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Review

Macromolecular crowding: qualitative and semiquantitative successes, quantitative challenges

Damien Hall, Allen P. Minton*

Section on Physical Biochemistry, Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, Building 8, Room 226, National Institutes of Health, US Department of Health and Human Services, Bethesda, MD 20892-0830, USA

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Abstract

The concept of excluded volume and the theory of effects of excluded volume on the equilibria and rates of macromolecular reactions in fluid media containing high total concentrations of macromolecules ('crowded' media) are summarized. Reports of experimental studies of crowding effects published during the last year are tabulated. Limitations of current excluded volume theory are discussed, and a determination is made of conditions under which this theory may and may not be validly applied. Recently suggested novel approaches to quantitative analysis of crowding phenomena, which may help to overcome some of the limitations of current theory, are summarized. Published by Elsevier B.V.

Keywords: Macromolecular solution; Excluded volume; Thermodynamic nonideality

1. Introduction

Physiological fluid media contain macromolecules collectively occupying between a lower limit of about 7% and an upper limit of about 40% of total fluid volume [1,2]. Such fluids are termed 'volume-occupied' or 'crowded' rather than concentrated, since no single macrosolute species may be concentrated.[1] The influence of high fractional volume occupancy on the rates and equilibria of macromolecular reactions taking place in crowded solutions has been recognized since the 1960s, but the biochemical and biophysical implications of these effects have only begun to be appreciated by the wider community of biomedical researchers within the last 10 years or so. During the last 3 years, several minireviews on the subject of macromolecular crowding have appeared [3–7]. While the qualitative and semiquantitative successes of crowding theory—and they are substantial—have been well documented, there remain important aspects of quantitation that in our opinion remain inadequately understood and analyzed. We shall discuss some of these challenging issues and call attention to some new approaches that may help to resolve conceptual ambiguities and enhance quantitative analyses of crowding phenomena.

This minireview is organized as follows. First, we provide a basic introductory tutorial to the concept of excluded volume, and its effect on chemical rates and equilibria in highly volume-occupied solutions resembling biological fluid media. Second, we present a tabulation of recently published experimental studies not cited in previous reviews. Third, we attempt to examine critically capabilities and, importantly, the very real limitations of current excluded volume theory. Finally, we review some new approaches to the quantitative analysis of macromolecular crowding.

2. The effect of nonspecific solute–solute interaction on the rates and equilibria of macromolecular reactions[2]

The influence of volume exclusion upon the thermodynamics of chemical reactions in volume-occupied media

* Corresponding author. Tel.: +1-301-496-3604.
E-mail address: minton@helix.nih.gov (A.P. Minton).

[1] The expression 'volume occupancy' denotes the occupancy of solution volume by solutes, and in the context of the present review refers more specifically to macromolecular solutes except when specified otherwise.

[2] This brief summary of basic relationships is presented for the benefit of readers not previously familiar with the subject, and to introduce nomenclature and notation utilized subsequently. More complete treatments may be found in the references cited below and in Ref. [2].

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becomes evident upon examination of a few simple but fundamental relationships [8,9]. Let us represent a generalized reaction in solution by the following scheme:

$$r_1R_1 + r_2R_2 + \ldots \rightleftarrows p_1P_1 + p_2P_2 + \ldots \quad (1)$$

where r_i is the stoichiometric coefficient of reactant species R_i, and p_i is the stoichiometric coefficient of product species P_i. The equilibrium concentrations of reactants and products at temperature T and pressure P are related by:

$$\frac{w_{P_1}^{p_1} w_{P_2}^{p_2} \cdots}{w_{R_1}^{r_1} w_{R_2}^{r_2} \cdots} \equiv K(T,P,\{w\}) = K^{\circ}(T,P)\ \Gamma(T,P,\{w\}) \quad (2)$$

where w_i denotes the equilibrium weight/volume concentration of solute species i, and $\{w\}$ denotes the composition of the solution (i.e., the equilibrium concentrations of all solutes). K denotes a composition-dependent *apparent* equilibrium constant, K° the true equilibrium constant, and Γ the "nonideality factor", a composition-dependent measure of solute–solute interactions, given by

$$\Gamma(T,P,\{w\}) = \frac{\gamma_{R_1}^{r_1}\gamma_{R_2}^{r_2}\cdots}{\gamma_{P_1}^{p_1}\gamma_{P_2}^{p_2}\cdots} \quad (3)$$

where γ_i denotes the thermodynamic activity coefficient of solute species i.[3] For transition-state rate-limited reactions, the effective forward and backward reaction rate constants are given by (Appendix of Ref. [10]):

$$k_f = k_f^{\circ}\frac{\gamma_{R_1}^{r_1}\gamma_{R_2}^{r_2}\cdots}{\gamma_T} \quad (4)$$

$$k_b = k_b^{\circ}\frac{\gamma_T}{\gamma_{P_1}^{p_1}\gamma_{P_2}^{p_2}\cdots} \quad (5)$$

where k_f° and k_b°, respectively, denote the forward and backward rate constants in the limit of high dilution of all macrosolutes, and γ_T denotes the thermodynamic activity coefficient of the transition-state complex. It has been argued [9] that due to the short-range nature of attractive intermolecular interactions in solution, the transition state of a self- or hetero-association reaction must be compact, suggesting that

$$\gamma_T \approx \gamma_{P_1}^{p_1}\gamma_{P_2}^{p_2}\cdots \quad (6)$$

from which it follows that for associations in solution, crowding is expected to affect primarily the forward rate constant: $k_f \approx k_f^{\circ}\Gamma$ and $k_b \approx k_b^{\circ}$. However, this is not necessarily the case for transition states in other types of reactions, such as isomerization or surface adsorption [10].

[3] The activity coefficient is a measure of solute–solute interaction in solution. Its formal definition is given below.

γ_i is a measure of the equilibrium average free energy of interaction in solution between a molecule of solute species i and all other solute molecules present:

$$RT\ln\gamma_i(T,P,\{w\}) = \left(\frac{\partial G}{\partial w_i}\right)_{T,P,\{w\}} - \left(\frac{\partial G}{\partial w_i}\right)_{T,P,\{w\}\Rightarrow 0} \equiv \left(\frac{\partial G}{\partial w_i}\right)^{*}_{T,P,\{w\}} \quad (7)$$

The asterisk attached to the rightmost term in Eq. (4) serves to remind us that the interaction energy defined by the activity coefficient is a *differential* interaction energy, i.e., the difference between (1) the energy of interaction between solute species i and the other solute molecules in the crowded solution, and (2) the energy of interaction between solute species i and the solvent molecules that were replaced by solute in the crowded solution. It follows from Eq. (7) that in the limit of high dilution of all solutes, the activity coefficient of each solute approaches unity, and subsequently the value of Γ approaches unity. Under these circumstances, the apparent equilibrium constant K becomes equal to the true equilibrium constant K°. In highly volume occupied solutions, on the other hand, the activity coefficient of each macrosolute—dilute as well as concentrated—may deviate from unity by as much as several orders of magnitude, with potentially major impact on reaction equilibria and rates in these solutions (see below).

Eq. (7) is exact and takes into account all (differential) solute–solute interactions. In the present minireview, we focus on one specific class of interactions, namely those arising from the mutual impenetrability of solutes and the consequent steric repulsion. We refer to these interactions as excluded volume interactions for reasons that will become clear in the following development. Depending upon experimental conditions, other types of solute–solute interactions may or may not contribute significantly to γ_i, but in crowded solutions, excluded volume effects are unavoidable, ubiquitous, and will almost always have significant energetic consequences [2,5].

In order to illustrate the concept of excluded volume, and its complement, available volume, we shall employ a macroscopic analogy. Consider a beaker filled to the brim with ball bearings of 5-mm diameter. The randomly close-packed ball bearings occupy about 65% of the volume of the beaker, leaving about 35% in the interstices between the ball bearings [11]. Even though the interstitial volume is 'empty', geometric constraints prevent the addition of even a single additional ball bearing to the beaker (Fig. 1A). The interstitial volume is said to be excluded to ball bearings. Alternately, the volume *available* to ball bearings (i.e., the total volume minus the excluded volume) has become zero. The interstitial volume is, however, available to particles that are sufficiently smaller, such as grains of sand. If we pour sand into the beaker, it will 'fill' the interstices between the ball bearings, but in reality occupy only about 65% of that volume (Fig. 1B). When the beaker is filled with sand in this fashion, the

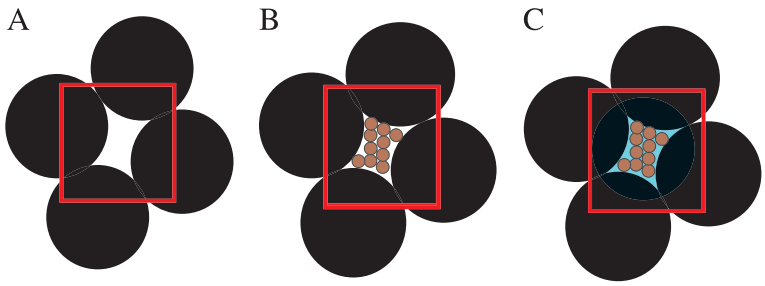

Fig. 1. Simplified example of the concept of a size-dependent available volume, as described in the text. Large black spheres depict ball-bearings, small brown spheres depict grains of sand, and blue field indicates water. The red border demarcates a region of unit volume.

volume remaining between grains of sand, corresponding to about 10% of the total volume of the beaker, is excluded to both ball bearings and grains of sand, but is available to yet smaller particles, and may be filled with water (Fig. 1C).

Consider next a fluid containing a volume fraction of hard particles significantly less than the close packing limit. In this fluid, the volume available to a given species of hard particle, i.e., the volume in which the center of an additional molecule of that solute may be placed, may, depending upon the abundance, sizes and shapes of all other particles present, be substantially less than the total volume minus fractional volume occupancy. As in the illustrative example above, this is simply due to the mutual impenetrability of the particles. For pure steric repulsion, the (entropic) work of particle insertion increases as available volume decreases, and the activity coefficient of species i may be expressed simply as [12,13]

$$\gamma_i = \frac{V_{\text{total}}}{V_{\text{available},i}} = \frac{1}{f_{\text{available},i}} \tag{8}$$

For model fluids consisting of hard convex particles, a number of approximate methods exist for calculating the volume available to another hard convex particle (reviewed in Ref. [14]).

The application of these concepts to solutions of biological macromolecules became practical when it was discovered that under commonly encountered conditions, experimentally measured activity coefficients of proteins in crowded solutions could be estimated surprisingly accurately using simple structural models, in which rigid globular proteins were represented by hard particles having a size and shape similar to that of the protein at low resolution (such as spheres or spherocylinders), and large random coil polymers were represented by a random matrix of long rigid rods [9,15–17]. Under these conditions, it is possible to use effective hard particle models to obtain realistic estimates of the activity coefficient of proteins and hence Γ for a variety of macromolecular reactions in crowded solutions of proteins and polymers, leading to a series of predictions of how crowding might affect the equilibria and rates of these reactions in different media [9].

We may estimate the activity coefficient of a dilute globular protein, called tracer T, in a solution containing a second globular protein of uniform size, called crowder C, occupying fraction ϕ of total volume by modeling both proteins as hard spherical particles. The result of a calculation carried out using the scaled particle theory of fluid mixtures [12] are plotted in Fig. 2. Two qualitative aspects of this result are notable. The first is the potentially extremely large activity coefficients—exceeding unity by as much as several orders of magnitude—that can be attained by proteins in solution at physiological levels of volume occupancy. For example, the dark circle indicates the activity coefficient of a sphere in a solution containing other spheres of equal size at a fractional volume occupancy of 0.3. This result, indicating an activity coefficient exceeding 100, corresponds closely to the experimentally measured activity coefficient of hemoglobin in a 350 g/l solution of hemoglobin, comparable to the contents of a red blood cell [16]. The second notable qualitative property of the result shown in Fig. 2 is the extremely nonlinear aspect of the dependence of γ_T on both the

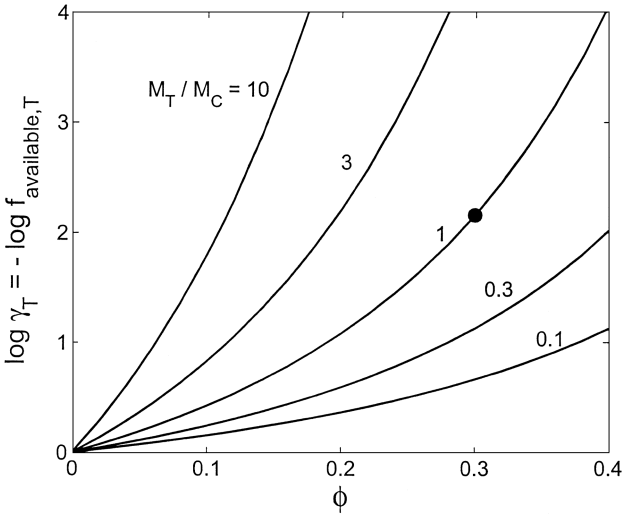

Fig. 2. Logarithm of activity coefficient of a spherical particle of species T (mass M_T) in a fluid of spherical particles of species C (mass M_C), plotted as a function of ϕ, the fractional occupancy of volume by C, and M_T/M_C. Significance of plotted point described in text. Calculations for this figure and Figs. 3 and 4 were performed using scaled particle theory of hard sphere mixtures [12]. It is stipulated here and elsewhere, unless explicitly stated otherwise, that all species have the same density, so that $M_i \propto r_i^3$.

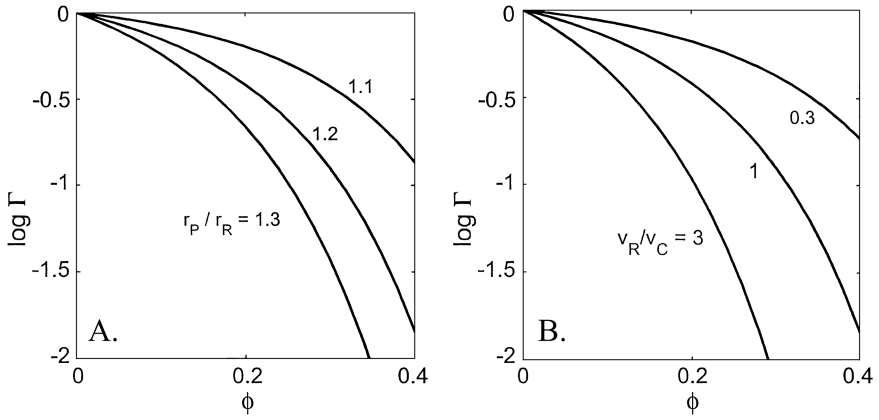

Fig. 3. Calculated effect of volume occupancy by inert species C on the equilibrium constant for the isomerization reaction R⇌P. (A) Effect of varying relative sizes of R and P for $v_R = v_C$. (B) Effect of varying relative sizes of R and C for $r_P = 1.2r_R$.

fractional volume occupancy and on the relative sizes of tracer and crowder.

It follows from Eqs. (2), (4), and (5) that the dependence of γ_T on the relative size and abundance of crowder shown above is manifested in a corresponding dependence of Γ and hence the equilibrium and rate constants of reactions of tracer species. Two examples are provided here. The first is a simple isomerization reaction resulting in a change in the effective volume of a dilute macrosolute

$$R \rightleftarrows P \tag{9}$$

The results of a calculation of Γ based upon the assumption that all macrosolute species (R, P, and C) may be represented as hard spherical particles are plotted in Fig. 3. It may be seen that any conformational change that increases the effective volume of the dilute species ($r_P > r_R$), such as protein unfolding, is progressively inhibited with increasing extent of crowding.

The second example is a simple self-association reaction involving formation of an n-mer from n monomers:

$$n\mathrm{R} \rightleftarrows \mathrm{P} \tag{10}$$

The results of a calculation of Γ based upon the assumptions that all macrosolute species (R, P, and C) may be represented as hard spherical particles, and that the volume of the hard spherical particle representing P is equal to n times the volume of R are plotted in Fig. 4. It may be seen that self-association is progressively facilitated with increasing extent of crowding, and that the magnitude of the crowding effect on self-association increases with the degree of self-association.

Before concluding this introduction to crowding theory, it must be noted that in addition to the thermodynamic consequences summarized above, substantial volume occupancy can considerably reduce the diffusional mobility of macromolecules—a hydrodynamic rather than thermodynamic consequence [2,8]. In some cases, this extra-thermodynamic effect may have a comparable or larger influence upon the extent or time course of a particular reaction in a crowded medium than the thermodynamic effect as reflected in the value of Γ. As the theoretical treatment of hydrodynamics in volume-occupied fluids is considerably more complex, and hence less developed than

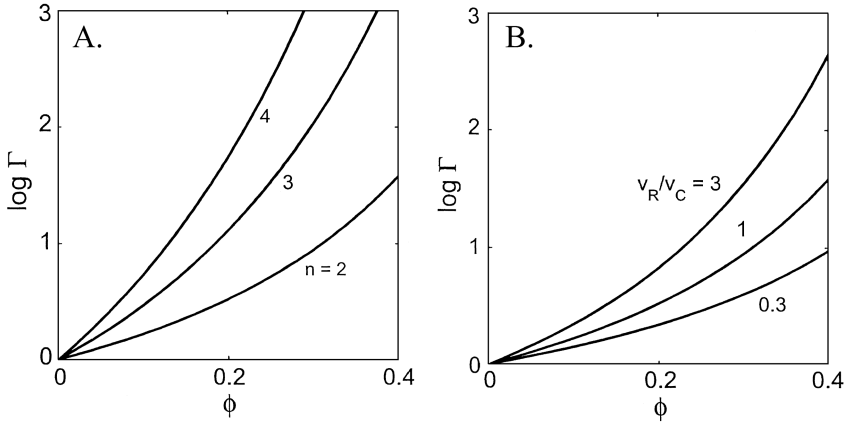

Fig. 4. Calculated effect of volume occupancy by inert species C on the equilibrium constant for the self-association reaction nR⇌P. It is assumed that the volume of P is n times the volume of R. (A) Effect of varying n for $v_R = v_C$. (B) Effect of varying relative sizes of monomer and crowder for $n = 2$.

Table 1
Summary of effects of excluded volume on macromolecular reactions in crowded solutions

Reaction	Effect of crowding	
	Equilibrium	Rate
Conformational isomerization	Biases reaction toward compact and against expanded and/or extended conformations	Either accelerates or retards compaction depending upon whether transition state is less or more compact than initial conformation
Association	Biases reaction(s) toward maximally associated state	Accelerates slower reactions not limited by rate of encounter; retards fast reactions limited by rate of diffusional encounter

the corresponding treatment of thermodynamics in such fluids, the extra-thermodynamic consequences of crowding, which have been extensively discussed elsewhere (see for example references cited in Refs. [2,18]), are necessarily restricted to semi-empirical analyses. The overall effects of crowding upon conformational isomerization and association of macromolecules are summarized qualitatively in Table 1.

3. Recent experimental findings

Over the last 40 years, a substantial number of experimental studies carried out in many laboratories (cited in Refs. [2,7,9]) have unequivocally established the potentially dramatic influence of excluded volume on the rates and equilibria of a wide variety of macromolecular reactions in solutions comparable in volume occupancy to biological media. During the past year, a number of interesting new findings have been reported by several laboratories. These are summarized in Table 2.

Of particular interest is the fact that several of the previously unobserved effects reported in this table were earlier predicted qualitatively on the basis of simple excluded volume theory. Moreover, in some cases, the magnitude of the observed effect agrees quite well with that calculated from the simple theory. We emphasize this point, since much of the remainder of the present review is devoted to a critical examination of the limits of this simple theory. This critical approach should not obscure the utility of the simple theory when applied under appropriate conditions, as will be discussed below.

4. Limits of the effective hard particle model for calculating excluded volume interactions

The success of the effective hard particle model in predicting and/or accounting semiquantitatively for a variety of experimentally observed excluded volume effects implies that under the conditions under which these effects were studied, approximations inherent in the simplified model were reasonably realistic. The effective hard particle model is based upon the assumption that the effective potential of interaction acting between macromolecules in solution may be realistically modeled by a simple hard

Table 2
Recent experimental studies of macromolecular reactions in crowded media

Observation	Comment	Reference
Several proteins refolding to native state spontaneously in dilute solution stringently require GroEL, Gro-ES and ATP in order to refold to native state in crowded solution		[34]
Formation of amyloid fibers by apolipoprotein C-II accelerated by added dextran	~ 8-fold acceleration in 150 g/l dextran T10; dependence of rate upon dextran concentration accounted for quantitatively by excluded volume model	[35]
Formation of fibrils by alpha-synuclein is accelerated by added proteins, polysaccharides and polyethylene glycol	~ 6-fold acceleration in 50 g/l lysozyme; ~ 5-fold acceleration in 60 g/l BSA	[36]
Formation of protofibrils and fibrils by alpha-synuclein is accelerated by added polyethylene glycol, dextran and ficoll		[37]
Addition of high concentrations of various crowding agents slows refolding of glucose-6-phosphate dehydrogenase and protein disulfide isomerase but does not decrease final yield of native protein. Addition of GroEL and ATP to crowded solutions increases both the refolding rate and final yield of native protein	Differs from observations of Ref. [34] in that final yield of native protein does not decrease in crowded medium, and chaperone activity of GroEL does not require GroES	[38]
Enzymes that catalyze proteolysis in dilute solution can catalyze peptide synthesis in sufficiently crowded solutions	Product must be significantly more compact than the reactants	[39]
Addition of dextran at high concentrations stabilizes lysozyme with respect to thermal denaturation	Dextran decreases entropy of denaturation, does not significantly alter enthalpy. Direction and magnitude of effect are consistent with predictions of excluded volume theory	[40]
Addition of dextran stabilizes compact molten globule (MG) state of cytochrome *c* at pH 2.0 relative to fully unfolded (U) state	Free energy of U-MG transition decreases by ca. 5 *RT* upon addition of 370 g/l dextran T35. Direction and magnitude of effect are consistent with predictions of excluded volume theory	[40]

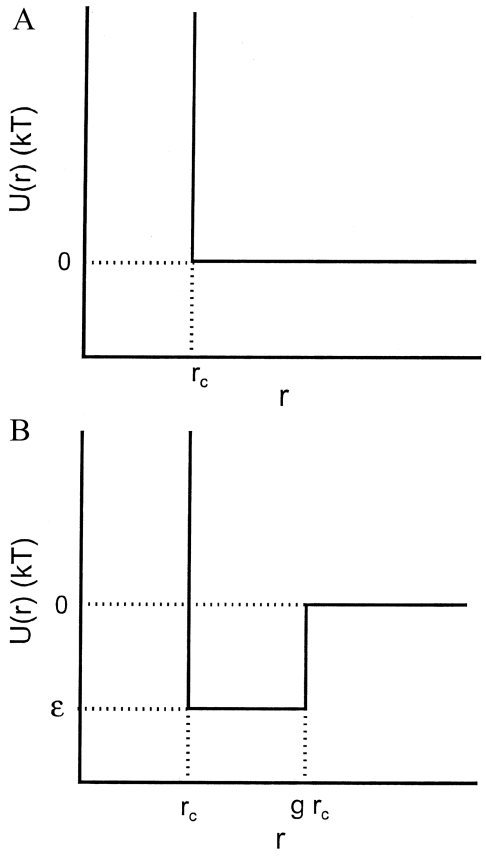

Fig. 5. (A) Hard sphere potential. (B) Square well potential.

particle (most simply, a hard sphere) potential, illustrated in Fig. 5A. According to this model, there is no interaction between macromolecules so long as the distance between their respective centers (r) exceeds the surface contact distance r_C, which is independent of relative orientation of the particles for the case of hard spheres. The mutually impenetrable molecules cannot approach more closely than the contact distance. In other words, the interaction potential at distances less than r_C is assumed to be infinitely positive. The validity of the hard particle model rests on several approximations, each of which must in itself be realistic if the overall model is to be considered realistic. We shall discuss each of these approximations in turn and indicate when a particular approximation may, and, perhaps more importantly, may not be expected to be realistic.

4.1. Solvent as continuum

The solvent (water + small molecule cosolvents) is treated as a continuum, which assumes that the effective potential of interaction between macrosolute molecules in solution is insensitive to the molecular (i.e., discontinuous) nature of solvent. This approximation is valid so long as the dimensions of the effective hard particle representing macrosolute are much greater than the range of significant variation in local density arising from the molecular nature of solvent, which may be estimated from measurements of the X-ray diffraction of water to have a range of ca. 3–4 Å [19]. Conversely, as the size of a solute species decreases and approaches that of a water molecule, the assumption that the effective potential of interaction between molecules of that solute and any other solute in water resembles a hard particle potential must become a progressively poorer approximation [20].

4.2. Influence of "soft" (nonsteric) interactions

It has been argued that to the extent that additional soft interactions such as electrostatic repulsion or attraction are significant, they may be incorporated into the effective hard particle model by appropriate adjustment of the radius of the effective hard particle [21,22]. This approximation was justified by the finding that an effective hard particle model could quantitatively account for the experimentally measured concentration dependence of the following colligative properties[4] of BSA solutions over a wide range of concentrations: osmotic pressure (to 100 g/l) [22], light scattering of BSA solutions (to 90 g/l) [21], and sedimentation equilibrium (to 200 g/l) [9,23], at pH values where the BSA molecule is known to be highly charged and significant electrostatic solute–solute interaction is expected. Moreover, the effective hard volumes obtained from analysis of different colligative properties, when measured under comparable experimental conditions (pH, ionic strength, temperature), are self-consistent (data not shown). The success of the effective hard particle model in accommodating these data—and the limitations of this model—may be understood in the context of the following analysis.

The activity coefficient of solute species i in a solution containing multiple solute species may be written as an expansion in powers of solute concentration

$$\ln\gamma_i = \sum_j B_{ij}c_j + \sum_j \sum_k B_{ijk}c_j c_k + \ldots \quad (11)$$

where B_{ij}, B_{ijk}, …, respectively denote two-body, three-body, and higher-order interaction coefficients that are functions of the effective potential of interaction (potential of mean force) between two solute species, three solute species, etc., in solution [24], and c_i is the molar concentration of species i. For the case of a single solute species, Eq. (11) reduces to

$$\ln\gamma = B_2 c + B_3 c^2 + \ldots \quad (12)$$

There exist simple thermodynamic relationships between solute activity coefficient and the colligative properties of the solution that enable one to express the colligative properties as expansions in powers of solute concentration

[4] Solution properties that are functions of the thermodynamic activity of solute.

(see, for example, Refs. [14,25]). For example, the osmotic pressure of a solution containing a single nondialyzable (i.e., macromolecular) solute species may be expanded in powers of the concentration of that solute:

$$\frac{\Pi}{RT} = c + C_2c^2 + C_3c^3 + \ldots \tag{13}$$

where $C_2 = 1/2B_2$, $C_3 = 2/3B_3$, etc. The statistical–thermodynamic theory of solutions [24] provides an analytical expression for each interaction coefficient as a function of the effective potential of interaction (potential of mean force) acting between two, three, and greater molecules of macrosolute in solution. For example, the two-body self-interaction coefficient B_2 may be expressed as the following function of a spherically symmetrical potential of interaction $U(r)$, where r is the distance between the centers of two interacting particles:

$$B_2 = 4\pi N_A \int_0^\infty \left[1 - \exp\left(-\frac{U(r)}{kT}\right)\right] r^2 dr \tag{14}$$

where N_A denotes Avogadro's number, k Boltzmann's constant and T the absolute temperature. The values of the first seven B_i have been analytically or numerically evaluated for the hard sphere (hs) potential shown in Fig. 5A [26].

Let us define the fractional contribution to the osmotic pressure

$$f_i = \frac{\sum_{j=1}^{i} C_j c^j}{\sum_{j=1}^{\infty} C_j c^j} \tag{15}$$

where $C_1 \equiv 1$. f_i so defined is a measure of the contribution of the first i terms of the osmotic expansion to the total osmotic pressure: f_1 is the ideal contribution, f_2 is the ideal plus two-body solute–solute interaction contribution, etc. The values of f_1–f_6, calculated for a hard sphere potential and a specific hard particle volume of 0.8 cm^3/g [2], are plotted in Fig. 6. It may be seen that at least 95% of the total osmotic pressure is accounted for by the first two terms in the osmotic expansion at concentrations of up to 100 g/l and by the first three terms at up to 200 g/l. It follows that over the concentration range where colligative properties (and by inference the thermodynamic activity) are governed by at most two-body interactions—up to about 100 g/l in the above example—the effective hard particle model is automatically valid, as the radius of the effective hard sphere is simply a parametric expression of C_2.[5]

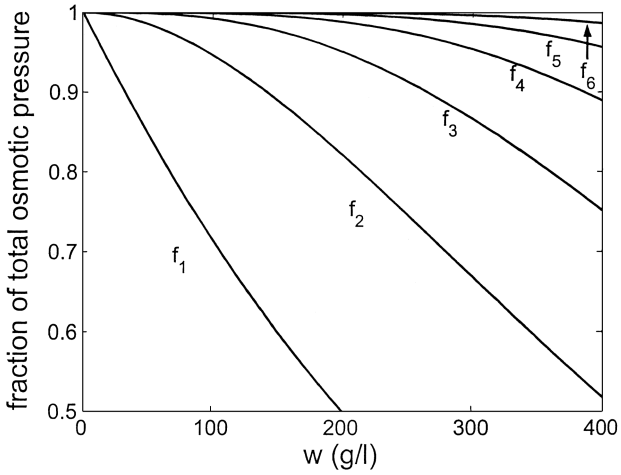

Fig. 6. Relative contribution of truncated expansions of osmotic pressure in powers of concentration to the total osmotic pressure, as described in text.

The question remains whether the effective hard particle model can be useful at higher solute concentrations, when the contributions of three-body and higher interactions to solute activity and colligative properties become significant. This is not a trivial question, since while the evaluation of B_2 for almost any model function $U(r)$ is simple, the corresponding expressions for B_3, B_4 and higher interaction coefficients (see, for example, Chapter 3 of Ref. [27]) become progressively more complex, and evaluation of the multiple integrals appearing in them becomes prohibitively difficult for all but the simplest forms of $U(\{r\})$, where $\{r\}$ denotes the relative positions of all interacting molecules.

The simplest potential containing a soft interaction, the square-well (sw) potential, is shown schematically in Fig. 5B. The strength of a "soft" interaction is parameterized by the well depth or height parameter ϵ,[6] and the range of the soft interactions parameterized by the width of the well, $r_C(g-1)$. Using expressions derived by Kihara [28] for the square-well potential, the unitless ratio $Q_{sw} \equiv C_3/C_2^2$ was calculated for different values of g and ϵ, and compared to Q_{hs}, the value obtained for $\epsilon = 0$, i.e., a hard sphere potential. A partial contour map of the results is displayed in Fig. 7. The ratio Q_{sw}/Q_{hs} is greater than unity for all $\epsilon < 0$, i.e., to the left of the vertical line indicating $\epsilon = 0$, and less than unity for all $\epsilon > 0$, i.e., to the right of the vertical line. The cross-hatched region indicates that region of $[g, \epsilon]$ space for which the values of Q_{sw} and Q_{hs} differ by less than 10%, i.e., the region in which an effective hard sphere model can closely emulate the square-well model.

Although the square-well model is a highly simplified representation of a realistic interaction potential between

[5] This conclusion is independent of the true form of $U(r)$. It must, however, be noted that if $U(r)$ does not resemble a hard sphere potential, the radius of the effective hard sphere may differ considerably from the average radius of the actual macromolecule, and structure alone will not provide a firm basis on which to predict thermodynamic activity.

[6] ϵ is negative for attractive and positive for repulsive soft interactions.

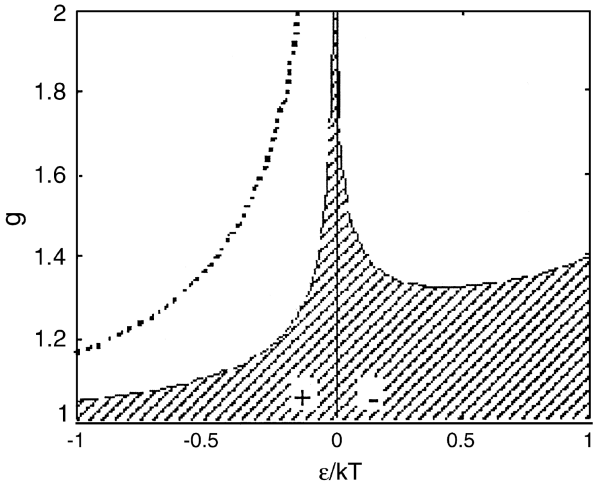

Fig. 7. Partial contour map of the function Q_{sw}/Q_{hs}. Crosshatched region covers all pairs of values of g and ϵ for which the absolute value of Q_{sw}/Q_{hs} is less than 1.1. Dashed line indicates pairs of [g, ϵ] for which C_2 calculated for the square-well potential equals 0, i.e., for which the function Q_{sw}/Q_{hs} diverges.

macromolecules in solution, it is nonetheless evident that the effective hard particle model can realistically emulate the behavior of particles with soft as well as hard interactions at higher concentrations (at least to ca. 200 g/l), so long as the range of the soft interactions is much smaller than the size of the hard repulsive core ($g<1.1$ in the case of weak attractive interactions, $g<1.3$ in the case of weak repulsive interactions). The converse of this finding is that one should not expect an effective hard particle model to account for colligative properties or to provide an accurate estimate of solute activity at high solute concentration when soft interactions between the solutes are significant over distances comparable to molecular dimensions.

4.3. Possible non-additivity of soft interactions

The effective hard particle model assumes that each solute species may be represented for the purpose of activity calculation by an equivalent rigid particle with dimensions that are fixed under a given set of experimental conditions. Under certain circumstances, this assumption may not be justifiable. As an example, consider a solution containing two globular proteins, one of which, denoted by 0, is essentially electroneutral at the pH of the solution, and one of which, denoted by +, is significantly positively charged. In the most general case, namely, arbitrarily large concentrations of each species, then the free energy of the system will contain contributions from three binary solute–solute interactions, 00, 0+, and ++. If an effective hard particle model is assumed, then the contact distance for a particular pair interaction is given by the sum of effective radii of each species:

$$r_C(00) = r_0 + r_0 \tag{16a}$$

$$r_C(0+) = r_0 + r_+ \tag{16b}$$

$$r_C(++) = r_+ + r_+ \tag{16c}$$

The first two interactions will be determined essentially entirely by hard steric repulsion between the macrosolutes, independent of the charge on +. But if electrostatic repulsion is significant, then r_C (++) may be significantly greater than $2r_+$, in which case Eqs. (16a)–(16c) cannot be simultaneously satisfied by any fixed values of r_0 and r_+ [9].[7] It follows that an effective hard particle model may not provide a realistic description of solute–solute interactions in a solution containing high concentrations of multiple macrosolute species having significantly different net charges.

4.4. When are structural details important?

The representation of a globular or fibrous macromolecular solute by an equivalent hard particle of fixed size and shape (i.e., independent of the nature of other solutes with which it may be interacting) embodies an implicit assumption that it is possible to realistically estimate the volume excluded by one macromolecule to another given only low-resolution structural information (i.e., gross size and shape). However, the degree of resolution required to provide a useful estimate of excluded volume depends upon the relative sizes and shapes of interacting particles. This is illustrated in the following example.

We wish to estimate the activity coefficient of dilute DNA (D) in a solution containing a globular protein P as a function of protein concentration. In such a solution, Eq. (11) reduces at low protein concentration to

$$\ln\gamma_D = B_{DP}c_P + \ldots \tag{17}$$

For the purpose of this example, we shall assume that both D and P may be represented by rigid particles interacting exclusively via volume exclusion. Under such circumstances, it can be shown very generally [28] that

$$B_{DP} \propto V_{DP} \tag{18}$$

where V_{DP} denotes the covolume of the particles representing D and P, i.e., the volume which one particle excludes to the center of mass of the second particle.

We shall assume that P may be represented by a hard sphere of radius r_P, and examine two models for DNA. The simpler of the two models (zeroth-order model) is a rigid cylinder of radius r_D, and the slightly less simple model (first-order model) is a rigid cylinder containing wedge-shaped grooves of angular width 2θ (Fig. 8A), which crudely represent the major and minor grooves of the double

[7] This problem does not occur when only one of the species is present at high concentration, since self-interaction of the dilute species is negligible and there exist only two relevant values of r_C, which may automatically be converted to self-consistent equivalent hard sphere radii.

helical molecule. The covolume per unit length of DNA, and hence B_{DP}, is proportional to the co-area of a circle of radius r_P and the cross-section of the DNA normal to the cylindrical axis. The co-area is just the area enclosed by the center of P when it is rolled completely around the surface of the cross-section of D. Co-areas calculated for the two models of D are drawn schematically in Fig. 8B and B′ for a protein that is small relative to the outer cylindrical dimension of DNA, and in Fig. 8C and C′ for a larger protein.

Letting the values of B_{DP} calculated using the simpler and less simple models of DNA be denoted, respectively, by $B_{DP}^{(0)}$ and $B_{DP}^{(1)}$, and letting $f = r_P/r_D$, the ratio $B_{DP}^{(1)}/B_{DP}^{(0)}$ is plotted as a function of the groove half-width θ in Fig. 9 for various values of f. For a fixed value of f, the value of $B_{DP}^{(1)}/B_{DP}^{(0)}$ decreases with increasing groove width, and this decrease may be taken as a measure of error in the calculation of B_{DP} due to the neglect of additional complexity in the shape of DNA (i.e., the presence of grooves). However, as the size of the protein increases relative to that of DNA, the fractional error incurred by ignoring a groove of fixed width decreases. For example, if $r_p = r_D$ ($f = 1$), essentially no error is incurred by neglecting a groove as wide as $2\theta = 45°$. In general, a realistic calculation of volume excluded by particle 1 to particle 2 requires specification of features of the surface of particle 1 that have a largest dimension comparable to or larger than the size of particle 2.

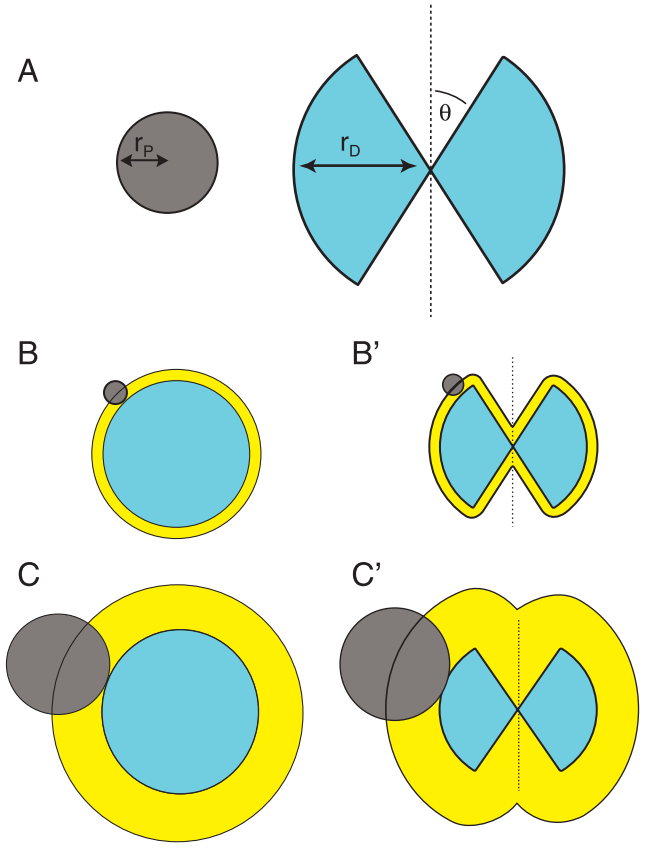

Fig. 8. Schematic depiction of models for hard particle interaction between a spherical cosolute and a zeroth and first-order model of DNA. (A) Cross-sections of cosolute, depicted as a gray circle of radius r_P, and the first-order model of DNA depicted as a blue circle of radius r_D with two symmetrical grooves of width 2θ. (B–C′) Sum of blue + yellow areas represent co-areas of small cosolutes (B, B′) or larger cosolutes (C, C′) and zero order (B, C) or first-order (B′, C′) models of DNA.

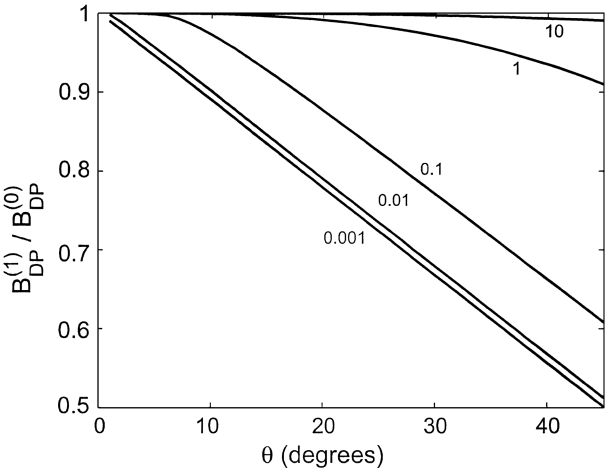

Fig. 9. Ratio of co-area calculated using first-order model for DNA to that using zeroth-order model for DNA, plotted as a function of groove half-width θ, for various values of r_P/r_D indicated in the figure.

5. Amplification of crowding effects by undetected ("hidden") cosolutes

When estimating the quantitative effect of an added inert macrosolute upon the activity coefficient of a second solute species, it is generally (if tacitly) assumed that other volume-excluding species are present at negligible concentrations. However, if one is studying the effect of crowding by a particular macrosolute in a biological fluid, the possibility exists that other solute species exercising their own significant crowding effects may be present without the investigators' knowledge. The following example calculations will demonstrate that the apparent effect of crowding arising from the addition of a single macrosolute species may be significantly altered by the presence of an additional crowding species.

For purposes of demonstration, we shall represent each solute species by an equivalent hard spheres with radius

$$r_i = \left(\frac{3M_i v}{4\pi N_a}\right)^{1/3} \tag{19}$$

where M_i is the molar mass of solute species i, v is the specific volume of the equivalent particle, assumed to be 0.8 cm^3/g for all species, and N_A Avogadro's number. The activity coefficient of each solute species in a mixture, the composition of which is specified by the w/v concentration of each solute species, is calculated using the scaled particle theory for fluid mixtures of hard spheres [12].

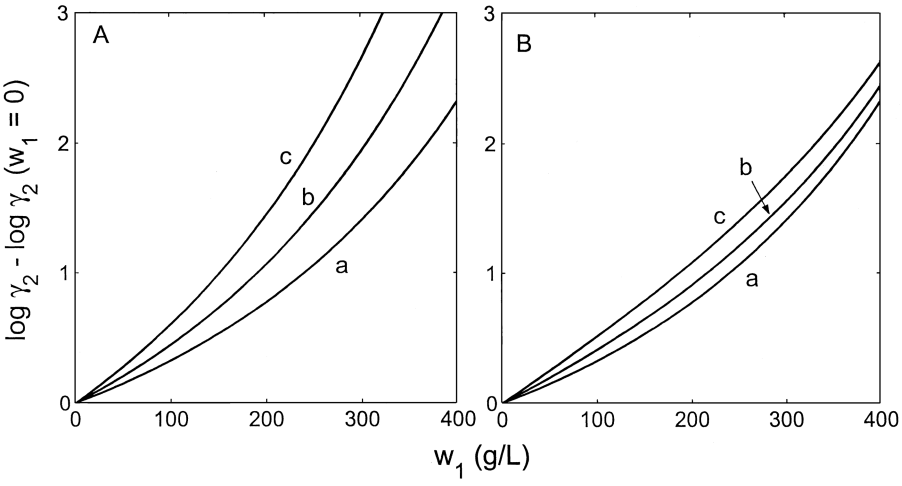

Fig. 10. Effect of adding species 1 (M = 70 K) upon the activity coefficient of dilute species 2 (M = 70 K) in the presence of various concentrations of species 3 (M = 10 K), calculated assuming a specific hard particle volume of 0.8 cm^3/g for all species. (A) Concentration of species 3 is held constant at (a) 0 g/l, (b) 50 g/l and (c) 100 g/l). (B) Species 3 is in dialysis equilibrium with species 3 in an exterior reservoir containing species 3 at fixed concentrations of (a) 0 g/l, (b) 50 g/l and (c) 100 g/l.

We wish to calculate the effect of adding an inert solute species 1 upon the activity coefficient of solute species 2 in the presence of a third "hidden" solute species 3 at various fixed concentrations. We arbitrarily set $M_1 = M_2 = 70\,000$, and $M_3 = 10\,000$. The results of this calculation are shown in Fig. 10A. The presence of a fixed concentration of hidden cosolute 3 very greatly magnifies the apparent effect of the specified cosolute 1 upon the activity coefficient of test species 2. The magnifying effect may be rationalized by recalling that the increase in the activity coefficient of species 2 is due to the reduction in volume available to species 2, and that available volume decreases sharply with increasing total volume occupancy by all solutes. In the presence of added species 3, the volume available to species 2 is already reduced, even before addition of species 1. The addition of a fixed quantity of 1 in the presence of 3 thus results in a larger *fractional* decrease in volume available to 2 than addition of the same quantity of 1 in the absence of 3.

Since excluded volume effects are likely to modulate biochemical equilibria and kinetics within cells [29], another possibility should be explored. The cell membrane is permeable with respect to certain solutes and impermeable with respect to others. Let us therefore consider the case in which a large impermeant cosolute (species 1) is introduced into a cell that contains a smaller permeant cosolute (species 3) that is in equilibrium with solute 3 at a fixed concentration in the external medium. As the concentration of 1 increases, the intracellular concentration of 3 would be expected to decrease correspondingly due to repulsive (excluded volume) interactions between 1 and 3. The combined effect of increasing the intracellular concentration of species 1 and the concomitant reduction of the intracellular concentration of species 3 upon the activity coefficient of species 2 is plotted in Fig. 10B. It is evident that the amplification in crowding effect arising from the presence of the hidden cosolute 3, highly evident in Fig. 10A, is greatly, although not entirely, suppressed when species 3 is allowed to redistribute in accordance with dialysis equilibrium.[8] Thus, a naive estimate of crowding effects that neglects the possible presence of additional undetected crowding cosolutes (for example, curves a in Fig. 10A and B) may considerably underestimate the consequences of crowding within the cell.

6. Buffering of crowding effects by undetected ("hidden") associations

In the preceding section, it was pointed out that the presence of significant concentrations of an uncharacterized ("hidden") macrosolute (species 3) in a biological fluid can, by decreasing total available volume, effectively amplify the effect of a known macrosolute (species 1) on the activity coefficients of other macrosolutes. One can ask a complementary question: what if the presence of macrosolute species 3 is known, rather than hidden, but its state of association is indeterminate and variable? Since volume occupancy favors association processes, as one adds increasing amounts of known macrosolute species 1 to the fluid, one would expect the degree of association of species 3 to increase. In doing so, the total volume excluded by species 3 to other macrosolutes decreases, suggesting that the ability of a particular macrosolute species to undergo association and/or dissociation should decrease the contribution of that species to crowding effects. Hall [30] has recently analyzed in some detail a biologically relevant example of this notion. Actin and tubulin, which are major components of the

[8] Quantitative expressions describing the redistribution of species 2 are presented in Appendix A.

cytoskeleton or cytomatrix, comprise a significant fraction of total intracellular protein in eukaryotic cells, and at any given time these proteins exist in both their dissociated (G-actin, free tubulin) and condensed (F-actin, microtubule) forms. Hall carried out simulations of the condensation equilibrium in highly nonideal solution, and demonstrated that condensation significantly reduces the effect of the condensing protein (at constant total concentration) on the activity coefficient of a trace protein. The results of his simulations apply equally to the case of an intracellular protein that is able to reversibly adsorb onto the surface of a structural element (a cytoskeletal fiber or membrane surface). Thus, intracellular condensation or surface adsorption provides, in principle, a mechanism for buffering changes in the thermodynamic activity of intracellular macrosolutes resulting from changes in cellular volume.

7. New quantitative approaches to the analysis of crowding phenomena

Although the effective hard particle model for macromolecular crowding has proven to be useful in a variety of qualitative and semiquantitative applications, its limitations are becoming increasingly evident as our attention turns from crowding in model systems to crowding in biological media. Whereas model systems are designed to facilitate the study of crowding by a single inert macrosolute and to minimize interactions other than excluded volume, biological media typically contain significant concentrations of more than one macrosolute species under conditions in which intermolecular interactions over and above simple hard core repulsion play an important role in determining the overall free energy of the system. Hence, nonadditivity of interaction (Section 4.3) becomes a major concern. Moreover, the relative concentrations of different macrosolutes in complex biological media such as cytoplasm may vary significantly with both time and position, and analyses based upon a static time- and space-averaged composition may not be realistic. Two novel attempts to deal with these complexities have appeared very recently.

Kinjo and Takada [31,32] have employed the density functional theory of fluids to explore excluded volume effects in inhomogeneous solutions. The authors postulate a system containing a dilute protein which may interconvert between native (N) and denatured (D) states, an inert crowder C and solvent S. Assuming that the sum of densities of all species is constant and uniform, the density of the *i*th species is specified as a function of position, $\phi^{(i)}(\mathbf{r})$, and the free energy of the system specified as a functional of all densities, $F(\{\phi(\mathbf{r})\})$, which takes into account model distance-dependent two-body repulsive and attractive interactions between each pair of species. The chemical potential of each species is then specified as the derivative of total free energy with respect to the density of each species at each point, $\mu^{(i)}(\mathbf{r}) = \delta F/\delta\phi^{(i)}(\mathbf{r})$. The equilibrium state of the system is obtained by numerically solving the resultant partial differential equations in an iterative fashion until each $\mu^{(i)}$ becomes spatially uniform and $\mu^{(N)} = \mu^{(D)}$. The approach is extended to treat dynamics by the introduction of expressions for the time dependence of densities at a fixed point which contain models for spatially dependent diffusion coefficients and rate constants for conversion of native to denatured and from denatured to native species. The approximations employed by these investigators are crude, and the resolution of their numerical solutions is low, but the results obtained suggest that this approach can provide some fresh qualitative insight into crowding-induced phase separation and the dynamics of crowding-induced aggregation.

An alternate approach that is conceptually more straightforward, but computationally much more intensive, has been introduced by Elcock [33], who employed Brownian dynamics to calculate the free energy of transfer of a rigid globular rhodanese molecule from the hollow interior of a GroEL chaperone protein into bulk solvent, as a function of the volume fraction of solvent that is occupied by an inert macromolecular crowding agent modeled as a rigid sphere (more precisely, a rigid spherical shell of small ''atomic'' beads). This calculation is equivalent to calculating the work of introducing a cavity the size of the rhodanese molecule into a fluid of crowder molecules. Such a calculation may be performed analytically when all molecular species are treated as hard particles, as described above. However, as Elcock correctly emphasizes, the Brownian dynamics approach may be readily extended to treat systems interacting via arbitrary model potentials under conditions where an effective hard particle model would not be realistic due to the presence of significant non-additivity and/or long-range intersolute interaction.

8. Summary/conclusions

While recent experimental findings generally provide support for the utility of the effective hard particle model in predicting and rationalizing effects of excluded volume under some conditions, the limitations of this model must be appreciated in order to avoid inappropriate application of the model under other conditions, when the underlying assumptions cannot be expected to be realistic. Such conditions include the following:

1. The major crowding species is much smaller than the probe species under study.
2. The range of soft interactions is comparable to the size of the smallest of the crowding species.
3. Multiple crowding species with possibly non-additive soft interactions are present.
4. When all solute species excluding significant amounts of volume to other species cannot be explicitly taken into account.

Some of the limitations of current excluded volume theory may be overcome by the application of new techniques, which, when employed with caution, may avoid oversimplification. Brownian dynamics (and other techniques of simulation) may provide new insight into reaction kinetics in crowded media. These benefits, however, are obtained only after a large computational investment and a concomitant loss of conceptual transparency.

Perhaps the most valuable aspect of the current review is the highlighting of recent experimental and theoretical advances in the area of non-ideal macromolecular solution chemistry. These works represent the leading edge of a field of research that is at long last beginning to have a significant impact on mainstream thinking in biochemistry, biophysics and cell biology [5].

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Appendix A. Dialysis equilibrium in a solution when membrane is permeable to only one of two solutes

Let a solution containing solute species 1 at concentration c_1^{in} and solute species 3 at concentration c_3^{in} be confined within a membrane that is permeable only to species 3. Let the external medium contain solute species 3 at concentration c_3^{out}. At dialysis equilibrium, the chemical potential of solute species 3 on both sides of the membrane must be equal:

$$\mu_3^{in} = \mu_3^{out} \quad (A1)$$

where

$$\mu_3^{in} = \mu_3^0(T,P) + v_3\Pi + RT\ln c_3^{in} + RT\ln\gamma_3^{in} \quad (A2)$$

$$\mu_3^{out} = \mu_3^0(T,P) + RT\ln c_3^{out} + RT\ln\gamma_3^{out} \quad (A3)$$

μ_3^o, v_3 and γ_3, respectively, denote the standard state chemical potential of 3, the partial molar volume, and the activity coefficient of species 3, and Π denotes the osmotic pressure of the inner solution, which is a function only of the concentration of impermeant solute 1. Eqs. (A1)–(A3) may be arranged to yield

$$c_3^{in} = c_3^{out}\exp[\ln\gamma_3^{out}(c_3^{out}) - \ln\gamma_3^{in}(c_1^{in}, c_3^{in}) - v_3\Pi(c_1^{in})] \quad (A4)$$

Given relations for calculating the activity coefficients and the osmotic pressure as a function of the indicated concentrations, Eq. (A3) may be iteratively solved for the value of c_3^{in} as a function of c_1^{in} and c_3^{out}. The results of such a calculation for two different values of c_3^{out} are presented in Fig. A1.

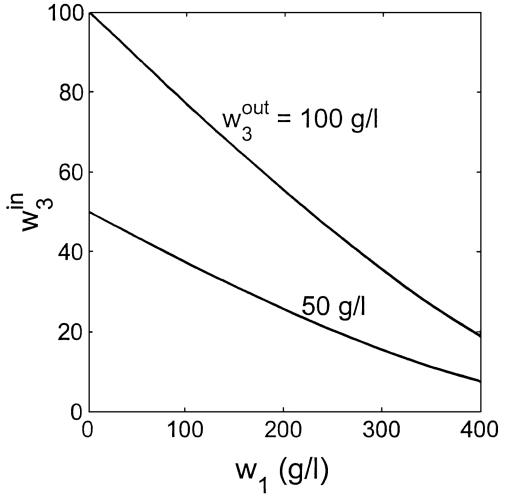

Fig. A1. Concentration of species 3 (M=10 K) in an 'inner' compartment containing concentration w_1 of species 1 (M=70 K), when species 3 is in dialysis equilibrium with species 3 in a second 'outer' compartment containing species 3 at the indicated concentrations. Calculation performed assuming a specific hard particle volume of 0.8 cm^3/g for all species.

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