# Low Viscosity in the Aqueous Domain of Cell Cytoplasm Measured by Picosecond Polarization Microfluorimetry

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*Abstract.* Information about the rheological characteristics of the aqueous cytoplasm can be provided by analysis of the rotational motion of small polar molecules introduced into the cell. To determine fluid-phase cytoplasmic viscosity in intact cells, a polarization microscope was constructed for measurement of picosecond anisotropy decay of fluorescent probes in the cell cytoplasm. We found that the rotational correlation time ($t_c$) of the probes, 2,7-bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein (BCECF), 6-carboxyfluorescein, and 8-hydroxypyrene-1,3,6-trisulfonic acid (HPTS) provided a direct measure of fluid-phase cytoplasmic viscosity that was independent of probe binding. In quiescent Swiss 3T3 fibroblasts, $t_c$ values were 20–40% longer than those in water, indicating that the fluid-phase cytoplasm is only 1.2–1.4 times as viscous as water. The activation energy of fluid-phase cytoplasmic viscosity was 4 kcal/mol, which is similar to that of water. Fluid-phase cytoplasmic viscosity was altered by <10% upon addition of sucrose to decrease cell volume, cytochalasin B to disrupt cell cytoskeleton, and vasopressin to activate phospholipase C. Nucleoplasmic and peripheral cytoplasmic viscosities were not different. Our results establish a novel method to measure fluid-phase cytoplasmic viscosity, and indicate that fluid-phase cytoplasmic viscosity in fibroblasts is similar to that of free water.

THE structure of cell cytoplasm has been a topic of major interest in cell biology (Fulton, 1982; Stossel, 1982; Taylor and Fechheimer, 1982; Porter, 1984). The view is now widely accepted that the cell cytoplasm is not a homogeneous solution but a gel-like structure composed of complex networks of actin filaments, microtubules, and intermediate filaments (Luby-Phelps et al., 1988). The aqueous domain of the cytoplasm that occupies the space between the cytoskeletal network contains macromolecules, and small organic and inorganic solutes (Horowitz and Miller, 1984; Bridgman and Reese, 1984; Paine, 1984). Many of the metabolic and enzymatic activities of living cells occur in the aqueous cytoplasm. Although the cytoskeletal network has been studied extensively, little information is available about characteristics of the aqueous domain of cell cytoplasm.

The physical state, or specifically the rheological characteristics of the aqueous domain of the cytoplasm, influences a number of intracellular dynamic processes, including solute transport, diffusion-limited enzyme kinetics, and cell motility. Current investigations on cell membrane transport and enzyme kinetics are based on the assumption that the cytoplasmic fluid has a physical state similar to that of aqueous solutions in vitro, so that thermodynamic information obtained in vitro can be applied to the biological activities in cell cytoplasm. In contrast, primarily based on proton nuclear magnetic resonance studies, the existence of "organized water" has been proposed (Clegg, 1984*a*; Parsegian and Rau, 1984). If the majority of cell water is organized by cytoplasmic macromolecules, thermodynamic information obtained in vitro would not be applicable to the cell (Clegg, 1984*b*). Because the rheological characteristics of organized water are probably very different from those of normal water (Keith et al., 1977*a*), the presence of organized water would have significant effects on intracellular dynamic processes.

The rheological characteristics of the aqueous domain of cell cytoplasm have been studied by several biophysical methods. However, because of methodological uncertainties, reported values of cytoplasmic viscosity vary in a range from 2 cP to >100 cP. One approach to measure cytoplasmic viscosity was the direct observation of the displacement of microinjected small magnetic particles and macromolecules in the cell (Valberg and Albertini, 1985; Dembo and Harlow, 1986). Values obtained by this method are higher than fluid-phase cytoplasmic viscosity because of mechanical barriers imposed by the meshlike structure of the cytoskeletal network. Another method was electron spin resonance (ESR).[1] From the translational diffusion of spin label probes, fluid-phase cytoplasmic viscosity values of 2–8 cP have been cal-

1. *Abbreviations used in this paper*: BCECF, 2,7-bis-(2-carboxyethyl)-5-(and-6-)carboxyfluorescein; BCECF-AM, BCECF-acetoxymethyl ester; 6-CF, 6-carboxyfluorescein; ESR, electron spin resonance; HPTS, 8-hydroxypyrene-1,3,6-trisulfonic acid.

culated (Keith et al., 1977*b*; Schobert and Marsh, 1982; Mastro et al., 1984). However, these values were based on uncertain estimates of the intracellular concentration of the spin probes. From the rotational motion of spin label probes, values of 2 to >50 cP have been calculated (Keith, 1973; Lepock et al., 1983: Matro and Keith, 1984); however, these calculations were based on the unproven assumption that the rotational motion of ESR probes is not hindered or blunted by binding of the probes to cytoplasmic macromolecules.

A more direct method to measure the fluid phase cytoplasmic viscosity is FRAP (Wojcieszyn et al., 1981; Salmon et al., 1984). Using a series of FITC-conjugated dextrans of different size, fluid-phase cytoplasmic viscosity was estimated to be 2–6 cP based on the extrapolation of diffusion coefficients to a dextran molecular size of zero (Luby-Phelps et al., 1986). These values may also be overestimated due to nonspecific binding of the probe to cytoplasmic macromolecules. Furthermore, the linear extrapolation to a probe size of zero may not give the correct value of fluid-phase cytoplasmic viscosity because of the possible nonlinear relationship between the probe size and viscosity. Lastly, steady-state fluorescence anisotropy was used to estimate the fluid-phase cytoplasmic viscosity (Lindmo and Steen, 1977; Hashimoto and Shinozaki, 1988; Dix and Verkman, 1990). The estimated values of 6–20 cP were in agreement with ESR values; however, it was necessary to assume that probe rotation is not restricted by binding to intracellular components.

The purpose of this study was to determine fluid-phase cytoplasmic viscosity without the ambiguities described above by measurement of time-resolved microfluorimetry of small polar fluorophores. We reasoned that the picosecond rotational motion of small probes would provide the most accurate measure of fluid-phase cytoplasmic viscosity, and that the use of fluorescence methodology would provide a rigorous approach to deconvolve effects of probe binding. In addition, the fluorescence method can be used to measure viscosity with subcellular spatial resolution. Swiss 3T3 fibroblasts were loaded with fluorescent probes and fluid-phase cytoplasmic viscosity was determined by measurement of fluorescence anisotropy decay using a new and powerful optical technique: multi-harmonic phase modulation fluorimetry. We examined effects of cell growth stage, temperature, cell volume change, cytoskeletal disruption, and phospholipase C activation on fluid-phase cytoplasmic viscosity, and compared the fluid-phase viscosity of cytoplasm and nucleoplasm.

## Materials and Methods

### Chemicals

2,7-Bis-(2-carboxyethyl)-5-(and-6-) carboxyfluorescein-acetoxymethyl ester, 688 $M_r$ (BCECF-AM), BCECF acid (520 $M_r$), 6-carboxyfluorescein, 376 $M_r$ (6-CF), and 8-hydroxypyrene-1,3,6-trisulfonic acid, 524 $M_r$ (HPTS) were purchased from Molecular Probes Inc. (Junction City, OR). Other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO).

### Cell Culture

Swiss 3T3 fibroblasts (American Type Culture Collection No. CL-101; Rockville, MD) were grown on 18-mm round glass coverslips in DME-H21 supplemented with 5% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml). Cells were maintained at 37°C in a 5% $CO_2$/95% air incubator. Exponentially growing cells or quiescent cells grown to confluence were studied. For fluorescence measurements, cells were perfused continuously in buffer containing (in millimoles): 0.7 $CaCl_2$, 1.1 $MgCl_2$, 2.7 KCl, 1.5 $KH_2PO_4$, 138 NaCl, 8.1 $Na_2HPO_4$ (buffer A) in a 200-μl perfusion chamber in which the cell free surface of the glass coverslip made contact with the immersion objectives (Chao et al., 1989). Experiments were performed at 24°C unless otherwise specified. To activate phospholipase C, 20 nM vasopressin was added to the perfusion solution. Cell volume was decreased by addition of sucrose. The cell cytoskeleton was disrupted by incubation of cells with 5 μg/ml cytochalasin B for 5 h.

### Labeling Protocol

For BCECF labeling, fibroblasts were incubated with buffer A containing 5 μM BCECF-AM for 15 min at 37°C. For 6-CF labeling, cells were incubated with 10 mM 6-CF, pH 6, for 15 min. 6-CF is relatively permeable to plasma membranes at pH 6, and is entrapped in cell cytoplasm at pH >7. 6-CF staining was stable for >30 min. For HPTS studies, cells were incubated with buffer A containing 10 mM HPTS for 15 min for uptake by fluid-phase endocytosis. The cells were then washed and incubated with buffer A for 2 h at 37°C. HPTS remain entrapped in the endocytotic vesicles for <30 min and was released into the cytoplasm by diffusion (Straubinger et al., 1990).

### Fluorescence Confocal Imaging System

Fluorescently labeled fibroblasts were viewed using an epifluorescence microscope (E. Leitz, Rochleigh, NJ) with a coaxial-confocal attachment (Technical Instrument Co., San Francisco, CA). The excitation source consisted of a 100-W Hg-arc lamp and 490 ± 5-nm six-cavity interference filter (Omega Optical, Inc., Brattleboro, VT). Light was reflected onto the perfusion bath by a 510-nm dichroic mirror. A 40× quartz objective (Leitz, N.A. 0.65, glycerol immersion, 0.35-mm working distance) was used. Emitted light was filtered by KV500 and GG530 cut-on filters and focused onto a variable gain, microchannel plate image intensifier (Videoscope International, Washington DC), and imaged with a solid-state CCD camera (Cohu Inc., San Diego, CA) operating at fixed gain. The output of the CCD camera was digitized with a frame grabber (DT2861; Data Translation, Marlboro, MA) and an auxiliary processing board (DT2858) in an 80287 computer.

### Time-resolved Fluorescence Measurements

Picosecond anisotropy decay of cytoplasmic fluorophores was measured by Fourier transform, multiharmonic fluorimetry by interfacing a fluorimeter (SLM Instruments, Inc., Urbana, IL) to a Nikon inverted epifluorescence microscope (Fig. 1; Verkman et al., 1991). Cells were excited with polarized light at 443 nm from a 25-mW He-Cd laser (Liconix, Sunnyvale, CA) or

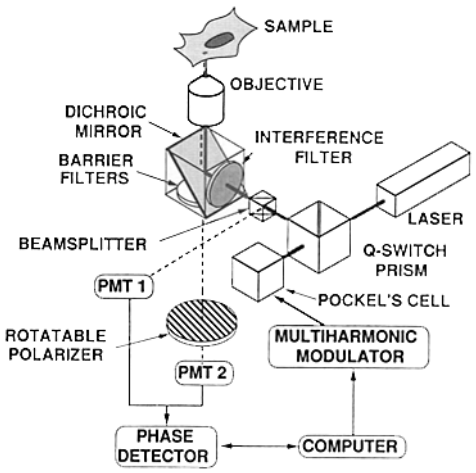


*Figure 1.* Schematic of apparatus for measurement of picosecond rotational correlation times by phase modulation microfluorimetry. The apparatus consists of a continuous wave laser, Q-switch prism, Pockel's cell, polarizers, fluorescence microscope, and phase sensitive photomultipliers.

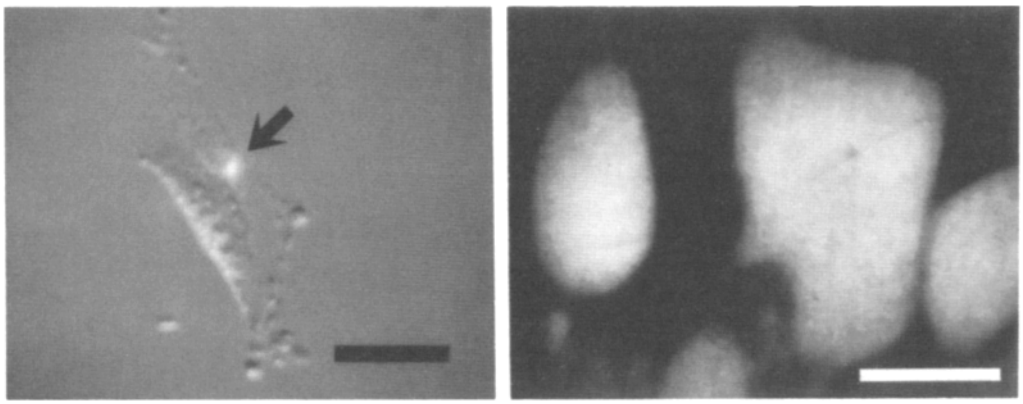

*Figure 2.* Micrographs of fibroblasts loaded with 5 μM BCECF-AM for 15 min at 37°C. (*Left*) Bright field Hoffman micrograph showing a 1-μm laser spot (*arrow*). (*Right*) Fluorescence confocal micrograph. Bar, 10 μm.

at 488 nm from a 4-W Argon laser (Coherent Inc., Palo Alto, CA). Light was modulated to form brief (1–2-ns) pulses by a Pockel's cell. 3% of the laser light was reflected by a beam splitter onto a reference photomultiplier via a glass fiber optic cable. The laser beam was reflected onto the cell chamber by a 1.5-mm square front surface mirror or a 510-nm fused silica dichroic mirror. Values of modulation amplitude ratios obtained with the dichroic mirror were corrected by a G-factor as described elsewhere (Fushimi et al., 1990). A 40× quartz objective and 20× objective (NA 0.75, 0.17-mm working distance) were used in anisotropy measurements; these objectives gave no measurable depolarizing effect (Axelrod, 1989). By use of lenses in the excitation path, the beam could be focused/defocused onto a <0.6-μm area in a single cell or onto one or more whole cells. Emitted light was filtered by serial KV500 and GG530 barrier filters and detected by a photomultiplier with an analyzing polarizer that could be rotated by 90°. The validity of the time-resolved anisotropy system was tested in every set of experiments by measuring solutions of known time-resolved anisotropy. Calibration solutions consisted of 20-μM BCECF in buffer A and in a 10% glycerol solution.

Differential phase angles and modulation amplitudes, representing the time-resolved motion of cytoplasmic fluorophores, were measured at 30–80 different modulation frequencies (4–280 MHz) in parallel by multiharmonic cross-correlation detection. Parameters for rotational models were fitted from differential phase angles and modulation amplitudes by a weighted nonlinear least squares procedure (Calafut et al., 1989). In studies of the kinetic response of cytoplasmic viscosity to various maneuvers, the complete multifrequency analysis was performed every 147 ms.

## Results

Fig. 2 shows a bright field (*left*) and confocal fluorescence micrograph (*right*) of fibroblasts labeled with BCECF. The arrow in the brightfield micrograph points to the focused laser beam. The fluorescence micrograph shows very little nonuniformity in the staining. Staining was also uniform in other focal planes and was not different after 30 min. Similar images were obtained for staining of fibroblasts with 6-CF and HPTS.

The viscosities determined from steady-state anisotropy and lifetime are upper limits to the true cytoplasmic viscosity because of the uncertain extent of fluorophore binding to cytoplasmic components. If fluorophore depolarizing rotations are blunted due to binding, then steady-state anisotropy is higher than that expected for an equivalent cytoplasmic viscosity in the absence of fluorophore binding. For an anisotropically rotating fluorophore, more than one rotational correlation time is required to describe the decay of anisotropy with time, $r(t)$,

$$r(t) = r_o \sum_i f_i \exp(-t/t_{ic}) \quad (1)$$

where $r_o$ is the maximum anisotropy in the absence of depolarizing rotations (0.39 for BCECF), and $f_i$ is the fractional component of anisotropy loss corresponding to rotational correlation time $t_{ic}$. Theoretically, the fastest component of the rotational motion should describe the rotational motion of the unbound fluorophore according to the Stokes-

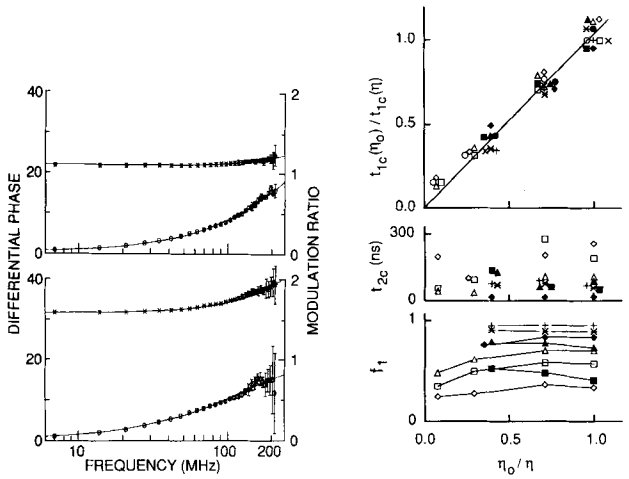


*Figure 3.* Anisotropy decay measurement of 10 μM BCECF, 10 μM 6-CF, and 20 μM HPTS in aqueous solutions containing glycerol and albumin measured by phase modulation microfluorimetry. (*Left*) Plot of differential phase angles (○) and modulation amplitude ratios (×) for BCECF in aqueous buffer (*top*) and in 10% glycerol + 0.3% albumin (*bottom*). Data were fitted to a two component anisotropic rotational model (Eq. 1). (*Right*) Dependence of rotational correlation times ($t_{1c}$, $t_{2c}$) and fraction of $t_{1c}$ ($f_1$) on the ratio of water-to-solution viscosity ($\eta_o/\eta$). Data are summarized for the three fluorophores in solutions containing different concentrations of glycerol to set $\eta_o/\eta$ and albumin or polylysine to alter binding. $f_1$ value of each fluorophore in PBS was 1.0. Symbols, which are displaced slightly in horizontal direction if necessary for clarity, indicate solution composition according to the legend: (○) BCECF in PBS; (△) BCECF in 0.3% albumin; (□) BCECF in 0.5% albumin; (◇) BCECF in 1.0% albumin; (×) 6-CF in PBS; (+) 6-CF in 0.3% albumin; (⁎) 6CF in 1.0% albumin; (●) HPTS in PBS; (▲) HPTS in 0.3% albumin; (■) HPTS in 1.0% albumin; and (◆) HPTS in 0.03% polylysine.

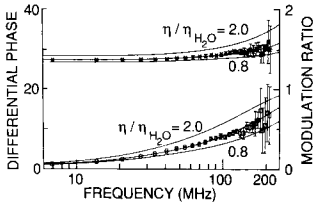


*Figure 4.* Plot of differential phase angles (○) and modulation ratios (×) (mean ± SD) of BCECF anisotropy decay in Swiss 3T3 fibroblasts. Data were fitted to a two component anisotropic rotational model. Fitted parameters: $t_{1c}$ 293 ps, $t_{2c}$ 18 ns, and $f_1$ 0.75 (chi-squared 0.9). Calculated viscosity relative to water was 1.23. Simulated curves for relative viscosities of 2.0 and 0.8 are shown for comparison.

Einstein equation, $t_{1c} = \eta V_h/kT$, where $\eta$ is the viscosity of fluid around the fluorophore; $V_h$ is the hydrated volume of the fluorophore molecule; k is the Boltzmann constant; $T$ is temperature. It should be noted that in macromolecule containing solutions, viscosity sensed by a small probe is almost equivalent to fluid-phase viscosity, whereas bulk viscosity may be higher (see below). The parameters $f_1$, $t_{2c}$, $t_{3c}$ . . . would depend upon the extent and characteristics of intracellular fluorophore binding. Methodology was therefore developed to measure $t_{1c}$ and $f_1$ for fluid-phase fluorophores in living cells and to validate the interpretation of $t_{1c}$ in terms of fluid-phase cytoplasmic viscosity. Because rotational correlation times of fluid-phase fluorescent molecules are often much less than 1 ns, specialized instrumentation was developed as described in Materials and Methods. Phase modulation fluorimetry was chosen because of its excellent resolution of short fluorescence lifetimes and rotational correlation times, and because of the rapidity in which measurements can be performed on photosensitive and labile biological samples.

To validate the interpretation of $t_{1c}$ in terms of fluid-phase viscosity, and $f_1$ in terms of fluorophore binding, anisotropy decay was determined in small droplets of perfusion buffer containing 10 μM BCECF, 10 μM 6-CF, or 20 μM HPTS. 0–64% glycerol was added to alter bulk viscosity and 0–1% BSA or 0–0.3% polylysine was added to alter fluorophore binding. In glycerol solutions, fluid-phase viscosity sensed by small fluorophores should be the same as the bulk viscosity because the size of glycerol molecules (92 $M_r$) is much smaller than that of the fluorophores (~400 $M_r$). As shown in Fig. 3 (*left*), phase angles and modulation amplitudes are sensitive to the glycerol and albumin. Fitted $t_{1c}$, $t_{2c}$, and $f_1$ are given in Fig. 3 (*right*) for experiments performed in solutions containing varying glycerol, albumin, and polylysine. Over a wide range of glycerol and albumin concentrations, and for the three fluorescent probes, $(t_{1c})^{-1}$ was linearly related to (viscosity)$^{-1}$ as predicted theoretically. The independence of $t_{1c}$ on protein concentration indicates that $t_{1c}$ is a good measure of fluid-phase viscosity even in the presence of considerable fluorophore binding. The strong dependence of $t_{2c}$ and $f_1$ on protein concentration indicates significant and complex fluorophore binding. The approximate independence of $f_1$ on glycerol concentration indicates that fraction of bound fluorophore does not depend upon solution viscosity. These results validate the interpretation of $t_{1c}$ in terms of fluid-phase viscosity.

Additional anisotropy decay studies were carried out to show that $t_{1c}$ for BCECF does not depend upon pH or excitation wavelength. At low pH ($<< pK_a$ = 6.3), BCECF is protonated, whereas at high pH (>> 6.3) BCECF has multiple negative carboxyl groups. $t_{1c}$ and $f_1$ were measured in droplets containing 10 μM BCECF in perfusion buffer titrated to pH 5.5 and 8.0, using an excitation wavelength of 443 nm (He-Cd laser) or 488 nm (argon laser). In the absence of glycerol, $t_{1c}$ was 267 ± 12 ps (pH 5.5, mean ± SD for five measurements) and 257 ± 8 ps (pH 8.0) at 443 nm, and 263 ± 11 (pH 5.5) and 249 ± 8 (pH 8.0) at 488 nm. In the presence of 32% glycerol, $t_{1c}$ was 596 ± 25 ps (pH 5.5) and 649 ± 20 ps (pH 8.0) at 443 nm, and 620 ± 22 (pH 5.5) and 668 ± 21 (pH 8.0) at 488 nm. Therefore, the rotational correlation times does not depend upon the extent of BCECF protonation and therefore does not depend upon intracellular pH or excitation wavelength. The (2.4 ± 0.1)-fold increase in $t_{1c}$ for a 2.5-fold increase in viscosity (0–32% glycerol) in Fig. 3 (*right*) provides further support for the interpretation of $t_{1c}$ in terms of fluid-phase viscosity.

Anisotropy decay of BCECF, 6-CF, and HPTS were measured in Swiss 3T3 fibroblasts. A typical phase modulation plot for BCECF is shown in Fig. 4. To evaluate the sensitivity of fitted parameters to viscosity values, curves corresponding to viscosities of 2.0 and 0.8 times that of water are shown. Rotational parameters and calculated viscosities are summarized in Table I. Viscosities calculated from $t_{1c}$ values of the three fluorophores show that fluid phase cytoplasmic viscosity is very low, only 20–40% more viscous than free water. Fluid-phase cytoplasmic viscosity values determined from the three fluorophores were in good agreement, providing strong support for the validity of our method. $f_1$ and $t_{2c}$ values were different for these fluorophores indicating different intracellular binding properties, as predicted, because of differences in fluorophore structure and polarity. Although control experiments showed a significant amount of the fluorophore binding in low concentration albumin solutions (Fig. 3, *right*), $f_1$ values in Table I indicate that only 20–40% of the fluorophores were bound in the cytoplasm,

*Table I. Cytoplasmic Viscosity in Swiss 3T3 Fibroblasts*

| | | $t_{1c}$ | $t_{2c}$ | $f_1$ | $t_f$ | $\eta/\eta_{H_2O}$ |
|---|---|---|---|---|---|---|
| | | *ps* | *ns* | | *ns* | |
| BCECF | ($n$ = 15) | 294 ± 7 | 16 ± 2 | 0.78 ± 0.01 | 3.4 ± 0.2 | 1.22 ± 0.03 |
| 6-CF | ($n$ = 4) | 180 ± 20 | 14 ± 3 | 0.65 ± 0.01 | 4.5 ± 0.2 | 1.37 ± 0.17 |
| HPTS | ($n$ = 5) | 181 ± 12 | 25 ± 2 | 0.59 ± 0.01 | 5.1 ± 0.3 | 1.42 ± 0.09 |

Rotational parameters of the time-resolved anisotropy decay of BCECF, 6-CF, and HPTS in fibroblasts are given. Phase modulation data were fitted to a two component anisotropic rotational model; $t_{1c}$ and $t_{2c}$ are shorter and longer rotational correlation times; and $f_1$ is the fractional anisotropy loss corresponding to $t_{1c}$. Fluid-phase cytoplasmic viscosity relative to that of water, $\eta/\eta_{H_2O}$, was calculated from $t_{1c}$. Lifetime values ($t_f$) were obtained by phase modulation lifetime analysis. Values are mean ± SEM for $n$ separate cells. Experiments were performed at 24°C.

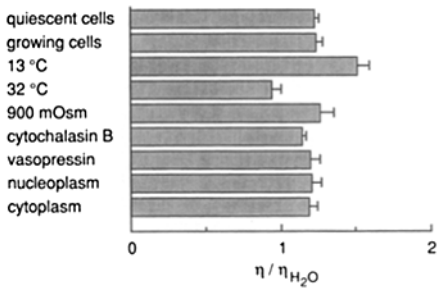


*Figure 5.* Fluid-phase cytoplasmic viscosity (mean ± SEM, for 3–5 measurements) in Swiss 3T3 cells measured by phase modulation microfluorimetry. Cells were loaded with 5 μM BCECF-AM at 37°C for 15 min. Anisotropy decay was fitted to the two component anisotropic rotational model. Fluid-phase cytoplasmic viscosities were calculated from $t_{1c}$. Data are shown for cells in different stages of growth, at low (13°C) and high (32°C) temperature, in hyperosmotic buffer (900 mOsm), and after incubation with 5 μg/ml cytochalasin B for 5 h. Viscosity in subcellular domains (nucleoplasm and peripheral cytoplasm) was measured using a focused laser spot. Unless specified, temperature was 24°C.

probably because intracellular proteins are much less efficient than albumin in fluorophore binding.

The fluid-phase cytoplasmic viscosity of fibroblasts was compared in a series of different conditions (Fig. 5). To examine the effect of cell growth on fluid-phase cytoplasmic viscosity, anisotropy decay of BCECF was measured in quiescent confluent cells and exponentially growing, nonmitotic cells. No significant difference in fluid-phase cytoplasmic viscosity was observed. The rotational correlation time was weakly temperature dependent. The activation energy for fluid-phase cytoplasmic viscosity determined from three to five measurements at different temperatures (13–32°C) was 4.3 ± 0.2 kcal/mol, not different from that of free water (4.1 kcal/mol).

The effects of modification of cytoskeletal components on fluid-phase cytoplasmic viscosity were examined (Fig. 5). When cell volume was decreased 1.5–2.5 times by addition of 300–600 mmol sucrose to perfusion solution, fluid-phase cytoplasmic viscosity increased by <5%. When cells were incubated with 5 μg/ml cytochalasin B for 5 h, fluid-phase cytoplasmic viscosity was decreased by 7%. Under these conditions, the organization of the actin cytoskeleton is altered from an isotropic to a focal distribution (Mastro and Keith, 1984; Cooper, 1987). To examine the effects of phospholipase C activation on fluid-phase cytoplasmic viscosity, 20 nM vasopressin was added and kinetics of anisotropy decay of BCECF was studied. In separate studies, vasopressin increased ionized calcium to >600 nM over a 0–90 s time course. Anisotropy decay was measured continuously every 0.14 s from 30 s before to 10 min after vasopressin addition to the perfusion solution. Vasopressin did not change fluid-phase cytoplasmic viscosity.

The spatial distribution of fluid-phase cytoplasmic viscosity in fibroblasts was examined by illuminating multiple small areas with a laser spot size of ~1 μm (Fig. 1, *left*). To avoid photobleaching, the laser beam was attenuated by neutral density filters and each set of measurements was completed within 30 s. Fluid-phase viscosity values were not different in cytoplasmic cortex and nucleoplasm. There were no significant differences in fluid-phase viscosity measured at multiple locations in nucleoplasm and peripheral cytoplasm.

As a reference for interpretation of fluid-phase cytoplasmic viscosity values, measurements of anisotropy decay of BCECF were made in 3–10% solutions of small molecules and macromolecules (Table II). Bulk viscosities of these solutions were determined by a Cannon-Fenske Viscometer (Fisher Scientific Co., Pittsburgh, PA). Table II shows that the rotational correlation time increases in parallel with bulk viscosity only in solutions containing small molecules but not in solutions containing macromolecules, indicating that fluorophore rotation provides an accurate measure of fluid-phase viscosity but not of bulk viscosity.

## Discussion

We have shown that the fluid-phase cytoplasm in fibroblasts is only 1.2–1.4 times more viscous than free water. Data were obtained by a novel time-resolved fluorescence anisotropy technique in which the rotational correlation time of fluid-phase probes was related directly to the fluid-phase viscosity. In addition, our experiments showed that cell growth stage, cell volume change, cytoskeletal disruption, and phospholipase C activation do not alter fluid-phase cytoplasmic vis-

*Table II. Fluid-Phase Viscosity and Bulk Viscosity of Solutions*

| | | $t_{1c}$ | $\eta/\eta_{H_2O}$ (BCECF rotation) | $\eta/\eta_{H_2O}$ (viscometer) |
|---|---|---|---|---|
| | | *ps* | | |
| PBS | | 255 ± 5 | 1.05 ± 0.02 | 1.05 ± 0.01 |
| 10% Alanine | (*89 D*) | 301 ± 12 | 1.24 ± 0.05 | 1.22 ± 0.02 |
| 10% Glycerol | (*92 D*) | 318 ± 10 | 1.31 ± 0.04 | 1.29 ± 0.02 |
| 10% Sucrose | (*342 D*) | 328 ± 12 | 1.35 ± 0.05 | 1.38 ± 0.02 |
| 10% Dextran | (*44 kD*) | 281 ± 19 | 1.16 ± 0.08 | 7.58 ± 0.05 |
| 3% Globin | (*68 kD*) | 320 ± 17 | 1.32 ± 0.07 | 1.56 ± 0.05 |
| 3% ALP | (*76 kD*) | 252 ± 15 | 1.04 ± 0.06 | 1.62 ± 0.04 |
| 10% γ-Globulin | (*150 kD*) | 261 ± 5 | 1.08 ± 0.02 | 1.30 ± 0.03 |
| 5% Mucin | | 268 ± 12 | 1.10 ± 0.05 | 1.50 ± 0.05 |
| 3% Muscle extract | | 254 ± 10 | 1.05 ± 0.04 | 1.33 ± 0.04 |

Fluid-phase viscosities of protein and dextran solutions were calculated from $t_{1c}$ values of BCECF rotational analysis. Bulk viscosities were measured by a Cannon-Fenske viscometer. All chemicals are obtained from Sigma Chemical Co. *ALP*, alkaline phosphatase. Data are mean ± SD for three measurements.

cosity significantly, and that fluid-phase viscosity of cytoplasm and nucleoplasm do not differ.

The time resolved fluorescence anisotropy method provided a quantitative value for viscosity of macromolecule-containing solutions like cytoplasm. The rotational correlation time of small polar fluorophores was directly proportional to viscosity as set by glycerol addition (Fig. 3). In the presence of albumin or polylysine, apparent fluorophore rotational motion was described well by a two-component model for anisotropic rotation. The fraction of anisotropy decay due to the faster rotational component decreased as the concentration of either BSA or polylysine increased. The shorter correlation time represented the rotation of unbound fluorophore whereas the longer correlation time represented the rotation of the protein-bound fluorophores, whose rotation was blunted and altered by binding (Lakowicz, 1983). Importantly, the relationship between the faster rotational correlation time and fluid-phase viscosity was similar, for all of the fluorophores used in this study, even in the presence of significant protein binding.

As shown in Fig. 2, cells labeled with BCECF showed quite uniform staining in the cytoplasm and the nucleoplasm. There was no evidence of fluorophore compartmentation 30 min after labeling. It is assumed that the submicroscopic distribution of fluorophores that do not bind to cytoplasmic structures is reasonably uniform. Diffusion of unbound fluorophore is not restricted geometrically since the size of these fluorophores, 10–12 Å, is much smaller than that of the cytoskeletal mesh (200–400 Å; Luby-Phelps et al., 1986). From the polar and hydrophilic characteristics, the unbound fluorophore molecules are expected to diffuse in the fluid-phase cytoplasm as small ionic and nonionic solutes.

Our value of fluid-phase cytoplasmic viscosity is significantly lower than the values estimated by ESR, FRAP, and steady-state anisotropy. In some ESR studies (Keith et al., 1977*a*; Schobert and Marsh, 1982; Mastro et al., 1984) and FRAP studies (Wojcieszyn et al., 1981; Salmon et al., 1984; Luby-Phelps et al., 1986), cytoplasmic viscosity values estimated from the diffusional movement of probes were generally higher than fluid-phase viscosity because of the non-Newtonian characteristics of cytoplasmic fluid. Mechanical barriers imposed by the cytoplasmic macromolecules and nonspecific low affinity binding of the probes to cytoplasmic structures alter probe diffusional motion (Luby-Phelps et al., 1988). In other ESR studies (Lepock et al., 1983; Mastro and Keith, 1984) and steady-state anisotropy studies (Lindmo and Steen, 1977; Hashimoto and Shinozaki, 1988; Dix and Verkman, 1990), cytoplasmic viscosity values estimated from probe rotation were too large because of probe binding. As shown in the present study, small, polar fluorophores do bind to cytoplasmic components appreciably. Contrary to the previous methods, time-resolved analysis of anisotropy provides information about the rotational movement of unbound fluorophores, a quantity that is related directly to fluid-phase cytoplasmic viscosity in a complex macromolecule solution.

Low fluid-phase cytoplasmic viscosity suggests that the diffusional movement of small solutes in cytoplasm is similar to that in a dilute aqueous solution. However, the bulk viscosity of aqueous cytoplasm is probably higher because of the presence of cytoplasmic macromolecules. As shown in Table II, small solutes increase the fluorophore rotational correlation time in parallel with bulk viscosity, whereas macromolecules increase bulk viscosity with minimal effect on fluorophore rotation. In aqueous cytoplasm, diffusion of small molecules is mainly limited by fluid-phase viscosity, whereas diffusion of macromolecules is limited by the interaction with other macromolecules and the cytoskeletal mesh (Mastro and Keith, 1984; Luby-Phelps et al., 1988). Therefore, our finding of low fluid-phase cytoplasmic viscosity indicates that intracellular dynamic processes involving small solutes are similar to those in free solution.

Similarly, the low fluid-phase cytoplasmic viscosity and the activation energy of ~4 kcal/mol suggest that the physical state of water molecules in cytoplasm is similar to that of free water. The physical state of cell water and the possibility of organized cell water have been debated. Based on nuclear magnetic resonance studies showing that the rotational movement of 10–50% of cell water molecules is slower than that in free solution, it was suggested that a significant fraction of cell water is "bound" or "organized" by macromolecules (Cameron et al., 1988). Although the rheological characteristics of organized water have not been defined clearly, our findings that the rheological characteristics of cell water are comparable to those of free water are not consistent with extensive organization of cell water. (The rotational correlation time of BCECF measured in ice was >15 times longer than that in free water. Therefore, the rotational motion of fluorophores is probably sensitive to physical state or organization of water.) However, there are several cautions in the assessment of water organization by measurement of probe rotation. First, if the fluorophore probes were selectively excluded from organized water, our measurement would not provide information about the physical characteristics of organized water. However, because the distribution of small polar fluorophores would be similar to that for small ions and solutes, fluorophore inaccessible regions in the cell might be biologically inactive. Second, even if fluorophores do diffuse into organized water, the rotational movement of fluorophore in organized water might be very slow and indistinguishable from the rotational movement of bound fluorophores. In this case, the $f_i$ values in Table I indicate that at most 20% of water molecules are organized.

Our findings that viscosity of fluid-phase cytoplasm is not different from that of nucleoplasm, and not modified by major cytoskeletal alterations, suggest little influence of the cytoskeletal network on the rheological characteristics of the fluid-phase cytoplasm. In previous studies, small changes of ESR probe movement by actin filament disruption were observed (Mastro and Keith, 1984); however, these changes might be caused by alteration of probe binding to cytoplasmic structures. Interestingly, whereas nucleoplasm has little filamentous structure and different solute composition compared to cytoplasm (Horowitz and Miller, 1984; He et al., 1990), it was found that fluid-phase viscosity of nucleoplasm and cytoplasm are similar.

In conclusion, we showed that fluid-phase cytoplasm and nucleoplasm in fibroblasts is only 1.2–1.4 times as viscous as free water and not influenced by major alterations in the cytoskeleton. The present study establishes an optical method to measure fluid phase cytoplasmic viscosity in selected regions of single cells. Our findings suggest that the majority of cell water is not organized and has physical properties similar to those of free water.

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