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Biophysical Chemistry 120 (2006) 106 – 113

Biophysical Chemistry

http://www.elsevier.com/locate/biophyschem

Negative second virial coefficients as predictors of protein crystal growth: Evidence from sedimentation equilibrium studies that refutes the designation of those light scattering parameters as osmotic virial coefficients

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Received 10 August 2005; received in revised form 4 October 2005; accepted 4 October 2005
Available online 21 November 2005

Abstract

The effects of ammonium sulphate concentration on the osmotic second virial coefficient (B_{AA}/M_A) for equine serum albumin (pH 5.6, 20 °C) have been examined by sedimentation equilibrium. After an initial steep decrease with increasing ammonium sulphate concentration, B_{AA}/M_A assumes an essentially concentration-independent magnitude of 8–9 ml/g. Such behaviour conforms with the statistical–mechanical prediction that a sufficient increase in ionic strength should effectively eliminate the contributions of charge interactions to B_{AA}/M_A but have no effect on the covolume contribution (8.4 ml/g for serum albumin). A similar situation is shown to apply to published sedimentation equilibrium data for lysozyme (pH 4.5). Although termed osmotic second virial coefficients and designated as such (B_{22}), the negative values obtained in published light scattering studies of both systems have been described incorrectly because of the concomitant inclusion of the protein–salt contribution to thermodynamic nonideality of the protein. Those negative values are still valid predictors of conditions conducive to crystal growth inasmuch as they do reflect situations in which there is net attraction between protein molecules. However, the source of attraction responsible for the negative virial coefficient stems from the protein–salt rather than the protein–protein contribution, which is necessarily positive.

Keywords: Second virial coefficients; Sedimentation equilibrium; Light scattering; Protein crystallization; Protein–protein interaction; Protein–salt interaction; Lysozyme; Equine serum albumin

1. Introduction

In recent years there has been considerable interest in the potential use of second virial coefficients as a diagnostic of solution conditions commensurate with protein crystal growth [1–7]. From measurements of the second virial coefficient for self-interaction (B) by "static" (i.e., total intensity) light scattering it has been noted that values within a fairly narrow range (-2×10^{-4} to -8×10^{-4} mol ml g^{-2}) seem to describe the thermodynamic nonideality of dilute solutions (less than 10 mg/ml) under conditions commensurate with crystal growth for a range of proteins (see e.g., Table 1 of Ref. [5]). Inasmuch as a negative second virial coefficient implies a dominance of attractive forces between protein molecules, those light scattering data seemingly imply the involvement of protein self-association as a general step in the pathway towards crystallization. However, although the presence of protein aggregates at the onset of lysozyme crystallization is well-documented [8–12], their existence in lysozyme solutions prior to that nucleation stage has been questioned [13]. More convincing evidence for the lack of self-association was provided subsequently [14] by a sedimentation equilibrium study of the enzyme under similar conditions (acetate-chloride buffer, pH 4.5, 20 °C). Although the second virial coefficient deduced by this method also exhibits an inverse dependence upon ionic strength, it remains positive and approaches a limiting value that is in reasonable agreement with that predicted on the statistical–mechanical basis of excluded

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doi:10.1016/j.bpc.2005.10.003

volume [15] for a solid sphere with the Stokes radius of lysozyme [14,16].

There is clearly a need to trace the source of the discrepancy between estimates of second virial coefficients obtained by light scattering and sedimentation equilibrium procedures. In that regard the parameter obtained by the latter technique has been identified as the second virial coefficient that applies when the protein chemical potential is defined under the constraints of constant temperature and chemical potential of solvent [16–18]. Moreover, this osmotic second virial coefficient is amenable to direct molecular interpretation on the statistical basis of excluded volume [15]. On the other hand, the second virial coefficient obtained by light scattering is measured under circumstances where constant temperature and pressure are the constraints imposed upon the protein chemical potential being monitored [19–21]. It is therefore likely that the estimate of B from light scattering measurements may not be amenable to the simple molecular interpretation that has been attempted thus far.

In this study we employ sedimentation equilibrium to quantify the effect of ammonium sulphate concentration on the osmotic second virial coefficient for equine serum albumin under conditions (acetate buffer, pH 5.6, 20 °C) similar to those used in a corresponding light scattering study [7]. As with lysozyme [14], this more rigorous method again yields results that conform with statistical–mechanical predictions [15,16] for a non-associating protein. The nature of the negative second virial coefficients that are obtained by light scattering therefore needs further examination.

2. Theory

In sedimentation equilibrium the parameter governing solute distribution is the molar thermodynamic activity, z_A, defined under conditions of constant temperature, T, and chemical potential of solvent, μ_S [16–18]. Consequently, sedimentation equilibrium distributions for a single protein species may be used to evaluate osmotic virial coefficients [14,16,22].

2.1. Statistical–mechanical description of osmotic pressure

In experimental studies the osmotic pressure, Π, of a single solute species (A) with molar mass M_A in solvent (species 1) is conventionally expressed as the virial expansion

$$[\Pi/(RT)]_{T,\mu_S} = c_A/M_A + B_{AA}(c_A/M_A)^2 + \ldots \quad (1)$$

in which B_{AA}, the second virial coefficient for self-interaction, is an experimental parameter derived from the dependence of osmotic pressure upon the weight-concentration of solute, c_A. Rigorous statistical–mechanical considerations also lead to Eq. (1) with B_{AA} now reflecting the potential-of-mean-force between pairs of solute molecules [23]. Furthermore, Eq. (1) must also describe situations in which the protein undergoes self-association because the distinction between monomers and dimers, etc., is a non-thermodynamic exercise for a system with a single solute component [24]. As demonstrated elsewhere [24–26], the notional separation between "associative forces" (dimerization, etc.) and "non-associative forces" (excluded volume interaction) may be incorporated into Eq. (1) by identifying M_A as the monomer molar mass and the second virial coefficient as

$$B_{AA} = B^*_{AA} - K_2 \quad (2)$$

where K_2 is the dimerization constant and B^*_{AA} the coefficient describing excluded volume interactions between monomers. An estimate of the latter parameter may be obtained from the expression [27]

$$B^*_{AA} = \frac{16\pi N R_A^3}{3} + \frac{Z_A^2(1+2\kappa R_A)}{2I(1+\kappa R_A)^2} \quad (3)$$

for the second virial coefficient of a rigid impenetrable solute sphere with radius R_A bearing net charge Z_A spread uniformly over its surface. Avogadro's number (N) is included to express B^*_{AA} on a molar rather than a molecular basis. The inverse screening length (κ) may be calculated as $3.27\times10^7\sqrt{I}$ (cm^{-1}) from the molar ionic strength I. On the grounds that B^*_{AA} can therefore only be positive, the return of a negative osmotic second virial coefficient in an experimental context necessarily implicates a contribution from solute dimerization which exceeds that from excluded volume interaction between monomers: i.e., $K_2 > B^*_{AA}$ in Eq. (2) [25,26]. The stance that the second virial coefficient for self-interaction can assume negative values in the absence of protein self-association [13] is therefore seemingly untenable from the statistical–mechanical viewpoint.

2.2. Measurement of the second virial coefficient by sedimentation equilibrium

On the basis of the Gibbs–Duhem equation ($\sum \mu_i dn_i = 0$), Hill [23] has established that

$$(\partial\Pi/\partial c_A)_{T,\mu_S} = M_A c_A (\partial\mu_A/\partial c_A)_{T,\mu_S} \quad (4)$$

where

$$(\mu_A)_{T,\mu_S} = (\mu^o_A)_{T,\mu_S} + RT\ln z_A = (\mu^o_A)_{T,\mu_S} + RT\ln(\gamma_A c_A/M_A) \quad (5)$$

describes the dependence of solute chemical potential in terms of a standard-state value, $(\mu^o_A)_{T,\mu_S}$, and the molar thermodynamic activity (z_A) expressed as the product of molar concentration (c_A/M_A) and the corresponding activity coefficient (γ_A). Differentiation of Eqs. (1) and (5) to obtain the required partial derivatives in Eq. (4) leads to the conclusion [23] that

$$\ln\gamma_A = (2B_{AA}/M_A)c_A + \ldots \quad (6)$$

whereupon the expression relating the thermodynamic activity ($M_A z_A$ on a weight-concentration basis) to the weight concentration of solute becomes

$$M_A z_A = c_A \exp[2(B_{AA}/M_A)c_A + \ldots] \quad (7)$$

In sedimentation equilibrium of a single solute species at constant temperature (T) and angular velocity (ω) the thermo-

dynamic activity, $M_A z_A(r)$, at radial distance r is related to that, $M_A z_A(r_F)$, at a selected reference radial position, r_F, by the expression [28]

$$M_A z_A(r) = M_A z_A(r_F)\psi_A(r) \tag{8a}$$

$$\psi_A(r) = \exp\left[M_A(1-\bar{\nu}_A\rho_S)\omega^2\left(r^2-r_F^2\right)/(2RT)\right] \tag{8b}$$

in which $\bar{\nu}_A$ refers to the partial specific volume of the protein and ρ_S to the solvent density. Combination of Eqs. (7) and (8a,b) then yields the relationship

$$c_A(r) = M_A z_A(r_F)\psi_A(r) - 2(B_2/M_A)[M_A z_A(r_F)\psi_A(r)]^2 + \ldots \tag{9}$$

where $B_2 \equiv B_{AA}$. Nonlinear regression analysis of the dependence of $c_A(r)$ upon $\psi_A(r)$, a transform of radial distance r, in accordance with Eq. (9) thus leads to evaluation of the reference thermodynamic activity, $M_A z_A(r_F)$ and the osmotic second virial coefficient, B_{AA}/M_A, expressed on a weight (l/g) rather than molar (l/mol) basis [16,22]: division of B_2 by M_A yields the equivalent of the parameter B that has been used [1–7] as a diagnostic of protein crystallization conditions. Furthermore, the requirement of magnitudes for M_A, $\bar{\nu}_A$, and ρ_S can be obviated by curve-fitting the sedimentation equilibrium distribution for a low protein concentration (such that $M_A z_A \approx c_A$ for all r) to Eqs. (8a,b) in order to deduce $M_A(1-\bar{\nu}_A\rho_S)$ from the coefficient of the exponent [22]. The important point to note is that the nonideality parameter obtained by analysis of sedimentation distributions is unequivocally the osmotic second virial coefficient.

2.3. Molecular crowding effect of a small cosolute

Protein crystallization frequently requires the presence of a high concentration of a cosolute such as sodium chloride or ammonium sulphate, in which case account needs to be taken of its effect on the thermodynamic activity of the protein. In the presence of a molar concentration C_M of an inert cosolute (M) the counterpart of Eq. (6) for the protein activity coefficient (γ_A) becomes

$$\ln\gamma_A = 2(B_{AA}/M_A)c_A + B_{AM}C_M + \ldots \tag{10}$$

where B_{AM} is the second virial coefficient (l/mol) reflecting the protein–cosolute interaction. Because of its small size, the cosolute can be regarded as part of the solvent by predialysis of the protein solution against cosolute-supplemented buffer [29]. Under those circumstances it may be shown [30] that the sedimentation equilibrium distribution is described by the relationships

$$c_A(r) = c_A(r_F)\psi'_A(r) - 2(B_2/M_A)\left[c_A(r_F)\psi'_A(r)\right]^2 + \ldots \tag{11a}$$

$$\psi'_A(r) = \exp[\{M_A(1-\bar{\nu}_A\rho_S) - B_{AM}C_M(r_F)M_M(1-\bar{\nu}_M\rho_S)\} \times \omega^2\left(r^2-r_F^2\right)/(2RT)] \tag{11b}$$

where $B_2 \equiv B_{AA}$. Furthermore, $C_M(r_F)$ may reasonably be identified with C_M, the molar concentration of small solute in the diffusate [$C_M(r) \approx C_M(r_F) \approx C_M$ for all r]. In the terminology of Casassa and Eisenberg [29]

$$\psi'_A(r) = \exp\left[M_A\left(1-\phi'_A\rho_d\right)\omega^2\left(r^2-r_F^2\right)/(2RT)\right] \tag{12}$$

where ρ_d denotes the density of the cosolute-supplemented diffusate, and ϕ'_A the apparent partial specific volume of the protein. From an experimental viewpoint, the magnitude of the exponent coefficient in $\psi'_A(r)$ is again deduced from the sedimentation equilibrium distribution obtained with a sufficiently dilute protein solution for $c_A(r)$ to be described adequately by the linear term in Eq. (11a).

3. Materials and methods

A commercial preparation of equine serum albumin (Sigma Chemical Co., St. Louis, MO) was dissolved in acetate buffer (0.10 M acetic acid, pH adjusted to 5.6 with NaOH) and the irreversibly dimerized material removed by size exclusion chromatography on the Pharmacia FPLC system: no detectable dimer reformed within the time frame of this study (2 weeks). The purity of the isolated monomer was checked by employing SEDFIT [31] to analyze the extent of boundary spreading in a sedimentation velocity experiment conducted at 60,000 rev./min and 20 °C. On the grounds that the molecular mass of 65.0 kDa thus deduced from the ratio of sedimentation and diffusion coefficients essentially matched that of 66.2 kDa determined from the amino acid sequence [32], the purified equine serum albumin monomer was taken to be homogeneous with respect to molecular size.

Solutions of the albumin monomer (1 ml, 3 mg/ml) were first dialyzed exhaustively (4×500 ml) against ammonium sulphate-supplemented acetate buffer (0.10 M sodium acetate/acetic acid, 0–2 M ammonium sulphate), pH 5.6. Samples of each were then spun in a Beckman XL-I ultracentrifuge operated at 32,500 rev./min and 20°C, the resulting sedimentation equilibrium distributions in these experiments of meniscus-depletion design [33] being recorded interferometrically. In addition, a sample of each dialyzed protein solution was diluted to about 0.5 mg/ml with the relevant diffusate before centrifugation at 10,000 rev./min to obtain a low-speed sedimentation equilibrium distribution [34]. These distributions, recorded spectrophotometrically at 280 nm, were used to calculate the buoyant molecular mass, $M_A(1-\bar{\nu}_A\rho_S)$ or $M_A(1-\phi'_A\rho_d)$, for the assignment of magnitudes to $\psi_A(r)$ [Eq. (9)] or $\psi'_A(r)$ [Eqs. (11a,b)] in the analysis of high-speed sedimentation equilibrium distributions. Specifically, the low-speed distributions were fitted to Eqs. (8a,b) with $A_{280}(r)$ substituted for $M_A z_A(r)$, and with the exponent defined by Eqs. (8b) and (12) in the absence and presence, respectively, of ammonium sulphate.

Rayleigh interferometric distributions from the high-speed experiments were corrected for baseline variation on the basis of a relatively minor linear radial dependence of the absolute fringe displacement. The weight-concentration (mg/ml) at each

radial distance, $c_A(r)$, was then calculated from the corrected absolute fringe displacement, $J(r)$, by means of the relationship $c_A(r)=J(r)/3.33$ [35]. Results were then analyzed by nonlinear least-squares curve-fitting to Eq. (9) or Eq. (11a) with $\psi_A(r)$ [or $\psi'_A(r)$] defined on the basis of the buoyant molecular mass obtained from the corresponding low-speed experiment.

4. Results and discussion

Because sedimentation equilibrium is a technique with which many in the protein crystallization field are unfamiliar, some practical aspects of the evaluation of virial coefficients by this method [16,22] are reiterated. The results of a low-speed [34] sedimentation equilibrium experiment (10,000 rev./min) on a dilute solution of equine serum albumin in acetate buffer (pH 5.6, 20 °C) is shown in Fig. 1a, where the protein concentration ranges between 0.15 and 0.75 mg/ml. Over this concentration range the effect of thermodynamic nonideality arising from albumin self-interaction is negligible [$\exp\{(2B_{AA}/M_A)c_A\} \approx 1$ in Eq. (7)]; and hence the results may be analyzed in terms of Eqs. (8a,b) with $A_{280}(r)$ substituted for $M_A z_A(r)$ to obtain the buoyant molecular mass, $M_A(1-\bar{\nu}_A\rho_S)$. Such treatment of the sedimentation equilibrium distribution yields

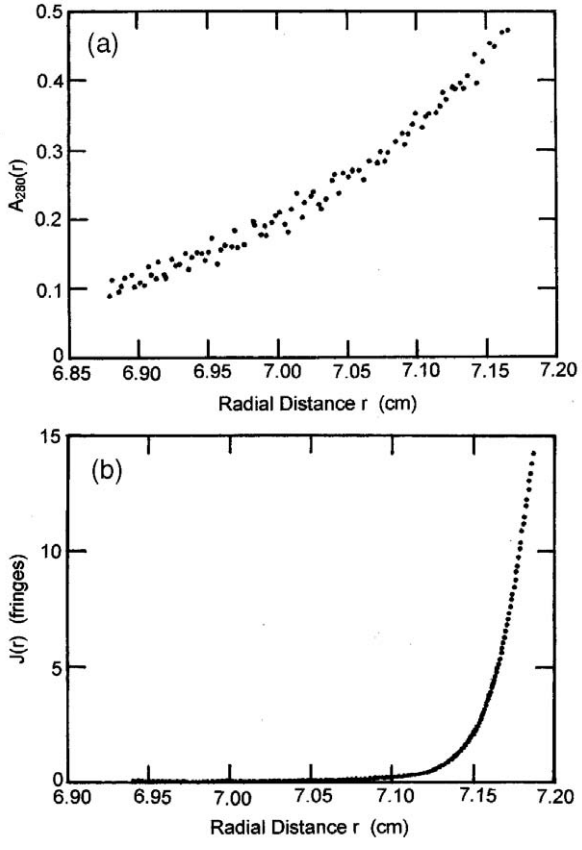

Fig. 1. Sedimentation equilibrium distributions used in the evaluation of the second virial coefficient for equine serum albumin (pH 5.6, I 0.05 M). (a) Low-speed (10,000 rev./min) sedimentation equilibrium distribution used to determine the buoyant molecular mass, $M_A(1-\bar{\nu}_A\rho_S)$, required for calculation of the psi function. (b) Rayleigh interferometric record of the high-speed (32,500 rev./min) sedimentation equilibrium distribution used to calculate the second virial coefficient.

a buoyant molecular mass of 17.1 (±0.2) kDa, which on combination with the partial specific volume of 0.736 ml/g calculated [36] from the amino acid composition [32] signifies a molecular mass of 65.0 (±0.8) kDa — a value that duplicates the estimate obtained above by SEDFIT analysis of sedimentation velocity distributions.

Of greater importance from the viewpoint of virial coefficient determination is that knowledge of the buoyant molecular mass allows calculation of $\psi_A(r)$ for the high-speed [33] sedimentation equilibrium experiment at 32,500 rev./min (Fig. 1b), in which the Rayleigh fringe number (J) ranges between zero and 15 ($0<c_A(r)<4.5$ mg/ml). The second virial coefficient for self-interaction ($B_2 \equiv B_{AA}$), as well as the reference thermodynamic activity $M_A z_A(r_F)$, may then be obtained from the expression for $c_A(r)$ as a designated quadratic in $\psi_A(r)$, Eq. (9). In principle, the magnitudes of $M_A(1-\bar{\nu}_A\rho_S)$, $M_A z_A(r_F)$ and B_{AA}/M_A may all be obtained by curve-fitting the [r, $c_A(r)$] data set to Eq. (9) with Eq. (8b) substituted for $\psi_A(r)$. However, such action would lead to greater uncertainty in the estimates as well as to increased correlation problems in the statistical assessment of errors associated with the parameter estimates. Furthermore, the separate low-speed experiment provides information on the extent of protein nonideality arising from the presence of high concentrations of ammonium sulphate. We consider that effect first.

4.1. Effect of ammonium sulphate on the thermodynamic activity of equine serum albumin

Although the presence of an additional component (ammonium sulphate) in the albumin solution subjected to sedimentation equilibrium precludes molecular mass determination because of the requirement for a value of the apparent partial specific volume ϕ'_A [29], the magnitude of the buoyant molecular mass, $M_A(1-\phi'_A\rho_d)$, is unequivocal in experiments with sufficiently small protein concentrations for self-interaction effects to be negligible. Furthermore, from Eqs. (11a,b) and (12) it is evident [30] that

$$M_A\left(1-\phi'_A\rho_d\right) = M_A(1-\bar{\nu}_A\rho_S) - B_{AM}C_M M_M(1-\bar{\nu}_M\rho_S) \tag{13}$$

which signifies the feasibility of extracting information on the protein–cosolute contribution ($B_{AM}C_M$) to the thermodynamic activity of the protein from the dependence of buoyant molecular mass upon cosolute concentration. The effect of ammonium sulphate concentration on the buoyant molecular mass of equine serum albumin in acetate buffer (pH 5.6) is very pronounced (Fig. 2). In that regard the curvilinear form of Fig. 2 certainly contrasts with the linear dependence observed in corresponding studies of proteins with sucrose as cosolute [30] — a finding which precludes the consideration of ammonium sulphate as a chemically inert cosolute in the present system. That observation is hardly surprising inasmuch as ammonium sulphate is an electrolyte that exhibits negative deviations from Raoult's Law (rather than the positive deviations symptomatic

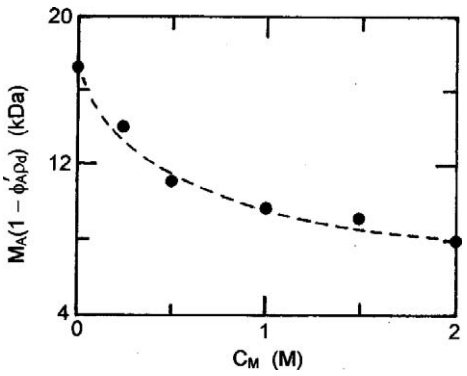

Fig. 2. Dependence of the buoyant molecular mass of equine serum albumin upon the concentration of ammonium sulphate included in the acetate buffer (pH 5.6).

of inertness) because of ion interactions with water. Nevertheless, the difference between $M_A(1-\bar{v}_A\rho_S)$ and $M_A(1-\phi'_A\rho_d)$ for a given ammonium sulphate concentration remains a quantitative measure of the contribution by the protein–cosolute interaction to protein nonideality [designated as $B_{AM}C_M$ in Eq. (13)].

To gain further insight into the size of the nonideality contribution ($B_{AM}C_M$) we can assign a value of 0.59 to $(1-\bar{v}_M\rho_S)=d\rho/dc_M$ from the concentration dependence of the density (ρ) of aqueous ammonium sulphate solutions that is reported in the Handbook of Chemistry and Physics, whereas M_M is 74 Da (the weight-average for NH_4^+ and $SO_4^=$ ions in 2:1 ratio) for ammonium sulphate. On those bases the magnitude of $B_{AM}C_M$, or ln (M_Az_A/c_A) [see Eq. (10)] is about 70 in the presence of 0.25 M ammonium sulphate, and increases a further two-fold on elevating the cosolute concentration to 2 M.

Although rigorous statistical–mechanical interpretation of the enhanced thermodynamic activity of equine serum albumin in the presence of ammonium sulphate as a solute–cosolute virial coefficient is precluded by the ionic nature of the cosolute, the quantification of that phenomenon in terms of its effect on the buoyant molecular mass of the protein (Fig. 2) renders possible progression to the next step—delineation of the effect of ammonium sulphate on the second virial coefficient (B_{AA}/M_A) for excluded volume self-interaction of equine serum albumin.

4.2. Effect of ammonium sulphate on the second virial coefficient for self-interaction of equine serum albumin

Analysis of the high-speed sedimentation equilibrium distribution for equine serum albumin in acetate buffer (Fig. 1b) in terms of Eq. (9) is presented in Fig. 3a. Values for $\psi_A(r)$ are based on the buoyant molecular mass for the protein in the absence of ammonium sulphate (Fig. 2) and the selection of a reference radial position (r_F) such that $c_A(r_F)=0.3$ mg/ml. Nonlinear least-squares curve-fitting of the $[\psi_A(r), c_A(r)]$ data set for $c_A(r)>2$ mg/ml yields the best-fit description shown (——) in Fig. 3a and a second virial coefficient, $B_2/M_A \equiv B_{AA}/M_A$, of 21.9 (±0.2) ml/g—the source of the downward curvature at higher $\psi_A(r)$ values. In that regard the decision to eliminate data at lower concentrations from the least-squares curve-fitting reflects their essential conformity with Eq. (9) truncated at the linear term, whereupon this region contains no information relevant to the magnitude of B_2/M_A. As required, however, the results in the low concentration range conform well with the best-fit description.

The corresponding analysis of the high-speed sedimentation equilibrium distribution for equine serum albumin in the presence of 2 M ammonium sulphate according to Eqs. (11a,b) is shown in Fig. 3b, about which two points are noted. First, the greater range of the $[\psi'_A(r), c_A(r)]$ data set for this distribution reflects the lower buoyant molecular mass under these conditions (Fig. 2) and hence a shallower concentration gradient that allows greater resolution of Rayleigh fringes. Secondly, because the curvilinearity of the plot remains convex to the $\psi'_A(r)$ axis, the second virial coefficient must still be positive. Indeed, nonlinear regression analysis of the $[\psi'_A(r), c_A(r)]$ data set for $c_A(r)>2$ mg/ml in terms of Eqs. (11a,b) yields a B_2/M_A of 8.8 (±0.1) mg/ml.

The dependence of B_2/M_A upon ammonium sulphate concentration is presented (●) in Fig. 4, together with the corresponding dependence (○) inferred from light scattering studies under similar conditions [7]. In that regard the disparity between estimates of B_2/M_A for equine serum albumin in the absence of ammonium sulphate reflects to some extent the use of a less appropriate concentration scale (g/ml solution rather than g/g solvent) in the light scattering studies [21]. Conse-

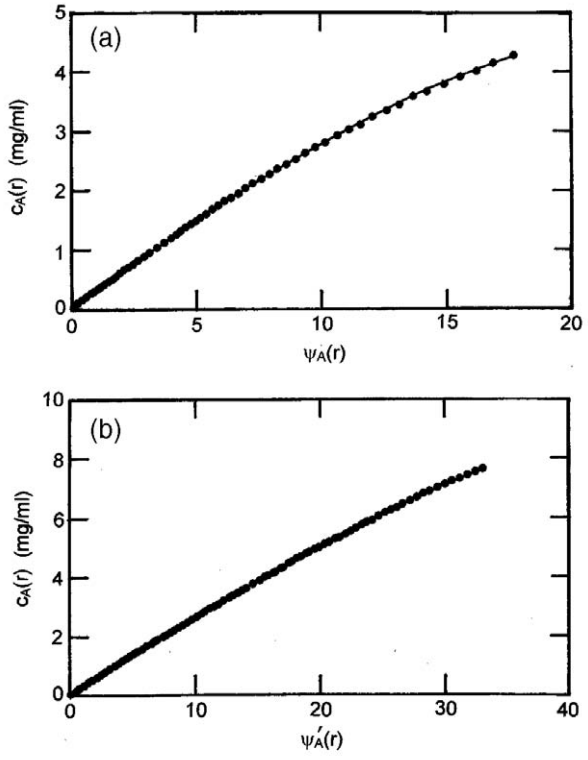

Fig. 3. Determination of the osmotic second virial coefficient for equine serum albumin. (a) Plot of results deduced from Fig. 1b according to Eq. (9). (b) Corresponding analysis of results obtained in the presence of 2 M ammonium sulphate [Eqs. (11a) and (12)].

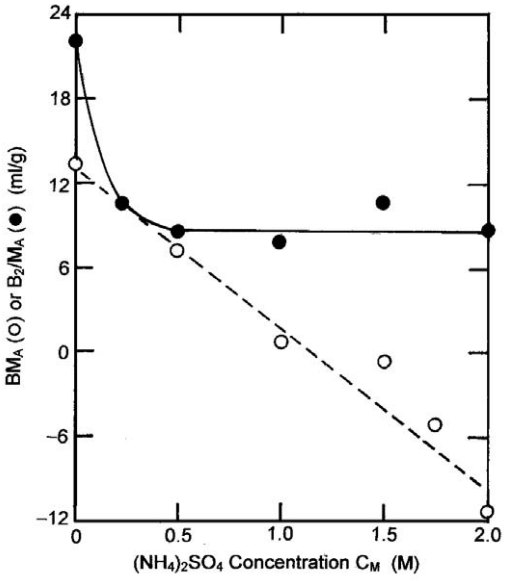

Fig. 4. Effect of ammonium sulphate concentration on the second virial coefficient for equine serum albumin in acetate buffer, pH 5.6. ●, present results; ○, results obtained by light scattering [7].

quently, the slope of the Debye plot (Kc_A/R_θ vs. c_A) underestimates B_2/M_A by at least twice the protein molar volume—see Eq. (49) of Hill [21]. Of greater concern, however, is the marked difference between the forms of the two concentration dependencies. After an initial steep decrease in B_2/M_A with increasing ammonium sulphate concentration, the current estimates of the second virial coefficient become essentially independent of cosolute concentration. As noted in Section 2, such behaviour conforms with the statistical–mechanical prediction that a sufficient increase in ionic strength should effectively eliminate the contribution of charge interactions to B_2 but have no effect on the covolume term [Eq. (3)]. Indeed, the asymptote in Fig. 4 corresponds to the limiting value of 8.4 ml/g for B_{AA}/M_A that is predicted for human serum albumin modelled as the oblate ellipsoid of revolution inferred [37,38] from the crystal structure [39] and a Stokes radius (R_A) of 3.5 nm: $2B_{AA}=10.262V_h$ where $V_h=4\pi NR_A^3/3$ (see [22]). Inasmuch as the results of the light scattering study [7] are clearly not reconcilable with such molecular interpretation on the statistical–mechanical basis of excluded volume, it must be concluded that the parameter termed B in that investigation is not the osmotic second virial coefficient for protein self-interaction.

4.3. Effect of sodium chloride on the second virial coefficient for lysozyme

Although the present study is the first to draw attention to a disparity between second virial coefficients deduced by light scattering and sedimentation equilibrium, there are separate literature reports that signify the existence of a similar problem in studies of the effect of sodium chloride inclusion on the thermodynamic nonideality of lysozyme in acetate buffer, pH 4.5 (Fig. 5). Open symbols denote results from published light scattering measurements [2,3,40] which establish conformity of lysozyme with the concept that a negative second virial coefficient is symptomatic of conditions favouring protein crystallization. Inasmuch as lysozyme is known to self-associate at neutral pH [41], this system could conceivably have reflected a situation in which the increase in ionic strength promoted enzyme self-association and hence rendered B_2 increasingly negative because of the contribution of the dimerization constant (K_2) to B_2 [see Eq. (2)]. However, results [14] obtained from sedimentation equilibrium distributions (●) bear a striking similarity to the present findings with equine serum albumin (Fig. 4) in that B_2/M_A approaches the lower-limiting positive value that is predicted on the statistical–mechanical basis of excluded volume [14,16]. The conclusion [41] that lysozyme does not self-associate under mildly acidic conditions is thus reinforced.

4.4. Second virial coefficients as predictors of protein crystallization conditions

The similarity of findings in studies with lysozyme and equine serum albumin adds weight to the above inference that the concentration dependence of the Rayleigh scattering ratio in the presence of cosolutes does not provide a measure of the osmotic second virial coefficient for protein self-interaction. In that regard it seems pertinent to draw on experience gained in the present study of equine serum albumin by sedimentation equilibrium, which renders possible a comparison of the relative contributions of $2(B_{AA}/M_A)c_A$ and cosolute-effected nonideality to the logarithm of the activity coefficient [Eq.

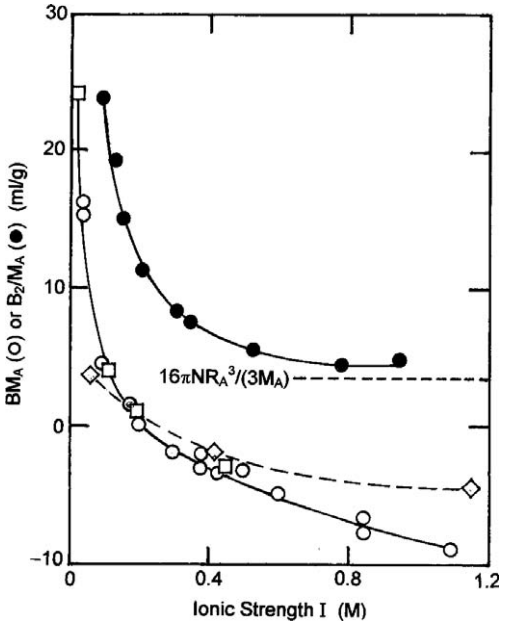

Fig. 5. Effect of ionic strength on the second virial coefficient for lysozyme in acetate buffer, pH 4.5. ●, results obtained from sedimentation equilibrium measurements [14]; □, ○, ◇, results obtained from light scattering measurements by Muschol and Rosenberger [2], Rosenbaum and Zukoski [3] and Bajaj et al. [40]. The lower-limiting asymptote for B_2/M_A ($\equiv B_{AA}/M_A$) is based on a radius (R_A) of 1.7 nm for lysozyme [14,16].

(10)]. For example, in the presence of 2 M ammonium sulphate $B_{AM}C_M$ is about 140, whereas $2(B_{AA}/M_A)c_A$ is only 0.084 for c_A=5 mg/ml. Excluded volume contributions from protein self-interaction are thus responsible for only a small proportion of the overall thermodynamic nonideality of the protein solution. It therefore seems probable that the negative B values obtained by light scattering reflect the sum of a positive contribution from the virial coefficient for protein self-interaction and a larger, negative contribution from the protein–cosolute interaction—a situation that has also been predicted by other thermodynamic reasoning [42].

Theoretical support for the above contention is provided by an early statistical–mechanical treatment of light scattering by a three-component system in which only one component (protein in the current context) contributes to the Rayleigh ratio [20]. From that treatment, developed in terms of molality to incorporate the constraints of constant temperature and pressure into the definition of the operative chemical potentials [19,20], the concentration dependence of the Rayleigh ratio, R_θ, is of the form

$$\frac{Kc_A}{R_\theta} \approx \frac{(1/M_A)+(1/M_A^2)\left[2B_{AA}-B_{AM}^2C_M/\left[1+C_M(\partial \ln\gamma_M/\partial C_M)_{C_M}\right]c_A\right.}{\left\{1-(M_M/M_A)B_{AM}C_M/\left[1+C_M(\partial \ln\gamma_M/\partial C_M)_{C_M}\right][(dn/dc_M)/(dn/dc_A)]\right\}^2} \quad (14)$$

where the final term in the denominator reflects the ratio of refractive index increments for cosolute and protein. This expression is presented as an approximation because of (i) slight differences in the earlier definition of the optical constant (K) and (ii) its failure to take into account the relatively minor errors associated with direct substitution of weight/volume concentrations and second virial coefficients for their molal counterparts [17,21,43]. The first point to be noted from Eq. (14) is that the slope of the dependence of Kc_A/R_θ upon c_A is certainly not governed solely by the second virial coefficient for protein self-interaction, which is the assumption upon which the reports of negative virial coefficients were based. Secondly, for each series of Rayleigh ratio measurements at constant cosolute concentration the denominator on the right-hand side of Eq. (14) is a constant (positive but less than unity) that affects equally the ordinate intercept and slope of the Debye plot (Kc_A/R_θ vs. c_A). For present purposes it suffices to regard its magnitude as effectively unity—a stance that is seemingly supported by the experimentally observed convergence of Debye plots for a series of cosolute concentrations to a common ordinate intercept. Under those circumstances the slope of the concentration dependence of Kc_A/R_θ underestimates the magnitude of B_{AA}/M_A^2 provided that $[1+C_M(\partial \ln\ \gamma_M/\partial C_M)_{C_M}]>-1$, a proviso that is fulfilled for both ammonium sulphate and sodium chloride at the concentrations employed. Furthermore, in view of the relatively large magnitudes of $B_{AM}C_M$ that have been calculated for the equine serum albumin–ammonium sulphate interaction, there is every likelihood that the negative protein–cosolute contribution to the slope of the Debye plot exceeds $2B_{AA}$. In other words, the negative virial coefficients obtained from light scattering measurements provide an overall indicator of protein nonideality.

The discrepancies between estimates of the second virial coefficient by sedimentation equilibrium and light scattering thus reflect differences in the nonideality parameter being monitored. Whereas the former provides an unequivocal measure of the osmotic second virial coefficient for protein self-interaction, the parameter obtained by light scattering also takes into account the contributions to protein nonideality arising from protein–cosolute interactions. Confusion has thus been caused by the misidentification [1–7,13,40] of the slope of the concentration dependence of Kc_A/R_θ in cosolute-supplemented solutions as twice the osmotic second virial coefficient (B_{AA}). As a measure of overall protein nonideality, the negative values of B obtained from light scattering studies do, indeed, signify the existence of a net attractive force between protein molecules, and hence there is a sound statistical–mechanical explanation for their correlation with conditions conducive to protein crystal growth.

5. Concluding remarks

This investigation has removed the paradox of negative osmotic second virial coefficients that do not reflect protein self-association [1–7]. Sedimentation equilibrium studies of equine serum albumin and lysozyme under conditions comparable with those for which the negative virial coefficients were obtained by light scattering have returned positive values of the osmotic second virial coefficient for protein self-interaction — values in keeping with their molecular interpretation on the statistical–mechanical basis of excluded volume. The negative virial coefficients obtained by light scattering reflect an anomaly in their description rather than a violation of statistical–mechanical theory.

Acknowledgements

Financial support from the United Kingdom Biotechnology and Biomolecular Sciences Research Council is gratefully acknowledged.

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