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Intracellular Viscosity of Lymphocytes Determined by a ^{15}N Spin Label Probe

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^{15}N-Tempone was synthesized from $(NH_4)_2SO_4$ and phorone and was used as a spin label probe to study the protoplasmic viscosity of lymphocytes. The intracellular viscosity was determined to be approximately two times the viscosity of water. ^{14}N-Tempone yielded a similar result. The value of the hyperfine coupling constant for ^{15}N-Tempone in lymphocytes was slightly less than that in water. The EPR signal of the intracellular spin label was observed to decrease exponentially as a function of time with a decay constant of 0.25 ± 0.18 min^{-1}.

INTRODUCTION

Spin labels are capable of providing information concerning certain aspects of their environment, such as molecular motion and orientation. It has been suggested that spin labels enriched in ^{15}N instead of the naturally abundant ^{14}N may be advantageous in probing biological systems (*1*). A ^{15}N nitroxide spin label has an inherently greater sensitivity than the equivalent ^{14}N molecule, since fewer lines are present in the EPR spectrum of the ^{15}N-labeled molecule. Additionally, for spin labels undergoing rapid rotation, longer correlation times can be measured if ^{15}N rather than ^{14}N is present in the nitroxide moiety. This difference exists because of the smaller amount of line broadening which occurs for a given tumbling rate with ^{15}N compared with ^{14}N spin labels (*1*). Thus far no studies which utilize a ^{15}N spin label in a biological system have been reported. In the present study the protoplasmic viscosity of lymphocytes determined by the freely diffusible spin labels 2, 2, 6, 6-tetramethyl-4-oxo-piperidine-^{15}N-oxyl (^{15}N-Tempone) and 2,2,6,6-tetramethyl-4-oxo-piperidine-^{14}N-oxyl (^{14}N-Tempone) are compared.

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MATERIALS AND METHODS

Synthesis of ^{15}N-Tempone Precursor

The ^{15}N-Tempone precursor (^{15}N-2,2,6,6-tetramethyl-4-oxo-piperidine or ^{15}N-triacetone amine) was synthesized according to a modified procedure reported by Briere *et al.* (*2*).

A mixture of phorone in methanol (25 g/30 ml) and potassium hydroxide (2 g) was added to a mixture of ^{15}N-ammonium sulfate in water (1 g/3 ml). The mixture was stirred at room temperature for 3 days. The solution was made acidic using 1 *N* H_2SO_4 and was extracted to remove starting materials. The aqueous solution was made alkaline using hydroxide and extracted with ether. The ether extract was washed with brine and solvent removed. The mother liquor, after ether extraction, was evaporated under reduced pressure and the residue extracted with ether to collect more of the triacetonamine. Total recovery of crude material was 0.84 g. The crude material was chromatographed over a neutral alumina column (30 g, 20 cm long) and eluted successively with hexane/benzene (1:1), benzene, and benzene/chloroform (1:1). Most of the required material was eluted out by benzene and benzene/chloroform. The weight of pure ^{15}N-triacetone amine recovered was 0.52 g.

Synthesis of 2,2,6,6-Tetramethyl-4-Oxo-Piperidine-^{15}N-Oxyl

To a solution of ^{15}N-triacetoneamine (0.240 g) in water (5 ml) was added EDTA (25 mg), sodium tungstate (25 mg), and H_2O_2 (2 ml). The mixture was left at room temperature in the dark for 3 days. The solution was saturated with sodium chloride and extracted with ether. The ether extract was washed once with 10 ml of saturated brine and dried over sodium sulfate and the solvent was removed. The crude material was applied on two silica-gel-coated plates and developed in a solvent system consisting of chloroform/ethyl acetate (1:1). The yellow band of nitroxide was cut and eluted with acetone. Removal of solvent gave pure nitroxide (0.19 g).

Isolation and Treatment of Lymphocytes

The spleen was removed from an anesthetized Holtzman rat and minced in a petri dish containing buffered saline at 0°C. The suspended cells were pipetted into a culture tube and centrifuged at 100*g* for 10 min. The cells were washed once in buffered saline before treating with a NH_4Cl and Tris buffer solution to lyse the RBC's (*3*). The lymphocytes were finally washed three times in buffered saline at room temperature. A typical yield was 50 to 100×10^6 cells with greater than 90% viability determined by the exclusion of trypan blue stain.

Cells were suspended in a small volume of an isotonic solution of $10^{-3}M$ Tempone, 0.07 *M* $NiCl_2$, and Tris buffer (pH 7.4). This solution provided a constant osmotic strength and prevented a change in cell volume during the experiment. The cell suspension was placed in a capillary tube (inside diameter 1.2 mm) which was centrifuged at 100*g* for 2 min, so that a cell pellet was formed. The EPR spectrum was recorded as a function of time on a Varian Century E–109 spectrometer

interfaced with a Nicolet Instrument Corporation Model 1180 Data System. The EPR spectrum of the supernatant was also obtained.

In order to determine the loss of viability of lymphocytes during the experiment, the cells were recovered after the EPR spectra had been recorded. These cells were washed three times in buffer and the viability was measured to be in the range of 70 to 80%, which indicates that no major loss of viability occurred during the experiment.

RESULTS

For ^{15}N-Tempone an isotropic g value of 2.00534 was determined by using ^{14}N-Tempone with a reported g value in water of 2.0054 (*4*) as an internal standard. The isotropic hyperfine coupling constant was 22.5 ± 0.1 G in water. Figure 1 shows that microwave power saturation for ^{15}N-Tempone occurs at approximately 30 mW. ^{14}N-Tempone power saturates at a similar value.

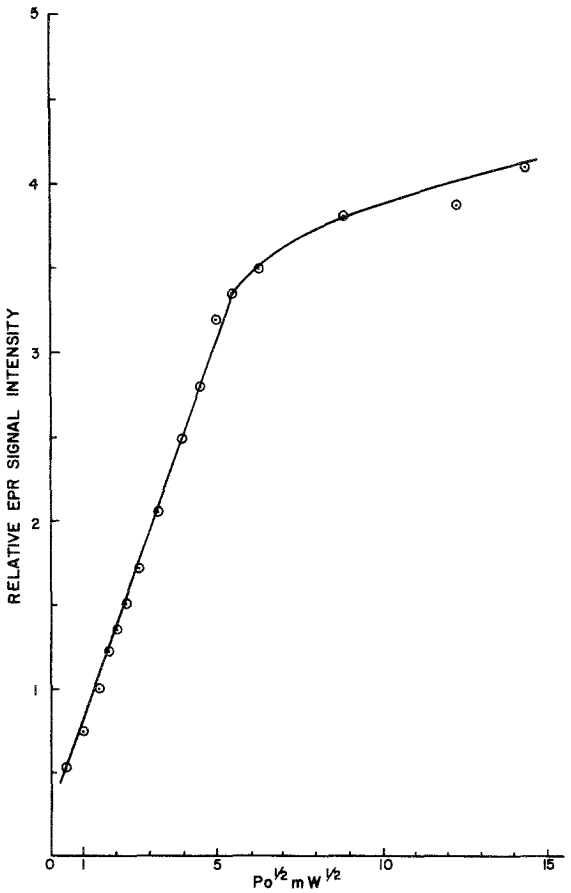

FIG. 1. Power saturation curve for 10^{-4} M ^{15}N-Tempone in water. The intensity (hW^2) of the low field EPR line was normalized to $P_0^{1/2} = 1.41$ $mW^{1/2}$.

For equal concentrations of spin label, the line for $m_I = -\frac{1}{2}$ in the ^{15}N-Tempone spectrum was measured to be 1.58 times greater in intensity (hW^2) than the line for $m_I = 0$ in the ^{14}N-Tempone spectrum. The relative concentrations were determined by uv absorption at 240 nm.

One potential use of spin labels is to obtain information about the freedom of molecular motion, expressed as rotational correlation time (τ_c) of the nitroxide molecule. Quantitatively, in the limit of rapid rotation, the correlation time for an ^{15}N spin label is determined by the following equation (*5*) which has been derived from the theory of McConnell (*6*) and Kivelson (*7*),

$$\tau_c = KW_{-1/2}[(h_{-1/2}/h_{1/2})^{1/2} - 1], \qquad [1]$$

where K is a constant that depends on the anisotropic g values and nitrogen hyperfine couplings for a specific spin label molecule. The parameters $h_{-1/2}$ and $h_{1/2}$ are, respectively, the heights of the low-field line and high-field line, and $W_{-1/2}$ is the peak-to-peak width in gauss of the low-field line on the first derivative absorption spectrum. The reported principal values of the gyromagnetic and hyperfine coupling tensors of ^{14}N-Tempone (*8*) were utilized to determine the respective tensors of ^{15}N-Tempone. By assuming the gyromagnetic tensors are identical and the components of the ^{15}N hyperfine coupling tensor are 1.4 times the corresponding ^{14}N components (this factor is the ratio of the gyromagnetic ratios of the nuclei), the constant K was evaluated to be 9.3×10^{-10} sec/G for an applied magnetic field of 3400 G.

In the present work with Tempone in aqueous solutions of uniform viscosity, the expression $W_{-1/2}[(h_{-1/2}/h_{1/2})^{1/2} - 1]$ has been found to vary as a function of microwave power in the range of 8 to 30 mW. (This is the microwave power range in which the EPR linewidths broaden with increasing power.) Such microwave power effects were eliminated by recording all spectra at 4 mW.

In order to demonstrate the sensitivity of ^{15}N-Tempone to different environments, the spin label was introduced into various concentrations of aqueous glycerol solutions and the EPR spectra were recorded. The EPR spectra showed dramatic changes in spectral characteristics with increasing glycerol concentration. Further,

TABLE 1

CORRELATION TIMES OF ^{14}N- AND ^{15}N-TEMPONE IN AQUEOUS GLYCEROL SOLUTIONS

% Glycerol (by volume)	^{14}N τ_c ($\times 10^{11}$ sec)	^{15}N τ_c ($\times 10^{11}$ sec)
0	1.85	2.00
41	4.37	4.41
50	6.63	6.5
59	8.15	8.83
69	13.3	14.6
78	23.7	24.7
91	74.3	69.4

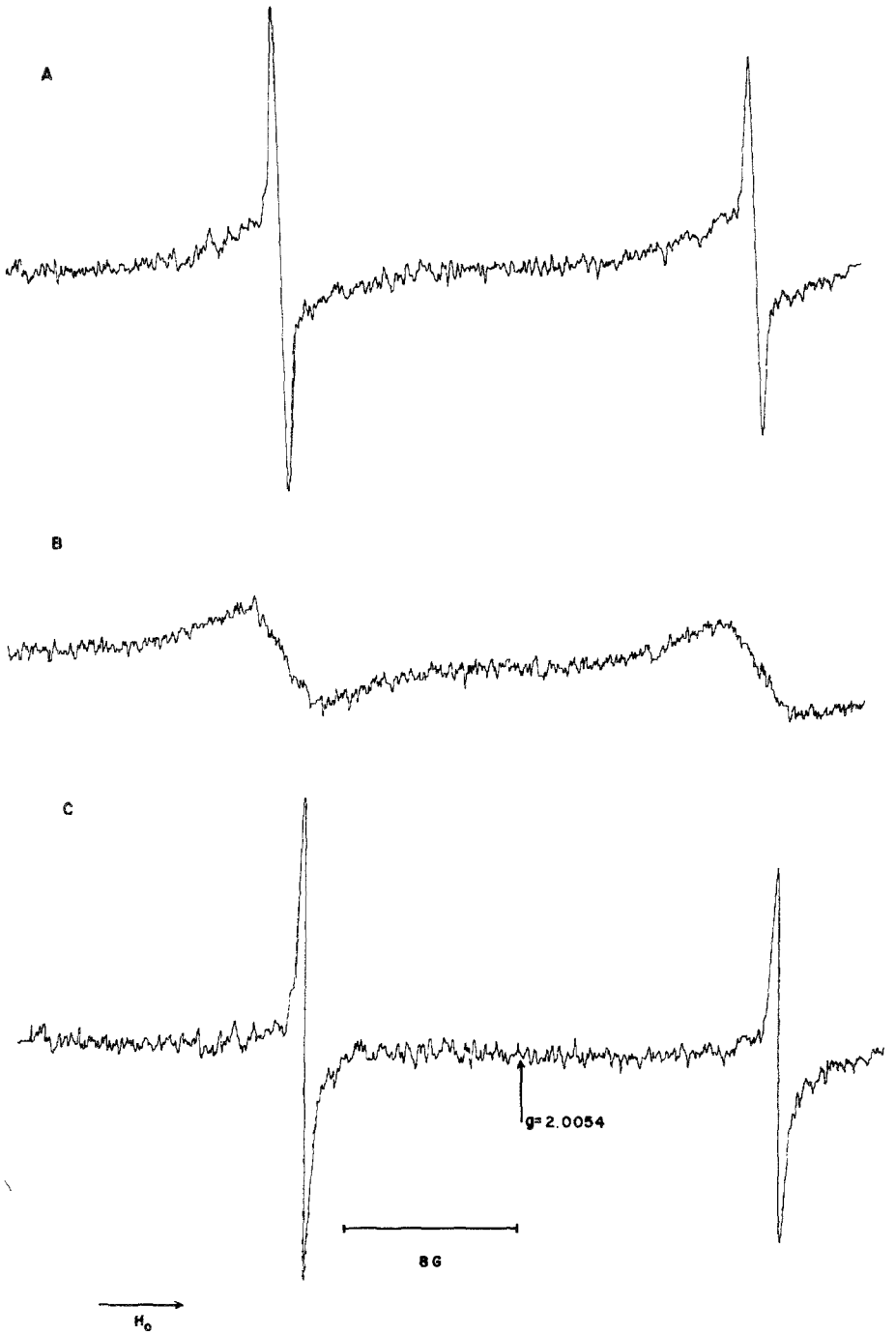

FIG. 2. (A) EPR spectrum of lymphocytes suspended in a buffered solution of spin label and nickel chloride. (B) EPR spectrum of the supernatant only. Amplifier gain equals 0.8 times that of spectrum A. (C) Figure 2B subtracted from Fig. 2A.

the calculated rotational correlations times listed in Table 1 agreed with those determined from ^{14}N-Tempone in the respective aqueous glycerol mixtures.

^{15}N-Tempone was used to probe the protoplasmic viscosity of lymphocytes using the method introduced by Keith and Snipes (*9*), with the modification that isotonic solutions were used. The EPR spectrum of cells suspended in the isotonic solution of spin label and nickel chloride showed a very broad component originating from nitroxide molecules outside the cell interacting with Ni^{2+} ions (*9*) and a narrow component attributed to spin label residing inside the cell, a region free of Ni^{2+} ions (see Fig. 2A). The EPR spectrum of heat inactivated lymphocytes (nonviable, but intact cells) did not differ from that of the supernatant alone. These experiments suggest that only viable cells contribute to the narrow-lined component. Since the presence of $NiCl_2$ at 0.07 *M* is not sufficient to broaden the extracellular signal completely, the supernatant contribution (Fig. 2B; scaled accordingly to account for displacement of solution by cells) must be subtracted from the EPR spectrum to yield a net intracellular spectrum (Fig. 2C).

The spectrum of the intracellular spin label showed a decrease in line height as a function of time. Figure 3 represents a typical decay curve. Regression analysis indicated the decay was exponential and yielded a decay constant 0.25 ± 0.18 min^{-1} for ^{15}N-Tempone and 0.29 ± 0.10 min^{-1} for ^{14}N-Tempone at 23°C. The difference

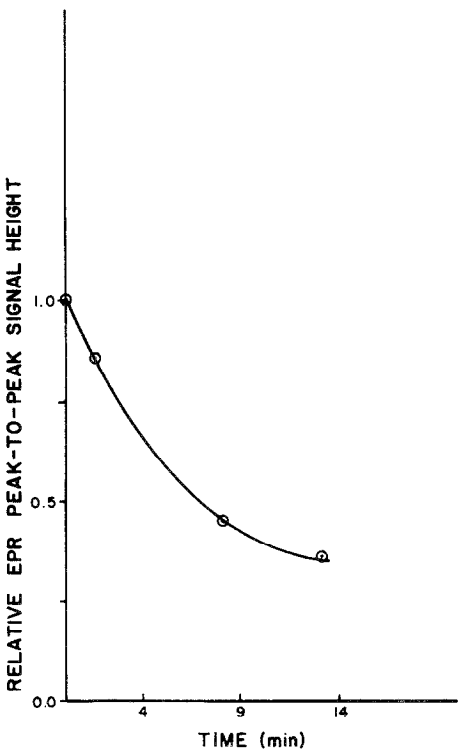

FIG. 3. Decay of the EPR signal as a function of time. The peak-to-peak height of the low-field line was measured for spectra obtained in the same manner as Fig. 2C.

TABLE 2

PROTOPLASMIC VISCOSITY OF LYMPHOCYTES MEASURED BY ^{14}N AND ^{15}N SPIN LABEL PROBES

Spin label	No. of samples	A_N (G)	τ_c ($\times 10^{11}$ sec)	η, Viscosity[a] (cP)
^{15}N-Tempone	5	22.0±0.1	3.63±0.45	2.02
^{14}N-Tempone	22	15.9±0.1	3.78±0.31	2.10

[a] It is possible to calculate the viscosity from correlation time by using the following equation (*10*)

$$\tau_c = \frac{4\pi \eta r^3}{3kT},$$

where η is the viscosity, r is the radius of the rotating molecule, k is Boltzmann's constant, and T is the temperature in °K. The radius was determined to be 2.6×10^{-8} cm by measuring the correlation time of ^{14}N-Tempone in water with a known viscosity (*11*).

between these two decay constants was not statistically significant as determined by the standard t-test ($P < 0.05$). Decay measurements were also made as a function of temperature. It was found that a 10°C decrease in temperature lowered the decay constant by a factor of 2.7. Since the relative heights of the lines changed during the acquisition of the spectrum, the height of the high-field line was corrected to the height it would have at the time the low-field line was recorded.

The correlation time measurements and intracellular viscosities as well as the hyperfine coupling constants A_N, for both ^{15}N-Tempone and ^{14}N-Tempone are presented in Table 2. The values obtained for the intracellular viscosity of lymphocytes are approximately twice that of pure water which has a value of 1.00 cP (*11*).

DISCUSSION

The decay of the EPR signal was more rapid than expected if loss of cell viability alone was responsible for the decrease of the signal. Another study in which the spin label 2,2,5,5-tetramethyl-pyrrolidine-1-oxyl-3-carboxylic acid (PCA) was substituted for Tempone has also yielded an exponential decay curve. However, the rate of decay for PCA was found to occur approximately 30 times slower than for Tempone. This evidence supports the hypothesis that biological reduction of ^{15}N-Tempone molecules was occurring inside the cell, thus eliminating their paramagnetic property. Since the decay constant differed markedly at two different temperatures, the reduction of ^{15}N-Tempone in lymphocytes may be an enzymatic process.

The hyperfine coupling constant for a particular spin label is changed by solvent interactions with the spin label. For example, A_N for ^{14}N-Tempone has been shown to vary from 16.3 G in water to 14.5 G in n-hexane (*4*). The hyperfine coupling constant decreases because of changes in the electron density of the unpaired electron on nitrogen produced by a reduction in the dielectric constant of the solution. The presence of physical structures as well as enzymes and other molecules within the cell would be expected to lower the dielectric constant compared with water and, consequently, yield a smaller hyperfine coupling constant. The value of

A_N in lymphocytes was only slightly less than that in water, which suggests the spin label was located in a predominantly aqueous environment.

The protoplasmic viscosity determined from the rotational correlation time was found to be approximately two times the viscosity of water. This relative value for the protoplasmic viscosity is lower than that reported for other cell types (*4, 9, 12–15*) which have been shown to have viscosities at least three times that of water. Experiments in this laboratory with *Escherichia coli* and yeast have also yielded protoplasmic viscosities greater than that obtained for lymphocytes. This difference may be attributed to the morphological characteristics of lymphocytes which include the disproportionally large volume occupied by the nucleus compared with the cytoplasm and the near absence of physical structures in the cytoplasm.

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