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Hindered diffusion of inert tracer particles in the cytoplasm of mouse 3T3 cells

(fluorescence recovery after photobleaching/cytoplasmic structure/cytomatrix)

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ABSTRACT **Using fluorescence recovery after photobleaching, we have studied the diffusion of fluorescein-labeled, size-fractionated Ficoll in the cytoplasmic space of living Swiss 3T3 cells as a probe of the physical chemical properties of cytoplasm. The results reported here corroborate and extend the results of earlier experiments with fluorescein-labeled, size-fractionated dextran: diffusion of nonbinding particles in cytoplasm is hindered in a size-dependent manner. Extrapolation of the data suggests that particles larger than 260 Å in radius may be completely nondiffusible in the cytoplasmic space. In contrast, diffusion of Ficoll in protein solutions of concentration comparable to the range reported for cytoplasm is not hindered in a size-dependent manner. Although we cannot at present distinguish among several physical chemical models for the organization of cytoplasm, these results make it clear that cytoplasm possesses some sort of higher-order intermolecular interactions (structure) not found in simple aqueous protein solutions, even at high concentration. These results also suggest that, for native cytoplasmic particles whose smallest radial dimension approaches 260 Å, size may be as important a determinant of cytoplasmic diffusibility as binding specificity. This would include most endosomes, polyribosomes, and the larger multienzyme complexes.**

The non-Newtonian properties of cytoplasm have been well documented during more than a century of study, but the physical chemical basis for the non-Newtonian properties of cytoplasm is not understood (for reviews, see refs. 1–12). While such macroscopic non-Newtonian phenomena as viscoelasticity and thixotropy imply that cytoplasm possesses some sort of submicroscopic intermolecular organization not found in a dilute, aqueous solution, the possible forms of this organization range from a liquid crystal structure due to the high concentration of protein in cytoplasm, to a meshwork of entangled filamentous proteins, to a crosslinked gel network. A fundamental problem in approaching this question has been the difficulty of studying living cells with high enough resolution. Until recently there has been no method of obtaining data on a molecular level without the necessity of first fixing the cells for electron microscopy or fractionating the cells for subsequent biochemical analysis. Each of these approaches contains the potential for artifacts that make it uncertain how far the results of such experiments can be extended to the structure and function of living cells. Two relatively new techniques have made it possible to study the behavior of specific molecules in living cells while keeping perturbation of the cells' normal structure and function to a minimum. Fluorescent analog cytochemistry (FAC) can be used to study the subcellular distribution of fluorescent derivatives (analogs) of specific molecules (13), and fluorescence recovery after photobleaching (FRAP) can be used to study quantitatively the mobility of these analogs within living cells (14–21). By combining these two techniques, the diffusion of inert fluorescent macromolecules within cells can be studied as an indicator of the properties of cytoplasm in living cells. Comparison of the diffusion of these probes in cytoplasm to their diffusion in carefully chosen model systems may eventually allow us to understand more fully the non-Newtonian properties of cytoplasm in terms of its physical chemistry.

In a Newtonian fluid at constant temperature, the diffusion coefficient (D) is proportional to $(R_H\eta)^{-1}$, where R_H is the hydrodynamic radius of the diffusing particle and η is the viscosity of the medium. Thus, the ratio of the diffusion coefficient of a particle in that fluid (D_{fluid}) to the diffusion coefficient of the same particle in water (D_{aq}) would be independent of the dimensions of the particle and would equal the inverse relative viscosity of the fluid at a given temperature. In contrast, since cytoplasm exhibits many non-Newtonian properties, one might expect to find that this is reflected in the diffusion of inert particles in the cytoplasm of living cells. In fact, we have recently reported that the relative diffusion coefficient (D_{cyto}/D_{aq}) for size-fractionated, fluorescein-labeled dextrans (fluorescein thiocarbamoyldextrans, or FTC-dextrans) diffusing in the cytoplasm of living Swiss 3T3 cells, rather than being constant, is a strongly decreasing function of the estimated radius of gyration of the dextran (22) (Fig. 1). The interpretation of these data was somewhat complicated by the fact that dextrans are flexible, quasi-random-coil molecules that do not have a well-defined size or shape. Therefore, we have repeated the experiments using size-fractionated, fluorescein-labeled Ficoll (FTC-Ficoll) as a probe particle. Compared with dextran, Ficoll behaves much more like a rigid sphere (23–26). Thus, the dimensions of the particles can be determined with more certainty, and deformability can be given less weight in interpreting the data. The results of these experiments, which are described in this report, are remarkably similar to the results of the previous experiments using dextran.

MATERIALS AND METHODS

Fluorescence Labeling of Ficoll. Ficoll 400 (Pharmacia Fine Chemicals) can be labeled with fluorescein isothiocyanate or tetramethylrhodamine isothiocyanate by a scaled-down version of the Williamson synthesis as described by Inman (27). Ficoll 400 (1.33 g; Pharmacia) was dissolved in 18.5 ml of freshly prepared 1.35 M sodium chloroacetate. Five milliliters of 10 M NaOH was added and the reaction mixture was brought to 25 ml with distilled water. After 30 min at 25°C, the

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Abbreviations: FRAP, fluorescence recovery after photobleaching; FTC, fluorescein thiocarbamoyl; TRTC, tetramethylrhodamine thiocarbamoyl.

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reaction was quenched with 0.2 ml of 2 M NaH_2PO_4 and the mixture was titrated to pH 7.0 with 6 M HCl. The activated Ficoll was dialyzed for several days vs. distilled water, then lyophilized and resuspended in distilled water at a concentration of 25 mg/ml. Ethylenediamine dihydrochloride was added at 5.7 mg/mg of Ficoll while a constant pH of 4.7 was maintained with 1 M NaOH. Next, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.5 mg/mg of Ficoll) was added over a 10-min period, and the mixture was stirred for 3.5 hr at room temperature while the pH was maintained at 4.6–4.8. The Ficoll was again dialyzed extensively vs. distilled water, lyophilized, and suspended in carbonate buffer (pH 9.0) at a concentration of 20 mg/ml for labeling with the fluorophore. For labeling with fluorescein isothiocyanate, dye was added to a concentration of 10 mg/ml and the labeling was allowed to proceed for ≥12 hr at 40°C, pH 9.0. For labeling with tetramethylrhodamine isothiocyanate, 0.1 mg of dye per mg of Ficoll was dissolved in carbonate buffer (pH 9.0) and was added dropwise to an equal volume of buffer containing the Ficoll at 40 mg/ml. After 30 min at 40°C, the reaction mixture was clarified and then desalted on Sephadex G-25 (Sigma). Labeled Ficolls were then dialyzed extensively against distilled water, lyophilized, and stored desiccated at 4°C until use. The substitution ratio of covalently bound dye per sugar residue obtained by this procedure was 0.004. The molar extinction coefficients used for this calculation were 68,000 for FTC at pH 8.0 and 55,000 for tetramethylrhodamine thiocarbamoyl (TRTC). Ninhydrin tests indicated that few if any free amino groups remained on the derivatized Ficoll after the labeling procedure. Flat-bed electrophoresis in nondenaturing agarose gels showed that the labeled Ficolls contained no free dye and had a negligible surface charge.

Fractionation of FTC-Ficoll. Labeled Ficoll at 12 mg/ml was loaded on a 2.8 × 100-cm column of Sepharose CL-6B (Pharmacia) equilibrated in 20 mM Tris Cl, pH 8.0/50 mM KCl/0.02% NaN_3. Elution was with the same buffer at 20–30 ml/hr, and 5-ml fractions were collected. Selected fractions of the included volume of the CL-6B column were concentrated by dialysis against distilled water, followed by lyophilization and suspension in a small volume of buffer. The void volumes from several column runs were pooled and chromatographed on Sepharose CL-4B to obtain size-fractions of large radius. Selected fractions were concentrated as above. The average hydrodynamic radius (R_H) of each selected size-fraction was determined from the aqueous diffusion coefficient as measured by FRAP.

FRAP. Aqueous diffusion coefficients for FTC-Ficoll were obtained by FRAP measurements on samples contained in flat glass capillaries (Vitro Dynamics) using the 488-nm line of an argon-ion laser operated at 200 mW (SpectraPhysics, Mountain View, CA). The radius of the laser spot, measured as previously described (28), was 50 μm. Data acquisition and analysis were performed with the aid of an IBM PC-AT linked to the photobleaching apparatus via an IBM I/O board. Fluorescence-recovery curves were fit using the method of Yguerabide *et al.* (21). Aqueous solutions of FTC-Ficoll were made in 2.5 mM Pipes, pH 7.0/0.05 mM $MgCl_2$/50 mM KCl. For FRAP of FTC-Ficolls in concentrated protein solution, ovalbumin (grade V, essentially salt-free; Sigma) or bovine serum albumin (fraction V; Sigma) was dissolved overnight in the same buffer to an approximate concentration of 35%. This solution was then dialyzed for 10 min vs. the buffer, using collodion bags (catalog no. 43-25300; Schleicher & Schuell). After dialysis, the protein concentration was determined by refractometry before dilution to the final concentration. Bulk viscosities for these solutions were determined by Cannon–Ostwald viscometry. For determination of cytoplasmic diffusion coefficients (D_{cyto}), small volumes of size-characterized FTC-Ficoll fractions were microinjected into the cytoplasm of living Swiss 3T3 fibroblasts as described (22, 28). The cells were allowed to recover for at least 4–6 hr before FRAP measurements were made using a laser spot 6 μm in radius. The area of the bleached region was thus <2% of total cell area. The fraction of total fluorescence bleached in this region was kept below 60% (usually 20–30%) to ensure accuracy of curve fitting and to avoid significant dilution of total cell fluorescence. During the measurements, the environment of the cells was maintained at pH 7.3 and 37°C.

RESULTS

Using the procedure outlined above, we were able to obtain FTC-Ficoll fractions ranging in average molecular hydrodynamic radius from 30 to 248 Å. Seven of these were selected for microinjection into living Swiss 3T3 cells (see Table 1). FTC-Ficoll was detected within living cells as long as 48 hr after microinjection and had no detectable effect on cell morphology or viability. FTC-Ficoll does not appear to interact significantly with intracellular components, since with the exception of the largest size fractions (see below), analysis of FRAP curves indicated 100% recovery of a single species. Cytoplasmic diffusion coefficients of FTC-Ficoll measured in living cells 48 hr after injection were the same as those measured 4–6 hr after injection. FTC-Ficoll was rarely found in intracellular vesicles, and then only 24–48 hr postinjection.

The relative diffusion coefficient (D_{cyto}/D_{aq}) for FTC-Ficoll is a strongly decreasing function of particle radius with a slope virtually identical to the slope of the curve for FTC-dextrans ≤140 Å (Table 1 and Fig. 1). Unlike the curve for FTC-dextrans, the curve for FTC-Ficolls does not show an inflection point at a radius of 140 Å. Over the range of particle radii we tested, the curve appears linear (correlation coefficient = −0.99) with an extrapolated x-intercept of 260 Å, suggesting that particles of radius larger than 260 Å are not freely diffusible in cytoplasm. Unfortunately, we have not been able to obtain useful amounts of size-fractionated FTC-Ficolls larger than 248 Å and so cannot yet test this hypothesis more rigorously by studying the diffusion of particles larger than 260 Å in radius. However, it is interesting that as the radius of FTC-Ficoll approaches 260 Å, an increasing percentage of it is immobile in cytoplasm (Table 1). Since even narrow size fractions of FTC-Ficoll are polydisperse, this may reflect the presence within the fraction of nondiffusible particles larger than 260 Å in radius. Alternatively, this may reflect the existence of subcellular domains where hindrance of diffusion occurs at a smaller radius than that predicted by the extrapolated x-intercept of the average data.

As a preliminary test to see whether size-dependent hindered diffusion of FTC-Ficoll in cytoplasm could be explained simply as the effect of the high concentration of protein in cytoplasm (15–30%, wt/vol), we studied the

Table 1. Diffusion of FTC-Ficoll in the cytoplasm of 3T3 cells

Radius, Å	D_{cyto}/D_{aq}	% mobile
32	0.277 ± 0.02	99.5 ± 0.4 (33)
62	0.223 ± 0.005	102.0 ± 1.1 (24)
106	0.167 ± 0.009	97.5 ± 1.6 (21)
140	0.116 ± 0.013	94.0 ± 1.5 (20)
180	0.098 ± 0.011	97.0 ± 0.8 (3)
227	0.037 ± 0.004	84.0 ± 3.9 (10)
248	0.034 ± 0.004	63.0 ± 4.8 (23)

Relative diffusion coefficient (D_{cyto}/D_{aq}) and % mobile fraction were determined for FTC-Ficoll fractions ranging in average radius from 32 to 248 Å. Values of D_{cyto}/D_{aq} are given plus or minus the standard error of the ratio. Values of % mobile are given as sample mean plus or minus the sample standard deviation. Numbers in parentheses indicate the sample sizes.

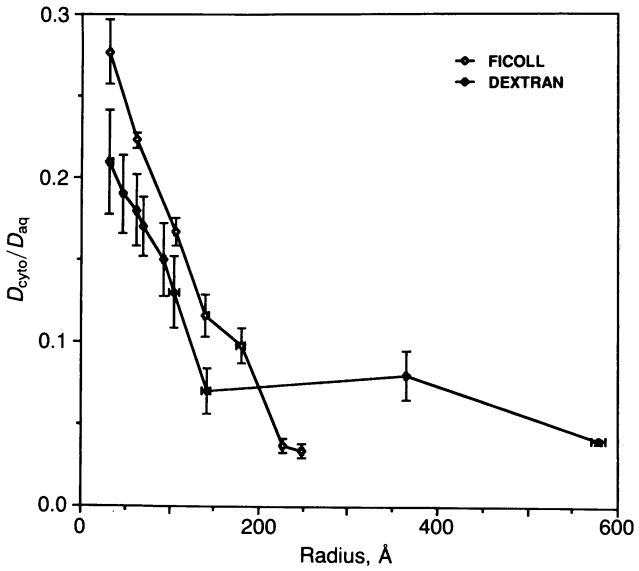

FIG. 1. Relative diffusion coefficient (D_{cyto}/D_{aq}) vs. tracer radius in Å for size-fractionated FTC-dextran (◆) and size-fractionated FTC-Ficoll (◇). Error bars represent standard error of the mean. Tracer radius for Ficoll was taken as the hydrodynamic radius calculated from D_{aq}. Tracer radius for dextran was taken as the radius of gyration estimated from D_{aq} (see ref. 22). The data indicate that the long-range diffusion of particles whose smallest radial dimension is >260 Å may approach zero. Differences between the two curves may reflect differences in flexibility between dextran and Ficoll.

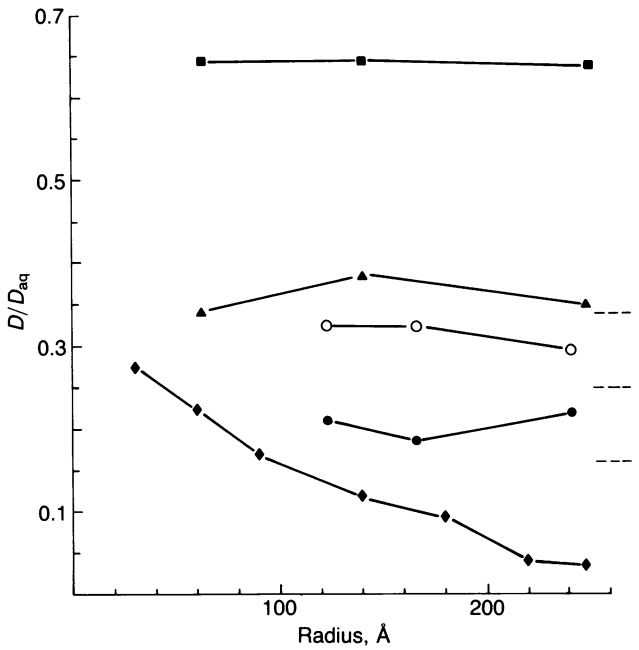

FIG. 2. Comparison of diffusion of FTC-Ficoll fractions in cytoplasm with diffusion in concentrated solutions of proteins. D/D_{aq} is plotted vs. hydrodynamic radius. Protein solutions were 10% (■), 20% (▲), or 24% (○) ovalbumin or 26% (●) bovine serum albumin. Data from Fig. 1 are replotted for comparison (◆ here, ◇ in Fig. 1). Horizontal dashed lines demarcate the inverse relative bulk viscosities of 20% and 24% ovalbumin and 26% bovine serum albumin. By this criterion, protein solutions of concentration in the range of those reported for cytoplasm appear Newtonian, whereas cytoplasm exerts a size-dependent effect on diffusion. This suggests that cytoplasm cannot be modeled as simply a concentrated protein solution.

diffusion of FTC-Ficoll in 10%, 20%, and 24% ovalbumin and 26% bovine serum albumin. In contrast to the diffusion of FTC-Ficoll in cytoplasm, the diffusion of FTC-Ficoll in these concentrated protein solutions did not appear to be size-dependent (Fig. 2). By this criterion, concentrated protein solutions appeared as Newtonian fluids, albeit of much higher viscosity than water.

DISCUSSION

There is considerable evidence in the literature that, thermodynamically and hydrodynamically, Ficoll approximates a hard sphere much more closely than dextran, which is a flexible, long-chain poly(D-glucose) with sparse, short branches (25, 26, 29–31). Ficoll is a highly branched copolymer of two short building blocks, sucrose (a disaccharide) and epichlorohydrin (a three-carbon crosslinker), making it less flexible and more compact than dextran on a molecular weight basis (23–25). While Ficoll may lack the strong intrachain hydrogen bonding that constrains a globular protein, it has been shown that the diffusion of Ficoll across Nuclepore (track-etched) porous membranes closely fits the accepted models for diffusion of a hard sphere through cylindrical pores (23, 24). This means that apparent hydrodynamic radius is most likely a reasonable descriptor of the dimensions of these particles, both in dilute aqueous solution and in complex systems, like cytoplasm, where passive obstructions to free diffusion may be significant. In this paper we have reported the use of size-fractionated FTC-Ficolls to eliminate some of the uncertainties in interpreting the results of a previous study in which size-fractionated FTC-dextrans were employed to probe the properties of cytoplasm (22).

The ratio (D_{cyto}/D_{aq}) for the diffusion of both dextrans and Ficolls in the cytoplasm of living cells is size-dependent, confirming that non-Newtonian properties of cytoplasm can be detected by this approach without perturbing the cell in any apparent way. The data also indicate that for particles ≥30 Å in radius, the effective viscosity of cytoplasm is at least 3–4 times higher than that of water. Lacking well-defined particles of radius less than 30 Å, we cannot determine the limit of D_{cyto}/D_{aq} as radius decreases toward zero. However, data from other laboratories suggest that even particles as small as 3 Å in radius experience a viscosity in cytoplasm 2–6 times that of water (32, 33). This effective viscosity should be a function not only of the true bulk viscosity of the solvent phase of cytoplasm but also of the volume fraction of dissolved macromolecular species and of hydrodynamic screening.

The slope of the size dependence of diffusion of dextrans and Ficolls in cytoplasm is virtually identical for both types of particle up to a radius of 140 Å. At this point the curve of D_{cyto}/D_{aq} vs. radius for dextrans levels off, while the curve for Ficoll continues with the same slope up to the largest particle radius available for this study (Fig. 1). If we make the assumption that this difference reflects the difference in flexibility of the two types of particle, the data suggest that rigid particles whose smallest radial dimension is larger than about 260 Å are nearly, if not completely, nondiffusible in the cytoplasmic space of living cells. This conclusion is supported by the emergence of an immobile fraction as tracer radius approaches 260 Å (Table 1). Hindered diffusion of particles in this size range is exactly what one would expect based on high-voltage electron microscopy of whole, unembedded cells, in which a network with a mesh in the range of 350- to 500-Å radius appears to fill the cytoplasmic space (9). Thus, it may be that all organelles, including most endosomes, polyribosomes, and even large multienzyme complexes, must be regarded as nondiffusible in cytoplasm purely on the basis of their size, regardless of their binding specificities. The use of test particles of radius greater than 260 Å will allow

us to better characterize the size limit for free diffusion in cytoplasm.

Various mathematical models have been constructed for the long-range tracer diffusion of an inert particle in a network of obstructions (34–44). In all these models the relative diffusion coefficient (D/D_0) for a tracer particle diffusing in the network vs. in a reference phase (the solvent phase of the network minus the obstructions) is found to be a decreasing function of tracer radius, decaying to zero at a finite radius or asymptotic at zero mobility. In a rigid gel, long-range diffusion must decrease to zero for tracer particles larger than the mesh or the percolation cut-off of the network. Asymptotic decay of D/D_0 would be observed in "dynamic" networks such as that formed in solutions of flexible long-chain polymers above the entanglement limit (44). While the present data clearly show that relative diffusion coefficient for tracer particles diffusing in cytoplasm is a strong function of tracer size, they do not allow us to choose between these two extremes because of the limited range of particle radii we were able to test. Strict application of the existing theory would be premature for at least two other reasons. All the models make different assumptions concerning the shape and statistical distribution of the obstructions, and it is not yet clear which of these assumptions will be most applicable to cytoplasm. In addition, lacking specific knowledge of the composition of the solvent phase of the cytoplasm, the reference phase used for our experiments was a dilute aqueous solution. Although the average protein concentration of cytoplasm is known, we cannot yet estimate how this protein is apportioned between the solid and solvent phases of the cytoplasm. The actual protein concentration of the solvent phase will determine its viscous properties, altering the relative diffusion coefficient by a factor that is not accounted for in the existing theories. More detailed study of the diffusion of Ficoll in concentrated protein solutions and in model systems reconstituted from biological components will be necessary before any existing theory can be applied confidently. In the meantime, the study of labeled Ficoll in living cells by FRAP and quantitative fluorescence microscopy can be used as an empirical tool to probe the effective viscosity of cytoplasm as an indicator of cytoplasmic structure and to look for spatial and temporal variations in cytoplasmic structure during a variety of cellular functions.

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