

# Spin Relaxation Measurements Using First-Harmonic Out-of-Phase Absorption EPR Signals: Rotational Motion Effects

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**A recent survey of nonlinear continuous-wave (CW) EPR methods revealed that the first-harmonic absorption EPR signal, detected 90° out of phase with respect to the Zeeman modulation ( $V_1$ -EPR), is the most appropriate for determining spin-lattice relaxation enhancements of spin labels (V. A. Livshits, T. Páli, and D. Marsh, 1998, *J. Magn. Reson.* 134, 113–123). The sensitivity of such  $V_1$ -EPR spectra to molecular rotational motion is investigated here by spectral simulations for nitroxyl spin labels, over the entire range of rotational correlation times. Determination of the effective spin-lattice relaxation times is less dependent on rotational mobility than for other nonlinear CW EPR methods, especially at a Zeeman modulation frequency of 25 kHz which is particularly appropriate for spin labels. This relative insensitivity to molecular motion further enhances the usefulness of the  $V_1$ -EPR method. Calibrations of the out-of-phase to in-phase spectral intensity (and amplitude) ratios are given as a function of spin-lattice relaxation time, for the full range of spin-label rotational correlation times. Experimental measurements on spin labels in the slow, intermediate, and fast motional regimes of molecular rotation are used to test and validate the method.** © 2000 Academic Press

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## INTRODUCTION

Spin-lattice relaxation of nitroxide spin labels currently finds increasing application in membrane biophysics. Structural information is obtained by measuring relaxation enhancements induced by paramagnetic relaxation agents, and sensitivity to slow dynamic processes is also very much enhanced (1–3). It is well known that information on spin relaxation rates may be obtained from progressive saturation experiments. These experiments use the conventional  $V_1$ -EPR spectra detected in phase with the first harmonic of the Zeeman modulation field. On the other hand, the absorption  $V_2$ -EPR spectra of spin labels detected at the second modulation harmonic, in phase quadrature with the modulation field (i.e., saturation transfer EPR), are also used to measure spin-lattice relaxation enhancements (3). These include relaxation enhancements induced by Heisenberg spin exchange or by dipole-dipole interactions with fast-relaxing paramagnetic species.

Continuous-wave (CW) EPR experiments, as compared to pulse measurements, have the advantage of relative accessibility and high concentration sensitivity. Therefore, it is useful to ex-

plore new nonlinear CW approaches to the determination of spin relaxation rates of spin-labeled molecules. Of these, the  $V_2$  saturation transfer EPR spectra, although being sensitive to  $T_1$ , are also very sensitive to molecular motion (4) and to  $T_2$  relaxation (5). In comparison, conventional CW progressive saturation parameters are less sensitive to  $T_1$ , and also they are dependent directly on  $T_2$ .

Recently, we proposed the use of the out-of-phase first-harmonic EPR absorption signals ( $V_1'$ ) for determining spin-lattice relaxation enhancements (6). This  $V_1'$ -EPR signal was first suggested by Hyde and Thomas (7) as a  $T_1$ -sensitive nonlinear EPR display. In Ref. (6) we showed both theoretically and experimentally that the  $V_1'$ -EPR amplitudes or double integrals are more sensitive to  $T_1$  and less dependent on  $T_2$  than are conventional in-phase  $V_1$ -EPR spectra. A survey of nonlinear CW EPR methods suggested that first-harmonic out-of-phase  $V_1'$ -EPR was the method of choice for determining  $T_1$  relaxation enhancements of spin labels. These results, however, were obtained in the absence of molecular motion and for a single-line spectrum.

For the practical application of this new CW approach, it is important to investigate the dependence of the  $T_1$ - and  $T_2$ -sensitive spectral parameters of the  $V_1'$ -EPR spectra on the dynamics of rotational motion. A dependence is to be expected for nitroxide spin labels under conditions of partial saturation. This is because molecular rotation induces spectral diffusion of microwave saturation and provides a mechanism for transverse spin relaxation by modulation of the Zeeman and hyperfine anisotropies. The aim of the present work is to extend the capability of out-of-phase first-harmonic  $V_1'$ -EPR measurements for determining the  $T_1$  relaxation times of spin labels to the whole range of rotational correlation times. Calibrations are given for the  $T_1$ -sensitive parameters of the  $V_1'$ -EPR spectra at different rotational mobilities. The applicability of this approach is demonstrated experimentally for spin labels in viscous glycerol-water mixtures in the rapid, intermediate, and slow motional regimes.

## MATERIALS AND METHODS

The spin label TEMPONE (4-oxo-2,2,6,6-tetramethylpiperidine-*N*-oxyl) was obtained from Eastman Kodak (Rochester, NY). Maleimide spin label, 3-maleimidoproxyl (5-MSL), was



$$V'_1 = \text{Im}\{V_{1,1}\}. \quad [10]$$

The conventional first-harmonic in-phase absorption spectrum is correspondingly given by

$$V_1 = \text{Re}\{V_{1,1}\}. \quad [11]$$

In Eqs. [10] and [11], the value of  $V_{1,1}$  is obtained from the solution of Eq. [8], which is expressed as  $\vec{\mathbf{I}}_1 = (U_{1,1}, V_{1,1}, Z_{1,1})$ . Inhomogeneous line broadening due to hyperfine interaction with methyl and environmental protons can be taken into account by convoluting the lineshapes of Eqs. [10] and [11] with a Gaussian function.

As noted in Ref. (11), use of a hyperfine resonance field given by Eq. [7] is an adiabatic approximation that is not applicable in the fast motion region ( $\tau_R < 3 \times 10^{-9}$  s) (12). In this region, an additional contribution to the spin-spin relaxation rate,  $T_2^{-1}$ , arises from rotational modulation of the anisotropic Zeeman and hyperfine interactions. This can be determined by time-dependent perturbation theory in which the full spin Hamiltonian is used (13). The resulting expressions for the linewidths of the hyperfine components are written in the form

$$T_2^{-1}(M_1) = T_{2,0}^{-1} + A + BM_1 + CM_1^2, \quad [12]$$

where  $T_{2,0}^{-1}$  is the residual homogeneous broadening arising from mechanisms other than rotational motion. For isotropic rotational reorientation, the  $A$ ,  $B$ , and  $C$  coefficients are (13)

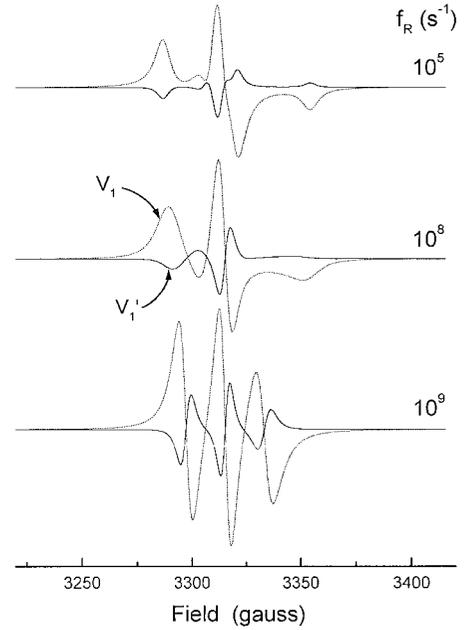
$$A = \frac{4}{45} [(\Delta\omega)^2 + 3(\delta\omega)^2] \tau_R \left( 1 + \frac{3}{4} \frac{1}{1 + \omega_e^2 \tau_R^2} \right) + \frac{7}{45} [(\Delta A)^2 + 3(\delta A)^2] \tau_R \times \left( \frac{1}{1 + \omega_e^2 \tau_R^2} + \frac{3}{7} \frac{1}{1 + \omega_n^2 \tau_R^2} \right) \quad [13]$$

$$B = \frac{8}{45} [\Delta\omega\Delta A + 3\delta\omega\delta A] \tau_R \left( 1 + \frac{3}{4} \frac{1}{1 + \omega_e^2 \tau_R^2} \right) \quad [14]$$

$$C = \frac{4}{45} [(\Delta A)^2 + 3(\delta A)^2] \tau_R \left( 1 - \frac{1}{8} \frac{1}{1 + \omega_e^2 \tau_R^2} - \frac{3}{8} \frac{1}{1 + \omega_n^2 \tau_R^2} \right), \quad [15]$$

where the tensor anisotropies  $\Delta\omega = [g_{zz} - \frac{1}{2}(g_{xx} + g_{yy})] \beta_e H_0 / \hbar$ ,  $\delta\omega = \frac{1}{2}(g_{xx} - g_{yy}) \beta_e H_0 / \hbar$ ,  $\Delta A = A_{zz} - \frac{1}{2}(A_{xx} + A_{yy})$ , and  $\delta A = \frac{1}{2}(A_{xx} - A_{yy})$  are in angular frequency units, and  $\omega_n \approx \frac{1}{2} a_N$  is the nuclear Larmor frequency.

We have performed comparative simulations of the  $V'_1$ -EPR spectra by using different approximations. These are: (i) Eq. [12] with the expressions for the  $A$ ,  $B$ , and  $C$  coefficients given



**FIG. 1.** Simulated first-harmonic out-of-phase  $V'_1$  (solid lines)- and in-phase  $V_1$  (dotted lines)-EPR spectra of nitroxide spin labels for different frequencies of isotropic rotation:  $f_R = 10^5$ ,  $10^8$ , and  $10^9$  s $^{-1}$ . Modulation frequency,  $\omega_m/2\pi = 100$  kHz;  $H_1 = 0.4$  G;  $\Delta H_0 (=1/\gamma_e T_{2,0}) = 3.0$  G;  $T_1 = 2 \times 10^{-6}$  s. The adiabatic approximation is used in these simulations.

by Eqs. [13]–[15] above; (ii) Eq. [12] and the above expressions for  $A$ ,  $B$ , and  $C$  without the terms dependent on nuclear frequency ( $\omega_n$ ); and (iii) the adiabatic approximation, with the hyperfine field given by Eq. [7]. For fixed values of residual linewidth,  $T_{2,0}^{-1}$ , the spectral parameters used for determination of  $T_1$  from the experimental spectra (i.e., the ratios of integrated intensities or low-field amplitudes of the out-of-phase and in-phase components) differ for the cases (i)–(iii). However, if separate values of  $T_{2,0}^{-1}$  are determined for each model by simulations of the low-field component of the conventional (linear) experimental spectra, then the  $T_1$  values obtained from the different approximations (i)–(iii) are very close to each other.

## RESULTS AND DISCUSSION

### Simulations of Out-of-Phase First-Harmonic $V'_1$ -EPR Spectra

Simulated first-harmonic in-phase and out-of-phase EPR spectra are given in Fig. 1. The simulations are for a nitroxide spin label undergoing isotropic rotation of different rates. In the case of moderate and high microwave saturation, the out-of-phase  $V'_1$ -EPR lineshape varies with rotational correlation time in a way similar to that of the in-phase  $V_1$ -EPR signal. This means that, in contrast to the second-harmonic out-of-phase spectra, the  $V'_1$ -EPR signals are not sensitive to slow rotational motions on the microsecond timescale.

The  $V'_1$ -EPR amplitudes decrease as  $H_1^2$ , with a decrease in microwave  $H_1$ -field intensity (see also Ref. 7). At low saturation

tion, a sharp change in the lineshape occurs. In the rapid motion region, this change is similar to that for a single-line spectrum; cf. Ref. (6). If the condition  $T_2/T_1 \ll 1$  is satisfied (where  $T_2^{-1}$  includes both the intrinsic and the rotationally induced spin–spin relaxation rate),  $T_1$  can be estimated in a way similar to that for the “no motion” case (6). This method uses the microwave field amplitude,  $H_1^*$ , at which the transition in lineshape occurs.

### Sensitivity of Out-of-Phase Intensities to $T_1$ : Dependence on Rotational Frequency

More generally, the spin–lattice relaxation times may be determined from the integrated intensities, or amplitudes, of the  $V_1$ -EPR spectra. In the absence of motion, useful  $T_1$ -sensitive parameters are dimensionless ratios of the double-integrated intensities,  $\rho'_1$ , and of the low-field amplitudes,  $\rho'_1(M_1 = +1)$ , for out-of-phase and in-phase spectra measured at the same value of  $H_1$  (6). These ratios are used again here for quantitation of the spectra in the presence of rotational molecular motion. For integrated intensities and amplitudes, the ratios are, respectively,

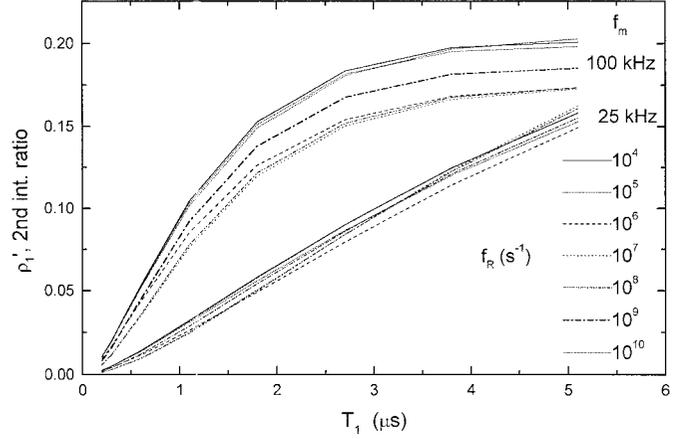
$$\rho'_1 = \frac{\int \int V'_1 d^2H}{\int \int V_1 d^2H} \quad [16]$$

$$\rho'_1(M_1 = +1) = \frac{V'_1(M_1 = +1)}{V_1(M_1 = +1)}. \quad [17]$$

In Eq. [17],  $V'_1(M_1 = +1)$  and  $V_1(M_1 = +1)$  are the maximum lineheights of the out-of-phase and in-phase spectra, respectively, in the low-field region. A local maximum (ignoring absolute sign) is well defined in the low-field region of the spectra, at all rotational frequencies (see Fig. 1). The  $T_1$  dependences of the integrated intensity ratio are presented in Fig. 2, for modulation frequencies of 100 and 25 kHz,  $H_1 = 0.5$  G, and different values of  $f_R$ . The values of the  $\rho'_1$  ratio have a nonmonotonic dependence on rotational frequency. They decrease with increasing  $f_R$  over the range  $10^6$ – $10^8$  s $^{-1}$ , but increase with increasing  $f_R$ , for  $f_R \geq 10^8$  s $^{-1}$ . Typical dependences of the intensity ratio  $\rho'_1$  and the absolute out-of-phase intensity  $\int \int V'_1 d^2H$  on  $f_R$  are given in Fig. 3, for  $T_1 = 3$   $\mu$ s and  $H_1 = 0.5$  G. The dependence for the in-phase integral  $\int \int V_1 d^2H$  is similar to that for the effective spin–spin relaxation rate,  $1/T_2^{\text{eff}}$ ; see Ref. (10). On the other hand, that for the out-of-phase integral  $\int \int V'_1 d^2H$  is relatively weaker and multiphasic (see Fig. 3b). This latter dependence means that rotational motion affects the value of the out-of-phase magnetization through changes additional to those in the value of  $1/T_2^{\text{eff}}$ . This will be analyzed in more detail below.

### Calibration of Integral Ratio $T_1$ Dependences

For all rotational frequencies in the range up to  $f_R = 10^{11}$  s $^{-1}$ , the dependence of the intensity ratio on  $T_1$  can be fitted by an



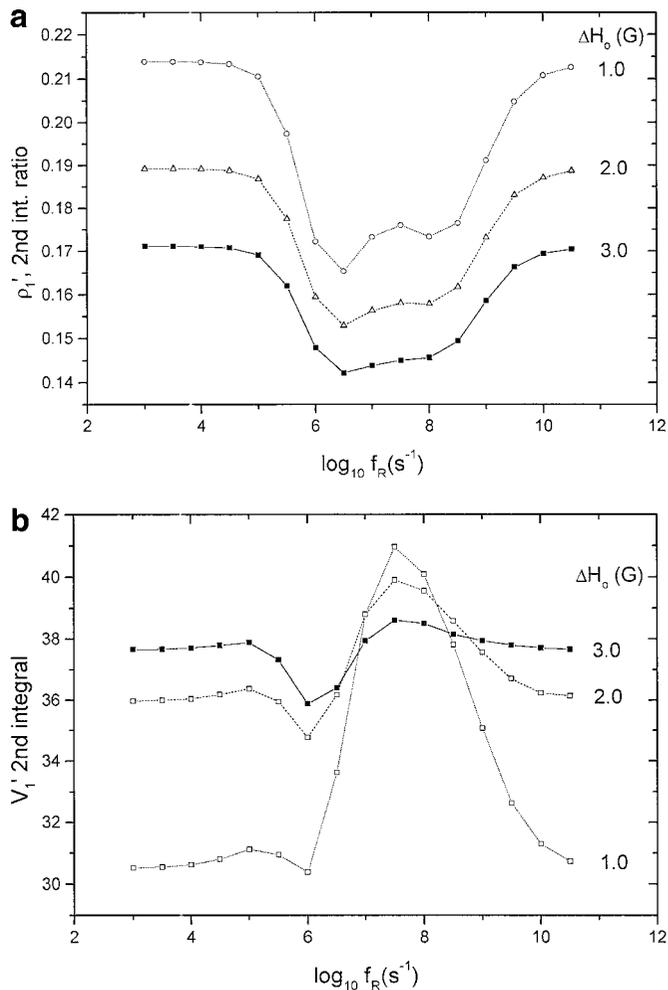
**FIG. 2.** Simulated dependences on spin–lattice relaxation time,  $T_1$ , of the ratio,  $\rho'_1$ , of the integrated intensities of the first-harmonic out-of-phase spectra to those of the in-phase spectra, for different rotational frequencies,  $f_R = 10^4$ – $10^{10}$  s $^{-1}$ , and modulation frequencies,  $\omega_m/2\pi$ , of 100 and 25 kHz, with  $H_1 = 0.5$  G,  $\Delta H_0 (=1/\gamma_e T_{2,0}) = 2.0$  G.

empirical expression of the same form as that used in the absence of molecular rotation (6),

$$\rho'_1 = \rho_1^{0'} + \frac{a'_1 T_1^m}{1 + b'_1 T_1^m}, \quad [18]$$

where the fit parameters  $a'_1$ ,  $b'_1$ ,  $\rho_1^{0'}$ , and  $m$  depend on  $f_R$ , as well as on  $H_1$ ,  $T_{2,0}$ , and  $\omega_m$ . For  $H_1 = 0.5$  G and  $\omega_m/2\pi = 100$  kHz, satisfactory fits were obtained for all values of  $f_R$  and  $T_{2,0}$  with  $m = 2$ . For a modulation frequency of  $\omega_m/2\pi = 25$  kHz, fitted values of the exponent are  $m = 1.3$ – $1.5$ , where the range of  $m$  applies to different  $T_1$  intervals.

The fit parameters,  $a'_1$  and  $b'_1$ , determine the slopes of the  $T_1$  dependences in relaxation-enhancement experiments. Therefore it is important to know their variation with the rotational frequency  $f_R$  and transverse relaxation time,  $T_{2,0}$  (or intrinsic linewidth,  $\Delta H_0 = 1/\gamma_e T_{2,0}$ ). Typical dependences of these parameters on rotational frequency for different intrinsic linewidths are given in Figs. 4a and 4b. The absolute values of these parameters, and ranges of  $f_R$ ,  $\Delta H_0$ , and  $T_1$  for which fits were performed, are given in Tables 1 and 2. These values were obtained with the adiabatic approximation, throughout the entire range of rotational frequencies. Their validity for the rapid motional region is established later, under Experimental Applications. The value of  $\rho_1^{0'}$  is close to zero over most of the range of  $f_R$ . The dependences of the  $a'_1$  and  $b'_1$  parameters on  $f_R$  are both similar to that for  $\rho'_1$ , at a modulation frequency of 100 kHz (see Fig. 3). The  $a'_1$  and  $b'_1$  parameters decrease approximately linearly with  $\Delta H_0$ , but their absolute values change by only 30–40% when  $\Delta H_0$  changes from 0.5 to 3.0 G (see Figs. 4a and 4b). Moreover, this dependence becomes even weaker when approaching the region of maximum spectral diffusion rate ( $f_R \approx 10^7$  s $^{-1}$ ), from both low and high rotational frequencies.



**FIG. 3.** Simulated dependences on rotational frequency,  $f_R$ , of (a) the ratio,  $\rho'_1$ , of the integrated intensities of the out-of-phase and in-phase spectra, and (b) the integrated intensities of the  $V'_1$  signal, for different spin-packet widths,  $\Delta H_0$  ( $= 1/\gamma_e T_{2,0}$ ), a modulation frequency of 100 kHz, and  $H_1 = 0.5$  G,  $T_1 = 3 \times 10^{-6}$  s.

The dependence of the intensity ratios on rotational frequency,  $f_R$ , is substantially reduced at lower modulation frequencies. This is seen in Fig. 2 for  $\omega_m/2\pi = 25$  kHz, in comparison with  $\omega_m/2\pi = 100$  kHz. The exponent,  $m$ , is smaller at lower modulation frequencies ( $m = 1.3$  for 25 kHz). Nevertheless, the dependences of the fit parameters  $a'_1$  and  $b'_1$  on  $f_R$  and  $T_{2,0}$  are qualitatively similar to those for 100 kHz. The linear dependences of  $a'_1$  and  $b'_1$  on  $T_{2,0}$  have smaller slopes at  $\omega_m/2\pi = 25$  kHz. In addition, these differences are to a large extent compensated in the absolute values of the out-of-phase intensities, at the lower modulation frequency. Again, as for  $\omega_m/2\pi = 100$  kHz, the dependence on  $T_{2,0}$  becomes weaker when approaching the region of maximum spectral diffusion rate. Thus, the sensitivity of the first-harmonic intensity ratio to  $T_{2,0}$  is weaker than that for the progressive saturation parameter (10), over the entire rotational frequency range. Moreover, it can be decreased even further by lowering the Zeeman modulation frequency.

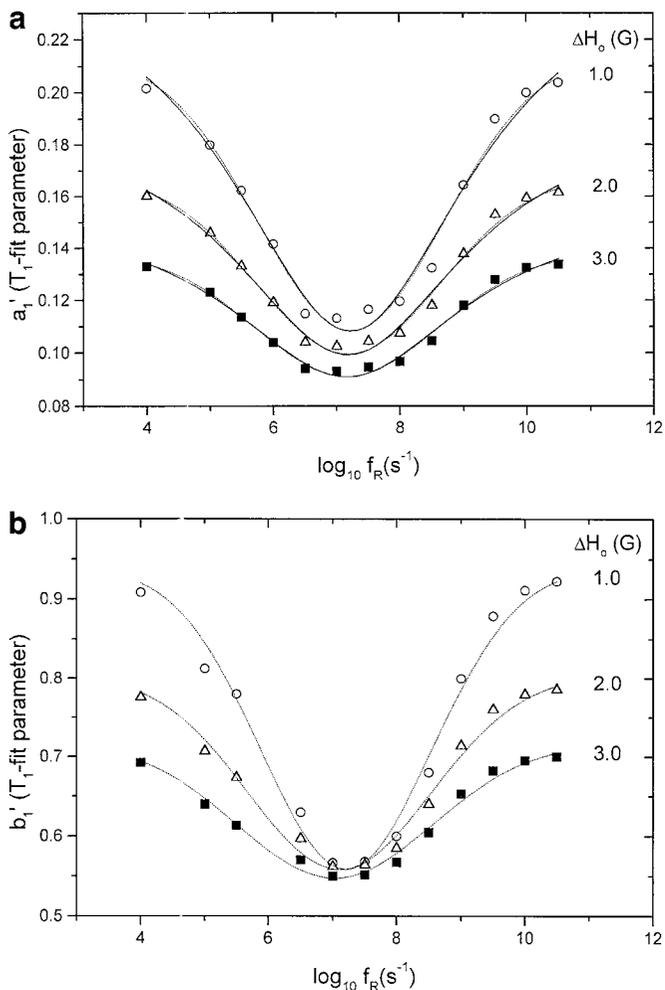
As seen in Fig. 3a, the values of the integral parameter,  $\rho'_1$ , are identical for no rotational motion and for very rapid rotation, even though the lineshapes are completely different for the two cases. This is because both spectral diffusion and the rotational contribution to the spin-spin relaxation rate are absent in these two limits.

#### Calibration of Amplitude Ratio $T_1$ Dependences

The dependence of the amplitude ratio,  $\rho'_1(M_I)$ , on  $T_1$  can be fitted by an expression of the form

$$\rho'_1(M_I) = \rho_1^{0'}(M_I) + \frac{a'_1(M_I)T_1^m}{1 + b'_1(M_I)T_1^m}. \quad [19]$$

This is similar to Eq. [18] for the integrated intensity ratio. Equation [19] applies over the whole range of rotational fre-



**FIG. 4.** Simulated dependence on rotational frequency,  $f_R$ , of the fit parameters, (a)  $a'_1$  and (b)  $b'_1$  in Eq. [18] with  $m = 2$  and  $T_1$  in  $\mu$ s, for different spin-packet widths,  $\Delta H_0$  ( $= 1/\gamma_e T_{2,0}$ ), and a modulation frequency of 100 kHz;  $H_1 = 0.5$  G. Dotted lines are fits to a Gaussian curve, and solid lines those to a Lorentzian.

TABLE 1

**$T_1$  Calibration of the Intensity Ratio Fitting Parameters in Eq. [18] (with  $m = 2$ ) for a Zeeman Modulation Frequency of 100 kHz, and Rotational Frequencies in the Range  $10^4$ – $3.16 \times 10^{10} \text{ s}^{-1}$**

$f_R \text{ (s}^{-1}\text{)}$	$\rho_1^0$	$a'_1$	$b'_1$
$10^4$	0.0134–0.0037	0.202–0.133	0.91–0.69
$10^5$	0.0146–0.0044	0.180–0.123	0.81–0.64
$3.16 \times 10^5$	0.0147–0.0047	0.162–0.114	0.78–0.61
$10^6$	0.0129–0.0042	0.142–0.104	0.78–0.62
$3.16 \times 10^6$	0.0096–0.0031	0.115–0.094	0.63–0.57
$10^7$	0.0053–0.0022	0.113–0.093	0.56–0.55
$3.16 \times 10^7$	0.0037–0.0016	0.116–0.094	0.57–0.55
$10^8$	0.0040–0.0017	0.120–0.097	0.60–0.57
$3.16 \times 10^8$	0.0053–0.0022	0.134–0.105	0.68–0.60
$10^9$	0.0076–0.0026	0.164–0.118	0.80–0.65
$3.16 \times 10^9$	0.0102–0.0031	0.190–0.128	0.88–0.68
$10^{10}$	0.0120–0.0034	0.200–0.133	0.91–0.69
$3.16 \times 10^{10}$	0.0127–0.0035	0.204–0.134	0.92–0.70

<sup>a</sup> Results are given for different values of the intrinsic linewidth,  $\Delta H_0$ , in the range 1–3 G, over the range  $T_1 = 0.2$ – $5.1 \mu\text{s}$  for which fitting was performed. The microwave field amplitude,  $H_1$ , is 0.5 G. Values of  $a'_1$  and  $b'_1$  are given for  $T_1$  in  $\mu\text{s}$ . The adiabatic approximation is used throughout for the simulations.

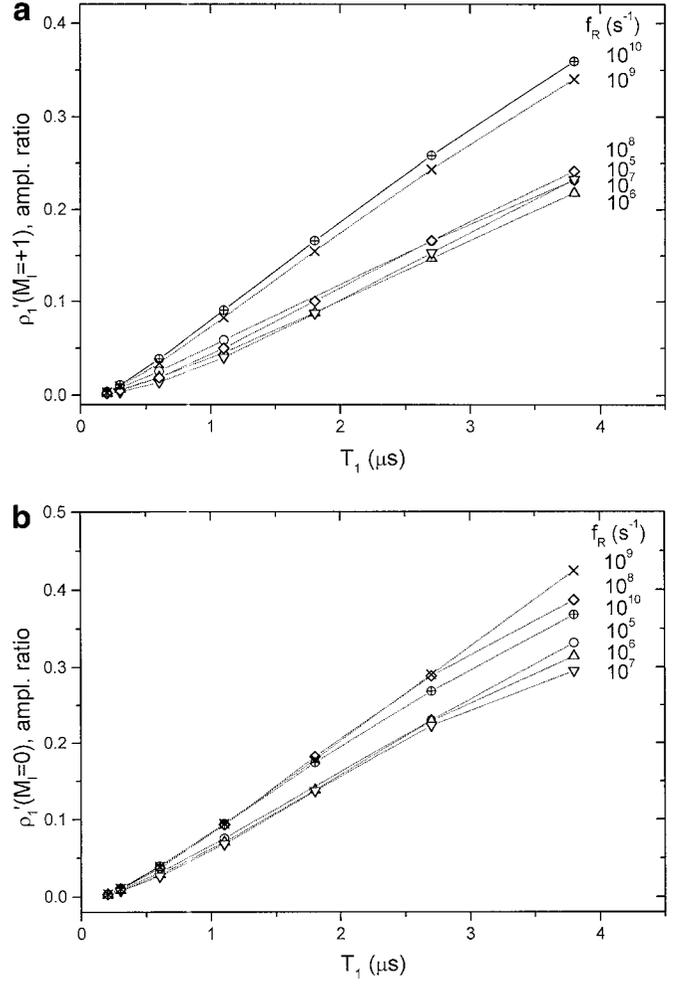
quencies, with  $m = 1.6$  for  $\omega_m/2\pi = 100 \text{ kHz}$  and  $m = 1.3$  for  $\omega_m/2\pi = 25 \text{ kHz}$ . Examples of the dependences on  $T_1$  are shown in Fig. 5, for the amplitudes of both the  $M_1 = +1$  and  $M_1 = 0$  hyperfine manifolds. Values of the fit parameters,  $\rho_1^0(M_1 = +1)$ ,  $a'_1(M_1 = +1)$ , and  $b'_1(M_1 = +1)$ , for the low-field (i.e.,  $M_1 = +1$ ) amplitude are given in Tables 3 and 4. The adiabatic approximation was used throughout, but the validity for the rapid motional regime is established later under Experimental Applications.

TABLE 2

**$T_1$  Calibration of the Intensity Ratio Fitting Parameters in Eq. [18] (with  $m = 1.3$ ) for a Zeeman Modulation Frequency of 25 kHz, and Rotational Frequencies in the Range  $10^4$ – $3.16 \times 10^{10} \text{ s}^{-1}$**

$f_R \text{ (s}^{-1}\text{)}$	$\rho_1^0$	$a'_1$	$b'_1$
$1 \times 10^4$	0.0007–0.0025	0.0361–0.0303	0.1051–0.0724
$1 \times 10^5$	0.0010–0.0023	0.0344–0.0291	0.1041–0.0701
$3.16 \times 10^5$	0.0013–0.0021	0.0310–0.0273	0.0928–0.0632
$1 \times 10^6$	0.0008–0.0023	0.0273–0.0254	0.0635–0.050
$3.16 \times 10^6$	–0.0013–0.0030	0.0278–0.0254	0.0486–0.0430
$1 \times 10^7$	–0.0030–0.0034	0.0300–0.0260	0.0490–0.0429
$3.16 \times 10^7$	–0.0035–0.0037	0.0309–0.0263	0.0510–0.0438
$1 \times 10^8$	–0.0032–0.0036	0.0305–0.0264	0.0542–0.0460
$3.16 \times 10^8$	–0.0023–0.0033	0.0309–0.0269	0.0664–0.0529
$1 \times 10^9$	–0.0012–0.0030	0.0334–0.0285	0.0868–0.0633
$3.16 \times 10^9$	–0.0002–0.0028	0.0355–0.0294	0.0990–0.0673
$1 \times 10^{10}$	–0.0003–0.0027	0.0363–0.0302	0.1033–0.0718
$3.16 \times 10^{10}$	–0.0005–0.0026	0.0366–0.0305	0.1047–0.0735

<sup>a</sup> Results are given for different values of the intrinsic linewidth,  $\Delta H_0$ , in the range 1–3 G, over the range  $T_1 = 0.2$ – $5.1 \mu\text{s}$  for which fitting was performed. The microwave field amplitude,  $H_1$ , is 0.5 G. Values of  $a'_1$  and  $b'_1$  are given for  $T_1$  in  $\mu\text{s}$ . The adiabatic approximation is used throughout for the simulations.



**FIG. 5.** Simulated dependences on  $T_1$  of the ratio,  $\rho'_1(M_1)$ , of the amplitudes of (a) the low-field ( $M_1 = +1$ ) and (b) the central ( $M_1 = 0$ ) hyperfine components of the out-of-phase and in-phase EPR spectra, for different values of rotational frequency  $f_R$  and a Zeeman modulation frequency of 25 kHz;  $H_1 = 0.5 \text{ G}$ ;  $\Delta H_0 (= 1/\gamma_e T_{2,0}) = 2.0 \text{ G}$ .

The central part of an EPR spectrum in the slow motion region contains the contributions from the  $M_1 = \pm 1$  hyperfine components. Nevertheless, amplitudes in this region ( $M_1 = 0$ ) are also useful  $T_1$ -sensitive parameters. The advantage of this central,  $M_1 = 0$ , region of the spectrum is that it has a larger amplitude than the low- and high-field components. It is seen from Fig. 5 that the sensitivity of this  $\rho'_1(M_1 = 0)$  amplitude ratio to  $T_1$  is about the same as for  $\rho'_1(M_1 = +1)$ , in the rapid motion region. In the slow motion region, the sensitivity is even greater than for the low-field amplitude ratio,  $\rho'_1(M_1 = +1)$ . Additionally, the  $\rho'_1(M_1 = 0)$  parameter is only relatively weakly sensitive to inhomogeneous broadening.

In comparison with the integrated intensities,  $\rho_1^0$ , the dependence of the amplitude ratio,  $\rho'_1(M_1 = +1)$ , on  $f_R$  is stronger and more asymmetric. This is also true for the individual  $V_1$  and  $V'_1$  amplitudes. This is evident in Fig. 5 for the amplitude ratios, and also in the dependences on rotational frequency of the fit parameters,  $a'_1(M_1 = +1)$  and

TABLE 3

$T_1$  Calibration of the  $M_1 = +1$  Amplitude Ratio Fitting Parameters in Eq. [19] (with  $m = 1.6$ ) for a Zeeman Modulation Frequency of 100 kHz, and Rotational Frequencies in the Range  $10^4$ – $3.16 \times 10^{10} \text{ s}^{-1}$ <sup>a</sup>

$f_R$ ( $\text{s}^{-1}$ )	$\rho_1^0(M_1 = +1)$	$a_1'(M_1 = +1)$	$b_1'(M_1 = +1)$
$1 \times 10^4$	−0.0060–−0.0116	0.352–0.260	0.629–0.560
$1 \times 10^5$	0.0007–−0.0065	0.337–0.250	0.623–0.555
$3.16 \times 10^5$	0.0007–−0.0080	0.269–0.216	0.544–0.487
$1 \times 10^6$	−0.0001–−0.0076	0.219–0.190	0.555–0.485
$3.16 \times 10^6$	−0.0060–−0.0095	0.175–0.171	0.473–0.452
$1 \times 10^7$	−0.0120–−0.0110	0.174–0.169	0.436–0.437
$3.16 \times 10^7$	−0.0134–−0.0108	0.188–0.177	0.461–0.451
$1 \times 10^8$	−0.0126–−0.0120	0.251–0.218	0.528–0.498
$3.16 \times 10^8$	−0.0173–−0.0156	0.370–0.285	0.494–0.481
$1 \times 10^9$	−0.0169–−0.0171	0.440–0.330	0.500–0.480
$3.16 \times 10^9$	−0.0151–−0.0175	0.464–0.352	0.507–0.483
$1 \times 10^{10}$	−0.0141–−0.0176	0.470–0.360	0.508–0.485
$3.16 \times 10^{10}$	−0.0139–−0.0176	0.470–0.361	0.508–0.486

<sup>a</sup> Results are given for different values of the intrinsic linewidth,  $\Delta H_0$ , in the range 1–3 G, a peak-to-peak inhomogeneous linewidth of 1.4 G, over the range  $T_1 = 0.2$ – $5.1 \mu\text{s}$  for which fitting was performed. The microwave field amplitude,  $H_1$ , is 0.5 G. Values of  $a_1'(M_1 = +1)$  and  $b_1'(M_1 = +1)$  are given for  $T_1$  in  $\mu\text{s}$ . The adiabatic approximation is used throughout for the simulations.

$b_1'(M_1 = +1)$  in Eq. [19]. The latter are presented in Fig. 6. The asymmetry is explained by the hyperfine- and  $g$ -tensor anisotropy. In the absence of motion, and at low rotational frequencies, this results in an anisotropic distribution of

TABLE 4

$T_1$  Calibration of the  $M_1 = +1$  Amplitude Ratio Fitting Parameters in Eq. [19] (with  $m = 1.3$ ) for a Zeeman Modulation Frequency of 25 kHz, and Rotational Frequencies in the Range  $10^4$ – $3.16 \times 10^{10} \text{ s}^{-1}$ <sup>a</sup>

$f_R$ ( $\text{s}^{-1}$ )	$\rho_1^0(M_1 = +1)$	$a_1'(M_1 = +1)$	$b_1'(M_1 = +1)$
$1 \times 10^4$	−0.0027–−0.0055	0.0677–0.0568	0.090–0.073
$1 \times 10^5$	−0.0017–−0.0053	0.0620–0.0540	0.087–0.063
$3.16 \times 10^5$	−0.0007–−0.0050	0.0530–0.0495	0.072–0.051
$1 \times 10^6$	−0.0016–−0.0057	0.0440–0.0458	0.040–0.036
$3.16 \times 10^6$	−0.0059–−0.0074	0.0439–0.0456	0.026–0.028
$1 \times 10^7$	−0.0084–−0.0082	0.0471–0.0465	0.027–0.029
$3.16 \times 10^7$	−0.0085–−0.0075	0.0491–0.0473	0.033–0.033
$1 \times 10^8$	−0.0074–−0.0077	0.0586–0.0537	0.056–0.050
$3.16 \times 10^8$	−0.0092–−0.0090	0.0846–0.0690	0.075–0.066
$1 \times 10^9$	−0.0081–−0.0093	0.0948–0.0780	0.079–0.071
$3.16 \times 10^9$	−0.0071–−0.0093	0.0972–0.0814	0.079–0.073
$1 \times 10^{10}$	−0.0066–−0.0092	0.0978–0.0826	0.079–0.074
$3.16 \times 10^{10}$	−0.0065–−0.0092	0.0978–0.0829	0.078–0.074

<sup>a</sup> Results are given for different values of the intrinsic linewidth,  $\Delta H_0$ , in the range 1–3 G, a peak-to-peak inhomogeneous linewidth of 1.4 G, over the range  $T_1 = 0.2$ – $5.1 \mu\text{s}$  for which fitting was performed. The microwave field amplitude,  $H_1$ , is 0.5 G. Values of  $a_1'(M_1 = +1)$  and  $b_1'(M_1 = +1)$  are given for  $T_1$  in  $\mu\text{s}$ . The adiabatic approximation is used throughout for the simulations.

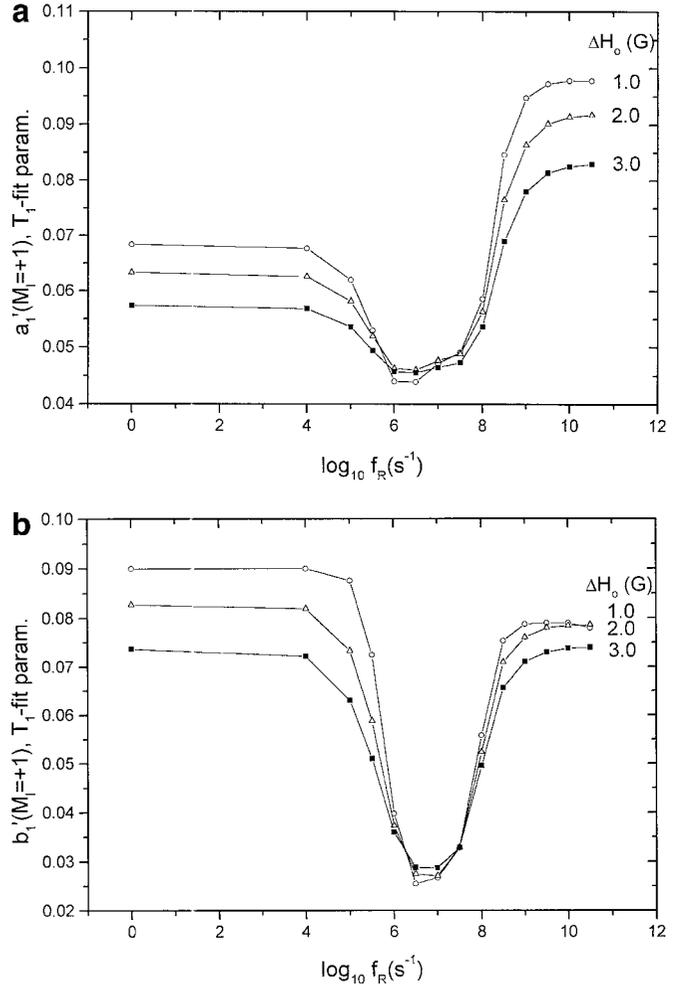


FIG. 6. Dependence on rotational frequency,  $f_R$ , of the fit parameters (a)  $a_1'(M_1 = +1)$  and (b)  $b_1'(M_1 = +1)$  for the  $T_1$  dependence of the amplitude of the low-field hyperfine line in Eq. [19] with  $m = 1.3$  and  $T_1$  in  $\mu\text{s}$ , for different values of  $\Delta H_0$  ( $= 1/\gamma_e T_{2,0}$ ) and a Zeeman modulation frequency of 25 kHz;  $H_1 = 0.5 \text{ G}$ .

resonance fields. At high rotational frequencies this anisotropic distribution is motionally averaged. The dependence of the  $\rho_1^0(M_1)$  ratio on  $f_R$  is determined by the  $f_R$  dependences of the individual  $V_1$  and  $V_1'$  amplitudes. The  $V_1'$  amplitude for  $\omega_m/2\pi = 100 \text{ kHz}$  is less dependent on  $f_R$  in the slow and intermediate motional regions. At higher rotational frequencies, however, it increases more strongly than does the in-phase amplitude. For the  $a_1'(M_1)$  parameter, this dependence is similar to that for  $\rho_1^0(M_1)$  itself. The minimum in the region  $f_R \approx 10^7 \text{ s}^{-1}$  is caused by the stronger effect of spectral diffusion on the  $V_1$  amplitude compared with that on the  $V_1'$  amplitude (see Fig. 6a). As for the integrated intensities, going to lower modulation frequencies (i.e., 25 kHz) results in weaker dependences on  $T_{2,0}$ . This is true both for the amplitude ratio,  $\rho_1^0(M_1 = +1)$ , and for the fit parameters,  $a_1'(M_1 = +1)$  and  $b_1'(M_1 = +1)$  (compare Tables 3 and 4).

### Experimental Applications

$V_1'$ - and  $V_1$ -EPR spectra were measured for TEMPONE and 5-MSL in glycerol–water mixtures of different viscosities. The measurements were performed with modulation frequencies of 100 and 25 kHz. To extract effective spin–lattice relaxation times from the experimental values of the  $\rho_1'$  and  $\rho_1'(M_1)$  ratios, calibrations of the fitting parameters for the appropriate rotational correlation times were used (Tables 1–4). Additionally, spectral simulations were performed for fitting to the experimental dependences of  $\rho_1'$  and  $\rho_1'(M_1 = +1)$  ratios on the microwave field intensity,  $H_1$ . Rotational correlation times were determined from the conventional  $V_1$ -EPR spectra by using calibrations or equations given in Refs. (12–14). As noted above, the dependence of the  $\rho_1'$ ,  $\rho_1'(M_1 = +1)$ , and  $\rho_1'(M_1 = 0)$  ratios on  $\Delta H_0$  is relatively unimportant at a modulation frequency of 25 kHz. However, this dependence must be taken into account at  $\omega_m/2\pi = 100$  kHz to obtain sufficiently precise  $T_1$  estimates. Residual (i.e., intrinsic) linewidths ( $\Delta H_0 = 1/\gamma_e T_{2,0}$ ) were determined from lineshape simulations of the low-field maximum in the conventional  $V_1$ -EPR spectra. Inhomogeneous Gaussian broadening was included explicitly in these simulations.

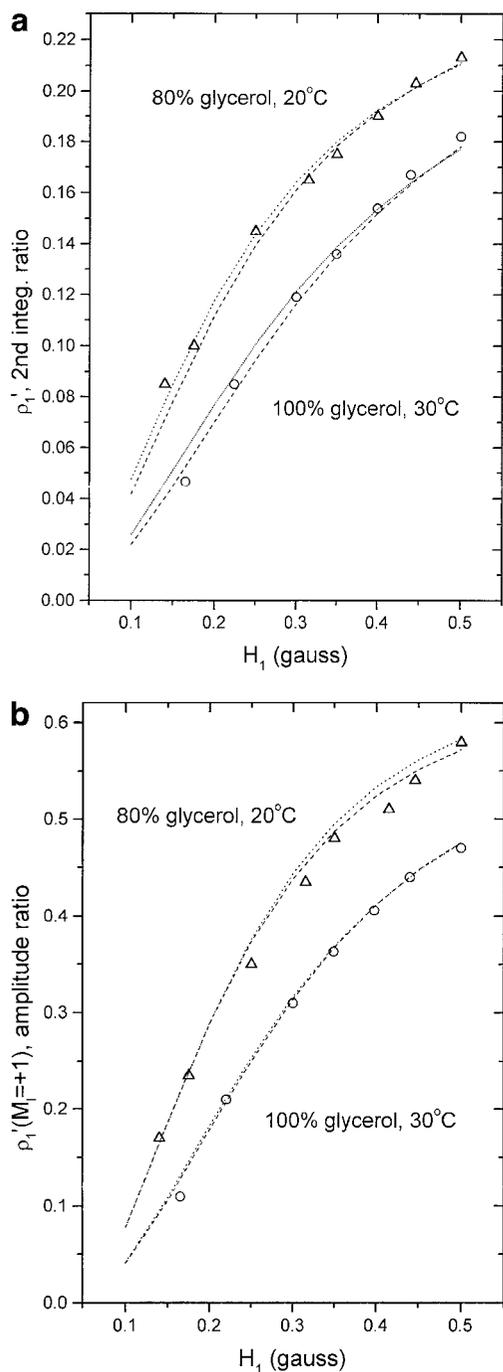
In the rapid motional regime, rotational correlation times,  $f_R^{-1}$ , were estimated from the ratio of low- and high-field lineheights, together with the central linewidth (14). The total uncertainty, allowing also for inhomogeneous broadening, is unlikely to exceed 20–30%. In the intermediate and slow motional regimes, the rotational correlation times were determined from the values of the outer hyperfine splitting, relative to rigid limit values from the literature (13). The error arising from uncertainties in these values is in the region of 20–30%. However, for small free spin labels in the slow and intermediate regimes, a much greater uncertainty arises from the motional model assumed for the calibrations. This can amount to an uncertainty of 200–300% (13). (Note that for spin-labeled macromolecules, the motion is Brownian diffusion and this uncertainty does not exist.) The residual homogeneous linewidths,  $\Delta H_0$ , were determined from lineshape simulations of the low-field spectral components in the conventional, low-power  $V_1$ -EPR spectra. Models appropriate to the different motional regimes were used in the simulations. Allowance was also made for inhomogeneous broadening. It is estimated that the error in  $\Delta H_0$  does not exceed 20%.

We have made error estimates for  $T_1$  arising from a 25% uncertainty in linewidth  $\Delta H_0$  and a 250% uncertainty in rotational frequency,  $f_R$ . This was done for the different motional regimes and for different ranges of  $T_1$ . The uncertainty in  $T_1$  from the linewidth measurements increases from 2 to 10% over the range  $T_1 = 0.3$ – $2.5$   $\mu\text{s}$ , with  $\omega_m/2\pi = 100$  kHz. For a modulation frequency of  $\omega_m/2\pi = 25$  kHz, this uncertainty is reduced to 1–4% over the extended range  $T_1 = 0.3$ – $6$   $\mu\text{s}$ . In both cases, this applies to rotational frequencies in the range  $f_R = 10^6$ – $10^9$   $\text{s}^{-1}$ . The uncertainty in  $f_R$  is maximum ( $\sim 250\%$ ) for values of  $f_R = 10^6$ – $10^7$   $\text{s}^{-1}$ . In this range, it results in a

maximum uncertainty in  $T_1$  of  $\sim 11\%$  for  $T_1 = 0.3$ – $3$   $\mu\text{s}$  with  $\omega_m/2\pi = 100$  kHz, and of  $\sim 10\%$  for  $T_1 = 0.3$ – $6$   $\mu\text{s}$  with  $\omega_m/2\pi = 25$  kHz. For higher rotational frequencies, the uncertainty in  $f_R$  is much lower and correspondingly the uncertainty in  $T_1$  is reduced. For  $f_R = 5 \times 10^7$ – $5 \times 10^9$   $\text{s}^{-1}$ , an uncertainty of 50% in  $f_R$  results in an uncertainty in  $T_1$  of  $\sim 2\%$  (for  $T_1 = 0.3$ – $3$   $\mu\text{s}$  with  $\omega_m/2\pi = 100$  kHz), or  $\sim 1\%$  (for  $T_1 = 0.3$ – $6$   $\mu\text{s}$  with  $\omega_m/2\pi = 25$  kHz).

The above error estimates were made for  $T_1$  determinations from the integrated intensity ratio,  $\rho_1'$ . For the amplitude ratios,  $\rho_1'(M_1)$ , the corresponding errors in  $T_1$  are of the same order of magnitude. However, unlike the integrated intensity ratios, the measured amplitude ratios also depend on the degree of Gaussian inhomogeneous broadening. Simulations using the present methods show that the effects of inhomogeneous broadening on the amplitude ratios are qualitatively similar to those for the no motion case (see Ref. 6). The accuracy of determining the inhomogeneous broadening for simulations is similar to that for the homogeneous broadening. This amounts to an uncertainty of 20–30%. For an uncertainty in inhomogeneous broadening of 25%, with  $\Delta H_0 = 1$  G, the uncertainty in  $T_1$  determination from the amplitude ratio is 3.5–7%, depending on the range of  $T_1$ . This applies to low and high rotational frequencies, i.e.,  $f_R = 10^6$  or  $10^9$ – $10^{10}$   $\text{s}^{-1}$ . At intermediate rotational frequencies ( $f_R = 10^8$ – $10^7$   $\text{s}^{-1}$ ), the above uncertainty in  $T_1$  decreases to 1–1.5%. This is because rotational relaxation broadening dominates in this regime. Correspondingly, the amplitude ratios are also less sensitive to inhomogeneous broadening at larger intrinsic homogeneous linewidths ( $\Delta H_0 > 1$  G).

*1. TEMPONE in glycerol–water mixtures.* The glycerol content and temperature were adjusted to obtain rotational correlation times corresponding to the slow ( $\tau_R \cong 33$  ns), intermediate ( $\tau_R \cong 10$  ns), and rapid ( $\tau_R \cong 0.5, 0.67,$  and  $0.9$  ns) motional regimes. The fit parameters presented in Tables 1–4 were obtained with the adiabatic approximation for the whole range of  $f_R$ . To check the accuracy of such an approach for the rapid motion region, additional simulations were performed that did not rely on this approximation. These further simulations used: (i) Eqs. [12]–[15] for the rotational contribution to the linewidth from time-dependent perturbation theory with the full spin Hamiltonian, or (ii) the same equations without the nuclear frequency-dependent terms. Experimental dependences of the  $\rho_1'$  and  $\rho_1'(M_1 = +1)$  ratios on  $H_1$  are given for TEMPONE in 100% glycerol at 30°C in Fig. 7a. Those for TEMPONE in 80% glycerol at 20°C are given in Fig. 7b. The simulated dependences of these parameters on  $H_1$ , calculated with method (i) and also with the adiabatic approximation, are given in the same figures. When values of  $\Delta H_0$  were determined consistently from simulations with the corresponding lineshape equations, the values obtained for  $T_1$  from the two approximations were closely similar (see Figs. 7a and 7b). The values determined for  $\Delta H_0$  from the two models are 0.75 and



**FIG. 7.** Experimental and simulated dependences on microwave field strength,  $H_1$ , of the ratio of (a) the integrated intensities,  $\rho'_1$ , and (b) the low-field amplitudes,  $\rho'_1(M_1 = +1)$ , of the first-harmonic out-of-phase and in-phase EPR spectra, for the TEMPONE radical in 100% glycerol at 30°C (O) and 80% glycerol at 20°C ( $\Delta$ ). The Zeeman modulation frequency is 100 kHz. Simulations were performed with the adiabatic (dotted lines) and second-order perturbation (dashed lines) models. The best-fit values of rotational frequency and spin-packet width to the linear  $V_1$ -EPR spectra for the two models are  $f_R = 1.1 \times 10^9 \text{ s}^{-1}$ ,  $\Delta H_0 = 0.95$  and  $0.75 \text{ G}$ , respectively, for 100% glycerol at 30°C, and  $f_R = 2.1 \times 10^9 \text{ s}^{-1}$ ,  $\Delta H_0 = 0.55$  and  $0.42 \text{ G}$ , respectively, for 80% glycerol at 20°C. The  $T_1$  values determined from the simulated  $H_1$  dependencies are (a)  $2.0$  and  $2.2 \times 10^{-6} \text{ s}$ , and (b)  $1.6$  and  $1.7 \times 10^{-6} \text{ s}$ , respectively, for 100% glycerol at 30°C, and (a)  $2.4$  and  $2.6 \times 10^{-6} \text{ s}$ , and (b)  $1.75$  and  $1.85 \times 10^{-6} \text{ s}$ , respectively, for 80% glycerol at 20°C.

0.95 G for TEMPONE in 100% glycerol, and 0.42 and 0.55 G for TEMPONE in 80% glycerol, respectively.

Satisfactory fits for the dependence of both  $\rho'_1$  and  $\rho'_1(M_1 = +1)$  on  $H_1$ , and agreement between the values deduced for  $T_1$ , are obtained from the two approximations. This result is of considerable importance. It means that the calibrations in Tables 1–4 can be used with confidence throughout the entire range of rotational frequencies. However, the absolute values of  $T_1$  determined from the amplitudes are somewhat lower than those obtained from the integrated intensities. The effect of inhomogeneous broadening is insignificant for TEMPONE because the inhomogeneous linewidth is very small for this nitroxide (0.215 G peak-to-peak) (15). The slight difference between the  $T_1$  values may be attributed to differences in  $T_1$  (and possibly in  $\Delta H_0$ ) for the three hyperfine components.

The spin relaxation parameters of the TEMPONE radical in different motional regimes were measured in 90% glycerol at various temperatures (20,  $-1$ , and  $-30^\circ\text{C}$ ). At lower temperatures, one predicts that the  $T_1$  values lie outside the region of optimum  $T_1$  sensitivity ( $T_1 \leq 4 \mu\text{s}$ ) for a modulation frequency of 100 kHz (see Fig. 2). Therefore, these measurements were performed with a modulation frequency of 25 kHz. The values of  $T_1$  for the intermediate and slow motional regimes were determined from the  $\rho'_1$  and  $\rho'_1(M_1 = +1)$  ratios measured at two values of  $H_1$ , 0.4 and 0.5 G, by using the calibrations in Tables 2 and 4. These values, together with the spin-packet widths ( $\Delta H_0$ ), are given in Table 5. The  $T_1$  values determined from the ratio of integrated intensities agree satisfactorily for the independent measurements with  $H_1 = 0.4$  and 0.5 G. Similar agreement is observed for the  $T_1$  values determined from the amplitude ratios,  $\rho'_1(M_1 = +1)$ . However, the latter  $T_1$  values are somewhat lower than the former ones, as noted above for TEMPONE in 100 and 80% glycerol solutions at 20°C.

Consistency is obtained between the values of  $T_1$  determined by fitting the  $H_1$  dependence for TEMPONE in 90% glycerol at 20°C, and those obtained from the calibrations given in Tables 1 and 2 for measurements with a different (i.e., 100 kHz) modulation frequency (see Table 5). At a modulation frequency of 25 kHz, the  $T_1$  values obtained from the adiabatic approximation and from simulation method (i) again both agree, as found already at a modulation frequency of 100 kHz. These results serve as further experimental validation of the simulation methods used. Also, the values of  $T_1$  obtained for TEMPONE in 80% glycerol at 20°C are very close to those obtained for small spin labels with similar rotational correlation times by saturation recovery methods (16). The value of  $T_1$  obtained for TEMPONE at a rotational correlation time of  $\tau_R = 10 \text{ ns}$  (see Table 5) is close to that estimated from the progressive saturation parameter and linewidth measurements for a similar small spin label in glycerol–water. For 4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl at  $\tau_R = 8.5 \pm 0.5 \text{ ns}$  we determined a value of  $T_1 = 4.8 \pm 0.6 \mu\text{s}$  by progressive saturation (10). More direct comparison with progressive sat-

TABLE 5

**Spin Relaxation Properties of TEMPONE in Glycerol–Water Mixtures of Different Viscosities Deduced from the Spectral Parameters of the Out-of-Phase and In-Phase First-Harmonic EPR Spectra at Modulation Frequencies of 100 and 25 kHz**

Glycerol (%)	$T$ (°C)	$\tau_R$ (ns)	$\Delta H_0$ (G)	$\omega_m/2\pi$ (kHz)	$H_1$ (G)	$\rho'_i$	$T_1$ ( $\mu$ s)	$\rho'_i(M_i)$	$T_1$ ( $\mu$ s)
80	20	0.48	0.55	100	0.15–	$f(H_1)^a$	2.4	$f(H_1)^a$	1.75
			0.42 <sup>b</sup>		0.5	2.6 <sup>b</sup>	1.85 <sup>b</sup>		
100	30	0.9	0.95	100	0.15–	$f(H_1)^a$	2.0	$f(H_1)^a$	1.6
			0.75 <sup>b</sup>		0.5	2.2 <sup>b</sup>	1.7 <sup>b</sup>		
90	20	0.67	0.9	100	0.5	0.195	2.85	0.62	2.35
			0.7 <sup>b</sup>		0.195	3.0 <sup>b</sup>	2.45 <sup>b</sup>		
90	20	0.67	0.9	25	0.15–	$f(H_1)^a$	3.0	$f(H_1)^a$	2.7
			0.7 <sup>b</sup>		0.5	3.1 <sup>b</sup>	2.8 <sup>b</sup>		
90	−1	10	1.8	25	0.4	0.148	5.4	0.262	4.8
					0.5	0.173	5.5	0.309	4.9
90	−30	33	2.0	25	0.4	0.17	6.5	0.278	5.7
					0.5	0.218	6.9	0.355	5.8

<sup>a</sup>  $T_1$  values are obtained from fitting the intensity ( $\rho'_i$ ) or amplitude ( $\rho'_i(M_i)$ ) ratios as a function of  $H_1$ , over the range given.

<sup>b</sup> Values determined from time-dependent perturbation theory. All other values are obtained by using the adiabatic approximation.

uration experiments for the fast and very slow motional regimes can be found in our previous paper (6).

Table 5 shows that it is sufficient to perform  $V'_1$ -EPR measurements at a single high microwave field intensity. Consistent values are obtained for  $T_1$  from single-point measurements at  $H_1 = 0.4$  G and  $H_1 = 0.5$  G. These values are also consistent with those obtained by fitting the complete  $H_1$  dependence. Routinely, therefore,  $V'_1$ -EPR measurements would be made at  $H_1 = 0.4$  or  $0.5$  G, and the calibrations of Tables 1–4 used.

2. *3-Maleimide proxyl in glycerol–water mixtures.* Similar  $T_1$  measurements were performed for the 5-MSL spin label in 90% glycerol–water mixtures, with a modulation frequency of 25 kHz. Again measurements were made in the rapid, intermediate, and slow motional regimes. For 5-MSL, these motional regimes correspond to higher temperatures than for TEMPONE (40, 20, and −2°C). The  $T_1$  values, as well as homogeneous linewidths and rotational correlation times, are given in Table 6. For this spin label the  $T_1$  values obtained from the integrated intensities agree satisfactorily with those determined from the amplitude ratios. The absolute values of

$T_1$  are found to be higher for MSL than for TEMPONE, at the same glycerol content and temperatures. The value of  $T_1$  at −2°C can be compared with the value of 6.6  $\mu$ s for this label bound to hemoglobin in a 90% glycerol–water mixture at 0°C (4).

## CONCLUSIONS

The advantages of the first-harmonic out-of-phase  $V'_1$ -EPR method that were previously established for CW  $T_1$  determinations are insensitivity to  $T_2$ , favorable signal strength, and ability to tune the range of sensitivity to  $T_1$  by varying the Zeeman modulation frequency (6). These results, however, were obtained only for spin labels in the very fast or very slow motional regimes and by theoretical simulations for single-line spectra. The present work extends the validity of these conclusions by simulations of the complete nitroxide spin-label  $V'_1$ -EPR spectrum and by investigating the full range of nitroxide spin-label rotational correlation times. An important further finding is that  $T_1$  relaxation time determinations by the  $V'_1$ -EPR method are less affected by rotational motion of the spin label

TABLE 6

**Spin Relaxation Properties of 3-Maleimide Proxyl Spin Label in Glycerol–Water Mixtures of Different Viscosities Deduced from the Spectral Parameters of the Out-of-Phase and In-Phase First-Harmonic EPR Spectra**

Glycerol (%)	$T$ (°C)	$\tau_R$ (ns)	$\Delta H_0$ (G)	$H_1$ (G)	$\rho'_i$	$T_1$ ( $\mu$ s)	$\rho'_i(M_i)$	$T_1$ ( $\mu$ s)
90	+40	1.1	1.6	0.4	0.068	2.5	0.195	2.4
			1.3 <sup>a</sup>			2.5 <sup>a</sup>		2.5 <sup>a</sup>
90	+20	10	2.0	0.4	0.1	3.7	0.202	3.7
				0.5	0.14	4.2	0.266	4.1
90	−2	33	2.1	0.4	0.15	5.5	0.283	5.6

Note. The modulation frequency is 25 kHz.

<sup>a</sup> Values determined from time-dependent perturbation theory. All other values are obtained by using the adiabatic approximation.

than are other nonlinear methods. This result reinforces the usefulness of  $V'_1$ -EPR as a "pure"  $T_1$ -sensitive display, as was suggested originally in Ref. (7). Particularly, this holds for Zeeman modulation frequencies in the region around 25 kHz that are appropriate for use with spin labels. Calibrations that are presented for the  $T_1$  dependence of the intensity and amplitude ratios for different correlation times considerably improve the precision of the method at modulation frequencies in the standard range around 100 kHz.

The results presented here should help to establish this new nonlinear CW EPR method on a firm basis, and to stimulate further applications. Its use in studying paramagnetic relaxation enhancement of spin-labeled fatty acids bound to bovine serum albumin will be reported elsewhere. One further extension that can be envisaged is to exploit the dependence of  $T_1$  sensitivity on modulation frequency to study heterogeneous systems with different  $T_1$  values that give unresolved or overlapped conventional EPR spectra.

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### REFERENCES

1. C. Altenbach, T. Marti, H. G. Khorana, and W. L. Hubbell, Transmembrane protein structure: Spin labelling of bacteriorhodopsin mutants, *Science* **248**, 1088–1092 (1990).
2. W. L. Hubbell, A. Gross, R. Landgren, and M. A. Lietzow, Recent advances in site-directed spin labeling of proteins, *Curr. Opin. Struct. Biol.* **8**, 649–656 (1998).
3. D. Marsh, Progressive saturation and saturation transfer ESR for measuring exchange processes of spin labelled lipids and proteins in membranes, *Chem. Soc. Rev.* **22**, 329–335 (1993).
4. D. D. Thomas, L. R. Dalton, and J. S. Hyde, Rotational diffusion studied by passage saturation transfer of electron paramagnetic resonance, *J. Chem. Phys.* **65**, 3006–3024 (1976).
5. T. Páli, V. A. Livshits, and D. Marsh, Dependence of saturation-transfer EPR intensities on spin-lattice relaxation, *J. Magn. Reson. B* **113**, 151–159 (1996).
6. V. A. Livshits, T. Páli, and D. Marsh, Spin relaxation measurements using first-harmonic out-of-phase EPR signals, *J. Magn. Reson.* **134**, 113–123 (1998).
7. J. S. Hyde and D. D. Thomas, New EPR methods for the study of very slow motion: Application to spin-labeled hemoglobin, *Ann. N.Y. Acad. Sci.* **222**, 680–692 (1973).
8. P. Fajer and D. Marsh, Microwave and modulation field inhomogeneities and the effect of cavity  $Q$  in saturation transfer ESR spectra. Dependence on sample size, *J. Magn. Reson.* **49**, 212–224 (1982).
9. M. A. Hemminga, P. A. de Jager, D. Marsh, and P. Fajer, Standard conditions for the measurement of saturation transfer ESR spectra, *J. Magn. Reson.* **59**, 160–163 (1984).
10. V. A. Livshits, T. Páli, and D. Marsh, Relaxation time determinations by progressive saturation EPR: Effects of molecular motion and Zeeman modulation for spin labels, *J. Magn. Reson.* **133**, 79–91 (1998).
11. R. C. McCalley, E. J. Shimshick, and H. M. McConnell, The effect of slow rotational motion on paramagnetic resonance spectra, *Chem. Phys. Lett.* **13**, 115–119 (1972).
12. J. Israelachvili, J. Sjosten, L. E. G. Eriksson, M. Ehrström, A. Gräslund, and A. Ehrenberg, ESR spectral analysis of the molecular motion of spin labels in lipid bilayers and membranes based on a model in terms of two angular motional parameters and rotational correlation times, *Biochim. Biophys. Acta* **382**, 125–141 (1975).
13. J. H. Freed, Theory of slow tumbling ESR spectra of nitroxides, in "Spin Labelling. Theory and Applications" (L. J. Berliner, Ed.), pp. 53–132, Academic Press, New York (1976).
14. S. Schreier, C. F. Polnaszek, and I. C. P. Smith, Spin labels in membranes. Problems in practice, *Biochim. Biophys. Acta* **515**, 375–436 (1978).
15. B. L. Bales, Inhomogeneously broadened spin-label spectra, in "Biological Magnetic Resonance" (L. J. Berliner and J. Reuben, Eds.), Vol. 8, Chap. 2, pp. 77–130, Plenum Press, New York/London (1989).
16. P. W. Percival and J. S. Hyde, Saturation-recovery measurements of spin-lattice relaxation times of some nitroxides in solution, *J. Magn. Reson.* **23**, 249–257 (1976).