Cytoarchitecture and Physical Properties of Cytoplasm: Volume, Viscosity, Diffusion, Intracellular Surface Area

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Classical biochemistry is founded on several assumptions valid in dilute aqueous solutions that are often extended without question to the interior milieu of intact cells. In the first section of this chapter, we present these assumptions and briefly examine the ways in which the cell interior may depart from the conditions of an ideal solution. In the second section, we summarize experimental evidence regarding the physical properties of the cell cytoplasm and their effect on the diffusion and binding of macromolecules and vesicles. While many details remain to be worked out, it is clear that the aqueous phase of the cytoplasm is crowded rather than dilute, and that the diffusion and partitioning of macromolecules and vesicles in cytoplasm is highly restricted by steric hindrance as well as by unexpected binding interactions. Furthermore, the enzymes of several metabolic pathways are now known to be organized into structural and functional units with specific localizations in the solid phase, and as much as half the cellular protein content may also be in the solid phase.

KEY WORDS: Cytoplasm, Viscosity, Diffusion, Partitioning, Particle Transport.

I. Introduction

Much of the current paradigm for cellular biochemistry has been extrapolated from studies of dilute solutions containing a single enzyme and a single substrate whose interaction is diffusion-limited. While this reductionist approach has led to many valuable insights over the past several decades, measurements of the physical properties of cells indicate that the interior

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of a cell departs from these ideal conditions in several important ways. Long considered a minority view, there now is a dawning awareness on the part of investigators that the nonideality of the cell interior may necessitate an alternate view of cellular biochemistry. In this chapter, we will address five assumptions implicit in the prevailing paradigm, review the extent to which they are valid for cells, and point out areas in need of further study. These assumptions are that: (1) the reaction volume is infinite, (2) the solution is dilute, (3) the concentrations of substrates are much higher than the concentrations of their enzymes, (4) the solution is well-defined, and (5) the solution is homogeneous. Each of these points has been raised by previous reviewers (for example, Srere, 1967; Fulton, 1982; Agutter *et al.,* 1995). This chapter will provide an overview of the field and focus on the most recent contributions. For the sake of brevity, the reader is referred to recent review articles for more detailed discussion of some areas, and I apologize to any author whose work is not cited individually.

II. Examining the Assumptions

A. Assumption 1: Infinite Volume

An underlying assumption of classical physical biochemistry is that the reaction volume is infinite. The familiar concept of concentration depends on this assumption. In reality, because a cell is surrounded by a limiting membrane, which is only selectively permeable, the assumption of infinite volume is patently false. The question is whether the finite volume of cells can be neglected, as it frequently is. Single cells range in size from the smallest mycoplasma (0.3 μm in diameter) to the eggs of the African clawed toad, *Xenopus laevis* (1 mm in diameter). The relevant volumes thus range from 1×10^{-17} liters to about one-half microliter. Bacteria typically have a volume of the order of 2×10^{-16} liter (Halling, 1989). The volume of an average mammalian tissue culture cell is approximately 4 picoliter (Alberts *et al.,* 1994). For eukaryotic cells, in which many biochemical pathways are sequestered within membrane-bounded compartments (organelles) of submicrometer dimensions, the reaction volume may be very small indeed (6×10^{-20} liters for a spherical vesicle 50 nm in diameter). In addition, the internal membrane-bounded compartments together occupy up to half the volume of the cell, further reducing the free volume available to reactants diffusing in the cytoplasm that bathes the organelles.

The small, finite volume of cells and intracellular compartments means that, at physiologically relevant molar concentrations, the number concentration of a particular molecular species may be surprisingly low. In the

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example given by Halling (1989), a species present in a single copy per bacterium will have a nominal concentration of nearly 10^{-8} M. In an average eukaryotic cell, a protein present at a whole cell concentration of 1 nM would have number concentration on the order of 1000 copies. At these low copy numbers, number fluctuations may limit the availability of a species at a given subcellular location and time. In addition, a large surface with even low affinity for that species could adsorb the entire population. Even for ions, number fluctuations can be significant if the volume is small enough. For example, the pH of the endosomal compartment in eukaryotic cells is maintained at around 6. For a typical, spherical endosome 250 nm in diameter, this amounts to one free proton. As the endosome matures, the interior becomes acidified to pH 5 by the action of a proton pump in the surrounding membrane. Assuming constant volume, this requires an increase of only 48 free protons. Thus, it may be more accurate to understand the interior of this compartment in terms of the protonation of specific residues rather than a global pH. In general, for small cells or intracellular compartments, the number concentration of a particular molecular species may be more informative than its molar concentration.

B. Assumption 2: The Cell Interior Is Dilute

A fundamental assumption of the physical chemistry of dilute solutions is that interactions between solute molecules can be neglected. However, the total concentration of macromolecules inside cells is very high, with proteins by far the most abundant species. As reviewed by Fulton (1982), the protein content of cells is in the range 17 to 35% by weight. Estimation of the protein concentration in the cytoplasm of mammalian tissue culture cells from refractive index measurements indicates a value of 20 to 30 g/100 ml (Lanni *et al.,* 1985). The low spatial resolution of these meaurements doesn't allow a distinction between structural and soluble proteins, and the proportion of the total that is actually soluble continues to be the subject of some debate, as discussed in more detail below.

A 20% solution of protein is very crowded and significant interactions between solute molecules can be expected: For a typical 50 kDa protein, this is well above the theoretical "overlap" concentration of 13 g/100 ml (Chang *et al.,* 1987). Although the physical volume fraction of protein at a concentration of 20% is well below the threshold for close packing of hard spheres (Kertesz, 1981), some proteins have been crystallized at concentrations in this range. How crowded a milieu the cytoplasm is can be appreciated visually from the drawings of Goodsell (1991, 1993), who has depicted the cell interior with macromolecular components at the correct concentration and drawn to scale (Fig. 1).

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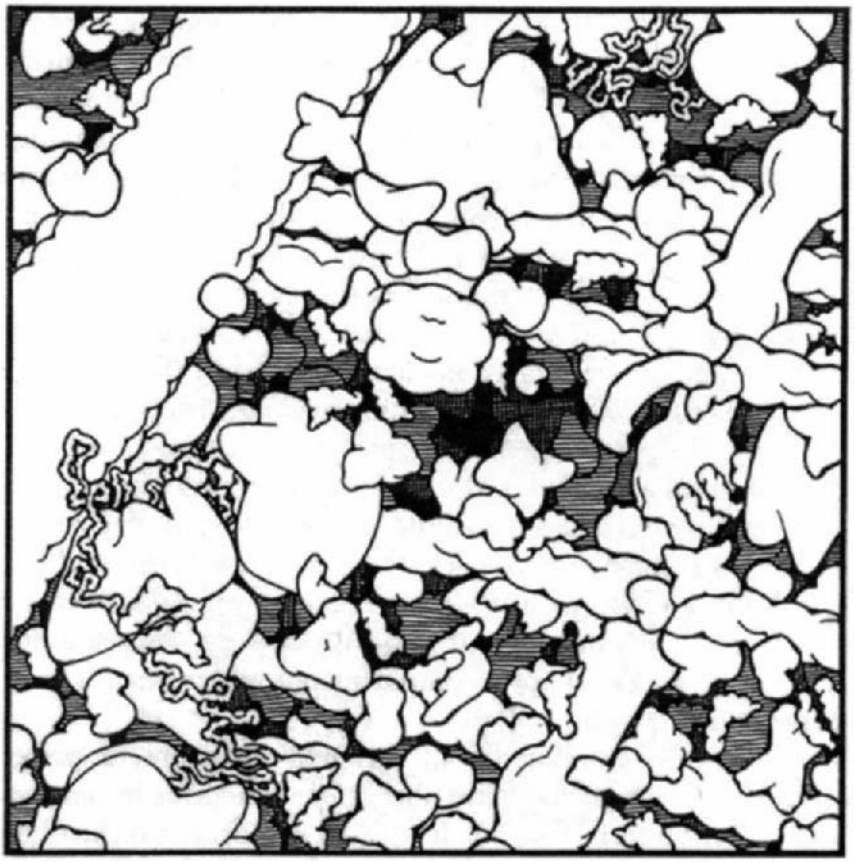

FIG. 1 A view of the cytoplasm of the yeast, *S. cerevisiae,* with components drawn to scale and at the correct concentrations. Soluble proteins, ribosomes with mRNA, microtubules, actin filaments, and intermediate filaments are all represented. Small solutes are omitted from the drawing. Magnification 1,000,000 ×. Reprinted with permission (Goodsell, 1993).

The biochemistry of crowded solutions can be shown both theoretically and experimentally to deviate from what occurs in dilute solution (Minton, 1997; see also chapter by Johansson *et al.* in this volume). The increased pathlength required to circumvent obstacles can retard significantly the translational diffusion of particles comparable in size to the crowding solute, slowing the rates of diffusion-limited reactions accordingly. The entropy benefit to the system arising from a reduction in particle number may drive association of components that associate only weakly or not at all in dilute solution. Excluded volume effects may drive conformation changes in a particular species. Because the threshold for crowding effects is steep, small

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changes in cellular volume may thus lead to large changes in the chemical activities of the solutes. Many examples of altered kinetics, specific activity or substrate specificity of enzyme-catalyzed reactions in crowded solutions can be found in the literature (Minton, 1997). Crowding has also been shown to drive self-assembly reactions and phase separations of biomolecules *in vitro* (Minton, 1997). For a more detailed discussion of the effects of macromolecular crowding, the reader is directed to the chapter by Johansson *et al.* in this volume.

C. Assumption 3: Substrate Concentrations are Greater than Enzyme Concentrations

Not only are macromolecules generally present in high concentration, but the concentration of a specific enzyme or protein is generally much higher in cells than in conventional *in vitro* assays, and is often much in excess of the K_d as determined *in vitro*. The Ca^{2+}-dependent regulatory protein, calmodulin (CaM), which binds to and activates a variety of target enzymes in response to intracellular Ca^{2+} signals, serves as a good illustration. The K_{act} of CaM for myosin light chain kinase, its major known target in smooth muscle, is around 1 nM, but the concentration of CaM in smooth muscle is on the order of 38 μM (Tansey *et al.,* 1994; Zimmermann *et al.,* 1995) The concentration of enzymes confined within organelles such as mitochondria or peroxisomes can be even higher. As pointed out some time ago by Srere (1967), if the K_ds *in vivo* are the same as measured *in vitro,* a substantial proportion of substrates may be bound to enzymes, making the concentration of available substrate rate limiting. (Srere, 1967). In order to proceed efficiently under these conditions, channeling of substrates from enzyme to enzyme in a particular metabolic pathway may be necessary. See the chapter to by Ovádi and Srere in this volume for further discussion.

D. Assumption 4: The Cytoplasm is a Simple, Well-defined Solution

An inspection of any metabolic pathway chart will show that the interior of the cell contains a complex web of enzyme pathways, many of which compete for common substrates. It appears from the number of different mRNAs that a typical eukaryotic cell synthesizes 10,000 to 20,000 different proteins (Alberts *et al.,* 1994). Because most of these have not been characterized biochemically, their effect on any given reaction pathway is unknowable. In addition, most regulatory enzymes and proteins are promiscuous, interacting with a large number of target proteins, at least *in vitro.* To

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continue with the example of calmodulin introduced above, it has more than 50 known targets, including kinases, phosphatases, ion channels, structural proteins, and transcription factors (Rhoads and Friedberg, 1997). *In vitro,* the affinities of most of these targets for binding by Ca^{2+}–CaM are within an order of magnitude. It is difficult to see how discrimination among competing interactions can occur in the intact cell without preexisting colocalization of enzyme and substrate, or regulatory protein and target. For some pathways, such as the tricarboxylic acid cycle, this has been achieved by sequestering them within an organelle or immobilizing the enzyme on a membrane. However, the majority of "soluble" proteins have long been assumed to be distributed homogeneously in the cell interior.

E. Assumption 5: The Cytoplasm is a Homogeneous Solution

Traditional chemistry depends on the assumption that the solution is homogeneous and can be treated as a continuum. This assumption is not valid for intact cells. To begin with, the presence of membrane-bounded subcompartments confers an intrinsic inhomogeneity on the interior of an eukaryotic cell. Additionally, the organelles themselves are not randomly distributed (see chapter by Aw in this volume). The localization of mitochondria near sites of high ATP consumption, for example, in striated muscle, is well known. The nonrandom disposition of organelles during differentiation can confer both structural and functional polarity on cells, as in the case of columnar epithelial cells or neurons. Cells expend a large proportion of their energy supply in establishing and maintaining this nonrandom array of organelles.

The cytoplasm that bathes the organelles also cannot be considered a homogeneous solution. It is permeated by a network of self-assembling polymeric protein fibers known collectively as the cytoskeleton. The three best studied of these are the microfilaments, the microtubules, and the intermediate filaments. The microfilaments are homopolymers 8 nm in diameter formed from the globular protein, actin. Actin is very abundant in most cells, with a concentration of about 4 mg/ml in a typical eukaryotic cell (Bray and Thomas, 1975). Roughly half this amount is polymeric, giving a value of 3×10^5 μm of filament per cell (based on 370 monomers per μm). Microtubules are heteropolymers of the related globular proteins, α- and β-tubulin. The subunits self-assemble to form a rigid, hollow tube 25 nm in diameter. There are typically about 150 microtubules in a tissue culture cell and each is 50 to 100 μm in length (Hiller and Weber, 1978). Intermediate filaments are highly stable polymers formed by lateral, coiled-coil associations between the polypeptides of a broad family that includes vimentin, the cytokeratins, and the

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neurofilaments (Chou *et al.,* 1997). The concentration of vimentin in a mammalian tissue culture cell of mesenchymal origin is on the order of 150 μg/ml, all of which is polymerized (Lai *et al.,* 1993), resulting in an estimated 10,000 μm filament per cell (based on 32 monomers per 10 nm diameter $\times$ 48 nm long association domain).

Although the three types of cytoskeletal filaments are often treated as independent systems, a mounting body of evidence supports the idea that they are physically interconnected. Whole-mount electron microscopy of tissue culture cells extracted with nonionic detergents revealed a three-dimensional network in which all three filament types could be identified with frequent connections among them (Webster *et al.,* 1978; Schliwa and van Blerkom, 1981). Similar views have been obtained by electron microscopy of rapidly frozen tissue from which the ice has been sublimed (Heuser and Kirschner, 1980). In living cells, treatment specifically targeting one of these three systems frequently leads to rearrangement of one or both of the other two. In a recent example, a peptide that specifically disassembles intermediate filaments *in vitro* leads to collapse of the microtubules and microfilament systems when microinjected into intact cells (Eriksson *et al.,* 1992). In addition, specific proteins, such as plectin and map2C, can be shown to crosslink one filament type to another both *in vitro* and *in vivo* (Chou *et al.,* 1997; Cunningham *et al.,* 1997). Thus, the cytoplasmic volume may be better described as an aqueous gel than as a homogeneous solution (Fig. 2).

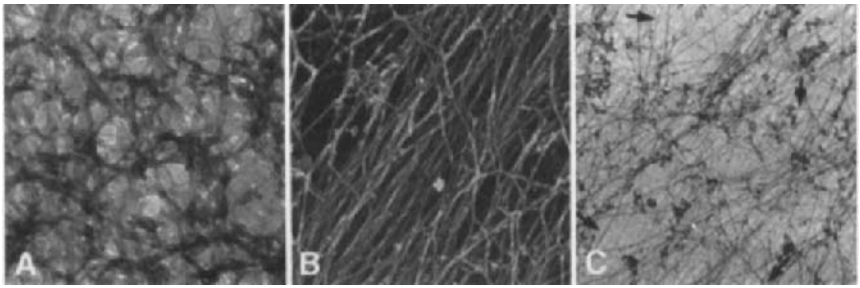

FIG. 2 Three views of the cytoplasm by electron microscopy. (A) The microtrabecular lattice in a freeze-dried, whole-mounted fibroblast. Magnification 80,000. Reproduced from *The Journal of Cell Biology,* 1984, vol. 99, pp. 3s–12s by copyright permission of The Rockefeller University Press. (B) The appearance of the cytoplasm in a platinum replica of a fibroblast that was extracted for 30 min with 0.5% Triton before freeze-drying. Magnification 70,000. Reproduced from *The Journal of Cell Biology,* 1980, vol. 86, pp. 212–234 by copyright permission of The Rockefeller University Press. (C) The appearance of the cytoplasm in a fibroblast lightly extracted with Triton, then critical point dried and whole mounted. Magnification 17,500. Arrows indicate microtubules. Reproduced from Schliwa and van Blerkom (1981) by copyright permission of The Rockefeller University Press.

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The individual cytoskeletal filaments themselves are not isotropically distributed within the network. The microfilaments form bundles known as stress fibers, and the ends of the bundles are often fixed at specific sites, such as adhesion plaques in the ventral membrane of a fibroblast or adherens junctions in the lateral membranes of adjoining epithelial cells. By whole-mount electron microscopy, stress fibers and smaller microfilament bundles appear as local inhomogeneities embedded in the larger cytoskeletal network (Small *et al.*, 1978; Provance *et al.*, 1993). In most cells, the majority of microtubules radiate from an organizing center, or centrosome, usually located near the cell nucleus. Intermediate filaments are often colinear with these microtubules, especially in the more peripheral regions of the cell.

The inhomogeneities contributed by the cytoskeleton vary temporally as well as spatially. The assembly state and subcellular distribution of all three types of cytoskeletal filament are physiologically regulated. Well-known examples of this include the disassembly of the radial array of microtubules and subsequent assembly of the microtubule spindle at mitosis, and the reorganization of the actin cytoskeleton in response to growth factor stimulation or tumor viruses. Recently, progress has been made in defining the signaling and control pathways that lead to these changes, and accessory protein candidates for mediating these changes have been identified (for reviews, see Maccioni and Cambiazo, 1995; Johansen, 1996; Lim *et al.*, 1996; Pereira and Schiebel, 1997; Tapon and Hall, 1997). In pure solutions, microfilaments and microtubules undergo spontaneous phase separations, forming bundles in the absence of strong binding interactions between filaments. Three types of phase separation are recognized: filament length- and concentration-dependent, entropy-driven formation of nematic phases embedded in the isotropic phase; excluded volume-driven demixing of filaments with different flexibilities or of filaments and spheres; and bundling of filaments in the presence of polycations analogous to the counterion condensation of DNA (Herzfeld, 1996; Tang and Janmey, 1996). The composition and type of phases can be modified by changing the length or concentration of the filaments; manipulating the free volume by crowding the solution with inert macromolecules (such as polyethylene glycol (PEG) or Ficoll); changing the concentration or availability of polycations; or stabilizing bundles with cross-linking proteins. Similar phase separations of cytoskeletal components *in vivo* may contribute to the inhomogeneous distribution described above. Specific proteins are known that regulate the length or total content of filaments, as well as proteins that stabilize bundles by cross-linking the filaments (Carlier and Pantaloni, 1997; Pereira and Schiebel, 1997). These proteins are presumed to be the immediate effectors of signal-initiated reorganization of the cytoskeleton. In addition to the

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regulated formation and dissolution of more or less ordered phases, individual microtubules in living cells have been shown to be dynamically unstable, alternately growing and shrinking (Waterman-Storer and Salmon, 1997). This means that local inhomogeneities fluctuate on a time scale of a few minutes.

The inhomogeneities described above present large intracellular surface areas upon which phase separations might occur. The surface area of specific membrane compartments has been measured in many cell types. The total outer surface area of mitochondria is on the order of a few hundred to a few thousand μm^2/cell (Mori *et al.*, 1982; Hoppeler *et al.*, 1987). At the other end of the spectrum, the total surface area of the abundant endoplasmic reticulum in secretory cells, such as the Leydig cells of the testis, may be as high as 30,000 μm^2/cell (Mori *et al.*, 1982). Altogether, for eukaryotic cells, the internal membrane surface area far exceeds the area of the plasma membrane. For hepatocytes, it is estimated to be approximately 100,000 μm^2/cell, which is 50 times the area of the plasma membrane (Alberts *et al.*, 1994). It is interesting to note that many cell types modify the surface area of their internal organelles in response to growth conditions. While this is often interpreted as a consequence of modifications of organelle volume, the corresponding changes in intracellular surface area are significant and could potentially play a regulatory role in metabolism.

The cytoskeleton also presents a significant surface area. Calculating from the known size, shape, and concentration of actin microfilaments, microtubules, and intermediate filaments, the naked cytoskeleton would contribute about 3000 μm^2 of surface area in a typical mammalian tissue culture cell. This may be an underestimate if other types of filaments exist or when one takes into account other components immobilized on the cytoskeleton. Whole-mount electron microscopy of unextracted cells reveals not the well-defined cytoskeleton that is observed after detergent extraction, but a three-dimensional network of irregularly shaped strands that was designated the microtrabecular lattice (MTL) by Porter and colleagues (Wolosewick and Porter, 1979) (Fig. 2C). Great care was taken to minimize fixation artifacts in the preparation of these specimens, suggesting that the MTL may accurately reflect the appearance of the cytoskeletal network in the living state. The surface area of the MTL estimated by morphometry of stereo electron micrographs is 69,000–91,000 μm^2 per cell (Gershon *et al.*, 1985). Thus, the surface area of the MTL and internal membranes together could be as high as 200,000 μm^2 per cell in some cell types under certain conditions. It has been proposed that many enzyme pathways are immobilized and localized on the surfaces of internal membranes and that substrates are transported to these enzymes by bulk streaming of the cytoplasm (Wheatley and Malone, 1993; Wheatley, 1998).

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III. Physical Properties of Cytoplasm (Measured)

It is obvious from the foregoing discussion that the nonideality of the cell interior could have profound effects on its physical and chemical properties. In this section, we will survey experimental data aimed at studying these properties in the cytoplasm of intact cells. Three areas of research that have seen the most activity are the behavior of intracellular water, the constraints on diffusion and partitioning of inert tracer particles, and the diffusibility of endogenous proteins and vesicles.

A. Water in Intact Cells

The idea that macromolecules in solution contain bound water molecules is a concept of long standing in physical biochemistry. As the crystal structures of more and more proteins have become available, it has become clear that not only is hydration necessary for the function of particular enzymes, but insertion or removal of water molecules can drive conformational changes and macromolecular associations. What is less commonly considered is the possibility that in addition to water that is an integral part of a protein's folded conformation, the mobility of water near hydrophobic surfaces may be reduced by the formation of hydrogen-bonded clathrate structures. Thermodynamically, reduced mobility of water translates into a reduced chemical activity compared with bulk water. The solvent properties of this perturbed water may also differ from bulk water in a solute-dependent manner. Conversely, dissolved macromolecules can also have water destructuring effects due to the dielectric field around charged or polar groups that attracts the polar water molecules and prevents hydrogen bonding (Cameron *et al.,* 1997). A different way of conceptualizing the same effects is to think in terms of water density (Wiggins, 1996). The local concentration of water molecules (and, hence, water activity) will be higher around charged (hydrophilic) surfaces and lower around hydrophobic surfaces.

The high concentration of macromolecules inside a living cell, and the large intracellular surface area presented by proteins and membranes, raises the question of whether a significant proportion of total cellular water differs from the bulk. This has been the subject of both experimentation and debate for many years (see chapter by Garlid in this volume). Opinions range from the point of view that only a low percentage of cellular water is bound (Cooke and Kuntz, 1974), to the claim that most cellular water is ordered to some degree (Ling, 1988; Ling *et al.,* 1993).

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Attempts to address this issue experimentally fall into two broad methodological classes: measurement of the properties of cellular water itself and measurement of the rotational mobility of solutes, either endogenous molecules or probes that have been introduced into living cells. Approaches to measuring the properties of intracellular water can be further divided into measurement of water mobility and measurement of water activity by studying the colligative properties of cells.

1. Measurements of Water Mobility

Two methods have been used to measure the mobility of cellular water: proton NMR and quasielastic neutron scattering (QENS). Both methods have the advantage of being noninvasive. Since a typical cell contains 70% water, the ^{1}H -NMR signal from cells is dominated by the water protons. In pure water, the transverse relaxation time (T_2) reflects the randomization of the proton dipoles subsequent to orientation in a magnetic field and is, thus, a measure of rotational diffusion. In more complex milieuxs, T_2 may be shortened due to local fields in the sample or exchange of protons among populations with different properties. T_2 measured from cells generally is about 1/50 that of pure water protons (Foster *et al.,* 1976). Assuming that the mechanism of accelerated relaxation is fast exchange of protons between two or more populations of water with different mobilities, T_2 is the weighted average of the relaxation times from all water fractions in the cell. When T_2 is corrected to 100% water content, it appears that the average rotational mobility of cellular water is reduced about twofold (Clegg, 1984; Rorschach *et al.,* 1991; Cameron *et al.,* 1997). However, because water molecules can diffuse over distances comparable to the dimensions of a cell in the relatively long time required for these measurements, the reduction in T_2 could reflect encounters with physical barriers in the cell rather than altered rotational mobility. QENS measurements can potentially resolve this ambiguity because of the short distance scale over which the measurements are made. Unfortunately, because the samples must be held under nonphysiological conditions for several days, the use of this technique has been limited to a few hardy cell and tissue types, such as the brine shrimp *Artemia* and frog sartorius muscle (Rorschach *et al.,* 1973; Trantham *et al.,* 1984). As found by NMR, the apparent self-diffusion coefficient of intracellular water measured by QENS is significantly reduced compared with pure water. In the cases where QENS data and NMR data are both available for the same cell or tissue, there is remarkable quantitative agreement between the two parameters (Cameron *et al.,* 1997). However, quantitative interpretation of these data is ambiguous unless the relaxation times and proportions of each water fraction can be measured by independent methods. Furthermore, it may not be correct to assume that exchange

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between water fractions is rapid. For example, in a study of water mobility in barnacle muscle, the tightly bound fraction was assayed independently by its inability to exchange with deuterium. A second, less tightly bound fraction was identified that exchanged with deuterium at an intermediate rate, but remained unfrozen at −34°C. Together these two fractions accounted for only 3% of the water protons in barnacle muscle, with the rest having the properties of bulk water (Foster *et al.,* 1976). It should also be kept in mind that alternate mechanisms of relaxation such as dipole interactions, exchange of water protons with the protons of proteins, and slow exchange of water across the plasma membrane can also reduce T_2 and cannot always be ruled out experimentally.

2. Measurement of Colligative Properties of Cells and Biomolecules

The colligative properties of a solution are directly related to water activity. These include osmotic pressure, freezing point depression, and vapor pressure. While all of these have been applied to study the properties of protein solutions, only the first two are readily applicable to living cells. The appropriate use of osmotic pressure measurement to study the activity of water in biological specimens recently was the subject of an excellent review (Parsegian *et al.,* 1995). We have already seen that a small fraction of water in barnacle muscle had a depressed freezing point, suggesting that its activity is significantly lower than that of bulk water (Foster *et al.,* 1976). Reports of the nonideal osmotic response of some cells indicate a much larger fraction of water with perturbed activity. The water content of lens fiber cells, *Xenopus* oocytes, sea urchin eggs, and frog sartorius muscle, was found to scale linearly as a function of inverse osmotic pressure (Π) over a limited range as expected for an osmometer (for review, see Cameron *et al.,* 1997). However, linear extrapolation of the data to infinite Π gave a nonzero intercept that was interpreted as "osmotically unresponsive" water. This fraction ranged from 30 to 90% of total water content of the specimens. A recent paper addressed water activity in neurons by combining ^{1}H-NMR imaging of single cells with osmotic perturbation (Hsu *et al.,* 1996). These investigators found that T_2 varied with changes in tonicity over the very limited range tested, as expected for a perfect osmometer. Under the same conditions, the apparent diffusion coefficient of the protons was unaffected.

Several ambiguities attend the use of osmotic approaches to study the activity of cellular water that are not a factor for the study of water activity in solution. For example, it is rarely possible to rule out active responses of the cell to changes in extracellular tonicity. In addition, volume changes may not be directly coupled to osmotic pressure, because the ratio of plasma

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membrane surface area to cell volume often is very large due to convolution of the membrane (Clegg, 1986).

Measurements of the colligative properties of specific proteins *in vitro* have also been used to predict the amount of perturbed water in cells (for review, see Cameron *et al.,* 1997). For example, the nonzero intercept of plots of water content vs inverse osmotic pressure for BSA indicate that this protein contains 4 g of osmotically unresponsive water per gram of protein. Assuming that BSA is representative of other cytoplasmic proteins, extrapolation of this result to the estimated protein concentration in cytoplasm suggests that the osmotic response of all cellular water molecules would be perturbed. Studies of ion partitioning and water flow through protein gels *in vitro* suggest that the activity of water in such gels is greatly reduced (Van Steveninck *et al.,* 1991, Wiggins *et al.,* 1991; Ito *et al.,* 1992). If the cytoskeleton renders the interior of an intact cell more like a gel than a solution, significant changes in water activity and mobility are predicted on the basis of these experiments.

3. Measurements of Solute Rotational Mobility

The second approach to examining the properties of cellular water has been to study the rotational mobility of solutes, which primarily reflects the viscosity of the solvent (water) with a negligible contribution from any barriers to long-range translational motion. The rotational mobility of endogenous glycolytic enzymes was measured in the yeast *Saccharomyces cerevisae* by biosynthetically labeling them with 5-fluorotryptophan (Williams *et al.,* 1997). NMR gave relaxation times for phosphoglycerate kinase and hexokinase that were consistent with a solvent viscosity between 1 and 2 cP. Similarly, the rotational mobility of deoxymyoglobin was measured by ^{1}H-NMR in bovine heart muscle by taking advantage of its unique proton signature. In this case, a value consistent with a solvent viscosity of 2.3 cP was measured (Livingston *et al.,* 1983). These values are in general agreement with the average reduction in water mobility measured by proton NMR.

An alternative approach taken by several laboratories is to measure the rotational mobility of small spin probes or fluorescent molecules that have been introduced into living cells. Values of cytoplasmic viscosity as high as 11 cP were estimated from the behavior of two small spin probes in tissue culture cells (Lepock *et al.,* 1983; Mastro *et al.,* 1984). Similarly high values were suggested based on the steady-state fluorescence polarization exhibited by small fluorescent probes (Lindmo and Steen, 1977; Hashimoto and Shinozaki, 1988). However, the binding of these probes to intracellular components and the subcellular location of the probes were not assessed in these studies, so it is not clear that the rotational behavior of the probes

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reflects cytoplasmic viscosity. In addition, the high steady-state polarization of the fluorescent probes could not be directly interpreted in terms of the rotational mobility of the probe since the fluorescence lifetime of the probe in the cells was not measured. More recently, a series of papers from the Verkman laboratory have reported the use of time resolved fluorescence anisotropy imaging of the small fluorescent probe BCECF to measure solvent viscosity (Fushimi and Verkman, 1991; Periasamy *et al.*, 1991, 1992). Although the probe showed significant binding to intracellular components, these investigators developed an algorithm for data analysis by which free probe could be distinguished from bound. Measurements of lifetime showed that the lifetime of the probe was unaffected by the intracellular environment. In several tissue culture cell lines, these studies indicated that cytoplasmic solvent viscosity is not significantly different from bulk water (Fushimi and Verkman, 1991). However, cells such as sea urchin eggs and kidney tubule cells, that adapt to anisoosmotic conditions by making small osmolytes, did exhibit a two- or threefold increased solvent viscosity (Periasamy *et al.*, 1991, 1992). No significant spatial variation in viscosity was detected and nuclear viscosity was not different from the cytoplasm. More recently, time-resolved fluorescence anisotropy imaging of sulforhodamine B was used to measure the solvent viscosity of a range of cell types (Srivastava and Krishnamoorthy, 1997). The free and bound fractions of the probe could be distinguished unequivocally by a marked difference in fluorescence lifetime. In all cell types studied, the apparent solvent viscosity was very similar to bulk water, with modest spatial variations on a distance scale of 1 μm. It was found that plant cells generally have a higher viscosity and more spatial variability than do animal cells. A somewhat different approach has been to utilize steady-state fluorescence ratio imaging to map solvent viscosity, using a viscosity-sensitive fluorophore attached to dextran (Luby-Phelps *et al.*, 1993). Again, solvent viscosity in the cytoplasm of two mammalian tissue culture cell lines did not appear significantly different from bulk water and no spatial variation was observed beyond that accounted for by systematic error.

These results would appear, at first glance, to be in conflict with data on water mobility from NMR and QENS. However, an inherent limitation of using solute rotation to probe water viscosity is that steric hindrance or insolubility may limit access of the probes to compartments where water is perturbed. In addition, the limited spatial resolution of standard imaging apparatus would allow surface associated layers of ordered water up to 200 nm thick to escape detection. The spatial resolution can be improved by using total internal reflection fluorescence (TIRF) imaging to measure the mobility of only those fluorescent probes within 50 nm of the ventral membrane of a cell. TIRF combined with time-resolved anisotropy indicated that the rotation of BCECF adjacent to the membrane was unimpeded

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relative to bulk water (Bicknese *et al.*, 1993). In summary, the average mobility of water in living cells does not appear to be greatly altered compared with pure water, but significant layers of more or less ordered water cannot be ruled out.

B. Hindrance of Long-Range Translational Diffusion

1. Diffusion of Protein-sized Tracer Macromolecules

In addition to possible effects of water structure on the rotational mobility of solutes, the long-range translational diffusion of macromolecules in the cytoplasm could be hindered by the excluded volume effects of crowding and by steric constraints imposed by obstacles such as the cytoskeleton. Studies of the translational diffusion of hydrophilic, electroneutral tracer particles suggest that such long-range effects are important in intact cells. Two different fluorescence microscopic methods have been used in these studies to obtain the cytoplasmic diffusion coefficients of fluorescent, size-fractionated dextrans and Ficolls that have been microinjected into intact cells. The first approach, fluorescence recovery after photobleaching (FRAP), uses a laser beam focused at the specimen plane of a fluorescence microscope to introduce a gradient of fluorescence in the sample by irreversible photolysis (bleaching) of fluorophores in the path of the laser beam. The subsequent exchange of bleached tracers with unbleached tracers diffusing in from outside the bleached region leads to relaxation of the gradient and recovery of fluorescence in the bleached region. The time constant (τ) that characterizes the recovery is inversely proportional to the effective diffusion coefficient (D) of the fluorescent tracer (Elson and Qian, 1989). In an alternative approach that avoids the possible artifacts associated with high-power laser illumination, low light-level, time-lapse video recording was used to monitor the spread of fluorescence down the axons of neurons that had been loaded with tracers by whole cell patch at the soma. D was calculated from the one-dimensional equation for diffusion (Popov and Poo, 1992).

Although dextrans and Ficolls do not appear to bind to intracellular components, the results of these studies show that they diffuse significantly more slowly in intact cells than in dilute solution (Luby-Phelps *et al.*, 1987; Hou *et al.*, 1990; Popov and Poo, 1992; Arrio-Dupont *et al.*, 1996; Seksek *et al.*, 1997). Comparison of the diffusion of the same tracers in concentrated solutions of unlabeled dextran or Ficoll suggests that the crowdedness of cytoplasm in fibroblasts resembles 12–13% dextran or Ficoll (Hou *et al.*, 1990). A similar result was obtained from FRAP studies of the small fluorescent dye, BCECF (Kao *et al.*, 1993). Although a significant fraction of

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BCECF was bound inside cells, this was corrected for in the analysis of the FRAP data. A crowdedness similar to 12–13% dextran or Ficoll would mean that roughly half of total cytoplasmic protein, or about 110 mg/ml, is in solution and the remainder is structural. An estimate of 135 mg/ml soluble protein was obtained by FRAP of fluorescent dextrans in cultured myotubes (Arrio-Dupont *et al.*, 1996). The hindrance on translational diffusion due to macromolecular crowding may be more severe in the vicinity of membrane surfaces. By combining TIRF with FRAP, the diffusion coefficient of BCECF within 50 nm of the ventral surface of epithelial cells in culture was found to be as much as tenfold lower than in water (Swaminathan *et al.*, 1996).

Superimposed on this apparent crowding effect, several groups have reported a significant size-dependence of the two-dimensional diffusion coefficient of tracers in cytoplasm relative to aqueous buffer (D/Do) (Fig. 3). In Swiss 3T3 fibroblasts, D/Do declined from 0.3 to 0.04 for particles ranging from 3 to 25 nm in hydrodynamic radius (Luby-Phelps *et al.*, 1987).

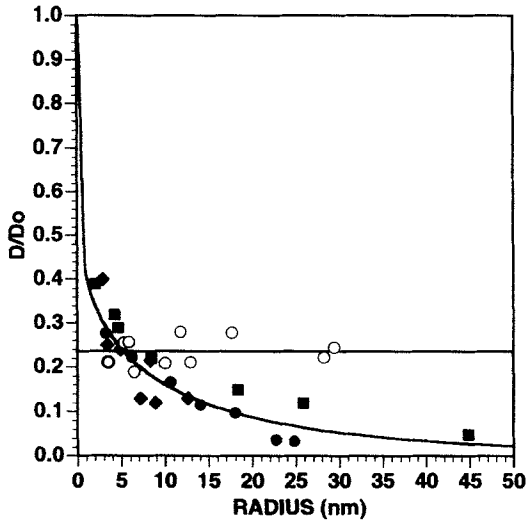

FIG. 3 Relative diffusion coefficients of inert fluorescent tracers in fibroblasts (filled circles, open circles), neurons (squares), and myotubes (diamonds). Data taken from Luby-Phelps *et al.* (1987), Popov and Poo (1992), Arrio-Dupont *et al.* (1996), and Seksek *et al.* (1997). Curve is nonlinear least-squares best fit of data from Luby-Phelps *et al.* (1987) (closed circles) to a model in which a network of filaments and a crowded background solution contribute independently to the hindrance on diffusion. The model predicts a network volume fraction of 0.11 and a protein concentration in the background of 110 mg/ml (Hou *et al.*, 1990). Line is linear least-squares fit to data from Seksek *et al.*, (1997) (open circles) with slope constrained to zero. The reason for the differing size-dependence of D/Do in the two studies of fibroblasts remains unexplained at present.

In cultured *Xenopus* neurons D/Do declined from 0.39 to 0.05 for particles ranging from 2 to 44.8 nm in hydrodynamic radius (Popov and Poo, 1992). In skeletal muscle myotubes, D/Do declined from 0.4 to 0.13 for particles ranging from 2.9 to 12.6 nm in radius (Arrio-Dupont *et al.*, 1996). Such size-dependence is consistent with sieving by a network of obstructions. The most likely candidate for such a network is the cytoskeleton. In support of this idea, in neurons the size-dependent constraint on diffusion was partially relieved by treating the cells with cytochalasin D, a drug that depolymerizes actin filaments (Popov and Poo, 1992).

The existence of sieving has been challenged in a recent paper which repeated earlier studies of the diffusion of dextrans and Ficolls in intact cells using a FRAP apparatus with greatly improved time resolution (Seksek *et al.*, 1997). In the more recent study, no size dependence of D/Do was found and these investigators concluded that the diffusion of macromolecules in cytoplasm is slowed only by crowding. It is not immediately obvious why an increase in time resolution should lead to such different results, and several differences in methodology need to be addressed before deciding which view of the cytoplasm is more accurate. For example, the interpretation of high time-resolution FRAP data potentially is complicated by photophysical effects, such as reversible photobleaching, that occur on the same timescale as the measurements. Anomalous temperature dependence of the rate of fluorescence recovery was observed in this study and interpreted as reversible photobleaching. The authors argued from circumstantial evidence that this was avoided by carrying out all measurements at room temperature rather than the physiological temperature of 37°C. Another potentially significant difference is the method of analyzing the FRAP recovery curves. Correct determination of τ dependent critically on the accurate estimation of the fluorescence intensity at infinite time, $F(\infty)$. Most algorithms for obtaining τ involve fitting the curve either to a truncated approximation of the nonconverging series that describes diffusive recovery (Axelrod *et al.*, 1976) or to an empirical relation that has been demonstrated to produce equivalent results (Yguerabide *et al.*, 1982), and then extrapolating to $F(\infty)$. In the more recent study, $F(\infty)$ was taken to be the apparent plateau value of the actual recovery curve at an arbitrarily long time after the bleach. If this plateau value was determined prematurely, τ will be underestimated and D overestimated. In addition, the fluorescence recovery will appear incomplete and the estimated percentage recovery will decline with increasing tracer size. In the previous studies, the fluorescence recovery of all but the largest tracers was nearly 100%. In the more recent paper, recoveries for tracers of all sizes were significantly less than 100% and declined with increasing tracer size. This was attributed to anomalous subdiffusion of tracer in subcompartments of cytoplasm whose viscosity differs from the bulk, perhaps due to some sort of phase separation.

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2. Size-dependent Partitioning of Tracers

Fluorescence ratio imaging of large and small tracers comicroinjected into well spread fibroblasts reveals the existence of subcompartments of the cytoplasm into which the large tracers do not partition (Fig. 4) (Luby-Phelps and Taylor, 1988). Whole-mount electron microscopy of these compartments shows them to be very flat and to contain numerous filament bundles embedded in a meshwork of thin filaments (Provance *et al.*, 1993). Immunofluorescence microscopy suggests that actin and actin-binding proteins are components of the bundles and meshwork, while microtubules and intermediate filaments are absent from the excluding compartment (Provance *et al.*, 1993). Mitochondria, endoplasmic reticulum, and small vesicles also are absent from the excluding compartments and are restricted to the perinuclear region or to channels defined by microtubules radiating from the perinuclear region to the peripheral membrane of the cell. The boundary between excluding and nonexcluding compartments was discernible as an abrupt change in ultrastructure and cell height, but no membrane or other physical barrier was evident. Overall, the excluding compartments appeared ultrastructurally similar to previously described "peripheral cytoplasmic domains" (Bridgman *et al.*, 1986) and to the leading lamellipodium of migrating cells (Abercrombie *et al.*, 1971; Small *et al.*, 1978).

The presence of a finely divided filament network in the excluding compartments suggested a molecular sieving mechanism for exclusion. However, the reduced cell height in these regions could not be ruled out as a mechanism. Using quantitative fluorescence ratio imaging to measure the partition coefficient for tracers into these regions as a function of tracer size, it was determined that sieving is the more likely mechanism (Janson *et al.*, 1996). While the estimated mean pore size of the sieve is 50 nm in radius, the partition coefficient decays as the cube of tracer radius, leading to significant exclusion of particles well below the nominal percolation cutoff (Janson *et al.*, 1996).

3. Diffusion of Vesicle-sized Particles

The restriction of the machinery for vesicle transport to nonexcluding compartments, and the size-dependent exclusion of large particles from excluding compartments, suggest that vesicle trafficking is restricted to nonexcluding compartments. In working out a complete model of vesicle trafficking, it is important to know whether and how far a vesicle can diffuse in this compartment. Inspection of the data for diffusion of tracers up to 30 nm in radius in nonexcluding regions suggests that diffusion of the much larger vesicles might be highly restricted. In fact, it has long been noted that Brownian motion of endogenous intracellular particles is highly

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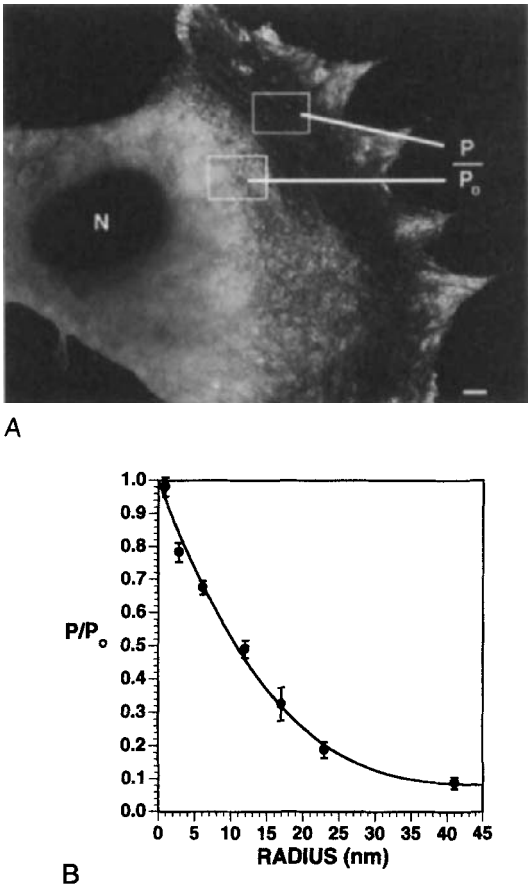

FIG. 4 Size-dependent partitioning of inert tracers in cultured fibroblasts. (A) Fluorescence ratio map of the distribution of 17 nm radius particles relative to 1 nm radius particles following injection into the cytoplasm of a single cell. Variations in intensity are directly proportional to the relative concentration of large to small particles in different regions of the cell. Regions of lower intensity are domains that exclude large particles. P is the fluorescence ratio value in excluding regions, and Po is the ratio value in nonexcluding regions. The nucleus (N) excludes large particles due to the finite size of pores in the nuclear envelope. Bar is 5 μm. (B) Partition coefficient (P/Po) plotted as a function of particle radius. Curve is nonlinear least-squares fit to a model in which exclusion is due to molecular sieving by a network of obstructions. Although the percolation size cutoff for partitioning into the excluding domains is 50 nm, the size-dependence is steep, resulting in significant exclusion of particles at sizes well below the percolation cutoff. (B) is reprinted with permission (Janson *et al.*, 1996).

constrained in living cells and that increased Brownian motion is an indicator of cellular distress (for example, Green, 1968; Wheatley and Malone, 1993). We have recently addressed this question directly by two-dimensional, single-particle tracking of 80 nm radius green fluorescent microspheres in living fibroblasts (Jones *et al.*, 1998). In order to minimize binding to intracellular components, the microspheres were made hydrophilic and electroneutral by derivatizing the surface with hydroxyls and preincubating them with rhodamine dextran to block exposed polystyrene surfaces. No adsorption of protein to the beads prepared in this way could be detected after incubation with concentrated cell lysates. By averaging the initial slopes of plots of mean squared displacement vs time, we obtained a mean D_{cyto} of 2.6×10^{-11} cm^2/sec for beads in SW3T3 fibroblasts and 4.0×10^{-11} cm^2/sec in CV1 fibroblasts. This is 500- to 1000-fold lower than the diffusion coefficient for the beads in aqueous solution. At this rate, in 1 min the bead would exhibit a mean displacement of <0.5 μm. D_{cyto} for the beads is very similar to values reported in the literature for the diffusion coefficient of endogenous vesicles in a variety of cell types. Using quasielectric light scattering, Felder and Kam (1994) obtained a mean value of 2.5×10^{-10} cm^2/sec for endogenous vesicles of unknown size in neutrophils. A value of 3×10^{-11} cm^2/sec has been reported for fluorescently stained chromaffin granules near the plasma membrane (Steyer *et al.*, 1997). Values ranging from 3.9×10^{-12} to 7.4×10^{-11} cm^2/sec were measured for secretory vesicles tagged with green fluorescent protein (Burke *et al.*, 1997). The very restricted diffusive mobility of vesicles necessitates their active transport on microtubules for efficient delivery to distant sites in the cell. On the other hand, it also ensures that beads detaching from a microtubule remain nearby, increasing the probability of reattachment.

In addition to slow diffusion due to percolation through a network of obstructions, it is possible that beads and vesicles might be confined ("caged") for some portion of their trajectory in the pores of the network. Tests for caging involve comparison of the actual area covered by an individual bead or vesicle over a long observation time with the mean squared displacement predicted from the diffusion coefficient measured over short observation times (Saxton 1995; Simson *et al.*, 1995). Applying these criteria to the trajectories of beads in CV1 and 3T3 cells indicates that some have a $>90\%$ probability of arising from caged diffusion (Jones *et al.*, 1998). The mean cage size estimated from this type of analysis is 1.5 μm in radius. The very slow diffusion coefficients measured over short times suggest that the cages are not simply fluid-filled pores, but contain additional constraints on diffusion.

To examine the role of the cytoskeleton in restricting the diffusion of vesicle-sized beads, we selectively disassembled each of the three main filament systems as independently as possible and examined the effects on

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bead diffusion (Jones *et al.*, 1998). Treatment of cells with nocodazole for times short enough to depolymerize most microtubules without noticeably affecting intermediate filaments did not remove the barrier to bead diffusion. This result is not surprising in view of the relatively low number concentration and wide interfilament spacing of the microtubules in these cell types. Decreasing the amount of polymerized actin by transient overexpression of the actin monomer-sequestering protein, thymosin, or by treatment with the actin-depolymerizing drug, latrunculin B, also had no effect on bead diffusion. Similarly, another actin-depolymerizing drug, cytochalasin D, did not alter the diffusivity of endogenous granules in neutrophils (Felder and Kam, 1994). However, treatment of cells with acrylamide, which has been shown to depolymerize intermediate filaments (Eckert, 1986) caused a nearly 10-fold increase in the mean diffusion coefficient of the beads. In qualitative agreement with this, bead diffusion decreased about 4-fold when vimentin intermediate filament protein was expressed in a vimentin-null background (Holwell *et al.*, 1997). However, even in the absence of intermediate filaments, the beads diffuse very slowly, suggesting that there are additional constraints on diffusion that do not involve the three major components of the cytoskeleton. In addition, preliminary analysis of the data suggests that caging of the beads is not abolished by any of these treatments.

4. Diffusion of Proteins and Other Macromolecules within Cytoplasm

As mentioned above, the intracellular diffusion of proteins that have hydrophobic domains and ionizable surface groups might be even slower than for inert tracer particles, due to binding interactions with intracellular components. In fact, fluorescent analogs of almost every protein ever studied by FRAP, including many that have no known binding sites inside cells, diffuse very slowly in intact cells (Table I). In general, the cytoplasmic diffusion coefficient is uncorrelated with the radius of the protein and a significant immobile component is evident in the FRAP recovery curves (Wojcieszyn *et al.*, 1981; Kreis *et al.*, 1982; Wang *et al.*, 1982; Jacobson and Wojcieszyn, 1984; Salmon *et al.*, 1984; Luby-Phelps *et al.*, 1986). Notable exceptions appear to be non-neuronal enolase (Pagliaro *et al.*, 1989; Arrio-Dupont *et al.*, 1997) and certain genetically engineered mutants of green fluorescent protein (Yokoe and Meyer, 1996; Swaminathan *et al.*, 1997). Both of these proteins recover at about the rate predicted for an inert spherical tracer of the same size—two to four times slower than in dilute aqueous solution. The crystal structure of GFP was recently reported to be a dimer "beta can" structure with very few ionizable or hydrophobic groups on its surface, which might explain its apparent lack of binding

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TABLE I

Diffusion Coefficients of Some Proteins in Living Cells[a]

Protein	Aqueous D[b] (μm^2/sec)	Radius (nm)	Cytoplasmic D(μm^2/sec)	D/Do	% mobile	Reference
Insulin	nd	1.6	0.9	(0.007)	87	Jacobson and Wojcieszyn (1984)
CaM	102	2.1	<4	0.039	81	Luby-Phelps *et al.*, (1985)
Lactalbumin	102	2.1	6.9	0.07	42	Luby-Phelps *et al.*, (1985)
Green fluorescent protein	nd		43	(0.49)	nd	Yokoe and Meyer (1996)
	87	2.5	27	0.31	82	Swaminathan *et al.*, (1997)
Ovalbumin	69	3.1	5.9	0.086	78	Luby-Phelps *et al.*, (1985)
BSA	nd	3.5	1.7	0.027	97	Jacobson and Wojcieszyn (1984)
	67	3.2	6.8	0.1	77	Luby-Phelps *et al.*, (1985)
Creatine kinase	65	3.3	<4.5	0.07	50–80	Arrio-Dupont *et al.*, (1997)
Enolase (non-neuronal)	60	3.6	7–11	0.11–0.18	100	Pagliaro *et al.*, (1989)
	56	3.8	13.5	0.24	100	Arrio-Dupont *et al.*, (1997)
Aldolase	47	4.6	6–11	0.12–0.23	>77	Pagliaro and Taylor (1988)
IgG	46	4.7	6.7	0.146	54	Luby-Phelps *et al.*, (1985)
Apoferritin	nd	6.1	1.6	(0.045)	96	Jacobson and Wojcieszyn (1984)

[a] Numbers in parentheses are calculated using the Stokes-Einstein relation to obtain the aqueous diffusion coefficient expected for a globular protein of the given radius.

[b] nd, not determined.

(Ormo *et al.*, 1996; Yang *et al.*, 1996). A more detailed examination of the binding of specific proteins is contained in the chapter by D. E. Brooks in this volume.

An interesting example of the binding of proteins in cytoplasm is calmodulin, which is commonly assumed to be freely diffusible at resting intracellular Ca^{2+} levels because, in simple, dilute solutions, its binding to most target proteins requires Ca^{2+}. However, FRAP of fluorescent analogs of CaM in fibroblasts and in unstimulated smooth muscle cells shows that at most 5 to 10% is freely diffusing even under conditions where Ca^{2+} is low and the fluorescent analog is in 50% excess of the endogenous concentration of CaM (Luby-Phelps *et al.*, 1985, 1995). Both slowly exchanging and transient binding interactions can be inferred from the complex photobleaching recovery curves for obtained fluorescent CaM in living cells. The low diffusibility of CaM at resting Ca^{2+} levels is also indicated by a study of the rapid kinetics of myosin light chain phosphorylation and force development in permeabilized portal vein smooth muscle cells upon activation by photolysis of caged ATP or Ca^{2+}. Based on the strong dependence of these parameters on exogenous calmodulin, it was calculated that only about 0.25 μM of endogenous calmodulin is freely diffusible under resting conditions (Zimmermann *et al.*, 1995).

It is not clear how much of the binding observed for proteins in intact cells reflects specific interactions of these proteins with targets defined *in vivo*. Several of the proteins tested are serum proteins that do not have known intracellular targets. However, given the modular organization of proteins, these proteins may contain protein–protein interaction domains that allow them to bind to heterologous targets inside the cell. Preliminary studies of the diffusion of site-directed mutants of CaM in living cells show that binding is not strongly dependent on the domains of CaM that have been implicated in binding to specific targets *in vitro*. This suggests that binding is mediated by alternative domains or is nonspecific. Regardless of whether the unexpected binding interactions of proteins in cytoplasm are specific, they are indicative of real effects that must be taken into account in understanding the behavior of proteins in the cytoplasm of intact living cells.

In favor of the specificity of such interactions, many proteins previously assumed to be homogeneously distributed in the aqueous phase of cytoplasm are now known to be resident on membranes or on the cytoskeleton, at least under certain conditions. Several examples are known of so-called scaffold or adaptor proteins that serve as docking sites for the assembly of multikinase signaling complexes at the plasma membrane near receptors (Pawson and Scott, 1997). In addition, kinases that exist as multiple isozymes with similar substrate specificity *in vitro* may utilize differential local-

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ization to discriminate between substrates *in vivo* (for example, Jaken, 1996; Mochly-Rosen and Gordon, 1998).

Another example of the localization of a pathway in the solid phase of the cytoplasm is glycolysis. Several key enzymes in this pathway have been shown to interact with actin filaments and microtubules *in vitro* (Masters, 1992; Knull and Minton, 1996; Ovádi and Srere, 1996; Reitz *et al.*, 1997). Binding domains on actin for aldolase, phosphofructokinase, and GAPDH have been identified antigenically (Mejean *et al.*, 1989) and an actin-binding domain on aldolase has been mapped by deletion and site-directed mutagenesis (O'Reilly and Clarke, 1993; Wang *et al.*, 1996). In some cases, enzyme activity is modified by binding to cytoskeletal filaments and the binding is modified by substrate, suggesting that glycolysis might be regulated by association with the cytoskeleton (Knull and Minton, 1996; Ovádi and Srere, 1996). There is evidence for similar interactions *in vivo*. Aldolase and GAPDH have been localized to actin-containing stress fibers in cultured fibroblasts (Pagliaro and Taylor, 1988; Minaschek *et al.*, 1992), and cell cycle changes in glycolytic activity are correlated with changes in actin filament content in some cell types (Bereiter-Hahn *et al.*, 1995). GAPDH, aldolase, creatine kinase, and glycerol-3-phosphate dehydrogenase (GPDH) are colocalized at the M-lines in skeletal muscle (Arnold and Pette, 1968; Arrio-Dupont *et al.*, 1997; Wojtas *et al.*, 1997). Mutation or deletion of the flight muscle-specific isoform of GPDH in *Drosophila* leads to flightlessness and is accompanied by mislocalization of GAPDH and aldolase (Wojtas *et al.*, 1997). Taken together, these data suggest the existence of a glycolytic complex localized at specific sites in the cytoplasm and perhaps regulated by interaction with the cytoskeleton.

Many RNA messages and polysomes also are localized to specific regions or structures of cells (for reviews, see Fulton and L'Ecuyer, 1993; Suprenant, 1993; Bassell and Singer, 1997). This has been studied most extensively in developing cardiac muscle, in migrating fibroblasts, in developing embryos, and in neurons. The mechanisms responsible for mRNA localization are just beginning to be understood. For several messages, the 3′ UTR has been shown to be both necessary and sufficient to confer specific localization of mRNA (Kislauskis and Singer, 1991; Wilson *et al.*, 1995; Ainger *et al.*, 1997). In many systems, localization has been shown to depend on an intact microtubule cytoskeleton (Suprenant, 1993) and, in neurons, microtubule-dependent transport of RNPs containing specific mRNAs has been directly observed (Ainger *et al.*, 1993; Knowles *et al.*, 1996).

Messenger RNAs are often colocalized with the proteins they encode, suggesting that translation of these messages occurs at the site of localization (Singer *et al.*, 1989; Fulton and L'Ecuyer, 1993; Cohen, 1996; Cohen and Kuda, 1996). In fact, mislocalization of messages has been shown to result in altered phenotypes (Kislauskis *et al.*, 1994; L'Ecuyer *et al.*, 1995). Consis-

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tent with this idea, a component of the translational machinery, elongation factor 1α, binds actin and colocalizes on the cytoskeleton with RNPs (Condeelis, 1995; Knowles *et al.,* 1996). Specific mRNAs and polysomes are found to copurify with microtubules from sea urchin egg extracts (Hamill *et al.,* 1994).

Such organization may be the rule rather than the exception. Early experiments involving the centrifugation of intact cells, in which stratification order reflects specific gravity, revealed very little cellular protein in the aqueous layer where soluble proteins should be found (Zalokar, 1960; Kempner and Miller, 1968a,b). More recently, the release of cellular contents following permeabilization of the plasma membrane of some cells has been reported to be incomplete or slower than expected for freely diffusing molecules. For example, mouse L929 cells released only 10–15% of cellular protein during 30 min of permeabilization with dextran sulfate despite the fact that molecules as large as 400,000 kDa were able to diffuse into the permeabilized cells from outside (Clegg and Jackson, 1988). When exogenous glucose and ATP were supplied, the permeabilized cells were found to carry out efficient glycolysis at linear rates. However, exogenously added glycolytic intermediates were inefficient in entering the glycolytic pathway (Clegg and Jackson, 1990). Intact cells dehydrated to 15% of their normal water content still carried out glycolysis at significant rates, suggesting that this pathway does not rely on a dilute aqueous phase (Clegg *et al.,* 1990). In experiments with bovine eye lenses or red blood cells permeabilized with nonionic detergents, as little as 1 to 7% of total protein was found to leak out over an 8 hr period (Kellermayer *et al.,* 1994; Cameron *et al.,* 1996). However, both these cell types are unusual in that they contain a single major protein at very high concentration. It is not clear whether proteins in most cells are similarly immobile. These findings have led some investigators to propose that nearly all cellular protein is bound to membrane and cytoskeletal surfaces within the cell and that the aqueous phase is dilute rather than crowded.

A different approach to this problem has been to implant a reference phase into intact cells as a means of assessing the diffusivity of proteins *in vivo* (Paine, 1984). In these experiments, a drop of molten gelatin was injected into *Xenopus* oocytes and allowed to gel. After waiting 20 h to allow equilibration of cellular proteins with the aqueous pores of the implanted gel, the cells were rapidly frozen at liquid nitrogen temperature, and the implanted gel and the cytoplasm were sampled by cryomicrodissection. The proteins in each sample were analyzed by two-dimensional polyacrylamide gel electrophoresis and silver staining. Based on the optical density of specific spots, it was estimated that 73% of the polypeptides analyzed exhibited some degree of binding that led to asymmetry of their distribution between the cytoplasm and the reference phase. For individual

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spots, the nondiffusive fraction ranged from 5 to 80%. At first glance, this does not seem as dramatic as the results of the permeabilization experiments. However, the long time allowed for equilibration in the reference phase experiment would favor the detection of only the highest affinity interactions, while the slow release of protein from permeabilized cells would include more transient interactions that retard diffusion. Such transient interactions have been detected for specific proteins by FRAP as described above.

IV. Concluding Remarks

There appears to be ample evidence that the interior of a living cell is not well described by the dilute solution paradigm. Although the average mobility of cellular water is no more than twofold reduced compared to bulk water, existing data do not rule out a small but significant population of immobilized water molecules immediately adjacent to membrane and cytoskeletal surfaces. The evidence suggests that the aqueous phase is crowded with macromolecules, yet at least 50% of cytoplasmic proteins appear to be resident in the solid phase. Brownian motion of particles in the aqueous phase is hindered by crowding and perhaps obstructed by the cytoskeletal network. Transient binding interactions further slow the long-range translational diffusion of proteins. For vesicle-sized particles, this hindrance renders long-range diffusion negligible and necessitates active transport. Several phenomena have been observed that might indicate the existence of microphases in the cytoplasmic volume, including the caged diffusion of microinjected beads and the size-dependent partitioning of inert tracers between excluding and nonexcluding domains of the cytoplasm. The potential impact of actual intracellular conditions on the kinetics, mechanisms, and regulation of metabolism make it imperative to reexamine continuum descriptions of cellular biochemistry that have been extrapolated from reductionist experiments carried out in dilute solution.

Acknowledgments

This chapter is dedicated to the memory of Keith R. Porter (1912–1997), who taught me to question accepted paradigms. I also gratefully acknowledge Dick McIntosh for first pointing out the finite volume problem to me several years ago, Ivan Cameron for helpful discussion of his data, and The National Science Foundation for their generous support of my research (MCB-9604594).

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