ELSEVIER

Biophysical Chemistry 104 (2003) 345–359

Biophysical Chemistry

www.elsevier.com/locate/bpc

Allowance for thermodynamic non-ideality in the characterization of protein self-association by frontal exclusion chromatography: hemoglobin revisited

Donald J. Winzor[a,*], Peter R. Wills[b]

[a]*Department of Biochemistry, School of Molecular and Microbial Sciences, University of Queensland, Brisbane, Qld 4072, Australia*

[b]*Department of Physics, University of Auckland, Auckland, New Zealand*

Received 10 September 2002; received in revised form 16 December 2002; accepted 16 December 2002

Abstract

This investigation re-examines theoretical aspects of the allowance for effects of thermodynamic non-ideality on the characterization of protein self-association by frontal exclusion chromatography, and thereby provides methods of analysis with greater thermodynamic rigor than those used previously. Their application is illustrated by reappraisal of published exclusion chromatography data for hemoglobin on the controlled-pore-glass matrix CPG-120. The equilibrium constant of 100/M that is obtained for dimerization of the $\alpha_2\beta_2$ species by this means is also deduced from re-examination of published studies of concentrated hemoglobin solutions by osmotic pressure and sedimentation equilibrium methods.

Keywords: Exclusion chromatography; Thermodynamic non-ideality; Hemoglobin self-association; Osmotic pressure; Sedimentation equilibrium

1. Introduction

There are many examples of experimental studies in which protein self-association has been characterized by frontal gel chromatography [1–14]. Most of those systems exhibited sufficiently strong self-association to allow their characterization on the basis of thermodynamically ideal behavior. For systems exhibiting weaker extents of self-association the protein concentration required for quantitative characterization extends to a range where effects of thermodynamic non-ideality must be taken into account. In that regard the analyses employed under those conditions [5,6] have been shown to lack rigor because of deficiencies in the empirical allowances made for effects of thermodynamic non-ideality [15]. An approximately linear concentration dependence of elution volume for a non-associating protein emanates from osmotic shrinkage of the beads comprising the gel phase of the chromatography column [15–18]—a phenomenon that stems from buffer efflux in response to the lower solvent chemical potential

*Corresponding author. Fax: +61-7-3365-4699.
E-mail address: d.winzor@mailbox.uq.edu.au (D.J. Winzor).

0301-4622/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0301-4622(03)00003-6

in an outer phase with higher protein concentration than that within the stationary phase. Osmotic shrinkage of the gel beads may thus be regarded as thermodynamic non-ideality arising from physical interaction between protein and gel matrix.

Although procedures are in place to make better allowance for the effects of osmotic shrinkage in the characterization of two-state protein self-association by frontal gel chromatography [15,18], the analysis is still open to criticism on two grounds. No account is taken of possible variation in the partition coefficients of monomeric and polymeric species as the result of changes in gel porosity due to bead shrinkage, nor is any allowance made for thermodynamic non-ideality as the consequence of protein–protein interactions. Fortunately, a change of chromatographic matrix to porous glass beads for studies of concentrated protein solutions obviates the complications stemming from changes in the void and stationary-phase volumes of the column; and thereby affords a simpler means of allowing for the effects of thermodynamic non-ideality arising from protein–protein and protein–matrix interactions.

Considerable progress has been made in the use of exclusion chromatography for the characterization of thermodynamically non-ideal protein self-association [19–22]. However, those determinations of self-association constants entail simplifying assumptions/approximations about activity coefficient relationships that require closer scrutiny. This communication presents a more critical appraisal of the steps involved in the characterization of relatively weak protein self-association by exclusion chromatography on incompressible molecular-sieve matrices such as porous glass beads. Results from a published study of hemoglobin self-association [20] are then used to illustrate a more rigorous thermodynamic analysis of frontal exclusion chromatography data.

2. Basic theoretical expressions

Allowance for the effects of thermodynamic non-ideality is to be made on the statistical–mechanical basis of excluded volume [23,24], a concept which allows quantitative expressions to be written for the activity coefficients of participating species in an equilibrium reaction. In order to pinpoint the weaknesses inherent in current applications of this approach to the characterization of weak protein self-association [19–22], it is appropriate to derive again from first principles the statistical–mechanical treatment of thermodynamic non-ideality in exclusion chromatography [19].

2.1. Partition behavior of a non-associating solute

Consider initially the distribution of a non-associating solute, A, between the mobile phase (α) and stationary phase (β) in the solute plateau region of a frontal chromatography experiment. Because the system is subject to the constraints of constant temperature and chemical potential of solvent (μ_s), the condition of partition equilibrium in terms of solute chemical potential ($\mu_A^\alpha = \mu_A^\beta$) dictates that the molar activities of solute (z_A) in the two phases are related by the expression

$$z_A^\beta / z_A^\alpha = \exp\left[\left\{\left[(\mu_A^0)^\alpha\right]_{T,\mu_s} - \left[(\mu_A^0)^\beta\right]_{T,\mu_s}\right\}/(RT)\right] \qquad (1)$$

where R is the universal gas constant and T the absolute temperature. The ratio of molar solute activities is thus a constant defined by the exponential of the difference between the standard chemical potentials of solute in the two phases. On the other hand, the experimentally determined partition coefficient (σ_A) is the ratio of the corresponding molar concentrations of solute (C_A), which varies because of the different concentration dependencies of the molar activity coefficients (γ_A) in the two phases. Specifically,

$$\sigma_A = C_A^\beta / C_A^\alpha = \left(z_A^\beta / z_A^\alpha\right)\left(\gamma_A^\alpha / \gamma_A^\beta\right) \qquad (2)$$

Description of the concentration dependence of the experimental partition coefficient thus requires a quantitative relationship for the concentration dependence of the activity coefficient ratio ($\gamma_A^\alpha / \gamma_A^\beta$).

In the statistical–mechanical approach [19–22] advantage is taken of the general expression

$$\gamma_i = \exp\left[2B_{ii}C_i + \sum B_{ij}C_j + (3/2)B_{iii}C_i^2 + \sum B_{iij}C_iC_j + \sum (1/2)B_{ijj}C_j^2 + \ldots\right] \quad (3)$$

where B_{ii} and B_{ij} are the respective second virial coefficients reflecting excluded volume interactions of species i with itself and other species: B_{iii} is the third virial coefficient for self-interaction between three like molecules, whereas B_{iij} and B_{ijj} complete the set of third virial coefficients for excluded-volume interactions involving species i and j. In the present context only the former type of interaction needs to be considered in the mobile (α) phase, whereupon

$$\gamma_A^\alpha = \exp\left[2B_{AA}C_A^\alpha + (3/2)B_{AAA}(C_A^\alpha)^2 + \ldots\right] \quad (4a)$$

However, the activity coefficient for solute in the other phase needs to be written as

$$\gamma_A^\beta = \exp\left[2B_{AA}C_A^\beta + (3/2)B_{AAA}(C_A^\beta)^2 + B_{AM}C_M^\beta + B_{AAM}C_A^\beta C_M^\beta + (1/2)B_{AMM}(C_M^\beta)^2 + \ldots\right] \quad (4b)$$

where the terms in C_M^β take into account the contributions of excluded-volume interactions between solute and the stationary-phase matrix (M), present at a concentration C_M^β. Combination of Eqs. (2), (4a) and (4b) yields the relationship

$$\begin{aligned}\sigma_A &= \left(z_A^\beta/z_A^\alpha\right)\exp\left\{2B_{AA}(C_A^\alpha - C_A^\beta) + (3/2)B_{AAA}\left[(C_A^\alpha)^2 - (C_A^\beta)^2\right] - B_{AM}C_M^\beta - B_{AAM}C_A^\beta C_M^\beta - (1/2)B_{AMM}(C_M^\beta)^2 + \ldots\right\} \\ &= \left(z_A^\beta/z_A^\alpha\right)\exp\left\{2B_{AA}(1-\sigma_A) - [B_{AAM}\sigma_A C_M^\beta]C_A^\alpha + (3/2)B_{AAA}(1-\sigma_A^2)(C_A^\alpha)^2 - B_{AM}C_M^\beta - (1/2)B_{AMM}(C_M^\beta)^2 + \ldots\right\}\end{aligned} \quad (5)$$

At very low solute concentration the terms containing C_A^α tend to zero, and hence the expression for the partition coefficient in the limit of zero solute concentration (σ_A^0) becomes

$$\sigma_A^0 = \left(z_A^\beta/z_A^\alpha\right)\exp\left[-B_{AM}C_M^\beta - (1/2)B_{AMM}(C_M^\beta)^2 + \ldots\right] \quad (6)$$

Substitution of this expression into Eq. (5) then gives

$$\sigma_A = \sigma_A^0\exp\left[2B_{AA}C_A^\alpha(1-\sigma_A) + (3/2)B_{AAA}(C_A^\alpha)^2(1-\sigma_A^2) - B_{AAM}C_M^\beta\sigma_A C_A^\alpha + \ldots\right] \quad (7)$$

as the relationship for the concentration dependence of the partition coefficient for solute A. An expression for σ_A that is independent of any matrix-term contribution can thus only be obtained by considering situations in which thermodynamic non-ideality is effectively restricted to nearest-neighbor interactions. Under those circumstances the truncated form of Eq. (7) becomes

$$\sigma_A = \sigma_A^0\exp\left[2B_{AA}C_A^\alpha(1-\sigma_A) + \ldots\right] \quad (8)$$

which is the expression derived previously [19] without realization of the limits of its applicability.

2.2. Incorporation of solute self-association

In the extension of this logic to exclusion chromatography of a self-associating solute, the experimentally measured partition coefficient, σ_w, becomes a weight-average quantity defined as

$$\sigma_w = \sum (iC_i^\beta)/\bar{C}_A^\alpha; \quad \bar{C}_A^\alpha = \sum (iC_i^\alpha) \quad (9)$$

where $i=1$ denotes monomer, $i=2$, dimer, etc.; and where $\bar{C}_A^\alpha$ is the base-molar concentration of solute (weight concentration divided by monomer molecular mass) in the liquid phase. Considerable simplification of the expression for σ_w can be achieved by choosing a stationary phase that excludes all polymers ($\sigma_i = 0$ for $i \geqslant 2$). Under those circumstances

$$\sigma_w = \sigma_1 C_1^\alpha / \bar{C}_A^\alpha \quad (10)$$

where σ_1 is the partition coefficient of monomer in a frontal experiment with total solute concentration $\bar{C}_A^\alpha$. Although the limiting value, σ_1^0, may be obtained by extrapolating σ_w to zero solute concentration, the parameter required for the determination of C_1^α from Eq. (10) is σ_1. An expression analogous to Eq. (8) is thus required for advantage to be taken of this approach to the evaluation of C_1^α and hence to the characterization of protein self-association.

The presence of dimer (and possibly higher oligomer) species in the mobile (α) phase requires the molar activity coefficient of monomer for that phase to be written as

$$\gamma_1^\alpha = \exp[2B_{11}C_1^\alpha + B_{12}C_2^\alpha + \ldots] \quad (11a)$$

to allow for steric interaction of monomer with dimer, etc. On the other hand, the selection of a stationary phase which excludes all polymers ensures that monomer is the sole solute species present in the stationary (β) phase, whereupon

$$\gamma_1^\beta = \exp[2B_{11}C_1^\beta + B_{1M}C_M^\beta + \ldots] \quad (11b)$$

when effects of thermodynamic non-ideality are restricted to nearest-neighbor interactions. Incorporation of these expressions for the monomer activity coefficients into the counterpart of Eq. (2) then gives rise to the expression

$$\sigma_1 = C_1^\beta / C_1^\alpha = \sigma_1^0 \exp\left[2B_{11}C_1^\alpha(1-\sigma_1) + B_{12}C_2^\alpha + \ldots\right] \quad (12)$$

for the partition coefficient of monomer in an experiment with total base-molar solute concentration $\bar{C}_A^\alpha$. Despite its rigor, this is not a very useful expression for σ_1 because its application requires knowledge of the self-association characteristics of the solute—the information being sought from the experiment.

The problem of evaluating the composition-dependence of activity coefficients was therefore side-stepped [19–22] by considering that all $B_{ij} = 2B_{11}$—an assumption akin to that introduced into sedimentation equilibrium theory by Adams and Fujita [25] when they invoked the relationship $\gamma_i = \exp(iBM_1)$ to describe the activity coefficient γ_i of i-mer. With that approximation Eq. (12) simplified to the form

$$\sigma_1 = \sigma_1^0 \exp\left[2B_{11}\bar{C}_A^\alpha(1-\sigma_1) + \ldots\right] \quad (13)$$

which allowed the direct specification of σ_1 for any $[\bar{C}_A^\alpha, \sigma_w]$ combination after assigning a magnitude to B_{11}; and hence of C_1^α from Eq. (12). As noted previously [26–28], a consequence of this Adams–Fujita approximation [25] is the self-cancellation of effects of thermodynamic non-ideality in the ratio of activity coefficients relating the apparent dimerization constant (K_2^{app}) to its thermodynamic counterpart (K_2). On the grounds that

$$K_2^{app} = C_2^\alpha / (C_1^\alpha)^2 = K_2(\gamma_1^\alpha)^2 / \gamma_2^\alpha = K_2 \quad (14)$$

the collected $[C_1^\alpha, \bar{C}_A^\alpha]$ data set was then amenable to analysis in terms of the expression

$$\bar{C}_A^\alpha = C_1^\alpha + 2K_2(C_1^\alpha)^2 + 3K_3(C_1^\alpha)^3 + \ldots \quad (15)$$

to obtain the equilibrium constants K_2, K_3, etc., characterizing the protein self-association.

Although adoption of the Adams–Fujita approximation [25] certainly afforded a tractable analysis for the characterization of solute self-association by exclusion chromatography, the approximations inherent in its use are highly questionable for isoelectric globular proteins [26]. We therefore seek a procedure that exhibits greater thermodynamic rigor.

2.3. A revised approach for characterizing solute self-association

The starting point for the revised approach to the characterization of non-ideal self-association is Eq. (10), which is combined with Eq. (2) to give the expression

$$\sigma_w \bar{C}_A^\alpha = C_1^\beta = z_1^\beta \exp\left[-2B_{11}(\sigma_w C_A^\alpha) + \ldots\right] \quad (16)$$

which is valid provided that non-ideality considerations can be restricted to effects of nearest-neighbor interactions. By assigning a magnitude to B_{11} it is thus possible to evaluate the thermodynamic activity of monomer (z_1^β) and hence $z_1^\alpha = z_1^\beta/\sigma_1^0$ from each ($\bar{C}_A^\alpha, \sigma_w$) measurement in exclusion chromatography studies where partition is restricted to the monomeric species. Characterization of the self-association, which is necessarily confined to that of dimerization because of the constraint imposed by restriction of non-ideality considerations to effects of nearest-neighbor interactions, now requires evaluation of C_1^α and hence C_2^α from the relationships

$$C_1^\alpha = z_1^\alpha \exp(-2B_{11}C_1^\alpha - B_{12}C_2^\alpha) \quad (17a)$$

$$C_2^\alpha = (\bar{C}_A^\alpha - C_1^\alpha)/2 \quad (17b)$$

which can be solved iteratively with $\bar{C}_A^\alpha$ as the initial estimate of C_1^α in the exponential term of Eq. (17a). The dimerization constant is then obtained from the expression

$$C_2^\alpha = K_2(z_1^\alpha)^2 \exp(-2B_{22}C_2^\alpha - B_{12}C_1^\alpha) \quad (18)$$

as the slope of the linear dependence of $C_2^\alpha \exp(2B_{22}C_2^\alpha + B_{12}C_1^\alpha)$ upon $(z_1^\alpha)^2$.

2.4. Other potential statistical–mechanical approaches

Whereas resort to the above statistical–mechanical approach leads to the expression of a species activity coefficient as a virial expansion containing the free concentration of every species present, Hill and Chen [29] regard solute self-association as merely another form of thermodynamic non-ideality. For a monomer–dimer system the molar activity of monomer may then be expressed as the following polynomial expansion in $\bar{C}_A^\alpha$ [28].

$$z_1^\alpha = \bar{C}_A^\alpha \exp\{2(B_{11}-K_2)\bar{C}_A^\alpha - (3/2)[4K_2^2 - 2K_2(4B_{11}-B_{12}) + B_{111} - 2K_3] \times (\bar{C}_A^\alpha)^2 + \ldots\} \quad (19)$$

where the polynomial coefficients now comprise a mixture of association constant (K_i) and excluded volume (B_i) terms. Alternatively, the same general concept can be used to express $\bar{C}_A^\alpha$ as a function of z_1^α [28,30–32]. Specifically

$$\bar{C}_A^\alpha = z_1^\alpha \exp[2(K_2 - B_{11})(z_1^\alpha)^2 + 3(K_3 - K_2B_{12} + 2B_{11}^2 - B_{111}/2) \times (z_1^\alpha)^3 + \ldots] \quad (20)$$

Although these approaches have the potential to obviate the iterative process entailed in the above analysis of $[z_1^\alpha, \bar{C}_A^\alpha]$, their successful application requires rapid convergence of the power series, which in the present context needs to be truncated at the second virial coefficient term. In general, the expression of z_1^α as a multinomial in $\bar{C}_A^\alpha$ offers greater potential for systems with non-ideality as the predominant phenomenon, whereas the reverse expansion assumes this role in instances where $K_2 \gg B_{11}$ [26,33]. It is therefore of interest to determine the extent to which either of these more direct approaches provides a viable alternative to the iterative approach described in Section 2.3.

2.5. Assignment of magnitudes to virial coefficients

Magnitudes of osmotic second virial coefficients B_{ii} and B_{ij} have been calculated by means of the expressions

$$B_{ii} = 16\pi N R_i^3/3 + Z_i^2(1+2\kappa R_i)/[4I(1+\kappa R_i)^2] \quad (21a)$$

$$B_{ij} = 4\pi N(R_i+R_j)^3/3 + Z_iZ_j(1+\kappa R_i + \kappa R_j)/[2I(1+\kappa R_i)(1+\kappa R_j)] \quad (21b)$$

which are based on the premise that the species can be modelled as hard spheres with radii R_i and R_j: for a monomer–dimer system ($i=1$, monomer; $j=2$, dimer) $R_2 = 2^{1/3}R_1$. Avogadro's number (N) converts the molecular covolume to a molar basis, and the charge–charge terms are expressed in classical Debye–Hückel nomenclature. Z_i and Z_j

are the respective net charges on species *i* and *j*; and at 20 °C the inverse screening length, κ (1/cm), may be calculated as $3.27\times10^7\sqrt{I}$, where I is the molar ionic strength of the solvent. Such consideration of proteins as rigid impenetrable spheres has been shown to be a reasonable approximation for a range of globular proteins [34].

For hemoglobin, which is essentially uncharged at neutral pH [35], R_1 has been taken as 3.13 nm, the Stokes radius of the 64.5 kDa ($\alpha_2\beta_2$) moiety obtained from measurements of the translational diffusion coefficient [36–40] and the sedimentation coefficient [41,42]; and also the value deduced as the effective covolume radius [15] from the osmotic data of Adair [43]. On this basis the three virial coefficients required for the application of Eqs. (16) and (17a) have been assigned the following magnitudes: $B_{11}=309$ l/mol; $B_{12}=892$ l/mol, $B_{22}=1234$ l/mol. In the reappraisal of osmotic pressure data on isoelectric hemoglobin, a value of 59 800 l^2/mol^2 has been ascribed to B_{111} on the basis that $B_{111}=160\pi^2N^2R_1^6/9$.

For ovalbumin, a non-associating protein, the second virial coefficient for self-interaction has been taken as 810 l/mol, the value calculated by assigning the protein a radius of 2.92 nm and a net charge of −16 [19] under the conditions of the experimental study (pH 7.4, 0.156*I*).

3. Reinterpretation of published exclusion chromatography and other data

There have only been four reported experimental studies of protein self-association by exclusion chromatography on porous glass beads. The possibility of allowing for effects of thermodynamic non-ideality was first illustrated with an analysis of results for bovine liver glutamate dehydrogenase on CPG-170 [19]. However, that application does not provide a stringent test of the approach because of the limited protein concentration range (0.05–5 mg/ml) examined. In subsequent exclusion chromatography studies of hemoglobin [20,22] and lysozyme [21] consideration certainly needs to be accorded the consequences of thermodynamic non-ideality in view of the protein concentrations (up to 225 mg/ml) employed. Here we focus on results from the initial investigation of hemoglobin on CPG-120, where concentration dependence of the partition coefficient (Table 1 of Ref. [20]) seemingly reflected a compromise between effects of self-association and thermodynamic non-ideality. This situation was confirmed in the subsequent experimental study of hemoglobin [22], which was undertaken in response to concerns expressed about the interpretation placed on the original findings [44]. The present aim is to ascertain whether the purported evidence of hemoglobin self-association survives more rigorous scrutiny in terms of allowance for the effects of thermodynamic non-ideality.

3.1. Range of accessible solute concentration

As noted in Section 2.1, the validity of the expression to be used in the allowance for effects of thermodynamic non-ideality (Eq. (8)) is conditional upon the adequacy of their description in terms of nearest-neighbor interactions (i.e. of second virial coefficients). It is therefore necessary to ascertain the likely range of protein concentration over which Eq. (4a) truncated at the $B_{AA}C_A$ term provides an adequate description of the molar activity coefficient, γ_A. For that purpose we take advantage of the relationships between the second (B_{AA}) and higher virial coefficients reported by Ree and Hoover [45] to obtain a more precise estimate of the concentration dependence of γ_A. The solid line in Fig. 1 summarizes the activity coefficient calculated by extending the power series in Eq. (4a) to the seventh virial coefficient term for an uncharged solute spherical solute with $R_A=3.13$ nm, the Stokes radius of hemoglobin. For solute concentrations up to 0.3 mM the activity coefficients calculated on the basis of Eq. (4a) truncated at the linear concentration term (---) is within 1% of the more accurate estimate; and the disparity is still only 5% at $C_A=0.7$ mM (45 mg/ml). We therefore regard this concentration as an upper limit for the validity of Eq. (8) in view of its inherent assumption that second virial terms suffice to describe the effects of thermodynamic non-ideality. In situations where the effects of non-ideality may be extended to include contributions from the third virial coefficient, the corresponding

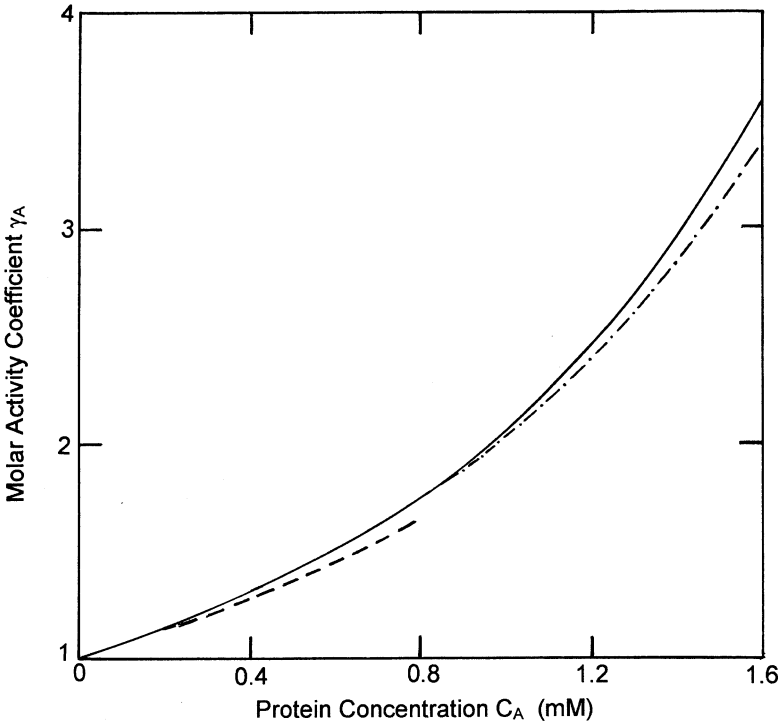

Fig. 1. Concentration dependence of the activity coefficient of an uncharged spherical solute with a radius of 3.13 nm. Dependence calculated on the basis of Eq. (4a) with the series taken to the sixth power of concentration (——). Corresponding dependence obtained by truncating the series at the linear concentration term (---). Dependence obtained by truncation after the term in C_A^2 (-•-•-).

upper limit of concentration is approximately 1.6 mM or 100 mg/ml (-•-•-, Fig. 1).

3.2. Consideration of oxyhemoglobin as a non-associating solute

Results of the first six experiments reported in Table 1 of Ref. [20] for the exclusion chromatography behavior of oxyhemoglobin on CPG-120 are presented (●) in Fig. 2. Although these results signify a positive value of the second virial coefficient (B_{AA}), the extent of the dependence of the partition coefficient upon protein concentration is much smaller than that predicted by Eq. (8) for a solute with the Stokes radius of hemoglobin (---). Furthermore, the estimate of 2.0 nm for R_A that can be deduced from the experimental dependence is unrealistic in that it is smaller than the radius, $[3M_A\bar{v}_A/(4\pi N)]^{1/3}$, of an unsolvated hemoglobin molecule. The previous inference [20,22] that these results must reflect a compromise between effects of thermodynamic non-ideality and solute self-association is seemingly thereby substantiated. However, Minton [46] has criticized the simplicity of the present partition model, and devised a more complicated version to account for the hemoglobin data without invoking self-association. Fortunately, the corollary that exclusion chromatography data for all non-associating solutes should be at variance with the predictions of Eq. (8) is readily discredited.

Partition results obtained in exclusion chromatography of ovalbumin on a column of CPG-75 equilibrated with 0.156*I* phosphate–chloride buffer, pH 7.4 [19], are presented as the second set of data (■) in Fig. 2. Also shown is the theoretical dependence predicted by Eq. (8) for a spherical solute with the radius (2.92 nm) and net charge

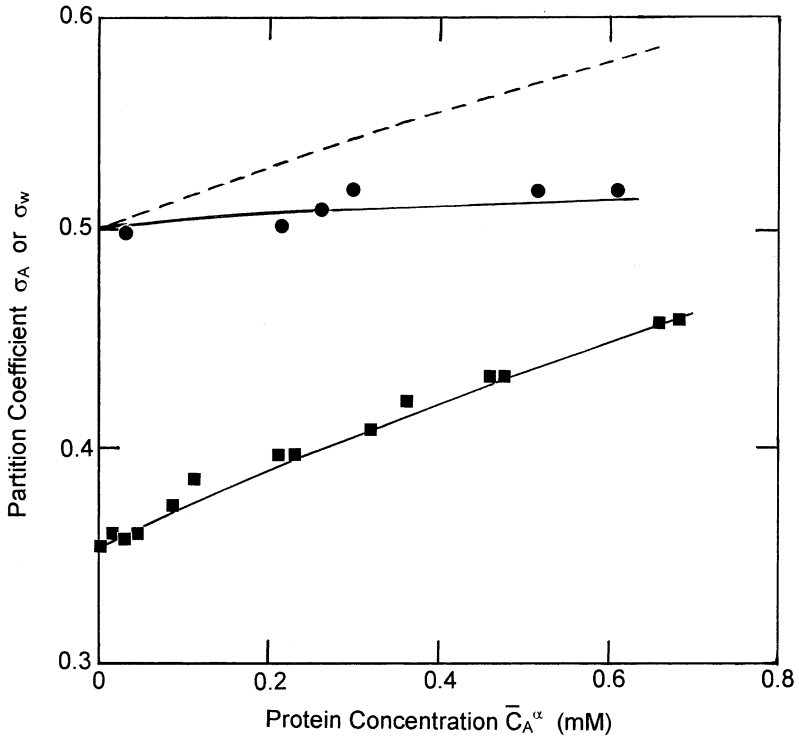

Fig. 2. Concentration dependence of the partition coefficient for oxyhemoglobin (●) and ovalbumin (■) in frontal exclusion chromatography of the proteins on columns of CPG-120 and CPG-75, respectively. For hemoglobin the broken line corresponds to the behavior predicted by Eq. (8) for a non-associating protein with the Stokes radius (3.13 nm) of the $\alpha_2\beta_2$ moiety, whereas the solid line is the dependence predicted by Eqs. (16) and (22b) for a monomer–dimer system with $K_2 = 102/\mathrm{M}$. The solid line through the ovalbumin data is based on Eq. (8) and the second virial coefficient (B_{AA}) calculated for a spherical solute with the Stokes radius and net charge of this protein under the conditions of the experiment (0.156I phosphate–chloride, pH 7.4). (Data for hemoglobin and ovalbumin taken from Refs. [20,19], respectively.)

(−16) of ovalbumin under these conditions [19]. For this non-associating protein there is excellent conformity of the experimental data with the behavior predicted by the simple partition model; and hence, by inference, poor agreement with that predicted by the more elaborate model invoked to account for the hemoglobin results [46]. To cover the possibility that the existence of a finite net charge on the ovalbumin may account for the difference in the partition behavior of the two proteins, exclusion chromatographic data for ovalbumin on Fractogel under isoelectric conditions (pH 4.6) is examined in Fig. 3, together with corresponding results for isoelectric ribonuclease and bovine serum albumin [47]. For all three proteins the close conformity of the results with the behavior predicted by Eq. (8) and the values of B_{AA} obtained from the Stokes radii again signifies the adequacy of the present partition model for a non-associating protein. On the grounds that these findings reinforce the conclusion that the partition of behavior of hemoglobin deviates from that of a non-associating protein, we resume the attempt to account for the exclusion chromatography behavior in terms of the compensating effects of thermodynamic non-ideality and solute self-association.

3.3. Consideration of the partition data in terms of hemoglobin dimerization

As noted in Section 3.2, the starting point for this analysis is estimation of the thermodynamic

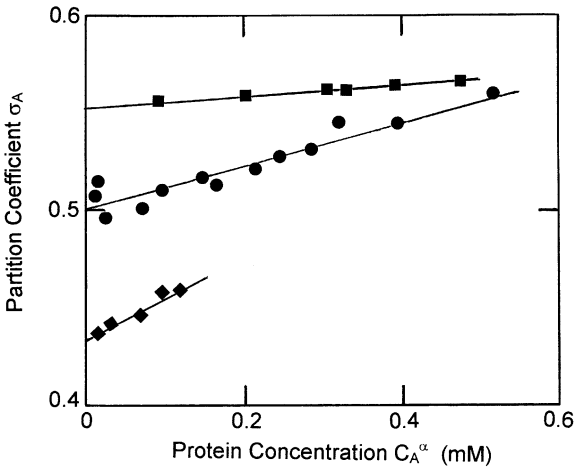

Fig. 3. Concentration dependence of the partition coefficients of ovalbumin (●), ribonuclease (■) and bovine serum albumin (♦) on Fractogel under isoelectric conditions. Lines denote the behavior predicted on the basis of Eq. (8) and the value of B_{ii} calculated from Eq. (21a) with $Z_i=0$ and the Stokes radius of the protein. (Data and Stokes radii taken from Ref. [47].)

activity of monomer from each $[\bar{C}_A^{\alpha},\sigma_w]$ measurement via Eq. (16) and the relationship $z_1^{\alpha}=z_1^{\beta}/\sigma_1^0$. The subsequent solution of Eqs. (17a) and (17b) to obtain the molar concentrations of monomer and dimer in the liquid phase $(C_1^{\alpha},C_2^{\alpha})$ proved to be relatively straightforward in that three iterations sufficed. A dimerization constant (± 2 S.D.) of 102 (± 5)/M is obtained from Fig. 4a by ascribing linearity to the dependence of $C_2^{\alpha}\exp[2B_{22}C_2^{\alpha}+B_{12}C_1^{\alpha}]$ upon $(z_1^{\alpha})^2$, in accordance with the prediction of Eq. (18).

To obtain the consequent dependence of partition coefficient upon total protein concentration $\bar{C}_A$ we note that

$$K_2=z_2^{\alpha}/(z_1^{\alpha})^2=\left[(\bar{C}_A^{\alpha}-C_1^{\alpha})/\{2(C_1^{\alpha})^2\}\right]\exp \times\left[(B_{22}-B_{12})\bar{C}_A^{\alpha} +(2B_{12}-4B_{11}-B_{22})C_1^{\alpha}\right] \quad (22a)$$

or, on making the approximation that $\exp(x)\approx 1+x+\ldots$,

$$K_2=\left[(\bar{C}_A^{\alpha}-C_1^{\alpha})/\{2(C_1^{\alpha})^2\}\right] \times\left[1+(B_{22}-B_{12})\bar{C}_A^{\alpha} +(2B_{12}-4B_{11}-B_{22})C_1^{\alpha}\right] \quad (22b)$$

Solution of this quadratic in C_1^{α} for any assigned magnitude of $\bar{C}_A^{\alpha}$ yields the value of C_1^{α} and hence, from Eqs. (17a) and (17b), the corresponding estimate of z_1^{α}. Substitution of $\sigma_1^0 z_1^{\alpha}$ for z_1^{β} in Eq. (16) then allows establishment of the dependence of σ_w upon $\bar{C}_A^{\alpha}$ that is predicted for such a dimerizing system (solid line in Fig. 2). The experimental results clearly conform much better with description in terms of non-ideal hemoglobin self-association.

Although Eqs. (19) and (20) seemingly provide a more direct means of characterizing the dimerization, they impose constraints on the relative magnitudes of the opposing effects of thermodynamic non-ideality and self-association on the concentration dependence of the partition coefficient: convergence of the power series in Eq. (19) is more rapid for systems with non-ideality as the predominant effect [26]. An analysis of the $[z_1^{\alpha},\bar{C}_A^{\alpha}]$ data set by this means is summarized in Fig. 4b, where the broken line signifies the dependence predicted for hemoglobin as a non-associating solute ($K_2=0$ in Eq. (19) truncated at the linear term of the exponent). A finite value of the dimerization constant is clearly required to describe the slope, $(2B_{11}-2K_2)$, of the experimental dependence of $\ln(z_1^{\alpha}/\bar{C}_A^{\alpha})$ upon $\bar{C}_A^{\alpha}$. An estimate (± 2 S.D.) of 116 (± 7)/M is obtained for K_2 (cf. 102/M from Fig. 4a), which signifies that relatively minor error is introduced by truncation of the series in Eq. (19) at the linear term. On the other hand, an apparent dimerization constant of 170 (± 17)/M is obtained from the corresponding dependence of $\ln(z_1^{\alpha}/\bar{C}_A^{\alpha})$ upon z_1^{α} (Eq. (20))—a finding that signifies a requirement for higher-order terms in the power series to obtain a reasonable estimate of K_2 by this approach. Such a conclusion is consistent with earlier observations

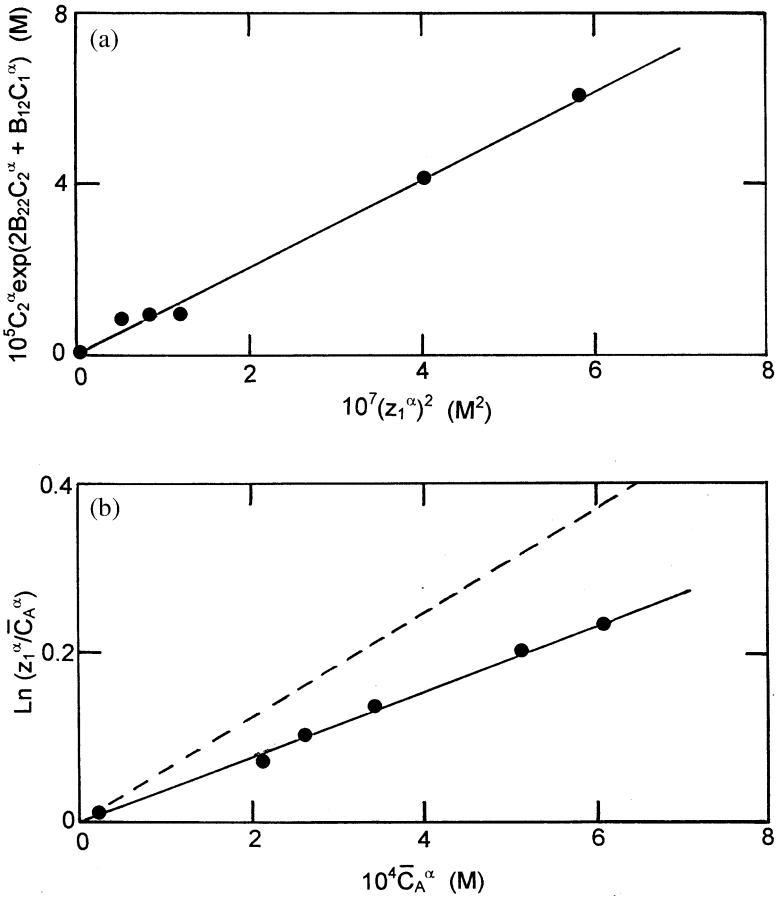

Fig. 4. Interpretation of the exclusion chromatography data for hemoglobin (Fig. 2) in terms of dimerization of the $\alpha_2\beta_2$ species. (a) Analysis of the monomer activity data determined from Eq. (16) in terms of Eq. (18). (b) Analysis of the same data in terms of the logarithmic form of Eq. (19).

that the expression of $\bar{C}_A^{\alpha}$ as a polynomial expansion in z_1^{α} is more appropriate when self-association is the predominant effect [26,33].

On the basis of the more accurate description of the experimental results in terms of Eq. (18), we consider that the self-association of oxyhemoglobin is governed by a dimerization constant of 100/M rather than that of 160/M deduced previously [20] on the basis of a questionable analytical procedure. We also note that resort to a more direct characterization by means of Eq. (19) also provides a reasonable estimate of K_2 for this example of weak, non-ideal protein self-association.

3.4. Characterization of hemoglobin self-association by other means

In a previous study [20] it was noted that the results of osmometry [43] and sedimentation equilibrium [48,49] studies may also be interpreted as indicating hemoglobin self-association inasmuch as the extent of the concentration dependence of the measured parameter (osmotic pressure and molecular mass) is less than that predicted for a non-associating $\alpha_2\beta_2$ species. We now attempt a quantitative rationalization of those results in terms of non-ideal hemoglobin dimerization to ascertain

the degree of correspondence between equilibrium constants deduced by the various procedures. For both methods we shall employ the Hill–Chen approach [29] wherein the virial coefficients reflect a mixture of covolume and dimerization constant terms—on the grounds that Eq. (19) provided a reasonable estimate of K_2 from the partition coefficient data for hemoglobin (Fig. 4b). Because the hemoglobin is confined to a single phase in either experimental design, the above necessity for truncation of the power series at the linear term ceases to apply. However, the unavailability of Hill–Chen expressions for the fourth and higher virial coefficients describing the multinomial in $\bar{C}_A^{\alpha}$ dictates that an upper limit of 1.6 mM (100 mg/ml) be used to retain accuracy of the calculated activity coefficient for a non-associating solute within 5% of the value obtained by extending the series to the seventh virial coefficient term (–•–•–, Fig. 1).

On the grounds that the dependence of osmotic pressure, Π, upon concentration C_A^{α} of a non-associating solute is given by

$$\Pi = RTC_A^{\alpha}\left[1 + B_{AA}C_A^{\alpha} + B_{AAA}(C_A^{\alpha})^2 + \ldots\right] \qquad (23a)$$

the corresponding expression for a solute undergoing reversible dimerization is

$$\Pi = RT\bar{C}_A^{\alpha}\left[1 + (B_{11} - K_2)\bar{C}_A^{\alpha} + \{4K_2^2 - 2K_2(4B_{11} - B_{12}) + B_{111}\}(\bar{C}_A^{\alpha})^2 + \ldots\right] \qquad (23b)$$

where the substitutions for the second and third virial coefficients are those in Eq. (19). From Fig. 5, which summarizes findings reported by Adair [43], it is evident that the behavior predicted by Eq. (23a) with $B_{AA} = B_{11} = 309$ l/mol and $B_{AAA} = B_{111} = 59\,800$ l^2/mol^2 (---) does not describe the results for sheep (●) and human (■) hemoglobins—the disparity noted previously [20]. However, good agreement with the experimental data is obtained by invoking Eq. (23b) with $K_2 = 102$/M for the theoretical description of the data (——). The osmotic pressure measurements are thus consistent with quantitative interpretation in terms of the model of hemoglobin dimerization

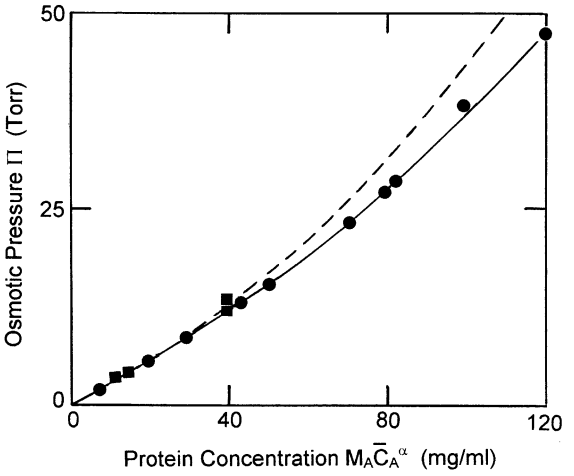

Fig. 5. Reappraisal of the concentration dependence of the osmotic pressure of sheep (●) and human (■) hemoglobins at neutral pH. The broken line signifies the dependence predicted by Eq. (23a) for a non-associating $\alpha_2\beta_2$ moiety, whereas the solid line is that predicted by Eq. (23b) for the monomer–dimer model with $K_2 = 102$/M. (Data taken from Ref. [43].)

deduced from the exclusion chromatography results.

Of the two sedimentation equilibrium studies of hemoglobin [48,49], both of which yielded similar results [20,50], we shall focus attention on that by Williams [49], whose approach to analysis of the data closely resembles that to be followed here. Because the apparent molecular mass of a non-associating solute, M_A^{app}, is related to the true molecular mass, M_A, by the relationship [51,52]

$$M_A^{app} = M_A/\left[1 + C_A\left(\partial \ln \gamma_A/\partial C_A\right)_{T,\mu_s}\right] \qquad (24)$$

incorporation of the relationship for the activity coefficient Eq. (3) leads to the conclusion that

$$M_A^{app} = M_A/[1 + 2B_2C_A + 3B_3C_A^2 + \ldots] \qquad (25)$$

where B_2 and B_3 are the second and third virial coefficients (B_{AA} and B_{AAA}, respectively). Dimerization of the solute is accommodated by making the Hill–Chen substitutions for these parameters. Specifically,

$$M_A^{app} = M_1/\left[1 + 2(B_{11} - K_2)\bar{C}_A + 3\{4K_2^2 - (4B_{11} - B_{12}) + B_{111}\}\bar{C}_A^2 + \ldots\right] \quad (26)$$

Analysis of the dependence of M_A^{app} upon $\bar{C}_A$ thus has the potential to yield K_2 on the basis of the magnitudes of the experimentally determined virial coefficients.

Williams [49] has analyzed sedimentation equilibrium data for carbonmonoxyhemoglobin in terms of the weight–concentration counterpart of Eq. (25) to obtain estimates of parameters termed the second, third and fourth virial coefficients. In that regard no constraints were imposed on the relative magnitudes of these parameters, which should therefore be regarded as polynomial curve-fitting coefficients. Nevertheless, the determination of coefficients for the C_A^2 and C_A^3 terms should improve the reliability of that for the linear concentration term as an estimate of the second virial coefficient B_2. However, interpretation of the findings is complicated by the use of an incorrect expression for the determination of molecular mass from a sedimentation equilibrium distribution [51,52]. Apparent molecular masses were determined by means of the relationship

$$d\ln C_A/dr^2 = M_A^{app}(1 - \bar{v}_A\rho)\omega^2/(2RT) \quad (27)$$

in the mistaken belief that the product of density and partial specific volume ($\bar{v}_A$) in the buoyancy terms referred to the solution density (ρ) rather than that of solvent (ρ_s). Inasmuch as

$$(1 - \bar{v}_A\rho) = (1 - \bar{v}_A\rho_s)(1 - M_A\bar{v}_AC_A) \quad (28)$$

for an incompressible solution, the parameter measured by Williams [49] is actually the product $M_A^{app}(1 - M_A\bar{v}_AC_A)$. The counterpart of Eq. (25) is then

$$M_A^{app}(1 - M_A\bar{v}_AC_A) \approx \frac{M_A}{(1 + M_A\bar{v}_AC_A)(1 + 2B_2C_A + 3B_3C_A^2 + \ldots)} \quad (29a)$$

which upon collection of terms as a power series in concentration becomes

$$M_A^{app}(1 - M_A\bar{v}_AC_A) = \frac{M_A}{1 + 2(B_2 + M_A\bar{v}_A/2)C_A + 3(B_3 + 2B_2M_A\bar{v}_A/3)C_A^2 + \ldots} \quad (29b)$$

The coefficient deduced as B_2 by nonlinear curve-fitting of the incorrectly determined molecular mass data to Eq. (25) should therefore be interpreted as

$$B_2 = B_{AA} + M_A\bar{v}_A/2 \quad (30a)$$

for a non-associating solute, or as

$$B_2 = B_{11} - K_2 + M_A\bar{v}_A/2 \quad (30b)$$

for a monomer–dimer system.

Conversion of the reported [49] second virial coefficient of 5.9×10^{-5} ml mol/g^2 to the corresponding molar counterpart entails its multiplication by M_A^2, whereupon $B_2 = 245$ l/mol. Incorporation of a value of 48 l/mol for the partial molar volume (product of a molar mass of 64.5 kg and a partial specific volume of 0.746 ml/g) into Eq. (30a)) signifies an apparent second virial coefficient (B_{AA}) of 221 l/mol, which markedly underestimates the calculated value of 309 l/mol (Section 2.5). On the other hand, its substitution into Eq. (30b) with B_{11} taken as 309 l/mol yields an estimate of 88/M for K_2. This dimerization constant, which cannot be assigned an uncertainty in the absence of any indication of the standard error in the estimate of B_2, is considered to signify quantitative correlation of the sedimentation equilibrium results [49] with the dimerization model ($K_2 = 100$/M) inferred from osmotic pressure [43] as well as exclusion chromatography [20] studies of hemoglobin.

4. Concluding remarks

This investigation has served to illustrate procedures whereby rigorous allowance for the effects of thermodynamic non-ideality may be incorporat-

ed into the characterization of protein self-association by exclusion chromatography on incompressible matrices such as porous glass beads. In that regard the method is confined to studies over the concentration range for which thermodynamic non-ideality in the stationary phase is described adequately in terms of nearest-neighbor interactions. This restrictive condition was not appreciated in the earlier interpretation [20,22] of exclusion chromatography results over a wider range of hemoglobin concentration in terms of isodesmic indefinite self-association as well as the dimerization model employed here. Nevertheless, the earlier conclusion that the $\alpha_2\beta_2$ hemoglobin species undergoes self-association remains unchanged by subjecting the exclusion chromatography data to an analysis with greater thermodynamic rigor. Furthermore, the concept of reversible dimerization of the $\alpha_2\beta_2$ moiety is reinforced by the deduction of essentially the same equilibrium constant (order of 100/M) from studies of concentrated hemoglobin solutions by osmotic pressure [43] and sedimentation equilibrium [49] as well as by exclusion chromatography.

By demonstrating the existence of relatively weak self-association of the tetramer ($\alpha_2\beta_2$) species, this investigation raises questions about the molecular state of hemoglobin at its much higher concentration ($\approx$300 mg/ml) in the red blood cell. As noted in relation to Eq. (8), the selection of 50 mg/ml as the upper concentration limit for the present analysis of published exclusion chromatography data [20] reflects an inability to make quantitative allowance for thermodynamic non-ideality beyond the effects of bi-molecular interactions in the protein–matrix contribution (Eq. (7)). This upper limit could be extended to 100 mg/ml for the analyses of osmotic pressure [43] and sedimentation equilibrium [49] data because of the availability of rigorous expressions for the base-molar concentration $\overline{C}_A^{\alpha}$ as a function of monomer activity z_1^{α} that encompass tri-molecular interactions requiring description in terms of third virial coefficients. Although such analysis of those osmotic pressure and sedimentation equilibrium measurements thus offers potential for the evaluation of a trimerization constant (K_3) as well as K_2, the results presented in Fig. 5 are still described adequately by a model in which self-association of the $\alpha_2\beta_2$ species is restricted to dimer formation ($K_3=0$). The situation pertaining at higher hemoglobin concentrations will require investigation by analyses that are still to be developed.

The major outcome of this reappraisal of published data for hemoglobin is thus consolidation of the concept (20, 22) that self-association extends beyond the $\alpha_2\beta_2$ species; and hence of the concept that the oligomeric state of hemoglobin in its physiological role as a transporter of oxygen remains to be established. May this investigation provide the stimulus for extension of the statistical–mechanical treatment of non-ideality in associating systems (28, 31) to allow the rigorous interpretation of thermodynamic data at even higher protein concentrations; and thereby to render feasible the prospect of quantifying hemoglobin self-association under conditions more closely resembling those in the red blood cell.

Acknowledgments

The financial support of this investigation by the Australian Research Council is gratefully acknowledged.

References

[1] D.J. Winzor, H.A. Scheraga, Studies of chemically reacting systems on Sephadex. 1. Chromatographic demonstration of the Gilbert theory, Biochemistry 3 (1963) 1263–1267.

[2] D.J. Winzor, H.A. Scheraga, Studies of chemically reacting systems on Sephadex. 2. Molecular weights of monomers in rapid association equilibrium, J. Phys. Chem. 68 (1964) 338–343.

[3] G.K. Ackers, T.E. Thompson, Determination of stoichiometry and equilibrium constants for reversibly associating systems by molecular sieve chromatography, Proc. Natl. Acad. Sci. USA 53 (1965) 342–349.

[4] L.W. Nichol, A.B. Roy, The sulfatase of ox liver. IX. The polymerization of sulfatase A, Biochemistry 4 (1965) 386–396.

[5] G.K. Ackers, Molecular sieve studies of interacting systems. I. Equations for transport of associating systems, J. Biol. Chem. 242 (1967) 3026–3034.

[6] E. Chiancone, L.M. Gilbert, G.A. Gilbert, G.L. Kellett, Dissociation of hemoglobin subunits: gel filtration studies, J. Biol. Chem. 243 (1968) 1212–1219.

[7] P.W. Chun, S.J. Kim, C.A. Stanley, G.K. Ackers, Determination of equilibrium constants of associating systems. III. Evaluation of the weight-fraction of monomer from the weight-average partition coefficient (application to bovine liver glutamate dehydrogenase), Biochemistry 8 (1969) 1625–1632.

[8] P.A. Baghurst, L.W. Nichol, W.H. Sawyer, Effects of deuterated water on the association of β-lactoglobulin, J. Biol. Chem. 247 (1972) 3198–3204.

[9] R. Valdes, G.K. Ackers, Thermodynamic studies on subunit assembly in human hemoglobin. Self-association of oxygenated chains (α^{SH} and β^{SH}): determination of stoichiometries and equilibrium constants, J. Biol. Chem. 252 (1977) 74–81.

[10] R. Tellam, J. de Jersey, D.J. Winzor, Evaluation of equilibrium constants for the binding of *N*-acetyl-L-tryptophan to monomeric and dimeric forms of α-chymotrypsin, Biochemistry 18 (1979) 5316–5321.

[11] M.A. Kukuruzinska, B.W. Turner, G.K. Ackers, S. Roseman, Subunit association of enzyme I of *Salmonella typhinurium* phosphoenolpyruvate:glycose phosphotransferase system, J. Biol. Chem. 259 (1984) 11679–11681.

[12] D. Beckett, K.S. Koblan, G.K. Ackers, Quantitative study of protein association at picomolar concentrations: the λ phage cI repressor, Anal. Biochem. 196 (1991) 69–75.

[13] L. Kiger, A.L. Klinger, L.D. Kwiatkowsky, et al., Thermodynamic studies on the equilibrium properties of a series of recombinant βW37 hemoglobin mutants, Biochemistry 37 (1998) 4336–4345.

[14] P.J. Darling, J.M. Holt, G.K. Ackers, Coupled energetics of λ *cro* repressor self-assembly and site-specific DNA operator binding: analysis of *cro* dimerization from nanomolar to micromolar concentrations, Biochemistry 39 (2000) 11500–11507.

[15] P.A. Baghurst, L.W. Nichol, A.G. Ogston, D.J. Winzor, Quantitative interpretation of concentration-dependent migration in gel chromatography of reversibly polymerizing systems, Biochem. J. 147 (1975) 575–583.

[16] E. Edmond, S. Farquhar, J.R. Dunstone, A.G. Ogston, The osmotic behaviour of Sephadex and its effect on chromatography, Biochem. J. 108 (1968) 755–763.

[17] L.W. Nichol, M. Janado, D.J. Winzor, The origin and consequences of concentration dependence in gel chromatography, Biochem. J. 133 (1973) 15–22.

[18] R. Tellam, D.J. Winzor, Concentration-dependent migration in gel chromatography of reversibly polymerizing systems: the osmotic behaviour of polyacrylamide gels, Biophys. Chem. 12 (1980) 299–306.

[19] L.W. Nichol, R.J. Siezen, D.J. Winzor, The study of multiple polymerization equilibria by glass-bead exclusion chromatography with allowance for thermodynamic nonideality effects, Biophys. Chem. 9 (1978) 47–55.

[20] L.W. Nichol, R.J. Siezen, D.J. Winzor, Chromatographic evidence for the self-association of oxyhemoglobin in concentrated solutions: its biological implications, Biophys. Chem. 10 (1979) 17–26.

[21] P.R. Wills, L.W. Nichol, R.J. Siezen, The indefinite self-association of lysozyme: consideration of composition-dependent activity coefficients, Biophys. Chem. 11 (1980) 71–82.

[22] R.J. Siezen, L.W. Nichol, D.J. Winzor, Exclusion chromatography of concentrated hemoglobin solutions: comparison of the self-association behavior of the oxy and deoxy forms of the $\alpha_2\beta_2$ species, Biophys. Chem. 14 (1981) 221–231.

[23] W.G. McMillan, J.E. Mayer, The statistical thermodynamics of multicomponent systems, J. Chem. Phys. 13 (1945) 276–305.

[24] T.L. Hill, Thermodynamics for Chemists and Biologists, Addison-Wesley, Reading, MA, 1968.

[25] E.T. Adams, H. Fujita, Sedimentation equilibrium in reacting systems, in: J.W. Williams (Ed.), Ultracentrifugal Analysis in Theory and Experiment, Academic Press, New York, 1963, pp. 119–128.

[26] A.G. Ogston, D.J. Winzor, Treatment of thermodynamic nonideality in equilibrium studies of associating solutes, J. Phys. Chem. 79 (1975) 2496–2500.

[27] M.P. Jacobsen, D.J. Winzor, Refinement of the omega analysis for the characterization of solute self-association by sedimentation equilibrium, Biophys. Chem. 45 (1992) 119–132.

[28] P.R. Wills, M.P. Jacobsen, D.J. Winzor, Direct analysis of solute self-association by sedimentation equilibrium, Biopolymers 38 (1996) 119–130.

[29] T.L. Hill, Y.D. Chen, Theory of aggregation in solution. I. General equations and application to the stacking of bases, nucleosides, etc., Biopolymers 12 (1973) 1285–1312.

[30] P.R. Wills, M.P. Jacobsen, D.J. Winzor, Analysis of sedimentation equilibrium distributions reflecting macromolecular associations, Biophys. J. 79 (2000) 2178–2187.

[31] P.R. Wills, D.J. Winzor, Studies of solute self-association by sedimentation equilibrium: allowance for effects of thermodynamic nonideality beyond the consequences of nearest-neighbor interactions, Biophys. Chem. 91 (2001) 253–262.

[32] P.R. Wills, D.J. Winzor, Exact theory of sedimentation equilibrium made useful, Prog. Colloid Polym. Sci. 119 (2002) 113–120.

[33] D.J. Winzor, P.R. Wills, The omega analysis and the characterization of solute self-association by sedimentation equilibrium, in: T.M. Schuster, T.M. Laue (Eds.), Modern Analytical Ultracentrifugation: Acquisition and Interpretation of Data for Biological and Synthetic Polymer Systems, Birkhäuser, Boston, 1994, pp. 66–80.

[34] D.J. Winzor, L.E. Carrington, S.E. Harding, Analysis of thermodynamic nonideality in terms of protein solvation, Biophys. Chem. 93 (2001) 231–240.

[35] L. Pauling, H.A. Itano, S.J. Singer, I.C. Wells, Sickle cell anemia: a molecular disease, Science 110 (1949) 543–548.
[36] O. Lamm, A. Polson, The determination of diffusion constants by a refractometric method, Biochem. J. 30 (1936) 528–541.
[37] V. Riveros-Moreno, J.B. Wittenberg, The self-diffusion coefficients of myoglobin and hemoglobin in concentrated solutions, J. Biol. Chem. 247 (1972) 895–901.
[38] W.W. Wilson, M.R. Luzzana, J.T. Penniston, C.S. Johnson, Pregelation aggregation of sickle cell hemoglobin, Proc. Natl. Acad. Sci. USA 71 (1974) 1260–1263.
[39] S.S. Alpert, G. Banks, The concentration dependence of the hemoglobin mutual diffusion coefficient, Biophys. Chem. 4 (1976) 287–296.
[40] C.R. Jones, C.S. Johnson, J.T. Penniston, Photon correlation spectroscopy of hemoglobin: diffusion of oxy-HbA and oxy-HbS, Biopolymers 17 (1978) 1581–1593.
[41] G. Kegeles, F.J. Gutter, The determination of sedimentation constants from Fresnel diffraction patterns, J. Am. Chem. Soc. 73 (1951) 3770–3777.
[42] H.K. Schachman, S.J. Edelstein, Ultracentrifuge studies with absorption optics. IV. Molecular weight determinations at the microgram level, Biochemistry 5 (1966) 2681–2705.
[43] G.S. Adair, A theory of partial osmotic pressures and membrane equilibria, with special reference to the application of Dalton's law to hemoglobin in the presence of salts, Proc. R. Soc. Lond. A 120 (1928) 573–603.
[44] A.P. Minton, Thermodynamic nonideality and the dependence of partition coefficient upon solute concentration in exclusion chromatography. Application to self-associating and non-self-associating solutes: application to hemoglobin, Biophys. Chem. 12 (1980) 271–277.
[45] F.H. Ree, W.G. Hoover, Reformulation of the virial series for classical fluids, J. Chem. Phys. 41 (1964) 1635–1645.
[46] A.P. Minton, Thermodynamic nonideality and the dependence of partition coefficient upon solute concentration in exclusion chromatography. II. An improved theory of equilibrium partitioning of concentrated protein solutions: application to hemoglobin, Biophys. Chem. 18 (1983) 139–143.
[47] K.E. Shearwin, D.J. Winzor, Thermodynamic non-ideality in macromolecular solutions: evaluation of parameters for the prediction of covolume effects, Eur. J. Biochem. 190 (1990) 523–529.
[48] R.W. Briehl, S. Ewart, Effects of pH, 2,3-diphosphoglycerate and salts on gelation of sickle cell deoxyhemoglobin, J. Mol. Biol. 80 (1973) 445–458.
[49] R.C. Williams Jr., Concerted formation of the gel of hemoglobin S, Proc. Natl. Acad. Sci. USA 70 (1973) 1506–1508.
[50] P.D. Ross, A.P. Minton, Analysis of non-ideal behavior in concentrated hemoglobin solutions, J. Mol. Biol. 112 (1977) 437–452.
[51] P.R. Wills, D.J. Winzor, Thermodynamic non-ideality and sedimentation equilibrium, in: S.E. Harding, A.J. Rowe, J.C. Horton (Eds.), Analytical Ultracentrifugation in Biochemistry and Polymer Science, Roy. Soc. Chem, Cambridge, UK, 1992, pp. 311–330.
[52] P.R. Wills, W.D. Comper, D.J. Winzor, Thermodynamic non-ideality in macromolecular solutions: interpretation of virial coefficients, Arch. Biochem. Biophys. 300 (1993) 206–212.