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
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 $1 \times 10^{-17}$ liters to about one-half microliter. Bacteria typically have a volume of the order of $2 \times 10^{-18}$ 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 ($6 \times 10^{-20}$ 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 example given by Halling (1989), a single bacterium will have a nominal concentration of substrate, and it would have number concentration on the order of copy numbers, number fluctuations, etc., at a given subcellular location with even low affinity for that species. Even for ions, number fluctuations can be quite significant. For example, the pH of the cell may be maintained at around 6. For a cell 10 μm in diameter, this amounts to one free proton per cell, the interior becomes acidified to pH 4 in the surrounding membrane. Assuming the increase of only 48 free protons, thus the interior of this compartment resides rather than a global pH. In these compartments, the number concentrations may be more informative than their molar concentration.

B. Assumption 2: The Cell Interior

A fundamental assumption of the phylogenetic model is that interactions between solute molecules can be expressed as total concentration of macromolecules by far the most abundant species. As the content of cells in the range 17 to 30 protein concentration in the cytoplasm as read from refractive index measurements is (Lanni et al., 1985). The low spatial resolution allows a distinction between structural and functional aspects of the total that is actually soluble debate, as discussed in more detail below.

A 20% solution of protein is very likely between solute molecules can be expressed. This is well above the theoretical "on" (Chang et al., 1987). Although the pH of a concentration of 20% is well below the hard spheres (Kertesz, 1981), some concentrations in this range. How crowd appreciated visually from the drawing depicted the cell interior with macromolecular concentration and drawn to scale (Fig
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 measurements 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).
changes in cellular volume may thus influence activities of the solutes. Many examples or substrate specificity of enzyme-catalyzed reactions can be found in the literature (Mintons shown to drive self-assembly reaction cules in vitro (Minton, 1997). For a detailed analysis of macromolecular crowding, the reader is referred to Johansson et al. in this volume.

C. Assumption 3: Substrate Concentrations

Not only are macromolecules generally present in cells than in conventional in vitro studies, the $K_d$ as determined in vitro. The calmodulin (CaM), which binds to and in response to intracellular Ca^{2+} signals, has a $K_{eq}$ of CaM for myosin light chain kinase in muscle, is around 1 mM, but the concentration is on the order of 38 mM (Tansey et al.).

The concentration of enzymes confined to subcellular organelles or peroxisomes can be even higher. Srere (1986), under conditions similar to in vivo conditions, the conversion of substrates may be efficiently catalyzed by the enzyme in a particular metabolic chapter by Övádi and Srere in this volume.

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

An inspection of any metabolic pathway of the cell contains a complex web of enzymes that compete for common substrates. It appears that a typical eukaryotic cell contains many proteins (Alberts et al., 1994). Because these proteins are typically biologically inert, their effect on the cell is negligible. In addition, most regulatory enzymes can interact with a large number of transduction pathways.
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 $K_d$s 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
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 \(3 \times 10^7\) 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 neurofilaments (Chou et al., 1997).

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Although the three types of cytoskeletal systems, a mounting box they are physically interconnected. V tissue culture cells extracted with a dimensional network in which all thr with frequent connections among the van Blerkom, 1981). Similar views ha copy of rapidly frozen tissue from whi and Kirschner, 1980). In living cells, t these three systems frequently leads the other two. In a recent example, a intermediate filaments in vitro leads microfilament systems when microinj 1992). In addition, specific proteins, shown to crosslink one filament type (Chou et al., 1997; Cunningham et al., may be better described as an aqueou (Fig. 2).

**FIG. 2.** Three views of the cytoplasm by electro in a freeze-dried, whole-mounted fibroblast. Journal of Cell Biology, 1984, vol. 99, pp. 36–1. University Press. (B) The appearance of the c that was extracted for 30 min with 0.5% Triton Reproduced from The Journal of Cell Biology, sion of The Rockefeller University Press. (C) I lightly extracted with Triton, then critical po 17,500. Arrows indicate microtubules. Reprod copyright permission of The Rockefeller Univ
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les vimentin, the cytokeratins, and the

neurofilaments (Chou et al., 1997). The concentration of vimentin in a mammalian tissue culture cell of mesenchymal origin is on the order of 150 \( \mu g/ml \), all of which is polymerized (Lai et al., 1993), resulting in an estimated 10,000 \( \mu m \) 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 Blerkum, 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 microtubular lattice in a freeze-dried, whole-mounted fibroblast. Magnification 80,000. Reproduced from The Journal of Cell Biology, 1984, vol. 99, pp. 36-12 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. 212-224 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 Blerkum (1981) by copyright permission of The Rockefeller University Press.
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 (Herfeld, 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 regulated formation and dissolution of microtubules in living cells have been alternately growing and shrinking (W). This means that local inhomogeneous

The inhomogeneities described above areas upon which phase separations mi membrane compartments have been m outer surface area of mitochondria is f few thousand μm²/cell (Mori et al., 198 end of the spectrum, the total surface reticulum in secretory cells, such as t as high as 30,000 μm²/cell (Mori et otic cells, the internal membrane surf plasma membrane. For hepatocytes, 100,000 μm²/cell, which is 50 times (Alberts et al., 1994). It is interesting the surface area of their internal orga. While this is often interpreted i organelle volume, the corresponding  are significant and could potentially p.

The cytoskeleton also presents a from the known size, shape, and con microtubules, and intermediate filam contribute about 3000 μm² of surface culture cell. This may be an understi or when one takes into account oht cytoskeleton. Whole-mount electron veals not the well-defined cytoskeletal extraction, but a three-dimensional ne that was designated the microtrabeu leagues (Wolosewick and Porter, 1975 minimize fixation artifacts in the prepara that the MTL may accurately reflect network in the living state. The surf morphometry of stereo electron micro (Gershon et al., 1985). Thus, the sur membranes together could be as high types under certain conditions. It ha pathways are immobilized and localiz branes and that substrates are transporing of the cytoplasm (Wheatley and M
The cytoskeleton varies temporally and subcellularly among all physiologically regulated cells. Disassembly of the radial array of the microtubule spindle in mitotic cytoskeleton in response to growth Recently, progress has been made in pathways that lead to these changes, mediating these changes have been and Cambiazo, 1995; Johansen, 1996; 1997; Tapon and Hall, 1997). In pure tubules undergo spontaneous phase absence of strong binding interactions as separation are recognized: filament, entropy-driven formation of nematic phase; excluded volume-driven demixing of filaments or spheres; and of polycations analogous to the cation, 1996; Tang and Janmey, 1996), an be modified by changing the length promoting the free volume by crowding as (such as polyethylene glycol (PEG) on or availability of polycations; or proteins. Similar phase separations may contribute to the inhomogeneous phase proteins are known that regulate the as well as proteins that stabilize bundles and Pantaloni, 1997; Pereira and resumed to be the immediate effectors the cytoskeleton. In addition to the 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 \( \mu m^2/cell \) (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 \( \mu m^2/cell \) (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 \( \mu m^2/cell \), 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 \( \mu m^2 \) of surface area in a typical mammalian tissue culture cell. This may be an underestimate if other types of filaments exist or if 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 \( \mu m^2 \) per cell (Gershon et al., 1985). Thus, the surface area of the MTL and internal membranes together could be as high as 200,000 \( \mu m^2 \) 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).
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).

PHYSICAL PROPERTIES OF CYTOPLASM

Attempts to address this issue exper- ological classes: measurement of the 1 measurement of the rotational mobili- cules or probes that have been intro- measuring the properties of intracel- measurement of water mobility and m- ing the colligative properties of cells.

1. Measurements of Water Mobility

Two methods have been used to me proton NMR and quasielastic neutro have the advantage of being noninvasi water, the $^1$H-NMR signal from cell: In pure water, the transverse relaxatio of the proton dipoles subsequent to c thus, a measure of rotational diffusion be shortened due to local fields in the s populations with different properties. about 1/50 that of pure water protos the mechanism of accelerated relaxatio two or more populations of water weighted average of the relaxation t cell. When $T_2$ is corrected to 100% wat rotational mobility of cellular water is Rorschach et al., 1991; Cameron et molecules can diffuse over distances in the relatively long time required for $T_2$ could reflect encounters with phy altered rotational mobility. QENS in this ambiguity because of the short di- ments are made. Unfortunately, beca nonphysiological conditions for sever been limited to a few hardy cell and t Artemia and frog sartorius muscle ( a, 1984). As found by NMR, the $T$ intracellular water measured by QEN with pure water. In the cases where av available for the same cell or tissue, th between the two parameters (Ca- tative interpretation of these data is a and proportions of each water fractic methods. Furthermore, it may not t
plasm (Measured)

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 thus, a measure of rotational diffusion. In more complex milieus, $T_2$ 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; Tranham 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
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 \(-34^\circ C\). 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 (II) over a limited range as expected for an osmometer (for review, see Cameron et al., 1997). However, linear extrapolation of the data to infinite II 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 osmotic pressure 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 osmolarity. In addition, volume changes may not be directly coupled to osmotic pressure, because the ratio of plasma membrane surface area to cell volume of the membrane (Clegg, 1986).

Measurements of the colligative properties have also been used to predict the anesthetic effect of water on cells. For example, plots of water content vs inverse osmotic pressure suggest that the osmotic effect of water is significant. If the osmotic effect is reduced (Van Steveninck et al., 1991), the osmotic effect can be predicted by the use of an equation based on the dependence of water on the internal water content. This equation can then be used to predict the anesthetic effects of water on the cells. Reviews of this subject are available (Clegg, 1986).

3. Measurements of Solute Rotation

The second approach to examining the rotational mobility of small spin probes has been to study the rotational mobility of small spin probes in living cells. As an example, the rotational mobility of small spin probes has been introduced into living cells. Various values have been estimated for the rotational mobility of small spin probes in living cells. As an example, the rotational mobility of small spin probes has been introduced into living cells. Various values have been estimated for the rotational mobility of small spin probes in living cells. As an example, the rotational mobility of small spin probes has been introduced into living cells. Various values have been estimated for the rotational mobility of small spin probes in living cells.
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PHYSICAL PROPERTIES OF CYTOPLASM

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 cyto-
plasm 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
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
ceresiae by biosynthetically labeling them with 5-fluorotryptophan (Wili-
ams 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 1H-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 pro-
on 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
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 insolvency 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 relative to bulk water (Bicknese et al., 1990). Mobility of water in living cells does compared with pure water, but significant water cannot be ruled out.

### B. Hindrance of Long-Range Trans

#### 1. Diffusion of Protein-sized Tracer

In addition to possible effects of water of solutes, the long-range translation of cytoplasm could be hindered by the and by steric constraints imposed by Studies of the translational diffusion particles suggest that such long-range Two different fluorescence microscopy studies to obtain the cytoplasmic diffusion coefficient of B shows that the exchange of bleaching or recovery of fluorescence in the b) that characterizes the recovery is in diffusion coefficient (D) of the fluoro In an alternative approach that avoids high-power laser illumination, low light was used to monitor the spread of fluorescent dye, BCECF (Kao et al., 1990). Although dextrans and FColl do components, the results of these studies also more slowly in intact cells than in dextran (Hou et al., 1990; Popov and Poo, 1997). Comparison of the diffusion solutions of unlabeled dextran or FColl cytoplasm in fibroblasts resembles (L. 1990). A similar result was obtained with a fluorescent dye, BCECF (Kao et al., 1990).
tion, the high steady-state polarization be directly interpreted in terms of the fluorescence lifetime of the probe; recently, a series of papers from the the use of time resolved fluorescence resonance probe BCECF to measure Van, 1991; Periasamy et al., 1991, 1992). At binding to intracellular components, orth for data analysis by which free und. Measurements of lifetime showed nfluenced by the intracellular environ- nes, these studies indicated that cyto- cically different from bulk water (Fus- or, cells such as sea urchin eggs and osmotic conditions by making small fold increased solvent viscosity (Peria- sant spatial variation in viscosity was ot different from the cytoplasm. More anisotropy imaging of sulfrothodamine viscosity of a range of cell types (Srivase- free and bound fractions of the probe by a marked difference in fluorescence e apparent solvent viscosity was very patial variations on a distance scale of rially have a higher viscosity and moreells. A somewhat different approach reservoir ratio imaging to map solvent icrophore attached to dextran (Luby-osity in the cytoplasm of two mamma- ppear significantly different from bulk observed beyond that accounted for by at glance, to be in conflict with data on S. However, an inherent limitation of xosity is that steric hindrance or, probes to compartments where water spatial resolution of standard imaging tiated layers of ordered water up to he spatial resolution can be improved resonance (TIRF) imaging to measure nt probes within 50 nm of the ventral d with time-resolved anisotropy indi- cent to the membrane was unimpeded 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
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).

![Graph showing relative diffusion coefficients of inert fluorescent tracers in fibroblasts](image)

**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 contributes 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.

**Physical Properties of Cytoplasm**

In cultured *Xenopus* neurons D/Do d ranging from 2 to 44.8 nm in hydrodynam

In skeletal muscle myotubes, D/Do d ranging from 2.9 to 12.6 nm in radi size-dependence is consistent with some of this idea, in neurons the size-depen
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rowding effect, several groups have considered the two-dimensional diffusion of aqueous buffer (D/Do) (Fig. 3) declined from 0.3 to 0.04 for particles of mic radius (Luby-Phelps et al., 1987).

- 25 30 35 40 45 50

DIUS (nm)

fluorescent tracers in fibroblasts (filled circles, open circles from Luby-Phelps Dupont et al. (1986), and Seksek et al. (1997)). The model predicts a network volume fraction of 0.110 mg/ml (Hou et al., 1990). Lins et al., (1997) (open circles) with slope constrained D/Do in the two studies of fibroblasts.

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 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 depends critically on the accurate estimation of the fluorescence intensity at infinite time, F(∞). 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 (Ygerabide et al., 1982), and then extrapolating to F(∞). In the more recent study, F(∞) 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.
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). Immuno-fluorescence 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 tracer ratio map of the distribution of 17 nm radius particles injection into the cytoplasm of a single cell. The relative concentration of large to small particles of lower intensity are domains that exclude in excluding regions, and Po is the ratio of particles due to the finite size. (B) Partition coefficient (P/Po) plotted as a least-squares fit to a model in which exclusions obstruct. Although the percolation size cutoff is 50 nm, the size-dependence is steep, result well below the percolation cutoff. (B) is represented.

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regions could not be ruled out as a scence ratio imaging to measure the these regions as a function of tracer the more likely mechanism (Janson an pore size of the sieve is 50 nm in s as the cube of tracer radius, leading well below the nominal percolation s.
" vesicle transport to nonexcluding at exclusion of large particles from et vesicle trafficking is restricted to ing out a complete model of vesicle ther and how far a vesicle can diffuse e data for diffusion of tracers up to ns suggests that diffusion of the much ed. In fact, it has long been noted us intracellular particles is highly

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 $D_{\text{cyto}}$ of $2.6 \times 10^{-11} \text{ cm}^2/\text{sec}$ for beads in SW3T3 fibroblasts and $4.0 \times 10^{-11} \text{ cm}^2/\text{sec}$ 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 $<0.5 \mu\text{m}$. $D_{\text{cyto}}$ 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 $2.5 \times 10^{-10} \text{ cm}^2/\text{sec}$ for endogenous vesicles of unknown size in neutrophils. A value of $3 \times 10^{-11} \text{ cm}^2/\text{sec}$ has been reported for fluorescently stained chromaffin granules near the plasma membrane (Steyer et al., 1997). Values ranging from $3.9 \times 10^{-12}$ to $7.4 \times 10^{-11} \text{ cm}^2/\text{sec}$ 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 $\mu\text{m}$ 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 bead diffusion (Jones et al., 1998). The times short enough to depolymerize n affecting intermediate filaments did n. This result is not surprising in concentration and wide interfilament cell types. Decreasing the amount of p repression of the actin monomer-sequen with the actin-depolymerizing d on bead diffusion. Similarly, another laisin D, did not alter the diffusiv of (Felder and Kam, 1994). However, which has been shown to depolymyb 1986) caused a nearly 10-fold increase the beads. In qualitative agreement about 4-fold when vimentin interacts a vimentin-null background (Holm absence of intermediate filaments, the that there are additional constraints three major components of the cytoske sis of the data suggests that caging of these treatments.

4. Diffusion of Proteins and Other within Cytoplasm

As mentioned above, the intracellular phobic domains and ionizable surface for inert tracer particles, due to bindin ponents. In fact, fluorescent analogs by FRAP, including many that have diffuse very slowly in intact cells (T diffusion coefficient is uncorrelated y significant immobile component is ev (Wojcieszyn et al., 1981; Kreis et al., 1 Wojcieszyn, 1984; Salmon et al., 1984 exceptions appear to be non-neuronal Dupont et al., 1997) and certain gene fluorescent protein (Yokoe and Mey Both of these proteins recover at at spherical tracer of the same size—tw aqueous solution. The crystal structu be a dimer "beta cat" structure with groups on its surface, which might e
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; Arri-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
TABLE I
Diffusion Coefficients of Some Proteins in Living Cells

<table>
<thead>
<tr>
<th>Protein</th>
<th>Aqueous D* (μm²/sec)</th>
<th>Radius (nm)</th>
<th>Cytoplasmic D(μm²/sec)</th>
<th>D/Do</th>
<th>% mobile</th>
<th>Reference</th>
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<td>Insulin</td>
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<td>0.9</td>
<td>(0.007)</td>
<td>87</td>
<td>Jacobson and Wojcieczyn (1984)</td>
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<tr>
<td>CaM</td>
<td>102</td>
<td>2.1</td>
<td>&lt;4</td>
<td>0.039</td>
<td>81</td>
<td>Luby-Phelps et al., (1985)</td>
</tr>
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<td>2.1</td>
<td>6.9</td>
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<td>42</td>
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<td>Green fluorescent protein</td>
<td>nd</td>
<td>87</td>
<td>43</td>
<td>(0.49)</td>
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<td>3.5</td>
<td>1.7</td>
<td>0.027</td>
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<tr>
<td>67</td>
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<td>6.8</td>
<td>0.1</td>
<td>77</td>
<td></td>
<td>Luby-Phelps et al., (1985)</td>
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<td>50–80</td>
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<td>7–11</td>
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</tr>
<tr>
<td>56</td>
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<td>0.24</td>
<td>100</td>
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<td>&gt;77</td>
<td>Pagliaro and Taylor (1988)</td>
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<td>IgG</td>
<td>46</td>
<td>4.7</td>
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<td>54</td>
<td>Luby-Phelps et al., (1985)</td>
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<td>Apoferritin</td>
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<td>1.6</td>
<td>(0.045)</td>
<td>96</td>
<td>Jacobson and Wojcieczyn (1984)</td>
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</tbody>
</table>

*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.

*nd, not determined.
PHYSICAL PROPERTIES OF CYTOPLASM

(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 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 multisite signaling complexes at the plasma membrane near receptors (Pawson and Scott, 1997). In addition, kinases that exist as multiple isoforms with similar substrate specificity in vitro may utilize differential local-

<table>
<thead>
<tr>
<th>BSA</th>
<th>Crease kinase</th>
<th>Enolase (non-neuronal)</th>
<th>Aldolase</th>
<th>IgG</th>
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<td>46</td>
<td>47</td>
<td>47</td>
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</table>

* Numbers in parentheses are calculated using the Stokes–Einstein relation to obtain the aqueous diffusion coefficient for a globular protein.
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; Knill and Minton, 1996; Óvádi 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 (Knill and Minton, 1996; Óvádi 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 glyceraldehyde 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-
Strategies in vivo (for example, Jaken, 1998). A pathway in the solid phase of enzymes in this pathway have been shown to involve microtubules in vitro (Masters, 1991; Srere, 1996; Reitz et al., 1997). 2, phosphofructokinase, and GAPDH (Jean et al., 1989) are known to act as nucleation points for microtubule assembly. In some cases, actin undergoes a similar interaction (Knoll and Minton, 1996; Ovadi, 1992). Calcium-dependent interactions in vivo occur through aldolase into containing stress fibers in cultured cells (Minaschek et al., 1992), and vimentin correlates with changes in vimentin (Bereiter-Hahn et al., 1995). The glycogen that localizes in skeletal muscle (Arnold, 1997; Wojtas et al., 1997). Mutation of the isofrom of GAPDH in Droso phila aned to localization of GAPDH taken together, these data suggest that the localization of specific sites in the cytoplasm with the cytoskeleton. Strands also are localized to specific regions of the cytoplasm. Fulton and L’Ecuyer, 1993; Suprenant, 1995; has been used most extensively in growing fibroblasts, in developing embryos, to understand mRNA localization are several messages, the 3' UTR has sufficient to confer specific localization in 191; Wilson et al., 1995; Ainger et al., 1995. Ainger et al., 1995), and in neurons, microtubule-ligning specific mRNAs has been directly demonstrated with the proteins they encode. cssages occurs at the site of localization (L’Ecuyer, 1993; Cohen, 1996; Cohen and colleagues have shown that related I., 1994; L’Ecuyer et al., 1995). Consis-

tent with this idea, a component of the translational machinery, elongation factor 1a, binds actin and localizes on the cytoskeleton with RNPs (Condellis, 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; Kempen 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 their cellular content 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 of 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 implanted 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
spots, the nondiffusible 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 tran-
sient 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,
mechanism, 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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that the interior of a living cell is a rather complex system. Although the average molecule is much larger than the size of a typical macromolecule, the volume of the cytoplasm is large enough to accommodate a significant fraction of the cell's total volume. The diffusion of molecules in the cytoplasm is limited by the presence of macromolecules, such as proteins, which can act as obstacles to the free movement of other molecules.

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PHYSICAL PROPERTIES OF CYTOPLASM


