

Probing the Structure of Cytoplasm

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Abstract. We have used size-fractionated, fluorescent dextrans to probe the structure of the cytoplasmic ground substance of living Swiss 3T3 cells by fluorescence recovery after photobleaching and video image processing. The data indicate that the cytoplasm of living cells has a fluid phase viscosity four times greater than water and contains structural barriers that

restrict free diffusion of dissolved macromolecules in a size-dependent manner. Assuming these structural barriers comprise a filamentous meshwork, the combined fluorescence recovery after photobleaching and imaging data suggest that the average pore size of the meshwork is in the range of 300 to 400 Å, but may be as small as 200 Å in some cytoplasmic domains.

BULK cytoplasm is a gel-like substance that exhibits viscoelastic and thixotropic behavior. That is, there is hysteresis in its recoil after stretching (or compression), and its viscosity is much higher at low, rather than at high, rates of shear. Cytoplasm appears to exist in three interconvertible states: solated, gelled, and contracted. Subcellular, localized transitions between these states are thought by some investigators to be intimately involved in cellular events such as cell division, phagocytosis, locomotion, and intracellular transport. (For reviews, see Porter, 1984; Pollard, 1984; Taylor and Condeelis, 1979; Stossel, 1982; for an overview of some current approaches to the study of cytoplasm, see *The Cytoplasmic Matrix*, 1984, *J. Cell Biol.* 99[1, Pt. 2].)

Among the primary candidates for the structural components of the cytoplasmic matrix are three types of filaments found abundantly in cytoplasm, F-actin, microtubules, and intermediate filaments, along with an assortment of accessory proteins that cross-link these filaments *in vitro* (e.g., Pollard, 1984; Lieska et al., 1985). Morphological studies have indicated that the filamentous components and their associated proteins in some combination may be interconnected into an isotropic network that pervades the cytoplasmic space, and confers on cytoplasm the properties of a viscoelastic, thixotropic, contractile gel (Wolosewick and Porter, 1979; Schliwa and Van Blerkom, 1981; Heuser and Kirschner, 1980; Penman et al., 1981; Stossel, 1982; Pollard, 1984; Taylor and Fehheimer, 1982; Ris, 1985). However, there is little direct evidence from intact, living cells as to precisely how the interaction of filaments and cross-linkers determines the properties of cytoplasm and what role their interaction plays in cellular events. The presence of a cross-linked network of filaments has not yet been demonstrated in living cells, the fluid phase viscosity of the cytoplasmic gel in living cells is not well understood, and the average "pore size" (better described as the permeation size parameter, or mesh) of the cytoplasmic gel in living cells is completely unknown.

Measurements of the fluid phase viscosity of cytoplasm in

several types of cells, by electron spin resonance and by low temperature autoradiography of small radiolabeled dextrans have uniformly indicated a value 2–6 times that of water at 22°C (Mastro et al., 1984; Lepock et al., 1983; Paine and Horowitz, 1980). While several of these studies were done specifically to look for cytoplasmic structure, no evidence was found that the cytoplasm is anything other than a fairly concentrated solution of protein. However, as noted by these authors, probes of the relatively small sizes used for these studies would not necessarily have detected higher order structure (Paine and Horowitz, 1980; Mastro et al., 1984; Lepock et al., 1983). In contrast, the resistance to displacement of magnetic particles introduced into intact, living cells suggests that large particles the size of organelles may experience an effective viscosity orders of magnitude greater in cytoplasm than in water (Crick and Hughes, 1950; Sato et al., 1983; Valberg and Albertini, 1985).

Fluorescence recovery after photobleaching (FRAP)¹ can be used both to measure the fluid phase viscosity of cytoplasm and to detect higher order structure (such as a cross-linked cytoplasmic gel) in living specimens, without the application of mechanical stress (shear) that damages or disrupts cytoplasmic structure. By using fluorescent molecules of a range of sizes as probes, the mesh of a cytoplasmic gel can be estimated from FRAP data as well. The ideal probes for such a study would be rigid compact spheres of known sizes that do not exhibit long-range interactions with or binding to intracellular components. Originally several laboratories attempted to use fluorescently labeled peptides and globular "control" proteins as probes (Wojcieszyn et al., 1981; Wang et al., 1982; Kreis et al., 1982; Jacobson and Wojcieszyn, 1984; Luby-Phelps et al., 1985). Unfortunately, the cyto-

1. *Abbreviations used in this paper:* AF-actin, iodoacetamidofluorescein-labeled actin; D_{aq} , aqueous diffusion coefficient; D_{cyto} , cytoplasmic diffusion coefficient; FRAP, fluorescence recovery after photobleaching; FTC, fluorescein thiocarbonyl; R_G , radius of gyration; R_H , hydrodynamic radius; TRTC, tetramethylrhodamine thiocarbonyl.

plasmic mobilities of these fluorescent peptides and proteins did not scale inversely with molecular size, suggesting to these investigators that their diffusion in cytoplasm is complicated by transient binding to some unidentified cytoplasmic component (Wojcieszyn et al., 1981; Wang et al., 1982; Gershon et al., 1985; Mastro et al., 1984; Jacobson and Wojcieszyn, 1984; Luby-Phelps et al., 1985).

Size-graded fluorescein thiocarbamyl-dextrans (FTC-dextrans) appear to be better probes for FRAP studies of cytoplasmic structure than fluorescent proteins, since dextrans do not appear to interact significantly with intracellular components (Paine and Horowitz, 1980). This is supported by our preliminary evidence: FTC-dextrans in the cytoplasm of living 3T3 cells exhibited a single component recovery after photobleaching and recovered to ~100% of the fluorescence intensity before photobleaching. The smallest dextrans indicated a value for cytoplasmic viscosity comparable to previous reports (see above), and mobility in cytoplasm was inversely dependent on molecular size, as predicted by diffusion theory (Luby-Phelps et al., 1985). The dextran backbone is electrically neutral, so the FTC-dextrans have only a very low charge density, conferred on them by the fluorescein residues, thus minimizing the potential for screened electrostatic interactions. FTC-dextrans are not particularly hydrophobic molecules and are readily soluble in water. Dextran does not have a well-defined configuration because it is a flexible, branched random-coil polymer, rather than a rigid, compact sphere. However, its size can be defined statistically by the radius of gyration (R_G). We have used fluorescein- and rhodamine-labeled dextrans to probe the fluid phase viscosity and higher order structure of cytoplasm in living 3T3 cells by FRAP and by video image analysis, as a first step toward understanding the structure and function of the cytoplasmic ground substance.

Materials and Methods

FRAP

Fluorescence photobleaching recovery was performed as described (Luby-Phelps et al., 1985). Briefly, an argon-ion laser (Spectra-Physics Inc., Mountain View, CA) was focused through a Zeiss Universal microscope with epifluores-

cence optics. The laser was operated at 150 mW, and was tuned to either the 488-nm line or the 514-nm line for bleaching of fluorescein or rhodamine, respectively. Bleaching pulses ranged from 1.8 ms to 1 s. Fluorescence recovery was monitored with the laser beam attenuated 3,600 times. Data were analyzed according to Yguerabide et al. (1982).

Cell Culture

Swiss 3T3 cells (American Type Culture Collection, Rockville, MD) were grown in Dulbecco's modified Eagle's medium and 10% calf serum (GIBCO, Grand Island, NY) as previously described (Luby-Phelps et al., 1985).

Preparation of Fractionated Dextrans

FTC-dextrans of various sizes were purchased from Sigma Chemical Co. (St. Louis, MO). FTC-dextran of M_r 157,000 was further fractionated by gel filtration on S-300 (Pharmacia Fine Chemicals, Piscataway, NJ). Elution of FTC-dextran from the column was monitored by absorbance at 495 nm. The resulting broad peak was divided into four equal parts and fractions within each part were pooled. Each pool was dialyzed against distilled water, lyophilized, and resuspended in 1 ml of injection buffer (2 mM Pipes, pH 7.0, 0.05 mM $MgCl_2$). The concentrations of the resulting fractions were determined by lyophilizing 100 μ l and weighing the dry powder. Aliquots of each fraction were stored frozen at $-70^\circ C$ until use. T40, T500, and T2000 dextran were purchased from Sigma Chemical Co. and were labeled with fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate according to the method of De Belder and Granath (1973). The labeled products were fractionated on Sepharose CL-4B (Pharmacia Fine Chemicals), dialyzed against distilled water, lyophilized, and resuspended in a small volume of injection buffer. Aliquots were stored frozen at $-70^\circ C$ until use. Substitution ratios (reported in Table I) were determined from dye absorbance at 495 nm (pH 8.0) or 550 nm, and the dry weight of the labeled dextran. Except in the case of tetramethylrhodamine thiocarbamyl-dextrans (TRTC-dextrans), the average hydrodynamic radius of each dextran fraction was estimated based on the diffusion coefficient calculated from FRAP in aqueous solution at $22^\circ C$ (D_{aq}). These values are reported in Table I. Radius of gyration (R_G) was estimated according to Tanford (1961) by dividing the observed hydrodynamic radius by 0.665. For calculation of D_{cyto}/D_{aq} , values of D_{aq} measured at $22^\circ C$ were corrected to $37^\circ C$.

Microinjection and Imaging

FTC- or TRTC-dextrans at a concentration of 5–10 mg/ml were injected into Swiss 3T3 fibroblasts as previously described (Luby-Phelps et al., 1985). For selective visualization of mitochondria, cells were stained with DiI_{C3}-(3) (Simms et al., 1974) at a concentration of 10^{-7} M for 5–10 min. Iodoacetamidofluorescein-labeled actin (AF-actin) was prepared as previously described (Wang and Taylor, 1980) and was microinjected at a concentration of 2–4 mg/ml. Fluorescence microscopy was performed with a Zeiss photomicroscope using either a $25\times$ (N.A. 0.8) or $63\times$ (N.A. 1.2) Neofluar water immersion lens. Specimens were maintained at $37^\circ C$ using a Sage air curtain and a thermoelectric stage (Cambridge Thermionic Corp., Cambridge, MA). For video image

Table I. FRAP of Fluorescent Dextrans in Aqueous Solution and in 3T3 Cell Cytoplasm

R_H	R_G	$D_{aq} \times 10^6$	$D_{cyto} \times 10^7$	D_{aq}/D_{cyto}^*	Substitution ratio
21.6	32.0	1.01 ± 0.2	2.9 ± 0.67 (13)	4.8	0.004
31.0	46.5	0.70 ± 0.03	1.8 ± 0.32 (4)	5.4	0.005, 0.01
TRTC [‡]	46.5		1.6 ± 0.75 (3)	6.1	0.004
41.5	62.0	0.52 ± 0.05	1.3 ± 0.33 (11)	5.6	0.004
47.0	70.5	0.46 ± 0.03	1.1 ± 0.29 (15)	5.9	0.004
62.0	93.0	0.35 ± 0.03	0.75 ± 0.23 (11)	6.5	0.01
70.0	105.0	0.31 ± 0.06	0.56 ± 0.18 (15)	7.6	0.01
94.0	141.0	0.23 ± 0.03	0.23 ± 0.11 (14)	14.0	0.01
TRTC [‡]	141.0		0.37 ± 0.07 (6)	8.7	0.002
243	364.5	0.088 ± 0.003	0.10 ± 0.04 (10)	12.32	0.01
386	579.0	0.056 ± 0.003	0.033 ± 0.003 (9)	23.75	0.01

R_H is apparent hydrodynamic radius. R_G is estimated radius of gyration (Tanford, 1961). D_{aq} is diffusion coefficient in aqueous solution at $22^\circ C$ (cm^2/s^{-1}). D_{cyto} is diffusion coefficient in 3T3 cell cytoplasm at $37^\circ C$ (cm^2/s^{-1}). D_{aq}/D_{cyto} was calculated after correction of D_{aq} to $37^\circ C$. Substitution ratio is number of dye molecules per glucose residue in the dextran. Diffusion coefficients are listed as the mean \pm SEM. Numbers in parentheses are the number of observations used to calculate the mean.

* D_{aq} corrected to $37^\circ C$.

[‡] Accurate hydrodynamic radius unknown: size estimated by gel filtration chromatography.

enhancement and analysis, cells were viewed with an ISIT video camera (Dage/MTI Inc., Michigan City, IN) or a Zeiss Venus three-stage intensified camera, and integrated images were acquired using a Hamamatsu Photonics image processor (Photonics Microscopy, Inc., Oak Brook, IL) or a Vicom image processor (Vicom Systems, Inc., San Jose, CA). In attempt to present the data in Fig. 3 in an unbiased manner, the fluorescein and rhodamine images were acquired at the same excitation intensity, camera gain, and integration time. Without adjusting the contrast or brightness of the display, 35-mm photographs of the two images were taken at the same *f* stop on the same roll of Ilford HP5 film and were developed in Diafine (Ilford Ltd., U.K.). Pictures were printed on the same grade of paper. Due to the loss of gray level resolution inherent in this process, differences in distribution of the two fluorophores appear more subtle in Fig. 3 than through the microscope.

Results

Characterization of Labeled Dextrans in Aqueous Solution

Aqueous diffusion coefficients for fractionated FTC-dextrans of a range of sizes were obtained by FRAP, using a spot 50 μm in radius to bleach solutions contained in 100- μm glass capillaries (Vitro Dynamics Inc., Rockway, N.J.) (Table I). As expected from diffusion theory, the diffusion coefficients were inversely dependent on molecular size. The values obtained were used to calculate the average hydrodynamic radius (R_H) of each dextran fraction from the Stokes-Einstein equation: $D = kT/6\pi\eta R_H$, where D is the diffusion coefficient, k is the Boltzmann constant, η is the solvent viscosity, and T is temperature in degrees Kelvin. These values of R_H are somewhat smaller than predicted for unbranched, random-flight polymers of the given molecular weights (Benoit, 1948). This may reflect the fact that dextrans do have a low percentage of short branches (Grotte, 1956; Ogston and Woods, 1953; Larm et al., 1971). For this reason, dextrans are listed in Table I by apparent hydrodynamic radius rather than molecular weight. Radii of gyration (R_G) were estimated from R_H according to the Kirkwood-Riseman theory ($R_G = R_H/0.665$; Tanford, 1961).

We were unable to obtain aqueous diffusion coefficients for TRTC-dextrans by FRAP for lack of sufficient laser power

at 514 nm. For this reason, the sizes of TRTC-dextran fractions were estimated from their K_{av} on Sepharose CL-4B, using fractionated FTC-dextrans as size standards.

Distribution of Labeled Dextrans in 3T3 Cell Cytoplasm

Labeled dextrans of various sizes were microinjected into living 3T3 cells and their subcellular distributions were compared 3–6 h later. All dextrans were excluded from mitochondria and small vesicles (Fig. 1). M_r 20,000 FTC-dextran (estimated $R_G = 32 \text{ \AA}$) permeated the cytoplasm and entered the nucleus where it was excluded from nucleoli (Fig. 1). Dextrans with an estimated $R_G > 46 \text{ \AA}$ permeated the cytoplasm, but did not enter the nucleus. Dextrans with estimated $R_G > 70 \text{ \AA}$ apparently were excluded from large stress fibers, allowing visualization of stress fibers in some cells by a negative staining effect (Fig. 2). Dextrans with $R_G > 100 \text{ \AA}$ appeared to be excluded additionally from some cytoplasmic domains (relative to the 32 \AA dextran) (Fig. 3).

FRAP of Dextrans in 3T3 Cell Cytoplasm

The mobilities of labeled dextrans of various sizes in the cytoplasm of 3T3 cells are presented in Table I. Spots 6 μm in radius were bleached in the perinuclear region, the extreme periphery, and halfway along a radius of each cell. The diffusion coefficient of the mobile species was determined from the recovery half-life (t) according to the equation $D = \gamma w^2/4t$, where w = Gaussian spot radius and γ = correction factor for % bleaching (Axelrod et al., 1976; Yguerabide et al., 1982). Within experimental error, the value of D obtained did not depend on the location of the bleached spot, although scatter in the data may reflect the occurrence of local regions of differing structure. As predicted by diffusion theory, the mobilities of dextrans were inversely dependent on molecular size. Dextrans of R_G smaller than 141 \AA exhibited 100% recovery of a single fluorescent component after photobleaching. In general, the effective viscosity indicated by labeled dextrans increased with increasing molecular size. A plot of

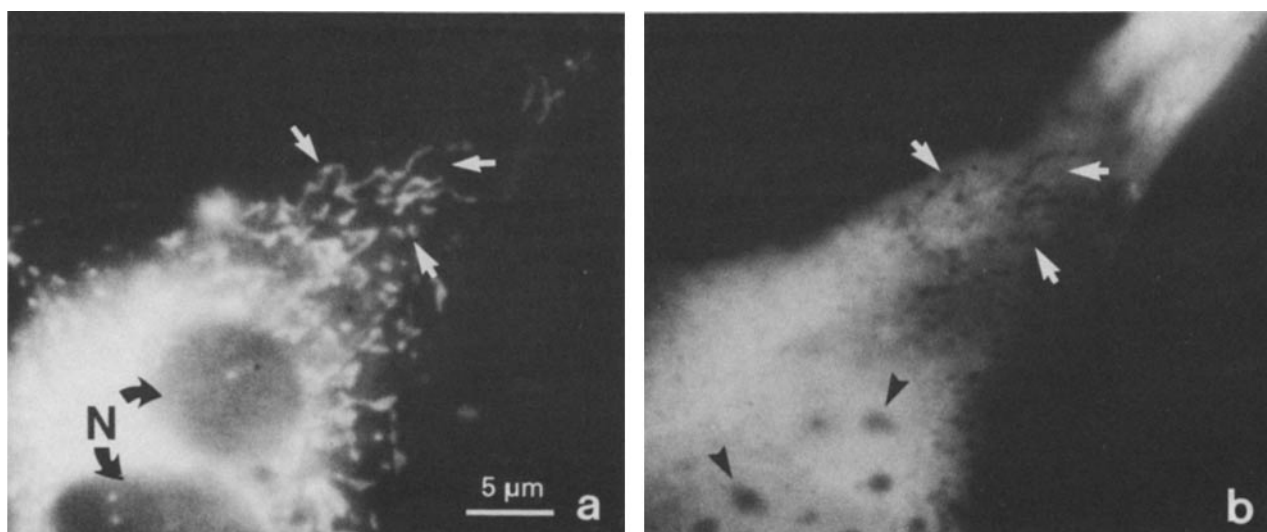


Figure 1. Fluorescent dextrans are excluded from mitochondria. Fluorescence images of a portion of a Swiss 3T3 cell. (a) Specific staining of mitochondria with DiIC₃(3). White arrows indicate region with distinct mitochondria. *N* denotes nucleus. (b) M_r 20,000 FTC-dextran in same cell, showing exclusion of dextran from mitochondria. This size dextran enters the nucleus, but is excluded from nucleoli (black arrowheads).

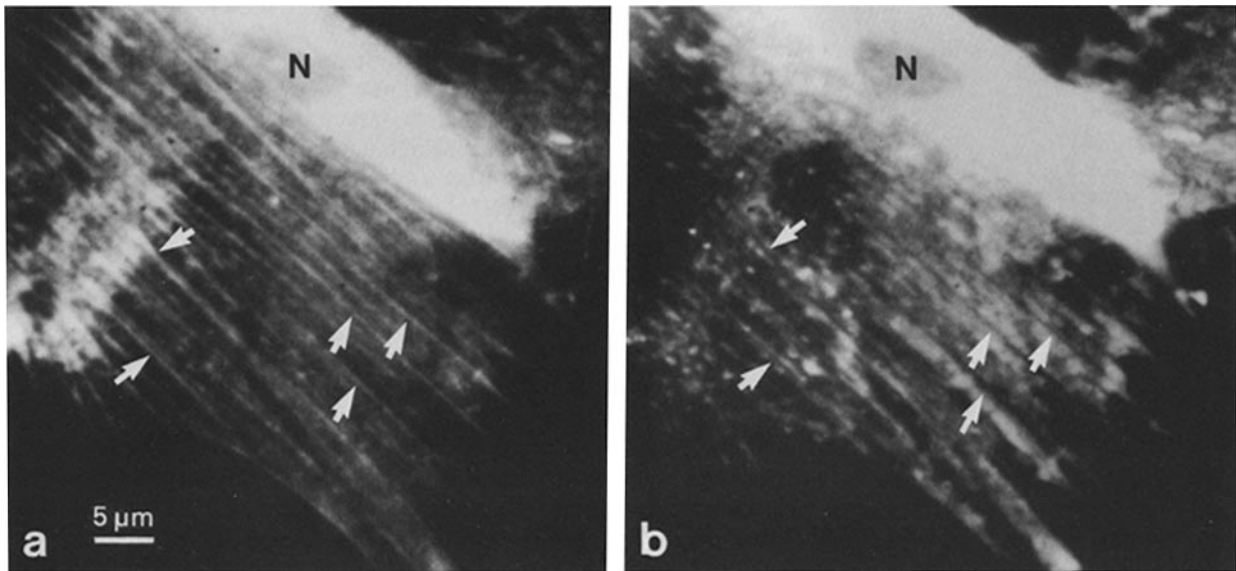


Figure 2. Dextran of $R_G > 70 \text{ \AA}$ are visibly excluded from actin-containing stress fibers. (a) Fluorescence image of AF-actin in a 3T3 cell. (b) Fluorescence image of 140 Å TRTC-dextran in the same cell. White arrows indicate stress fibers. *N* denotes the nucleus, which excludes both AF-actin and 140 Å TRTC-dextran.

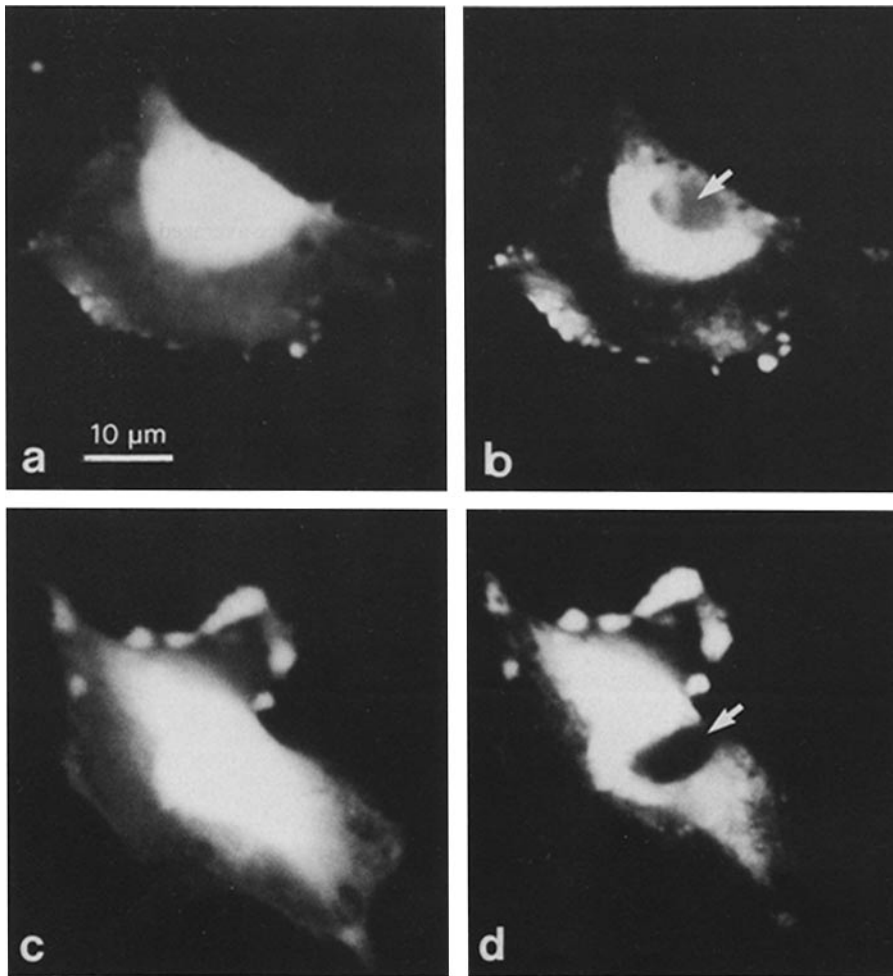


Figure 3. Dextran of $R_G > 100 \text{ \AA}$ are relatively excluded from some cytoplasmic domains. Comparison of the distributions of M_r 10,000 TRTC-dextran (estimated $R_G < 30 \text{ \AA}$) and 580 Å FTC-dextran in the same cells. (a and c) TRTC-dextran. (b and d) FTC-dextran. White arrows indicate nuclei, which exclude the large dextran.

($D_{\text{cyto}}/D_{\text{aq}}^{37^\circ\text{C}}$) vs. R_G decreased linearly up to $R_G = 141 \text{ \AA}$ (Fig. 4). Beyond this point $D_{\text{cyto}}/D_{\text{aq}}^{37^\circ\text{C}}$ was nearly constant, or slowly decreasing, and in some cases fluorescence did not recover to 100% of its initial value before photobleaching.

Extrapolation of $D_{\text{cyto}}/D_{\text{aq}}$ to zero radius gives an estimate of 4 cp for the effective viscosity experienced by a point particle diffusing in the cytoplasm. This should be equivalent to the fluid phase viscosity of cytoplasm when a small correction is

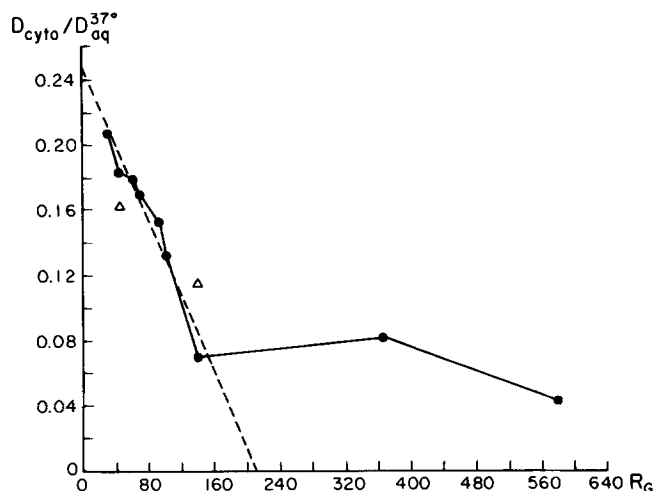


Figure 4. Effective viscosity experienced by dextrans in cytoplasm is size dependent. Plot of $1/\text{effective viscosity } (D_{\text{cyto}}/D_{\text{aq}})$ vs. estimated radius of gyration (R_G). Values of D_{aq} were corrected to 37°C . R_G was estimated from apparent hydrodynamic radius according to Tanford (1961). Filled circles are FTC-dextrans, open triangles are TRTC-dextrans. $1/\text{effective viscosity}$ decreases linearly up to $R_G = 140 \text{ \AA}$. Dashed line is best-fitting line calculated from data by regression analysis (correlation coefficient = -0.97). See text for discussion.

Table II. FRAP of FTC-Dextrans in Concentrated Protein Solutions

R_H	D/D_0	20% BSA	7% BSA	Egg white
31		0.159	0.366	0.345
70		0.208	0.518	0.377
386		0.217	0.625	
Bulk viscosity (cp)		3.6	1.36	3.77

R_H is apparent hydrodynamic radius calculated from FRAP in aqueous solution without added protein. D/D_0 is the ratio of diffusion coefficient in concentrated protein solution to diffusion coefficient in aqueous solution with no protein. Bulk viscosity is the viscosity of each protein solution determined by Cannon/Ostwald viscometry.

made for the volume fraction occupied by the structural elements of the cytoplasm (Lauffer, 1961). Varying the substitution ratio (fluorescein/glucose) (Table I) or the concentration of labeled dextran injected did not significantly alter these results. Although the diffusion coefficients in cytoplasm observed for TRTC-dextrans are not identical with those of FTC-dextrans nominally of the same size, the results are qualitatively similar. A likely reason for the discrepancy is that since we were unable to obtain diffusion coefficients for TRTC-dextrans by FRAP in aqueous solution, we do not know their hydrodynamic radii accurately. Relatively small errors in estimation of size would alter considerably their position on the graph (Fig. 4) since D_{aq} is also dependent on size.

To see whether the increase in effective viscosity with increasing size of dextran could be explained simply by the effects of a concentrated protein solution on diffusion of the dextran, we examined the diffusion of FTC-dextrans in 7% BSA, 20% BSA, and a low-speed supernatant of egg white (13% protein by refractive index) by FRAP. In all three solutions, the ratio of diffusion coefficient in concentrated protein solution to diffusion coefficient in water (D/D_0) trended weakly upward with increasing radius of dextran,

rather than downward as was observed in cytoplasm (Table II). At this point, no simple model adequately explains this result, but the apparently non-Stokes-Einstein behavior of dextrans diffusing in concentrated protein solutions qualitatively resembles observations made by Phillies and his colleagues, who have studied diffusion in complex solutions of macromolecules (Phillies, 1985; Ullman et al., 1985a, b). It is possible that a treatment based on the more general Einstein relation is required to explain these observations.

Discussion

This study represents the first demonstration in living cells that the cytoplasm contains barriers restricting the free diffusion of macromolecules in a size-dependent manner. The ratio between the diffusion coefficient of a fluorescent dextran in aqueous solution and its diffusion coefficient in the cytoplasm of living cells ($D_{\text{aq}}^{37^\circ\text{C}}/D_{\text{cyt}}$) increases with increasing molecular size, indicating that the effective viscosity of the cytoplasm depends on the size of the probe. This phenomenon is not a function of the density or total number of fluorescent labels per molecule, since dextrans of the same size with a threefold difference in substitution ratio did not exhibit significantly different diffusion within cytoplasm (see Table I). This phenomenon is also not dependent on the nature of the fluorophore, since dextrans labeled with TRTC instead of FTC produce similar results.

Video images of cells co-injected with large and small dextrans show that the larger size fractions are relatively excluded from extensive cytoplasmic domains of some cells (Fig. 3). Calculating from the space-averaged values for D_{cyto} measured by FRAP, ample time was allowed for these dextrans to diffuse the distance from the site of injection to any point on the cell periphery. This was confirmed by the presence of the large dextrans in ruffles at the cell margin (Fig. 3). Thus, the differential distribution of large and small dextrans in the cytoplasm, like the FRAP data, indicate that otherwise undifferentiated cytoplasm contains barriers that hinder the free diffusion of dextrans in a size-dependent manner, and shows further that this property of the cytoplasm may exhibit local variation in space, and perhaps also in time. It is not possible based on our data to distinguish between thermodynamic and kinetic mechanisms for the differential partitioning of large and small dextrans into cytoplasmic domains.

The restricted diffusion of dextrans in cytoplasm cannot be explained simply by the effects of high concentrations of dissolved protein in cytoplasm, since the effect of concentrated protein solutions on the diffusion of dextrans in experiments *in vitro* showed a weaker size dependence, which tended in the opposite direction. The most likely source of the cytoplasmic obstructions are the abundant filamentous components of the cytoplasm, although other formed elements such as membrane systems may play a role. It cannot presently be determined from our data whether these components are interconnected into a cross-linked network. However, the data do not fit well the exponential relationship between ($D_{\text{cyto}}/D_{\text{aq}}$) and radius predicted for diffusion of particles in solutions of non-cross-linked, long chain polymers (Laurent et al., 1963; Ogston et al., 1973; Cukier, 1984). More extensive study of dextrans in reconstituted model systems by FRAP will enable us to compare the effect of cross-linked vs.

non-cross-linked filaments on diffusion of particles, and will be essential for the further interpretation of FRAP studies of living cells.

When the curve in Fig. 4 is extrapolated to zero radius, a value of 4 cp is obtained for the viscosity experienced by a point particle diffusing in the cytoplasm. This value approximates the viscosity of the fluid phase of the cytoplasmic ground substance and falls within the range of values (2–6 cp) for cytoplasmic microviscosity previously measured by other methods (Mastro et al., 1984; Lepock et al., 1983; Paine and Horowitz, 1980). Peters (1984) has reported that FTC-dextran with $R_H = 55 \text{ \AA}$ (estimated $R_G = 82 \text{ \AA}$) has a mobility in the cytoplasm of cultured hepatocytes that is 20 times slower than in water. In contrast, the most comparable dextran used in our study, which had an apparent $R_H = 62 \text{ \AA}$ (estimated $R_G = 93 \text{ \AA}$), was only 6.5 times less mobile in the cytoplasm of 3T3 cells than in water. This discrepancy could reflect differences inherent in cell type.

Estimates of the effective mesh (permeation size parameter) of the aqueous cytoplasm can be made from the data presented in Fig. 4. If the initial portion of the curve is extended as a straight line, the x-intercept, where diffusion in cytoplasm drops to zero, occurs at a radius of $\sim 207 \text{ \AA}$. This means that mesh would be twice that, or $\sim 400 \text{ \AA}$. However, the change in slope of the curve at a radius of $\sim 141 \text{ \AA}$ indicates the mesh may actually be closer to 280 \AA . In any case, an explanation is needed for why the diffusion of dextrans having radius greater than 141 \AA did not drop to zero. One possibility is that flexible random coils like dextrans can pass by reptation through pores that do not admit a rigid molecule of similar average dimensions, although at a greatly reduced rate (Bohrer et al., 1984). Another possibility is that the size and position of pores in the cytoplasm varies on a timescale fast enough to permit some diffusion of particles larger than the average mesh. The imaging data suggest that the mesh in some regions of the cell may be as small as 200 \AA , since dextrans of radius greater than 100 \AA are excluded from portions of the cytoplasm.

The above estimates of mesh depend on the assumption that we have correctly estimated the relevant size parameter for determining passage of a particle through a pore. The radius of gyration (R_G), which is a configurational average of the mass distribution of the particle, is the parameter that describes the degree of interference between two random-coil molecules (Tanford, 1961). A large configurational entropy decrease occurs when the center-of-mass separation between two random flight polymers in solution decreases to within twice this dimension (Tanford, 1961). We have assumed that R_G is also the range of interaction between a random coil and a barrier. Mean external diameter may be a more suitable size parameter than radius of gyration (Casassa, 1985). At present we can only estimate the relative mean external diameter for dextrans, but it is slightly larger than twice the radius of gyration (Casassa, 1985). In principle, gel filtration chromatography can be used to determine this parameter absolutely when appropriate size standard particles are available to calibrate the column. Our estimates of radii of gyration were made from apparent R_H on the basis of the Kirkwood–Riseman relationship between R_H and R_G for unbranched, random-flight polymers (Tanford, 1961). In fact, since dextrans are known to be branched, the exact relation between

R_H and R_G may differ from the Kirkwood–Riseman result. While these uncertainties may make our estimate of pore size inaccurate it probably doesn't vary more than 10–15% from the estimated value. We are currently working to develop inert fluorescent probes that are rigid, compact spheres with radii ranging from 20 \AA to $1,000 \text{ \AA}$ so that we may more accurately estimate cytoplasmic mesh.

We have performed some calculations to see whether our estimates of cytoplasmic mesh are plausible based on the little that is known (or inferred) about cytoplasmic structure. The range of mesh dimensions estimated from our data is considerably smaller than the mesh estimated from computer morphometry of the microtrabecular lattice in high voltage electron micrographs. Gershon et al. (1985) calculated from digitized electron micrographs that the average size of pores in the microtrabecular lattice is $700\text{--}1,000 \text{ \AA}$, whereas we estimate that the mesh in the cytoplasm of living 3T3 cells is $200\text{--}400 \text{ \AA}$. The two estimates of mesh do not necessarily conflict, when one considers that the high voltage electron micrograph images represent the dehydrated morphology of the cytoplasm. In contrast, the mesh estimated in living cells includes any water of hydration adjacent to the barriers, as well as the mutual effect of macromolecules dissolved in the aqueous phase of the cytoplasm.

It is interesting to note that the axial repeat distance along F-actin filaments (360 \AA) falls into the range of estimated mesh, as do the lengths of some proteins thought to form cross-links between filaments (e.g., muscle and nonmuscle alpha-actinin = 380 \AA , Fechheimer et al., 1982; microtubule-associated proteins = $100\text{--}600 \text{ \AA}$, Pollard et al., 1984; Kim et al., 1979). To see whether such a mesh is reasonable based on current knowledge of cytoplasmic composition, we have calculated the weight fraction of F-actin required to obtain a mesh of 400 \AA , and arrived at a figure of 13 mg/ml . Estimates of the concentration of F-actin in most cells range from $2\text{--}20 \text{ mg/ml}$ (see Blikstad et al., 1978; Bray and Thomas, 1975). Since determinations of the concentration of actin in cells generally don't take into account the volume displaced by membrane-bounded organelles, including the nucleus, mitochondria, and endoplasmic reticulum, the actual concentration of F-actin in the cytoplasmic ground substance may be higher than these measurements suggest. Thus, although F-actin alone may not account for a mesh of 400 \AA , this mesh is not unreasonable, especially when other filamentous components such as microtubules, intermediate filaments and, possibly, $2\text{--}3\text{-nm}$ filaments (Schliwa and Van Blerkom, 1981) are considered, and when the possibility is taken into account that various membrane systems in the cytoplasm may be integrated into the cytomatrix (Wolosewick and Porter, 1979).

The results of the present study have profound implications for intracellular biochemistry. Although most monomeric globular proteins are small enough to penetrate pores of 400 \AA diameter, the translational diffusion of unbound species in cytoplasm may be slowed four- to sixfold relative to their diffusion in water, depending on their sizes (see Table I). This would significantly alter the rates of diffusion-limited interactions between macromolecules. Larger particles such as oligomeric proteins, multi-enzyme complexes, long chain polymers (including mRNA), ribosomes, and viruses may diffuse very slowly or not at all over extended distances within the cytoplasm. This would necessitate site-directed synthesis of

components of macromolecular complexes (e.g., Penman et al., 1981; Fulton, 1982; Porter and Anderson, 1980) and active transport of many intracellular components according to a complex addressing system.

The use of FRAP to study fluorescent protein analogs in living cells presents an even more immediate need for better understanding of the structure and properties of cytoplasm (Taylor et al., 1984). It is impossible to relate FRAP data to the function of a specific protein in vivo without first knowing how the composition and structure of cytoplasm are likely to affect the mobility of macromolecules within the cell. Small molecules should diffuse at rates dependent on the viscosity of the fluid phase of the cytoplasm, whereas the mobility of large molecules may additionally be constrained by structural barriers within the cytoplasm. These parameters must be understood in order to make optimum use of FRAP to study the binding and/or polymerization of fluorescent analogs in vivo. Cytoplasmic diffusion coefficients need to be corrected for the higher viscosity of the fluid phase of the cytoplasm (4 cp in 3T3 cells). For large particles, a correction is also needed to account for the effect of obstructions on the diffusing particle. In lieu of inert rigid particles for which the sizes are more accurately known, fluorescent dextrans can be used as standards to calculate the expected time constant of recovery for the freely diffusing particle. Recoveries with time constants longer than this cannot represent free diffusion of monomeric species. This may indicate the presence of freely diffusing oligomers, uncharacterized long-range interactions, or transient binding to fixed targets (chemical exchange) (Elson and Reidler, 1979; Koppel, 1981). For example, monomeric AF-actin (radius = 28 Å; Lanni and Ware, 1984) should diffuse about five times slower in the cytoplasm of 3T3 cells than in water. The actual half-life of recovery of the high-mobility species of AF-actin in 3T3 cells is four times longer than this estimate (Luby-Phelps et al., 1985). This suggests that either diffusible actin exists as a freely diffusing oligomer (or other complex) in the cytoplasm, or diffusion of monomeric actin is complicated by transient binding to fixed targets.

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