Minireview

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 14, Issue of April 6, pp. 10577–10580, 2001
Printed in U.S.A.

The Influence of Macromolecular Crowding and Macromolecular Confinement on Biochemical Reactions in Physiological Media*

Published, JBC Papers in Press, February 15, 2001, DOI 10.1074/jbc.R100005200

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Detailed knowledge of the rates, equilibria, and mechanism of biochemical reactions has traditionally been acquired through experiments conducted on solutions containing low concentrations (less than about 1 mg/ml) of total protein, nucleic acid, and/or polysaccharide together with buffer salts, low molecular weight substrates, and cofactors as required. In contrast, biochemical reactions in living systems take place in media containing substantially greater total concentrations (50–400 mg/ml) of macromolecules that may be present in solution and/or in indefinitely large arrays (e.g. cytoskeletal fibers) (1, 2). Because no single macromolecular species may be present at high concentration, but all species taken together occupy a significant fraction of the volume of the medium, such media are referred to as "crowded" (3) and/or "confining" (4) rather than "concentrated," depending upon whether the macrosolutes are soluble and/or structured. Fig. 1 provides a schematic illustration of crowding and confinement in eukaryotic cytoplasm. In such media, nonspecific interactions between macrosolutes contribute significantly to the total free energy of the medium. High concentrations of "background" macromolecules that do not participate directly in a particular test reaction have been observed to induce order-of-magnitude or greater changes in the rates and equilibria of numerous test reactions (see below). To properly assess the physiological role of a particular reaction or set of reactions characterized *in vitro,* it is important to consider the possible influence of crowding and/or confinement upon the reaction in its physiological milieu.

Nonspecific Interaction

A nonspecific interaction between a pair of macromolecules does not depend strongly upon details of the primary, secondary, or tertiary structure(s) of the interacting macromolecules but rather upon global properties such as net charge, dipole or multipole moment, the polarity of surface residues, and macromolecular "shape." Nonspecific interactions may be either repulsive (steric, electrostatic) or attractive (electrostatic, hydrophobic) and are generally substantially weaker on a pairwise basis than specific interactions between reaction partners.

The concept of "nonspecific interaction" is widely misunderstood. Many if not most biomedical researchers still regard such interaction as an artifact of a particular experimental system that interferes with the acquisition of meaningful data. Strategies such as extrapolation of results to zero macromolecular concentration are devised for the reduction or elimination of the influence of nonspecific interaction on a test reaction. Although such procedures may be appropriate in certain specific experimental situations, they do not necessarily provide results that are more meaningful in a biological context. On the contrary, significant nonspecific interaction is an unavoidable consequence of crowding and confinement in most or all physiological fluid media. To understand molecular processes in such media one must therefore take account of nonspecific interactions rather than attempt to eliminate them.

Effect of Nonspecific Solute-Solute Interaction upon Chemical Equilibria

The contribution of a particular solute species X to the total free energy of the system is a function of an effective concentration, called the *thermodynamic activity* of X, denoted by a_x. Thermodynamics teaches that equilibrium constants are generally expressed in terms of equilibrium activities rather than actual concentrations. As a simple example, consider a protein molecule that may reversibly self-associate to form a dimer. The equilibrium association constant for this reaction is $K^o_{12} = (a_2/a_1^2)$, where subscripts 1 and 2 refer to monomer and dimer, respectively. Biochemists are accustomed to seeing equilibrium constants written as ratios of equilibrium concentrations. However, the so-called equilibrium constant written in terms of concentrations, K_{12}, is actually an apparent constant related to the true equilibrium constant, K^o_{12}, by $K_{12} \equiv (c_2/c_1^2) = K^o_{12}(\gamma_1^2/\gamma_2)$, where γ_i denotes the ratio of effective to actual concentrations of species i, termed the *activity coefficient*. The activity coefficient has a precise definition in terms of nonspecific solute-solute interaction, $\ln\gamma_i = \langle g_i \rangle / kT$, where $\langle g_i \rangle$ denotes the (composition-dependent) equilibrium average free energy of nonspecific interaction between a molecule of species i and all of the other macrosolutes present in the medium, k is the Boltzmann constant, and T is the absolute temperature.

Excluded and Available Volume

Steric repulsion is the most fundamental of all interactions between macromolecules in solution and is always present at finite concentration, independent of the magnitude of additional electrostatic or hydrophobic interactions. Because solute molecules are mutually impenetrable, the presence of a significant volume fraction of macromolecules in the medium places constraints on the placement of an additional molecule of test macrosolute that depend upon the relative sizes, shapes, and concentrations of all macrosolutes in the medium. Fig. 2 depicts a region, demarcated by a square outline, in a solution containing spherical "background" macrosolutes of radius r_b, colored *black*, that occupy ~30% of the total volume (v_{tot}) of the specified region. The available volume ($v_{a,T}$) is defined to be that part of the volume of the region which may be occupied by *the center of mass* of a molecule of a spherical test species T of radius r_t added to the solution. If the test species is very small relative to the background species (Fig. 2*A*), then the available volume, indicated in *blue*, is approximately equal to that part of the total volume not occupied by the background species, *i.e.* ~0.7 v_{tot}. However, if the size of the test species is comparable

* This minireview will be reprinted in the 2001 Minireview Compendium, which will be available in December, 2001.

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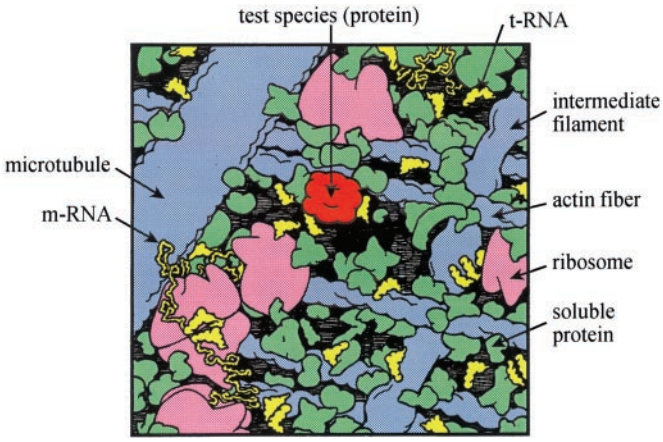

FIG. 1. **Cartoon of eukaryotic cytoplasm at a magnification of 1,000,000 ×.** The test protein molecule (*red*) is in a fluid medium that is crowded by soluble proteins (*green*), RNA species (*yellow*), and ribosomes (*pink*) and confined by cytoskeletal fibers (*blue*). Modified from Ref. 47 and reproduced with permission of the copyright holder.

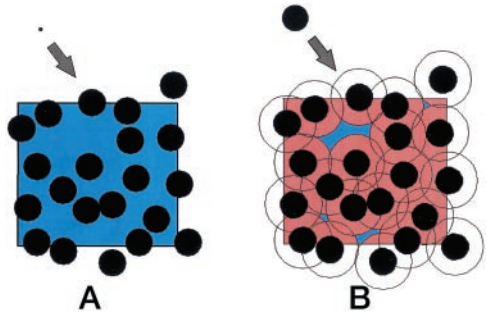

FIG. 2. **Excluded (*pink* and *black*) and available (*blue*) volume in a solution of spherical background macromolecules.** *A,* volume available to a test molecule of infinitesimal size; *B,* volume available to a test molecule of size comparable with background molecules.

with (or larger than) the background species (Fig. 2*B*), the available volume is substantially smaller, as the center of a molecule of the test species can approach the center of any background molecule to no less than the distance, denoted by r_C, at which the surfaces of the two molecules contact each other.[1] One may visualize this restriction by drawing a circular shell with radius r_C about each background molecule. Then the volume available to the test species, indicated by the *blue-colored regions* in Fig. 2*B*, is that part of the total volume which is not occupied by any background molecule or by any shell. It is evident upon inspection of Fig. 2, *A* and *B,* that the available volume is a sensitive function of the relative sizes (and shapes) of test and background molecules and the number density of background molecules.[2]

Volume may be excluded to a test particle by the surfaces of immobile structures as well as by individual background macrosolutes (4, 5), as illustrated in Fig. 3, which depicts a pore with square cross-section.[3] The center of a spherical test molecule whose diameter is comparable with the largest dimension of the pore (Fig. 3*B*) is excluded from the *pink-colored region*,

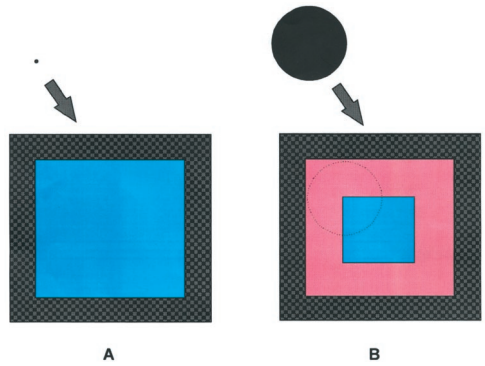

FIG. 3. **Excluded (*pink*) and available (*blue*) volume in a pore of square cross-section.** *A,* volume available to a test molecule of infinitesimal size; *B,* volume available to a test molecule of size comparable with pore dimensions.

which in this instance represents a significant fraction of the total volume of the solution enclosed in the pore.

Available Volume, Free Energy, and Chemical Reactivity

In a solution of macromolecules interacting exclusively via steric repulsion there exists an extremely simple relationship between the effective and actual concentration of each solute species (6), $\gamma_i \equiv (a_i/c_i) = (\nu_{tot}/\nu_{a,i})$, where ν_{tot} and $\nu_{a,i}$ denote the total volume and volume available to species *i,* respectively. The thermodynamic activities of macromolecules in fluid media may be measured by several physical-chemical methods. In Fig. 4, the experimentally measured ratio of the effective to actual concentration of hemoglobin, under experimental conditions comparable with those encountered in a red blood cell, is plotted as a function of the actual concentration. The first remarkable feature of this dependence is its highly non-linear nature; the effective concentration of hemoglobin exceeds the actual concentration by a factor of >10 at 200 g/liter and a factor approaching 100 at 300 g/liter. (For reference, the concentration of hemoglobin within a normal red blood cell typically exceeds 300 g/liter.) The second remarkable feature is that the experimentally measured dependence may be accounted for quantitatively over the entire concentration range by a simple geometrical model for available volume, in which each hemoglobin molecule is represented by a rigid spherical particle of radius ~29.5 Å, *i.e.* a particle closely resembling a "shrink-wrapped" hemoglobin molecule (7, 8).

The ratio of effective to actual concentration (*i.e.* activity coefficient) of a protein within a polymer gel may be calculated from the extent to which the protein partitions between the gel and bulk solution (4, 5). In Fig. 5, this ratio, measured experimentally in a dextran gel occupying about 3% of total solution volume, is plotted for a variety of globular proteins as a function of molar mass. We note that the dependence of activity coefficient upon molar mass is reasonably independent of the identity of the protein, indicating that it is a property primarily of protein size and is insensitive to small changes in shape or composition. The *solid curve* was calculated using a simple geometrical model for available volume (9), in which each protein is modeled as a hard spherical particle with a radius proportional to the cube root of mass, and polymer is modeled as a random matrix of hard cylindrical rods.

Estimated Magnitude of Crowding Effects on Association Equilibria

We present a simple example of how the difference between activity and concentration in a crowded medium may qualita-

[1] For markedly non-spherical molecules, r_C is a function of the mutual orientations of test and background molecules. For approximately spherical molecules, r_C may be treated as a constant equal to the sum of the average radii of test and background molecules.

[2] Although Fig. 2, *A* and *B,* reflects a static distribution of background molecules, these conclusions hold also for a dynamic distribution, assuming equivalence of spatial and time averages.

[3] This pore is one possible idealized representation of a small element of volume bounded by large macromolecular assemblies, such as interstices within a lattice of rodlike fibers or lamellar space between adjacent membrane surfaces.

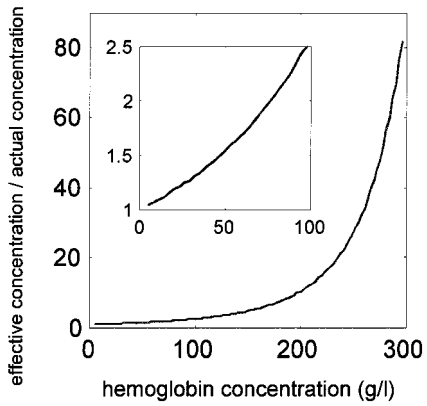

FIG. 4. **The effect of hemoglobin concentration on its effective concentration (thermodynamic activity), calculated from concentration dependence of the osmotic pressure (7).** *Inset* is a magnification of the low concentration regime.

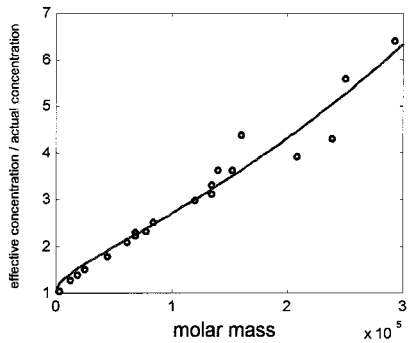

FIG. 5. **The dependence of effective concentration on the molar mass of quasispherical test proteins in a dextran G-200 gel particle of fractional volume occupancy of ~0.03.** *Symbols* are calculated from experimentally measured partition coefficients presented in Ref. 48. *Solid curve* indicates best fit of the excluded volume model of Ogston (9).

tively influence association equilibria.[4] Consider the dimerization reaction introduced above, with real and apparent equilibrium constants defined in the first two equations. For the sake of illustration, we set the molar mass of A equal to 100,000 and assume that both A and A_2 have roughly spherical shape.[5] Using the same geometrical model for excluded volume and the same size and shape parameters used to fit the data in Fig. 5 (9), the values of γ_1 and γ_2 may be estimated to be about 3×10^2 and 1×10^4, respectively, for a fractional volume occupancy ϕ of 0.2, and about 1×10^4 and 1×10^6, respectively, for $\phi = 0.3$. It follows from the second equation that the experimentally observed equilibrium constant, K_{12}, would be expected to exceed K^o_{12} (the value of K_{12} in the limit of high dilution) by a factor of ~10 in a medium of $\phi = 0.2$ and ~100 in a medium of $\phi = 0.3$. Although this estimate is only qualitative, the large magnitude of the predicted effect of excluded volume transcends the crudeness of the theoretical model. Indeed, similar but somewhat more refined predictions have been confirmed, in some cases quantitatively, by experimental observation (see references in Ref. 11 and in Table I).

Effect of Excluded Volume on Macromolecular Association Reaction Rates

There are two opposing effects of excluded volume on reaction rates (12). If the overall rate of the reaction is limited by the rate with which a transition state complex decays to products, then crowding would be expected to enhance the relative abundance of the transition state complex and hence the forward reaction rate. Under these conditions, the forward rate constant may be increased by up to the equilibrium enhancement factor, depending upon details of the particular reaction. However, if the overall rate of the reaction is limited by the rate with which reactant molecules encounter each other through diffusional motion, then crowding, which retards diffusional motion (13, 14), would be expected to lower the forward reaction rate. In the limit of high fractional volume occupancy, all association reactions are expected to be diffusion limited and hence slowed by crowding (11). Hence, depending upon the nature of a particular reaction, one of two types of behavior may be observed as the fractional volume occupancy of background molecules increases: the forward rate for a macromolecular association may decrease monotonically or may initially increase, pass through a maximum, and then decrease. A bimodal dependence of reaction rate on crowder concentration has been observed experimentally (15).

Macromolecular Reactions Affected by Excluded Volume

Macromolecular crowding and/or confinement by background molecules or structures can in principle affect the equilibrium and kinetics of any macromolecular reaction in which there exists a significant difference between the volume excluded to reactants and the volume excluded to products. Such reactions include self- or heteroassociation, condensation (crystallization, nucleation-controlled fiber formation), binding of macromolecules to specific surface sites, nonspecific surface adsorption, and protein isomerization, including folding/unfolding (4, 10, 11, 16–18). Crowding may also affect enzyme-catalyzed reactions of small molecules if the mechanism of catalysis involves significant conformational change of the enzyme (3, 10). Many such effects have indeed been observed experimentally. Most of the older observations are cited in Ref. 11, and some more recent observations are listed in Table I.

Broader Physiological Ramifications

In recent years increased attention has been paid to the functioning of ever larger macromolecular assemblies and systems of interacting components, sometimes referred to as molecular machines (19). As larger and more complex systems have come under closer scrutiny, a growing number of biomedical researchers have emphasized the extremely broad ramifications of macromolecular crowding and confinement for biochemistry in the intact cell (see for example Refs. 20–25). It is becoming more widely appreciated that under physiological conditions of crowding or confinement, the size- and shape-dependent reduction of volume available to every species of macromolecule results in major shifts in the rates and equilibria of a broad range of macromolecular reactions relative to those measured in dilute solution. We now recognize that nonspecific interactions, including (but not limited to) steric repulsion, provide a substantial contribution to the free energy balance of a physiological system such as an intact cell or tissue.

It seems likely that the constituent elements of these systems have evolved to function optimally under normal physiological (*i.e.* crowded and/or confined) conditions and that the proper functioning of the system depends upon maintenance of the free energy balance established *under those crowded and/or confined conditions*. Excluded volume theory predicts that at the high level of macromolecular fractional volume occupancy characteristic of all living cells (*i.e.* >0.20–0.30), the reactivity of almost every soluble macromolecular species, dilute as well as concentrated, will depend sensitively upon its

[4] A more complete treatment is presented in Ref. 10.
[5] Although the dimer is unlikely to be spherical, its deviation from sphericity will not be so large that treatment as an approximate sphere will introduce a qualitative error into the present estimate (10).

TABLE I

Some recent reports of experimentally observed crowding and confinement effects on macromolecular reactions

Earlier observations are tabulated in Zimmerman and Minton (11).

Observation[a]	Magnitude
Enhancement of spectrin self-association by PEG, dextran (35, 36)[b]	10-fold increase of K_{12} in 20% dextran
Enhancement of actin polymerization by dextran and PEG (37)	3-fold decrease in solubility in 15% dextran
Enhancement of binding of HU protein to *E. coli* DNA by PEG and non-DNA binding proteins (38, 39)	12% PEG increases affinity of DNA for HU by >10-fold
Stabilization of supercoiled conformations of DNA by PEG (40)	
Sequestration of protein molecules in hydrated sol-gel glass stabilizes them with respect to thermal denaturation (41)	T_{50} for α-lactalbumin increased by >25 °C
Self-association of fibrinogen induced by bovine serum albumin (42)	Doubling of weight-average molar mass in >5% bovine serum albumin
Enhancement by dextran of limited self-association of tubulin under conditions not permitting microtubule assembly	>2-fold increase in weight-average molar mass in 10% dextran
Enhancement of self-association of FtsZ by bovine serum albumin, hemoglobin (43)	2-fold increase in weight-average molar mass in 30% albumin or hemoglobin
Enhancement of unimolecular condensation of large linear DNA by PEG (44)	>10-fold increase in 2-state equilibrium constant at 18% PEG
Enhancement of productive refolding and assembly of GroEL by Ficoll 70 (45)	>3-fold increase in recovery of ATPase activity in presence of >10% Ficoll
Reduction in solubility of deoxy sickle cell hemoglobin by dextran (46)	~15-fold decrease in 21% dextran

[a] PEG, polyethylene glycol.
[b] Numbers in parentheses are references.

available volume, which, in turn, depends sensitively upon the total volume fraction of macromolecules. It follows that relatively small changes in the fractional volume occupancy of the cellular interior are expected to have major effects on the equilibria and kinetics of a broad variety of intracellular reactions (26, 27). These considerations help us to understand two very general properties of living cells. 1) Relatively modest changes of cellular volume in animal cells (*i.e.* concentration of intracellular macromolecules) are associated with changes in the rates of a broad spectrum of diverse intracellular processes that are much too large to be accounted for on the basis of simple mass action (28). 2) Every type of cell so far examined, from bacterial to human, is equipped with one or more mechanisms (varying widely among different types of cells) for the maintenance or restoration of cellular volume, water content, and/or turgor pressure in response to changes in composition of the extracellular fluid (29).

The examples presented here are only a few of many supporting the hypothesis that macromolecular crowding and confinement play important and perhaps essential roles in cell biology and physiology (11, 24, 30–34). Effects of excluded volume in physiological media are of sufficient magnitude to mandate careful consideration when postulating a role *in vivo* for any macromolecular reaction characterized *in vitro*.

Acknowledgments—I thank the following colleagues for reviewing early drafts of this communication: Damien Hall, Nancy Nossal, Herbert Tabor, Reed Wickner, and especially Steve Zimmerman for incisive comments.

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