Diffusion of Fluorescently Labeled Macromolecules in Cultured Muscle Cells

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ABSTRACT Myotubes were obtained from culture of satellite cells. They had a sarcomeric organization similar to that of muscle. The diffusion in the direction perpendicular to the fibers of microinjected fluorescein isothiocyanate–dextran of molecular weight ranging from 9500 to 150,000 was examined by modulated fringe pattern photobleaching. On the time scale of the observation, 10–30 s, all of the dextrans were completely mobile in the cytoplasm. The diffusion coefficients were compared to the values obtained in water. The ratio $D_{\text{cytoplasm}}/D_w$ decreased with the hydrodynamic radius $R_h$ of the macromolecules. The mobility of inert molecules in muscle cells is hindered by both the crowding of the fluid phase of the cytoplasm and the screening effect due to myofilaments: $D_{\text{cytoplasm}}/D_w = (D_{D_w})_{\text{protein crowding}} \times (D_{D_w})_{\text{filament screening}}$. The equation $(D_{D_w})_{\text{filament screening}} = \exp(-k_L R_h)$ was used for the contribution of the filaments to the restriction of diffusion. A free protein concentration of 135 mg/ml, a solvent viscosity of cytoplasm near that of bulk water, and a calculated $k_L$ of 0.066 nm$^{-1}$, which takes into account the sarcomeric organization of filaments, accurately represent our data.

INTRODUCTION

For many cellular processes, small metabolites and macromolecules, proteins, and mRNAs are required to move to intracellular sites, where these molecules are involved in cell metabolism. The structure of cell cytoplasm has been a subject of several studies during the last several years, which have led to contradictory interpretations (reviewed by Luby-Phelps, 1994). Cell cytoplasm is a complex medium comprising a fluid medium, a high concentration of proteins, and a network of cytoskeletal filaments. The viscosity of the fluid phase has been a subject of debate, and values for viscosity in the range 2 to 20 cP have generally been reported (Kushman and Podolsky, 1969; Clegg, 1984; Lang et al., 1986; Luby-Phelps et al., 1988). On the other hand, recent studies gave values closer to water viscosity (1 cP) (Kao et al., 1993; Luby-Phelps et al., 1993). The effect of molecular crowding on biochemical reactions has recently been reviewed (Zimmerman and Minton, 1993): crowding effects influence a variety of biochemical processes and must be taken into account when observations made in vitro are tentatively extrapolated to physiological processes. By studying the diffusion of inert tracers in the cytoplasm of living cells by a FRAP technique, Luby-Phelps et al. (1986, 1987) showed that the diffusion of polymers in cytoplasm decreased with increasing polymer size. They proposed (Luby-Phelps et al., 1988; Hou et al., 1990) that the cytoplasm may be described as a network of entangled fibers interpenetrated by a fluid phase containing soluble proteins at high concentration. The dynamics of the cytoskeletal network was among the subjects raised by this representation of cell cytoplasm.

Striated muscle is one of the most highly ordered of all biological tissues (reviewed by Pollack, 1990). Besides the contractile elements, thick filaments of myosin, and thin filaments of actin and associated proteins, whose spacing has been accurately described by electronic microscopy and x-ray diffraction (Sosa et al., 1994), a cytoskeletal lattice maintains the structure of muscle cells. The proteins of the M-line and Z-line serve as structural integrators of the myofilaments and the longitudinal lattice components. Two giant proteins, titin and nebulin, are both the elastic filament system in skeletal muscle and molecular rulers specifying the length of the contractile filaments (reviewed by Trinick, 1994). Furthermore, a muscle membrane cytoskeleton has been described (reviewed by Small et al., 1992). In this highly organized network, small metabolites, proteins, and mRNAs must move to maintain the life of the cell, the contractile activity, and the renewal of the cell constituents. It was shown that the diffusivities of small ions (except Ca$^{2+}$) and small molecules are reduced by a factor of 2 relative to diffusivities in aqueous medium (Kusmerick and Podolsky, 1969; Yoshizaki et al., 1990). In the case of proteins, very few observations are available. Most of them were obtained from studies of the diffusion of glycolytic enzymes out of skinned fibers (Maughan and Lord, 1988). Direct measurement of the diffusion of myoglobin, a protein involved in the intracellular transport of oxygen, was recently reported (Jurgens et al., 1994). The diffusivity of proteins in cytoplasm is reduced by a factor of 6 to 20 relative to diffusivity in aqueous medium. Some of the proteins used in these studies have an affinity for intrasarcolemmal sites, other glycolytic enzymes, and actin. For this reason, it is better to study in a first stage the diffusivity of inert tracers to investigate how their size affects their mobility in the network of sarcomeres. The FRAP technique...
is a suitable way to measure diffusion constants in intracellular medium (Jacobson and Wojcieszyn, 1984; Peters, 1984). To this end, we have microinjected fluorescein isothiocyanate-labeled dextrans (FITC-dextrans) of various sizes in cultured muscle cells and measured the diffusion of the tracers by modulated fringe pattern photobleaching.

**MATERIALS AND METHODS**

**Muscle cells culture**

Rabbit satellite cells were cultured from a slow muscle, semimembranous proprius (Barjot et al., 1993). Satellite cells were isolated by pronase digestion of muscle fibers (Alterio et al., 1990). After exhaustive washing, the cells, suspended in Dulbecco's modified Eagle's culture medium (DMEM) containing 20% fetal calf serum, 100 units/ml penicillin, and 1 mg/ml streptomycin, were seeded on 100-mm petri dishes (Falcon) at a density of 2 × 10^5/cm^2 and grown to about 80% confluence in a humidified incubator at 37°C and 6% CO₂. Cells were trypsinized afterward and either frozen at −80°C in fetal calf serum plus 10% dimethyl sulfoxide or reseeded in culture dishes at a maximum split of 1:3. The reseeded cells were grown nearly to confluence, and then the medium was changed to DMEM containing antibiotics, 2% fetal calf serum, 5 µg/ml insulin, 5 µmol/ml transferrin, and 5 nmol/ml sodium selenite (ITS medium). In ITS medium cells fused and differentiated into myotubes. The ITS medium was changed every 3 days. For indirect immunofluorescence assays, muscle cells were cultured on microscopic coverslips (either several 22 mm × 22 mm coverslips per Ø 10-cm dishes or one coverslip per well in 6-well plates). To perform FRAP experiments, cells were grown in Ø 6-cm dishes, the bases of which were replaced by sealed glass coverslips allowing microscopic observation. Neither the petri dishes nor the glass surfaces were coated, but an incubation overnight in DMEM medium plus 20% serum before seeding increased the adhesion of the cells. For microinjections and FRAP experiments, an ITS medium devoid of phenol red and buffered by 20 mM HEPES was used.

The stage of differentiation was followed by indirect immunofluorescence, using monoclonal antibodies directed against desmin (Boehringer), titin (Sigma), and myosin heavy chains (developmental, neonatal and fast, Novo Castra; slow, Biosys). FITC-labeled goat anti-mouse IgG was used as a secondary antibody.

**Dextrans**

FITC dextrans of mean molecular mass varying from 9.5 to 150 kDa (FD 10S, FD 20S, FD 40S, FD 70S, and FD 150) were obtained from Sigma. The FD 150 polymer was very dispersed, so it was fractionated before use by FPLC on a Superose 12 HR 10/30 column (Pharmacia). One hundred microliters of a 20 mg/ml dextran was dissolved in the KNa phosphate buffer used for microinjections (buffer A, containing 48 mM K₂HPO₄, 14 mM Na₂HPO₄, and 4.5 mM KH₂PO₄, pH 7.2) and loaded on the column. Elution of FITC-dextran from the column was monitored at 254 nm, and the resulting peak was divided into three parts: the ascending part, the maximum of the peak, and the descending part, to an absorbancy at 254 nm, half that of the maximum. The resulting fractions were concentrated on Microcon 10 (Amincon).

**Microinjection**

Dextrans were introduced into myotubes by pressure injection. Sterile Femnotips (Eppendorf) were filled with 2 µl of a solution of dextran in buffer A. The concentration of dextrans was 20 mg/ml, and before use the solutions were centrifuged for 20 min at 100,000 × g in a Beckman airfuge. A filled Femnotip was inserted into the needle holder of a micro-manipulator Leitz and connected to a pressure Microinjector 5242 (Eppendorf). The total volume injected was smaller than 10% of the cell volume, so that the intracellular concentration of dextran was not high enough to change the osmotic pressure and thus the myofilament spacing (Matsubara et al., 1984; Baggi et al., 1994). For myotubes of length greater than 200 μm, small volumes were injected into several places in the cell. The myotubes were allowed to equilibrate for several hours before starting the FRAP experiments.

**Translational diffusion measurements**

The modulated fringe pattern photobleaching technique takes advantage of a spatial and temporal modulation that allows direct recording of the contrast modulation data (Davoust et al., 1982). The experiments were performed with an apparatus described earlier (Morrot et al., 1986). This apparatus is built around a fluorescence Zeiss IM-35 inverted microscope, an argon laser (Coherent, Innova 90-5) tuned to 488 nm as the excitation source, and a Victor computer for signal analysis. The modulated fringe pattern photobleaching produces a bleaching pattern of interference fringes. Unless stated, the fringes were oriented perpendicular to the muscle fibers. Decay of fluorescence contrast between bleached and unbleached regions was treated by the Padé-Laplace formalism, which allowed an immediate multieponential analysis. An example of decay of fluorescence contrast and its semilogarithmic transformation is shown in Fig. 1. Translational diffusion coefficients (D) were deduced from the relation D = i^2/4π^2τ, where τ is the time constant of the exponential decay and i the interference spacing. The experiments were performed at 20°C in a temperature-regulated room.

Diffusion coefficients of dextrans in free solution were measured for dilute solutions in buffer A, placed as a thin layer between a glass slide and a coverslip. The diffusion rate of the smaller dextrans was decreased by adding various sucrose concentrations to increase the viscosity of the medium. The values were then extrapolated to the buffer viscosity.

**RESULTS**

**Cultured muscle cells**

The differentiation to myotubes of fused satellite cells was followed by the identification of some specific proteins by indirect immunostaining.

After 10 days of differentiation, the myotubes were cross-striated and contained peripherally located nuclei (Fig. 2 A). Almost all of the sarcoplasm was occupied with striated myofilibrils. Titin was found to be localized in a striated fashion, and a cross-striation was observed with antibodies against desmin, indicating the presence of Z-lines (not shown). At this stage of differentiation, myotubes expressed myosin heavy chains. Fig. 2 B shows evidence of neonatal

![FIGURE 1 Decay of the fluorescence contrast for FITC-dextran FD 40S in muscle cell at 20°C. (Left) Curve corresponding to the direct recording. (Right) Curve corresponding to the semilogarithmic transformation. The interference was 17.6 μm. The decay constant is 0.72 s. This gives a diffusion constant of D_{cyt} = 11 μm² s⁻¹.](image-url)
heavy chain; at this stage adult myosins were also observed. These cultured myotubes were similar to a young muscle.

Most of the FRAP experiments were performed on myotubes after 10 to 17 days of differentiation. The myotubes were 10 to 40 μm wide and up to 1 mm in length.

Properties of FITC-dextran in aqueous medium

In solution the dextran molecule is essentially in a random-coil conformation. However, it has been proposed that to a first approximation, the hydrodynamic properties of dextrans are well modeled by those of hard hydrated spheres (Peters, 1986). To test this proposition, the diffusion constants $D$ of FD 10S, FD 20S, FD 40S, and FD 150 were determined in media of various viscosities. The diffusion coefficients of dextrans were proportional to the inverse of the viscosity. The values were then extrapolated to the buffer viscosity. According to the Stokes-Einstein equation $D = kT / 6πηR_n$, the hydrodynamic radius, $R_n$, may be calculated from the diffusion constants (Table 1) in aqueous medium. The values of $D_w$ varied as the square root of the inverse of the molecular weight (Fig. 3), rather than as the cubic root one could expect for a compact conformation.

The loose conformation of dextrans was also shown by chromatography of FD 150 (mean molecular mass 147,800 Da) on Superose 12 HR 10/30. Most of the ascending part of the elution peak was in the void volume of the column, and the maximum of the peak corresponded to a partition coefficient of 0.06. On the same column, the partition coefficient of a globular protein of equivalent molecular mass, aldolase (140 kDa), was 0.25.

Diffusion of FITC-dextran in cultured muscle cells

FRAP experiments were performed at least 10 h after FITC-dextran microinjection. After this incubation time, the dextrans were equally distributed along the myotubes; dextrans of $R_n$ greater than 4 nm (i.e., molecular mass higher than 40,000) were excluded from nuclei. The measurements were performed on different cultures of satellite cells. For each dish, 5–10 myotubes were observed. As the interfringes were set in the range 10–20 μm, the mobilities were averaged on several sarcomeres (see Fig. 1). FRAP experiments performed at different places on the same myotube gave the same dextran mobility. No significant differences were observed when the fringes were oriented oblique to the myofilaments; parallel orientation was hardly ever performed, as myofilaments are usually too thin. The values of the diffusion coefficients are presented in Table 1. The diffusion coefficients of dextrans in the cytoplasm of myotubes were much lower than in aqueous medium and were not inversely proportional to hydrodynamic radius, as would be expected for the mobility of inert macromolecules in a simple fluid (Fig. 4). The relative diffusion coefficient $D_{cyt}/D_w$ decreased with the hydrodynamic radius of dextrans, $R_n$ (Fig. 5). In the graph we have indicated the values obtained by $^{31}$P NMR spectroscopy by Yoshizaki et al. (1990) for the mobility of two small molecules, ATP and creatine phosphate, in the cytoplasm of heart cells. The extrapolation of $D_{cyt}/D_w$ to zero radius gives an estimate of 2.3 cP for the viscosity of the fluid phase of cytoplasm. The data may fit an exponential relationship between $D_{cyt}/D_w$ and $R_n$: $D_{cyt}/D_w = 0.43 \exp(-κI R_n)$, where the screening constant, $κ_I$, is 0.110 nm$^{-1}$.

### TABLE 1 Diffusion of FITC-dextran in aqueous solutions and in muscle cells

<table>
<thead>
<tr>
<th>Average MW FITC-dextran</th>
<th>$D_w$ (μm²/s)</th>
<th>$R_n$ (nm)</th>
<th>$D_{cyt}$ (μm²/s)</th>
<th>$D_{cyt}/D_w$</th>
</tr>
</thead>
<tbody>
<tr>
<td>9400</td>
<td>75 ± 3</td>
<td>2.9 ± 0.04</td>
<td>29.9 ± 0.7</td>
<td>0.40 ± 0.03</td>
</tr>
<tr>
<td>17,200</td>
<td>64 ± 2</td>
<td>3.4 ± 0.1</td>
<td>16.0 ± 1.8</td>
<td>0.25 ± 0.08</td>
</tr>
<tr>
<td>35,600</td>
<td>44 ± 5</td>
<td>4.9 ± 0.6</td>
<td>10.7 ± 0.9</td>
<td>0.24 ± 0.05</td>
</tr>
<tr>
<td>71,200</td>
<td>30 ± 2</td>
<td>7.15 ± 0.5</td>
<td>4.0 ± 0.7</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>148,000 (total)</td>
<td>18 ± 1</td>
<td>11.9 ± 1</td>
<td>n.d.</td>
<td></td>
</tr>
<tr>
<td>148,000 (f3)</td>
<td>26 ± 2</td>
<td>8.3 ± 0.6</td>
<td>5.6 ± 1.5</td>
<td>0.215 ± 0.09</td>
</tr>
<tr>
<td>148,000 (f2)</td>
<td>24 ± 2</td>
<td>8.9 ± 0.6</td>
<td>2.8 ± 0.6</td>
<td>0.12 ± 0.06</td>
</tr>
<tr>
<td>148,000 (f1)</td>
<td>17 ± 3</td>
<td>12.6 ± 2.2</td>
<td>2.2 ± 0.6</td>
<td>0.13 ± 0.06</td>
</tr>
</tbody>
</table>

Small molecules*

<table>
<thead>
<tr>
<th></th>
<th>$D_{cyt}$ (μm²/s)</th>
<th>$D_{cyt}/D_w$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP, 507</td>
<td>450</td>
<td>0.48</td>
</tr>
<tr>
<td>Creatine-P, 250</td>
<td>590</td>
<td>0.36</td>
</tr>
</tbody>
</table>

The values are given ± SD; $R_n$ is apparent hydrodynamic radius, in nanometers, calculated from the Stokes-Einstein equation: $D = kT / 6πηR_n$.

*Values determined by $^{31}$P-NMR (Yoshizaki et al., 1990).
FIGURE 4 Analysis of dextran mobility in terms of the Stokes-Einstein equation. The translational diffusion coefficients in dilute buffer ($D_w$) and in the cytoplasm of myotubes ($D_{cyt}$) were plotted versus the reciprocal hydrodynamic radius.

Geometric considerations

The hydrodynamic model of diffusion of Brownian spheres in rod polymer solutions (Cukier, 1984) may be applied to muscle cells. In this model, the polymer molecules (here, myofilaments) are modeled as prolate ellipsoids of major ($L$) and minor ($b$) axes, with $L \gg b$. If the rodlike polymers are fixed in space, they would exert a frictional force on the fluid (here, the aqueous part of the cytoplasm). The screening constant, $\kappa_L$, measures the resistance to fluid flow exerted by fixed rods and is related to the friction experienced by the rods. If the solution is diluted in rods, the $\kappa_L$ must be of the form $\kappa_L = \xi_L n_L/\eta$, where $\xi_L$ is the friction coefficient for one rod and $n_L$ the number density of the rods. $\xi_L$ (Tanford, 1961) is equal to $(6πη(L/2))/Ln(Lb)$. Cukier described a semi-empirical relationship between the friction coefficient $f$ of a Brownian particle with hydrodynamic radius $R_h$ in the filament network and the friction coefficient of this particle in the solvent: $ff_o = \exp(\kappa_L R_h)$. This relationship may be applied to the diffusion of dextran polymers in myotubes: $(D_{cyt}/D_w) = (\eta_{buffer}/\eta_{aqueous part of the cytoplasm}) \times \exp(-\kappa_L R_h)$. $\kappa_L$ may be estimated from the known structure of a sarcomere (Fig. 6) (Matsubara et al., 1984; Funatsu et al., 1993; Sosa et al., 1994): In a volume 2.5 $\mu$m $\times$ 0.045 $\mu$m $\times$ 0.040 $\mu$m, i.e., 0.0045 $\mu$m$^3$, there are at least one thick filament of myosin, $L = 1.63$ $\mu$m, $\phi = 0.025$ $\mu$m; four thin filaments of actin plus tropomyosin, troponins, and nebulin, $L = 1.12$ $\mu$m, $\phi = 0.008$ $\mu$m; and four titin filaments, $L = 1.25$ $\mu$m, $\phi = 0.002$ $\mu$m. A value of 66 $\mu$m$^{-1}$ (or 0.066 nm$^{-1}$) is obtained by taking only these filaments into account.

DISCUSSION

Before analyzing the diffusion of proteins in muscle fibers and their interactions with myofilaments, it is necessary to determine how inert tracers diffuse in these cells. The use of FITC-dextran as inert tracer particles within cells has been criticized by Luby-Phelps et al. (1987), who finally prefer fluorescein-labeled Ficoll. According to these authors, dextrans behave as flexible polymers and Ficoll much more like rigid spheres. Peters (1986) indicated that in solution dextran molecules were essentially in a random-coil conformation and that, to a first approximation, the hydrodynamic properties of dextrans were well modeled by hard hydrated polymers.
spheres. We have observed that in aqueous medium the diffusion coefficient of dextrans varied as \(1/M^{1/2}\), indicating that dextrans behave as random-coil polymers (Tanford, 1961). They are more loosely packed than equivalent proteins, and their hydrodynamic radius was higher than those of proteins of equivalent molecular weight; for instance, an FITC-dextran of molecular mass 148,000 has a \(R_h\) of 8 nm, two times higher than that of aldolase, which has the same molecular mass. The random-coil conformation of dextrans corresponded to that of hard hydrated spheres.

The diffusion of inert macromolecules has been studied in a few cellular species, namely fibroblasts 3T3 by Luby-Phelps et al. (1986, 1987) and hepatocytes by Peters (1986). Unlike these cells, which are characterized by a dynamic cytoskeletal network, muscle cells present a stable myofilament organization with thin and thick filaments of definite length, in which the dynamics is restricted to sliding of these filaments during contraction. Modulated fringe pattern photobleaching is a suitable technique for studying the diffusion of macromolecules in muscle cells, because fringes may be oriented, giving access to a possible anisotropy of diffusion in the sarcomeres. Another advantage of the technique is that the measurement is independent of motion of the cells, for example, beating of the myotubes. Nevertheless, we did not observe any significant difference in dextran diffusion when the orientation of the fringes was changed. Theoretical considerations by Han and Herzfeld (1993) suggested a possible anisotropy of diffusion of hard spheres in oriented polymers; this anisotropy becomes significant at a high volume fraction of filaments and in the cases where the diameter of the tracer is much larger than the diameter of the filaments. In our case, the volume fraction of myofilaments is 0.23 and the hydrodynamic diameter of tracers was higher than the titin diameter and varied from 0.5 to 2.5 times that of thin filaments (see Fig. 6 B). Assuming that the theory may be applied to heterogeneous parallel filaments, the maximum anisotropy of diffusion cannot be higher than 1.4. Another suggestion of the Han and Herzfeld theory is that the hindrance to diffusion of spherical particles among rodlike particles is lower when the rods are parallel to each other than when they are randomly oriented. The hindrance to diffusion is lower in skeletal muscle cells, where the filaments are parallel, than in 3T3 (Luby-Phelps et al., 1986, 1987) or in hepatocytes (Peters, 1986), where the cytoskeletal network is entangled.

We have observed that the distribution of fluorescent dextrans is homogeneous, except for the fact that nuclei are unlabeled for dextrans of \(R_h\) higher than 4 nm. Similar results have been reported on 3T3 fibroblasts (Luby-Phelps et al., 1986) and on hepatocytes (Peters, 1984; Lang et al., 1986), which can be expected, considering the size of nuclear pores. Note that in contrast to these latter cells, muscle cells do not present vesicularization, except in the process of dying.

Lang et al. (1986) have shown that in the case of dextran diffusion in the cytoplasm of hepatoma tissue culture cells, there was a linear relationship between \(D\) and \(1/R_h\). From this observation, they deduced a cytoplasmic viscosity of 6.6 cP. A similar relationship was not observed in the case of dextrans in cultured muscle cells.

The extrapolation to \(R_h = 0\) for the plot of \(D_{cyt}/D_w\) versus the hydrodynamic radius gave a value of 0.43. This corresponded to a viscosity of 2.3 cP for the aqueous part of the cytoplasm. This result is in good agreement with the result of \(^{31}\)P NMR spectroscopy (Yoshizaki et al., 1990), which showed that the diffusion of ATP and creatine-P is two times slower in heart cells than in water. This is also supported by the report (Kushmerick and Podolsky, 1969) that diffusivities of small cations and anions are reduced by a factor of 2 inside a muscle cell.

Muscle cells consist of a highly organized network of myofilaments and cytoskeleton. In a simplified view of the organization of the cytoplasm one could expect to observe, in the case of inert macromolecules of increasing size, a cutoff value of the hydrodynamic radius when its value is equivalent to the interfilament spacing. This naive hypothesis does not correspond to the present observations, where dextrans of diameter greater than the interfilament spacing are able to move in myotubes and where the decrease in \(D_{cyt}/D_w\) as a function of \(R_h\) is better fitted by an exponential function than by a linear one. The hydrodynamic model of diffusion of Brownian spheres in rod polymer solutions (Cukier, 1984) is here applied to muscle cells. In this model:

\[
D_{cyt}/D_w = \frac{\eta_{buffer}}{\eta_{aqueous \ part \ of \ the \ cytoplasm}} \times \exp(-\kappa_L R_h),
\]

where \(\kappa_L\) is a screening constant due to the filaments in the cytosol. Our observations correspond to a screening constant of 0.110 nm\(^{-1}\) and a viscosity of the aqueous phase of the cytoplasm 2.3 times higher than that of bulk water. The screening constant, \(\kappa_L = 0.066\) nm\(^{-1}\), evaluated by taking into account the known sarcomeric filaments (see Geometric considerations), is smaller than the experimental value. The dotted line in Fig. 5 illustrates that a 2.3-cP viscosity of the aqueous part of the cytosol and the evaluated screening constant do not adequately correspond to our experimental data. It is possible that other filaments of the cytoskeleton have to be included for estimation of the screening constant, but the introduction of two more titin filaments per sarcomeric unit gave only a low increase in the screening constant: from 0.066 to 0.069 nm\(^{-1}\). Furthermore, the 2.3-cP viscosity of the aqueous part of the cytoplasm did not agree well with recent papers showing that the averaged cytoplasmic solvent viscosity is not significantly different from bulk water (Kao et al., 1993; Luby-Phelps et al., 1993), and the fit did not take into account the crowding of the fluid phase of the cytoplasm of the myotubes. The cytoplasm of muscle cells contains a high amount of proteins, mainly glycolytic enzymes, creatine phosphokinase, and adenylate kinase. Therefore, this high concentration of biological macromolecules has to be taken into account when fitting the results (Zimmerman and Minton, 1993; Luby-Phelps, 1994). It is more realistic to consider the fluid phase of the cytoplasm as a crowded medium rather than a dilute solution of proteins.
in a water solvent of viscosity higher than that of bulk water. An experimental approach to this problem has been made by Hou et al. (1990) by studying the diffusion of fluorescently labeled tracers into a medium of crowded particles. These authors proposed that in cell cytoplasm,

$$D_{\text{cytoplasm}}/D_w = (D/D_w)^{\text{protein crowding}} \times (D/D_w)^{\text{filament screening}}.$$  

In the case of well-organized myofilaments, Cukier's equation may be used for the contribution of the filaments to the restriction of diffusion of macromolecules in myotubes with the calculated 0.066 nm$^{-1}$ value of $k_f$:

$$(D/D_w)^{\text{filament screening}} = \exp(-0.066 R_h).$$  

The experimental relation given by Hou et al. (1990) for the restriction to diffusion due to protein crowding has been applied here, with a free protein concentration of 135 mg/ml in the cytoplasm, which corresponds to the known value for muscle (Szent-Györgyi, 1960). The solvent viscosity of cytoplasm was assumed to be near that of bulk water. With these parameters, the data were accurately represented (Fig. 5, dashed line).

Thus, the mobility of inert molecules in muscle cells is size dependent, hindered by both the crowding of the fluid phase and the screening effect due to myofilaments. In the case of proteins, both specific binding to cellular structures and non-specific interactions due to electrostatic charges, for instance, are factors further contributing to intracellular mobility.

*Note added in proof:* In a recent study, Kraft et al. (1995) have shown that the time required for the equilibration of protein in muscle fibres was found to vary widely, from a few minutes to several days. This report emphasizes the importance of preliminary work with inert tracers.

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