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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

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An improved theory for the partitioning of protein between concentrated bulk solution and solution sequestered in a porous medium is presented. The theory is based upon the assumption that the sequestered solution may be formally represented as a sum of three compartments: (1) a compartment which is inaccessible to protein, but accessible to solvent and small molecule solutes; (2) a surface layer immediately adjacent to the pore boundary, within which protein molecules are constrained to quasi-two-dimensional motion; and (3) the remaining volume accessible to protein, within which the protein molecules behave as if in bulk solution. The dependence of the partition coefficient of hemoglobin upon protein concentration over the range 10–225 g/l, calculated using the theory presented, is found to agree quantitatively with experimental data presented previously (R.J. Siezen, L.W. Nichol and D.J. Winzor, Biophys. Chem. 14 (1981) 221) without invoking self-association of hemoglobin molecules.

1. Introduction

The equilibrium partitioning of protein between bulk solution and solution sequestered in a porous medium, such as a polymer gel or porous glass, is influenced by both self-association of protein molecules and deviations from thermodynamic ideality. In the absence of nonideal effects, self-association causes the partition coefficient to decrease with increasing protein concentration [1], and in the absence of self-association, protein nonideality causes the partition coefficient to increase with increasing protein concentration [2]. It is of considerable theoretical interest and practical importance to inquire whether the dependence of partition coefficient upon protein concentration which is observed experimentally may be unambiguously decomposed into separate contributions arising from nonideality and self-association.

Nichol and co-workers [3] have introduced a theoretical model for the effect of excluded volume upon the chemical potential of protein in the sequestered solution. By employing this model in the analysis of experimental measurements of the partition coefficient of hemoglobin in solutions of up to 170 g/l concentration, they concluded that hemoglobin self-associates to a substantial extent as protein concentration increases over this range [3]. It was subsequently pointed out that the theoretical model of Nichol and co-workers [3] was highly oversimplified, and that by using a slightly less oversimplified theoretical model for nonideal

effect (henceforth referred to as APM I), it was possible to account for essentially all of their experimental data without postulating self-association [4]. It was stated in ref. 4 that residual discrepancies between the results of model calculations and the experimental data of Nichol et al. [3], small as they were, were more likely due to the inadequacy of APM I than to the neglect of hemoglobin self-association. Nichol and co-workers [5] have recently contested this assertion, and argue that the inability of APM I to generate plots of partition coefficient versus hemoglobin concentration which are concave upwards (as suggested by the data) constitutes firm evidence for the presence of substantial hemoglobin self-association in these solutions.

We present here an improved theoretical model for the effect of excluded volume upon the chemical potential of protein in a sequestered solution. It will be shown that by using this model, the dependence of partition coefficient upon hemoglobin concentration reported by Nichol and coworkers [3,5] may be accounted for quantitatively without the assumption of self-association.

2. Theory

In the present treatment, as in its predecessors [3,4], protein nonideality will be assumed to arise exclusively from volume exclusion (which includes the effect of electrostatic repulsion), and that volume exclusion may be accounted for on the assumption that globular proteins behave as effective hard spherical particles (for a recent review, see ref. 6).

The quantity which is to be calculated and compared with experiment is the partition coefficient of a single species of protein

$$\alpha = c_S / c_M \tag{1}$$

where c_M is the equilibrium concentration of protein in the bulk or mobile phase, and c_S the equilibrium concentration of protein in the sequestered or stationary phase, averaged over all elements of volume in this phase which are accessible to solvent or a small molecule marker. It was previously pointed out [4] that due to the heterogeneous fine structure of the porous medium in which the stationary phase is sequestered, the local concentration of protein in this phase is expected to vary with position *. In a formal sense one may represent the stationary phase as a mixture of compartments, within each of which the concentration of protein is uniform. In the interest of mathematical tractability it is necessary to determine the minimum number of compartments which can provide a physically realistic representation of the distribution of protein within the stationary phase.

The total volume of the stationary phase may be divided into a fraction which is accessible to all components of the protein solution (protein, small molecule solutes, and solvent) and a fraction which is accessible to small molecules but inaccessible to protein.

In a Monte Carlo study of hard-sphere fluids in the vicinity of a hard wall, Snook and Henderson [7] found that the mean density of spherical particles immediately adjacent to the wall was significantly greater than the bulk density of the fluid, and that these particles tended to follow quasi-two-dimensional trajectories rather than the three-dimensional trajectories followed by particles more than a particle diameter away from the surface. They characterized the surface layer as a two-dimensional (hard disc) fluid. In accordance with these findings we shall divide the fraction of volume in the stationary phase which is accessible to protein into a surface layer, having the thickness of a single spherical protein molecule, and the remaining volume accessible to protein.

Thus, our minimal model of the stationary phase consists of three compartments:

$$v_S = v_I + v_{SL} + v_{RA} \tag{2}$$

where v_S is the total volume of the stationary phase and v_I, v_{SL} and v_{RA} the volumes of the inaccessible compartment, the surface layer and the remaining accessible compartment, respectively. These compartments are shown schematically in fig. 1.

* The stationary phase is thus not a true phase in the thermodynamic sense. However, we shall continue to use this term in accordance with established chromatographic practice.

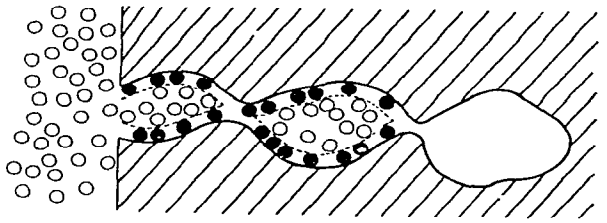

Fig. 1. Schematic illustration of the distribution of protein molecules within the stationary phase. Molecules residing in the surface layer are colored black, and those in the remaining accessible compartment or in the mobile (exterior) phase are uncolored.

It may be easily shown that

$$\sigma = f_I \sigma_I + f_{SL}\sigma_{SL} + f_{RA}\sigma_{RA} \tag{3}$$

where $f_X \equiv v_X/v_S$ and $\sigma_X \equiv c_X/c_M$. By definition $\sigma_I = 0$ for all c_M. Protein molecules in the remaining accessible compartment do not interact in any fashion whatsoever with the boundaries of the stationary phase, and are thus thermodynamically indistinguishable from protein molecules in bulk solution. Hence, $\sigma_{RA} = 1$ for all c_M, and eq. 3 reduces to

$$\sigma = f_{SL}\sigma_{SL} + f_{RA} \tag{4}$$

At equilibrium the chemical potential of protein in the surface layer must equal that in the bulk phase. It follows that [3,4]

$$\sigma_{SL} = \sigma^0_{SL}\frac{\gamma_M}{\gamma_{SL}} \tag{5}$$

where σ^0_{SL} is the partition coefficient for the surface layer in the limit of infinite dilution (thermodynamic ideality), and γ_M and γ_{SL} the activity coefficients for protein in the mobile phase and surface layer, respectively.

The value of γ_M may be calculated according to the hard-sphere approximation using the following virial expansion [6]

$$\ln \gamma_M = \sum_{i=2} R_i \phi_M^{i-1} \tag{6}$$

where the R_i are dimensionless coefficients given in table 1 and ϕ_M is the fraction of volume of the mobile phase occupied by equivalent spherical particles representing protein molecules.

Table 1

Values of the coefficients defined in eqs. 6 and 9

i	R_i	S_i
2	8.00	6.00
3	15.00	10.56
4	24.48	19.18
5	35.30	33.80
6	47.40	58.09

Since molecules in the surface layer are assumed to exclude volume to each other in two rather than three dimensions, we shall calculate γ_{SL} using the following virial expansion for hard-disc fluids [8]

$$\ln \gamma_{SL} = \sum_{i=2} \lambda_i \rho_{SL}^{i-1} \tag{7}$$

where the λ_i are dimensionless coefficients tabulated in ref. 8, and ρ_{SL} is the fraction of surface area occupied by the circular cross-sections of equivalent spherical particles representing protein molecules. Since the thickness of the surface layer is defined to be one particle diameter, it may be readily shown that

$$\rho_{SL} = \tfrac{1}{2}\phi_{SL} \tag{8}$$

where ϕ_{SL} is the fraction of the volume of surface layer occupied by equivalent spherical particles. It follows from eqs. 7 and 8 that

$$\ln \gamma_{SL} = \sum_{i=2} S_i \phi_{SL}^{i-1} \tag{9}$$

where the coefficients S_i ($= \lambda_i(3/2)^{i-1}$) are given in table 1.

Eqs. 5, 6 and 9 may be combined to yield

$$\sigma_{SL} = \sigma^0_{SL} \exp\left[\sum_{i=2}\left(R_i\phi_M^{i-1} - S_i\phi_{SL}^{i-1}\right)\right] \tag{10}$$

Since $\phi = vc$, where v is the specific volume of equivalent spherical particles and c the weight concentration of protein, it follows from eqs. 1 and 10 that

$$\sigma_{SL} = \sigma^0_{SL} \exp\left\{\sum_{i=2}(vc_M)^{i-1}\left[R_i - S_i(\sigma_{SL})^{i-1}\right]\right\} \tag{11}$$

The parameters f_{SL} and f_{RA} may be calculated as functions of f_I, σ^0_{SL} and σ^0 (the value of σ extrapolated to $c_M = 0$) using the following constraining relations derived from eqs. 3 and 4, respectively:

$$f_{SL} + f_{RA} = 1 - f_I \tag{12a}$$

$$\sigma^0 = f_{SL}\sigma^0_{SL} + f_{RA} \tag{12b}$$

Thus, σ may be calculated as a function of c_M using eqs. 4, 11 and 12 together with values of the parameters v, f_I, σ^0_{SL} and σ^0.

3. Results and discussion

Nichol and co-workers [3,5] have presented experimental data on the dependence of σ upon c_M for oxyhemoglobin in phosphate buffer and oxy- and deoxyhemoglobin in Bistris-chloride buffer. Their data are plotted in fig. 2 together with curves calculated using eqs. 4, 11 and 12 together with the parameter values given in the figure caption *. While these particular sets of parameter values provide excellent agreement with the corresponding data sets, other sets of significantly different (but physically reasonable) parameter values can lead to equally good agreement, and so the data do not uniquely determine the values of all four parameters. For this reason, and because of the obviously simplistic nature of the present theoretical model, it would be unwise to attach physical significance to these parameter values. It is, however, relevant to note that the upward curvature evident in the data, taken by Nichol and co-workers [5] as a firm indication of hemoglobin self-association, is faithfully reproduced by the calculated curves. According to the present model, the upward curvature is due not to self-association, but rather to the quasi-two-dimensional behavior of protein molecules in the surface layer, which results in a reduction in volume exclusion in this layer relative to that in the bulk solution.

In conclusion, we reassert (cf. ref. 4) that while neither the present analysis nor any other of which we are aware can exclude the possibility that hemoglobin does indeed self-associate under the conditions studied by Nichol and co-workers [3,5], it is not necessary to invoke self-association in order to account for the data which they have so far presented. Hence, these data do not provide evidence for the existence of self-association.

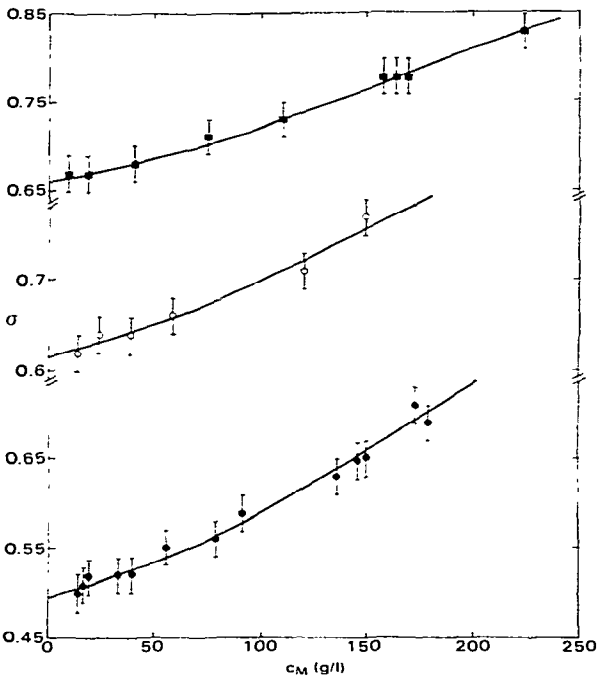

Fig. 2. Dependence of the partition coefficient of hemoglobin at pH 7.3, 20°C upon hemoglobin concentration. Data were taken from ref. 5. Curves were calculated as described in text with $f_I = 0.1$, $v = 0.92$ ml/g, and additional parameter values given below. Lower: bovine and human oxyhemoglobin in 0.156 *I* phosphate, $\sigma^0 = 0.495$, $\sigma^0_{SL} = 0.195$. Middle: human oxyhemoglobin in 0.15 *I* Bistris-chloride, $\sigma^0 = 0.615$, $\sigma^0_{SL} = 0.27$. Upper: human deoxyhemoglobin in 0.15 *I* Bistris-chloride, $\sigma^0 = 0.66$, $\sigma^0_{SL} = 0.22$.

* For the purpose of calculation, the infinite sum indicated in eq. 11 was truncated after the term for $i = 6$. Convergence of the truncated sum was verified at the highest protein concentration encountered in each case.

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