# CYTOPLASMIC SOLVENT STRUCTURE OF SINGLE BARNACLE MUSCLE CELLS STUDIED BY ELECTRON SPIN RESONANCE

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ABSTRACT A free radical probe was introduced into single barnacle muscle cells, and its freedom of motion inferred from the spin resonance spectra. The probe reported an average local viscosity of 5–10 cp compared with 1 cp for pure water. From a comparison of the temperature dependence of the probe's tumbling rate in model aqueous systems and in the muscle we concluded that in the muscle the probe was undergoing fast exchange between sites of different mobility. Thus 10 cp must be taken as an upper limit for the viscosity of most cell water.

## INTRODUCTION

The structural features of cell water are of intimate interest to biologists since water constitutes 75–80% of living cells. Work on isolated proteins has indicated that they may bind water, up to 1 g/g protein, in a sufficiently immobilized state to affect the water's nuclear magnetic resonance (NMR) (1) and dielectric (2) relaxations, diffusion constant (3), and other properties. (See ref. 4 for a recent review).

Since cells contain approximately 5 g of water per gram of protein, the question arises as to whether the remaining water (above about 1 g/g) is structured in some way. This problem has been well studied in muscle by NMR (5–9), with the most convincing data showing an average water diffusion constant half that of pure water. This factor of two probably represents occlusion of the diffusion path and exchange with water of hydration (3). From electrical and osmotic measurements on barnacle muscle, Hinke (10) concluded that 75–80% of muscle water has the solvation properties of normal water.

We chose to examine the solvent structure of single barnacle muscle cells using as a probe a water soluble nitroxide free radical. The tumbling rate of the probe (calculated from its electron paramagnetic resonance [EPR] spectrum) measured structuring of the probe's environment. If all the cell water were ordered on a nanosecond time scale, we expected to observe slow tumbling. If only a fraction of the cell water were ordered, then during dehydration the more mobile fraction of

the radical should have become immobilized as the more fluid (higher vapor pressure) water disappeared.

For the sake of comparison, several model systems were studied to estimate the properties of the probe in bulk water and in water containing various interacting and noninteracting solutes.

## METHODS

We used single muscle fibers from the barnacle *Balanus nubilus* in this study. Preparation of the muscle bundles (11) and the procedure for isolation of single muscle fibers (12) have been described. The spin probe (4-hydroxy-2,2,6,6-tetramethylpiperidine-*l*-oxyl, TEMPOL) was introduced into the cell by microinjection or by immersing the fiber for about 1 h in saline (12) containing the probe at a concentration of $10^{-3}$–$10^{-4}$ M. After soaking, the fiber was rinsed with saline, (or saline containing $10^{-2}$ M ascorbate to reduce extracellular TEMPOL) and then blotted. For dehydration studies, the fiber was tied at both ends and introduced into a preweighed 10 cm polyethylene tube (Intramedic PE260). The tube was attached to a source of dry $N_2$ or perfusion fluid, and inserted into the cavity of a Varian E-3 EPR spectrometer (Varian Associates, Palo Alto, Calif.). All EPR measurements were made at 25°C unless otherwise noted.) During $N_2$ dehydration the tube containing the fiber was periodically removed from the cavity and weighed. At the end of each experiment, the fiber was dried in vacuum in the presence of $P_2O_5$. For variable temperature studies, the fibers were sealed in capillaries and maintained at constant temperature (±1°C) by a Varian E4557-9 temperature controller.

Microinjections were performed by introducing a capillary tube (100–150 μm diameter) inside a 1–2 mm diameter fiber and slowly injecting a maximum of 2 μl of probe solution to give a final concentration of about $5 \times 10^{-4}$ M inside the muscle fiber.

We measured the amount of muscle water accessible to TEMPOL as follows. Muscles were equilibrated with a 10 mM solution of the probe. The muscles were then blotted and weighed, and the probe was eluted from the muscle into a 200-fold excess of methyl alcohol. The amount of TEMPOL in the alcohol was then measured by comparing spectral peak heights with those of a standard. The mean probe concentration in the muscle was calculated from the muscle weight and the amount of probe present.

## ANALYSIS OF SPECTRA

Spectra were analyzed in the simplest possible manner following the approach of Stone et al. (13) in the following form:

$$\tau_1 = -2211 \times W_0 \times R_-/H_0 \quad (1)$$

$$\tau_2 = 0.65\, W_0 \times (R_+ - 2), \quad (2)$$

where $\tau_1$ and $\tau_2$ are correlation (tumbling) times in nanoseconds, $W_0$ is the line width of the central peak ($G$), and $H_0$ is the magnetic field ($G$). $R_\pm = [(h_0/h_{+1})^{1/2} \pm (h_0/h_{-1})^{1/2}]$, where $h_{0,+1,-1}$ = the height of the middle, low, and high field peaks, respectively. ($\tau_1$ and $\tau_2$ usually agreed to within 25%.) The correlation time, $\tau_c$, was taken as the average of $\tau_1$ and $\tau_2$, a procedure which seemed to reduce random

errors. Our analysis ignored nonsecular contributions to the linewidth, a reasonable procedure when $\tau_c$ is greater than 40 ps (14). We assumed the lines to be perfect Lorentzians which allowed us to measure peak heights rather than widths, and we ignored the proton dipolar contribution to the linewidths. Finally, we used the $g$ tensor and hyperfine tensor components derived from di-*t*-butyl nitroxide (15), rather than the actual values found in our solutions. Poggi and Johnson (14) in a careful analysis of nitroxide correlation times, found that such simplifying assumptions produce errors which tend to cancel, and that $\tau_c$ calculated in the simplest way may be expected to be in error by 20%. Furthermore, we calculated that the activation energy for tumbling for TEMPOL in 85% glycerol was 10.9 kcal/mol, whereas Goldman et al. (16) using an extensive spectral analysis, calculated 10.9–11.5 kcal/M for a similar molecular weight nitroxide in the same solution.

## RESULTS

The spectra of TEMPOL in fully hydrated muscles were indicative of rapidly tumbling radicals (Fig. 1). Spectra of fresh muscles gave a mean correlation time of 113 ± 7 ps. Since correlation time is proportional to viscosity via the Stokes' law relationship (17), a comparison of $\tau_c$ in water (20 ± 10 ps) to that in the muscles, suggested that the local viscosity of hydrated muscles was 5 cp, five times that of pure water. The metabolic state of the cell seemed to have some effect on the spectrum since cells from starved barnacles exhibited shorter correlation times (~60 ps).

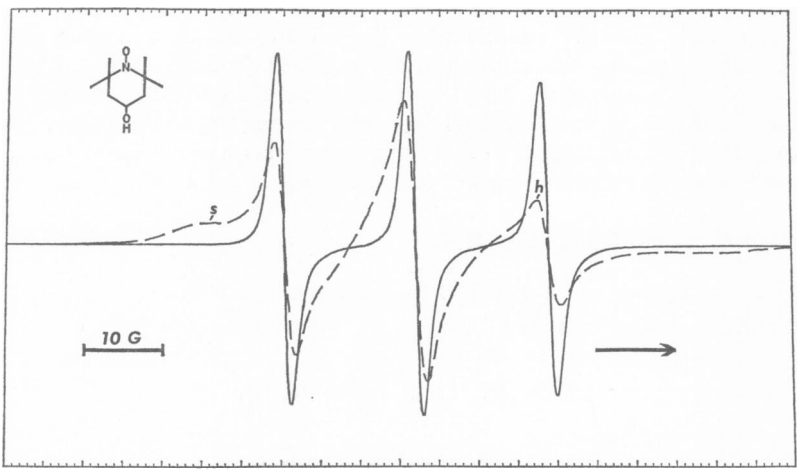


FIGURE 1 The ESR spectrum of TEMPOL (structure at upper left) in single barnacle muscle fibers. The solid line is the spectrum at normal hydration. The dotted line is the spectra at 5% of muscle water (note the spectrometer gain at 5% is 4.7 times that at 100%). The symbols *s* and *h* indicate, respectively, the low field peak of the strongly immobilized component and the high field peak of the rapidly tumbling component. The arrow indicates the direction of increasing field. Muscles were dehydrated with $N_2$ and loaded with TEMPOL by diffusion.

Equilibration of muscles with TEMPOL solutions, as described in Methods, showed that on a weight basis, the probe was 0.69 ± 0.10 times as concentrated in the muscle as in the soaking solution. The muscle water was 0.76 of the wet weight, and allowing for 7% of that as extracellular space (10), we calculated that TEMPOL was dispersed through 90% of intracellular water (asssuming no significant binding). Two additional experiments suggest that binding of the probe is not responsible for its presence within the cell. We compared the amount of probe in an equilibrated muscle with that in a capillary of the same approximate dimensions filled with the labeling solution. The signals were of approximately the same amplitude in accordance with the equilibration studies cited above. Thus the muscle can store a relatively large (1 mM or more) average concentration. Were this amount to be sequestered into a minor volume of the cell (the cell membrane, mitochondria, particular proteins, etc.) then concentration broadening due to radical-radical interactions would have become large and shown up in the spectrum. In fact, using different concentrations of labeling solution, we showed that concentration broadening in the muscle was comparable to that in the labeling solution. Thus, the probe cannot be reporting on a small part of the cell's volume. As a check that the probe was in fact intracellular, we injected muscles with 60 mM K ascorbate, an impermeant reducing agent which rapidly destroys TEMPOL. When these ascorbate injected cells were soaked in TEMPOL solutions and tested, they showed 100 times less signal than noninjected controls.

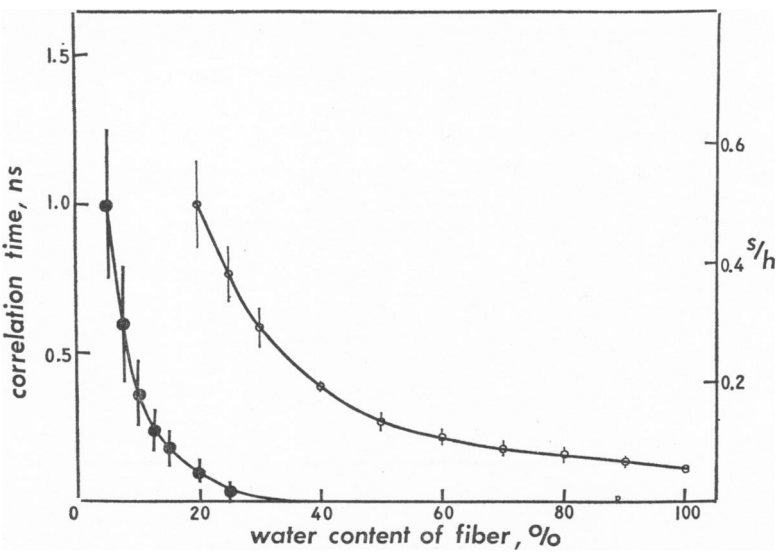


FIGURE 2 The correlation time (open circles), and $s/h$ (filled circles) of TEMPOL in single barnacle muscle fibers as a function of hydration. $s/h$ represents the relative proportion of strongly immobilized (SI) to liquid-like probe. The bars represent standard errors of the mean. Note that since the SI spectrum in much broader than the liquid-like one, the ratio of peak heights underemphasizes the proportion of SI probes.

As the muscles were dehydrated, either by a flow of dry $N_2$ or by hypertonic sucrose solutions, $\tau_c$ gradually increased (Fig. 2). With extensive drying using $N_2$, a second, strongly immobilized component (SI) appeared in the ESR spectrum (Fig. 1). As shown in Fig. 2, the SI fraction, measured by the ratio of the low field peak of SI to the high field peak of the liquid fraction, was a sharp function of water content. The spectrum at low water content (Fig. 1) clearly indicates the presence of two environments for the label and not just an average increase in immobilization. The coexistence of two phases may represent inhomogeneous drying of the specimen (some areas dry, and some wet). However, both components were observed under equilibrium conditions at 80% relative humidity. The SI component was never observed with hypertonic dehydration, probably because of the limited range of osmotic pressures obtainable with sucrose. By changing the orientation of fully dried muscles with respect to the magnetic field, the SI component was seen to be

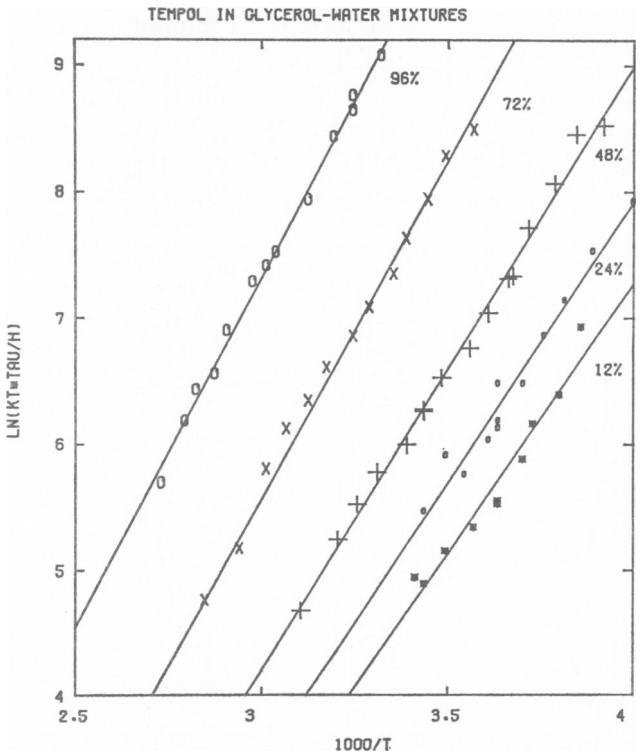


FIGURE 3 Arrhenius plot of the tumbling (correlation) time of TEMPOL in glycerol-water solutions. The ordinate is natural log of the "thermal frequency," $kT/h$, times $\tau_c$, the correlation time. ($kT/h = 6.3 \times 10^{12}$/s at 25°C). The abscissa is in inverse absolute temperature units and percentages refer to the weight percent of glycerol in the mixture. Straight lines were fitted to the data points by a least squares computer program. The slope of each line is proportional to the activation enthalpy, and the intercept to activation entropy.

slightly oriented, favoring an orientation with the N—O bond perpendicular to the muscle axis. The fully dried muscles were hard and brittle (similar to beef jerky), and the collapsed filament lattice undoubtedly provided a strong anisotropic environment accounting for the observed probe anisotropy. The spectral changes associated with dehydration were quickly reversible with wet $N_2$ or normal tonicity solutions.

Several muscles were macerated with a small quantity of label and slowly dehydrated with $N_2$. The results were similar to those for the intact muscles.

The results of the study on normally hydrated muscles left no doubt that the probe was moving quite freely in muscle cytoplasm. The probe did, however, appear to be moving more slowly than in pure water, and to better understand the reasons for this apparent increased viscosity, we decided to examine the temperature dependence of $\tau_c$ in both muscles and model systems. Glycerol-water mixtures were studied as simple model systems (Fig. 3). The temperature dependence was characterized as an activation energy, $E_a$, by an Arrhenius relationship: $\tau_c = \tau_{c_0} \exp (E_a/RT)$. $E_a$ was divided into enthalpic, $\Delta H$, and entropic, $\Delta S$, terms following the dielectric relaxation formulation of the transition state theory (18):

$$\tau_c = (h/kT) \exp (\Delta H/RT) \cdot \exp (-\Delta S/R),$$

where $h$ = Planck's constant, $k$ = Boltzmann's constant, $R$ = gas constant, and

TABLE I

THE RESULTS OF TEMPERATURE STUDIES ON BARNACLE MUSCLE AND SOME MODEL SYSTEMS

$\Delta H$ and $\Delta S$ were calculated from the slope and intercept, respectively, of the appropriate Arrhenius plots over the indicated temperature range. The correlation time at 25°C was calculated from the best fit straight line of the Arrhenius plot. (cf. Figs. 2 and 3).

| Preparation | $\tau_c(25°)$ | $\Delta H$ | $\Delta S$ |
|---|---|---|---|
| | *ps** | *kcal/mol* | *cal/mol·degree* |
| 96% Glycerol | 1,770 | 11.1 | 18.7 |
| 72% " | 282 | 10.7 | 20.9 |
| 48% " | 59 | 9.6 | 20.3 |
| 24% " | 25 | 8.9 | 19.8 |
| 12% " | 15 | 8.6 | 19.7 |
| Water (extrap.) | 10 | 8.4 | 20 |
| 20% Gelatin | 26 | 8.6 | 18.7 |
| Barnacle muscle, hydrated (0–30°) | 57 | 2.0 | −5.1 |
| Barnacle muscle, hydrated (30–94°) | 4 | −0.3 | −12.6 |
| 20% BSA (12–50°) | 104 | −2.0 | −19.6 |
| 20% BSA (−2–12°) | 66 | 2.7 | −2.4 |
| 6 M Guanidine HCl | 14 | 8.0 | 17.9 |
| 20% BSA in 6 M guanidine HCl | 61 | 5.1 | 5.5 |

* Calculated from $\tau_c = (h/KT) \exp (\Delta H/RT) \cdot \exp (-\Delta S/R)$.

$T$ = absolute temperature. Measurement of $E_a$ for TEMPOL in water was difficult due to its rapid tumbling and the consequent insensitivity of the $\tau_c$ formulas. $E_a$ in water was calculated on the assumption that $E_a$ for a mixture was a linear combination of the $E_a$'s for pure substances (18), thus:

$$E_a = N_1 E_{a1} + N_2 E_{a2},$$

where $N_{1,2}$ represent the mole fractions of components 1 and 2, respectively, and $E_{a1,2}$ the respective activation energies. The above expression was well fitted by glycerol-water mixtures from 12.5 to 75% glycerol, yielding a value of 8.42 ± 0.05 kcal/mol for water, which gave $\tau_c$ = 10 ps at 25° C. The results of the temperature studies are shown in Table I.

As expected, the activation energy of TEMPOL in glycerol and other solutions of small molecules increased with $\tau_c$ (i.e. viscosity). Contrary to expectations, the muscles yielded a relatively long $\tau_c$ but a low $E_a$ (Fig. 4). This anomalous behavior of the muscle led us to try some model protein systems. Results are shown in Table I. Gelatin gave a temperature dependence similar to that observed with glycerol, suggesting that gelatin was an inert solute. Since we suspected that radical binding was involved in the anomalous muscle temperature dependence, we decided to use

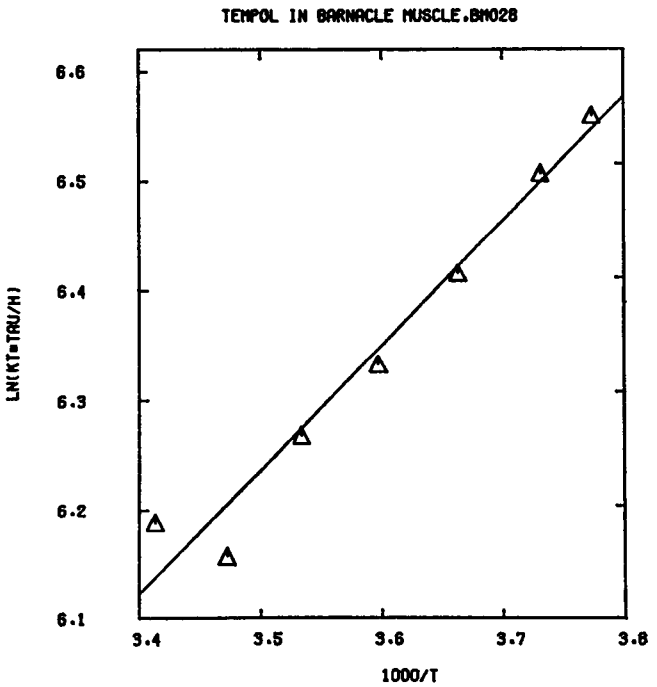


FIGURE 4 Arrhenius plot of the correlation time of TEMPOL in a barnacle muscle cell. Notation as in Fig. 3.

bovine serum albumin (BSA) as a model system since BSA is known to bind neutral molecules (19). The binding of TEMPOL to BSA was measured by equilibrium dialysis, and yielded an equilibrium constant (assuming single site binding) of about 200 for the association, or 3.1 kcal/mol for the free energy of binding. We measured the octanol-water partition coefficient of TEMPOL for comparison with the BSA binding predicted by Helmer et al. (19), and found that our partition coefficient of 2.83 (octanol: water) yielded, by their relationship, a binding energy of 3.17 kcal/mol.

The temperature dependence of TEMPOL tumbling in 20% BSA (a protein concentration roughly equal to the protein concentration of muscle), was of the anomalous sort seen in the muscles. The tumbling time, $\tau_c$, was fairly long, 60–100 ps, but the temperature dependence was slight, and the Arrhenius plot was nonlinear (Table I).

To see whether the anomalous behavior was related to conformation, BSA was denatured in 6 M guanidine HCl. Compared to guanidine alone, addition of BSA increased $\tau_c$ of TEMPOL in the solution, but lowered the apparent activation energy (Table I). The Arrhenius plot was linear, but the paradoxical increase in $\tau_c$ coupled with a decrease in the temperature dependence persisted.

## DISCUSSION

The initial part of the study shows unequivocally by the narrow linewidths of the probe in muscle, that the bulk of muscle water is, at most, weakly structured. The temperature dependence of the correlation times allowed us to place limits on the extent of cell water structuring

Most of the model systems studied had the expected temperature dependence: increased viscosity corresponding to increased activation energy for tumbling of the probe. Furthermore, the activation entropy changes were the same for these substances over a wide range of concentrations suggesting that the structural organization of the transition state was characteristic of the solvent rather than the solute, i.e. the probe did not directly interact with the solutes. For the muscle and BSA, however, we found increases in viscosity associated with lower activation energies and even nonlinear Arrhenius plots. These results suggested that we were not observing a single process and thus the calculated correlation times could not be taken at face value. A simple model that would explain the anomalous data is a fast exchange of the probe between immobilized and rapidly tumbling fractions. This sort of a model has been invoked in most of the NMR studies of cells and polymeric hydrated systems (7, 9). Basically, the idea is that if the probe is moving sufficiently rapidly between several sites which have different properties, then the probe will report an environment which is an average over all the local environments (weighted by the residence times). Since the relaxation rates are averaged in a fast exchange process, we will rewrite the equations for $\tau_c$ in terms of the relaxation rates of the three hyperfine lines. The relaxation times are proportional to the square root of the peak heights (13).

Writing Eq. 1 in terms of peak heights we have:

$$\tau_1 \alpha [(h_0/h_{+1})^{1/2} - (h_0/h_{-1})^{1/2}] W_0 .$$

Substituting relaxation times, $T$, for the square root of the peak heights, $h$, and factoring $h_0$ :

$$\tau_1 \alpha [(1/T_{+1}) - (1/T_{-1})] T_0 W_0 , \tag{3}$$

where the $T$'s represent the transverse relaxation times for the corresponding nuclear spin states and $W_0$ is the linewidth of the center line. (Eq. 2 may be handled similarly). The product $T_0 W_0$ is a constant. If we write the fast exchange condition as $1/\bar{T} = \Sigma_i f_i/T_i$ (7), where $\bar{T}$ is the mean relaxation time, $T_i$ is the relaxation time in the $i$th state, and $f_i$ is the fraction of time spent in the $i$th state, then Eq. 3 can be rearranged to show that:

$$\tau_{av} = \sum_i f_i \tau_i ,$$

where $\tau_{av}$ is the average correlation time over all $i$ states and $\tau_i$ is the correlation time in the $i$th state. In other words, the mean correlation time is simply a weighted average of the correlation times in each environment. The nature of the relaxation process weights long correlation times very heavily, so that even if the probes spend a small fraction of their time in a stiff environment, the average correlation time can be considerably lengthened. The net effect of probe binding is thus to emphasize that the observed correlation times are, in fact, minima for the probe in its unbound environments. In our BSA experiments we used a total probe concentration of 0.1 mM and total BSA concentration of 3 mM (20%). With a binding constant of 200, simple equilibrium considerations predict that the radical is bound 37% of the time. The mean correlation time at 25° for the BSA was about 100 ps, and if we assume that in the BSA solution the radical can move no faster than it can in pure water ($\tau_c$ = 10 ps), then a two-compartment fast exchange model predicts that in the bound state the probe is tumbling with a correlation time less than 260 ps. This rate is much faster than the rotational rate of the BSA itself (13) and thus indicates that the probe is probably not inside the BSA molecule, but bound to the surface, in accord with our observation that denaturation does not remove the anomalous temperature effects which we have attributed to binding.

In the muscles the same kind of binding data doesn't exist, but since the equilibration studies indicated that the muscle contained less probe than an equivalent volume of the labeling solution, the binding must be quite weak in general. The excess correlation time of the probe in the muscles, as opposed to bulk water, is thus probably due to a contribution of a small number of relatively stiff binding sites. This means that the bulk of the cytoplasmic water must have a viscosity less

than 10 cp. Estimates of ion translational mobility in barnacle muscle inferred from conductance measurements suggest a 5 cp value (21). These values seem insufficiently rigid to account for the ion exchange properties proposed for cell water (20). The temperature dependence of $\tau$ in a fast exchange situation will reflect temperature effects on the equilibrium between the various states. Recent NMR work (9) suggests that three or more correlation times are required to describe the behavior of water in muscle. We are inclined to favor the same view with regard to the electron spin resonance (ESR) probe relaxation. Extensive modeling of our system is unwarranted in view of the number of variables, but the muscle behavior (temperature and hydration) has been fit with a two compartment fast exchange model using binding energies of several kilocalories per mole.

We have assumed that motional restriction is the only source of broadening of the spectrum. Other sources of broadening such as variation in the contact hyperfine interaction between different environments (22) would only serve to emphasize that the motional broadening factor is less than we have calculated, and the probe must be moving even more freely than indicated. This data suggests that cytoplasmic structures have little influence on the bulk solvent structure and is supported by our results on dehydration which showed that as much as 75% of the cell's water could be removed without producing a qualitative change in the spectrum.

The ESR spin probe techniques used here may be applied to other tissues and to the use of other probes. Preliminary data has been obtained for TEMPOL in the giant nucleated red blood cells of the salamander *Amphiuma*. These ESR spectra are indicative of an average viscosity of 7 cp (more than the barnacle muscle), but the activation enthalpy appears to be 0 above 15°, and 2 kcal below 15°. Again, the fast exchange model seems appropriate. It would be interesting to extend this technique to neoplastic cells where changes in the NMR relaxation times of cell water have been correlated with carcinogenesis (23).

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