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Diffusion of a small molecule in the cytoplasm of mammalian cells

(cytoplasmic viscosity/electron spin resonance/cytoskeleton/fibroblasts)

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ABSTRACT **Electron spin resonance was used to measure the diffusion of a small (M_r 170) spin label in the aqueous cytoplasm of mammalian cells. Translational and rotational motion were determined from the same spectra. Based on measurements made in model systems, it was hypothesized that calculations of the apparent viscosity from either rotational or translational motion would distinguish between the effects of cytoplasmic viscosity or cytoplasmic structure on diffusion. The diffusion coefficient calculated from spin label collision frequency, averaged 3.3×10^{-6} cm^2/sec in several cell lines. It was greater in growing cells and in cells treated with cytochalasin B than in quiescent cells. The viscosity of the cytoplasm calculated from the translational diffusion coefficient or the rotational correlation time was 2.0–3.0 centipoise (1 P = 0.1 Pa·sec), about 2–3 times that of the spin label in water. Therefore, over the dimensions measured by the technique, 50–100 Å, solvent viscosity appears to be the major determinant of particle movement in cells under physiological conditions. However, when cells were subjected to hypertonic conditions, the translational motion decreased by 67%, while the rotational motion changed less than 20%. These data suggested that the decrease in cell volume under hypertonic conditions was accompanied by an increase in cytoplasmic barriers and a decrease in the spacing between existing components. In addition, a comparison of reported values for diffusion of a variety of molecules in water and in cells indicates that cytoplasmic structure plays an important role in the diffusion of proteins such as bovine serum albumin.**

Movement in cell cytoplasm has been a topic of investigation since early observations of cytoplasmic streaming. The study of movement has been intimately associated with a study of the structure of the cytoplasm. As the use of transmission and high-voltage electron microscopes revealed a complex and ordered structure, theories about how molecules move within the cytoplasm have had to be modified to take this structure into account.

Over the years various approaches have been taken to measure the movement of large probes placed into cells by phagocytosis or by microinjection. For example, Crick and Hughes (1) examined the cytoplasm of fibroblasts that had phagocytized iron filings. Others have used radiolabeled molecules to measure diffusion in oocytes, muscle fibers, or axons (2–6). More recently, the techniques of microinjection and of fluorescence recovery after photobleaching have been combined to estimate the translational diffusion of proteins such as bovine serum albumin in cell cytoplasm (7, 8).

ESR techniques have been used to measure the rotational diffusion of small probes in cytoplasm (9). The rotational correlation time, τ_c, of the spin label is closely related to local cytoplasmic viscosity. However, the translational motion also can be calculated from the same spectra when an appropriate concentration range of spin label is used. In this study we used ESR to calculate the translational diffusion coefficient, D, of a small probe in mammalian cells. We had previously shown (10, 11), using a model system, that the presence of barriers can affect translational motion more than rotational motion; the apparent viscosity, η, calculated from spin label collision frequency is greater than that calculated from rotational motion parameters. Therefore, a comparison of translational and rotational diffusion was used in the present study to try to detect the presence of barriers within distances of ≈50–100 Å. Additionally by comparing these data with that presented in the literature for the D of other molecules in aqueous solutions and in cells, we have further estimated the relative roles of viscosity and barriers in controling movement in the cytoplasm.

MATERIALS AND METHODS

Cells. Swiss 3T3 and simian virus 40 (SV40)-transformed Swiss 3T3 cells were used for most experiments. Two clones of BALB/c 3T3, two clones of SV40-transformed BALB/c 3T3, a clone of methylcholanthrene (MCA)-transformed BALB/c 3T3, and BHK cells were also tested. Stock cultures were maintained at low cell density in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (10 mg/ml). To obtain quiescent cultures, 3T3 cells were grown to confluency in medium with 5% serum. Cells in G_1 phase were obtained by changing the medium to one containing 10% serum and waiting 12–16 hr. "Growing cells" were in exponential growth phase. Transformed cells were always growing and were used at the same density as the untransformed cells on the day of the experiment.

Spin Labeling Procedure. The technique for labeling of cells with spin label has been described (11). In most experiments, the spin label 2,2,5,5-tetramethyl-3-methanolpyrroline-*N*-oxyl, designated PCAOL, was used. Where indicated, deuterated 2,2,6,6-tetramethylpiperidine-*N*-oxyl, designated tempone, was the spin label. $NiCl_2$ was used to quench extracellular spin label signal. This treatment was not toxic to the cells (11). Measurements were taken at four concentrations of spin label between 3 and 40 mM.

The change in midfield linewidth (ΔH) of the ESR signal was plotted vs. spin label concentration. The D was determined from the slope of this line by using the equation: $D = K\Delta H/M$.

ΔH is the concentration-dependent linewidth component contributed by the spin label and is calculated from $\Delta H_M - \Delta H_{min}$, where ΔH_M is the linewidth at a given molar concentration of spin label and ΔH_{min} is the minimum linewidth of a very dilute spin label solution. M is the molarity of the spin label and K is a constant of proportionality relating spin label collision frequency with molar concentration (12).

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Abbreviations: MCA, methylcholanthrene; SV40, simian virus 40.

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The spin label molecules were synthesized in our laboratory (13). Other compounds were reagent grade.

RESULTS

In order to measure changes in line broadening (ΔH), PCAOL was added to cells at several concentrations. τ_c was calculated from the same spectra at the lowest spin label concentration, usually 3–4 mM. The change in ΔH with spin label concentration was about 50% less in cells than in water (Fig. 1). An average D of $3.9 \pm 0.2 \times 10^{-6}$ cm^2/sec was calculated for PCAOL in G_1-phase cells and $3.4 \pm 0.4 \times 10^{-6}$ cm^2/sec for quiescent cells. The τ_c values ($\approx 0.9 \times 10^{-10}$ sec) were $\approx$2.5 times higher in cells than in water, corresponding to a 40% decrease in the diffusion coefficient calculated from rotational motion.

The same experiment was repeated with several lines of cells in a quiescent or growing state or with cells transformed by SV40 or MCA. The average value of D for all of the cells was 3.3×10^{-6} cm^2/sec (Table 1). In general, this value did not vary greatly among the different clones or with growth parameters. More specifically, however, when quiescent Swiss 3T3 cells were compared with Swiss 3T3 cells in G_1 phase in the same experiment, there was a small but reproducible difference in D between the two cultures (Fig. 1). In five separate experiments, we saw that the D of PCAOL in the G_1-phase cells was always greater than that in quiescent cells.

The SV40-transformed cells showed little difference in diffusion parameters on average compared with their nontransformed counterparts, although in paired experiments differences in D were seen (11). BALB/c 3T3 cells, whether quiescent, growing, or transformed by MCA or SV40, all showed approximately the same values of D (Table 1).

Several experiments were done to determine if the movement of spin label could be varied by changing the cytoplasmic structure with trypsin (0.25%), colcemid (1 μM), or vinblastin (1 μM). There was no consistent change in ΔH with these three treatments (data not shown). However, treatment of cells with cytochalasin B allowed the PCAOL to diffuse more rapidly (Fig. 2). There was an $\approx$20% increase in the translational and rotational diffusion of PCAOL in the cytochalasin B-treated cells compared with untreated control cells. Similar results were seen when SV40-transformed 3T3 and BHK cells were treated in the same way. There was a consistent increase in the diffusion of PCAOL with cytochalasin B treatment when values were compared within the same experiment.

The greatest changes in D were seen when cells were subjected to hypertonic conditions (Fig. 3). As the volume of cells in the highest salt solution decreased by a factor of $\approx$2, D decreased by $\approx$4, probably because the aqueous volume decreased more than the whole-cell volume. In contrast to the change in D, τ_c decreased by only $\approx$20%. When the apparent viscosities calculated from spin label collision frequency and from rotational motion (τ_c) were plotted vs. cell

Table 1. Diffusion of PCAOL in several mammalian cell lines

Cell line	n	$D \times 10^6$, cm^2/sec[6]	Viscosity,* cP
Swiss 3T3 (Q)	13	3.4 ± 0.4	2.0
Swiss 3T3 (S)	5	3.4 ± 0.5	2.0
Swiss SV40 3T3 (G)	7	3.2 ± 0.4	2.1
BALB/c 3T3† (Q)	3	3.4 ± 0.2	2.0
BALB/c SV40 (G)	3	3.2 ± 0.4	2.1
BALB/c MCA 3T3 (G)	1	3.5 ± 0.3	1.9
BALB/c 3T3‡ (Q)	1	3.3 ± 0.3	2.1
BALB/c SV40 3T3 (G)	1	2.2 ± 1.3	3.1
BHK (G)	5	3.6 ± 0.3	1.8

PCAOL was added to cells and D was calculated from ΔH. The value shown is the average D ± SD calculated for the number of experiments indicated. The τ_c for all the cells was approximately 0.90×10^{-10} sec. Q, quiescent; S, serum stimulated; G, growing; n, number of experiments.

*Calculated from Stokes–Einstein equation: $\eta = \frac{kT}{6\pi r D \cdot f/f_0}$.

†Clone of BALB/c; gift from R. Scott, Univ. Minn.
‡Clone of BALB/c; gift of P. Beall, Baylor Univ.

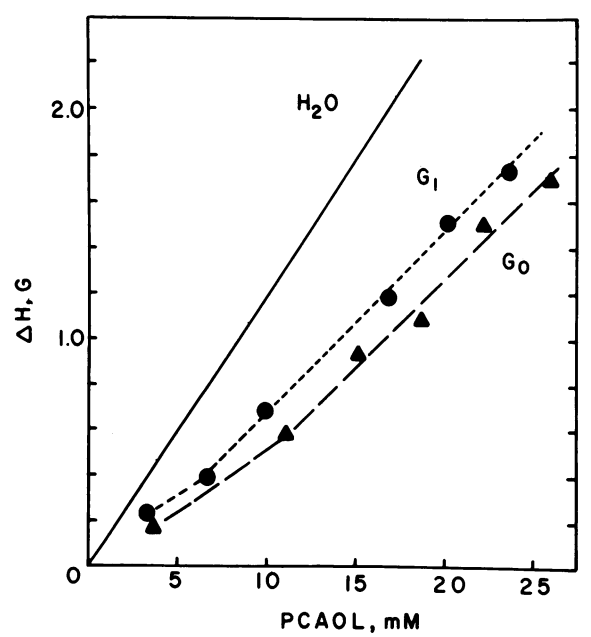

FIG. 1. Diffusion of PCAOL in 3T3 cells. Quiescent or serum-stimulated cultures of 3T3 cells were labeled with PCAOL at the concentrations shown. The results of one experiment are shown. This experiment was repeated five times with similar results. The line broadening (ΔH) in gauss (0.1 G = mT) of PCAOL in water is given as a reference. ▲, PCAOL in quiescent G_0 cells: $D = 3.4 \times 10^{-6}$ cm^2/sec, $\tau_c = 0.97 \times 10^{-10}$ sec; ●, PCAOL in serum-stimulated G_1 cells: $D = 3.9 \times 10^{-6}$ cm^2/sec, $\tau_c = 0.90 \times 10^{-10}$ sec; —, PCAOL in water: $D = 6.4 \times 10^{-6}$ cm^2/sec, $\tau_c = 0.36 \times 10^{-10}$ sec.

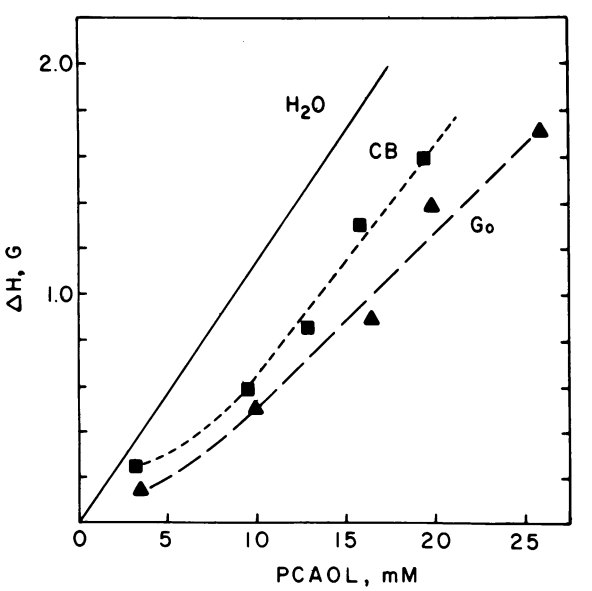

FIG. 2. Diffusion of PCAOL in cells treated with cytochalasin B. Quiescent cultures of Swiss 3T3 cells were treated with cytochalasin B (5 μg/ml in 0.4% dimethyl sulfoxide) for 5 hr at 37°C before being prepared for spin labeling. Dimethyl sulfoxide alone had no effect on ΔH. ▲, Untreated; ■, cytochalasin B (CB); —, water.

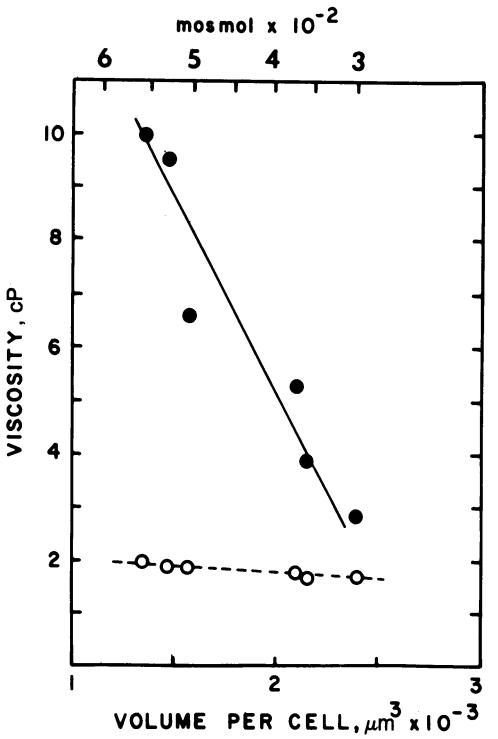

FIG. 3. Apparent viscosities of cells under hypertonic conditions. BHK cells were labeled with deuterated tempone. $NiCl_2$ (75 mM) and increasing concentrations of KCl were added to increase the hypertonicity of the medium. The osmotic pressure ranged from 290 to 550 milliosmoles (mosmol). Spin label was used at 10 mM to measure D and at 1 mM to measure τ_c. The τ_c for deuterated tempone is smaller than for PCAOL, but the values of each are proportional in cells compared with water. At 290 mosmol, $D = 2.3 \times 10^{-6}$ cm^2/sec and $\tau_c = 0.57 \times 10^{-10}$ sec; at 550 mosmol, $D = 6.7 \times 10^{-6}$ cm^2/sec and $\tau_c = 0.68 \times 10^{-10}$ sec. Volumes of cells were calculated from the data of Raaphorst and Kruv (14). Viscosities were calculated from the Stokes–Einstein equation, $\eta = kT/(6\pi rD \cdot f/f_o)$. ●, η calculated from D; ○, η calculated from τ_c.

volume, this difference was clear (Fig. 3). The apparent viscosity from D decreased 4-fold, whereas the apparent viscosity calculated from τ_c changed little. This difference suggests that the longer-range translational motion represented by D was impeded by barriers rather than by an increase in the local fluid viscosity as the cell volume decreased. This finding of a change in D without a change in τ_c is similar to that seen when spin label was trapped in porous beads (10).

For comparison, we also measured D for PCAOL in aqueous solutions of 10% protein. D changed by 20% or less compared with that in water alone (Table 2).

DISCUSSION

We have used ESR to calculate the D of a small molecule in the aqueous cytoplasm of several mammalian cell lines. The D for PCAOL, the spin label probe, was about 3.3×10^{-6} cm^2/sec. This is about half of the D for PCAOL in bulk water measured by ESR. If the decrease in D is due to an increase in the viscosity of cytoplasm compared with water, then from a consideration of the D of PCAOL in aqueous sucrose solutions (11), the viscosity of cytoplasm corresponds to that of a solution of 15% sucrose, about 2.0 cP (1 P = 0.1 Pa·sec). Calculation of viscosity from measurements of τ_c of PCAOL in the cells supports this interpretation.

Table 2. Diffusion of PCAOL in aqueous solutions of protein

Protein	$M_r \times 10^{-3}$	$D \times 10^6$ cm^2/sec	Viscosity, cP
None		6.4 ± 0	1.1
Cytochrome *c*	12.4	6.0 ± 0.2	1.1
RNase	13.7	5.6 ± 0.4	1.2
Lysozyme	14.4	5.6 ± 0.4	1.2
Bovine serum albumin	68.0	5.4 ± 0.5	1.3

PCAOL was added to solutions of protein (10% wt/vol) in water. PCAOL was used at 14, 20, 27, and 33 mM final concentration. D was calculated from the ΔH values at each concentration. The average D ± SD is given.

However, the cytoplasm is not a homogeneous sucrose solution. It contains structures that can make it appear to be very crowded (15). There is no evidence to suggest that the signal of PCAOL is due to its sticking to these structures. For example, if the protein content of cytoplasm is similar to that of serum, about 7%, (see ref. 7), one can ask if the protein accounts for the apparent viscosity. This was not the case (Table 2). In solutions of 10% protein, the D of PCAOL changed little (7–20%) compared with that in water—far less than the 2- to 3-fold change seen in cells. Furthermore, in previous studies we found that PCAOL did not bind to DNA or to synthetic polymers (10).

There is a great deal of debate as to the structural organization of the cytoplasm and to what extent water exists in a free or bound state. We considered whether PCAOL preferentially localized in the free or bound water. In a model system with phospholipid multilayers, we had determined previously that a water-soluble spin label partitioned nonpreferentially between bound and free water (16). It exchanged freely between the two, but because the exchange rate is greater than the spin lattice relaxation time, two distinct signals were seen. In a system containing both, the spectrum is an arithmetic average of the two; but in equal volumes of free and bound water, it is dominated by the signal from the free water because of the more narrow linewidths, even though the integrated intensities are equal. In the mammalian cell, the shape and intensity of the signal indicated that the spin label was predominately in free water. Furthermore, based on the integrated intensity of the signal for a given spin label concentration both in isotonic and hypertonic conditions, a calculation of the volume of this free-water domain suggested that it was about 90% of the water in the cell.

Another possible explanation of the lowered D of PCAOL in cells is that structural barriers limits movement. Evidence for such barriers is plentiful from electron microscopic studies. In a model system, we used ESR to distinguish between barriers and increased viscosity. When spin label was added to beads of various pore sizes, the translational movement was slowed considerably as the pore spaces became smaller. The rotational motion was largely unaffected (10). Therefore, if barriers alone limited the movement of PCAOL in cytoplasm, the rotational parameter, τ_c, should be relatively unchanged compared with that of water, while D should decrease. This was not the case. D and τ_c changed by about the same amount. The viscosity calculated from either measurement was 2–3 cP. Therefore, there is no evidence to support the idea that PCAOL movement is blocked by barriers under normal physiological conditions and over the dimensions detectable with 3–40 mM PCAOL—≈50–100 Å.

On the other hand, changes in the diffusion of PCAOL caused by treatment with cytochalasin B indicated that microstructure plays some role. The strongest evidence for this role was seen when cells were subjected to hypertonic conditions (Fig. 3). As cell volume decreased, the translational D decreased, and the apparent viscosity of the cytoplasm increased. However, the rotational diffusion changed much less (Fig. 3). This differential effect on D vs. τ_c is similar to that seen when spin label is sequestered in porous beads and is most likely explained by the presence of physical barriers. The spaces between structural barriers would become smaller as the cell volume decreased. Porter and Tucker (17) have

Table 3. *D*s of various molecules in cytoplasm

Compound	M_r	Radius, Å	$D \times 10^7$, cm²/sec		$\frac{D_{H_2O}}{D_{cells}}$	Viscosity, cP		Refs.
			D_{H_2O}	D_{cells}		η_{H_2O}	η_{cells}	
PCAOL	170	3.2	64	33	1.9	1.1	2.1	This work
Sorbitol	182	2.5*	94	50	1.9	0.9	1.7	5
Methylene blue	320	3.7*	40	15	2.6	1.5	4.0	6
Sucrose	324	4.4	52†	20	2.6	1.0	2.5	2
Eosin	648	6.0*	40	8	5.0	0.9	4.5	6
Dextran	3,600	12.0	18.	3.5	5.0	1.0	5.2	4
Inulin	5,500	13.0‡	15.	3.0	5.0	1.1	5.6	3
Dextran	10,000	23.3	9.2	2.5	3.7	1.0	3.7	4
Dextran	24,000	35.5	6.3	1.5	4.2	1.0	4.1	4
Actin	43,000	23.2‡	5.3§	0.03	167.0	1.1	179.0	8
Bovine serum albumin	68,000	36.0	6.9	0.10	71.0	0.9	81.0	7, 8
				0.06	111.0			
IgG	153,000	35.0‡	4.0§	0.09	43.5	1.1	63.0	7, 8
				0.06	66.7			

*Calculated from structure.
†CRC Handbook of Chemistry and Physics.
‡Calculated for a sphere of equivalent volume.
§CRC Handbook of Biochemistry and Molecular Biology.

reported that the lattice spacing of the cytoplasmic matrix decreases under hypertonic conditions. Theoretical calculations of the spacing between microtrabeculae under hypertonic conditions suggest that these alone would not account for these barriers. If the lattice spacings decrease in size relative to the decrease in the aqueous compartment, ≈75% for a 50% decrease in cell volume (18), the interlattice spaces would still exceed 500 Å—too large to be detected by PCAOL. However, as the aqueous volume decreases, the concentration of protein and other structural elements increases, leading to increased associations and more compact configurations (15). Thus, the data reported here suggest that, under hypertonic conditions, barriers appear with spacings in the range of 100 Å. Schobert and Marsh (19) have reported decreases in cell volume and apparent increases in cytoplasmic viscosity and density when algae were exposed to hypertonic conditions. Mansell and Clegg (18) have reported a compression of the cytoplasmic ground substance of fibroblasts under hypertonic conditions.

A better indication of the role of barriers can be drawn from a comparison of the diffusion of PCAOL with that of other molecules both in aqueous solutions and in cells (see ref. 20) (Table 3). The translational diffusion of molecules ranging in size from sorbitol (M_r 182) to IgG (M_r 153,000) have been determined for various cells by a variety of techniques. The distance over which diffusion was measured ranged from micrometers for low-temperature autoradiography and for fluorescence recovery after photobleaching to millimeters for dye-diffusion methods.

The *D* of a molecule in an aqueous solution varies approximately inversely with the radius as predicted from diffusion theory (Table 3). In spite of the variety of methods and cell systems, this general relationship holds true for the small molecules and the dextran spheres in the cytoplasm. The *D* in the cytoplasm is about 1/2 to 1/5th that in water. The same relationship between radius and *D* does not hold true for the three largest proteins, actin, bovine serum albumin, and IgG. This deviation is not surprising for actin measured under conditions in which it was largely immobile in the cells (8). Mobility and immobility were calculated from the fractional recovery of fluoresence after photobleaching. The *D* for actin is about 1/160th that in water. Bovin serum albumin and IgG had *D*s 1/100th that in water, although they were reported to be >90% mobile.

Using the Stokes–Einstein equation, $D = kT/(6\pi r\eta \cdot f/f_o)$, one can calculate the apparent viscosity (η) of each molecule in water and in cytoplasm. A plot of η vs. radius shows that all of the molecules in water experience a viscosity of about 1 cP (Fig. 4). The viscosity of cytoplasm appears to be between 2 and 6 cP for the small molecules and dextrans (Fig. 4). However, it appears to be ≈60–80 cP for bovine serum albumin and IgG and nearly 200 cP for actin as predicted from its association with existing actin structures. Therefore, the movements of bovine serum albumin and IgG are slowed to a much greater extent than would be expected on the basis of cytoplasmic viscosity alone.

One possible explanation for this affect is that the bovine serum albumin and IgG molecules encounter barriers. Another is that they bind with low affinity to cytoplasmic structures. If the binding were reversible with rate constants of

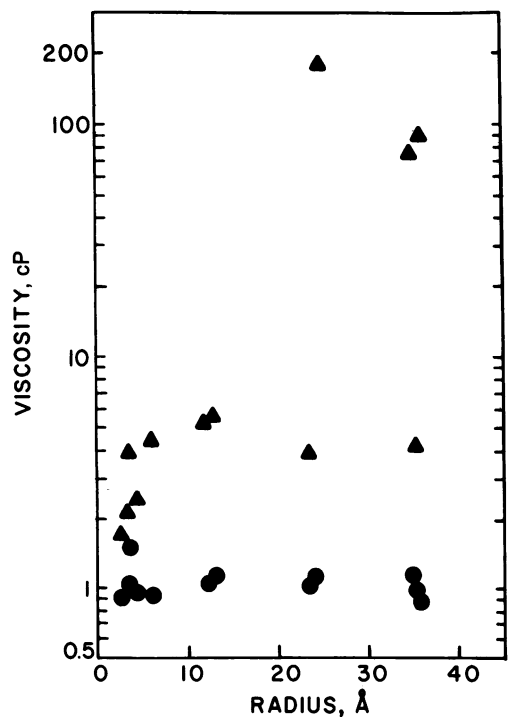

FIG. 4. A comparison of *D* in water and cytoplasm for molecules of various sizes (see Table 3). ●, Water; ▲, cytoplasm.

the same order as the rate of diffusion, the effect of continual binding and release would be seen as an apparent decrease in the *D*. This "chromatographic" effect was described for the movement of cysteamine phosphate in oocyte cytoplasm (21). Gershon *et al.* (22) also concluded on the basis of theoretical considerations of cytomatrix spacing that bovine serum albumin injected into cells binds to cytoplasmic proteins. In either case, barriers or binding or a combination would lead to the mistaken conclusion that bovine serum albumin or IgG were in a more viscous solution.

The largest dextran molecule tested has about the same radius as bovine serum albumin, yet it has a much higher *D* (Table 3). If barriers were responsible for the *D* of bovine serum albumin in cells, it would be expected that dextran would encounter the same barriers. However, there are differences between the molecules and the methods used to measure *D*. Dextran is a carbohydrate polymer chosen for its lack of binding (4). Bovine serum albumin is a protein that binds specifically and nonspecifically to other molecules. Furthermore, the *D* of dextran was measured in oocytes by low-temperature autoradiography, whereas movement of bovine serum albumin was measured in fibroblasts by fluorescence recovery after photobleaching. It also should be noted that when the *D*s of bovine serum albumin and ovalbumin were measured by fluorescence recovery after photobleaching in amoebae and mammalian cells (23), the compounds differed much more rapidly in the amoebae.

Determining rotational diffusion constants for bovine serum albumin and IgG in cells would be helpful in distinguishing between effects of viscosity and of barriers. Additionally, the development of an ESR technique that would allow the use of concentrations of label in the range of 10–100 nM in cells would make it possible to estimate barriers spaced about 100–1000 nm apart. This is the estimated distance between microtrabeculae in cells (24), a possible network of barriers to diffusion in the cell cytoplasm.

Note Added in Proof: After this manuscript was accepted for publication Lepock *et al.* (25) reported that the cytoplasmic viscosity of V79 cells was ≈3.6 times greater than that of water, as calculated from ESR measurements of the rotational diffusion of tempone.

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