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Interpretation of thermodynamic non-ideality in sedimentation equilibrium experiments on proteins

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Abstract

This investigation re-examines theoretical aspects of the allowance for effects of thermodynamic non-ideality on the sedimentation equilibrium distribution for a single macromolecular solute, and thereby resolves the question of the constraints that pertain to the definition of the activity coefficient term in the basic sedimentation equilibrium expression. Sedimentation equilibrium results for ovalbumin are then presented to illustrate a simple procedure for evaluating the net charge (valence) of a protein from the magnitude of the second virial coefficient in situations where the effective radius of the protein can be assigned. Finally, published sedimentation equilibrium results on lysozyme are reanalysed to demonstrate the feasibility of employing the dependence of the second virial coefficient upon ionic strength to evaluate both the valence and the effective radius of the non-interacting solute.

Keywords: Sedimentation equilibrium; Virial coefficient; Excluded volume; Net charge; Ovalbumin; Lysozyme

1. Introduction

A recent investigation [1] has again drawn attention to the measurement of the second virial coefficient by direct analysis of sedimentation equilibrium distributions for a single macromolecular solute [2–4]. Although the derivation of thermodynamic identities presented in [1] has earlier, equivalent counterparts [4–7], there are aspects of their derivation and application that merit closer scrutiny. Specifically, we have now developed a means of casting the basic sedimentation equilibrium equation in a form that establishes the nature of the thermodynamic activity being monitored, without resort to use of virial

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expansions. As well as examining theoretical aspects, this investigation employs the results of sedimentation equilibrium studies of ovalbumin under isoelectric conditions (pH 4.59, I 0.16) and at pH 8.5, where the protein bears a net negative charge of −14 [2], in order to illustrate a much simpler application of the theory for determining the second virial coefficient.

2. Theory

For a solution of single solute with molecular mass M_A the criterion for sedimentation equilibrium at constant temperature, T, can be written [6] in terms of the chemical potential of solute, μ_A, as

$$d\mu_A - M_A\omega^2 r dr = 0 \quad (1)$$

where ω is the angular velocity and r the radial distance. The differential $d\mu_A$ can then be expressed in terms of the independent variables pressure, P, and solute molal concentration, m_A. Specifically,

$$d\mu_A = (\partial\mu_A/\partial P)_{T,m_A} dP + (\partial\mu_A/\partial m_A)_{T,P} dm_A \quad (2)$$

On noting that $(\partial\mu_A/\partial P)_{T,m_A} = M_A\bar{v}_A$ and that $dP = \omega^2 r\rho(r)dr$, we then obtain

$$M_A[1 - \bar{v}_A\rho(r)]\omega^2 r = (\partial\mu_A/\partial m_A)_{T,P}(dm_A/dr) \quad (3)$$

as the description of the sedimentation equilibrium distribution for a single solute with partial specific volume, $\bar{v}_A$, in terms of the molal concentration gradient, dm_A/dr, and $\rho(r)$, the solution density at radial distance r. Adaptation of Eq. (3) for compatibility with the optical recording of the sedimentation equilibrium distribution requires a transition from the molal to the molar concentration scales. The simplest way to effect this transition is to express the solute chemical potential in terms of partial derivatives with respect to pressure and molar concentration, i.e.

$$d\mu_A = (\partial\mu_A/\partial P)_{T,C_A} dP + (\partial\mu_A/\partial C_A)_{T,P} dC_A \quad (4)$$

For incompressible solutions, i.e. those for which the solution density is pressure independent, the result is [6]

$$M_A[1 - \bar{v}_A\rho(r)]\omega^2 r dr = (\partial\mu_A/\partial C_A)_{T,P} dC_A \quad (5)$$

Two difficulties are encountered in attempts to use this equation. First, the composition dependence of $\rho(r)$ has to be taken into account to achieve the separation of variables required for integration [1,4,5,7]. Secondly, the form of the differential $(\partial\mu_A/\partial C_A)_{T,P}$ is odd in that it entails differentiation of a molal chemical potential with respect to molar concentration — a situation that is discussed again later. Whereas Behlke and Ristau [1] have employed virial expansions and the condition of solution incompressibility to render the problem tractable, we wish to illustrate a much more direct approach to the problem.

Instead of expressing the solute chemical potential in terms of Eq. (4), we choose to express it in terms of solvent chemical potential (μ_s) and solute molar concentration (C_A) as [8]

$$d\mu_A = (\partial\mu_A/\partial\mu_s)_{T,C_A} d\mu_s + (\partial\mu_A/\partial C_A)_{T,\mu_s} dC_A \quad (6)$$

the form of expression more often associated with osmotic pressure studies. The description of osmotic equilibrium defines a thermodynamic activity z_A on the molar scale. Because

$$\mu_A(T,\mu_s,C_A) = \mu_A^o(T,\mu_s) + RT\ln z_A(C_A) \quad (7)$$

we identify the second term in Eq. (6) as $RT\,d\ln z_A$, whereupon the condition for sedimentation equilibrium becomes

$$M_A\omega^2 r dr - (\partial\mu_A/\partial\mu_s)_{T,C_A} d\mu_s = RT\,d\ln z_A \quad (8)$$

Using the same condition for solvent, $d\mu_s = M_s\omega^2 r dr$, we have

$$\left[M_A - M_s(\partial\mu_A/\partial\mu_s)_{T,C_A}\right]\omega^2 r\,\mathrm{d}r = \mathrm{d}\ln z_A \tag{9}$$

The partial derivative on the left-hand side can be found by differentiating Eq. (5) with respect to μ_s at constant T and C_A to obtain

$$(\partial\mu_A/\partial\mu_s)_{T,C_A} = (\partial\mu_A/\partial P)_{T,C_A}(\partial P/\partial\mu_s)_{T,C_A} \tag{10}$$

where the corresponding differential of μ_A with respect to C_A has disappeared because $(\partial C_A/\partial\mu_s)_{T,C_A} = 0$. Our general thermodynamic expression for the condition of sedimentation equilibrium thus becomes

$$\left[M_A - M_s(\partial\mu_A/\partial P)_{T,C_A}(\partial P/\partial\mu_s)_{T,C_A}\right]\omega^2 r\,\mathrm{d}r = \mathrm{d}\ln z_A \tag{11}$$

Introducing the condition of solution incompressibility at this stage allows us to make the identifications

$$(\partial\mu_A/\partial P)_{T,C_A} = M_A\bar{v}_A \tag{12a}$$

$$(\partial P/\partial\mu_s)_{T,C_A} = 1/(M_s\bar{v}_s) = \rho_s/M_s \tag{12b}$$

in which case the basic sedimentation equilibrium expression becomes [4,5,8]

$$M_A(1-\bar{v}_A\rho_s)\omega^2 r\,\mathrm{d}r = RT\,\mathrm{d}\ln z_A \tag{13}$$

where ρ_s is the solvent density. The advantages of Eq. (13) as the expression for sedimentation equilibrium are twofold. First, the separation of variables allows its integration to yield

$$z_A(r) = z_A(r_F)\exp\left[M_A(1-\bar{v}_A\rho_s)\omega^2(r^2-r_F^2)/(2RT)\right] \tag{14}$$

where r_F is any chosen reference radial position. Secondly, the relevant activity coefficient is defined as $\gamma_A = z_A/C_A$, the parameter relevant to statistical-mechanical interpretation in terms of the McMillan–Mayer theory [9].

We now wish to rationalise further the conclusion that molar activity, z_A, provides the best description of sedimentation equilibrium, despite the fact that Eq. (3) written as

$$M_A[1-\bar{v}_A\rho(r)]\omega^2 r\,\mathrm{d}r = RT\,\mathrm{d}\ln a_A \tag{15}$$

seemingly implies that the relevant thermodynamic parameter is the molal activity, a_A, defined by the expression

$$\mu_A(T,P,m_A) = \mu_A^o(T,P) + RT\ln a_A(m_A) \tag{16}$$

This apparent paradox is readily resolved.

Consider the differentiation of Eq. (2) with respect to molar concentration C_A under conditions of constant temperature T and chemical potential of solvent μ_s. Because the fundamental thermodynamic expression [10] relating chemical potential to numbers of moles, n_i, entropy S, volume V, temperature and pressure, namely, $n_s\mathrm{d}\mu_s + n_A\mathrm{d}\mu_A = S\mathrm{d}T + V\mathrm{d}P$, dictates that

$$\mathrm{d}P = C_A(\partial\mu_A/\partial C_A)_{T,\mu_s}\mathrm{d}C_A \tag{17}$$

the chosen differential form of Eq. (2) becomes

$$(\partial\mu_A/\partial C_A)_{T,\mu_s} = (\partial\mu_A/\partial m_A)_{T,P}(\partial m_A/\partial C_A)_{T,\mu_s} + C_A(\partial\mu_A/\partial P)_{T,m_A} \times (\partial\mu_s/\partial C_A)_{T,\mu_s} \tag{18}$$

bearing in mind the fact that $C_A = n_A/V$.

For incompressible solutions the molal and molar concentration scales are related by

$$m_A = C_A/[\rho_s(1-M_A\bar{v}_A C_A)] \tag{19a}$$

and the differential of molality with respect to molarity is the perfect derivative,

$$\mathrm{d}m_A/\mathrm{d}C_A = \rho_s/(1-M_A\bar{v}_A C_A)^2 \tag{19b}$$

Eq. (18) then becomes

$$(\partial\mu_A/\partial C_A)_{T,P} = (\partial\mu_A/\partial C_A)_{T,\mu_s}(1-M_A\bar{v}_A C_A) \tag{20}$$

Furthermore, the composition-dependent buoyancy factor for an incompressible solution may be written [1,4,5,7,8]

$$[1-\bar{v}_A\rho(r)] = (1-\bar{v}_A\rho_s)(1-M_A\bar{v}_AC_A) \quad (21)$$

Substitution of Eqs. (20) and (21) into Eq. (3) then leads to cancellation of the $(1-M_A\bar{v}_AC_A)$ factors and hence to the derivation of Eq. (13) as the basic sedimentation equilibrium expression for incompressible solutions.

2.1. Statistical-mechanical interpretation

The interpretation of Eq. (13) is best understood by considering the differentiation of Eq. (17) with respect to molar concentration,

$$(\partial\Pi/\partial C_A)_{T,\mu_s} = C_A(\partial\mu_A/\partial C_A)_{T,\mu_s} \quad (22)$$

where the pressure P has been replaced by osmotic pressure Π [10] on the grounds that $\Pi = P - P_o$, where P_o is the constant reference pressure on the solvent side of the semipermeable membrane in an osmotic experiment ($dP \equiv d\Pi$). It then follows from Eq. (17) that

$$\begin{aligned}(\partial\mu_A/\partial C_A)_{T,\mu_s} &= RT\,d\ln z_A/dC_A \\ &= RT[(1/C_A) + d\ln\gamma_A/dC_A]\end{aligned} \quad (23)$$

The McMillan–Mayer theory [9], which relates the variation in osmotic pressure to molecular parameters, can therefore be used directly to interpret effects of non-ideality on sedimentation equilibrium.

The virial expansion for osmotic pressure in terms of solute concentration can be written as

$$\Pi/(RT) = C_A + \mathbf{B}_2C_A^2 + \mathbf{B}_3C_A^3 + \ldots \quad (24)$$

where the virial coefficients $\mathbf{B}_2$, $\mathbf{B}_3$, etc., have exact definitions in terms of the potential-of-mean-force between individual solute molecules [9–12]. The corresponding expansion of the molar activity coefficient γ_A in terms of the same parameters is [12]

$$\ln\gamma_A = 2\mathbf{B}_2C_A + (3/2)\mathbf{B}_3C_A^2 + \ldots \quad (25)$$

which follows directly from Eq. (22).

The most direct procedure for extracting the magnitudes of virial coefficients from the sedimentation equilibrium distribution for a single solute entails the combination of Eqs. (13), (14) and (22)–(25) to obtain [8]

$$\begin{aligned}\ln\psi_A(r) = &-\ln z_A(r_F) + \ln C_A(r) \\ &+ 2\mathbf{B}_2C_A(r) + (3/2)\mathrm{B}_3[C_A(r)]^2 + \ldots\end{aligned} \quad (26a)$$

where

$$\begin{aligned}\psi_A(r) = \exp\big[&M_A(1-\bar{v}_A\rho_s)\omega^2(r^2-r_F^2) \\ &/(2RT)\big]\end{aligned} \quad (26b)$$

is a renormalisation of the independent variable r when the buoyant molecular mass, ω, and T are already specified. Alternatively, advantage may be taken of the expansion of molar concentration as a power series in molar activity [8,13–15],

$$\begin{aligned}C_A(r) = &z_A(r) - 2\mathbf{B}_2[z_A(r)]^2 \\ &+ \big[6\mathbf{B}_2^2 - (3/2)\mathbf{B}_3\big][z_A(r)]^3 + \ldots\end{aligned} \quad (27)$$

which may be expressed in terms of the $\psi_A(r)$ function as

$$\begin{aligned}C_A(r) = &z_A(r_F)\psi_A(r) - 2\mathbf{B}_2[z_A(r_F)\psi_A(r)]^2 \\ &+ [6\mathbf{B}_2 - (3/2)\mathbf{B}_3][z_A(r_F)\psi_A(r)]^3 + \ldots\end{aligned} \quad (28)$$

Inasmuch as $z_A(r_F)$ and the $\mathbf{B}_i$ are constants, $C_A(r)$ is a specified polynomial in the single independent variable $\psi_A(r)$. Evaluation of virial coefficients by sedimentation equilibrium thus merely entails non-linear regression analysis of the radial dependence of solute concentration in terms of Eq. (28) to obtain $z_A(r_F)$ and the virial coefficients $\mathbf{B}_2$, $\mathbf{B}_3$, etc., as curve-fitting parameters. In practice it transpires that the accuracy with which present analytical ultracentrifuges describe the $C_A(r) - \psi_A(r)$ dependence dictates the truncation of Eq. (28) at the quadratic term: $\mathbf{B}_2$ is thus the only virial coefficient to be obtained by such analysis.

3. Experimental

Prior to sedimentation equilibrium experiments the salt-free, crystalline preparation of ovalbumin (Sigma Grade V) was dissolved in either veronal–chloride buffer (0.04 M sodium diethylbarbiturate–0.01 M diethylbarbituric acid–0.07 M sodium chloride; pH 8.5, I 0.11), or acetate–chloride buffer (0.01 M sodium acetate–0.01 M acetic acid–0.15 M sodium chloride; pH 4.59, I 0.16); and then dialysed against more of the same buffer to obtain a solution with chemical potential defined under the constraint of constant chemical potential of solvent [16]. The dialysed ovalbumin solutions (approx. 2 mg/ml) were then subjected to sedimentation equilibrium at 23 000 rev./min and 20°C in a Beckman XL-I ultracentrifuge — a speed sufficient to ensure experiments of the meniscus-depletion type [17]. 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 fringes for a 1-mg/ml protein solution in a 12-mm cell [18]. The evaluation of $\psi_A(r)$ from Eq. (26b) has been based on a molecular mass of 44 kDa and a partial specific volume of 0.736 ml/g for ovalbumin [19], together with measured buffer densities of 1.0037 and 1.0042 g/ml for the veronal–chloride and acetate–chloride buffers, respectively.

4. Results

Equilibrium solute distributions obtained by subjecting solutions of ovalbumin in acetate–chloride (pH 4.59, I 0.16) and veronal–chloride (pH 8.5, I 0.11) buffers to centrifugation at 20°C and 23 000 rev./min are presented in Fig. 1a,b, respectively. Despite the use of a rotor speed that sufficed to decrease the solute concentration at the meniscus to effectively zero, the sensitivity of the Rayleigh interference optical system sufficed to resolve the solute distribution up to a protein concentration of approximately 10 mg/ml — a range commensurate with good definition of the second virial coefficient. Analyses of these high-speed sedimentation equilibrium distributions [17] in terms of Eq. (28) are summarised in Fig. 2a,b, where the solid lines denote the best-fit descriptions obtained by non-linear curve-fitting. A value (± 2 S.D.) of 221 (± 4) l/mol is obtained for the second virial coefficient, $\mathbf{B}_2$, for isoelectric ovalbumin (pH 4.59, I 0.16), whereas a larger $\mathbf{B}_2$ of 417 (± 8) l/mol is obtained for ovalbumin in veronal–chloride buffer (pH 8.5, I 0.11) because of the effect of the net negative charge on the protein under these conditions.

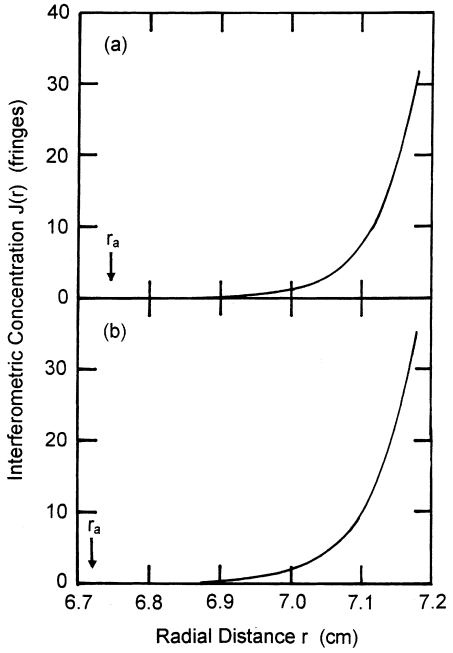

Fig. 1. High-speed [17] sedimentation equilibrium distributions obtained by centrifugation (20°C, 23 000 rev./min) of ovalbumin in (a) acetate–chloride buffer (pH 4.59, I 0.16), and (b) veronal–chloride buffer (pH 8.5, I 0.11). r_a denotes the radial position of the air–liquid meniscus.

Consideration of the thermodynamic non-ideality on the statistical-mechanical basis of excluded volume [9] gives rise to the expression [20]

$$\mathbf{B}_2 = 16\pi N R_A^3/3 + \left[Z_A^2/(4I)\right]\left[(1+2\kappa R_A)/(1+\kappa R_A)^2\right] \quad (29)$$

for the osmotic second virial coefficient. R_A is the radius of the protein modelled as a rigid impene-

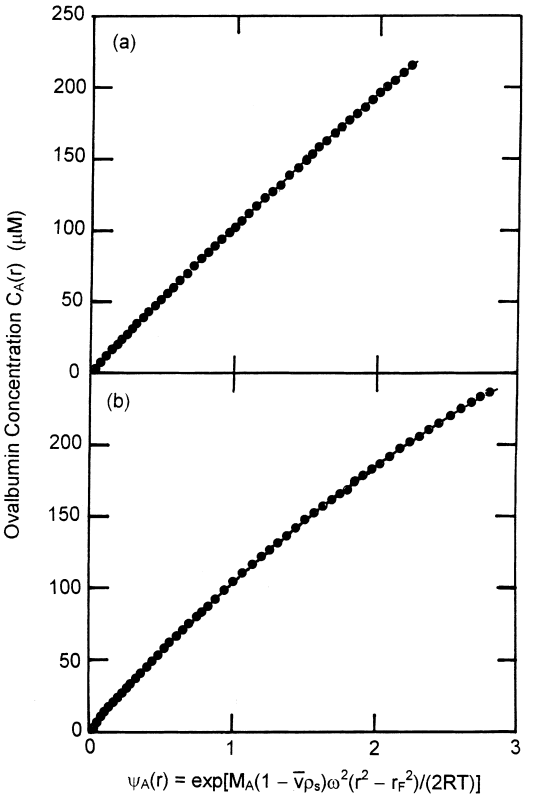

Fig. 2. Non-linear regression analysis of the sedimentation equilibrium distributions for ovalbumin (Fig. 1) in terms of Eq. (28) with r_F selected to correspond to a protein concentration of 102.4 μM [$J(r_F) = 15$ fringes], the solid lines being the best-fit descriptions. (a) Isoelectric ovalbumin (pH 4.59, I 0.16): $z_A(r_F) = 107.5$ (±0.2) μM, $\mathbf{B}_2 = 221$ (±4) l/mol; (b) ovalbumin in veronal–chloride buffer (pH 8.5, I 0.11): $z_A(r_F) = 113.0$ (±0.6) μM, $\mathbf{B}_2 = 417$ (±8) l/mol.

trable sphere with net charge Z_A distributed uniformly over its surface; and N is Avogadro's number. κ, the inverse screening length (cm^{-1}), may be calculated as $3.27 \times 10^7\sqrt{I}$, where I is the molar ionic strength. In that regard we note that ovalbumin is more appropriately represented as a triaxial ellipsoid [21], a shape for which the covolume term in Eq. (29) is available [22]. However, the consequences of uncertainties in the extent of protein solvation far outweigh those emanating from changes in the relative magnitudes of the three axes [21], and there is no corresponding charge repulsion term available for a triaxial ellipsoid. We therefore proceed with analysis of the results in terms of spherical geometry on the basis that this approximation should have minimal effect on the statistical-mechanical interpretation of the magnitude of $\mathbf{B}_2$.

Substitution of the above estimate of $\mathbf{B}_2$ for isoelectric ovalbumin ($Z_A = 0$) into Eq. (29) leads to a magnitude of 2.80 (±0.05) nm for the effective solvated radius of ovalbumin. This value is marginally smaller than the previous estimates of 2.92 (±0.03) nm deduced by omega analysis [23] of sedimentation equilibrium distributions for isoelectric ovalbumin [2,3], but is encompassed by the estimate of 2.84 (±0.15) nm obtained by frontal exclusion chromatography [24]. Interpretation of the second virial coefficient for ovalbumin in the veronal–chloride buffer in terms of Eq. (29) and the present estimate of 2.80 nm for R_A signifies a net anionic charge of 14.1 (±0.6), which essentially duplicates the Z_A of −14 deduced [2] from pH-titration and chloride-binding studies. In that regard, the much higher anionic charge ($Z_A = -27$) reported by Behlke and Ristau [1] on the basis of their application of Eq. (29) to results for ovalbumin under similar conditions (pH 8.5, I 0.148) is difficult to reconcile with either the known amino acid sequence [25] or the electrophoretic behaviour [26] of ovalbumin. In fact the high estimate of Z_A merely reflects the assignment of a low magnitude (2.5 nm) to the protein radius.

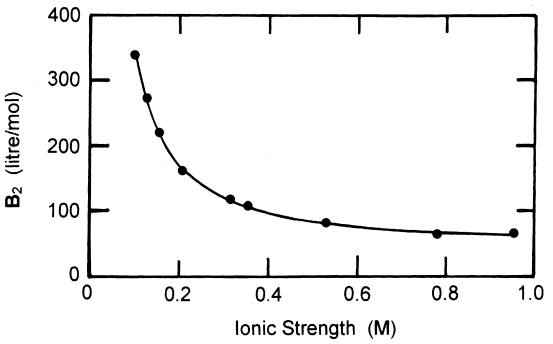

Fig. 3. Dependence of the second virial coefficient $\mathbf{B}_2$ of lysozyme in acetate–chloride buffer (pH 4.5) upon ionic strength, the data being taken from Fig. 2 of Behlke and Ristau [1]. The solid line is the best-fit description obtained by non-linear regression analysis in terms of Eqs. (28) and (29) with the effective radius (R_A) and net charge (Z_A) of the protein as curve-fitting parameters.

The above results for ovalbumin have illustrated the feasibility of evaluating the net charge of a protein from the magnitude of the second virial coefficient provided that a reliable estimate can be assigned to the protein radius R_A. We now employ the results of Behlke and Ristau [1] for lysozyme at pH 4.5 to explore the possibility of deducing both Z_A and R_A by interpreting the ionic-strength dependence of $\mathbf{B}_2$ in terms of Eq. (29). The experimental results from Fig. 2 of Behlke and Ristau [1] are presented in Fig. 3, together with the best-fit description (—) obtained by non-linear regression analysis in terms of Eq. (29) with R_A and Z_A as curve-fitting parameters. The fact that the returned estimate (± 2 S.D.) of 14.1 (± 0.2) for the net charge duplicates that of 14.1 (± 1) deduced by Behlke and Ristau [1] reflects the close agreement between the present estimate of 1.69 (± 0.06) nm for R_A and that of 1.72 nm assigned to this parameter in the original analysis [1]. Although pH-titration data [27] signify a nett charge of $+12$ at this pH, that estimate does not take into account the effects of proton release that seems to accompany lysozyme dissociation into monomer in the acid pH range [28,29].

5. Discussion

This probe into the evaluation of the second virial coefficient for a single protein solute by sedimentation equilibrium has served three major roles. First, it has confirmed the correctness of the original formulation of the basic sedimentation equilibrium equation [6,7], which came into question as the result of our earlier demonstration [4,5] that the sedimentation equilibrium distribution for incompressible solutions reflects the molar solute activity, z_A, a parameter normally associated with chemical potential of solute defined under the constraint of constant solvent chemical potential. This reconsideration of the theoretical aspects of sedimentation equilibrium has also resolved the disparity between the constraints imposed on the activity coefficient term in the Australasian [4,5] and Wisconsin [6,7] versions of the basic sedimentation equilibrium equation. Whereas the use of Eqs. (3), (15) and (16) gives rise to the definition of an activity coefficient $y_A = a_A/m_A$, or $y'_A = a_A/C_A$ as Eq. (5) would dictate, the activity coefficient relevant to the integrable form of the sedimentation equilibrium equation for incompressible solutes [Eq. (13)] is $\gamma_A = z_A/C_A$. Although there have been differences in the nomenclature for activity coefficients, it is now clear that the odd form y'_A, which entails division of a molal activity by a molar concentration, is indeed the activity coefficient appearing in the original versions of sedimentation equilibrium theory [6,7,30–32] and the recent analysis by Behlke and Ristau [1].

The second role of this investigation has been the illustration of a means of evaluating $\mathbf{B}_2$ that is much simpler than the procedure used by Behlke and Ristau [1]. In that regard the decision to replace the conventional expansion of $z_A(r)$ as a power series in $C_A(r)$ by its counterpart with $C_A(r)$ as a power series in $z_A(r)$ [8,13–15] is a major source of simplification.

Thirdly, the magnitude of $\mathbf{B}_2$ obtained by sedimentation equilibrium of ovalbumin under isoelectric conditions (Fig. 2a) has allowed the assignment of a covolume radius (R_A) to this protein — an assignment which has afforded a means of estimating a net charge of -14 for ovalbumin in veronal–chloride buffer (pH 8.5, I 0.11) on the basis of the larger $\mathbf{B}_2$ for the protein under the more alkaline conditions. In that regard the inference [1] that our earlier estimate of the net charge [2] was unreasonably low because of the use of an overestimate for the protein radius is incorrect: our values of Z_A then and now are based on experimental estimates of R_A. Clearly, great care is required in the assignment of a magnitude to R_A if the object of the exercise is to evaluate solute valence (Z_A) from the magnitude of $\mathbf{B}_2$. Indeed, inasmuch as methodology is still available for unequivocal estimation of protein valence [33,34] it can be argued that the magnitude of $\mathbf{B}_2$ should preferably be used to evaluate R_A on the basis of a known value of Z_A. Alternatively, estimates of both R_A and Z_A may be obtained under favourable circumstances by statistical-mechanical interpretation of the ionic-

strength dependence of the second virial coefficient (Fig. 3).

To conclude, we wish to emphasise the importance of evaluating parameters such as Z_A and R_A for protein solutes because of their relevance to allowance for effects of thermodynamic non-ideality in the characterisation of interactions between dissimilar macromolecular reactants. Characterisation of such interactions is conditional upon knowledge of these two parameters for the individual reactants — knowledge that also allows more confident assignment of the required values of the effective radius and net charge of the postulated complex(es) formed between the dissimilar protein species. It is hoped that the present investigation and the precursor [1] that prompted this more detailed examination of the issues may stimulate further evaluation of virial coefficients by sedimentation equilibrium; and thereby set the stage for quantitative characterisation of relatively weak associative interactions, for which effects of thermodynamic non-ideality cannot be ignored.

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