_{22} accounts for published reports of negative osmotic second virial coefficients as indicators of conditions conducive to protein crystal growth. Finally, textbook assertions about the equivalence of second virial coefficients obtained by sedimentation equilibrium and light scattering reflect the restriction of consideration to single-solute systems. Although sedimentation equilibrium distributions for buffered protein solutions are, indeed, amenable to interpretation in such terms, the same situation does not apply to light scattering measurements because buffer constituents cannot be regarded as part of the solvent: instead they must be treated as non-scattering cosolutes.

Keywords: Second virial coefficients; Sedimentation equilibrium; Light scattering; Protein crystallization; Lysozyme; Ovalbumin; Urate oxidase

1. Introduction

Although the thermodynamic nonideality of protein solutions can be quantified in terms of a second virial coefficient obtained from either sedimentation equilibrium or static light scattering studies, the two methods do not yield the same parameter. In the presence of high salt concentrations the second virial coefficients obtained from light scattering measurements on lysozyme [1,2] and equine serum albumin [3] are negative, but the values determined from sedimentation equilibrium distributions under comparable conditions are positive [4,5]. This disparity has been attributed [5] to the fact that the parameter deduced by sedimentation equilibrium is the osmotic second virial coefficient for protein self-interaction [6–10], whereas that obtained by light scattering seems to reflect the combined effects of protein−protein and protein−buffer interactions [11,12].

In this study we modify the thermodynamic treatment of Rayleigh scattering [11,12] to obtain a quantitative expression for the description of experimental data in the form of a Debye plot with concentration expressed on a weight/volume scale. The analysis highlights the effect of even small molecular cosolutes, like buffer components, and raises the question whether light scattering measurements ever yield the second virial coefficient for protein self-interaction in an ordinary buffered solution. This problem is addressed by comparing second virial coefficients obtained from sedimentation equilibrium and light scattering measurements on two proteins, ovalbumin and lysozyme, over a range of ionic strengths (0−0.25 M) that is commonly used in biochemical studies. The predicted nonequivalence of second virial coefficients obtained from sedimentation equilibrium and light scattering studies of buffered protein solutions is thereby demonstrated.

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2. Materials and methods

2.1. Materials

Crystalline preparations of ovalbumin obtained from Sigma Chemical Co. (St. Louis, MO) were dissolved directly in acetate buffer, pH 4.6, I 0.04 M (0.04 M sodium acetate, pH adjusted with acetic acid), or in the same buffer supplemented with either sodium chloride (0.04−0.20 M) or sucrose (0.025−0.20 M). Although the ovalbumin preparations contained contaminants with much higher molecular mass, the contaminants were separated from the 44 kDa protein by the size-exclusion chromatography step that precedes light scattering measurements. As in previous sedimentation equilibrium studies [13,14], zonal chromatography on a Biosep-SEC-S3000 column was also used to prepare contaminant-free solutions in dialysis equilibrium with the appropriate buffer medium.

2.2. Light scattering procedure

Second virial coefficients were determined by the Wyatt procedure [15] in which Rayleigh scattering and concentration detectors are used in series to monitor the effluent from a size-exclusion chromatography column. As noted by Bajaj and coworkers [16], a potential deficiency of this procedure is its neglect of the change in concentration distribution that occurs during passage of the solute zone through the inter-detector volume. However, subsequent computer software that makes allowance for band-broadening in the inter-detector volume [17] has been used to minimize the consequences of this phenomenon.

After preequilibration of the TSK G-3000 PW column with acetate buffer (pH 4.6, I 0.04 M) or the same buffer supplemented with either sodium chloride or sucrose at 20 °C and a flow rate of 0.8 ml/min, ovalbumin solution (100 μL, 100 mg/ml) was injected onto the size-exclusion chromatography column, the effluent from which flowed through a DAWN EOS multi-angle light scattering detector (Wyatt Technology Corp., Santa Barbara, CA) as well as an extended-range interferometric refractometer (Wyatt Optilab REX) to monitor the protein concentration of the column eluate (a maximum of 8 mg/ml). Because the elution profile signified the presence of material with much higher molecular mass, the data file for apparent molecular weight and virial coefficient determination was restricted to the trailing side of the ovalbumin zone — the procedure adopted earlier by Bajaj and coworkers (see. e.g., Fig. 4B of [16]).

2.3. Sedimentation equilibrium

Solutions of ovalbumin (approx. 2 mg/ml) in dialysis equilibrium with the appropriate buffer medium were subjected to sedimentation equilibrium at 23,000 rpm and 20 °C in a Beckman XL-I ultracentrifuge — a rotor speed that sufficed to ensure experiments of the meniscus-depletion design [18]. The resulting sedimentation equilibrium distributions were recorded interferometrically and converted to corresponding weight-concentration distributions on the basis of a calibration factor of 3.33 Rayleigh fringes for a 1 mg/ml protein solution [19]. The second virial coefficient for protein self-interaction (B_{22}) was then determined by the procedure used previously [5,13] except that the low concentration region of the same experiment [($c(r)<$ 0.5 mg/ml] was used to obtain the buoyant molecular mass.

2.4. Calculation of osmotic virial coefficients

Magnitudes of second virial coefficients for protein self-interaction have been obtained from the expression

$$B_{22} = \frac{16\pi N_A R_2^3}{3} + \frac{Z_2^2(1+2\kappa R_2)}{4I(1+\kappa R_2)^2} \quad (1)$$

which describes the excluded volume interaction between two spherical molecules (species 2) with radius R_2 and net charge Z_2 spread uniformly over the surface [9,10]. Avogadro's number (N_A) is included to convert the excluded volume from a molecular to a molar basis. The inverse screening length, κ, has been calculated from the molar ionic strength I as $3.27\times10^7\sqrt{I}$ cm^{-1}. R_2 may reasonably be identified with the Stokes radius, taken as 2.92 nm for ovalbumin [20] and 1.7 nm for lysozyme [4]. In the studies of ovalbumin at pH 4.6 the protein is isoelectric [21,22], and hence there is no contribution from the charge repulsion term [$Z_2=0$ in Eq. (1)]. However, its contribution is substantial for lysozyme under similar conditions (pH 4.5) because of the net charge of +14 borne by the enzyme [4,8].

For excluded-volume interaction between dissimilar species the counterpart of Eq. (1) for the second virial coefficient is [9,10]

$$B_{23} = \frac{4\pi N_A(R_2+R_3)^3}{3} + \frac{Z_2Z_3(1+\kappa R_2+\kappa R_3)}{2I(1+\kappa R_2)(1+\kappa R_3)} \quad (2)$$

where R_3 and Z_3 refer to the radius and net charge of the cosolute (species 3). Our choice of nonelectrolytes ($Z_3=0$) as cosolutes leads to the situation wherein the osmotic second virial coefficient for protein−cosolute interaction is simply the covolume [the first term on the right-hand side of Eq. (2)]. For these calculations the radius of sucrose (R_3) has been taken as 0.32 nm, the value deduced [7,23] from isopiestic measurements of the concentration dependence of its activity coefficient [24].

2.5. Light scattering in a three-component system

The thermodynamic analysis of light scattering due to density fluctuation in solutions of macromolecules [11,12] necessitates consideration of the variation of components' chemical potentials under conditions of constant temperature, pressure and quantities of different components — a situation which renders molality as the most convenient scale for the initial specification of concentrations [6,7,25]. For a solution comprising protein (molal concentration m_2) and a cosolute (concentration m_3) in solvent (weight-concentration c_1) the Rayleigh

excess ratio for vertically polarized light with wavelength λ is given by the relationship [12]

$$R_\theta = \frac{4\pi^2 n^2 (\partial n/\partial m_2)^2 m_2}{N_A \lambda^4 c_1}\left[\frac{[1-\beta_{23}\Psi m_3/(1+\beta_{33}m_3)]^2}{1+m_2[\beta_{22}-\beta_{23}^2 m_3/(1+\beta_{33}m_3)]}+\ldots\right] \quad (3)$$

or, upon making the simplifying approximation that $(1+x)^n \approx 1+nx+..$, in order to obtain results correct to first order in concentrations,

$$R_\theta = \frac{4\pi^2 n^2 (\partial n/\partial m_2)^2 m_2}{N_A \lambda^4 c_1}[1-2\beta_{23}\Psi m_3-\beta_{22}m_2 + (2\beta_{22}\beta_{23}\Psi+\beta_{23}^2)m_2 m_3+..]. \quad (4)$$

In these expressions n is the refractive index of the solution, $(\partial n/\partial m_2)_{T,P,m_3}$ its first derivative with respect to molal solute concentration, and Ψ the ratio of molal refractive index increments for cosolute and solute, $(\partial n/\partial m_3)_{T,P,m_2}/(\partial n/\partial m_2)_{T,P,m_3}$. N_A is Avogadro's number and the required β-parameters are defined by the relationship

$$\beta_{2j} = (\partial \ln y_2/\partial m_j)_{T,P,m_{i\neq j}} \quad (5)$$

where y_2 is the molal activity coefficient for solute, i.e., the activity coefficient relevant to measurement of chemical potential under the constraint of constant pressure [6,7,25]: j is 2 and 3 for β_{22} and β_{23} respectively. In this exploratory investigation we shall restrict considerations of thermodynamic nonideality essentially to those arising from nearest-neighbour interactions — in keeping with attempts to make allowance for first order effects of the phenomenon.

The activity coefficient derivatives in Eq. (4), β_{22} and β_{23}, are eliminated by expressing the logarithm of the molal activity coefficient for solute as an expansion in powers of concentrations,

$$\ln y_2 = 2C_{22}m_2 + C_{23}m_3 + C_{223}m_2 m_3 + \ldots \quad (6)$$

where C_{22} and C_{23} are the respective molal second virial coefficients for protein self-interaction and protein–cosolute interaction, etc. [10]. Inclusion of the term involving a coefficient with the dimensions (characteristics) of the third virial coefficient for solute–solute–cosolute interaction (C_{223}) is necessary in order to describe β_{22} and β_{23} correct to first order in solute and cosolute concentrations. However, in the absence of a proper account of heterogeneous three-body interactions, we regard C_{223} as a curve-fitting parameter, there being precedents [11,26,27] for such action. Upon incorporation of the truncated forms $\beta_{22}=2C_{22}+C_{223}m_3$ and $\beta_{23}=C_{23}+C_{223}m_2$ the expression for R_θ becomes

$$R_\theta = \frac{4\pi^2 n^2 (\partial n/\partial m_2)^2 m_2}{N_A \lambda^4 c_1}[1-2C_{23}\Psi m_3-2C_{22}m_2 + \{4C_{22}C_{23}\Psi-C_{223}(1+2\Psi)+C_{23}^2\}m_2 m_3+..]. \quad (7)$$

This relationship now requires conversion into a form that is suitable for analysis of Rayleigh scattering data with concentrations expressed on a weight/volume basis.

On the grounds that aqueous solutions are regarded as being essentially incompressible, the concentration of solvent (c_1) may be written in terms of the weight concentrations of solute and cosolute as

$$c_1 = \rho_1(1-\bar{v}_2 c_2-\bar{v}_3 c_3) \quad (8)$$

where ρ_1 is the solvent density and $\bar{v}_2$, $\bar{v}_3$ the partial specific volumes of solute and cosolute respectively. Assumed solution incompressibility also allows substitution of the relationship

$$m_i = c_i/[\rho_1 M_i(1-\bar{v}_2 c_2-\bar{v}_3 c_3)] \quad (9)$$

for the molal concentrations of solute and cosolute. Upon incorporation of those substitutions the expression for Rayleigh excess scattering may be written in the form

$$\frac{4\pi^2 n^2 (\partial n/\partial m_2)^2 c_2}{M_2 \rho_1^2 N_A \lambda^4 R_\theta} = 1 + \frac{2(C_{23}/\rho_1)\Psi-2M_3\bar{v}_3}{M_3}c_3 + \frac{2(C_{22}/\rho_1)-2M_2\bar{v}_2}{M_2}c_2 + \frac{\Lambda}{M_2 M_3}c_2 c_3 \quad (10a)$$

$$\Lambda = (C_{223}/\rho_1^2)(1+2\Psi) + 2M_2M_3\bar{v}_2\bar{v}_3-2(C_{23}/\rho_1)\Psi M_2\bar{v}_2-2(C_{22}/\rho_1)M_3\bar{v}_3 + 4(C_{22}/\rho_1)(C_{23}/\rho_1)\Psi-(C_{23}^2/\rho_1^2). \quad (10b)$$

We now need to accommodate experimentally entrenched conventions that the optical constant (K) in the Debye plot, Kc_2/R_θ versus c_2, is defined in terms of the refractive index of cosolute-supplemented solvent, n_s, and the specific refractive index increment for solute, $\chi_2=(\partial n/\partial c_2)_{T,P,c_3}$. Also required is the switch from $(\partial n/\partial m_2)^2$ to $(\partial n/\partial c_2)^2$, as is the modification of Ψ, the ratio of molal refractive index increments for solute and cosolute, to its molar counterpart (see Appendix A).

Incorporation of Eqs. (A2), (A6), (A7) and (A8) into Eqs. (10a), (10b) then gives

$$\frac{Kc_2}{R_\theta} = \frac{A_1}{M_2} + 2A_2 c_2 \quad (11)$$

where

$$A_1 = 1 + 2(\chi_3/\chi_2)[(C_{23}/\rho_1)/M_2 + \bar{v}_2]c_3 \quad (12)$$

$$A_2 = [(C_{22}/\rho_1) + M_2\bar{v}_2-M_2(\chi_2/n_s) + 1/2\Omega c_3/M_3]/M_2^2 + (1/2)\Omega c_3 \quad (13)$$

$$\Omega = (C_{223}/\rho_1^2)-(C_{23}/\rho_1)^2 + 2[(C_{22}/\rho_1)-(C_{23}/\rho_1)]M_3\bar{v}_3 + 4(C_{22}/\rho_1)M_3(\chi_3/\chi_2)\bar{v}_2] + 2\Psi_0[(C_{223}/\rho_1^2) + 2(C_{22}/\rho_1)(C_{23}/\rho_1) + 2(C_{23}/\rho_1)M_2\{\bar{v}_2-(\chi_2/n_s)\}] + M_2[6(\chi_3/\chi_2)\bar{v}_2^2-4(\chi_3/n_s)\bar{v}_2] \quad (14)$$

in which K is the usual optical constant, $4\pi^2 n_s^2(\partial n/\partial c_2)^2/(N_A\lambda^4)$. The two molal virial coefficients are related to their osmotic (molar) counterparts by the expressions

$$(C_{22}/\rho_1) = B_{22} - M_2\bar{v}_2 \tag{15}$$

$$(C_{23}/\rho_1) = B_{23} - M_2\bar{v}_2 - M_3\bar{v}_3. \tag{16}$$

In that regard it should be noted that Eq. (16) amends an error in Eq. (14d) of [23], which had been derived [6] on the basis of a non-standard definition of C_{23}. As noted above, in the absence of a satisfactory theoretical expression for C_{223}, this quantity is treated as a curve-fitting parameter.

3. Results and discussion

In sedimentation equilibrium the protein thermodynamic activity being monitored is that defined under the constraints of constant temperature and chemical potential of solvent [6–10], whereupon dialyzable solutes such as buffer constituents and supporting electrolytes may be regarded as part of the solvent [13,28]. However, as noted in the previous section, Rayleigh scattering from a macromolecular solute is affected by the concentrations of all solutes, irrespective of their ability to scatter light. Inasmuch as studies of proteins invariably entail the use of buffered solutions to maintain pH stability, it is important to establish the extent to which theory for a single-solute system is applicable to light scattering measurements. To address this question we compare values of second virial coefficients obtained by light scattering with those obtained from sedimentation equilibrium measurements for two proteins, ovalbumin and lysozyme, over a range of ionic strength (0.04–0.25 M) that is commonly used in biochemical studies.

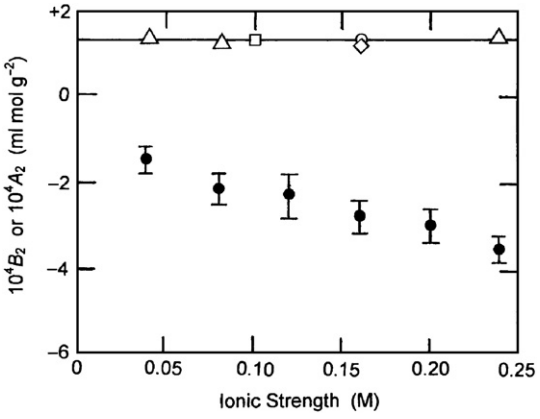

Fig. 1. Nonequivalence of estimates of the second virial coefficient for isoelectric ovalbumin obtained by sedimentation equilibrium (B_2, open symbols) and light scattering (A_2, ●) over the range of ionic strength commonly employed in biochemical studies, the solid line denotes the ionic-strength dependence of the osmotic second virial coefficient, B_{22}/M_2^2, calculated from Eq. (1) with R_2=2.92 nm [20] and Z_2=0 [21,22]. Sources of sedimentation equilibrium data: Δ, current data; ○, Jeffrey et al. [20]; ◇, Wills et al. [8]; □, Winzor et al. [14].

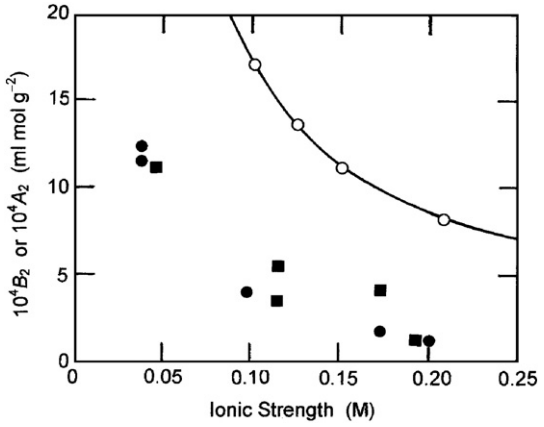

Fig. 2. Disparity between estimates of the second virial coefficient for lysozyme (pH 4.5) obtained by sedimentation equilibrium (B_2, open symbols) and light scattering (A_2, solid symbols). ○, Sedimentation equilibrium data of Behlke and Ristau [4], together with their best-fit description of the dependence in terms of Eq. (1) with Z_2=14.1, R_2=1.7 nm. ■, ●, Corresponding estimates inferred from light scattering studies by Muschol and Rosenberger [1] and Rosenbaum and Zukoski [2].

3.1. Effect of ionic strength on the second virial coefficient for proteins

For the present study isoelectric ovalbumin (pH 4.6) has been chosen initially as the model system to avoid any ambiguity about the magnitude of the charge-repulsion contribution to the second virial coefficient for protein self-interaction, B_{22} [see Eq. (1)]. From the open symbols in Fig. 1, which refer to second virial coefficients ($B_2=B_{22}/M_2^2$) obtained in this and previous [8,14,20] studies by sedimentation equilibrium, there is clearly substantial agreement between experiment and theoretical prediction (—) based on the statistical–mechanical concept of excluded volume. In other words, these results re-emphasize that the nonideality parameter derived from sedimentation equilibrium distributions is, indeed, B_{22}, the osmotic second virial coefficient for protein self-interaction.

A completely different outcome ensues from the corresponding measurements of the second virial coefficient, A_2, that are deduced from the slopes of Debye plots (●, Fig. 1). The return of negative values is clearly inconsistent with interpretation in terms of Eq. (13) with c_3=0. Instead, it signifies the existence of sizable negative contributions to A_2 from the c_2c_3 terms in Eq. (14) reflecting protein–cosolute interactions, even at the lowest ionic strength (I=0.04 M) investigated. From these results it would appear that there is no practicable range of small cosolute concentration over which light scattering measurements on protein solutions are amenable to interpretation in terms of single-solute theory.

Confirmation of that conclusion is provided by published results [4] for lysozyme under similar conditions (pH 4.5, I=0.04 – 0.20 M). The much larger values of $B_2=B_{22}/M_2^2$ obtained by sedimentation equilibrium [4] for this system (○, Fig. 2) compared with those for ovalbumin (open symbols, Fig. 1) reflect the contribution of the charge-repulsion term to B_{22} arising from the cationic nature of lysozyme (Z_2=+14) under these conditions. Although the values of A_2 obtained

from the slopes of Debye plots [1,2] remain positive over this range of ionic strength, their magnitudes clearly do not describe the ionic strength dependence of B_{22} based on the statistical–mechanical basis of the intermolecular potential of mean force (—, Fig. 2). A similar variation of the light scattering virial coefficient for lysozyme under the same conditions has been reported by Lenhoff and coworkers [29], who have accounted for the phenomenon by a more detailed molecular model [30] than that used here. Our aim has been to restrict modelling to the minimum required for description of the thermodynamics of the system.

The important point to emerge from Figs. 1 and 2 is the experimental demonstration that the nonideality parameter emanating from a Debye plot of light scattering measurements should not be regarded as the osmotic second virial coefficient for protein self-interaction. Its misidentification as such [1–3,31–33] undoubtedly reflects definitive statements to that effect in standard textbooks [34–36] — a consequence of restricting theoretical consideration to single-solute systems on the grounds that buffer constituents can surely be regarded as part of the solvent. Although that presumption turns out to provide for a valid analysis of sedimentation equilibrium studies of proteins, it clearly does not apply to light scattering. We therefore reach the conclusion that the textbook dogma about the equivalence of second virial coefficients from the two techniques is misleading in that it is only a reasonable approximation for single-solute systems, for which the relationship between A_2 and the osmotic virial coefficient is [from Eqs. (15) and (13) with $c_3=0$]

$$A_2 = [B_{22} - M_2(\mathrm{d}n/\mathrm{d}c_2)/n_s]/M_2^2 \qquad (17)$$

a result which would signify only slight underestimation (2–3%) of B_{22} for an isoelectric protein the size of ovalbumin. However, because of the necessity to regard buffer constituents as additional cosolutes in light scattering (but not sedimentation equilibrium), this previously unqualified statement no longer obtains.

3.2. Effect of poly(ethylene glycol) on A_2 for urate oxidase

A limitation of the quantitative expressions for the magnitudes of the ordinate intercept, $A_1/M_2=(1/M_2)_{app}$, and slope, $2A_2$, of a Debye plot [Eqs. (11)–(14)] obtained for a protein in the presence of cosolute is their description of thermodynamic nonideality on the statistical–mechanical basis of excluded volume. This restriction precludes detailed quantitative consideration of the effects of electrolytes, which exhibit negative deviations from Raoult's Law (B_{33} negative instead of positive) because of solvent–cosolute interactions. Although electrolytes such as sodium chloride and ammonium sulphate have been the favoured cosolutes in studies of proteins by Rayleigh scattering techniques, there are also investigations in which polyethylene glycol has been used as the solution supplement [37–42]. Furthermore, the physicochemical properties of solutions comprising mixtures of proteins and polyethylene glycol (PEG) may be rationalized on the statistical–mechanical basis of excluded volume [23,43–45]. We therefore illustrate predictions of the current theory by simulating the effects of this polymer on the light scattering second virial coefficient (A_2) for an uncharged (isoelectric) protein the size of bacterial urate oxidase ($M_2=128$ kDa) — a protein–polymer system for which second virial coefficients have been obtained under essentially isoelectric conditions ($Z_2\approx -4$) by the closely allied technique of low-angle X-ray scattering [42].

Values of the required second virial coefficients have been obtained on the basis of the expressions reported in Eqs. (1), (2), (15) and (16) with an enzyme radius of 3.5 nm, a value [42] based on the crystal structure. Respective magnitudes of 1.7 and 2.6 nm have been assigned to R_3 for PEG 3350 and PEG 8000, the two cosolutes used by Vivarès and Bonneté [42] on the basis [43] that $R_3=0.0292\sqrt{M_3}$ for polyethylene glycols in this molecular mass range. Refractive index parameters were accorded the following magnitudes: $\chi_2\approx \mathrm{d}n/\mathrm{d}c_2=0.185$ ml/g (the value for proteins incorporated into the DAWN software (Wyatt Technology Corp., Santa Barbara, CA); $\chi_3=\mathrm{d}n/\mathrm{d}c_3=0.134$ ml/g [43]; $\chi_2/n_s=\chi_2/[1.333+c_3(\mathrm{d}n/\mathrm{d}c_3)]$ ml/g. A typical protein value of 0.735 ml/g has been assigned to the partial specific volume of urate oxidase ($\bar{v}_2$), whereas $\bar{v}_3$ has been taken as 0.84 ml/g for polyethylene glycol [46,47]. In view of our tentative treatment of C_{223}/ρ_1^2 as a curve-fitting parameter, we initially set this parameter to zero in order to establish the extent to which it is required to provide a reasonable theoretical description of the data.

Predicted dependencies (with $C_{223}/\rho_1^2=0$) of the Debye slope parameter, A_2, upon concentration of the two polyethylene glycols are summarized in Fig. 3, which also presents the experimental virial coefficients (●, ○) reported by Vivarès and Bonneté [43]. Because of the disparity between the theoretical and observed virial coefficients in the absence of cosolute (a point already noted in relation to Figs. 1 and 2)), the calculated values have all been corrected by subtracting this difference to allow readier comparison of the experimentally observed

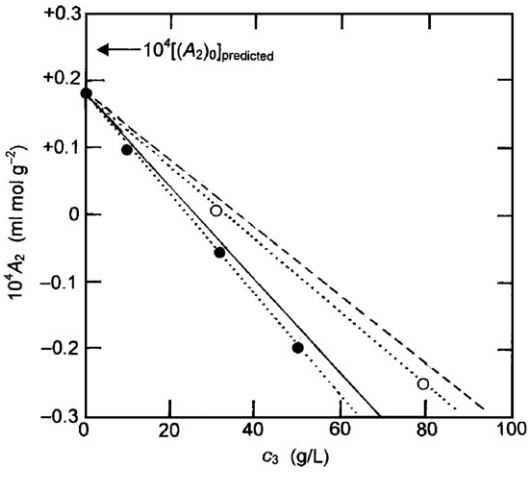

Fig. 3. Comparison of the effect of the concentration of polyethylene glycol (species 3) on the light scattering second virial coefficient for urate oxidase with that predicted for an isoelectric protein with the molecular size characteristics of the enzyme. ●, —, PEG 8000; ○, – – –, PEG 3350. Dotted lines are the theoretical dependencies predicted by Eq. (14) with assigned magnitudes of −19,100 and −6600 L^2 mol^{-2} to the empirical curve-fitting parameter C_{223}/ρ_1^2 for the PEG 8000 and PEG 3350 systems respectively (see the text).

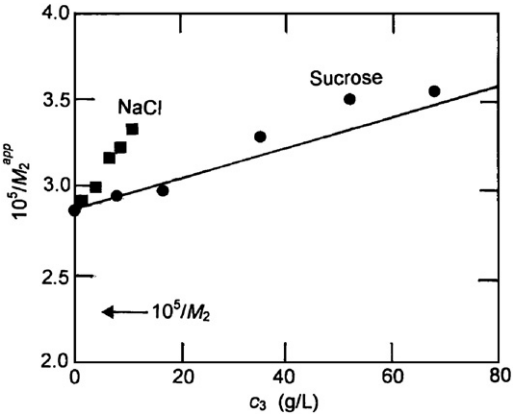

Fig. 4. Comparison of the effect of sucrose concentration on the ordinate intercept ($1/M_2^{app}$) of Debye plots for isoelectric ovalbumin (●) with the dependence (—) predicted by Eq. (12). Also shown are experimental results (○) for the corresponding effect of sodium chloride on $1/M_2^{app}$ for isoelectric ovalbumin in the same medium (pH 4.6, I 0.04 M).

declines in A_2 with the cosolute concentration dependence predicted by the $\Omega c_3/M_3$ term in Eq. (14). For both cosolutes the agreement between theoretical prediction and experiment is considered reasonable in that the decrease in $[(A_2)_3 - (A_2)_0]$ is only underestimated by 12%, a relatively minor disparity that can be eliminated by assigning respective values of −6600 and −19,100 L^2 mol^{-2} to C_{223}/ρ_1^2 for the experiments with PEG 3350 and PEG 8000 as the respective cosolutes (dotted lines in Fig. 3).

Although present theory is clearly holding for the interactions of urate oxidase with PEG 3350 and PEG 8000, a point at issue is whether a much smaller, dialyzable nonelectrolyte must also be regarded as a cosolute. In that regard the larger poly (ethylene glycol) samples used by Vivarès and Bonneté [42] were chosen deliberately to enhance the extent of the decline in A_2, as Kulkarni and coworkers [39] had already demonstrated the potential of PEG 400 for decreasing the magnitude of the light scattering second virial coefficient for lysozyme. We employ the effect of sucrose on A_2 for ovalbumin to examine in more detail the consequences of small solute inclusion on the characterization of proteins by light scattering.

3.3. Effect of sucrose on the Debye plot for isoelectric ovalbumin

In this section we report results of light scattering measurements on isoelectric ovalbumin solutions (pH 4.6, I=0.04 M) supplemented with sucrose, a cosolute which exhibits the positive deviations from Raoult's Law that are conducive to consideration in terms of excluded-volume interaction [7,23,24]. Values of the two second virial coefficients have been calculated from Eqs. (1), (2), (15) and (16) with a protein radius (R_2) of 2.92 nm [20,22] and a cosolute radius (R_3) of 0.32 nm for sucrose [23]. A protein specific refractive index increment (χ_2) of 0.185 ml/g was again assigned to ovalbumin, whereas a value of 0.143 ml/g was used for χ_3 [48,49]. The molecular mass (M_2) and partial specific volume ($\bar{v}_2$) of ovalbumin were taken as 44 kDa and 0.734 ml/g respectively [50], and the corresponding values for sucrose as 342 (the formula molecular mass) and 0.614 ml/g [51].

The first phenomenon examined is the effect of sucrose inclusion on the ordinate intercept of the Debye plot, a parameter usually considered to define $1/M_2$, but which is predicted to exhibit positive linear dependence upon c_3 [Eq. (12)]. Although there was clearly initial surprise at inability to observe a predicted effect of salt concentration on the ordinate intercept, the disparity between theory and experiment was attributed to seeming negligibility of the c_3 term for many protein systems [52]. Fig. 4 presents the dependence of the ordinate intercept ($1/M_2^{app}$) upon sucrose concentration (●), as well as its dependence upon sodium chloride concentration (○) in the experiments reported in Fig. 1. The dependence predicted by Eqs. (11) and (12) [—, Fig. 4] is clearly similar to that observed experimentally. A corresponding comparison between experiment and theory for the effect of NaCl concentration on $1/M_2^{app}$ is precluded by inapplicability of simple excluded-volume theory to an electrolyte, but a linear dependence of the ordinate intercept upon cosolute concentration is again observed. These observations clearly also reinforce the conclusion that the Rayleigh scattering by proteins should not be interpreted in terms of single-solute theory, which predicts an invariant intercept ($1/M_2$) for a system comprising protein and solvent.

Because of the failure of light scattering data to yield B_{22}/M_2^2 in the absence of sucrose (Fig. 1), results deduced from the slopes of Debye plots are reported as $[(A_2)_3 - (A_2)_0]$, where the subscripts denote measurements in the presence (subscript 3) and absence (subscript 0) of cosolute. Such action also has the advantage of isolating the term containing the protein−cosolute contribution to the light scattering second virial coefficient in that

$$2[(A_2)_3 - (A_2)_0] = (\Omega c_3/M_3)/M_2^2. \quad (18)$$

Results of those light scattering experiments on isoelectric ovalbumin are summarized (●) in Fig. 5, which clearly conforms qualitatively with Eq. (18) in that the difference in measured virial coefficients exhibits the inverse dependence

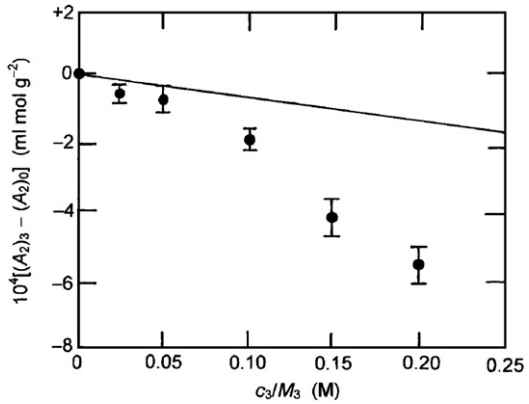

Fig. 5. Effect of buffer supplementation with sucrose (species 3) on the second virial coefficient of isoelectric ovalbumin (pH 4.6, I 0.04 M) determined by light scattering. Also shown (—) is the concentration dependence predicted by Eq. (14) with C_{223}/ρ_1^2 taken as zero.

upon sucrose concentration which should result from a value of A_2 that is dominated by negative contributions to the Ω term. However, the inverse dependence differs from that predicted (—) by Eq. (14) in two respects: (i) the extent greatly exceeds the theoretical prediction with C_{223}/ρ_1^2 taken as zero (in view of its relatively minor effect for the urate oxidase system); and (ii) the dependence is decidedly curvilinear. The increasing divergence at higher sucrose concentrations can be rationalized on the basis of superimposed ovalbumin self-association in the highly nonideal cosolute environment. Indeed, we note an earlier report of ovalbumin undergoing weak self-association [53]. Inasmuch as the theoretically predicted dependence provides a reasonable description of the experimental results at the low sucrose concentrations (where any sucrose-mediated self-association of ovalbumin should be minimal), we consider the results presented in Fig. 5 to verify the acceptability of Eqs. (11)–(14) as a quantitative description of cosolute effects on the second virial coefficient for an inert (nonassociating) protein that is obtained from the dependence of Rayleigh scattering ratio upon protein concentration.

3.4. Virial coefficient determination by sedimentation equilibrium

The results presented in Figs. 1 and 2 have clearly demonstrated the nonequivalence of second virial coefficients obtained by sedimentation equilibrium and light scattering. Thus far the major purpose of this investigation has been to establish that the parameter obtained from the Debye plot (A_2) is not B_{22}/M_2^2, the osmotic second virial coefficient that emanates from sedimentation equilibrium studies [4,8,14,20]. At this stage it seems appropriate to expand upon the earlier statement that small cosolutes can be regarded as part of the solvent in sedimentation equilibrium — the situation that justifies interpretation of the distributions in terms of single-solute theory.

In an experiment conducted at temperature T and angular velocity ω the equilibrium distribution of solute (protein) is described by the relationship [6–10]

$$z_2(r) = z_2(r_F)\exp[M_2(1-\bar{v}_2\rho_1)\omega^2(r^2-r_F^2)/(2RT)] \quad (19)$$

for the radial dependence of molar thermodynamic activity of protein, $z_2(r)$, as a function of that at a selected radial distance, r_F: ρ_1 is the density of unsupplemented solvent. For a mixture of protein and cosolute the molar activity coefficient for protein. $\gamma_2(r)$, is

$$\gamma_2(r) = \exp[2B_{22}c_2(r)/M_2 + B_{23}c_3(r)/M_3 + \ldots] \quad (20)$$

or, in an experiment with very low protein concentration. $[c_2(r)\rightarrow 0]$,

$$\gamma_2(r) \approx \exp[B_{23}c_3(r)/M_3 + \ldots]. \quad (21)$$

Furthermore, the cosolute concentration can be expressed as

$$c_3(r) \approx c_3(r_F)\exp[M_3(1-\bar{v}_3\rho_1)\omega^2(r^2-r_F^2)/(2RT)] \quad (22)$$

on the grounds that the relatively minor extent of small-cosolute redistribution at sedimentation equilibrium ensures essential constancy of the cosolute activity coefficient $[\gamma_3(r)\approx\gamma_3(r_F)]$. Combination of Eqs. (19), (21), and (22) then gives

$$c_2(r) = c_2(r_F)\exp[\{\omega^2(r^2-r_F^2)/(2RT)\} \times \{M_2(1-\bar{v}_2\rho_1)-(1-\bar{v}_3\rho_1)B_{23}c_3(r_F)\}] \quad (23)$$

as the relationship describing the protein distribution in a sedimentation equilibrium experiment conducted on a very dilute solution of protein supplemented with cosolute [13]. From the logarithmic form of Eq. (23) it follows that

$$(2RT/\omega^2)\mathrm{d}\ln c_2(r)/\mathrm{d}r^2 = M_2(1-\bar{v}_2\rho_1)-(1-\bar{v}_3\rho_1)B_{23}c_3(r_F) \quad (24)$$

where $c_3(r_F)$ may reasonably be identified as the cosolute concentration (c_3) in the mixture subjected to sedimentation equilibrium. The protein–cosolute contribution to nonideality thus gets incorporated into the buoyant molecular mass of the protein — the term on the left-hand side of Eq. (24). Indeed, it is possible to determine B_{23} experimentally from the slope of the dependence of $(2RT/\omega^2)\mathrm{d}\ln c_2(r)/\mathrm{d}r^2$ upon c_3 [13]. The concept of considering the cosolute to be part of the solvent stems from Casassa and Eisenberg [28], who expressed Eq. (24) in the form

$$(2RT/\omega^2)\mathrm{d}\ln c_2(r)/\mathrm{d}r^2 = M_2(1-\phi_2'\rho_d) \quad (25)$$

where ϕ_2' is the apparent specific volume of the protein and ρ_d the density of the cosolute–supplemented solvent.

From the above discussion it is evident that consideration of a small cosolute as part of the solvent is conditional upon use of the buoyant molecular mass, $(2RT/\omega^2)\mathrm{d}\ln c_2(r)/\mathrm{d}r^2$, that is derived either from a separate experiment with very low protein concentration or from the low concentration region of a high-speed sedimentation equilibrium experiment. With that proviso, the basic expression for sedimentation equilibrium [Eq. (19)] can be written as

$$c_2(r) = M_2 z_2(r_F)\exp[\{M_2(1-\phi_2'\rho_d)\omega^2(r^2-r_F^2)/(2RT)\} -2B_{22}c_2(r)/M_2 + \ldots] \quad (26)$$

whereupon the remaining parameters of unknown magnitude, $z_2(r_F)$ and B_{22}, may be determined by nonlinear curve-fitting of the $[r, c_2(r)]$ data from the high-concentration region of a sedimentation equilibrium experiment to Eq. (26) — a procedure illustrated in a previous study of equine serum albumin [5].

This section has shed further light on the combination of circumstances that render possible the evaluation of osmotic second virial coefficients for protein self-interaction from sedimentation equilibrium distributions for buffered protein solutions. Inasmuch as those distributions reflect the radial dependence of protein thermodynamic activity (z_2), they necessarily include the nonideality contribution arising from

protein–buffer interaction as well as that from protein self-interaction. However, the former is incorporated into the magnitude of the buoyant molecular mass of protein that is used for analysis of the sedimentation equilibrium distribution in terms of the expression for a single solute. In other words, sedimentation equilibrium measurements allow separate assessment of the effects of the two coefficients, B_{22} and B_{23}, that describe thermodynamic nonideality in a buffered protein solution. This situation also sets sedimentation equilibrium apart from osmometry, another technique for which measurements are made under the *constraint* of constant solvent chemical potential. Because osmotic pressure measurements provide only a single assessment of thermodynamic nonideality, the second virial coefficient obtained therefrom also includes the effects of nonideality arising from protein interactions with small molecules as well as those from self-interaction; and therefore also becomes increasingly negative with increasing ionic strength [54]. The correspondence between virial coefficients obtained by light scattering and osmometry was, of course, noted in the early stages of light scattering studies [55].

4. Concluding remarks

This investigation has drawn attention to the nonequivalence of second virial coefficients obtained for proteins by light scattering and sedimentation equilibrium. Whereas the second virial coefficient determined by light scattering (A_2) reflects the combined contributions of protein self-interaction and protein–buffer interactions to thermodynamic nonideality, that deduced from sedimentation equilibrium is B_{22} (or B_{22}/M_2^2), the osmotic second virial coefficient describing nonideality arising solely from protein self-interaction. The outcome of protein–buffer interaction is an effective attraction between protein molecules that opposes the repulsive force stemming from protein–protein interaction, especially in a polymer-containing medium [26,27]. Consequently, the value of the second virial coefficient obtained by light scattering underestimates B_{22}/M_2^2, a theoretical prediction [Eqs. (11)–(14)] that has also been observed experimentally (Figs. 1 and 2).

Misidentification of the parameter derived from light scattering as B_{22}/M_2^2 accounts for the reports of negative osmotic second virial coefficients that correlate with conditions conducive to protein crystal growth [1–3,31–33]. That indicator of potentially favourable conditions for protein crystallization (A_2 slightly negative) identifies situations wherein the repulsive force between identical protein molecules is outweighed by the consequences of protein−buffer and protein−cosolute interactions. Inasmuch as the net force between protein molecules then becomes an attraction, the use of a slightly negative light scattering virial coefficient as a diagnostic of potentially favourable conditions for protein crystal growth has a logical statistical–mechanical basis. However, it should not be termed the second virial coefficient for protein self-interaction, despite its determination from the dependence of Rayleigh scattering upon protein concentration.

Finally, as noted above, the erroneous identification of the second virial coefficient obtained by light scattering as B_{22}/M_2^2 almost certainly reflects the treatment accorded the technique in standard texts [34–36]. What is not stressed sufficiently is the fact that the quantitative expressions being presented therein refer specifically to single-solute systems (with omission of the refractive index contribution), the implication being that buffer components can be regarded as part of the solvent. In retrospect, it transpires that description of the virial coefficient as the osmotic second virial coefficient (B_{22}) is correct for sedimentation equilibrium but not for light scattering studies of buffered protein solutions.

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Appendix A. Adaptation of the light scattering equation to accommodate experimental protocol

The basic expression describing the Rayleigh excess scattering, Eq. (10a), (10b), requires modification to accommodate experimental conventions that the optical constant (K) is defined (i) in terms of the refractive index of the cosolute-supplemented solvent (n_s) rather than that of the solution (n), and (ii) in terms of the specific refractive index increment for solute, $\chi_2=(\partial n/\partial c_2)_{T,P,c_3}$ rather than its molal counterpart, $(\partial n/\partial m_2)_{T,P,m_3}$.

In order to express the refractive index of a protein solution, n, in terms of that for cosolute-supplemented solvent, n_s, and the specific refractive index increments of solute (χ_2) and cosolute (χ_3) we introduce the substitution

$$n = n_1 + \chi_2 c_2 + \chi_3 c_3 + \ldots = n_s[1 + (\chi_2/n_s)c_2 + \ldots] \qquad \text{(A1)}$$

where $n_s=n_1+c_3\chi_3$, correct to first order in concentration. Continuing in the same vein it follows that

$$(n_s/n_1)^2 = 1-2(\chi_2/n_s)c_2 + \ldots \qquad \text{(A2)}$$

To make the switch from $(\partial n/\partial m_2)^2$ to $(\partial n/\partial c_2)^2$ (i.e., χ_2^2) we need to use an extended expression relating the two concentration scales. Specifically,

$$c_i = \frac{\rho_1 M_i m_i}{1 + \rho_1 M_2 \bar{v}_2 m_2 + \rho_1 M_3 \bar{v}_3 m_3} \qquad \text{(A3)}$$

$$\approx \rho_1 M_i m_i (1-\rho_1 M_2 \bar{v}_2 m_2 - \rho_1 M_3 \bar{v}_3 m_3 + \rho_1^2 M_2^2 \bar{v}_2^2 m_2^2 + 2\rho_1^2 M_2 \bar{v}_2 M_3 \bar{v}_3 m_2 m_3 + \ldots)$$

where $i=2, 3$. Combination of these expressions with Eq. (A1) then gives(A4)

$$\begin{aligned} n = {} & n_1 + \chi_2\rho_1 M_2 m_2 \\ & + \chi_3\rho_1 M_3 m_3 - \chi_2 \bar{v}_2 \rho_1^2 M_2^2 m_2^2 \\ & - (\chi_3 \bar{v}_2 + \chi_2 \bar{v}_3)\rho_1^2 M_2 M_3 m_2 m_3 \\ & + (\chi_3 \bar{v}_2^2 + 2\chi_2 \bar{v}_2 \bar{v}_3)\rho_1^3 M_2^2 M_3 m_2^2 m_3 + .. \end{aligned} \quad \text{(A4)}$$

which on differentiation with respect to m_2 yields

$$\begin{aligned} \frac{(\partial n/\partial m_2)}{\chi_2\rho_1 M_2} = {} & 1 - 2\rho_1 M_2 \bar{v}_2 m_2 - \rho_1 M_3 [(\chi_3/\chi_2)\bar{v}_2 + \bar{v}_3] m_3 \\ & + 2\rho_1^2 M_2 M_3 [(\chi_3/\chi_2)\bar{v}_2^2 + 2\bar{v}_2 \bar{v}_3] m_2 m_3 + .. \end{aligned} \quad \text{(A5)}$$

Use of Eq. (9) to replace $\rho_1 M_i m_i$ by c_i then leads to the expression

$$\begin{aligned} \frac{(\chi_2\rho_1 M_2)^2}{(\partial n/\partial m_2)^2} = {} & 1 + 4\bar{v}_2 c_2 + 2[(\chi_3/\chi_2)\bar{v}_2 + \bar{v}_3] c_3 \\ & + 10[(\chi_3/\chi_2)\bar{v}_2^2 + \bar{v}_2 \bar{v}_3] c_2 c_3 + .. \end{aligned} \quad \text{(A6)}$$

The final quantity to be modified is the ratio of molal refractive index increments, Ψ, which becomes

$$\Psi = \Psi_0 [1 - \{(\chi_2/\chi_3)\bar{v}_3 + \bar{v}_2\} c_2 + \ldots] \quad \text{(A7)}$$

$$\Psi_0 = (\chi_3 M_3)/(\chi_2 M_2). \quad \text{(A8)}$$

The basic light scattering expression for experimental application can now be obtained by making the appropriate substitutions of Eqs. (A2), (A6), (A7) and (A8) into Eq. (10a), (10b).

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