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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

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The theory of partition of macromolecular solute between mobile and stationary phases under highly nonideal conditions is reexamined. Expressions are derived for the dependence of the partition coefficient of a non-self-associationg solute, and the weight average partition coefficient of a dimerizing solute, upon the total concentration of solute in the mobile phase. These expressions are based upon a treatment of nonideality in the stationary phase which is more realistic than that proposed earlier [L.W. Nichol, R.J. Siezin and D.J. Winzor, Biophys. Chem. 9 (1979) 17]. It is found that the chromatographic data on oxyhemoglobin in the above paper may be accommodated by the present theory without invoking self-association.

1. Introduction

It has been known for some time that macromolecular self-association may lead to a dependence of the partition coefficient upon concentration of macromolecular solute in exclusion chromatography [1]. More recently, it has been recognized that thermodynamic nonideality may also influence the partition coefficient at high concentrations of solute [2,3]. When equilibrium constants for self-association are large, the experiments required to characterize the self-association process may be carried out at solute concentrations which are sufficiently low so that nonideal effects may be neglected. However, when equilibrium constants for self-association are small, experiments must be carried out at high solute concentration. Under these conditions, the validity of any quantitative analysis of chromatographic data in terms of self-association will be heavily dependent upon the validity of assumptions which are made in evaluating nonideal contributions to the thermodynamic activity of solute in the mobile and stationary phases.

Nichol et al. [3] have presented a theoretical treatment of the effect of nonideality upon the concentration dependence of partition coefficient in exclusion chromatography. They have employed their results to analyze data obtained from the chromatography of hemoglobin solutions ranging in concentration up to over 160 g/ℓ. They concluded that in order to accomodate the data it was necessary to postulate that at the highest concentrations encountered in their experiments over one-third of the total hemoglobin present was in the form of dimers or higher oligomers.

We present here a theoretical treatment of partition of a macromolecular solute between mobile and stationary phases which is based upon assumptions regarding nonideal behavior that are more realistic than those employed by Nichol et al. [3]. It is shown that the data of Nichol et al. [3] can be accommodated by the present theory without postulating self-association of hemoglobin.

2. Average and local concentrations of solute in the stationary phase

The quantity which is measured in a conventional frontal exclusion chromatography experiment is the partition coefficient, σ, defined by

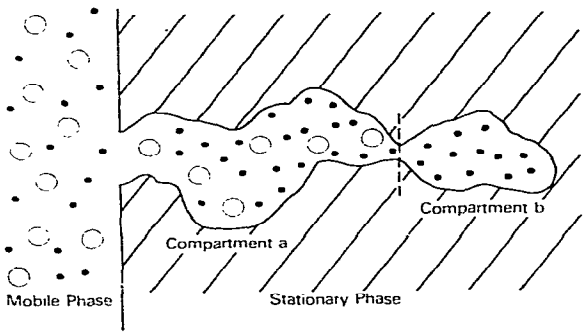

Fig. 1. Schematic representation of the distribution of small molecule solute (small black dots) and macromolecular solute (open circles) in the stationary phase matrix.

$$\sigma \equiv (V - V_0)/(V_t - V_0). \tag{1}$$

where V is the elution volume of the macromolecular solute, V_0 the void volume of the column, as measured by the elution volume of a solute which is too large to penetrate into the matrix containing the stationary phase, and V_t is the elution volume of a small molecule solute which is presumed to distribute equally in all elements of volume which are accessible to solvent, whether in the mobile or stationary phases. It may be readily shown that if one assumes that mobile and stationary phases are in dynamic equilibrium, then

$$\sigma = c_S/c_M \tag{2}$$

where c_M is the concentration of macromolecular solute in the mobile phase and c_S is the concentration of macromolecular solute in the stationary phase averaged over all elements of volume which are accessible to the small molecule solute used to define V_t in eq. (1).

Consideration of the structure of pores in the matrix on a molecular level suggests that their interior dimensions are subject to considerable fluctuation in space. Thus it is highly probable that there exist regions of pores or lacunae in the matrix which are accessible to the small molecule solute but not to the macromolecular solute (fig. 1). Thus one may distinguish between c_S, which represents a macromolecular concentration averaged over the entire volume accessible to small molecule solute (the sum of the volume of compartments a and b in fig. 1), and c_S^*, a local concentration of macromolecular solute defined for a particular region, which may be zero (as in compartment b of fig. 1) or greater than c_S (as in compartment a of fig. 1). The distinction between local and average concentration of macromolecular solute in the stationary phase is employed in the treatment of nonideal behavior which follows.

3. Concentration dependence of the partition coefficient for a nonassociating solute

We may write the chemical potentials of solute in the mobile and stationary phases in the conventional manner

$$\mu_M = \mu_M^0 + RT \ln c_M + RT \ln \gamma_M(c_M), \tag{3}$$

$$\mu_S = \mu_S^0 + RT \ln c_S + RT \ln \gamma_S(c_S), \tag{4}$$

where μ_M^0 and μ_S^0 are the standard state potentials of solute in mobile and stationary phases respectively, and γ_M and γ_S are the activity coefficients of solute in mobile and stationary phases, respectively. It is assumed that the activity of matrix is constant through out and that excluded volume and other interactions between matrix and individual solute molecules will manifest themselves in the difference between μ_M^0 and μ_S^0.

Assuming that in the plateau region, solute in the mobile phase is in equilibrium with stationary phase,

$$\mu_M = \mu_S. \tag{5}$$

Combining equations (2)–(5), we obtain

$$\sigma = \exp\left(\frac{\mu_M^0 - \mu_S^0}{RT}\right) \frac{\gamma_M(c_M)}{\gamma_S(c_S)} = \sigma_0 \frac{\gamma_M(c_M)}{\gamma_S(c_S)}. \tag{6}$$

In the limit of infinite dilution of solute, both γ_M and γ_S approach 1, and σ approaches σ_0.

Eq. (6) shows that the concentration dependence of the partition coefficient for a non-associating solute reflects differences between the activity coefficients of solute in the mobile and stationary phases. In order to employ eq. (6) in the analysis of experimental data, it is necessary to formulate expressions for γ_M and γ_S which are realistic under well-defined conditions which can be attained or approximated experimentally.

It has recently been demonstrated that long-range electrostatic interactions between globular protein molecules are essentially entirely damped out in solutions of moderate ionic strength at pH values in the vicinity of the isoelectric point [4]. Under these conditions (but not necessarily otherwise), the concentration dependence of thermodynamic and hydrodynamic properties of bulk solutions may be quantitatively described over the entire experimentally accessible range of concentrations by models developed for suspensions of hard quasispherical particles [4–7]. The activity coefficient of a suspension of hard spheres may be written as a virial expansion in powers of the concentration. It was found that for solutions of hemoglobin with concentrations less than 300 g/ℓ, this expansion could be truncated after five terms with no significant loss of precision [4]:

$$\ln \gamma = \sum_{i=2}^{6} \frac{1}{i-1} B_i c^{i-1}, \tag{7}$$

where $B_2 = 7.0\ v$; $B_3 = 22.0\ v^2$; $B_4 = 43.45\ v^3$; $B_5 = 67.74\ v^4$; and $B_6 = 95.97\ v^5$, and v is the volume of the equivalent hard spherical particle in units of reciprocal concentration. It is stressed that v is not constrained to be equal to either the partial specific volume of the protein or the effective hydrodynamic volume of the protein, which includes the volume of a hydration shell (Appendix 1).

Following Nichol et al. [3], we shall employ eq. (7) to describe the dependence of γ_M upon c_M:

$$\ln \gamma_M = \sum_{i=2}^{6} \frac{1}{i-1} B_i c_M^{i-1} \tag{8}$$

The description of γ_S as a function of c_S is complicated by the variability of the internal dimensions of the volume elements in the matrix which are accessible to solute:

(1) The local standard state chemical potential of a solute particle increases rapidly as the internal dimensions of the volume element shrink toward the external dimension of the particle [8].

(2) The volume which a particle excludes to other particles depends upon its position relative to the boundaries of the volume element.

(3) As pointed out in section 2, the local concentration and hence the local activity coefficient will vary from volume element to volume element in the matrix.

Nichol et al. [3] have assumed that the activity coefficient of solute in the matrix has the same functional dependence upon average concentration of solute in the matrix as the activity coefficient of solute in the mobile (bulk) phase has upon the concentration of bulk solute. This assumption lacks physical justification.

As the internal dimensions of a volume element increases, the ratio of surface to volume decreases, boundary effects will diminish, and the local concentration will asymptotically approach a limiting value. Let us consider the largest volume elements extant in the matrix and write the chemical potential of the hemoglobin in these elements as

$$\mu_{S'} = \mu_{S'}^0 + RT \ln c_{S'} + RT \ln \gamma_{S'}(c_{S'}), \tag{9}$$

where S′ denotes the subset of largest accessible volume elements in the stationary phase, and $c_{S'}$ is the local concentration of solute in these elements. To the extent that boundary effects upon activity coefficient are negligible in this subset of volume elements, we may use the same expression to describe the dependence of *local* activity coefficient upon *local* concentration which was used to describe the dependence of activity coefficient upon concentration in the mobile (bulk) phase.

$$\ln \gamma_{S'}(c_{S'}) = \sum_{i=2}^{6} \frac{1}{i-1} B_i c_{S'}^{i-1}. \tag{10}$$

Because the local standard state chemical potential of solute decreases as the size of the volume element increases, solute in the matrix will probably be preferentially distributed in the large volume elements. Thus we may set

$$c_{S'} = \alpha c_S, \tag{11}$$

where α is expected to exceed unity. Substituting (10) and (11) into (9), we obtain

$$\mu_{S'} = \mu_{S'}^0 + RT \ln \alpha + RT \ln c_S + RT \sum_{i=2}^{6} \frac{1}{i-1} B_i \alpha^{i-1} c_S^{i-1}. \tag{12}$$

By virtue of the requirement that solute in all volume elements be in equilibrium with mobile phase and hence

with solute in all other volume elements, $\mu_S' = \mu_S$, and eq. (12) is seen to be equivalent to eq. (4), with $\mu_S^0 = \mu_S'^0 + RT \ln \alpha$,

$$\ln \gamma_S = \sum_{i=2}^{6} \frac{1}{i-1} B_i \alpha^{i-1} c_S^{i-1}. \quad (13)$$

Substituting eqs. (8) and (13) into eq. (6), we obtain after rearrangement

$$\sigma = \sigma_0 \exp\left\{ \sum_{i=2}^{6} \frac{1}{i-1} B_i c_M^{i-1} [1 - (\alpha\sigma)^{i-1}] \right\}. \quad (14)$$

When $\alpha = 1.0$, eq. (14) reduces to eq. (3) of Nichol et al. [3], which was used by these workers to calculate the theoretical dependence of σ upon c_M for non-associating solutes. As we have pointed out above, α is expected on physical grounds to exceed 1.0.

It is stressed that eq. (14) is an approximation, derived on the assumption that in the largest elements of volume in the stationary phase matrix which are accessible to macromolecular solute, boundary effects are sufficiently small that a bulk treatment of nonideal behavior is applicable. While admittedly oversimplified, this treatment is clearly more realistic than that of Nichol et al. [3], who (a) ignored the difference between local and average concentration in the stationary phase, and (b) assumed that a bulk treatment of nonideality was uniformly applicable throughout the stationary phase.

4. Concentration dependence of the partition coefficient for a dimerizing solute

Consider a solute which dimerizes in bulk solution with the equilibrium constant K (inverse molar). Then we may write for the mobile phase

$$K = n_2^M \gamma_2^M / (n_1^M \gamma_1^M)^2, \quad (15)$$

$$n_2^M = K(n_1^M \gamma_1^M)^2 / \gamma_2^M, \quad (16)$$

where n_i^M and γ_i^M respectively denote molar concentrations and activity coefficient in the mobile phase, and the subscripts 1 and 2 respectively denote monomer and dimer.

In general, γ_1 and γ_2 are both functions of both n_1^M and n_2^M. The virial expansion of $\ln \gamma$ for a system containing two or more species becomes quite unwieldy beyond second order terms (see, for example, eq. (9) of ref. [9]), and the evaluation of higher order virial coefficients involving interactions between m_1 particles of species 1 and m_2 particles of species 2 is in general a problem of prohibitive mathematical complexity.

An approximate method for calculating activity coefficients in a suspension of several species of differently sized hard spherical particles is provided by the scaled particle theory [10]. The results of calculations obtained using this theory for binary mixtures of hard spheres agree well with the results of Monte Carlo computer simulations [10], lending confidence in the semiquantitative validity of the results. The required relations are presented in simplified form as eqs. (1) and (3) of ref. [11]. Let us schematically represent these equations as:

$$\gamma_1^M = F_1^{SP}(n_1^M, n_2^M, r_1, r_2),$$

$$\gamma_2^M = F_2^{SP}(n_1^M, n_2^M, r_1, r_2). \quad (17)$$

where r_1 and r_2 are the radii of the spherical particles representing monomer and dimer respectively.

Let c denote the total weight concentration of solute in the mobile phase:

$$c = M_1 [n_1^M + 2n_2^M], \quad (18)$$

where M_1 is the molecular weight of monomer. Given the values of c, M_1, K, r_1 and r_2, eqs. (16), (17) and (18) may be numerically solved for the values of n_1^M, n_2^M, γ_1^M and γ_2^M.

The calculation of γ_1^S and γ_2^S proceeds in a manner analogous to the calculation of γ^S in the preceding section. Let us designate the local concentrations of monomer and dimer in the largest volume elements accessible to them in the matrix by $\alpha_1 n_1^S$ and $\alpha_2 n_2^S$. α_2 is expected to be greater than α_1 since dimer will be preferentially excluded from smaller volume elements. If it is assumed that boundary effects in the largest elements are sufficiently small that a bulk treatment of nonideality is applicable, then

$$\gamma_1^S = F_1^{SP}(\alpha_1 n_1^S, \alpha_2 n_2^S, r_1, r_2),$$

$$\gamma_2^S = F_2^{SP}(\alpha_2 n_1^S, \alpha_2 n_2^S, r_1, r_2). \quad (19)$$

Each species is presumed to be in equilibrium between mobile and stationary phases:

$$\mu_1^M = \mu_1^S, \quad \mu_2^M = \mu_2^S.$$

By a procedure entirely analogous to that employed in the derivation of eq. (5) we obtain

$$\sigma_1 = \sigma_1^0 \gamma_1^M(n_1^M, n_2^M)/\gamma_1^S(n_1^S, n_2^S),$$

$$\sigma_2 = \sigma_2^0 \gamma_2^M(n_1^M, n_2^M)/\gamma_2^S(n_1^S, n_2^S). \quad (20)$$

The quantity measured experimentally is the weight average partition coefficient [2,3]

$$\bar{\sigma} = (\sigma_1 n_1^M + 2\sigma_2 n_2^M)/(n_1^M + 2n_2^M). \quad (21)$$

Given the values of $c, M_1, K, r_1, r_2, \alpha_1, \alpha_2, \sigma_1^0$, and σ_2^0, eqs. (16)–(20) may be numerically solved to obtain the values of $n_i^M, n_i^S, \gamma_i^M, \gamma_i^S$, and σ_i, from which the value of $\bar{\sigma}$ may be calculated via eq. (21).

The difficulty inherent in using the experimentally measured dependence of $\bar{\sigma}$ upon c to determine K is apparent. Of the input parameters required to calculate $\bar{\sigma}$, only c, M, and σ_1^0 (= $\lim_{c\to 0} \sigma$) can be unambiguously determined by independent measurement. The value of r_2 may be estimated to be equal to $2^{1/3} r_1$. Even so, five undetermined parameters (K, $r_1, \sigma_2^0, \alpha_1$ and α_2) remain [‡].

One may attempt to evaluate these parameters by least-squares fitting of the model equations to the data ($\bar{\sigma}$ as a function of c). However, in a situation involving so many disposable parameters it is unlikely that any of them will be well determined by the data, except in the special case of strong self-association, where the effects of self-association will be manifested at concentrations so low that nonideal effects may be neglected. In this case the equations above simplify enormously and reduce to well-known results [1].

Nichol et al. [2,3] have argued that substances such as controlled pore glass provide a matrix which can be chosen so as to exclude all species of solute except monomer from the stationary phase. In view of the intrinsic point-to-point variability of pore size on a molecular scale, it is difficult to see how any matrix which admits particles of volume v_1 or radius r_1 can be expected to rigorously exclude particles of volume $2v_1$ or radius $1.26\, r_1$.

[‡] Strictly speaking, only four of these five are independently variable. Initial deviations from ideality observed at low concentration in the measurement of any colligative property (for example, osmotic pressure) may be used to fix a relation between the values of K and r_1, but cannot be used to evaluate either independently [12].

Let us investigate how the above assumption simplifies the theoretical analysis. Since no dimer is admitted to the stationary phase, $n_2^S = \sigma_2 = 0$, and

$$\bar{\sigma} = \sigma_1^0 \frac{n_1^M}{n_1^M + 2n_2^M} \frac{\gamma_1^M(n_1^M, n_2^M)}{\gamma_1^S(n_1^S)}, \quad (22)$$

where γ_1^S is given by

$$\gamma_1^S = F_1^{SP}(\alpha n_1^S, 0, r_1, r_2). \quad (23)$$

For this simplified case, given the values of $c, K, M_1, r_1, r_2, \alpha$, and σ_1^0, one may numerically solve eqs. (16), (17), (18), (22), and (23) for the value of n_i^M, $n_1^S, \gamma_i^M, \gamma_1^S$ and $\bar{\sigma}$. Although this simplification reduces the number of independently variable parameters to three, it remains to be established that a small value of K may be reliably distinguished from zero by fitting the simplified equations to data, particularly in view of the approximate nature of (a) the calculation of activity coefficients at high concentration, especially in the stationary phase, and (b) the assumption of total exclusion of dimer from the stationary phase.

5. Comparison of theory with experiment

The dependence of σ upon c calculated according to eq. (14) is plotted in fig. 2 for three different values of α and a value of v equal to that obtained from the analysis of the sedimentation equilibrium of hemoglobin by Ross et al. [6] on the assumption that hemoglobin does not self-associate. Also plotted in fig. 2 are the data of Nichol et al. [3] on the concentration dependence of the partition coefficient of hemoglobin. It may be seen that the dependence calculated from eq. (14) with $\alpha = 1.15$ agrees with essentially all of the chromatographic data to within the experimental uncertainty indicated by the error bars, and it is in much better agreement with the data than the curve calculated with $\alpha = 1.0$ (i.e., the equation of Nichol et al. [3]).

Nichol et al. [3] claimed that the disagreement between their calculated curves and the data constituted evidence for the self-association of hemoglobin molecules at high concentration. The foregoing treatment shows that a more realistic treatment of nonideal effects in the stationary phase can greatly improve the degree of agreement between theory and experiment, without postulating self-association of hemoglobin. It

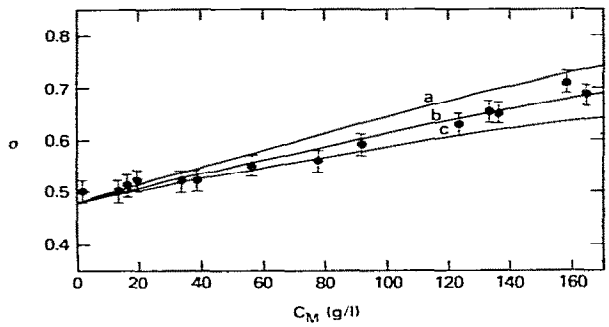

Fig. 2. Dependence of the partition coefficient of hemoglobin upon concentration of hemoglobin in the mobile phase. Points: data of Nichol et al. [3]. Curves: calculated from equation (14) with $\sigma_0 = 0.48$, $v = 0.92$ ml/g, B_i as given in eq. (7), and $\alpha = 1.0$ (curve a), 1.15 (curve b), and 1.3 (curve c).

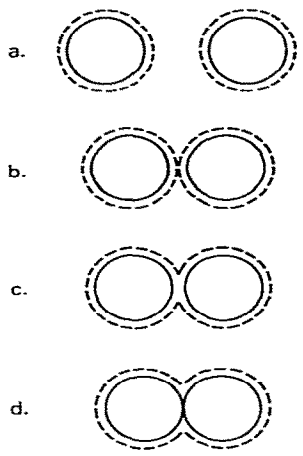

Fig. 3. Schematic representation of two molecules in solution surrounded by "hydration layers". (a) Molecules separated by bulk solvent. (b) Molecules separated by the thickness of two hydration layers. (c) Molecules separated by the thickness of one hydration layer. (d) Molecular surfaces in close contact.

is highly likely that any residual discrepancy between the present theory and the experimental data, small as it is, may be attributed to the necessarily simplistic nature of the treatment of nonideality rather than failure to take self-association into account.

In conclusion, our present view on the possibility of self-association of hemoglobin molecules remains as before, when we stated [4]: "The present analysis does not exclude the possibility that the intermolecular interactions between hemoglobin molecules in salt solutions are more complex than represented by the hard particle model. However, we have demonstrated that it is not necessary to invoke more complex interactions in order to account for the experimental data which are presently available".

Appendix

Distinctions between the partial specific volume, the effective hard sphere volume, and the effective hydrodynamic volume of a globular macromolecule

The difference between effective thermodynamic and hydrodynamic volumes of protein has been discussed previously [7,13]. However, in view of recent confusion between these quantities [3] it seems worthwhile to review this topic in some detail.

The partial specific volume of a macromolecule, $\bar{v}$ is defined as the differential change in solution volume per unit dry weight of protein added at fixed protein concentration, temperature, and pressure. On a molecular scale, this quantity corresponds to the amount of volume per protein molecule which is excluded to *solvent*.

The radius of the effective hard sphere, r, is defined to be one-half of the average distance of closest approach (contact distance) between two identical molecules. Interactions between molecules at distances greater than the contact distance are assumed to be of short range and highly dependent upon the mutual orientation of the two molecules. Such interactions will not significantly contribute to the volume excluded by the molecule [4]. The volume of the effective hard sphere, v, is of course $4\pi r^3/3$.

Hydrodynamic theory permits the calculation of hydrodynamic properties (such as intrinsic viscosity) of rigid ellipsoids in the limit of infinite dilution in a continuum fluid. The effective hydrodynamic volume of a globular macromolecule, v_h, is the volume of an equivalent particle whose theoretically calculated properties in the limit of infinite dilution are equal to those measured in solutions of the macromolecule.

Let us consider the differences between $\bar{v}$, v and v_h for a specific molecule. In an earlier publication [7] we

noted that for hemoglobin, $\bar{v} = 0.75$ cm^3/g, $v = 0.92$ cm^3/g, and $v_h = 1.30$ cm^3/g, the latter two values being calculated using the approximation of a spherical equivalent particle.

An inspection of a space-filling molecular model of hemoglobin reveals the presence of crevices between the subunits which will admit water molecules. Thus the volume per molecule from which solvent is excluded ($\bar{v}$) would certainly be less than the volume from which other hemoglobin molecules are excluded (v). This latter quantity would more closely correspond to the volume enclosed by the external boundary of the molecule at low resolution.

The effective hydrodynamic volume corresponds roughly to the combined volume of the molecule plus the volume of a layer of solvent surrounding the molecule which moves with the molecule (the "hydrodynamic hydration layer"). Fig. 3a schematically shows two molecules surrounded by their respective hydration layers. If the molecules could approeach each other to no closer than twice the thickness of the hydration layer (fig. 3b), then the effective hard sphere volume v would be equal to the effective hydrodynamic volume v_h. If, however, the two molecules may approach each other more closely (figs. 3c and 3d), then v will be less than v_h. There is no obvious reason why the two molecules should not be able to approach each other to within the distance of a single hydration layer (fig. 3c) or even more closely (fig. 3d).

We conclude that simple physical arguments can rationalize the observation that $\bar{v} < v < v_h$.

Acknowledgement

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