LETTER TO THE EDITOR

# *Temperature Dependence of Spin-Label Intensity in Solutions and its Implication in Spin-labeled Erythrocyte Membrane Studies*

**Dear Sir:**

Human erythrocyte membrane proteins alkylated with the nitroxide spin label, *N*-(1-oxyl-2, 2, 6, 6-tetramethyl 4-piperidinyl) maleimide (Mal-6), exhibit multicomponent electron paramagnetic resonance (EPR) signals. The conventional, first harmonic ($V_1$) spectra of these membrane samples contain broad and narrow line components. The broad component, which is the major signal, and has a large hyperfine splitting, comes from labels that are strongly immobilized by the host proteins; the narrow component, with a smaller hyperfine splitting, comes from labels that are weakly immobilized. The amplitudes of these two signals, $W$ and $S$, are convenient parameters, and the $W/S$ ratio has been used by many workers in the analysis of the EPR spectra (1–4). This ratio is very useful in studying intracellular molecules binding to membrane surfaces on the cytoplasmic side, and has been used in this laboratory to study a very low affinity hemoglobin-membrane association at physiological pH (5). Quantitatively, we have used a two-state model to interpret the $W/S$ values obtained from the binding studies.

However, Rifkind and co-workers have recently observed an increase in the integrated EPR signal intensity upon increasing temperature (6), and have suggested that there exists a state that is EPR silent at low temperature due to dipolar interactions, but becomes EPR active at higher temperatures. They suggested that a model of at least three states is more appropriate (6). We disagree with this interpretation, at least for spin-labeled erythrocyte studies. In this letter we present data showing that the increase in intensity upon increasing temperature is primarily related to EPR cavity sensitivity rather than to an increase in concentration of the bound spin labels that are EPR active.

In an EPR measurement, the cavity sensitivity depends on the $Q$ of the cavity, the filling factor, the magnetic moment of the sample, factors affecting either the noise level or the signal level, etc. (7). In general, the cavity sensitivity is a multifunctional quantity. The determination of absolute spin concentration is quite a complicated exercise. For example, recent work shows that many correction factors including the lens effect of a quartz Dewar flask insert and the lens effects of the solvent (8), are needed to compare integrated EPR signal intensities in lossy solutions. The $Q$ of the cavity also depends on the dielectric constant of the sample inside the cavity.

In this study, we have kept many factors related to cavity sensitivity constant, and have varied only the temperature of samples with different dielectric constants, including spin-labeled human erythrocyte membranes. Under this condition, the cavity sensitivity should depend mainly on the magnitude of the dielectric constant of the sample, and is a function of temperature if the dielectric constant is a function of temperature. Otherwise, the cavity sensitivity is relatively independent of temperature.

White membrane ghosts of human erythrocyte were labeled with Mal-6 following standard procedures (4). Human hemoglobin (Hb) was prepared from fresh red cells by standard procedures (5) and spin labeled as follows. 2 ml of 5% Hb solution at pH 6.7 in 5 mM phosphate was mixed with 1.6 mg Mal-6. The mixture was stirred in the cold for 45 min, and excess Mal-6 removed by Sephadex G-25 column chromatography (Pharmacia Fine Chemicals Div., Pharmacia, Inc., Piscataway, NJ). 1 ml of 10% unlabeled Hb was added to the spin-labeled Hb solution and stirred gently. This spin-labeled Hb solution was then lyophilized. For pure spin-label solutions, Mal-6 was dissolved in chloroform, hexane, phosphate-buffered saline (PBS), or water to give a final concentration of $10^{-4}$M. Mal-6 was also prepared in chloroform at $10^{-5}$ M.

For one set of EPR experiments, 50-$\mu$l capillaries (non-heparinized microhematocrit tubes; Dade Div., American Hospital Supply Corp., Miami, FL) were used as EPR sample tubes. A 3-mm quartz tube (Varian Associates, Palo Alto, CA) partially filled with silicon fluid for thermostability, was positioned and remained in the cavity throughout the experiments at all temperatures. The EPR sample tubes were placed concentrically inside this quartz tube. This sample tube arrangement ensured that all samples were positioned in the same location inside the cavity. This precaution was important since the cavity sensitivity varies depending on the location inside the cavity.

In a second set of experiments, quartz tube and capillary tube had the same arrangement but the quartz tube was used as sample tube and remained inside the cavity throughout the experiment. The capillary tube inserted inside the quartz tube was either empty or filled with pure chloroform or water. We chose a $10^{-5}$ M Mal-6 chloroform solution as the sample for this set of experiments due to the relatively large sample volume in the 3-mm quartz tube as compared with that of the 50-$\mu$l capillary tube used in the first set of experiments.

Standard EPR spectrometer settings were used (4). The

temperature of the sample was measured with a thin-wire copper-constantan thermocouple, which was placed inside the capillary tube, with the thermocouple junction just above the surface of the sample. First harmonic conventional EPR signals were fed into a computer for double integration. Spectral baselines were carefully adjusted to minimize integration error. Asymmetry of derivative signals, probably due to phase changes of the microwave magnetic field in lossy samples (8), was also minimized by careful tuning of the cavity. Signal intensities obtained by double integration of the signals of all samples at various temperatures were converted to apparent spin-label concentrations at 20°C for presentation convenience. The conversion factor was obtained from the spectrum of a Mal-6 sample with a known concentration in water at 20°C.

Fig. 1 *a* shows the temperature dependence of spin-label signal intensity of samples with Mal-6 in water, on human erythrocyte membranes and in PBS. The spin-label concentration was chosen in the range of $10^{-3}$ to $10^{-4}$M and is not identical between samples. When equal amounts of spin label were dissolved in water or PBS, the signal intensities at 20°C, as determined by double integration, are about the same. The intensity temperature dependence of each sample in Fig. 1 *a* was analyzed by linear regression, which shows linear behavior with correlation coefficients > 0.94 for all three samples. The spin-label signal intensity values calculated from the regression parameters (intercepts and slopes) for temperatures at 4 and 37°C differed substantially (Table I). Under these experimental conditions, the signal intensity increased ~45% upon increasing the temperature from 4 to 37°C for Mal-6 in distilled water, ~39% for the Mal-6–labeled membrane sample, and ~64% for Mal-6 in PBS (Table I).

Similar experiments were performed on samples with Mal-6 in chloroform and in hexane. However, little temperature dependence was observed for these samples (Fig. 1 *b* and Table I). The dielectric constant of hexane is relatively low and has a very weak temperature dependence (1.91 at 4°C and 1.87 at 37°C) (9). Chloroform has a higher dielectric constant, with a temperature dependence somewhat higher than that of hexane (4.77 at 4°C and 4.27 at 37°C) (9). When equal amounts of Mal-6 were dissolved in water, chloroform, and hexane, the hexane samples gave the largest signal intensity, followed by chloroform and then water. Samples used in Fig. 1 *a* and *b* were all in 50-μl capillary tubes. The EPR intensity for lyophilized spin-labeled Hb also showed a similar temperature independence. As expected with a solid sample, the cavity $Q$ changes little as a function of temperature, and the intensity is therefore independent of temperature.

To determine whether nitroxide-solvent interactions played any role in this behavior, the sample geometry was reversed, a $10^{-5}$M Mal-6 chloroform solution was placed in the 3-mm quartz tube, and 50-μl tubes containing air, chloroform or water were inserted concentrically, as above.

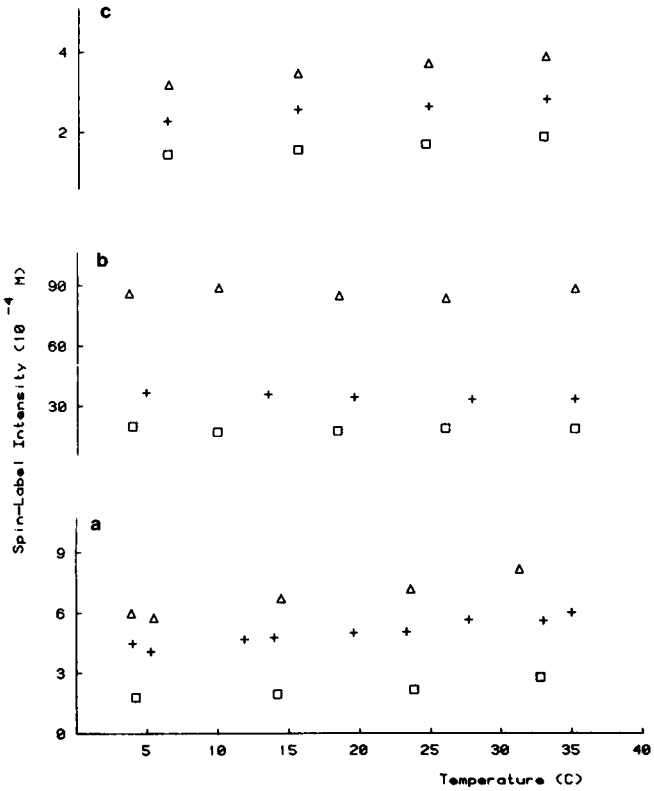


FIGURE 1 Spin-label intensities as a function of temperature for several Mal-6 samples. For presentation convenience, the intensity measurements have the unit of spin-label concentration at 20°C. See text for more detailed discussion. Results of the linear regression analysis for these data are given in Table I. (*a*) Data for Mal-6 spin label in water (Δ) and in phosphate-buffered saline (PBS) (□). Spin-labeled human erythrocyte membranes (+) show a temperature dependence similar to those in water or PBS samples. (*b*) Data for Mal-6 in hexane (Δ), in chloroform (□), and for lyophilized spin-labeled hemoglobin (+). These samples show little temperature dependence in spin-label intensity. (*c*) Data of Mal-6 in chloroform with different capillaries inserted: (Δ) for empty capillary, (+) for capillary with pure chloroform and (□) for capillary with pure water. Difference in intensity in these samples is due to the difference in dielectric constants of solutions inside the capillary tubes.

The highest signal intensity was obtained when an empty capillary was inserted (Fig. 1 *c*). The signal intensity decreased when a capillary with pure chloroform was inserted, and the capillary with distilled water gave the lowest signal intensity. In this experiment, the three samples have precisely the same volume of Mal-6 in chloroform and the same position in the cavity. Therefore, the comparison of signal intensities in these samples is rather straightforward. The $Q$ of the cavity, using the air-filled capillary as a reference, is lowered by a dielectric material in the capillary, for example, pure chloroform, thus the signal intensity is lowered. The $Q$ of the cavity is further lowered by pure water in the capillary, and the signal intensity decreases further, even though there is an equal number of spins in the cavity circuitry in all three samples.

Our results show that the EPR signal intensities of

TABLE I
LINEAR REGRESSION ANALYSIS OF SPIN-LABEL SIGNAL INTENSITY.
TEMPERATURE DEPENDENCE OF DATA IN FIG. 1

| System | Slope | Calculated intensity* (4°C) | (37°C) | (% change) |
|---|---|---|---|---|
| Water | 0.080 | 5.81 | 8.45 | 45 |
| Membrane | 0.050 | 4.24 | 5.95 | 40 |
| PBS | 0.033 | 1.68 | 2.76 | 64 |
| Hexane | −0.017 | 8.79 | 8.23 | 6 |
| Hb | −0.009 | 3.65 | 3.36 | 8 |
| Chloroform | 0.002 | 1.84 | 1.90 | 3 |
| c-empty† | 0.029 | 3.12 | 4.08 | 31 |
| c-chloroform | 0.022 | 2.25 | 2.98 | 32 |
| c-water | 0.019 | 1.38 | 2.00 | 45 |

*Intensity unit for water, membrane, PBS, c-empty, c-chloroform and c-water is $10^{-4}$M and for hexane, hemoglobin, and chloroform is $10^{-3}$M. The unit is defined in text.

†C-empty, c-chloroform and c-water represent empty capillary, capillary with chloroform, and capillary with water, respectively.

samples with equivalent amounts of Mal-6 decrease in the order hexane > chloroform > water. Thus, the higher the dielectric constant, the lower the sensitivity of the cavity and the lower the signal intensity. Due to the weak temperature dependence of the hexane dielectric constant, we observe little temperature dependence in the signal intensities of Mal-6 in hexane (Fig. 1 *b*). Chloroform has a relatively low dielectric constant that has a moderate temperature dependence. Thus, at high concentration ($\sim 10^{-4}$M) the signal intensity temperature dependence is small (Fig. 1 *b*), but at low concentration ($\sim 10^{-5}$M) the temperature dependence becomes more obvious (Fig. 1 *c*).

Water has a very high dielectric constant (86.15 at 4°C), which is also strongly temperature dependent (73.85 at 37°C) (10). Most biological systems are not in pure water, but in buffer solutions. Salt ions modify the water dipoles, which affect the solution dielectric constant. At low salt concentration, the dielectric constant decreases slightly as the salt concentration increases. At high salt concentration, the salt ion pairs are like permanent dipoles, and thus the salt increases the dielectric constant (11). However, the dielectric constant of 0.5 M NaCl in water is only slightly lower than that of pure water (8, 12). The dielectric constant of PBS, which contains 0.15 M NaCl, is just slightly lower than that of water.

Our spin-labeled membranes in buffer solution are in aqueous medium. They show a signal intensity temperature dependence similar to that for Mal-6 in water (or PBS), which indicates that the spin-label concentration at 37°C remains the same as 4°C, even though the signal intensity at 37°C increases 40% from the value at 4°C. Thus, we conclude that, at least for erythrocyte membrane samples, there are no "EPR silent" spin labels at either 37 or 4°C. This study does not show an increase in spin-label concentration in erythrocyte membranes upon increases in temperature. Changes in integrated signal intensity with temperature are primarily due to dielectric-induced changes in cavity sensitivity. We believe that the previously proposed two-state model (5) is a simple and convenient model for analysis of conventional, first harmonic, absorption EPR results, and is a realistic interpretation of the EPR results.

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