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Diffusion of injected macromolecules within the cytoplasm of living cells

(erythrocyte-mediated microinjection/photobleaching/human fibroblast/cytoplasmic viscosity/cytoskeleton)

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ABSTRACT **The diffusion of macromolecules introduced into the cytoplasm of human fibroblasts by erythrocyte-mediated microinjection was measured by the fluorescence recovery after photobleaching technique. The apparent diffusion coefficients for fluorescein-labeled IgG and fluorescein-labeled bovine serum albumin were $\approx 10^{-8}$ cm^2/sec at 22°C, consistent with the kinetics of spreading of the fluorescent probe following microinjection and $\approx 1/70$ the values in aqueous buffer. The diffusion of labeled bovine serum albumin was shown to be strongly dependent on temperature and, in fact, similar to that expected in a 61% aqueous sucrose solution. However, the marked reduction in diffusion at 5°C could be fully reversed by incubation with 0.1 mM colchicine. These findings suggest that cytoplasmic diffusion rates are reduced relative to rates in aqueous media as a result of increased aqueous phase viscosity or the impedence provided by structural elements. Several simple models to account for the data are presented.**

The organization of the cytoplasm is a relatively unexplored area of modern cell biology. Measurement of the translational diffusion of probe molecules placed within the cytoplasm should offer information concerning the resistance to diffusion that the cytoplasm provides, as well as indicate the rates at which various components are transported within the cell. Thus far, the rotational diffusion of small probe molecules has been measured by spin-label techniques (for review, see ref. 1) and by fluorescence polarization (2, 3). Cytoplasmic diffusion rates have been calculated for a variety of radiolabeled probes following pipette microinjection, fixation, and autoradiography (4). However, translational diffusion coefficients of molecules in the cytoplasm of living cells have not been measured. In the last several years, biophysical and biological techniques have been developed that make this measurement feasible.

We have introduced fluorescein-labeled macromolecules into the cytoplasm of living human fibroblasts by using the technique of erythrocyte-mediated microinjection (5) and then measured the apparent translational diffusion of these probe molecules within single cells by using the fluorescence recovery after photobleaching (FRAP) method (6, 7). The impedence provided by cytoskeletal and membraneous structures within the cell, superimposed on the intrinsic viscosity of the cytoplasmic aqueous phase, might be expected to reduce the translational mobility from the values measured in simple aqueous solutions. Such is the case as demonstrated here, illustrating that these transport measurements can be used to provide insight into the structure of the cytoplasm.

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MATERIALS AND METHODS

Cells. Human neonatal foreskin diploid fibroblast cells, strain BG-9 (passage 29–35), were grown in Eagle's minimal essential medium with Earle's salts (GIBCO), 10% heat-inactivated fetal calf serum (KC Biologicals, Kansas City, MO.), penicillin (150 units/ml), and streptomycin (50 mg/ml). Stock cells were subcultured into 35-mm Petri dishes and cultured for 24 hr prior to the fusion experiments. Monolayers were subconfluent at the time of fusion.

Reagents. All chemicals used were reagent grade. Fluorescein isothiocyanate-labeled normal goat IgG (F-IgG) was purchased from Cappel Laboratories (Cochranville, PA), and the salt was removed by gel filtration on a Sephadex column. Bovine serum albumin (BSA) was coupled with fluorescein isothiocyanate, and the F-BSA and free isothiocyanate were separated on a Sephadex column.

Methods. Loading of human erythrocytes and microinjection of human fibroblasts was performed according to Schlegel and Mercer (5). The FRAP technique has been described (6). For these measurements, a X25/0.5 NA objective was used to give ae^{-2} diameter of 3.4 μm. No image plane diaphragm was used. Bleach times were typically 20–40 ms and the bleaching beam was attenuated by 10,000 for the measurement of fluorescence recovery.

As we studied large, highly spread fibroblasts, we could use a relatively low-power objective in which the beam maintained almost constant width as it traversed the cytoplasm along the optical axis (see equation 3 of ref. 6). If we further assume that the beam diameter is small compared with the size of the cell, we can apply the two-dimensional treatment of Axelrod *et al.* (8); in this cylindrically symmetric situation, diffusion along the optical axis does not contribute to the observed recovery. In this respect, one can look at FRAP measurements on thin regions of the fibroblast cytoplasm bounded by nearly parallel cell surfaces as a simple extension of photobleaching measurements on molecular solutions (6, 8, 9).

RESULTS

Erythrocyte-mediated microinjection is the technique by which macromolecules are introduced into mammalian cells via fusion with mammalian erythrocytes loaded with those macromolecules. Efficient transfer of probe molecules into fibroblasts was achieved at 22°C, with >60% of the BG-9 cells becoming flu-

Abbreviations: FRAP, fluorescence recovery after photobleaching; *D*, diffusion coefficient; BSA, bovine serum albumin; F-BSA, fluorescein isothiocyanate-labeled BSA; F-IgG, fluorescein isothiocyanate-labeled IgG.

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orescent. Approximately 30–40% of the cells showed a rounded morphology within 1 hr after the standard fusion protocol. The criteria of confinement of fluorescence within the boundary defined by the plasma membrane and its exclusion from the nucleus were used to judge cytoplasmic localization. Cells that were cultured for at least 24 hr after seeding were most efficiently microinjected; however, confluent cultures were avoided as they tended to be more refractory to the fusion protocol.

Entry of F-IgG and F-BSA into a fibroblast from an erythrocyte typically began 2 or 3 min after the polyethylene glycol used as fusogen was diluted. As injection proceeded, a gradient of fluorescence was observed originating at the site of a fused erythrocyte and diminishing toward the more distal portions of the cell. The spread of fluorescence throughout the cell could be monitored photometrically as a gradual increase in emission with time by placing the measuring beam of the FRAP microscope at a point distant from the site of a bound erythrocyte (Fig. 1). In some cells, the fluorescence intensity reached a plateau in ≈5 min; such spreading kinetics are consistent with a diffusion coefficient (D) of $\approx 10^{-8}$ cm^2/sec, similar to that obtained by the FRAP technique (Table 1). Other cells did not display any increase in fluorescence, indicating that they had not fused with erythrocytes.

Cells that met the criteria for cytoplasmic staining outlined above were chosen for FRAP measurements. The measuring beam was placed distal to any bound erythrocytes and between the nucleus and the cell boundary, and measurements were made 5–40 min after fusion, a period during which no discrete localization of the probe was observed at 22°C–37°C. Measurements were made on the 60–70% of the cell population that retained a flattened morphology after the fusion protocol.

A typical recovery curve for F-BSA in the fibroblast cytoplasm at 22°C is shown in Fig. 2. The shaded area reflects the actual data (with associated noise) taken from a storage oscilloscope. Computer analysis of the recovery data (Fig. 1, solid line) was consistent with diffusional recovery for a single species. Preliminary curve fitting of some of the F-BSA at 5°C (>60 min) data suggests that a small fraction of a slow component might exist. It is also possible that a minor directed-flow component exists; such processes could be detected most conveniently in selected biological systems known to prominently display cytoplasmic flow or streaming.

At 5°C, no localization within cytoplasmic organelles was noted up to 60 min after microinjection; however, at 22°C, some

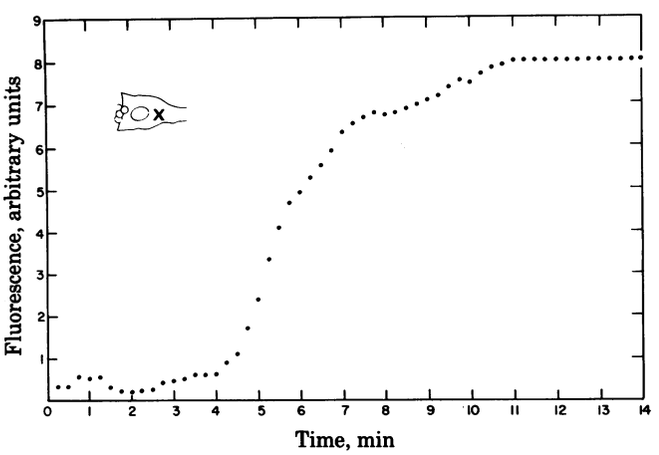

FIG. 1. Kinetics of fluorescence increase after microinjection. BG-9 human fibroblasts were fused with erythrocytes loaded with F-BSA. The measuring beam ($1/e^2$ diameter, 3.4 μm; 488-nm argon laser line) was positioned distal to bound erythrocytes as shown by the **X** in the *Inset*.

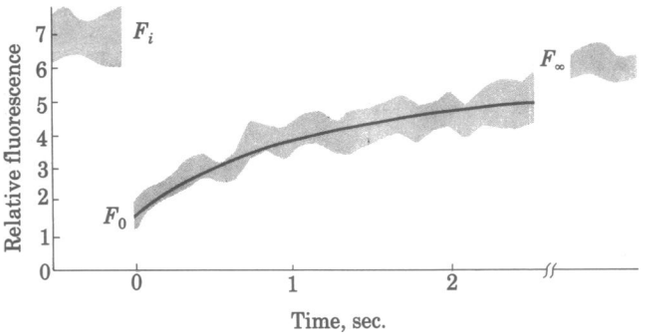

FIG. 2. Kinetics of FRAP for erythrocyte-mediated microinjected F-BSA in the cytoplasm of living BG-9 human fibroblasts at 22°C. Actual data and associated noise traced from a storage oscilloscope photograph are indicated by shading. The recovery curve is consistent with one-component diffusion, as shown by the solid line with $D = 0.9 \times 10^{-8}$ cm^2/sec. F_i, initial fluorescence; F_0, fluorescence intensity immediately after bleaching; and F_∞, final fluorescence.

perinuclear staining was observed at and beyond this point. By limiting the FRAP measurements to times when the intracellular fluorescence staining remained diffuse, the percent recovery (or mobile fraction) was 80–100%. The autofluorescence of the untreated cultured fibroblast was typically <10% of the total fluorescence seen in microinjected cells, and this endogenous fluorescence did not recover after photobleaching. In addition, cultured cells microinjected with unloaded erythrocytes gave results identical to those of untreated cells, indicating that the residual hemoglobin in these erythrocytes did not affect our results.

FRAP measurements of several different microinjected probe molecules were made; their apparent D values are given in Table 1. For IgG and BSA, these values were $\approx 10^{-8}$ cm^2/sec. For comparison, the D of BSA in buffer is $\approx 68 \times 10^{-8}$ cm^2/sec at 20°C or nearly 70-fold greater. Diffusion of carboxyfluorescein (M_r 374) was too rapid ($D > 4 \times 10^{-8}$ cm^2/sec) to measure with our instrument, a finding consistent with a previous observation on its cytoplasmic diffusion in L1210 cells (unpublished data).

The temperature dependence of D for F-BSA is shown in Fig. 3. Over the range from 5°C to 37°C, a 5.3-fold increase in D was observed. For comparison, the dotted line describes the temperature dependence of the D of BSA in 61% sucrose; it is seen to match the limited data, except at higher (>30°C) temperatures.

The low-temperature value of D for F-BSA (3×10^{-9} cm^2/sec) doubled on prolonged incubation (>60 min) prior to the FRAP measurement. Although the change was only marginally significant, this cold sensitivity suggests a possible dependence of D on microtubule depolymerization (10). For this reason, cells were treated with colchicine, which has been shown to disorganize microtubules. Cells microinjected with F-BSA and incubated with 0.1 mM colchicine for 15 min prior to FRAP

Table 1. Cytoplasmic Ds at 22°C

Probe	M_r	$D \times 10^8$, cm^2/sec
F-IgG	160,000	0.9 ± 0.1 (35)
F-BSA*	68,000	1.0 ± 0.2 (19)
Carboxyfluorescein	374	>4.0 (5)

Human erythrocytes were loaded with the indicated probe and fused with BG-9 cells as described by Schlegel and Mercer (5). Ds were determined from the FRAP measurement. Results represent mean ± SEM; numbers in parentheses represent trials.

* For comparison, for F-BSA in buffer, $D = 68.0 \times 10^{-8}$ cm^2/sec (9).

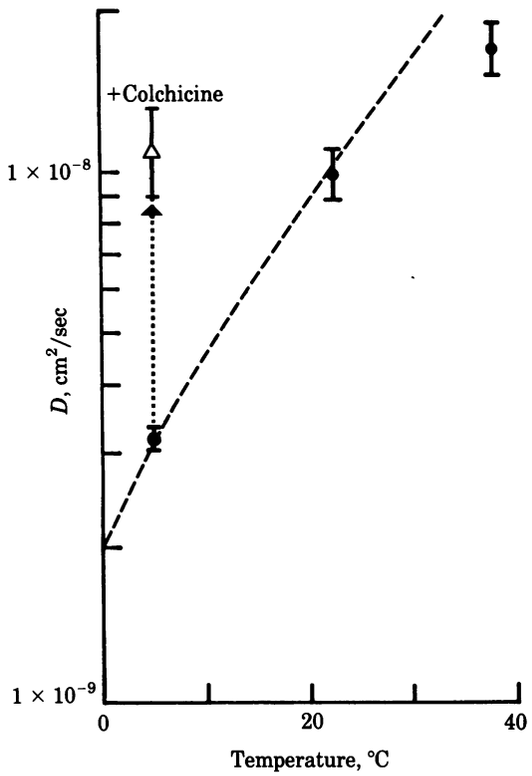

FIG. 3. Temperature dependence of the cytoplasmic D of F-BSA. Data points represent mean ± SEM. For comparison, the dashed line is D calculated for F-BSA in a 61% sucrose solution assuming that the Stokes–Einstein equation is applicable. The dotted line headed by an arrow at 5°C terminates at the point showing the effect of 0.1 mM colchicine on the diffusion of F-BSA at 5°C.

determination displayed an F-BSA D of $1.1 \pm 0.2 \times 10^{-8}$ cm^2/sec at 5°C (Fig. 3, higher value at 5°C above arrow); this value was >3 times as fast as the normal 5°C value and similar to the normal room temperature value.

In this case, treatment of the cells with colchicine did not increase the fraction of rounded cells above the control values and, again, only flat cells meeting the criteria for microinjection cited above were used for the FRAP measurements.

DISCUSSION

The macromolecules introduced into the cytoplasm of living cells in this study were capable of translational diffusion. At 20°C, the rate of diffusion was ≈1/70 that in simple buffer solutions (Table 1). In fact, below ≈30°C, the temperature dependence of cytoplasmic BSA diffusion was similar to that expected in a 61% sucrose solution (Fig. 2). At the very least, these results suggest that the cytoplasm could be viewed as possessing a considerably greater viscosity than simple aqueous solutions. However, the situation is more interesting because colchicine reversed the marked reduction in BSA diffusion observed at 5°C, suggesting that cytoplasmic structure is in some way a determinant of the observed diffusion rates. Several simplified models can be proposed to account for the observed diffusion rates. Although undoubtedly naive, such models should assist in illuminating the viscous and structural factors responsible for limiting the diffusion of macromolecules in the cytoplasm.

The simplest model would be to assert that the cytoplasm of the human fibroblast behaves as a simple solution having considerably greater viscosity (η) than water. Assuming the applicability of the Stokes–Einstein equation ($D \propto \eta^{-1}$), the viscosity of a 61% sucrose solution (≈70 cP at 20°C) would reduce the BSA D from the aqueous value (68×10^{-8} cm^2/sec at 20°C) to its cytoplasmic value. In the cell, one might assume that the cytoplasmic protein concentration would, perhaps through water ordering (11), make the cytoplasm more viscous. If, for the sake of argument, we assume that the soluble protein concentration was that of serum (≈7%), the cytoplasmic viscosity would only be ≈2 cP (12). At the other extreme, if the total cellular protein was assumed to be in solution within a cellular volume, protein concentrations would be 20–50%. For comparison, studies on normal erythrocytes indicate that the erythrocyte interior (27% hemoglobin) has a viscosity of up to 50 cP (13, 14).

The literature provides some evidence for increased cytoplasmic viscosity (Table 2). The possibility that both phospholipid membranes and soluble proteins alter the aqueous solvent structure to increase the viscosity is indicated by probe measurements in small unilamellar vesicles (15) and in erythrocytes (16); in both cases, the effect is modest and not enough to account for the apparent viscosity estimated from our measurements. Classical methods applied to frog muscle and lobster nerve yield cytoplasmic viscosity values 5–30 times that of water. Spin-label methods yield viscosities several times that of water in tissue culture cells (J. Lepock, personal communication; ref. 17).

Certainly, the evidence supporting relatively low cytoplasmic viscosities, in general, and the colchicine effect in our studies suggests that the various elements of cytoplasmic structure should also be considered in accounting for the low diffusion rates. These elements are of two major classes: filaments and internal membraneous organelles. Filamentous structures include the microtubules, microfilaments, and intermediate filaments (for review, see ref. 20). Although the organization of some of these elements would seem too coarse to appreciably affect the diffusion of proteins, the interplay of all of them and the cytoplasmic ground substance, the microtrabecular lattice (see ref. 21 and references therein), could serve to restrict diffusion. Furthermore, the sensitivity of this lattice to colchicine could qualitatively account for the changes in diffusion we observe (K. Porter, personal communication).

There are two extreme mechanisms by which molecular transit through the structured cytoplasm could occur. In the first, diffusive steps would occur in the aqueous spaces between the structural elements of the cytoplasmic matrix but the overall transport would be greatly hindered by collisions with filamentous and membranous elements of the cytoplasm, which would act either as barriers to be circumvented by the diffusant or as gates that open stochastically, allowing passage of the diffusant

Table 2. Some literature estimates of cytoplasmic viscosity

Cell	Viscosity,* cP	Measurement technique
Unilamellar phospholipid vesicle interior	3–4†	Fluorescence polarization (15)
Erythrocyte interior	2–3	Electron spin resonance (16)
V-79 Chinese hamster lung fibroblast	3–5	Electron spin resonance (J. Lepock, personal communication)
Baby hamster kidney	≈2	Electron spin resonance (17)
Lobster nerve	5.5	Oil drop movement (18)
Frog muscle	15–30	Oil drop movement (19)

* At 20°C.

† Estimated from the 3- to 4-fold increase in the probe rotational relaxation time.

to the adjoining aqueous compartment. In the other extreme, a much greater affinity of the diffusant for the structural elements of the cytoplasm would be assumed, resulting in essentially linear or two-dimensional transport involving the macromolecule hopping or sliding along the cytoplasmic fibers and membranes.

The first model, which assumes no marked affinity of the diffusant for the matrix, reconciles the present translational diffusion measurements and rotational relaxation measurements that indicate a low cytoplasmic viscosity. Each region in which molecular rotation occurs can be envisioned as an aqueous-like compartment having a viscosity approaching that of water; on the other hand, translational diffusion requires transit across these regions as well as circumvention of the intervening matrix barriers. This latter step provides the rate limitation to translational diffusion.

Possibly, diffusion within various Sephadex beads can be used as an experimental model for cytoplasmic diffusion or theoretical models (22, 23) for diffusion through porous materials could be applied to provide an average pore radius that would describe macromolecular transport through the cytoplasmic matrix.

This report has demonstrated the feasibility of measuring the cytoplasmic diffusion rates of microinjected molecules. In the future, we can expect that additional measurements of this type with different sized probes, as well as the investigation of possible anisotropic diffusion, will offer a new way to study structure in the cytoplasm. Furthermore, although our measurements can be adequately modeled by diffusive processes, it should be possible to detect energy-dependent "vectored" transport in selected biological systems such as, for example, in axoplasmic flow (24). Thus, we can anticipate that the FRAP technique applied to cytoplasmic diffusion will provide important new information concerning this aspect of the living cell.

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