ROTATIONAL DIFFUSION OF TEMPONE IN THE CYTOPLASM OF CHINESE HAMSTER LUNG CELLS

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ABSTRACT The correlation time for rotational diffusion (τ_R) of 2,2,6,6-tetramethyl-4-piperidone-*N*-oxide (TEMPONE) in Chinese hamster lung (V79) cells has been measured. For these cells in an isosmotic solution at 20°C, τ_R = 4.18×10^{-11} s, ~3.6 times greater than $\tau_R = 1.17 \times 10^{-11}$ s in water. The relationship between τ_R and viscosity was investigated in a number of glycerol-water (0–50%) and sucrose-water (20–40%) solutions and a constant Stokes-Einstein volume of 44 Å^3 was found for TEMPONE in solutions of <20% glycerol and sucrose. This gives an average shear viscosity (for rotation of a small molecule) of 0.038 poise for the cytoplasm. When nonsecular terms were used in the calculation of τ_R, the activation energies for rotation of TEMPONE in the above solutions correlated well with the activation energies for shear viscosity. The viscosity increases as the cell is shrunk in hypertonic solutions. It also increases with decreasing temperature with an activation energy of 3.7 kcal/mol, about the same as the activation energy for the viscosity of pure water. The rotational correlation times were carefully calculated considering inhomogeneous line broadening, non-Lorentzian line shapes, the need for accurate tensor values and nonsecular terms.

INTRODUCTION

In recent years it has become apparent that the cytoplasm of mammalian cells is highly structured on a gross scale by several filamentous systems that can undergo polymerization and depolymerization under various conditions (1, 2). There is some evidence that at least one system, the microtrabecular network, directly interacts with subcellular organelles and structures as small as ribosomes (3). Small scale organization has not been demonstrated, but theories have been advanced that the cytoplasmic water is strongly "structured" by the surfaces of proteins and other macromolecules (4). The motional behavior of molecules within the cytoplasm, depending upon their size, might be affected by any of these systems.

There are a number of ways of studying the physical properties of the cytoplasm. Water can be studied directly by proton nuclear magnetic resonance (NMR) (5). There is some controversy about how these measurements should be interpreted, but it appears that a small fraction of water is considerably hindered while the majority of water molecules behave like those in bulk water. The shear viscosity of cytoplasm from lysed bacterial cells has been directly measured and found to be very large (6). This lysate is not stable and it is difficult to interpret these measurements on a molecular scale because of the presence of membrane and nucleic acid fragments, but translational diffusion constants of sucrose, dextran, and β-galactosidase were considerably larger than expected for the measured value of shear viscosity. In addition, the effective viscosity for diffusion was found to increase as the size of the diffusing molecule increased.

Another approach taken to investigate cytoplasmic viscosity has been to incorporate spin-label or fluorescent-probe molecules into cells and measure parameters related to their rates of motion. This has some advantages since one is ultimately interested in how the physical properties of the cytoplasm affect the motion of cellular molecules, but this approach is prone to misinterpretation since the relationship between the parameters measured and the rate of motion is not trivial. Steady state fluorescent polarization measurements on the protozoan *Euglena gracilis* (7), the yeast *Saccharomyces cerevisiae* (8), human lymphocytes (9), and human cervical carcinoma NH1K 3025 cells (10), using fluorescein diacetate, gave polarization values that correspond to cytoplasmic viscosities of 6.3 to 13.6 centipoise (cp). Pure water has a bulk viscosity of ~1 cp at room temperature. Nearly all fluorescent molecules rotate anisotropically, even in isotropic fluids, and fluorescein almost certainly behaves similarly (11). This can lead to uncertainties in the determination of viscosity when the ratios of the rotational rates about the symmetry axes changes with viscosity, which appears to occur for some aromatic fluorescent molecules (11). In addition, although the extracellular fluorescein diacetate is nonfluorescent, there can be sufficient leakage of fluorescein, except at very low temperatures, after intracellular cleavage of the acetate groups, to interfere with the intracellular signal (12).

A number of studies have investigated the rotation of small, water soluble spin-label probes in cells using various paramagnetic species to eliminate the extracellular signal. This technique was introduced by Keith and Snipes who

measured the rotational correlation time (τ) of TEMPONE in a number of different cells (13). Measurements using TEMPONE and other spin labels in red blood cells (14, 15), mammalian neurons (16), BHK cells (17), and spinach thylakoids (18) gave τ values corresponding to four to tenfold increases in microviscosity over that of pure water. These results agree with those using fluorescence polarization, although measurements in some cells have shown a much greater viscosity (13).

Wojcieszyn et al. have measured the translational diffusion of proteins in mammalian cells by the technique of fluorescence recovery after photobleaching (19). They observed that the diffusion coefficients for F-IgG and F-BSA are ~70 times less than those in water. This corresponds to a much greater viscosity than that experienced by small molecules for rotation. Thus, either the effective viscosity for translation is much greater than that for rotation or molecules of different sizes experience considerably different effective viscosities. It appears that the structures and factors controlling translational diffusion may be different than those controlling rotation since recently Livingston et al. have measured the rotational diffusion of myoglobin in situ to be only 2.3 times less than that in a dilute aqueous solution (20). In this study we have used electron spin resonance spectroscopy to investigate the rotational diffusion of the spin label TEMPONE in Chinese hamster lung (V79) cells. The determination of accurate correlation times of rotation (τ_R) and the relationship between τ_R and viscosity is carefully considered.

MATERIALS AND METHODS

Cell Culture

Chinese hamster lung fibroblasts were grown in Basal Medium Eagle's (BME) supplemented with *L*-glutamine, 15% fetal calf serum, and the antibiotics penicillin and streptomycin. Cells to be used for experimental purposes were grown in roller bottles at pH = 7.4 and harvested in exponential phase using 0.25% trypsin. After removal from the roller bottles, the cells were suspended in spinner flasks containing calcium, magnesium-free BME supplemented with 10% fetal calf serum for 2 h at 37°C to repair possible trypsin damage. V79 cells do not divide but can be maintained for long periods in suspension.

ESR Sample Preparation (Cells)

For each measurement, an aliquot of cells was removed from the spinner flask, the cells pelleted and resuspended in a solution (pH = 7.4) consisting of potassium ferricyanide (80 mM), Hepes buffer (25 mM), 2,2,6,6-tetramethyl-4-piperidone-*N*-oxide (TEMPONE, 1 mM), and sodium chloride (various amounts). The sodium chloride content was varied to change the tonicity. Solutions labeled 0.73X (3.4 mM NaCl, 259 mOsM), 1X (66 mM NaCl, 355 mOsM), 1.38X (145 mM NaCl, 489 mOsM), 1.85X (222 mM NaCl, 658 mOsM), 2.26X (300 mM NaCl, 802 mOsM), 2.65X (378 mM NaCl, 941 mOsM), and 3.32X (534 mM NaCl, 1,179 mOsM) were used where the osmolality was measured by freezing point depression. The cells were then immediately pelleted and the pellet taken up in a small capillary. Electron spin resonance (ESR) spectra were then obtained of the cell pellet using a Varian E-12 spectrometer (Varian Associates, Inc., Palo Alto, CA). A maximum time of ~10 min was required from addition of the ferricyanide solution until two spectra were recorded. These were then averaged.

Rotational Correlation Time Calculations

An accurate correlation time of rotation (τ_R) can be calculated for spin labels when the rotation is isotropic and sufficiently rapid. This is true of TEMPONE in many solvents (21), but a careful analysis is required. Essentially the method of Poggi and Johnson (22) and Hwang et al. (21) has been followed. Correlation times were calculated using $\tau_B(1 + \frac{3}{4}u) = K_B[\Delta H(-1) - \Delta H(+1)]$ and $\tau_C(1 - \frac{1}{3}u) = K_C[\Delta H(+1) + \Delta H(-1) - 2\Delta H(0)]$, where ΔH is the intrinsic Lorentzian line width, K_B and K_C are constants determined from the B and C coefficients of $1/T_2(m) = A + Bm + Cm^2$, and the term containing u is the nonsecular contribution, where $u = 1/(1 + \epsilon\omega_o^2\tau_{CB}^2)$, where ω_o is the microwave frequency and $\epsilon = 1$ for Debye spectral densities. For both TEMPONE and peroxylamine disulfonate, at $\tau < 10^{10}$ s, there is anomalous line width behavior, which has been attributed to the nonsecular spectral densities (21, 23–25). The use and justification of ϵ as a correction for the Debye spectral densities is given in reference 25. The constants can be expressed as

$$K_B = \frac{-15\sqrt{3}\ \pi g|\beta|}{8\,h\,b\,\Delta\gamma\,B_o}$$

$$K_c = \frac{4\sqrt{3}\ \pi g|\beta|}{h\,b^2}$$

$$\Delta\gamma = \frac{|\beta|}{h}[g_{zz} - 1/2(g_{xx} + g_{yy})]$$

$$b = \frac{4\pi}{3}[A - B],$$

where the notation used is the same as Poggi and Johnson (22). Correlation times were also calculated using $\tau'_B = K_B[\Delta H'(-1) - \Delta H'(+1)]$ and $\tau'_c = K_c[\Delta H'(+1) + \Delta H'(-1) - 2\Delta H'(0)]$, where the line widths ($\Delta H'$) were measured directly from the first derivative ESR spectra, and K_B and K_c are as previously defined. In the calculation of τ'_B and τ'_c the nonsecular contribution was ignored.

The value of $\Delta\gamma$ is dependent upon the g tensor anisotropy, and b is dependent upon the hyperfine tensor anisotropy. These must be accurately known for the spin label in the particular solvent used for an accurate determination of correlation times. The tensor values are often determined from computer simulations of the rigid-limit spectrum or in principle the A_{zz} and g_{zz} values can be measured directly from the rigid-limit spectrum and $1/2(g_{zz} + g_{yy})$ and $(A_{xx} + A_{yy})$ determined from these values and the averaged values of the motionally narrowed spectrum. There was too much broadening of the protonated TEMPONE spectrum in the protonated solvents used in this study to accurately determine A_{zz} and g_{zz}, so the values determined by Hwang et al. for perdeuterated TEMPONE in 85% deuterated glycerol and water were used (21). These gave values of $K_B = 5.62 \times 10^{-10}$ s/G (gauss) and $K_c = 4.85 \times 10^{-10}$ s/G. This probably leads to a small inaccuracy in the calculation of τ_R in the solvents used in this study and in cells, since the tensor values should be somewhat different under these conditions. For example g and A_N were found to increase by 0.015% and 0.91% between 50% glycerol and pure water, respectively.

The intrinsic Lorentzian line width was extracted from the inhomogeneously broadened, measured first-derivative line width through the use of a calibration curve (22). The 12 methyl protons give an envelope of 13 Lorentzian lines, with a binomial intensity distribution. The amplitude of this envelope was calculated taking $a_H = 0.1$ G (which gave curves that fit the shape of the experimental spectra). The hyperfine splitting of the methylene protons is only one tenth that of the methyl protons, and hence, negligible (26, 27). This broadened absorption curve was differentiated using a differential equal to the modulation width. The peak-to-peak line width was then measured from this first derivative curve and the relationship between the intrinsic Lorentzian line width and first derivative line width found. This also allows instrumental broadening to be partially accounted for.

Nonsecular contributions are significant for small spin labels undergoing rapid rotation. A computer using an iterative technique was used to solve the equations for τ_B and τ_c. To obtain consistent correlation times, non-Debye spectral densities had to be assumed. This implies that additional relaxation mechanisms exist. Poggi and Johnson (22) used $\epsilon = 1$ for TEMPONE in *n*-butyl alcohol, and Hwang et al. used $\epsilon = 4$ for TEMPONE in an 85% glycerol-water mixture. We also found that $\epsilon = 4$ gave the most consistent results for all the solutions used. The value of ϵ is quite sensitive to that particular g and A tensor values used and appears to be related to the molar transition energy scale (25).

Survival Curves

Cells were harvested from the roller bottles as described and seeded onto tissue culture plates at 200 cells per plate. The cells were given 3 h to attach at 37°C, the medium removed and replaced with either the ferricyanide solutions or medium containing 0.5 mM or 1.0 mM TEMPONE for the specified time intervals. The ferricyanide solution or medium containing TEMPONE was then removed and the cells washed twice with complete medium before adding fresh medium and incubating at 37°C for 8–10 d for colony formation. Survival was then calculated as the ratio of the number of colonies formed in the treated plates to the number of colonies of the control plates (no ferricyanide or TEMPONE treatment). The plating efficiency of the controls was ~90%.

RESULTS

Solution Measurements

Before any definite conclusions can be made about the magnitude of cytoplasmic viscosity, some knowledge is needed of how well τ_R of TEMPONE mirrors the viscosity of simple solutions. Measurements of τ_R were made as a function of temperature in glycerol-water and sucrose-water solutions, which allow two independent ways of varying shear viscosity: composition and temperature. By varying composition, specific solvent-TEMPONE interactions become apparent. The values of τ_B and τ_c were within a few percent of each other in all solutions at all temperatures. This indicates that the rotational motion of TEMPONE is isotropic or very close to isotropic and that the rotational correlation times calculated are consistent and should be accurate. The actual rotational correlation time was taken as τ_c and will be represented by τ_R.

Arrhenius plots of τ_R in the glycerol-water mixtures are shown in Fig. 1. The spectrum of TEMPONE in water is shown in Fig. 4. The curves are fitted by a least-squares fit of ln τ_R vs. $1/T$, although in general there appears to be a slight upward curvature (sometimes hidden by the scatter of the points), which is also seen if the shear viscosity (values from reference 28) of the glycerol-water mixtures is plotted in the Arrhenius fashion. The curvature is very small over the temperature range of 0–50°C and has no effect upon the results. It can be corrected by using a relationship of the Arrhenius form but second order in T for the curve fitting or by allowing the activation energy to vary with temperature.

The activation energies for rotational diffusion of TEMPONE in the glycerol-water mixtures and in 20%, 30%, and 40% sucrose in water (correlation times not shown) are given in Table I. The activation energies calculated from

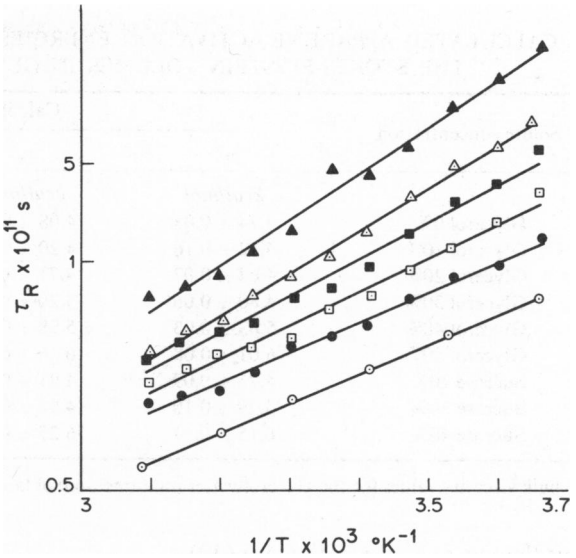

FIGURE 1 Arrhenius plots of τ_R in 0% (○), 10% (●), 20% (□), 30% (■), 40% (△), and 50% (▲) glycerol by weight in water. The straight lines were determined from a linear regression fit of ln τ_R vs. $1/T$.

the shear viscosities are also given and are an average value over this temperature range from 0–50°C, but from the small values of the standard deviations clearly the variation in activation energy over this temperature range is small. The activation energies for rotational diffusion of TEMPONE are quite close to those for shear viscosity and imply that similar mechanisms control both processes.

The rotational correlation times of TEMPONE in glycerol and sucrose solutions were also calculated neglecting nonsecular contributions. These values are referred to as τ'_B and τ'_c, and in general τ'_B did not equal τ'_c and the ratio τ'_c/τ'_B varied as a function of temperature. The activation energies calculated from τ'_B and τ'_c are also given in Table I. The activation energies from τ'_B and τ'_c are usually less than and greater than, respectively, the activation energies from shear viscosity for the various solutions. The correlation between these activation energies is much poorer than the correlation between the activation energies from τ_R and the shear viscosity. This good correlation between activation energies is a strong argument for the necessity of including nonsecular terms in the calculation of the rotation correlation time for TEMPONE in this motional range ($\tau_R < 7 \times 10^{-11}$ s).

The rotational correlation time of a generalized ellipsoid about each of the three axes can be related to the frictional resistance to rotation by a Stokes-Einstein relation of the form (29, 30) $\tau_{Ri} = (\beta_i)/(6kT)$ where $\beta_i = V_{SE_i}\eta$ and V_{SE_i} is commonly called the Stokes-Einstein volume and will in general be equal to the molecular volume only for a sphere with a volume much larger than the fluid boundary layer. TEMPONE can be approximated by a prolate ellipsoid with the major axis, $a = 4.2$ Å, and the minor axis, $b = 2.9$ @ (31). For a prolate ellipsoid the Stokes-Einstein volumes

TABLE I

CALCULATED APPARENT ACTIVATION ENERGIES OF THE BULK VISCOSITY (η) AND T_R OF TEMPONE, AND THE STOKES-EINSTEIN VOLUMES IN GLYCEROL-WATER AND SUCROSE-WATER MIXTURES

Solute concentration	Calculated activation energies				Stokes-Einstein volume
	η^*	τ_R	τ'_B	τ'_c	
	kcal/mol	*kcal/mol*	*kcal/mol*	*kcal/mol*	*Å³*
Glycerol 0%	3.74 ± 0.08	4.08 ± 0.05	3.16 ± 0.13	4.35 ± 0.26	44 ± 1
Glycerol 10%	4.32 ± 0.16	4.20 ± 0.15	3.31 ± 0.13	5.04 ± 0.19	44 ± 1
Glycerol 20%	4.64 ± 0.07	4.71 ± 0.21	3.75 ± 0.29	5.53 ± 0.22	44 ± 2
Glycerol 30%	4.80 ± 0.06	5.20 ± 0.25	4.17 ± 0.34	5.87 ± 0.29	44 ± 2
Glycerol 40%	5.63 ± 0.03	5.98 ± 0.13	5.17 ± 0.31	6.94 ± 0.24	33 ± 1
Glycerol 50%	6.01 ± 0.08	6.46 ± 0.27	6.27 ± 0.27	7.23 ± 0.27	33 ± 1
Sucrose 20%	4.75 ± 0.09	4.91 ± 0.17	3.89 ± 0.16	5.76 ± 0.17	43 ± 2
Sucrose 30%	5.29 ± 0.10	4.98 ± 0.21	4.21 ± 0.24	5.61 ± 0.20	32 ± 1
Sucrose 40%	6.15 ± 0.11	6.27 ± 0.09	5.89 ± 0.19	6.87 ± 0.08	27 ± 1

*The bulk viscosity values for the glycerol-water mixtures were obtained from reference 28 and those for the sucrose-water mixtures from reference 32.

about the two axes of rotation are (30)

$$V_{SE_a} = \frac{32\pi b^2(a^2 - b^2)}{3(2a - b^2S)}$$

$$V_{SE_b} = \frac{32\pi (a^4 - b^4)}{3[(3a^2 - b^2)S - 2a]}$$

where

$$S = \frac{2\ln\{[a + (a^2 - b^2)^{1/2}]/b\}}{(a^2 - b^2)}.$$

For TEMPONE V_{SE_a} = 130 Å³ and V_{SE_b} = 173 Å³. The average Stokes-Einstein volume, $\bar{V}_{SE} = (V_{SE_a} V_{SE_b})^{1/2}$ = 150 Å³, is close to the molecular volume of 148 Å³ because of the small deviation of TEMPONE from a sphere.

TEMPONE should have two different rotational rates about the two axes with a ratio equal to V_{SE_b}/V_{SE_a} = 1.33, which is too small of a difference to detect (21). Thus τ_R is actually a measure of the average of the two rotational rates where $\tau_R = \eta(\bar{V}_{SE})/(kT)$.

Fig. 2 contains plots of τ_R vs. η/T for the glycerol-water mixtures, and Fig. 3 contains similar plots for 20%, 30%, and 40% sucrose (32) in water (the original data for sucrose are not given). The relationship between τ_R and η/T is linear to within the accuracy of the measurements for most of the curves, although this simple relationship might not hold over a larger range of values of τ_R and η. The Stokes-Einstein volumes are given in Table I and, in general, they are different for each solution. Thus it is possible to predict η from τ_R, but $\bar{V}_{SE}$ of the probe must be known. In a solution of unknown composition, such as the cell cytoplasm, this will generally not be known.

The volumes given in Table I are considerably smaller than $\bar{V}_{SE}$ = 150 Å³ calculated from geometric considerations. One assumption made in deriving the Stokes-Einstein relation is that the rotating body carries a boun-

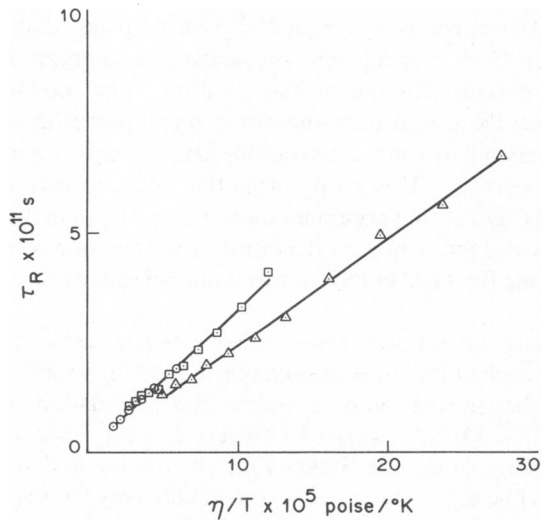

FIGURE 2 Plots of τ_R vs η/T for 0% (○), 30% (□), and 50% (△) glycerol in water. The 10%, 20%, and 30% curves fall on the 0% curve and the 40% curve falls on the 50% curve. The points for the 10% and 20% curves are not shown.

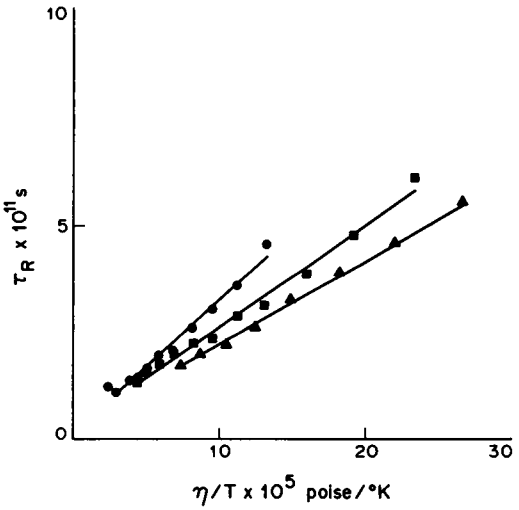

FIGURE 3 Plots of τ_R vs. η/T for 20% (●), 30% (■), and 40% (▲) sucrose by weight in water.

dary layer of fluid with it. This is sometimes called rotation with the sticking boundary condition (33). The other extreme is rotation with no interaction with the solvent, which gives an effective Stokes-Einstein volume of zero for a sphere (slipping rotation). Most small molecules in aqueous solutions undergo rotation similar to slipping rotation unless they can form several hydrogen bonds with the solvent (11). Even when undergoing slipping rotation, a prolate ellipsoid will experience frictional resistance because of hindered rotation. (The solvent molecules must be displaced for rotation to occur). The Stokes-Einstein volume can be calculated under these conditions, and using the calculations of Hu and Zwanzig (33) for the ratio of the frictional coefficients for slip and stick, one arrives at a value of $\overline{V}_{SE}$ = 13 Å^3 for TEMPONE undergoing slipping rotation only.

The volumes given in Table I are larger than this, indicating that TEMPONE rotates with a combination of slip and stick, the relative proportions of which are solvent dependent. As the glycerol concentration exceeds 30% and sucrose concentration exceeds 20%, the interaction of TEMPONE with the solvent decreases and slipping rotation becomes more important. The activation energy measured will depend upon both types of rotation and will include probe-solvent interactions for the sticking rotation. The strengths of these interactions are obviously similar to those between solvent molecules because of the similarity of activation energies. The activation energy for rotation of an asymetric molecule by slip alone should be determined by solvent interactions only.

The change in probe-solvent interaction, seen by a change in $\overline{V}_{SE}$ introduces a difficulty in determining viscosity of solutions in which $\overline{V}_{SE}$ is unknown. For viscosity determinations of the cytoplasm, we have taken $\overline{V}_{SE}$ = 44 Å^3 since this value is constant for sucrose and glycerol concentrations below 20%. The solute concentration within the cell is in this range although it is not clear what effect cellular macromolecules have upon $\overline{V}_{SE}$.

Cell Measurements

When added to a cell suspension TEMPONE rapidly diffuses across the plasma membrane and the ESR spectrum obtained is a sum of the intra and extracellular components. The extracellular component can be effectively removed by addition of a paramagnetic species with a short relaxation time capable of broadening the spectrum. The use of the ferricyanide ion, which for short exposures is nontoxic to V79 cells and can be buffered to pH = 7.4, for broadening is illustrated in Fig. 4. The spectral intensity of TEMPONE is reduced over 200-fold by 80 mM potassium ferricyanide due to an increase in line width from ~0.5 G to 7 G (Fig. 4 *A* and *B*). When cells are present only the 0.5 G line width spectrum is observed (Fig. 4 *C*). Upon making the cells permeable by a freeze-thaw cycle, the spectral intensity is reduced by a factor of 9 (Fig. 4 *D*). This remaining signal is probably due to a small

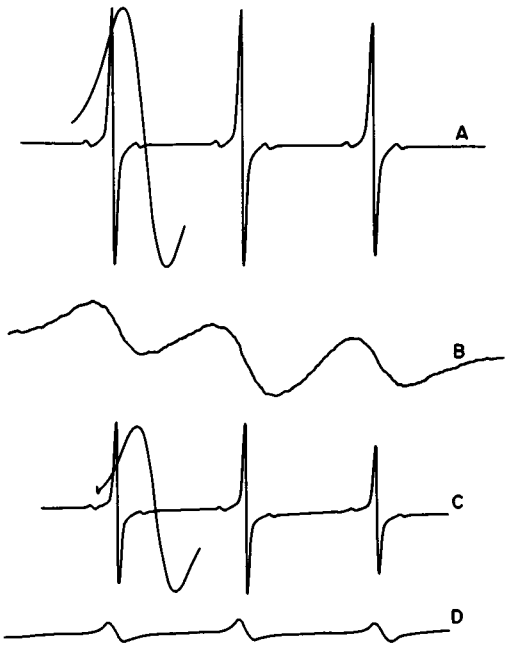

FIGURE 4 ESR spectra of TEMPONE (1 mM) in (*A*) water (gain = 1), (*B*) solution 1X, $K_3Fe(CN)_6$ = 80 mM (gain = 60), (*C*) V79 cells in solution 1X (gain = 4), and (*D*) same as *C* except the cells have undergone a freeze-thaw cycle. The mid-field line is expanded by a factor of 10 in *A* and *C*.

fraction of cells not made permeable, resistant compartments inside cells (i.e., mitochondria) and some TEMPONE partitioned into membranes.

A potassium ferricyanide concentration of 80 mM was found to produce sufficient broadening to eliminate the extracellular spectrum. The sodium chloride concentration was then varied so that the V79 cells had the same median volume as in normal culture medium, which is ~1,500 μm^3 (34). The volume was measured using a Celloscope cell sizer (Particle Data, Inc., Elmhurst, IL). This was labeled the 1.0X broadening solution that had a measured tonicity of 355 mOsm, somewhat greater than the 328-mOsm tonicity of culture medium. Cells tend to swell in high potassium solutions, so the greater tonicity needed to maintain their normal volume is expected (34).

In the 1.0X solution at 20°C, τ_R was found to equal $4.18 \pm 0.09 \times 10^{-11}$ s compared with $\tau_R = 1.17 \times 10^{-11}$ s in water at the same temperature. The Stokes-Einstein volume of TEMPONE in solutions of up to 30% glycerol and 20% sucrose is ~44 Å^3. At higher concentrations $\overline{V}_{SE}$ decreases, but assuming that TEMPONE undergoes similar interactions in the cytoplasm, a viscosity of 3.8×10^{-2} poise (p) is found. At 20°C, using $\tau_R = 1.17 \times 10^{-11}$ s, a viscosity is found for water of $\sim 1.1 \times 10^{-2}$ p, a factor of ~3 1/2 less.

The cell volume can be changed by placing the cells in solutions of varying tonicities. This was done and the tonicity was varied from 259 to 1,180 mOsM. The values of τ_R obtained at 24°C are given in Table II. There is an

TABLE II
VALUES OF τ_R IN V79 CELLS AT 23°C IN SOLUTIONS OF VARYING TONICITY

Solution*	Osmolality	$\tau_R \times 10^{11}$ s
	mOsM	
0.73X	259	3.71 ± 0.17
1.00X	355	4.18 ± 0.09
1.38X	489	4.61 ± 0.11
1.85X	658	6.29 ± 0.32
2.26X	802	6.64 ± 0.24
2.65X	941	7.69 ± 0.19
3.32X	1,180	7.50 ± 0.23

*Composition of each solution is given in Materials and Methods.

increase in τ_R with increasing tonicity, or decreasing volume. This is shown more clearly in Fig. 5, where τ_R is plotted as a function of cell volume. The cell volumes were calculated assuming a volume of 1,523 μm^3 at 355 mOsM and a volume of 380 μm^3 at infinite tonicity (34). The volume at 259 mOsM may be an overestimation since cells are able to somewhat adjust their volume back to a more normal value under hypotonic conditions (34).

If η, and hence τ_R, is proportional to the concentration of macromolecules and solutes within the cell, then τ_R should increase as the cell volume decreases. From Fig. 5 this clearly occurs. Measurements were made as a function of temperature from 8° to 37°C in the 1.0X solution (isotonic) and an Arrhenius plot of τ_R is shown in Fig. 6. There is a decrease in τ_R with increasing temperature with an activation energy of 3.71 ± 0.32 kcal/mol. This is within experimental error of the values obtained for pure water from both shear viscosity and τ_R.

Survival Curves

The 80 mM potassium ferricyanide solution is a nonphysiological environment. Survival curves of the V79 cells in some of the ferricyanide solutions are shown in Fig. 7. The fraction survival is measured as a plating efficiency. The plating efficiency is between 90 and 100% for cells maintained in phosphate buffered saline or the 1.0X solution for 30 min. There is some killing of the cells in the 2.65X and 3.32X solutions with time. The plating efficiency is ~83% in both solutions after 10 min, the maximum time necessary for the measurements. Values of τ_R of TEMPONE in cells in the 1.0X solution were constant for a period of at least 1 h. Thus, the toxicity, as determined by reproductive survival, of the potassium ferricyanide should have no effect on the measurement of τ_R.

Spin labels can also be cytotoxic after prolonged exposure (Fig. 8). Little killing occurs during the first 20 min of a 1 mM TEMPONE exposure, but there is greatly increased killing after this time. The precursor (2,2,6,6-tetramethyl-4-piperidone hydrochloride) of TEMPONE, before oxidation to the *N*-oxide form, at 1 mM concentration and 0.5 mM TEMPONE are not cytotoxic for exposures up to at least 100 min (Fig. 8). Thus, the threshold concentration for reproductive killing of V79 cells by TEMPONE over a period of a few hours exposure is between 0.5 and 1.0 mM. The *N*-oxide radical is necessary for killing. Reproductive death is measured 7–10 d after exposure and only signifies that a cell is unable to divide. This could be due to extensive structural damage or to a much more subtle damage, such as injury to DNA, that only impairs the ability to divide. The targets for either *N*-oxide radical or ferricyanide ion killing are not known.

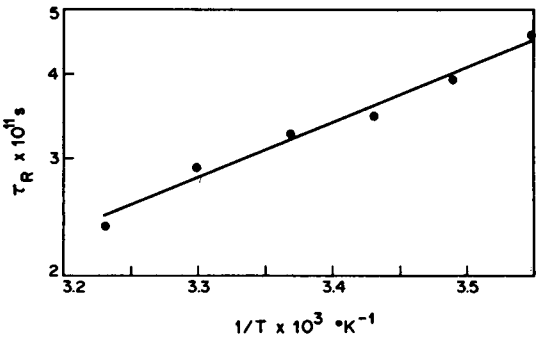

FIGURE 6 Arrhenius plot of τ_R vs. $1/T$ in V79 cells at isotonic volume.

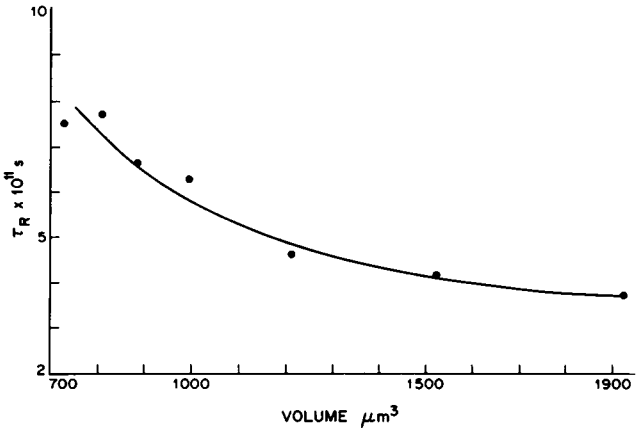

FIGURE 5 Plot of τ_R vs. cell volume of V79 cells at 20°C.

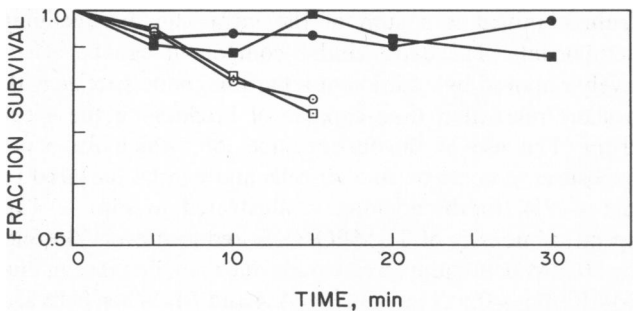

FIGURE 7 Fraction survival of V79 cells vs. time of exposure to phosphate buffered saline; no $K_3Fe(CN)_6$ (•), solution 1.0X, 80 mM $K_3Fe(CN)_6$ (■), solution 2.65X, 80 mM $K_3Fe(CN)_6$ (○), solution 3.32X, 80 mM $K_3Fe(CN)_6$ (□).

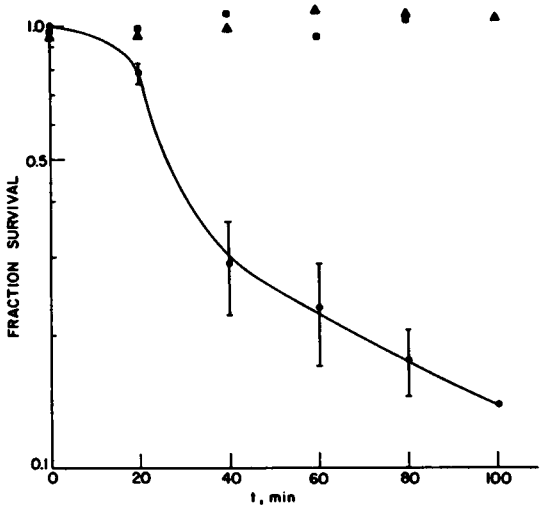

FIGURE 8 Fraction survival of V79 cells vs. time of exposure, in complete medium, to 0.5 mM TEMPONE (▲), 1.0 mM TEMPONE (•), and 1.0 mM 2,2,6,6-tetramethyl-4-piperidone hydrochloride (■).

DISCUSSION

To determine physical properties of materials, such as viscosity, using extrinsic probes, one must be able to both accurately determine the motional characteristics of the probe and be able to relate probe motion to the solvent properties that control it. The latter point requires a clear understanding of probe-solvent interactions. In addition, the probe must not perturb or damage the material being studied. It is possible to obtain accurate rotational correlation times for TEMPONE under conditions of rapid and isotropic rotation (21). TEMPONE is known to undergo isotropic, or nearly isotropic, rotation in many solvents (21). This also holds for glycerol and sucrose solutions of <50% solute concentration in water. To determine τ_R one must consider: (*a*) inhomogeneous line broadening by the 12 methyl protons surrounding the nitroxyl group, (*b*) non-Lorentzian line shapes, (*c*) the need for accurate $\bar{A}$ and $\bar{g}$ tensor values for TEMPONE in the solvent of interest, and (*d*) nonsecular terms including non-Debye spectral densities. In using all of the above τ_B becomes equal to τ_c, which is a necessary, but not sufficient, condition for accurate correlation times.

TEMPONE does not rotate by pure Brownian diffusion in the sense of carrying a boundary layer of solvent with it, but by a combination of slip and stick. There is some direct interaction with aqueous solvents, probably primarily through hydrogen bonds, that decreases in higher concentrations of glycerol and sucrose. This shows up as a decrease in the Stokes-Einstein volume, the value of which is needed for a determination of viscosity. Assuming that TEMPONE rotates with the same combination of slip and stick in the cell cytoplasm as in a 20% glycerol or sucrose solution, a viscosity value of ~3 1/2 times that of water is found for the cytoplasm of V79 cells over the temperature range of 5° to 37°C. The assumption of $\bar{V}_{SE}$ = 44 Å^3 is major, since there is no apparent direct method for determining $\bar{V}_{SE}$ of TEMPONE in the cytoplasm.

The cytoplasmic viscosity is dependent upon the concentration of molecules within the cell and increases as the cells are shrunk. The cytoplasmic viscosity of cells of normal size also varies with temperature with an activation energy of 3.71 kcal/mol, very close to that for water. Thus, a small molecule like TEMPONE experiences an effective viscosity to rotation in the cytoplasm only a few times greater than that in water. No evidence is seen of a large scale ordering or structuring of water. The rotation of TEMPONE is hindered by the interaction of TEMPONE with water molecules and by the necessity for transverse fluctuations or diffusion of water molecules due to the nonspherical shape of TEMPONE. This last term is small but becomes significant under conditions when TEMPONE forms weaker or fewer hydrogen bonds.

The viscosity obtained is reasonably consistent with, although somewhat less than, the values obtained using fluorescent probes in eucaryotic cells (7–10). Both the spin label and fluorescent probes are small molecules. The effective viscosity that limits translational diffusion of large proteins appears to be greater by a factor of 20 (19). Thus, as originally suggested by Lehman and Pollard (6), one may not be able to assign a single viscosity to the cytoplasm but one should think of viscosity as a function of the size of the diffusing molecules. Then $\eta = \eta(V)$, the volume of a diffusing species. However, this may not be necessary for rotational diffusion since the effective viscosities for rotation for both small spin labels and at least one large protein, myoglobin, are similar (20).

The viscosity may vary in different regions or in different organelles in the cell. This would be difficult to determine using intact cells and spin labels, but may be amenable to solution by isolating closed organelles. This has been done for spinach thylakoids, which gave a twofold increase in τ over that in water (18), although measurements were not made in intact plant cells for a comparison. Also the effective viscosity for rotation appears not to equal that for translation, especially if translational diffusion is measured over a large distance. An additional complication may occur, especially for translational diffusion, due to oriented structures within the cell. In this case diffusion is not only a function of size, but also of direction of motion (19). Thus it is apparent that a single viscosity value may not be sufficient for a complex fluid such as the cellular cytoplasm, but distributions of viscosity may be needed for a complete description.

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REFERENCES

1. Kirschner, M. W. 1978. Microtuble assembly and nucleation. *Cytology.* 54:1–37.

2. Lazarides, E. 1980. Intermediate filaments as mechanical integrators of cellular space. *Nature* (*Lond.*). 283:249–256.
3. Wolosewick, J. J., and K. R. Porter. 1979. Microtrabecular lattice of the cytoplasmic ground substance. *J. Cell Biol.* 82:114–139.
4. Ling, G. N., C. Miller, and M. M. Ochsenfeld. 1973. The physical state of solutes and water in living cells according to the association-induction hypothesis. *Ann. NY Acad. Sci.* 204:6–50.
5. Foster, K. R., H. A. Resign, and A. N. Garroway. 1976. Bounds on "bound water": Transverse nuclear magnetic resonance relaxation in barnacle muscle. *Science* (*Wash DC*). 194:324–326.
6. Lehman, R. C., and E. Pollard. 1965. Diffusion rates in disrupted bacterial cells. *Biophys. J.* 5:109–119.
7. Burns, V. W. 1969. Measurement of viscosity in living cells by a fluorescence method. *Biochem. Biophys. Res. Commun.* 37:1008–1014.
8. Burns, V. W. 1971. Microviscosity and calcium exchange in yeast cells and effects of phenethyl alcohol. *Exp. Cell Res.* 64:35–40.
9. Cercek, L., and B. Cercek. 1976. Effects of osmomolarity, calcium and magnesium ions on the structuredness of cytoplasmic matrix (SCM). *Radiat. Environ. Biophys.* 13:9–12.
10. Lindmo, T., and H. B. Steen. 1977. Flow cytometric measurement of the polarization of fluorescence from intracellular fluorescein in mammalian cells. *Biophys. J.* 18:173–187.
11. Mantulin, W. W., and G. Weber. 1977. Rotational anisotrophy and solvent fluorophore bonds: an investigation by differential polarized phase fluorometry. *J. Chem. Phys.* 66:169–176.
12. Thomas, J. A., R. N. Buchsbaum, A. Zimniak, and E. Racker. 1979. Intracellular pH measurements in Erlich ascites tumor cells utilizing spectroscopic probes generated in situ. *Biochemistry.* 18:2210–2218.
13. Keith, A. D., and W. Snipes. 1974. Viscosity of cellular protoplasm. *Science* (*Wash. DC*). 183:666–668.
14. Morse, P. D., II. 1977. Use of the spin label TEMPAMINE for measuring the internal viscosity of red blood cells. *Biochem. Biophys. Res. Commun.* 77:1486–1491.
15. Morse, P. D., II, D. M. Lusczakoski, and D. A. Simpson. 1979. Internal microviscosity of red blood cells and hemoglobin-free resealed ghosts: a spin-label study. *Biochemistry.* 18:5021–5029.
16. Haak, R. A., F. W. Kleinhaus, and S. Ochs. 1976. The viscosity of mammalian nerve axoplasma measured by electron spin resonance. *J. Physiol.* (*Lond.*). 263:115–137.
17. Mastro, A. M., and A. C. Keith. 1981. Spin label viscosity studies of mammalian cell cytoplasm. *In* The Transformed Cell. I. L. Cameron and T. B. Pool, editors. Academic Press, Inc., New York. 327–345.
18. Berg, S. P., D. M. Lusczakoski, and P. D. Morse. 1979. Spin label motion in the internal aqueous compartment of spinach thylakoids. *Arch. Biochem. Biophys.* 194:138–148.
19. Wojcieszyn, J. W., R. A. Schlegel, E.-S. Wu, and K. A. Jacobson. 1981. Diffusion of injected macromolecules within the cytoplasm of living cells. *Proc. Natl. Acad. Sci. USA.* 78:4407–4410.
20. Livingston, D. J., G. N. La Mar, and W. D. Brown. 1983. Myoglobin diffusion in bovine heart muscle. *Science* (*Wash. DC*). 220:71–73.
21. Hwang, J. S., R. P. Mason, L.-P. Hwang, and J. H. Freed. 1975. Electron spin resonance studies of anisotropic rotational reorientation and slow tumbling in liquid and frozen media. III. Perdeuterated 2,2,6,6,-tetramethyl-4-piperidone-*N*-oxide and an analysis of fluctuating torques. *J. Phys. Chem.* 79:489–511.
22. Poggi, G., and C. S. Johnson, Jr. 1970. Factors involved in the determination of rotational correlation times for spin labels. *J. Magn. Reson.* 3:436–445.
23. Goldman, S. A., G. V. Bruno, G. F. Polnaszek, and J. H. Freed. 1972. An ESR study of anisotropic rotational reorientation and slow tumbling in liquid and frozen media. *J. Chem. Phys.* 56:716–735.
24. Goldman, S. A., G. V. Bruno, and J. H. Freed. 1973. ESR studies of anisotropic rotational reorientation and slow tumbling in liquid and frozen media. II. Saturation and nonsecular effects. *J. Chem. Phys.* 59:3071–3091.
25. Zager, S. A., and J. H. Freed. 1982. Electron-spin relaxation and molecular dynamics in liquids. I. Solvent dependence. *J. Chem. Phys.* 77:3344–3359.
26. Kreilick, R. W. 1967. NMR studies of a series of alipathic nitroxide radicals. *J. Chem. Phys.* 46:4260–4264.
27. Briere, R., H. Lemaire, A. Rassat, P. Rey, and A. Rousseau. 1967. Resonance magnetique nucleaire de radicaux libres nitroxydes piperidiniques. *Bull. Soc. Chim. France.* 1967:4479–4484.
28. Timmermans, J. 1959. Physico-Chemical Constants of Binary Mixtures in Concentrated Solutions. John Wiley and Sons, Inc., New York. 4:261–262.
29. Perrin, F. 1934. Movement Brownien d'un ellipsoide (I). Dispersion dielectrique pour des molecules ellipsoidales. *J. Phys. Radium.* 5:497–511.
30. Freed, J. H. 1964. Anisotropic rotational diffusion and electron spin resonance line widths. *J. Chem. Phys.* 41:2077–2083.
31. Shibaeva, R. P., L. P. Atovmian, and M. G. Nejgauv. 1972. The crystal structure of the nitroxide free radical 2,2,6,6,-tetramethyl-4-piperi-dinone-1-oxyl. *Zh. Strukt. Khim.* 13:887–893.
32. Meade, G. P., and J. C. P. Chen. 1977. Cane Sugar Handbook. John Wiley and Sons, Inc., New York. 839.
33. Hu, C.-M., and R. Zwanzig. 1974. Rotational friction coefficients for spheroids with the slipping boundary condition. *J. Chem. Phys.* 60:4354–4357.
34. Raaphorst, G. P., and J. Kruuv. 1979. Effects of salt, sucrose and dimethylsulfoxide solutions on the water content and water structure of tissues and cultured cells. *In* The Aqueous Cytoplasm. A. D. Keith, editor. Marcel Dekker, Inc., New York. 91–136.