Annu. Rev. Biophys. Biomol. Struct. 1993. 22:27–65

MACROMOLECULAR CROWDING: Biochemical, Biophysical, and Physiological Consequences[1]

Steven B. Zimmerman

Section on Physical Chemistry, Laboratory of Molecular Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 5, Room 330, Bethesda, Maryland 20892

Allen P. Minton

Section on Physical Biochemistry, Laboratory of Biochemical Pharmacology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Building 8, Room 226, Bethesda, Maryland 20892

KEY WORDS: excluded volume, cytoplasm, cell volume, diffusion, intermolecular interactions, proteins, DNA, nucleic acids, enzymes

CONTENTS

PERSPECTIVES AND OVERVIEW 28
Nomenclature 30
THEORETICAL CONCEPTS 31
The Effect of Macromolecular Crowding on Solution Equilibria 31
The Effect of Macromolecular Crowding on Reaction Rates 36
The Effect of Macromolecular Crowding on Diffusive Transport of Solutes 37
The Effective Specific Volume of a Macromolecule—What Is It? 40

[1] The US government has the right to retain a nonexclusive, royalty-free license in and to any copyright covering this paper.

EXPERIMENTAL MEASUREMENT OF THE EFFECT OF VOLUME OCCUPANCY ON THE THERMODYNAMIC ACTIVITY OF A TRACER PROTEIN 43
Solutions Containing a Single Macrosolute 43
Solutions Containing a Tracer Protein and a Single Background Species 43
EXPERIMENTALLY OBSERVED EFFECTS OF CROWDING ON EQUILIBRIA AND REACTION RATES IN MODEL SYSTEMS 46
Extended Ranges of Enzymatic Activities Under Crowded Conditions 46
Alteration of Reactants or Products Under Crowded Conditions 47
EXPERIMENTALLY OBSERVED EFFECTS OF CROWDING UPON TRANSPORT PROPERTIES IN MODEL SYSTEMS 51
Viscosity of a Protein Solution 51
Self-Diffusion of Proteins in Membranes 51
Self-Diffusion of Proteins in Aqueous Solution 54
Tracer Diffusion of a Protein in Solutions of a Second Protein 54
Tracer Diffusion of Probe Molecules in Polymers 55
CROWDING IN CYTOPLASM 56
Experimental Approaches to the Calculation of Volume Exclusion in Cytoplasm 56
Rationalization of Discrepancies Between Phenomena Observed in Vitro and in Vivo 56
Diffusion of Probe Molecules in Cytoplasm 57
RAMIFICATIONS, SPECULATIONS, CONCLUSIONS 58
Possible Role of Crowding in Regulation of Cellular Volume 58
Efficacy of Macromolecular Drugs 58
Synthesis by Degradative Enzymes 59
Macromolecular Crowding as a Laboratory Tool 59
Osmotic Remedial Mutants 59
Crowding as a Factor in Cellular Evolution 59
Crowding Versus Confinement 60
Homeostasis or Metabolic Buffering 60
Crowding and the Functional Importance of Condensed DNA 61
Crowding and Cytoplasmic Structure 61

PERSPECTIVES AND OVERVIEW

Measurements of biochemical rates and equilibria are conventionally carried out under relatively idealized conditions selected to minimize effects of nonspecific interactions between the constituents of the reaction unit (i.e. the reactants, products, and transition-state complexes) and other species that do not directly participate in the reaction under study. Concentration-dependent apparent rate or equilibrium constants are then extrapolated to the limit of infinite dilution in order to extract intrinsic quantities reflecting the properties of isolated reactant and product molecules in an idealized bath of solvent.

Biological media differ from the idealized solvent bath in two important ways. First, a biological medium is likely to contain a high total concentration of nominally soluble macromolecules. Sometimes a single species at a high concentration predominates, such as hemoglobin within red cells (~350 g/liter) (see 3). More commonly, a medium may contain a variety of macromolecular species, none of which taken individually may be present at high concentration, but which taken collectively occupy a substantial fraction of the total volume of the medium [e.g. ~340 g/liter

of total RNA + protein in the cytoplasm of *Escherichia coli* (172)] (see also 19, 39). We refer to such a medium as crowded. Second, particularly in eukaryotes, a biological medium is also likely to be structured at the molecular level by the presence of a network of extended structures such as F-actin, microtubules, intermediate filaments, and membranous boundaries (40). We refer to those soluble or structural macromolecules that do not directly participate in a particular reaction as background species.

The local environment will influence reactions taking place in a biological medium when reactants, transition-state complexes, and products interact unequally with background species, as illustrated in Figure 1, and/or when interactions with background species alter the mobilities of constituents of the reaction unit. This applies to interactions of all types, including steric-repulsion, electrostatic, hydrophobic, and van der Waals interactions. In the present review, we focus on the steric-repulsive interactions deriving from the fundamentally impenetrable nature of molecules, as these interactions are always present in addition to any other interactions that may or may not be present.

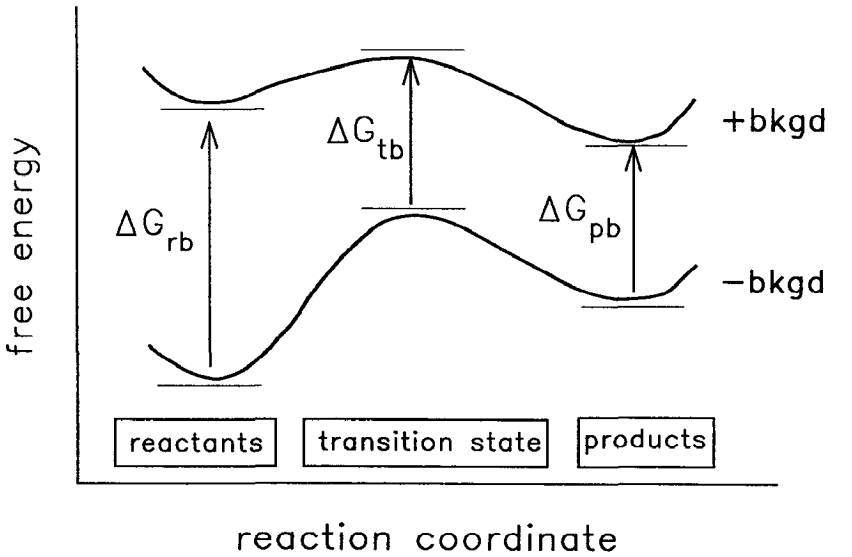

Figure 1 Schematic free-energy profile of a simple chemical reaction in the absence (*lower curve*) and presence (*upper curve*) of background molecules. ΔG_{rb}, ΔG_{tb}, and ΔG_{pb}, respectively, denote the free energies of nonspecific interaction between reactants and background molecules, between transition-state and background molecules, and between products and background molecules. To the extent that these three nonspecific interaction energies are unequal, the rate and/or equilibrium constants characterizing the reaction will differ in the absence and presence of background molecules.

Obviously, a living organism bears scant resemblance to a bath of solvent, yet it is not generally appreciated that, from a quantitative point of view, biochemical rates and equilibria in a living organism may likewise bear scant resemblance to those measured in a bath of solvent. Only relatively recently have investigators recognized that steric repulsion between macromolecules in a crowded medium can strongly influence both the rate and extent of a variety of macromolecular reactions, and that crowding effects must be taken into account when attempting to relate biochemical and biophysical observations made in vitro to physiological processes observed in vivo (93–95).

Thermodynamic evidence for the presence of substantial repulsive intermolecular interactions in solutions of proteins and mixtures of proteins and polymers comes from studies, dating back to the early part of this century, of colligative and partitioning properties of such solutions (2). Not until much later did researchers show that the thermodynamic data were accounted for semiquantitatively (or quantitatively in favorable cases) by simple geometric models presupposing only the existence of short-range steric repulsion between macromolecules (36, 132). These models have subsequently proven to be useful for interpreting the results of a wide variety of measurements of steady-state and time-dependent behavior in crowded media, as well for predicting the dependence of various measurable reaction rates and equilibria upon the fraction of volume occupied by macromolecules within a given medium (see below). Most recently, a growing body of experimental evidence testifies to the variety of biochemical processes that can be profoundly influenced by macromolecular crowding.

This review places particular emphasis upon research on the thermodynamic aspects of crowding done since 1983, when the last comprehensive review of this subject was presented (95), and upon topics of current interest to the authors. The reader is also referred to other discussions of crowding and crowding-related phenomena (5, 24, 29, 39, 43, 82, 124, 140).

Nomenclature

We refer frequently to the behavior of individual *tracer* or *probe* particles (molecules) in an environment comprised of many other particles (molecules) of various species, which are termed *background* particles (molecules). *Excluded volume* refers to the volume of a solution that is excluded to the center of mass of a probe particle by the presence of one or more background particles in the medium. *Fractional volume-occupancy* (ϕ) denotes the fraction of the total volume occupied by macromolecules.

THEORETICAL CONCEPTS

The Effect of Macromolecular Crowding on Solution Equilibria

Consider the general reaction

$$n_1X_1+n_2X_2+\cdots+n_jX_j \rightleftharpoons n_{j+1}X_{j+1}+n_{j+2}X_{j+2}+\cdots+n_qX_q,$$

where n_i is the number of molecules of X_i participating in the reaction. The condition of equilibrium at constant temperature and pressure requires that

$$K^\circ(T,P) \equiv \prod_{i=j+1}^{q} a_i \Big/ \prod_{i=1}^{j} a_i,$$

where K° is the thermodynamic association constant; a_i is the thermodynamic activity of solute species i, which may be written as a product of the concentration of species i, c_i, and an activity coefficient, γ_i.[2] It follows that the conventional equilibrium constant, expressing an equilibrium relation between concentrations rather than activities, is itself a function of solution composition (denoted by $\{c\}$):

$$K(T,P,\{c\}) \equiv \prod_{i=j+1}^{q} c_i \Big/ \prod_{i=1}^{j} c_i = K^\circ(T,P)\times\Gamma(T,P,\{c\}),$$

where

$$\Gamma \equiv \prod_{i=1}^{j} \gamma_i(T,P,\{c\})^{n_i} \Big/ \prod_{i=j+1}^{q} \gamma_i(T,P,\{c\})^{n_i}. \qquad 1.$$

The activity coefficient γ_i is a measure of nonideal behavior arising from interactions between solute molecules:

$$\gamma_i(T,P,\{c\}) = \exp[G_i^{\mathrm{NI}}(T,P,\{c\})/RT], \qquad 2.$$

where $G_i^{\mathrm{NI}}(T,P,\{c\})$ denotes the average free energy of interaction between a molecule of species i and all of the other solute molecules present in a real solution whose composition is denoted by $\{c\}$.[3] For the case of simple volume exclusion, G_i^{NI} is greater than 0, and γ_i is greater than 1 (95). The magnitude of γ_i strongly depends upon the relative sizes and shapes of the

[2] The concentration may be expressed in any units. In the present review, we express concentrations in units proportional to the number density of molecules, i.e. molar or weight/volume, depending on the specific application.

[3] As the solution approaches the limit of infinite dilution, intersolute interactions of all types become negligible, $\gamma_i \to 1$ for all i, $\Gamma \to 1$, and $K \to K^\circ$.

probe and background species as well as the concentrations of background species.

It follows from Equations 1 and 2 that

$$RT \ln \Gamma = \sum_{i=j+1}^{q} n_i G_i^{NI} - \sum_{i=1}^{j} n_i G_i^{NI}.$$

Thus, the apparent equilibrium constant K is increased or decreased relative to the ideal equilibrium constant K° by a factor that reflects the difference between the free energies of nonspecific interaction of molecules of products with all solute species and molecules of reactants with all solute species (Figure 1) (see also 95).

The logarithm of the activity coefficient may be expanded in powers of the concentrations of solute(s):

$$\ln \gamma_i = \sum_j B_{ij} c_j + \sum_j \sum_k B_{ijk} c_j c_k + \ldots, \qquad 3.$$

where the indices $j,k, \ldots$ can have any value from 1 to q. The terms $B_{ij}, B_{ijk}, \ldots$ are referred to as two-body, three-body, . . . interaction (or virial) coefficients, respectively. Exact formal expressions for the virial coefficients as functions of the effective potential of interaction between two or more molecules of solute in a bath of solvent have been derived via statistical thermodynamics (55, 89). In practice, evaluation of the values of the virial coefficients is only possible for small numbers of particles interacting via very simple interaction potentials. The simplest of these is the hard-particle potential, which is equal to zero for all interparticle distances (defined with respect to the particle centers) above a certain contact distance,[4] and infinite for all interparticle distances equal to or less than the contact distance. Thus it is a common (but not necessarily realistic) practice to describe macromolecular solutes as effective rigid hard particles with simple shapes for the purpose of calculating virial coefficients and estimating the value of the activity coefficient (107, 108, 110, 113, 161). Fortunately, this approximation seems to work reasonably well for solutions of a single globular protein in solutions of moderate ionic strength, i.e. under conditions such that long-range electrostatic interactions are largely damped out (95, 132). Approximate corrections for the presence of nonnegligible electrostatic interactions have been presented (18, 100, 161).

Even when macrosolutes may be reasonably approximated by rigid hard

[4] If at least one of the interacting particles is nonspherical, then the contact distance will be a function of the relative orientations of the particles as well as the distance between particle centers.

particles of simple shape, calculation of virial coefficients above the two-body coefficient is difficult to impossible for particles other than uniformly sized hard spheres. Thus, evaluation of activity coefficients via Equation 3 is limited to solutions in which all solute species are sufficiently dilute so that three-body and higher-order contributions would be negligible, or in which the size of background particles is sufficiently small relative to the size of the probe particle (see below).

An alternate approach to the calculation of activity coefficients of solutes in the hard-particle approximation is based upon the relation

$$\gamma_i(\{c\}) = \frac{1}{P_i(\{c\})},$$

where $P_i(\{c\})$ is the probability that a randomly selected point in the solution of composition $\{c\}$ can accommodate a probe molecule of species i, i.e. the point lies at the center of a solute-free region (or hole) that is at least as large as the probe molecule, as illustrated in Figure 2.

Although the value of $P_i(\{c\})$ cannot be calculated exactly, two approximate methods have proven useful. The first method, introduced by Ogston (110), which we refer to as available volume theory, or AVT (see also 23, 32, 158, 171), is based upon the assumption that centers of background particles are distributed randomly, so that the number of centers of background particles within a given element of area or volume is governed by a Poisson distribution. This approximation (which is equivalent to neglecting the interaction between molecules of background species) leads to expressions of the form of Equation 3 truncated after two-body interaction terms, and thus predicts that the logarithm of activity of each species varies linearly with the concentrations of all species; that is:

$$\ln \gamma_i = \sum_j B_{ij} c_j.$$

The second approximate method utilizes an equation of state derived from scaled particle theory, or SPT, to calculate $P_i(\{c\})$ and γ_i (41; note corrections to 41 in 21; 76, 125). According to SPT, the logarithm of the activity coefficient of species i may be expressed as a finite series in powers of R_i, the radius (or characteristic dimension) of the probe particle

$$\ln \gamma_i = \sum_{k=0}^{d} A_k R_i^k,$$

where d is the dimensionality of the solution and the A_k are positively valued functions of the number densities, characteristic dimensions, and shapes of all macrosolute species in solution. SPT has been applied to the calculation of protein activities and crowding effects in isotropic solutions

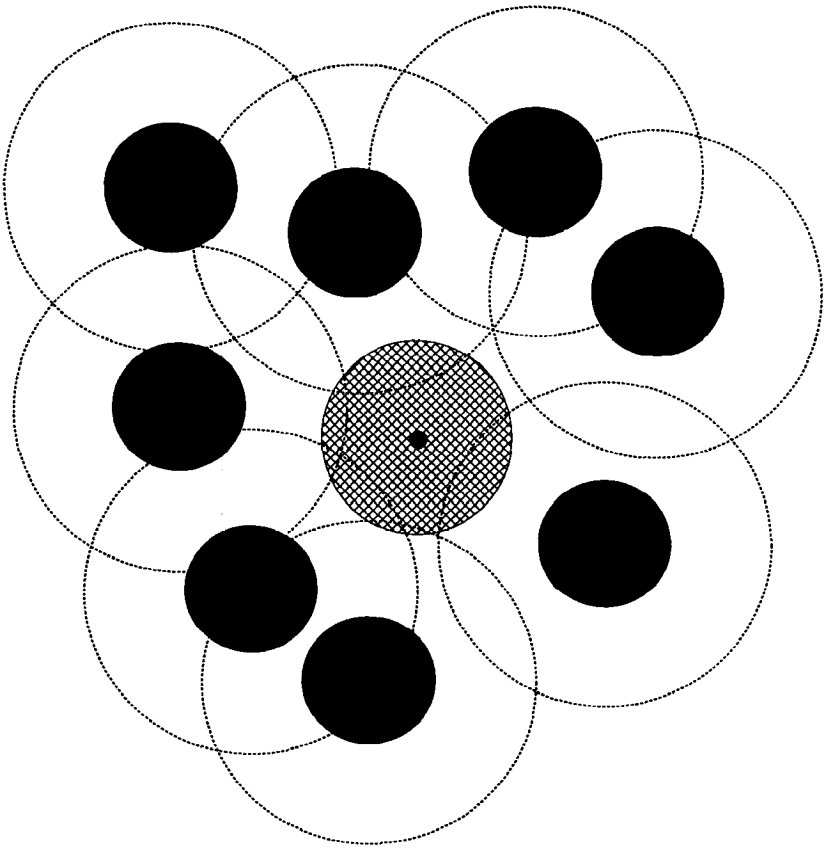

Figure 2 Illustration of volume available to a probe particle (*crosshatched sphere*) in a solution containing background particles (*solid spheres*). Each background particle lies at the center of a spherical region, indicated by a dashed surface, the radius of which is equal to the sum of the radii of background and probe particles. This region, representing the covolume of the background and probe particles, is inaccessible to the center of mass of the probe particle, indicated by a black dot. The volume available to the probe, defined as equal to the volume accessible to the center of mass of the probe, is thus the volume exterior to all such regions (*dashed surfaces*). If the probe is of species i, then P_i is equal to the ratio of the available to the total volume.

(94, 132, 133) and in solutions of proteins undergoing polymerization to highly elongated aggregates that spontaneously form nematic and other liquid-crystalline phases (83, 173). In both AVT and SPT, the solvent (water) is treated as a featureless continuum, but such a treatment will become increasingly less realistic as the size of background molecules

approaches the size of solvent molecules (15). A modified SPT calculation of crowding effects was recently presented in which water molecules are explicitly represented by an additional species of effective hard spherical particles (15).

Statistical-mechanical models for enumerating the possible configurations of a polymer chain in the vicinity of a compact impenetrable protein molecule (54) provide an approximate means for calculating the negentropic work associated with the insertion of a protein molecule into a polymer solution. A recent model of this type allows for the presence of short-range attractive interactions between segments of the polymer chain and the surface of the protein (12).

Model calculations of the effect of crowding upon association and conformational equilibria have been carried out for membranes modeled as two-dimensional fluids (45) and for aqueous solutions (69, 93–95, 108, 133). The major qualitative predictions of equilibrium excluded-volume analysis may be summarized as follows, where ϕ denotes the fraction of the total volume occupied by macromolecules:

1. For self- and heteroassociation reactions, the nonideal correction factor Γ increases monotonically with increasing ϕ, and the value of $d\Gamma/d\phi$ increases monotonically with increasing ϕ.
2. For fixed ϕ, the value of Γ increases with the degree of association. For example, Γ for a hypothetical monomer-tetramer self-association reaction would be expected to exceed that for a monomer-dimer self-association of the same species subunit.
3. Increasing volume occupancy tends to favor compact or globular conformations (or assemblies) relative to highly anisometric conformations (or assemblies).
4. For fixed ϕ, the value of Γ for association reactions increases with decreasing molecular weight (i.e. increasing number density) of background species.[5]
5. Volume occupancy by macrosolutes has a greater influence on association equilibria in three dimensions than the same degree of volume (area) occupancy has upon the corresponding reaction equilibria in two dimensions.
6. For fixed ϕ, the effect of crowding upon associations between macromolecules is expected to be much greater than upon associations between a macromolecule and a small molecule.
7. Finally, and most importantly, under conditions of macromolecular

[5] As pointed out above, this result would only be valid so long as background species are large relative to solvent, i.e. are also macromolecules.

volume occupancy comparable to those found in vivo ($0.1 < \phi < 0.5$), macromolecular association constants are predicted to exceed those in dilute solution by as much as several orders of magnitude. In fact, the contribution of volume exclusion in a crowded medium to the overall standard free energy change of a reaction can, under some circumstances, equal or exceed the ideal contribution that is intrinsic to the reaction unit (132).

Excluded volume theory has also been used recently to analyze the effects of small-molecule solutes (or cosolvents) upon the equilibrium properties of macrosolutes (141, 163). This analysis provides a microscopic or mechanistic alternative to traditional analyses of these effects in the context of macroscopic models of the preferential interaction of macrosolute with either water or cosolvent (see e.g. 6).

The Effect of Macromolecular Crowding on Reaction Rates

Macromolecular crowding affects the rates of different types of biochemical reactions in distinctly different ways.

FORMATION OF MACROMOLECULAR COMPLEXES Consider the association of two macromolecules forming a homo- or heterodimer. If the rate-determining step for dimer formation is the conversion of activated complex to fully formed dimer, then separated reactants and activated complex may be treated to a first approximation as if they are in equilibrium. Under these conditions, crowding is predicted to lower the free energy of activation and increase the rate of formation of complexes roughly to the same extent that crowding increases the equilibrium constant for dimer formation (94, 95). However, if the rate-determining step for dimer formation is the formation of activated complex (i.e. the rate with which the two reactant molecules encounter each other), then the reaction is said to be diffusion-limited, and the overall rate depends upon the sum of the diffusion coefficients of the reactants (16). Because crowding lowers the diffusion coefficient (see below), it is predicted to lower rates of association in diffusion-limiting circumstances (94, 97). Since the encounter rate represents an absolute upper limit for the rate of any bimolecular reaction, ultimately the overall bimolecular reaction rates must decrease with increasing fractional volume-occupancy (independent of the effect of crowding at low fractional volume-occupancy) when crowding becomes sufficiently great (97). Figure 3 schematically illustrates the overall effect of increasing volume occupancy on bimolecular association rate.

ENZYME-CATALYZED REACTIONS The effect of macromolecular crowding upon the rate of a prototypical enzyme-catalyzed reaction (substrate →

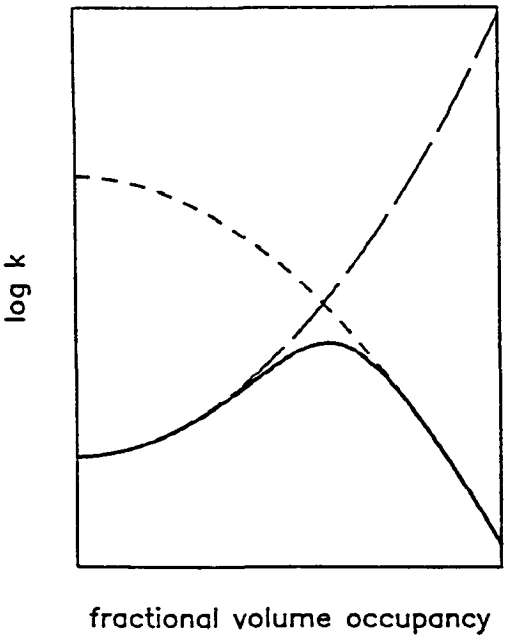

Figure 3 Schematic dependence upon fractional volume occupancy of transition state–limited rate constant (*long-dashed curve*), diffusion-limited rate constant (*short-dashed curve*), and overall rate constant (*solid curve*). The overall reaction rate is transition-state limited at low volume occupancy and diffusion limited at high volume occupancy. Reproduced from Ref. 97.

product) has been analyzed for the cases in which each of the following elementary steps is rate-limiting: (*a*) association of substrate and enzyme (94), (*b*) conversion of enzyme-bound substrate to enzyme-bound product (69, 94), and (*c*) dissociation of enzyme-bound product (94). In addition, if the enzyme exists in several states of association that have different intrinsic catalytic activities (see e.g. 116), crowding can affect the equilibrium-average state of association and hence the equilibrium-average catalytic activity (94, 101).

The Effect of Macromolecular Crowding on Diffusive Transport of Solutes

In the absence of an externally applied potential field, the motion of individual compact tracer macromolecules in dilute solution can be described by a random walk, so that

$$\langle r^2 \rangle = 2 \cdot d \cdot D^\circ \cdot t,$$

where $\langle r^2 \rangle$ is the squared displacement of position observed after elapsed time t, averaged over many observations; d is the dimensionality; and D°, the intrinsic diffusion coefficient of species i, is given by the Einstein relation

$$D_i^\circ = kT/f_i^\circ,$$

where f_i° is the frictional coefficient characteristic of an isolated tracer particle of species i in a solvent of given viscosity (148). In the absence of a macroscopic concentration gradient of background molecules, the coefficient of tracer or probe diffusion is defined by

$$D^{tr} = \langle r^2 \rangle_{tr}/(2 \cdot d \cdot t),$$

where the subscript tr indicates that the average of r^2 is taken over all of the tracer molecules in the solution.[6] The coefficient of tracer diffusion is equal to the coefficient of self-diffusion D^{self} when tracer and background species are identical.

The influence of background molecules on the diffusive motion of a probe molecule is a phenomenon that is extremely complex to analyze theoretically, and is far less tractable than the parallel phenomenon of the influence of background particles on the thermodynamic activity of a probe molecule. In principle, D_i^R, the reduced tracer diffusion coefficient of species i, may be expanded in a power series of concentrations, similar to Equation 3:

$$D_i^R \equiv \frac{D_i^{tr}}{D_i^\circ} = 1 + \sum_j Q_{ij} c_j + \sum_j \sum_k Q_{ijk} c_j c_k + \ldots,$$

where D_i^{tr} and D_i° respectively denote the tracer and intrinsic diffusion coefficients of species i, and the coefficients $Q_{ij}, Q_{ijk}, \ldots$ are measures of two-body, three-body, and higher-order interactions. Each interaction coefficient reflects contributions from direct interactions between solute particles (short-range steric repulsions in the present instance) and from hydrodynamic interactions.[7] Attempts to incorporate both direct and hydrodynamic interactions into statistical-mechanical theories of the liquid state have led to complex formal relations that permit numerical com-

[6] In principle, the tracer diffusion coefficient may be a function of time and distance (138, 140). In the context of the present discussion, we will take this quantity to be equal to the limiting value at long time and large distance, as that is the quantity determined in most experiments.

[7] Hydrodynamic interaction is the effect on the motion of the tracer particle of transient flows of solvent at the position of the tracer caused by motions of the background particles. These effects may be compared to the effect of the wake of a ship on the motion of a second nearby ship.

putation only in the limiting case of self-diffusion of hard spherical particles at low fractional volume-occupancy (87, 122). Three general approaches to the calculation of the effect of arbitrary concentrations of a compact background species upon tracer diffusion in the absence of hydrodynamic interaction have been developed.

A statistical-mechanical theory has been developed that permits one to calculate the coefficient of tracer diffusion of species i in the presence of a given concentration of background species j, over a broad range of background concentrations, provided that the equilibrium pair-correlation function $g_{ij}(r)$ is known (or can be calculated) for each concentration of species j (115). This theory has been adapted for two-dimensional self-diffusion of membrane proteins (1). The two-dimensional pair-distribution function $g_{ii}(r)$ was computed for hard disks at fractional volume- (area-) occupancies of 0.25 and 0.50 via Monte Carlo simulation. Using these distribution functions, the reduced two-dimensional self-diffusion coefficient of hard disks was computed at the two fractional volume-occupancies (1) and found to be in good agreement with the results of simulation of walks of hexagonal particles on a hexagonal lattice for different levels of fractional lattice occupancy (120, 137). A great deal of numeric calculation is required to compute the self-diffusion coefficient for even a single value of the fractional volume-occupancy, and at the present time, rapid computation of the reduced tracer-diffusion coefficient as a function of the size and volume occupancy of background species via fundamental theories of the liquid state does not appear to be feasible in two dimensions, much less three dimensions.

The second method for obtaining information about the effect of volume occupancy on tracer diffusion is simulation of random walks of particles on lattices of varying volume occupancy. Simulations have been carried out for two-dimensional particles on planar lattices, in which tracers and background particles are characterized by different intrinsic jump rates (diffusion coefficients) and different sizes (120, 137). These simulations have yielded insight into the effect of mobile obstacles on the motion of random walkers and serve as standards against which approximate theories should be compared. However, each simulation carried out for a single set of parameters (lattice type, particle sizes, intrinsic jump rates, lattice occupancy, etc) is computationally intensive, and we are unaware of comparable calculations carried out for a three-dimensional model at this time.

A third approach to calculating the effect of crowding on tracer diffusion employs a semiempirical free-volume model, according to which an isolated tracer molecule in solution diffuses by undergoing a Brownian displacement of average distance Δr on an average of once every Δt seconds. When background molecules are added to the solution, the probability of

undergoing a displacement is assumed to be proportional to the probability that the target volume (the element of volume into which the tracer would be relocated in the event of a successful jump) is free of any part of a background molecule. It follows that

$$D_i^R = P_i^V(\{c\}) = \exp[-\Delta G_i^V(\{c\})/RT],$$

where P_i^V is the probability that the target volume is vacant, and ΔG_i^V is the (negentropic) free energy associated with the creation of a vacancy of the required size and shape. For the case of a globular protein undergoing diffusion in a solution of random-coil polymers, the value of P_i^V has been estimated via AVT (112), and for globular proteins diffusing in solutions of other proteins, ΔG_i^V was estimated using SPT (106). Free-volume models provide a rapid means of estimating crowding effects in both two and three dimensions via back-of-the-envelope calculations (see also 96, 114), but because they are semiempirical and contain one or more adjustable parameters, they must be calibrated against experimental data.

All of the above-described theories and models for diffusion of a globular protein in a protein solution predict that D_i^R decreases more rapidly with increasing volume fraction of background protein $j(\phi_j)$ than expected on the basis of a simple decaying exponential in ϕ_j, and reaches zero at a value of $\phi_j < 1$ that is determined by the relative volumes of species i and j. The free-volume theory also predicts that smaller proteins will have a larger effect per unit weight on the diffusion of larger proteins than vice versa. In contrast, free-volume theory for diffusion of a globular protein in a polymer solution (112) predicts that D_i^R decreases as a decaying exponential in the square root of ϕ_j.

The Effective Specific Volume of a Macromolecule—What Is It?

Although the representation of macromolecules by equivalent rigid particles of simple shape for the purpose of calculating macromolecular activities in crowded solutions is an appealing and useful concept, the volume of the equivalent particle is an inherently ambiguous quantity that is more often than not evaluated by fitting approximate theoretical relations (of possibly dubious applicability) to experimental data and letting the results fall where they may. In principle, one would like to perform calculations that have predictive—as opposed to purely hand-waving—value. Thus, a discussion of the factors affecting the volume of an equivalent particle, and methods for estimating the "best" volume for a particular calculation is in order.

Hard-particle models are generally based upon several assumptions: (*a*)

the solvent may be treated as a continuum (but see 15 for an exception); (*b*) a macrosolute may be treated—at low resolution—as a rigid convex particle of simple shape or as an assembly of such particles; and (*c*) the potential of average force acting between the actual molecules in solution is short range and effectively zero (i.e. $\ll kT$) beyond some contact distance defined as a function of the geometry and relative orientations of the interacting particles. To the extent that these assumptions are valid, the size and shape (at low resolution) of actual macromolecules do in fact closely resemble those of the equivalent particles best accounting for concentration-dependent activity coefficients (106, 130, 132). Conversely, if significant attractive or repulsive interactions exist between molecules of macrosolute in a solution containing only a single macrosolute, the equivalent hard particle may be significantly smaller or larger than the actual size of the molecule (95, 100).

Even more confounding is the fact that, in the case of multiple solute species interacting via electrostatic or other nonadditive potentials, one cannot in principle define a unique equivalent hard particle corresponding to each solute species. For instance, consider a solution containing two quasispherical protein species, A and B, of approximately the same mass and density. In the absence of any long-range interaction, we could validly represent both of these proteins by equivalent hard spheres of radius r. However, if one of the proteins, say A, bears a significant net charge, then the interaction between two molecules of A will be more repulsive than the interaction between two molecules of B, which will be equal to the interaction between a molecule of A and one of B. The reader may readily verify that no values of r_A and r_B, effective or otherwise, will simultaneously satisfy these three relations in the context of a pure hard-particle model.

In the absence of a theoretical guide to the appropriate choice of effective particle size, it is commonly assumed that the effective molar volume is proportional to the molar mass of a macrosolute; the constant of proportionality is referred to as the effective specific volume or v (19, 101, 172). Uncertainty in v can easily be the largest source of error in crowding calculations. Past studies have variously assumed v values based upon partial specific volumes ($\sim$0.6–0.8 ml/g) or hydrodynamic volumes ($\sim$1.3 ml/g), although neither of these assumptions are correct in principle for excluded-volume interactions (92). We have estimated effective specific volume values for several proteins, for cell extracts, and for cytoplasmic macromolecules based upon a variety of experimental data (Table 1). The values range from 0.8 to 1.7 ml/g, in part a reflection of differences in experimental conditions. Values of v used in recent volume-occupancy calculations are 0.77 ml/g (19), and 1.0–1.3 ml/g (172).

Table 1 Effective specific volumes derived from experiment

Experimental material	Analysis	Effective specific volume, v (ml/g)[a]	Reference
Protein crystals	Unit cell volume per molecular mass	1.0[b]	86
	Bound water[c]	1.0	86
PEG-induced precipitates of proteins and cell extracts	Compositional analysis	1.0–1.4	172
Protein solutions	Fitting solution parameters to a hard-sphere model		
Hemoglobin			
pH 7.8[d]	Scaled particle theory	0.77	19
pH 7.0	Virial expansion	0.79	A. P. Minton[e]
Serum albumin			
pH 5.1	Virial expansion	0.8	100
pH 6.1	Virial expansion	1.3	100
pH 7.6	Virial expansion	1.7	100
Cells of *E. coli*	Cytoplasmic compositions as function of osmotic conditions	0.9	103
		1.1,[f] 1.4[g]	19
	Growth rate as function of osmotic conditions	1.2[h]	19

[a] To provide a uniform albeit approximate way of converting analytical information of various types to estimates of v, we assume a hard-sphere model, an average partial specific volume for proteins of 0.73 ml g. and an average partial specific volume for *E. coli* cytoplasmic macromolecules of 0.7 ml/g (corresponding to assumed protein and nucleic weight fractions in cytoplasm of 0.70 and 0.30 with partial specific volumes of 0.73 ml/g and 0.58 ml/g, respectively). For example, for 0.5 g of water of hydration per gram dry weight macromolecule, $v \approx (0.5 \text{ ml} + 0.7 \text{ ml})/1 \text{ g} = 1.2$ ml/g.

[b] Value of v for most common value of unit cell volume/molecular mass (V_M); range is approximately threefold in V_M values.

[c] Based on estimated average value for protein crystals of ~0.25 g bound water/g protein; definitions of bound water are discussed in Ref. 67.

[d] M. T. Record, Jr, personal communication.

[e] A. P. Minton, personal communication (cited in 35).

[f] Value of v based upon extrapolation to 0.4 ml cytoplasmic water/g dry weight at infinite external osmolarity.

[g] Based upon volume fraction in cytoplasm inaccessible to water, additional cell composition data, and assumptions for the magnitude of the noncytoplasmic volume fraction and the constancy of this fraction over a range of external NaCl concentrations.

[h] For an estimated value of $\sim 0.5 \pm 0.2$ ml cytoplasmic water/g cell dry weight at limiting conditions for growth.

EXPERIMENTAL MEASUREMENT OF THE EFFECT OF VOLUME OCCUPANCY ON THE THERMODYNAMIC ACTIVITY OF A TRACER PROTEIN

Solutions Containing a Single Macrosolute

A solution containing a single macrosolute is a special case of the solution containing a tracer plus a single background species; in this instance the tracer species is identical to the background species. The thermodynamic activity or the activity coefficient of a macromolecular solute may be determined as a function of concentration by measurements of membrane osmotic pressure, Rayleigh light scattering, or sedimentation equilibrium (148). The measured dependence of the logarithm of the thermodynamic activity of globular proteins upon protein concentration in solutions containing a single protein solute is well described over a wide concentration range by an expansion of the form of Equation 3, in which interaction coefficients are calculated according to a hard-particle model. Higher-order interaction terms become increasingly important as protein concentration increases, and at the highest protein concentrations for which activities have been measured, six or seven terms may be required (130, 132). Equivalent expressions derived from SPT also provide satisfactory descriptions (132). Under conditions of moderate ionic strength and at pH values such that the protein is not highly charged, the size of the effective hard particle is similar to that of the actual protein molecule (95, 132). Under conditions such that the protein bears substantial net charge, the size of the effective hard particle significantly exceeds that of the actual molecule. An approximate theory for the dependence of equivalent hard-particle size upon pH (or net charge) has been presented (100).

Solutions Containing a Tracer Protein and a Single Background Species

It was observed as early as the 1940s that viral particles and proteins could be precipitated out of solution by addition of water-soluble polymers such as heparin, hyaluronic acid, dextran, and polyethylene glycol (25, 61, 68). A series of studies carried out in the 1960s by Ogston, Laurent, and coworkers (28, 36, 68, 71, 111) led to the recognition that these and related effects resulted primarily from exclusion of the protein from part of the volume of the polymer solution.[8] More recently, the volume-excluding

[8] These early observations are the basis for a relatively direct assay for certain excluded volume parameters based upon the distribution coefficients of proteins, nucleic acid–related materials, and cell extracts in a two-phase system (167, 171, 172).

properties of proteins as well as polymers have become recognized (93–95).

PROTEIN IN THE PRESENCE OF A BACKGROUND POLYMER The interaction between proteins and polymers has been characterized experimentally by means of four methods. The first is the measurement of the equilibrium distribution, or partitioning, of a protein between a polymer-free medium (medium I) and a polymer-containing medium (medium II). If the protein is sufficiently dilute, then at equilibrium

$$\gamma^{II}_{protein} = c^{I}_{protein}/c^{II}_{protein}$$

to a good approximation. Experiments have been performed in which the two media were separated by a dialysis membrane (36), by phase immiscibility (in which case phase I may contain a relatively low amount of polymer) (64, 171), and by crosslinking the polymer to create an insoluble gel (13). By and large, the results of these experiments accord qualitatively with predictions of AVT for effective hard particles (171). Some polymers (e.g. dextran) are better modeled as rod-like particles (68, 95), while others [e.g. polyethylene glycol (PEG)] are satisfactorily modeled as effective spherical particles (171). It has been suggested on the basis of analysis using a lattice-statistical model for protein-polymer interaction that a small attractive interaction exists between at least some proteins and polymers (13).

A gel chromatographic method has been used to characterize the nonideal interaction between a small polymer (PEG 4000) and each of two proteins (156). This method has two advantages over dialysis equilibrium, to which it is thermodynamically equivalent: measurements are much more rapid, and the technique may be utilized even when the two solutes are not completely separable, so long as they partition sufficiently distinctly on a given size exclusion gel matrix.

A third method for the characterization of the interaction between proteins and polymers is the measurement of protein solubility as a function of the concentration of added polymer (9, 47, 57, 90). If it is assumed that the precipitate contains only protein and that the composition of the precipitate is independent of the concentration of polymer, then at equilibrium

$$d \ln \gamma_{protein}/dc_{polymer} = -d \ln s_{protein}/dc_{polymer},$$

where $s_{protein}$ denotes the solubility. Measurements of the effect of dextran and PEG upon the solubility of a variety of proteins indicate that, when the solubility of protein is small enough so that protein-protein interactions in solution are negligible, $\ln \gamma_{protein}$ increases linearly with polymer con-

centration over the entire experimentally measurable range of protein concentration, i.e. over as much as three orders of magnitude in solubility (9). For a fixed weight/volume concentration of dextran, the solubility of protein is independent of the molecular weight of polymer (68), as would be predicted by a rigid rod representation of polymer in AVT. However, for a fixed weight/volume concentration of PEG, the solubility decreases (and $\ln\gamma$ increases) with increasing molecular weight of PEG (9), in contradiction to the predictions of AVT, and in contradiction to the results of partition experiments (9). Anomalously small extents of exclusion calculated from the protein solubility data in the presence of PEG were attributed to the presence of some attractive interaction between proteins and PEG (95).

Knoll & Hermans (65) measured the excess light scattering of bovine serum albumin (BSA) in solutions of polyethylene glycol and from their data calculated the limiting value of $d\ln\gamma_{BSA}/dc_{PEG}$ as $c_{PEG} \to 0$ for PEG 1000, PEG 6000, PEG 20,000, and PEG 100,000. They found that $d\ln\gamma_{BSA}/dc_{PEG}$ decreased with increasing molecular weight of PEG, as predicted by AVT and by Hermans' polymer excluded-volume model (54).[9] Using radioactive tracer measurements, Knoll & Hermans measured the amount of PEG in the condensed phase and found substantial penetration of the condensed phase by smaller PEGs. From this finding, they argue that the assumption of constant protein activity in the condensed phase that is used to relate solubility to the protein activity coefficient (see above) is not valid for protein in the presence of PEG. Alternatively, the presence of PEG in the condensed phase may be evidence of nonspecific attractive interactions between protein and PEG.[10]

In general, three-body and higher-order interaction terms in the virial expansion (Equation 3) are not required to describe the dependence of the activity coefficient of protein on polymer concentration, even at high polymer concentration. The physical basis of this observation, which accords better with the predictions of AVT than those of SPT, is not entirely clear, as the assumption in AVT that background particles are distributed randomly is strictly valid only in the limit of massless particles, i.e. points or infinitely thin rods. The apparent ability of AVT to account for protein activity in the presence of relatively high concentrations of real polymers (but not in the presence of high concentrations of other proteins of comparable size), may possibly result from the difference between the

[9] The quantity $d\ln\gamma_{BSA}/dc_{PEG}$ is equal to the interaction factor B_{12} in Equation 3, where BSA is component 1 and PEG is component 2.

[10] Amounts of PEG in precipitates of proteins have been found to vary greatly with the salt composition of the supernatant fluid (appendix table in 172).

cross-sectional area of a typical globular protein and the cross-sectional area of a single polymeric chain; as the size of a protein increases, the approximation that polymer excludes volume to protein as would a random array of thin rods becomes increasingly realistic. By the same reasoning, one would expect that Equation 3, truncated after two-body terms, would become an increasingly realistic description of the dependence of the activity of a globular tracer species *i* in the presence of a large excess of a compact (i.e. not polymeric) background species *j* as the size of species *j* diminishes relative to that of species *i*. The results of several measurements of the effect of high concentrations of smaller solutes (such as sugars) on protein activity (141) seem to agree with this expectation.

PROTEIN IN THE PRESENCE OF A BACKGROUND PROTEIN The solubility of deoxygenated sickle hemoglobin (HbS) is reduced in the presence of other proteins that do not coprecipitate with the hemoglobin (14, 145). This effect has been interpreted in the context of excluded-volume models (133, 145). Also, excluded volume must be taken into account in order to quantitatively account for the effects on HbS solubility of other hemoglobin variants that coprecipitate with HbS to varying degrees (35, 91, 109, 145). In principle, the excluded volume interaction between proteins of two different species could be characterized quantitatively via measurement of the osmotic pressure or sedimentation equilibrium of protein mixtures (see e.g. 22, 71). However, we are unaware of published experimental studies of this kind.

EXPERIMENTALLY OBSERVED EFFECTS OF CROWDING ON EQUILIBRIA AND REACTION RATES IN MODEL SYSTEMS

We have summarized several observations of crowding effects on reaction equilibria in Table 2; Figure 4 shows an example (the effects of crowding on the equilibrium association of ribosomal particles). Similarly, we have summarized several reported effects of crowding upon reaction rates in Table 3; Figure 5 shows an example (the effects of crowding on the rate of enzyme-catalyzed exchange labeling of DNA termini). Certain entries in the tables are discussed below.

Extended Ranges of Enzymatic Activities Under Crowded Conditions

Crowding has been shown to greatly extend the range of solution conditions under which several enzymes or proteins are functional. The examples cited here all involve interactions with nucleic acids, probably

because of the large crowding effects on reactions between macromolecules. We anticipate that such effects are much more widespread.

DNA POLYMERASE AND DNA REPLICATION SYSTEMS The introduction of the use of hydrophilic polymers for in vitro DNA replication systems by Fuller et al (38) was an important development in the study of those and related systems (66; see references cited in Table 3). Fuller et al (38) suggested that the excluded-volume effects of the polymers might increase effective concentrations of macromolecular reactants, a prediction borne out by subsequent studies in which polymer requirements were removed by increasing the concentrations of reactants (62, 75).

In studies with isolated enzymes, synthetic activities and associated nuclease activities of DNA polymerases increased in a range of otherwise inhibitory salt concentrations (166). The increased activity seems to be a direct result of increased binding of polymerase to DNA. Crowding also enables DNA polymerase I of *E. coli* to remain active under a variety of otherwise inhibitory conditions (e.g. unfavorable pH or temperatures, inhibitory concentrations of urea, formamide, or ethidium bromide), leading to a general proposal of crowding as a source of homeostasis (169) (see below).

DNA LIGASES T4 DNA ligase and *E. coli* DNA ligase are both inhibited at higher salt concentrations under uncrowded conditions. In the presence of PEG, however, DNA ligation in these salt concentrations can be greatly stimulated (52, 53).

The temperature optimum of a DNA ligase from the thermophilic organism *Thermus thermophilus* is increased from 37°C in uncrowded media to a more normal temperature optimum for that organism (~55–65°C) in 20% PEG 6000 (147). Takahashi & Uchida (147) also note previous studies (53, 168) of DNA ligases from *E. coli* and rat liver that suggest crowding-induced shifts in temperature optima into temperature ranges characteristic for the organism.

recA PROTEIN Addition of PEG or polyvinyl alcohol (PVA) extended the functional range of a *recA* protein-promoted DNA strand–exchange system (73). Crowding allowed high rates of product formation at otherwise suboptimal Mg^{2+} concentrations and also approximately doubled the concentration of NaCl required for half-dissociation of *recA* protein from etheno M13 DNA (73).

Alteration of Reactants or Products Under Crowded Conditions

Enzymes of nucleic acid metabolism have provided several examples of changes in substrates or products under crowded conditions. These are discussed below.

Table 2 Crowding effects on equilibria

Observation	Crowding agent (background species)	Magnitude of effect	Comment	Reference
Reduction in solubility of deoxyhemoglobin S	Bovine serum albumin, crosslinked hemoglobin A	10-fold reduction in 250 g/liter protein	Excluded-volume interpretation in Ref. 133	14
Reduction in solubility of proteins	PEG, dextran			9, 47, 68, 68a
Self-association of apomyoglobin	Lysozyme, β-lactoglobulin, ribonuclease		Monomer in absence of added protein, mostly dimer in presence of >200 g/liter added protein	160
Stabilization of thrombin against thermal denaturation	Ribonuclease		Attributed to crowding-induced formation of heat-stable oligomers of thrombin	102
Thickening of fibrin fibrils	Bovine serum albumin	Twofold increase in birefringence at 70 g/liter albumin	Increased regularization of aggregate	154
Bundling of actin fibers	PEG			146

Lowering of critical concentration for actin fiber formation	PEG	Twofold in 50 g/liter PEG		33
	PEG	Threefold in 80 g/liter PEG		150
Self-association of pyruvate dehydrogenase	PEG	50–90% of ~22S species converted to ~55S species in 30 g/liter PEG	Excluded volume interpretation in Ref. 108	17
Enhancement of trypsin inhibition by bovine serum albumin	Dextran		Attributed to enhanced formation of enzymatically inactive trypsin-albumin complex	69
Enhancement of association of ribosomal particles	PEG, Dextran, Ficoll	>10-fold increase in equilibrium constants	Effects used to estimate particle volumes of polymers (cf 15)	170
Association of T4 gene 45 protein and gene 44/62 protein complex	PEG, Dextran	50-fold increase in association constant at 75 g/liter of PEG 12,000		59
Stabilization of double-stranded nucleic acids against thermal denaturation	PEG, Dextran	T_m of poly(dAT) increased ~10°C in 210 g/liter PEG 6000	Effect increases with increasing molecular weight of polymer	72
		T_m of poly(rI)·poly(rC) increased ~3°C in 90 g/liter PEG 20,000		164

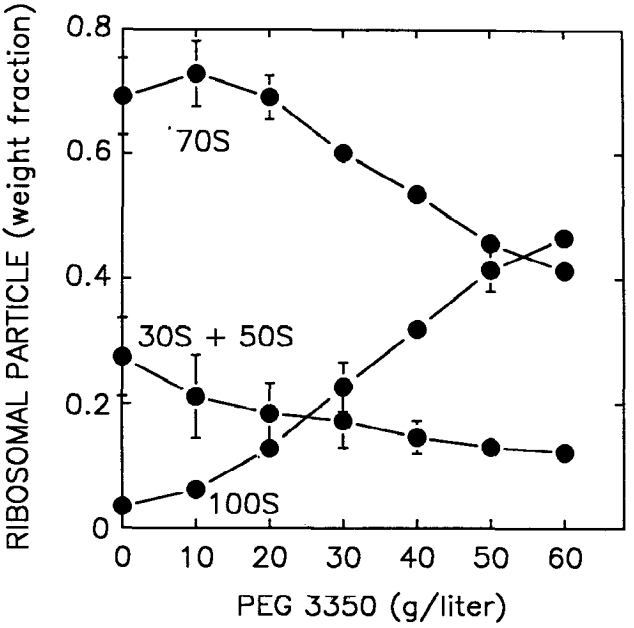

Figure 4 Effect of concentration of PEG 3350 on the equilibrium distribution of ribosomal species isolated from *E. coli* (based upon data in Figure 2 of Ref. 170).

ALTERED SUBSTRATE SPECIFICITY Absolute or relative substrate-specificity can be changed by crowding: (*a*) Blunt-end ligation by *E. coli* and rat-liver ligases was undetectable under normal assay conditions but became a strong reaction under crowded conditions (168), probably because of the condensation of the DNA under crowded conditions. (*b*) The relative rates of phosphorylation of blunt or recessed DNA termini by T4 polynucleotide kinase was increased under crowded conditions (49, 50).

ALTERED REACTION PRODUCTS Mixtures of cyclic and linear ligation products from both DNA ligases (118) and the T4 RNA ligase (48) shift strongly toward linear products under crowded conditions, which may reflect the increased thermodynamic activity of DNA termini under crowded conditions (cf 34). In agreement with such a mechanism, Sobczak & Duguet (143) obtained increases in intermolecular ligation by T4 DNA ligase resulting from an increase in the DNA concentration that are comparable to those obtained by crowding with PEG (see also 11).[11] The

[11] Lower molecular weight PEGs tend to favor circle formation in many of these ligation systems for unknown reasons.

similarities between the DNA ligases and T4 RNA ligase occur despite the difference in substrates (duplex DNA vs single-stranded DNA or RNA, respectively).

DECREASED SOLUBILITY OF NUCLEIC ACID COMPONENTS DNA (and perhaps RNA) can undergo intramolecular collapse in the presence of polymer and salt ("psi" DNA) (see 8, 77, 78). Such preparations are often readily sedimentable (48, 49, 118). It would be interesting to know how well the various salt and temperature effects on PEG stimulation of DNA (or RNA) ligases or *recA* protein (see above) correlate with the presence of psi DNA (or RNA), as measured, for example, by ready sedimentation of the DNA (or RNA).

EXPERIMENTALLY OBSERVED EFFECTS OF CROWDING UPON TRANSPORT PROPERTIES IN MODEL SYSTEMS

Viscosity of a Protein Solution

The viscosity of human hemoglobin has been measured as a function of concentration at concentrations up to 450 g/liter. The dependence of viscosity upon concentration is quantitatively accounted for by a semi-empirical equation (131) that was originally formulated to describe the concentration dependence of the viscosity of suspensions of rigid colloidal particles (105). This finding supports the appealingly simple notion that the effects of volume exclusion on both hydrodynamic and thermodynamic properties of solutions of compact globular proteins may be accounted for in a reasonably realistic fashion by models in which the protein molecules are treated as rigid particles of regular shape.

Self-Diffusion of Proteins in Membranes

Scalettar & Abney (140) recently reviewed this subject comprehensively. The concentration dependence of the two-dimensional self-diffusion of proteins in membranes, or irreversibly adsorbed onto membranes, has been measured by fluorescence recovery after photobleaching (117, 149, 153). The coefficient of self-diffusion decreases more strongly with increasing protein concentration than predicted by any model or simulation presuming only hard-particle interactions between diffusing molecules (140). However, one can model the experimental results by assuming that patches of the membrane surface are inaccessible to diffusing proteins, by assuming that there are immobile obstacles to surface diffusion, or by assuming the presence of long-range attractive or repulsive forces between diffusing proteins (136, 137, 139, 140). Apparently, the systems studied so

Table 3 Crowding effects on reaction rates

Observation	Crowding agent (background species)	Magnitude of effect	Comment	Reference
Reduction in specific activity of glyceraldehyde 3-phosphate dehydrogenase	Ribonuclease, β-lactoglobulin, bovine serum albumin	30-fold reduction in 300 g/liter protein	Attributed to crowding-induced formation of low-activity tetramer of high-activity subunits	101
Reduction in specific activity of hyaluronate lyase	PEG	Six- to eightfold reduction in 125 g/liter polymer		69
Acceleration of actin polymerization	PEG 6000	Threefold at 80 g/liter polymer		150
Acceleration of actin filament growth at barbed end	Dextran, PEG, ovalbumin	Threefold at 100 g/liter dextran		33
Acceleration of deoxyhemoglobin S polymerization	Hemoglobin F		Hemoglobin F does not copolymerize with hemoglobin S	145
Acceleration of fibrin gel formation	Bovine serum albumin, ovalbumin, hemoglobin, γ-globulin	Five- to sevenfold at 80 g/liter protein		159
	Bovine serum albumin	Five- to sixfold at 70 g/liter		154
Acceleration of DNA renaturation	PEG, dextran sulfate	50-fold at 175 g/liter dextran sulfate 500,000; 90-fold at 175 g/liter PEG 8000		142, 158a
Acceleration of cohesion of restriction fragments of lambda DNA	Bovine serum albumin, PEG, Ficoll	100-fold at 250 g/liter albumin; > 1000-fold at 150 g/liter PEG 8000		165
Acceleration of cohesion of lambda DNA	Bovine serum albumin, PEG, Ficoll			165
	PEG	~ 10-fold at 126 g/liter PEG 6000, 55°C	Rate approximates in vivo rate	79

Acceleration of DNA ligase reaction	PEG, bovine serum albumin, Ficoll	Orders of magnitude depending on system and polymer		4, 7, 48, 52, 53, 118, 123, 147, 151, 152, 168
Acceleration of enzymatic catenation of DNA circles	PEG, PVA	No measurable reaction in absence of crowding agent		80
Acceleration of enzymatic supercoiling of DNA by topoisomerase I of *Sulfolobus acidocaldarius*	PEG		Lack of reaction intermediates suggests processive mechanism in PEG solution	37
Acceleration of DNA replication system from *E. coli*	PEG, PVA, methyl cellulose	Absolute requirement for crowding agent at lower reactant concentrations (cf 62, 75)		38
Acceleration of in vitro replication system for λdv DNA	PEG	Eightfold increase at 60 g/liter PEG 20,000		155
Acceleration of in vitro transposition of bacteriophage Mu	PVA	155-fold increase at 50 g/liter PVA 49,000		104
Acceleration of in vitro replication of single-stranded DNA	PVA	Fourfold increase at 50 g/liter PVA 24,000		74
Acceleration of nuclease and polymerase activities of DNA polymerase	PEG, Ficoll, dextran, bovine serum albumin	Large stimulations of rate under variety of inhibitory conditions	Attributed to increased binding of enzyme to DNA under otherwise dissociating conditions	166, 169
Acceleration of T4 polynucleotide kinase	PEG, glycogen, Ficoll 70	Orders of magnitude stimulation depending on DNA substrate and polymer	Stimulates both forward and back reactions; attributed to stabilization of oligomeric form of enzyme	49, 50
Acceleration of *recA*-protein promoted DNA strand exchange at low Mg^{2+} concentration	PEG, PVA	Stimulation depends upon product type and reaction conditions	Reduction in Mg^{2+} requirement under crowded conditions	73

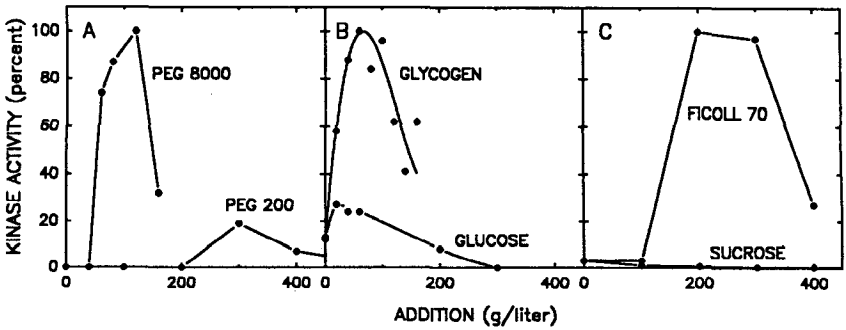

Figure 5 Effects of macromolecules and low molecular weight derivatives on the rate of exchange labeling by T4 polynucleotide kinase of recessed DNA termini (redrawn from Ref. 50).

far do not provide a satisfactory experimental model for the study of pure crowding effects.

Self-Diffusion of Proteins in Aqueous Solution

The concentration dependence of three-dimensional self-diffusion of proteins in aqueous solution has been measured with classical methods (reviewed in 46). Results obtained for several proteins are plotted in Figure 6, together with curves calculated using a semiempirical free-volume model (106) with best-fit values of parameters given in the figure caption. The self-diffusion coefficient typically decreases by a factor of 10 as the protein concentration increases to $\sim$300 g/liter.

Tracer Diffusion of a Protein in Solutions of a Second Protein

The diffusion of trace amounts of myoglobin, hemoglobin, and chromophorically labeled bovine serum albumin and aldolase has been measured as a function of the concentration of each of several different background proteins (ribonuclease, ovalbumin, serum albumin, aldolase) at concentrations up to 200 g/liter (106). For all proteins, D^{tr} decreases with increasing weight/volume concentration of background protein. Plots of D^{tr} vs the concentration of background protein resemble the data plotted in Figure 6. For three of the four proteins, the dependence of D^{tr} upon background-protein concentration becomes stronger as the molecular weight of the background protein decreases, as predicted by the free-volume model for tracer diffusion (106). Extrapolation of the data to higher concentrations using the free-volume model suggests that the tracer diffusion coefficient of large proteins could be reduced by two or more orders of magnitude in solutions with $\phi > 0.3$.

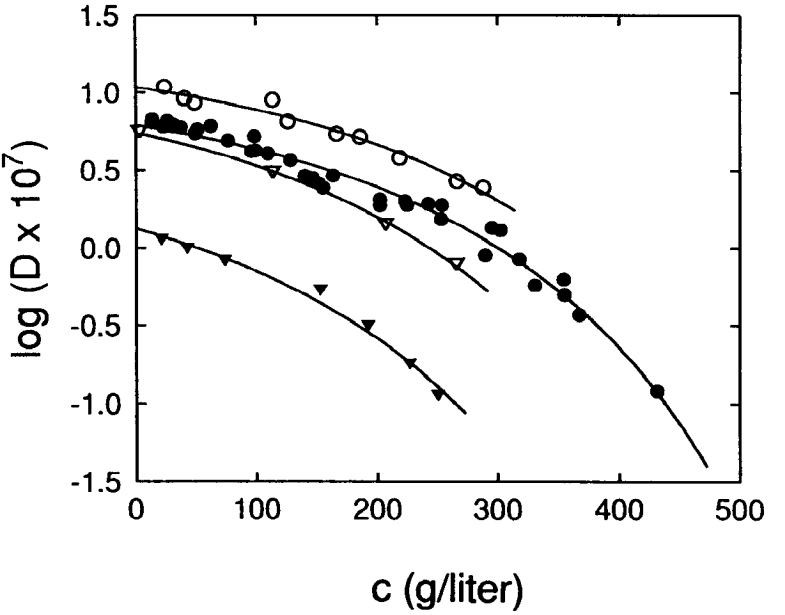

Figure 6 Self-diffusion coefficient of globular proteins plotted as a function of protein concentration. Symbols represent the combined data of several authors, summarized by Gros (46). The curves are best-fits of Equation 6 of Muramatsu & Minton (106) to each of the data sets, with v assumed equal to 0.8 ml/g for all proteins. The values of best-fit parameters for each protein are as follows: Myoglobin (*open circles*), log $(D_0 \times 10^7) = 1.04$, $\Delta r' = 4.34 \times 10^{-8}$ cm. Hemoglobin (*closed circles*), log $(D_0 \times 10^7) = 0.792$, $\Delta r' = 7.26 \times 10^{-8}$. Ovalbumin (*open triangles*), log $(D_0 \times 10^7) = 0.742$, $\Delta r' = 8.36 \times 10^{-8}$ cm. Invertebrate hemoglobin (*closed triangles*), log $(D_0 \times 10^7) = 0.126$, $\Delta r' = 4.54 \times 10^{-7}$ cm.

Tracer Diffusion of Probe Molecules in Polymers

The diffusion of compact tracer particles (proteins and latex spheres) in random coil polymers has been studied using boundary spreading (70, 121), inelastic laser light scattering (reviewed in 119), and fluorescence recovery after photobleaching (FRAP) (44). The diffusion coefficient of a dilute probe particle in a particular polymer solution may be described to within experimental error by an empirical relation

$$D/D^\circ = \exp(\alpha \cdot c^\nu \cdot R^\delta \cdot M^\gamma),$$

where D° is the diffusion coefficient of the probe in the absence of polymer,

c is the weight/volume concentration of polymer, R is the radius of the probe particle, M is the molecular weight of polymer, and α, ν, δ, and γ are fitting parameters. The parameter α increases with the net electrostatic charge of polymer (44). According to Phillies et al (119), inelastic light-scattering measurements for a broad range of probe-polymer combinations yield ν in the range 0.6–1.0, $\gamma = 0.7$–0.9, and $\delta \approx 0$. However, other techniques and other probe-polymer combinations have yielded ν as approximately 0.5 and δ significantly greater than zero (44, 70, 121). No single theory can account for these varied results. However, all studies reveal a large retarding effect of the polymer; the diffusion coefficient of a protein may be reduced by one or two orders of magnitude at polymer concentrations less than 100 g/liter.

CROWDING IN CYTOPLASM

Experimental Approaches to the Calculation of Volume Exclusion in Cytoplasm

Two groups (19, 20, 172) recently estimated the effect of volume occupancy upon the thermodynamic activity of a globular protein in the cytoplasm of *E. coli*. The two approaches are similar in outline, both starting with molecular weight distributions representing the cytoplasmic complement of macromolecules of *E. coli* and estimated or measured values for the actual cytoplasmic concentration of those macromolecules. The molecular-weight distributions are then converted to molecular-volume distributions by application of values for effective specific volumes (see above) and the molecular-volume distributions are finally expressed at cytoplasmic macromolecule concentrations to obtain a working description of the cytoplasmic background macromolecules for use in SPT calculations of activity coefficients. The two studies differ at each stage in the way parameters are chosen and the extent to which parameters are experimentally based.

Rationalization of Discrepancies Between Phenomena Observed in Vitro and in Vivo

Binding of the *lac* repressor to the *lac* operator (10, 127, 162) and RNA polymerase binding to the λP_R promoter (128, 129) are both exquisitely sensitive to salt inhibition in vitro, yet Richey et al (126) found no significant salt effect on either system in vivo. Both Cayley et al (19) and Zimmerman & Trach (172) agree that crowding is one of the keys to resolving the apparent discrepancy, but their respective analyses differ in the type of data on which their respective conclusions are based and in the

emphasis placed upon the contributions from various factors, as described below.

Zimmerman & Trach (172) calculate activity coefficients for test particles of arbitrary size under anticipated cytoplasmic crowding conditions over a range of values of the effective specific volume parameter. Application of these activity coefficients to parameters characterizing the dilute solution behavior of the *lac* repressor-operator system resulted in calculated trends of *lac* operator function that agree in general with the results of Richey et al (126) in vivo: crowding greatly decreases the expected salt dependence of *lac* operator function. According to this interpretation, crowding effects in vivo at any external osmolarity cause very large increases in the affinity of *lac* repressor for both specific and nonspecific binding sites in DNA. Hence under crowded conditions, an increase in internal salt concentration caused by shifts in external salt levels will not cause significant dissociation of *lac* repressor from DNA binding sites—even though such salt concentrations readily cause dissociation in dilute solution conditions in vitro. If essentially all protein is bound, then the expression of the *lac* operator is largely controlled by the (salt-independent) ratio of specific to nonspecific DNA sites.

The interpretation of Cayley et al (19) is based upon the decreased cytoplasmic volumes they observe as extracellular salt concentrations are raised; decreased cytoplasmic volumes lead to increased concentrations of cytoplasmic macromolecules and hence increased crowding effects. These increased crowding effects are suggested to balance the protein-dissociating effects of the increased salt, resulting in approximate salt independence in vivo. They estimate changes in the activity coefficient of RNA polymerase of at least two orders of magnitude in response to an increase in (RNA + protein) of 275 to 440 mg/ml cytoplasm, yielding final values consistent with the salt independence of the RNA polymerase–λP_R promoter interactions observed by Richey et al (126).

Diffusion of Probe Molecules in Cytoplasm

Mastro, Keith, and coworkers (84, 85) measured the translational and rotational diffusion of a small spin probe in the cytoplasm of mammalian cells by measuring electron spin resonance. They found that the translational mobility was about half that in water but was greatly retarded upon cell shrinkage in hypertonic media, whereas rotational mobility was only slightly affected, suggesting that the spin probe was distributed within compartments in the cytoplasm. The mobility of the spin probe within a given compartment is thought to be similar to that in a dilute aqueous solution, but translational diffusion between compartments is hindered by structural elements of the cytomatrix. The extent of hindrance, and thus

the overall long-range translational diffusion of the probe, is likely to reflect alterations in cytomatrix structure. Jacobson & Wojcieszyn (58) measured the apparent diffusion coefficients of several fluorescently labeled proteins in fibroblast cytoplasm via FRAP. No obvious dependence of the rate of diffusion upon the size of the protein was observed, leading them to suggest that associations with structural components of the cytomatrix, rather than hindrance due to volume exclusion, were the main factors regulating protein diffusion. Luby-Phelps, Lanni, and coworkers (56, 81, 82) have measured the diffusion of size-fractionated fluorophore-labeled Ficoll in the cytoplasm of mammalian cells by using FRAP. The diffusion coefficient of Ficoll in cytoplasm decreased with increasing polymer size in a fashion that could not be unambiguously interpreted in the context of any simple theoretical model, but which could be mimicked by the diffusion of Ficoll in mixtures of F-actin and concentrated unlabeled Ficoll or bovine serum albumin. On the basis of these findings, they propose that the cytoplasm may be described as a network of entangled fibers interpenetrated by a fluid phase containing a high concentration of soluble proteins.

RAMIFICATIONS, SPECULATIONS, CONCLUSIONS

Possible Role of Crowding in Regulation of Cellular Volume

In a variety of eukaryotic cell types, small changes in cellular volume can result in the activation of compensatory mechanisms that restore the original volume (135). Volume changes of a few percent reportedly can result in disproportionately large increases in ion fluxes (26, 60), prompting questions regarding the nature of the volume-change signal and mechanisms of response (30, 60). Recent studies demonstrated that in resealed dog red cell ghosts containing protein mixtures, activation of swelling and shrinkage-activated membrane ion transporters correlates with concentration of total cytoplasmic protein, as opposed to cell volume per se, or the concentration of any particular cytoplasmic protein constituent (26, 27). Because of macromolecular crowding in cytoplasm, small changes in volume (i.e. total macromolecular concentration) result in large changes in the thermodynamic activity of all macromolecular species, dilute as well as concentrated, and changes in the thermodynamic activity of one or more soluble regulatory proteins could trigger activation of compensatory mechanisms (26; also suggested in 166). Quantitative models for swelling-activated transporter-mediated ion flux embodying this notion were proposed recently (98a, 99).

Efficacy of Macromolecular Drugs

The binding of macromolecular ligands to their complementary sites is affected by the volume occupancy of the surrounding medium (94, 95, 98a,

99). Apparent binding constants may be altered by one or more orders of magnitude. Thus, we believe that the screening of potential macromolecular drugs or any other macromolecular species for pharmacological activity should take place under conditions that mimic the crowding effects of the physiological medium in which ligand binding would actually occur. Surrogate crowding solutions that mimic estimated prokaryotic cytoplasmic backgrounds may be useful adjuncts (172).

Synthesis by Degradative Enzymes

Given the enhanced tendency of macromolecules to associate to form compact complexes in crowded media, it seems probable that at least some enzymes that catalyze degradative reactions such as limited proteolysis in vitro (i.e. in dilute solution) might actually catalyze the reverse reaction in vivo, and thus function primarily as synthetic rather than degradative enzymes.

Macromolecular Crowding as a Laboratory Tool

Inert volume-occupying macromolecules such as PEG have been added to increase the efficiency of common laboratory procedures involving nucleic acids, such as labeling DNA termini, ligation of DNA fragments, and renaturing of DNA (134, 142).

Osmotic Remedial Mutants

Auxotrophic or temperature-sensitive mutants of prokaryotes commonly become functional in media of high osmolarity (31, 51). Cayley et al (19) showed that the steady-state cytoplasmic volume of growing cells decreased with increasing external osmolarity. They proposed that this enhances intracellular crowding effects at high osmolarity. Resulting shifts in intracellular equilibria and reaction rates could potentially compensate for a variety of mutational defects.

Crowding as a Factor in Cellular Evolution

Living cells have high total macromolecular content—we can think of no exceptions to this statement. Does this mean that macromolecular crowding is essential to life? Life is thought to have originated in a primordial soup of prebiotic organic molecules; was that soup crowded? Reversible interactions that evolved in a crowded soup—or in a living system—would be characterized by affinities that are no stronger than they need to be in order to confer function in the crowded environment. If the cell is lysed and total macrosolute concentration significantly reduced, the resulting reduction in thermodynamic activities would lead to the dissolution of

some relatively weak interactions that are present in the intact cell (88) and the loss of high-level structural information.

Berg (15) has suggested that intracellular crowding may act as an evolutionary force tending to bias conformational equilibria toward compact conformations. In this context, we note that native structures of proteins, ribosomes, and other intracellular moieties tend to be compact; even DNA, perhaps the most intrinsically anisometric of all biological materials, tends to occur in highly compact structures such as nucleoids or nucleosomes (157), and may even function in a condensed conformation (142).

Crowding Versus Confinement

In the present review, the term crowding has been used to denote the exclusion of solution volume to macrosolute molecules by other macrosolute molecules. Solution volume may also be excluded to macrosolutes by stationary structural elements, such as membranes and fiber lattices, that restrict the translational and rotational freedom of soluble species. The cytoplasm of most eukaryotic cells contains a significant volume fraction of immobile[12] structural elements of various types, collectively referred to as the cytomatrix (40). A statistical-thermodynamic theory of the thermodynamic activity of rigid particles in confined spaces (42) was recently used to calculate the effects of confinement on various types of reaction equilibria (98). Confinement was found to substantially affect thermodynamic activity of a particular solute species when the characteristic spacing between confining boundaries became smaller than about three times the maximum dimension of the confined particle.

If confinement is uniform throughout the fluid, then confinement enhances association equilibria. Unlike crowding, the extent to which confinement affects associations depends substantially upon the relationship between the shape of the aggregate and the shape of the confining boundaries. If confinement is nonuniform, then solutes (and aggregates) will partition so that larger particles will be preferentially found in larger spaces.

Homeostasis or Metabolic Buffering

The increased binding often caused by crowding may be a global tendency in cells that offsets a variety of perturbing influences, such as increased salt levels, drug binding, or pH changes (166, 169). This projected homeostatic tendency, termed metabolic buffering, could in principle apply to equi-

[12] That is, the structural elements are immobile on the time scale of translational motions of individual molecules of macrosolute.

librium and kinetic aspects of isomerization reactions, associations in solution, phase equilibria, and binding of soluble ligands to surface sites. A quantitative example has been presented (172).

Crowding and the Functional Importance of Condensed DNA

Sikorav & Church (142) demonstrated that factors such as the addition of volume-occupying polymers that favor the formation of condensed DNA (8, 77, 78) greatly accelerate the rate of renaturation of single-stranded DNA and can also support a protein-free DNA strand–exchange reaction. They suggest that the process of matching complementary base-pair sequences is more efficient in the condensed phase than in solution, and that condensed rather than extended DNA may be the physiologically relevant structure.

Crowding and Cytoplasmic Structure

The two major qualitative manifestations of crowding are enhancement of macromolecular associations and hindrance of macromolecular diffusion. Assuming that most of the cytoplasm is characterized by a fractional volume occupancy that is at least as great as the whole-cell average value, we speculate that a typical mature, functioning protein molecule within the cytoplasm is likely to exist as part of a complex rather than as a free-floating moiety, and consequently would have a greatly reduced rate of long-range translational diffusion (cf 24, 63). Because diffusion of small molecules is affected only slightly by macromolecular crowding, metabolism would therefore be expected to proceed predominantly via the diffusion of small-molecule metabolites between the various active sites of relatively immobile enzymes catalyzing sequential reactions. While these considerations do not necessarily mandate the formation of ordered complexes of enzymes catalyzing successive metabolic reactions, such as the metabolons proposed by Srere (144), they do render such complexes teleologically attractive: if proteins are to be complexed (as favored by crowding), would not evolution select for the formation of ordered complexes that could speed metabolic throughput and enhance control mechanisms (144) over the formation of random, functionally neutral complexes?

ACKNOWLEDGMENTS

We thank Philip Ross, NIH, and M. Thomas Record, Jr., University of Wisconsin, for helpful comments on a preliminary draft of this review, and Lizabeth Murphy, NIH, for assistance in its preparation.

Literature Cited

1. Abney, J. R., Scalettar, B. A., Owicki, J. C. 1989. *Biophys. J.* 55: 817–33
2. Adair, G. S. 1928. *Proc. R. Soc. London Ser. A* 120: 573–603
3. Altman, P. L. 1961. *Blood and Other Body Fluids*. Washington, DC: FASEB
4. Aoufouchi, S., Hardy, S., Prigent, C., Philippe, M., Thiebaud, P. 1991. *Nucleic Acids Res.* 19: 4395–98
5. Aragón, J. J., Sols, A. 1991 *FASEB J.* 5: 2945–50
6. Arakawa, T., Timasheff, S. N. 1985. *Biochemistry* 24: 6756–62
7. Arrand, J. E., Willis, A. E., Goldsmith, I., Lindahl, T. 1986. *J. Biol. Chem.* 261: 9079–82
8. Auer, C. 1978. *The influence of the ionic environment on the structure of DNA*. PhD thesis. Vanderbilt Univ., Nashville, Tenn. 323 pp.
9. Atha, D. H., Ingham, K. C. 1981. *J. Biol. Chem.* 256: 12108–17
10. Barkley, M. D. 1981. *Biochemistry* 20: 3833–42
11. Barringer, K. J., Orgel, L., Wahl, G., Gingeras, T. R. 1990. *Gene* 89: 117–22
12. Baskir, J. N., Hatton, T. A., Suter, U. W. 1987. *Macromolecules* 20: 1300–11
13. Baskir, J. N., Hatton, T. A., Suter, U. W. 1989. *J. Phys. Chem.* 93: 2111–22
14. Behe, M. J., Englander, S. W. 1978. *Biophys. J.* 23: 129–45
15. Berg, O. G. 1990. *Biopolymers* 30: 1027–37
16. Berg, O. G., von Hippel, P. H. 1985. *Annu. Rev. Biophys. Biophys. Chem.* 14: 131–60
17. Bosma, H. J., Voordouw, G., De Kok, A., Veeger, C. 1980. *FEBS Lett.* 120: 179–82
18. Brenner, S. L. 1976. *J. Phys. Chem.* 80: 1473–77
19. Cayley, S., Lewis, B. A., Guttman, H. J., Record, M. T. Jr. 1991. *J. Mol. Biol.* 222: 281–300
20. Cayley, S., Lewis, B. A., Record, M. T. Jr. 1992. *J. Bacteriol.* 174: 1586–95
21. Chatelier, R. C., Minton, A. P. 1987. *Biopolymers* 26: 507–24
22. Chatelier, R. C., Minton, A. P. 1987. *Biopolymers* 26: 1097–1113
23. Chun, P. W., Thornby, J. I., Saw, J. G. 1969. *Biophys. J.* 9: 163–72
24. Clegg, J. S. 1984. *Am. J. Physiol.* 246: R133–51
25. Cohen, S. S. 1942. *J. Biol. Chem.* 144: 353–62
26. Colclasure, G. C., Parker, J. C. 1991. *J. Gen. Physiol.* 98: 881–92
27. Colclasure, G. C., Parker, J. C. 1992. *J. Gen. Physiol.* 100: 1–11
28. Comper, W. D., Laurent, T. C. 1978. *Biochem. J.* 175: 703–8
29. Comper, W. D., Laurent, T. C. 1978. *Physiol. Rev.* 58: 255–315
30. Cossins, A. R. 1991. *Nature* 352: 667–68
31. Csonka, L. N. 1989. *Microbiol. Rev.* 53: 121–47
32. Doi, M. 1975. *J. Chem. Soc. Faraday Trans.* 2 71: 1720–29
33. Drenckhahn, D., Pollard, T. D. 1986. *J. Biol. Chem.* 261: 12754–58
34. Dugaiczyk, A., Boyer, H. W., Goodman, H. M. 1975. *J. Mol. Biol.* 96: 171–84
35. Eaton, W. A., Hofrichter, J. 1990. *Adv. Protein Chem.* 40: 63–279
36. Edmond, E., Ogston, A. G. 1968. *Biochem. J.* 109: 569–76
37. Forterre, P., Mirambeau, G., Jaxel, C., Nadal, M., Duguet, M. 1985. *EMBO J.* 4: 2123–28
38. Fuller, R. S., Kaguni, J. M., Kornberg, A. 1981. *Proc. Natl. Acad. Sci. USA* 78: 7370–74
39. Fulton, A. B. 1982. *Cell* 30: 345–47
40. Gershon, N. D., Porter, K. R., Trus, B. L. 1985. *Proc. Natl. Acad. Sci. USA* 82: 5030–34
41. Gibbons, R. M. 1969. *Mol. Phys.* 17: 81–86
42. Giddings, J. C., Kucera, E., Russell, C. P., Myers, M. N. 1968. *J. Phys. Chem.* 72: 4397–4408
43. Goodsell, D. S. 1991. *Trends Biochem. Sci.* 16: 203–6
44. Gorti, S., Ware, B. R. 1985. *J. Chem. Phys.* 83: 6449–56
45. Grasberger, B., Minton, A. P., DeLisi, C., Metzger, H. 1986. *Proc. Natl. Acad. Sci. USA* 83: 6258–62
46. Gros, G. 1978. *Biophys. J.* 22: 453–68
47. Haire, R. N., Tisel, W. A., White, J. G., Rosenberg, A. 1984. *Biopolymers* 23: 2761–79
48. Harrison, B., Zimmerman, S. B. 1984. *Nucleic Acids Res.* 12: 8235–51
49. Harrison, B., Zimmerman, S. B. 1986. *Anal. Biochem.* 158: 307–15
50. Harrison, B., Zimmerman, S. B. 1986. *Nucleic Acids Res.* 14: 1863–70
51. Hawthorne, D. C., Friis, J. 1964. *Genetics* 50: 829–39
52. Hayashi, K., Nakazawa, M., Ishizaki, Y., Hiraoka, N., Obayashi, A. 1985. *Nucleic Acids Res.* 13: 7979–92
53. Hayashi, K., Nakazawa, M., Ishizaki, Y., Obayashi, A. 1985. *Nucleic Acids Res.* 13: 3261–71
54. Hermans, J. 1982. *J. Chem. Phys.* 77: 2193–2203
55. Hill, T. L. 1960. In *An Introduction to*

Statistical Thermodynamics, pp. 340–70. Reading, MA: Addison-Wesley
56. Hou L., Lanni, F., Luby-Phelps, K. 1990. *Biophys. J.* 58: 31–43
57. Ingham, K. C. 1978. *Arch. Biochem. Biophys.* 186: 106–13
58. Jacobson, K., Wojcieszyn, J. 1984. *Proc. Natl. Acad. Sci. USA* 81: 6747–51
59. Jarvis, T. C., Ring, D. M., Daube, S. S., von Hippel, P. H. 1990. *J. Biol. Chem.* 265: 15160–67
60. Jennings, M. L., Schulz, R. K. 1990. *Am. J. Physiol.* 259: C960–67
61. Juckes, I. R. M. 1971. *Biochim. Biophys. Acta* 229: 535–46
62. Kaguni, J. M., Kornberg, A. 1984. *Cell* 38: 183–90
63. Kempner, E. S., Miller, J. H. 1968. *Exp. Cell Res.* 51: 150–56
64. Kim, C. W. 1986. *Interfacial condensation of biologicals in aqueous two-phase systems*. PhD thesis. MIT, Cambridge, Mass. 291 pp.
65. Knoll, D., Hermans, J. 1983. *J. Biol. Chem.* 258: 5710–15
66. Kornberg, A., Baker, T. A. 1992. In *DNA Replication*, pp. 314, 485, 525. New York: Freeman. 2nd ed.
67. Kuntz, I. D. Jr., Kauzmann, W. 1974. *Adv. Protein Chem.* 28: 239–345
68. Laurent, T. C. 1963. *Biochem. J.* 89: 253–57
68a. Laurent, T. C. 1963. *Acta Chem. Scand.* 17: 2664–68
69. Laurent, T. C. 1971. *Eur. J. Biochem.* 21: 498–506
70. Laurent, T. C., Björk, I., Pietruszkiewicz, A., Persson, H. 1963. *Biochim. Biophys. Acta* 78: 351–59
71. Laurent, T. C., Ogston, A. G. 1963. *Biochem. J.* 89: 249–53
72. Laurent, T. C., Preston, B. N., Carlsson, B. 1974. *Eur. J. Biochem.* 43: 231–35
73. Lavery, P. E., Kowalczykowski, S. C. 1992. *J. Biol. Chem.* 267: 9307–14
74. LeBowitz, J. H., McMacken, R. 1984. *Nucleic Acids Res.* 12: 3069–88
75. LeBowitz, J. H., Zylicz, M., Georgopoulos, C., McMacken, R. 1985. *Proc. Natl. Acad. Sci. USA* 82: 3988–92
76. Lebowitz, J. L., Helfand, E., Praestgaard, E. 1965. *J. Chem. Phys.* 43: 774–79
77. Lerman, L. S. 1971. *Proc. Natl. Acad. Sci. USA* 68: 1886–90
78. Lerman, L. S. 1973. In *Physico-Chemical Properties of Nucleic Acids*, ed. J. Duchesne, 3: 59–76. New York: Academic
79. Louie, D., Serwer, P. 1991. *Nucleic Acids Res.* 19: 3047–54
80. Low, R. L., Kaguni, J. M., Kornberg, A. 1984. *J. Biol. Chem.* 259: 4576–81
81. Luby-Phelps, K., Castle, P. E., Taylor, D. L., Lanni, F. 1987. *Proc. Natl. Acad. Sci. USA* 84: 4910–13
82. Luby-Phelps, K., Lanni, F., Taylor, D. L. 1988. *Annu. Rev. Biophys. Biophys. Chem.* 17: 369–96
83. Madden, T. L., Herzfeld, J. 1992. *Mater. Res. Soc. Symp. Proc.* 248: 95–100
84. Mastro, A. M., Babich, M. A., Taylor, W. D., Keith, A. D. 1984. *Proc. Natl. Acad. Sci. USA* 81: 3414–18
85. Mastro, A. M., Keith, A. D. 1984. *J. Cell Biol.* 99: 180s–87s
86. Matthews, B. W. 1977. In *The Proteins*, ed. H. Neurath, R. L. Hill, 3: 403–590. New York: Academic. 3rd ed.
87. Mazur, P. 1987. *Faraday Discuss. Chem. Soc.* 83: 33–46
88. McConkey, E. H. 1982. *Proc. Natl. Acad. Sci USA* 79: 3236–40
89. McMillan, W. G. Jr., Mayer, J. E. 1945. *J. Chem. Phys.* 13: 276–305
90. Middaugh, C. R., Tisel, W. A., Haire, R. N., Rosenberg, A. 1979. *J. Biol. Chem.* 254: 367–70
91. Minton, A. P. 1977. *J. Mol. Biol.* 110: 89–103
92. Minton, A. P. 1980. *Biophys. Chem.* 12: 271–77
93. Minton, A. P. 1980. *Biophys. J.* 32: 77–79
94. Minton, A. P. 1981. *Biopolymers* 20: 2093–2120
95. Minton, A. P. 1983. *Mol. Cell. Biochem.* 55: 119–40
96. Minton, A. P. 1989. *Biophys. J.* 55: 805–8
97. Minton, A. P. 1990. *Int. J. Biochem.* 22: 1063–67
98. Minton, A. P. 1992. *Biophys. J.* 63: 1090–1100
98a. Minton, A. P. 1993. In *Cellular and Molecular Physiology of Cell Volume Regulation*, ed. K. Strange. Boca Raton, FL: CRC. In press
99. Minton, A. P., Colclasure, G. C., Parker, J. C. 1992. *Proc. Natl. Acad. Sci. USA* In press
100. Minton, A. P., Edelhoch, H. 1982. *Biopolymers* 21: 451–58
101. Minton, A. P., Wilf, J. 1981. *Biochemistry* 20: 4821–26
102. Minton, K. W., Karmin, P., Hahn, G. M., Minton, A. P. 1982. *Proc. Natl. Acad. Sci. USA* 79: 7107–11
103. Mitchell, P., Moyle, J. 1956. In *Bacterial Anatomy*, *6th Symp. Soc. General Microbiology*, pp. 150–80. Cambridge, UK: Cambridge Univ. Press
104. Mizuuchi, K. 1983. *Cell* 35: 785–94
105. Mooney, M. 1951. *J. Colloid Sci.* 6: 162–70

106. Muramatsu, N., Minton, A. P. 1988. *Proc. Natl. Acad. Sci. USA* 85: 2984–88
107. Nichol, L. W., Jeffrey, P. D., Winzor, D. J. 1976. *J. Phys. Chem.* 80: 648–49
108. Nichol, L. W., Ogston, A. G., Wills, P. R. 1981. *FEBS Lett.* 126: 18–20
109. Noguchi, C. T., Schechter, A. N. 1985. *Annu. Rev. Biophys. Biophys. Chem.* 14: 239–63
110. Ogston, A. G. 1958. *Trans. Faraday Soc.* 54: 1754–57
111. Ogston, A. G., Phelps, C. F. 1961. *Biochem. J.* 78: 827–33
112. Ogston, A. G., Preston, B. N., Wells, J. D. 1973. *Proc. R. Soc. London Ser. A* 333: 297–316
113. Ogston, A. G., Winzor, D. J. 1975. *J. Phys. Chem.* 79: 2496–500
114. O'Leary, T. J. 1987. *Biophys. J.* 52: 137–39
115. Ohtsuki, K. 1982. *Physica A* 110: 606–16
116. Ovadi, J., Batke, J., Bartha, F., Keleti, T. 1979. *Arch. Biochem. Biophys.* 193: 28–33
117. Peters, R., Cherry, R. J. 1982. *Proc. Natl. Acad. Sci. USA* 79: 4317–21
118. Pheiffer, B. H., Zimmerman, S. B. 1983. *Nucleic Acids Res.* 11: 7853–71
119. Phillies, G. D. J., Ullman, G. S., Ullman, K., Lin, T.-H. 1985. *J. Chem. Phys.* 82: 5242–46
120. Pink, D. A. 1985. *Biochim. Biophys. Acta* 818: 200–4
121. Preston, B. N., Snowden, J. M. 1973. *Proc. R. Soc. London Ser. A* 333: 311–13
122. Pusey, P. N., Tough, R. J. A. 1983. *Faraday Discuss. Chem. Soc.* 76: 123–36
123. Rabin, B. A., Chase, J. W. 1987. *J. Biol. Chem.* 262: 14105–11
124. Ralston, G. B. 1990. *J. Chem. Educ.* 67: 857–60
125. Reiss, H. 1965. *Adv. Chem. Phys.* 9: 1–84
126. Richey, B., Cayley, D. S., Mossing, M. C., Kolka, C., Anderson, C. F., et al. 1987. *J. Biol. Chem.* 262: 7157–64
127. Riggs, A. D., Bourgeois, S., Cohn, M. 1970. *J. Mol. Biol.* 53: 401–17
128. Roe, J.-H., Burgess, R. R., Record, M. T. Jr. 1984. *J. Mol. Biol.* 176: 495–521
129. Roe, J.-H., Record, M. T. Jr. 1985. *Biochemistry* 24: 4721–26
130. Ross, P. D., Briehl, R. W., Minton, A. P. 1978. *Biopolymers* 17: 2285–88
131. Ross, P. D., Minton, A. P. 1977. *Biochem. Biophys. Res. Commun.* 76: 971–76
132. Ross, P. D., Minton, A. P. 1977. *J. Mol. Biol.* 112: 437–52
133. Ross, P. D., Minton, A. P. 1979. *Biochem. Biophys. Res. Commun.* 88: 1308–14
134. Sambrook, J., Fritsch, E. F., Maniatis, T. 1989. In *Molecular Cloning: A Laboratory Manual*, 1: 1.70–1.71, 4.35. Cold Spring Harbor, NY: Cold Spring Harbor Lab. 2nd ed.
135. Sarkadi, B., Parker, J. C. 1991. *Biochim. Biophys. Acta* 1071: 402–27
136. Saxton, M. J. 1982. *Biophys. J.* 39: 165–73
137. Saxton, M. J. 1987. *Biophys. J.* 52: 989–97
138. Saxton, M. J. 1989. *Biophys. J.* 56: 615–22
139. Saxton, M. J. 1990. *Biophys. J.* 58: 1303–6
140. Scalettar, B. A., Abney, J. R. 1991. *Comments Mol. Cell. Biophys.* 7: 79–107
141. Shearwin, K. E., Winzor, D. J. 1988. *Arch. Biochem. Biophys.* 265: 458–65
142. Sikorav, J.-L., Church, G. M. 1991. *J. Mol. Biol.* 222: 1085–1108
143. Sobczak, J., Duguet, M. 1988. *Eur. J. Biochem.* 175: 379–85
144. Srere, P. A. 1987. *Annu. Rev. Biochem.* 56: 89–124
145. Sunshine, H. R., Hofrichter, J., Eaton, W. A. 1979. *J. Mol. Biol.* 133: 435–67
146. Suzuki, A., Yamazaki, M., Ito, T. 1989. *Biochemistry* 28: 6513–18
147. Takahashi, M., Uchida, T. 1986. *J. Biochem. (Tokyo)* 100: 123–31
148. Tanford, C. 1961. *Physical Chemistry of Macromolecules*. New York: Wiley & Sons
149. Tank, D. W., Wu, E.-S., Meers, P. R., Webb, W. W. 1982. *Biophys. J.* 40: 129–35
150. Tellam, R. L., Sculley, M. J., Nichol, L. W. 1983. *Biochem. J.* 213: 651–59
151. Teraoka, H., Tsukada, K. 1987. *J. Biochem. (Tokyo)* 101: 225–31
152. Tessier, D. C., Brousseau, R., Vernet, T. 1986. *Anal. Biochem.* 158: 171–78
153. Tilton, R. D., Gast, A. P., Robertson, C. R. 1990. *Biophys. J.* 58: 1321–26
154. Torbet, J. 1986. *Biochemistry* 25: 5309–14
155. Tsurimoto, T., Matsubara, K. 1982. *Proc. Natl. Acad. Sci. USA* 79: 7639–43
156. Van Damme, M.-P. I., Murphy, W. H., Comper, W. D., Preston, B. N., Winzor, D. J. 1989. *Biophys. Chem.* 33: 115–25
157. Watson, J. D., Hopkins, N. H., Roberts, J. W., Steitz, J. A., Weiner, A. M. 1987. *Molecular Biology of the Gene*, Vol. 1. Menlo Park, CA: Benjamin Cummings. 4th ed.

158. West, R. 1988. *Biopolymers* 27: 231–49
158a. Wieder, R., Wetmur, J. G. 1981. *Biopolymers* 20: 1537–47
159. Wilf, J., Gladner, J. A., Minton, A. P. 1985. *Thromb. Res.* 37: 681–88
160. Wilf, J., Minton, A. P. 1981. *Biochim. Biophys. Acta* 670: 316–22
161. Wills, P. R., Nichol, L. W., Siezen, R. J. 1980. *Biophys. Chem.* 11: 71–82
162. Winter, R. B., Berg, O. G., von Hippel, P. H. 1981. *Biochemistry* 20: 6961–77
163. Winzor, D. J., Wills, P. R. 1986. *Biophys. Chem.* 25: 243–51
164. Woolley, P., Wills, P. R. 1985. *Biophys. Chem.* 22: 89–94
165. Zimmerman, S. B., Harrison, B. 1985. *Nucleic Acids Res.* 13: 2241–49
166. Zimmerman, S. B., Harrison, B. 1987. *Proc. Natl. Acad. Sci. USA* 84: 1871–75
167. Zimmerman, S. B., Murphy, L. D. 1992. *Biopolymers.* 32: 1365–73
168. Zimmerman, S. B., Pheiffer, B. H. 1983. *Proc. Natl. Acad. Sci. USA* 80: 5852–56
169. Zimmerman, S. B., Trach, S. O. 1988. *Biochim. Biophys. Acta* 949: 297–304
170. Zimmerman, S. B., Trach, S. O. 1988. *Nucleic Acids Res.* 16: 6309–26; Erratum. *Nucleic Acids Res.* 16: 9892
171. Zimmerman, S. B., Trach, S. O. 1990. *Biopolymers* 30: 703–18
172. Zimmerman, S. B., Trach, S. O. 1991. *J. Mol. Biol.* 222: 599–620
173. Taylor, M. P., Herzfeld, J. 1991. *Phys. Rev. A* 43: 1892–1905

Annual Review of Biophysics and Biomolecular Structure
Volume 22, 1993

CONTENTS

STRUCTURAL PRINCIPLES

Macromolecular Crowding: Biochemical, Biophysical, and Physiological Consequences, *Steven B. Zimmerman and Allen P. Minton* 27

The Control of Protein Stability and Association by Weak Interactions with Water: How Do Solvents Affect These Processes?, *Serge N. Timasheff* 67

Models of Lipid-Protein Interactions in Membranes, *Ole G. Mouritsen and Myer Bloom* 145

The Design of Metal-Binding Sites in Proteins, *Lynne Regan* 257

Hydrogen Bonding, Hydrophobicity, Packing, and Protein Folding, *George D. Rose and Richard Wolfenden* 381

Mechanisms of Membrane Fusion, *Joshua Zimmerberg, Steven S. Vogel, and Leonid V. Chernomordik* 433

STRUCTURE AND FUNCTION

Prolyl Isomerase: Enzymatic Catalysis of Slow Protein-Folding Reactions, *Franz X. Schmid* 123

Functional Bases for Interpreting Amino Acid Sequences of Voltage-Dependent K^+ Channels, *Arthur M. Brown* 173

The Effects of Phosphorylation on the Structure and Function of Proteins, *L. N. Johnson and D. Barford* 199

The Structure of the Four-Way Junction in DNA, *David M. J. Lilley and Robert M. Clegg* 299

Structure and Function of Human Growth Hormone: Implications for the Hematopoietins, *James A. Wells and Abraham M. de Vos* 329

DYNAMICS

Interaction of Proteins with Lipid Headgroups: Lessons from Protein Kinase C, *Alexandra C. Newton* 1

Realistic Simulations of Native-Protein Dynamics in Solution and Beyond, *V. Daggett and M. Levitt* 353

(*continued*)

Glycoprotein Motility and Dynamic Domains in Fluid Plasma Membranes, *Michael P. Sheetz* 417

Fast Crystallography and Time-Resolved Structures, *Janos Hajdu and Inger Andersson* 467

EMERGING TECHNIQUES

The Two-Dimensional Transferred Nuclear Overhauser Effect: Theory and Practice, *A. P. Campbell and B. D. Sykes* 99

What Does Electron Cryomicroscopy Provide that X-Ray Crystallography and NMR Spectroscopy Cannot?, *W. Chiu* 233

Artificial Neural Networks for Pattern Recognition in Biochemical Sequences, *S. R. Presnell and F. E. Cohen* 283

INDEXES

Subject Index 499

Cumulative Index of Contributing Authors, Volumes 18–22 511

Cumulative Index of Chapter Titles, Volumes 18–22 513