

Frequency Dependence of Electron Spin Relaxation of Nitroxyl Radicals in Fluid Solution<sup>†</sup>

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Electron spin lattice relaxation rates ( $1/T_{1e}$ ) of nitroxyl radicals for tumbling correlation times between about 0.1 and 10 ns in water:glycerol or water:sorbitol mixtures at room temperature were measured by saturation recovery at X-band (9.2 GHz), S-band (3.1 GHz), and L-band (1.9 GHz) for natural abundance 2,2,6,6-tetramethylpiperidiny-1-oxy (tempol), tempol- $d_{17}$ , and  $^{15}\text{N}$ -tempol- $d_{17}$  and for a spin-labeled derivative of ethylenediaminetetraacetic acid. Tumbling correlation times were calculated from the continuous wave EPR line shapes at X-band. The dependence of  $T_{1e}$  on tumbling correlation time was modeled with contributions from modulation of nitrogen nuclear hyperfine and  $g$  anisotropy, from spin rotation and from one or more thermally activated processes. At these microwave frequencies, modulation of nitrogen hyperfine anisotropy makes a substantially larger contribution than modulation of  $g$  anisotropy. Spin rotation makes a very small contribution to  $T_{1e}$  at the tumbling correlation times examined in these studies. Replacement of  $^{14}\text{N}$  by  $^{15}\text{N}$  decreases the relaxation rates as predicted for samples in which modulation of nuclear hyperfine anisotropy contributes to relaxation. Deuteration of the solvent does not affect  $T_{1e}$ , which indicates that the electron–nuclear dipolar interaction with solvent nuclei does not make a significant contribution to  $T_{1e}$  of nitroxyl radicals in this motional regime. The contributions to relaxation from one or more thermally activated processes that occur at rates comparable to the microwave frequency dominate the relaxation at tumbling correlation times longer than about 2 ns at X-band and S-band. The contributions from the thermally activated process(es) and from modulation of nitrogen nuclear hyperfine anisotropy increase as the microwave frequency is decreased, and the contribution from hyperfine anisotropy dominates at L-band.

## Introduction

Electron spin–spin relaxation times ( $T_2$ ) of spin labels in fluid solution, especially as revealed by continuous wave (CW) electron paramagnetic resonance (EPR) line shapes, are useful monitors of motion and environment of the radicals.<sup>1,2</sup> As commonly applied in spin labeling of biomolecules, the interpretation of CW EPR line shapes of nitroxyl radicals has become very sophisticated.<sup>3</sup> Development of in vivo EPR and imaging<sup>4</sup> requires a firm basis for predicting the frequency dependence of EPR spectra and electron spin relaxation times, and this in turn requires understanding of the physical mechanisms of electron spin relaxation. Understanding of nitroxyl  $T_{1e}$  in solution is less developed than is the understanding of  $T_2$  in solution.<sup>5,6</sup>

Freed and co-workers studied spin lattice relaxation by CW saturation at X-band for peroxyamine disulfonate (PADS) in water and water:glycerol mixtures<sup>7</sup> and for perdeuterated tempol (2,2,6,6-tetramethylpiperidiny-1-oxy- $d_{17}$ ) in toluene- $d_8$  and in glycerol- $d_3$ : $\text{D}_2\text{O}$  mixtures.<sup>8</sup> For  $\tau_R < 10^{-11}$  s, the dependence of  $T_{1e}$  on viscosity was consistent with spin rotation (eq 1)<sup>9</sup>

$$\frac{1}{T_{1e}^{\text{SR}}} = \frac{\sum_{i=1}^3 (g_i - g_e)^2}{9\tau_R} \quad (1)$$

where  $\tau_R$  is the tumbling correlation time and  $g_e$  is the free electron  $g$  value, 2.0023. For  $\tau_R > 10^{-11}$  s,  $T_{1e}$  was shorter than predicted by eq 1 and the dependence  $\tau_R^{-0.25}$  or  $\tau_R^{-0.33}$  was observed for PADS<sup>7</sup> and perdeuterated tempol,<sup>8</sup> respectively. It was suggested that an additional process contributed to the relaxation at longer correlation times<sup>7</sup> and the possible role of internal molecular motions was suggested.<sup>8</sup> The values of  $T_{1e}$  for the three nitrogen hyperfine lines of tempol differed by less than about 20% for most of the conditions examined.<sup>8</sup>

Although early measurements of relaxation times were performed by CW saturation, direct measurements of  $T_{1e}$  by saturation recovery (SR) are preferred because spectral diffusion processes including nitrogen nuclear spin flips have less impact on SR than on CW saturation curves.<sup>10</sup> Percival and Hyde used saturation recovery at 8.62 GHz to study tempol, perdeuterated tempone (4-oxo-2,2,6,6-tetramethylpiperidiny-1-oxy- $d_{16}$ ), and  $^{15}\text{N}$ -tempone in degassed *sec*-butyl benzene between  $-78$  and  $+31$  °C.<sup>10</sup> Over the full temperature range studied, there was little difference in  $T_{1e}$  between perdeuterated tempone and  $^{15}\text{N}$ -tempone, which showed that the nitrogen quadrupole moment and the protons had little impact on spin lattice relaxation. Values of  $\tau_R$  were estimated using the Stokes–Einstein equation and the literature values for viscosity of *sec*-butyl benzene. The values of  $1/T_{1e}$  predicted based on spin rotation (eq 1) were smaller than the experimental values at all temperatures examined. The discrepancy ranged from about a factor of 3 at low viscosity to about a factor of 20 at high viscosity. Although some of the discrepancy at low viscosity (fast tumbling) might be due to the difference between microscopic and macroscopic viscosity, it was evident that spin rotation alone could not

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explain the relaxation rates. On the basis of the work of Wilson and Kivelson,<sup>11</sup> it was proposed<sup>10</sup> that modulation of  $g$  and  $A$  anisotropy by molecular tumbling would result in a contribution to  $1/T_{1e}$  as given in eq 2

$$\frac{1}{T_{1e}}(m_I) = (0.39 - 1.91m_I + 2.89m_I^2) \frac{\tau_R}{1 + \epsilon\omega^2\tau_R^2} \times 10^{16} \text{ s}^{-1} \quad (2)$$

where  $m_I$  refers to the nitrogen nuclear spin state ( $-1, 0$ , or  $1$ ),  $\omega$  is the electron Larmor frequency, and  $\epsilon$  is an adjustable parameter proposed by Freed and co-workers.<sup>8</sup>

The numerical coefficients in eq 2 were calculated for the  $g$  and  $A$  values of tempol. The strong dependence on nitrogen nuclear spin state predicted by eq 2 was not consistent with the experimental relaxation rates, which showed little dependence on  $m_I$ . Even for  $m_I = 0$  the predictions of eq 2 were not consistent with experiment. It was concluded that the mechanism of relaxation was not known, but the possible role of intramolecular motions including methyl rotation and inversion of the pyramidal nitroxyl nitrogen were mentioned.<sup>10</sup> In 1979, Hyde again concluded that the dominant relaxation mechanism for nitroxyl spin–lattice relaxation was unknown.<sup>12</sup>

In reviews of electron spin relaxation processes, Bertini et al. proposed,<sup>5,13</sup> based on the work of Kivelson,<sup>14</sup> that for  $S = 1/2$  species the contribution to  $1/T_{1e}$  due to modulation of  $g$  and  $A$  anisotropy is given by eq 3. The spectral density function for molecular tumbling multiplies terms that reflect the contributions from  $g$  anisotropy,  $A$  anisotropy, and a cross term involving  $g$  and  $A$  anisotropy. The contributions from terms that involve  $g$  anisotropy increase with increasing external magnetic field

$$\frac{1}{T_{1e}^{g,A}} = \frac{2}{5\hbar^2} \left[ \frac{\tau_R}{1 + \omega^2\tau_R^2} \right] \left\{ \left[ \frac{(\Delta g)^2}{3} + \delta g^2 \right] \mu_B^2 B^2 + (7I(I+1) - m_I^2) \left( \frac{\Delta A^2 + \delta A^2}{8} \right) - (\Delta g \Delta A + 3\delta g \delta A) \mu_B B m_I \right\} \quad (3)$$

where  $\omega$  is the resonant frequency,  $B$  is the external magnetic field,  $\tau_R$  is the molecular tumbling correlation time,  $\mu_B$  is the Bohr magneton,  $I$  and  $m_I$  are the nuclear spin quantum numbers,  $\Delta g = g_z - 1/2(g_x + g_y)$ ,  $\delta g = 1/2(g_x - g_y)$ ,  $\Delta A = A_z - 1/2(A_x + A_y)$ , and  $\delta A = 1/2(A_x - A_y)$ .

The cross term involving the product of  $g$  and  $A$  anisotropy in eq 3 is not present in the models discussed in the following paragraphs. Also, NMR models for nuclear spin relaxation do not include cross-terms in nuclear hyperfine interaction and chemical shift anisotropy,<sup>15</sup> so the inclusion of this cross-term requires careful scrutiny. This model does not appear to have been compared with experimental  $T_{1e}$  data for nitroxyl radicals.

In 1994, Robinson and co-workers proposed<sup>1</sup> that nitroxyl spin–lattice relaxation could be modeled as the sum of contributions from spin rotation (eq 1), modulation of electron nuclear dipolar interaction ( $A$  anisotropy) (eq 4), and nuclear spin diffusion (eq 5)

$$\frac{1}{T_{1e}^{\text{END}}} = \frac{1}{6} \sum_{i=1}^3 (A_i - \bar{A})^2 \frac{\tau_R}{1 + \omega^2\tau_R^2} \quad (4)$$

where  $A_i$  are the components of the nitrogen nuclear hyperfine matrix and other terms are defined as in previous equations.

Equation 4 is for  $^{15}\text{N}$  and the numerical coefficient includes a factor of  $I(I+1) = 0.75$

$$\frac{1}{T_{1e}^{\text{sd}}} = R_{1e,\text{max}}^{\text{sd}} \left[ \frac{2\omega_X\tau_d}{1 + (\omega\tau_d)^2} \right]^{1/4} \quad (5)$$

where  $\omega_X$  is the X-band microwave frequency,  $\omega$  is the frequency at which the experiment is performed, and  $\tau_d$  is the diffusion correlation time.<sup>16</sup>

The sum of eqs 1, 4, and 5 was used to model  $1/T_{1e}$  as a function of  $\tau_R$  for  $^{15}\text{N}$ -tempol in water and water:glycerol mixtures and in *sec*-butyl benzene as a function of temperature.<sup>1</sup> The nuclear spin diffusion term (eq 5), which reflects modulation of dipolar interaction with solvent protons, was added because the sum of contributions from spin rotation (eq 1) and modulation of hyperfine anisotropy (eq 4) was not large enough to match the experimental  $1/T_{1e}$  data. The model was in good agreement with the experimental data. In 1999, Robinson et al. developed their model further and concluded that  $T_{1e}$  would not depend on the nitrogen nuclear spin state.<sup>17</sup> For the case of isotropic tumbling, the general equations in ref 17 reduce to eq 4 for modulation of hyperfine anisotropy and to eq 6 for modulation of  $g$  anisotropy.<sup>18</sup> Equation 6 is the same as the term for modulation of  $g$  anisotropy in eq 3

$$\frac{1}{T_{1e}^g} = \frac{2}{5\hbar^2} \left[ \frac{\tau_R}{1 + \omega^2\tau_R^2} \right] \left[ \frac{(\Delta g^2)}{3} + \delta g^2 \right] \mu_B^2 B^2 \quad (6)$$

In 1999, Andreozzi et al. measured spin–lattice relaxation rates by longitudinally detected ESR (LODESR) for natural isotope abundance nitroxyl spin labels in glassy *o*-terphenyl, where the motion is anisotropic.<sup>19,20</sup> The relaxation rates were modeled in terms of contributions from spin rotation and modulation of  $g$  and  $A$  anisotropy by molecular tumbling. The relationship between relaxation rate and tumbling correlation time was used to calculate tumbling correlation times as a function of temperature. If the motion is assumed to be isotropic, their equation for the contribution to relaxation from  $g$  and  $A$  anisotropy, which is for  $I = 1$  and  $m_I = 0$ , reduces to eq 7

$$\frac{1}{T_{1e}^{g,A}} = \frac{2}{5\hbar^2} \left[ \frac{\tau_R}{1 + \omega^2\tau_R^2} \right] \left\{ \left[ \frac{(\Delta g^2)}{3} + \delta g^2 \right] \mu_B^2 B^2 + \frac{20}{9} \left( \frac{\Delta A^2}{3} + \delta A^2 \right) \right\} \quad (7)$$

where the parameters are defined as in eq 3.

The contribution from modulation of  $g$  anisotropy in eq 7 is the same as in eq 3, but the coefficients for the contribution from modulation of hyperfine anisotropy in eq 7 are different from the coefficients in eq 3.

Thus, there is only partial agreement among published equations for contributions to  $1/T_{1e}$  and few checks of these equations against experimental results over a sufficiently wide range of physical parameters to test validity of models. The contributions to spin lattice relaxation due to modulation of  $g$  and  $A$  anisotropy by molecular tumbling predict a dependence of spin lattice relaxation on microwave frequency. However, most measurements of nitroxyl relaxation times have been performed at X-band. Reported X-band values of  $T_{1e}$  for nitroxyl spin labels at room temperature are in the range of  $0.3\text{--}8 \mu\text{s}$ .<sup>6</sup> In 1990, Hyde and co-workers<sup>21</sup> reported that the spin lattice relaxation time of a nitroxyl radical was  $2.54 \mu\text{s}$  at X-band (ca. 9 GHz) and  $0.94 \mu\text{s}$  at S-band (2–4 GHz). This frequency

dependence was described as “notable and unexpected.” Hyde and co-workers have now reported nitroxyl  $T_{1e}$  values that increase monotonically from 2 to 35 GHz.<sup>22</sup> We assigned the temperature dependence of spin lattice relaxation rates for tempol doped in a crystalline solid lattice between 20 and 293 K at X- and S-band to contributions from a Raman process and a thermally activated process, which was described by eq 8<sup>23,24</sup>

$$\frac{1}{T_{1e}^{\text{therm}}} = C_{\text{therm}} \frac{\tau_{\text{therm}}}{1 + \omega^2 \tau_{\text{therm}}^2} \quad (8)$$

where  $\tau_{\text{therm}}$  is the correlation time for the dynamic process =  $\tau_c^0 \exp(E_a/RT)$ ,  $E_a$  is the activation energy, and  $\tau_c^0$  is the preexponential factor.  $C_{\text{therm}}$  (with units of  $\text{s}^{-2}$ ) is the coefficient for the contribution from the thermally activated process, which is proportional to the magnitude of the inequivalence that is averaged by the process. To account for the observed isotope effects,  $C_{\text{therm}}(\text{H})$  and  $C_{\text{therm}}(\text{D})$  were defined for the natural isotope abundance and deuterated spin labels, respectively.

Analogous to the modulation of  $g$  and  $A$  anisotropy by molecular tumbling, the contribution from the thermally activated process is described in terms of a spectral density function and has its maximum impact when the correlation time for the dynamic process is  $\sim 1/\omega$ . The activation energy for the thermally activated process (1100 K) for tempol in the solid host is in good agreement with the activation energy for rotation of the nitroxyl ring methyl groups (1100 K) obtained by ENDOR for tempone in a solid matrix<sup>25</sup> so the thermally activated process was tentatively assigned as methyl group rotation.<sup>24</sup>

Saturation recovery studies of  $T_{1e}$  for semiquinones as a function of solvent, temperature and viscosity<sup>26–29</sup> are consistent with

$$\frac{1}{T_{1e}} = \frac{A\tau}{\eta} + B \exp\left(-\frac{\Delta E}{RT}\right) \quad (9)$$

where  $A$  and  $B$  are adjustable parameters,  $\eta$  is solvent viscosity and  $\Delta E$  is an activation energy of the order of 1 kcal/mol (500 K).

When tumbling is isotropic the first term in eq 9 is equivalent to the spin rotation contribution to spin lattice relaxation expressed in eq 1.<sup>29</sup> The second term in eq 9 has the form of  $B/\tau$  where  $\tau = \exp(\Delta E/RT)$ , which is equivalent to the general expression for a thermally activated process (eq 8) at constant microwave frequency in the limit where  $\omega^2 \tau^2 \gg 1$ , which is the regime in which many of the semiquinone studies were performed. In the semiquinone studies, the thermally activated process was attributed to hindered rotation within a solvent cage<sup>26,27,28</sup> or to an additional spin-rotation term arising from anisotropic motion.<sup>29</sup> The X-band  $T_{1e}$  values at room temperature for 2,5-di-*tert*-butyl-1,4-benzosemiquinone in ethanol or *n*-butanol were independent of concentration in the range of 1 to 15 or 20 mM.<sup>27</sup> Similarly, X-band  $T_{1e}$  values for tetra-chloro-1,4-benzosemiquinone and tetra-bromo-1,4-benzosemiquinone in ethanol at room temperature were independent of concentration over the range examined (0.1–1.8 mM).<sup>29</sup> The absence of concentration dependence of  $T_{1e}$  in these hydrogen-bonded solvents was attributed to solvent cages around the radicals, which decrease dipolar interactions.<sup>27</sup>

In this study, we seek to more clearly define the processes that contribute to nitroxyl spin lattice relaxation in solution over the range of tumbling correlation times that are likely to be observed for spin-labeled biomolecules. Relaxation times were measured at three microwave frequencies: X-band (9.2 GHz),

S-band (3.1 GHz), and L-band (1.9 GHz). In some prior studies, tumbling correlation times were varied by changing temperature. However, if a thermally activated process contributes to relaxation, its contribution will vary with temperature so in this study tumbling correlation time was varied by changing solvent composition at constant temperature. Water:glycerol and water:sorbitol mixtures were used to maintain the hydrogen bonding character of the solvent while varying viscosity. Tempol was chosen for study because it exhibits slower motion for a given water:glycerol mixture than, for example, tempone. The slower motion of tempol is attributed to hydrogen bonding with the hydroxylic solvents. To extend the motional range accessible in water:glycerol mixtures, the larger spin-labeled ethylenediamine tetraacetic acid (EDTA-SL)<sup>30</sup> molecule was studied. Both tempol and EDTA-SL contain six-membered ring nitroxyls. The dependence of  $1/T_{1e}$  on tumbling correlation time was then compared with that predicted by the models in the literature, including the effects of spin rotation, modulation of  $g$  and  $A$  anisotropy, and thermally activated processes.

## Experimental Methods

Tempol (Aldrich Chemical Co. Milwaukee, WI), perdeuterated tempol- $d_{17}$  (CDN Isotopes, Quebec, Canada), and perdeuterated  $^{15}\text{N}$ -tempol- $d_{17}$  (CDN Isotopes, Quebec, Canada) were used as received. EDTA-SL was synthesized by Dr. Kundalika More in our laboratory.<sup>30</sup> Glycerol was Aldrich anhydrous grade and sorbitol was Aldrich reagent grade. Water:glycerol mixtures ranging from 60% glycerol to 95% glycerol, by volume, were prepared gravimetrically based on a density of 1.26 g/mL for glycerol. Sorbitol:water mixtures ranging from 70 to 81%<sup>31</sup> were prepared gravimetrically. The nitroxyl tumbling correlation times in sorbitol:water mixtures were found to be strongly dependent on sample history. To obtain reproducible preparations, the sorbitol:water mixtures were aged overnight at  $-20^\circ\text{C}$  then heated to  $50^\circ\text{C}$  to facilitate transfer to the EPR tubes. At X-band, samples were 0.5 to 1.0 mM and were contained in thin-wall Teflon tubing with 1.15 mm i.d. At S-band, nitroxyl concentrations were 1.0 to 2.2 mM and samples were contained in 1.35 mm i.d. tubing. The Teflon tubing was supported in a 4 mm o.d. quartz tube. To remove oxygen from the solutions, nitrogen gas was passed over the samples via a thin Teflon tube positioned in the quartz tube alongside the sample-containing Teflon tube. At L-band, nitroxyl concentrations were 1.0–2.0 mM; samples were contained in 4 mm i.d. quartz tubes and were degassed by freeze-pump thaw. Systematic checks for concentration dependence of  $T_{1e}$  were performed for solutions of  $^{15}\text{N}$ -tempol- $d_{17}$  with tumbling correlation times of 0.22, 1.4, and 4 ns. There was no detectable difference between CW line shapes or values of  $T_{1e}$  in 0.5 and 1.0 mM solutions at X-band, or between 1.0 and 2.0 mM solutions at S-band or L-band. The absence of concentration dependence of  $T_{1e}$  in these hydrogen-bonded water:glycerol or water:sorbitol solutions in these concentration ranges are consistent with results obtained for semiquinone radicals.<sup>27,29</sup>

The X-band SR spectrometer<sup>32</sup> used a loop-gap resonator for 4 mm o.d. tubes as described in ref 33. The S-band spectrometer was described in ref 34 and used a crossed loop resonator<sup>35</sup> to minimize the dead time between the end of the saturating pulse and the collection of the first data point. The L-band spectrometer was described in ref 36 and used a crossed loop resonator.<sup>37</sup> The length of the saturating pulse was increased until it did not affect the recovery time constant. The observe power that was used to detect the signal was decreased until it did not affect the recovery time constant. Within the noise levels of the



experimental saturation recovery curves, the data could be fit with single exponentials, and the time constant was identified as  $T_{1e}$ . The uncertainties in values of  $1/T_{1e}$  are about 10%.

For each of the solutions that were prepared, the nitroxyl tumbling correlation time was determined from the X-band CW line shape. Values of  $\tau_R$  in the range of 0.1–3.5 ns were calculated from the ratios of peak heights using the expressions of Wilson and Kivelson,<sup>11,14</sup> as presented by Chasteen and Hanna.<sup>38</sup> The  $g$  and  $A$  values were determined from glassy solution spectra at  $-160^\circ\text{C}$  that were simulated using locally written software ( $g_x = 2.0103$ ,  $g_y = 2.0067$ ,  $g_z = 2.0032$ ,  $A_x = 18$ ,  $A_y = 22.5$ , and  $A_z = 103$  MHz for  $^{14}\text{N}$ -tempol in water: glycerol mixtures and  $g_x = 2.010$ ,  $g_y = 2.0080$ ,  $g_z = 2.0031$ ,  $A_x = 20.4$ ,  $A_y = 21.6$ , and  $A_z = 106$  MHz for  $^{14}\text{N}$ -tempol in water:sorbitol). Values of  $\tau_R$  less than about 2 ns could be calculated from the peak height ratios with relatively small uncertainty, but there may be systematic errors due to the limitations of the line shape models and the assumption of isotropic tumbling. Tumbling correlation times in the range of 2.5–20 ns were determined by simulation of the X-band spectra using the NLSL program of Budil et al.,<sup>39</sup> which is based on expressions derived by Freed and co-workers.<sup>2</sup> Simulations were based on isotropic motion and were not improved by introducing anisotropy in the motion. To compare the values of  $\tau_R$  calculated by the two methods, NLSL simulations also were used to analyze a sampling of the CW spectra with  $\tau_R$  less than 3 ns. Good agreement between the two methods was obtained for  $\tau_R$  less than about 2.5 ns for  $^{15}\text{N}$ -tempol- $d_{17}$  and less than about 1.5 ns for tempol- $d_{17}$ . For plotting and analyzing the spin lattice relaxation data, the values of  $\tau_R$  less than 2 ns were calculated from the peak height ratios. Between  $\tau_R$  about 2.5 (for  $^{15}\text{N}$ -tempol) or 1.5 (for tempol- $d_{17}$  or tempol) and 4 ns, there were systematic differences between values of  $\tau_R$  calculated using the NLSL program and values obtained from peak height ratios. Based on these differences, correction factors were calculated to convert values of  $\tau_R$  obtained with NLSL to be comparable to values obtained from peak heights. To make all of the values of  $\tau_R$  comparable, these corrections also were applied at longer values of  $\tau_R$ , which could be estimated only with NLSL. The correction ranged from about 1 ns at  $\tau_R = 3$  ns to about 3 ns at  $\tau_R = 18$  ns. However, because of the weak dependence of  $1/T_{1e}$  on  $\tau_R$  for  $\tau_R > 5$  ns, these corrections did not have a large impact on the conclusions.

The experimental values of  $1/T_{1e}$  as a function of viscosity and microwave frequency were modeled as the sum of the contributions from spin rotation, modulation of nitroxyl  $g$  anisotropy and nitrogen nuclear  $A$  anisotropy by molecular tumbling, and a thermally activated process

$$\frac{1}{T_{1e}} = \frac{1}{T_{1e}^{\text{SR}}} + \frac{1}{T_{1e}^{g,A}} + \frac{1}{T_{1e}^{\text{therm}}} \quad (10)$$

The contribution from spin rotation was calculated using eq 1 and the experimental  $g$  values for tempol in 90% glycerol at  $-160^\circ\text{C}$ . Each of the prior studies of nitroxyl spin lattice relaxation used this standard expression for the spin rotation contribution.<sup>1,5,6,9</sup> The expressions for the contribution from modulation of  $g$  anisotropy are the same in ref 5 and 19 (i.e., in eqs 3, 6, and 7). Because of the modest  $g$  anisotropy for nitroxyl radicals, this process makes a very small contribution to spin lattice relaxation at X-band and lower frequencies. It was included in this study for completeness and calculated using the experimental parameters for tempol in 90% glycerol. Prior studies differ in the expressions that were used to calculate the

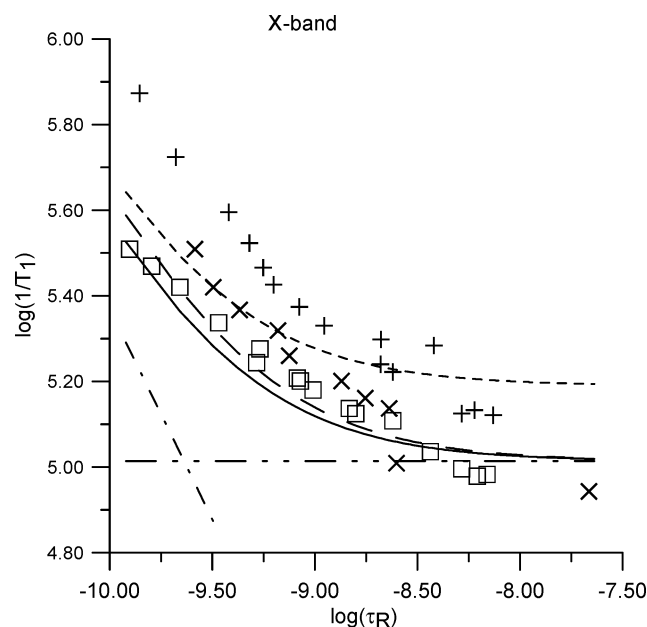
contribution to spin lattice relaxation from modulation of nitrogen hyperfine interaction. Therefore, the expressions in eqs 3 and 4 plus 6 or 7 were compared with the experimental data. Since the thermally activated process has not been assigned, the coefficients  $C_{\text{therm}}(\text{H})$  and  $C_{\text{therm}}(\text{D})$  were treated as adjustable parameters. The values of  $E_a$  and  $\tau_c^0$  were fixed at the values observed for tempol doped into solid 4-hydroxy-piperidine. Because of the wide range of values of tumbling correlation times, log–log plots of relaxation rates vs  $\tau_R$  were used to compare calculated and experimental rates.

## Results and Discussion

Robinson and co-workers<sup>1</sup> showed by electron–electron double resonance and SR that for tumbling correlation times of the order of  $10^{-8}$  s the nitroxyl  $^{15}\text{N}$  nuclear spin relaxation rate is substantially faster than the electron spin relaxation rate,  $1/T_{1e}$ , and that if a short pump time is used in saturation recovery experiments, the nuclear spin relaxation contributes to the recovery curve. For nitroxyl radicals Hyde and co-workers showed that if the pump time is long enough to equilibrate the populations of the nitrogen nuclear spin levels (long-pump saturation recovery), then the observed recovery time constant is  $T_{1e}$ .<sup>40</sup> The nitroxyl  $T_{1e}$  values reported here were measured by long-pump continuous wave saturation recovery.

Figures 1–3 display the electron spin relaxation rates ( $1/T_{1e}$ ) measured at  $20 \pm 1^\circ\text{C}$  at 9.22, 3.1, and 1.9 GHz as a function of tumbling correlation time for natural abundance tempol or EDTA-SL, tempol- $d_{17}$ , and  $^{15}\text{N}$ -tempol- $d_{17}$ . The overall pattern is slower relaxation rates at longer tumbling correlation times and weaker dependence on  $\tau_R$  for  $\tau_R > \sim 3$  ns than for shorter values of  $\tau_R$ , consistent with prior studies.<sup>1,6</sup> There are several features of the data that provide clues to the relaxation processes. (i)  $1/T_{1e}$  increases in the order X-band < S-band < L-band for all tumbling correlation times examined, which indicates that relaxation is dominated by processes that are described by spectral density functions. The ratio of relaxation rates at S- and X-bands is similar to that reported by Hyde and co-workers for doxyl spin labels,<sup>21</sup> which contain five-membered rings, showing that the observed frequency dependence of  $1/T_{1e}$  has some generality for biophysical studies. (ii)  $\text{D}_2\text{O}$ :glycerol- $d_8$  mixtures were used instead of natural isotope-abundance solvent for X-band measurements at  $\tau_R = 0.2$ , 2.0, and 5.2 ns and at S-band for  $\tau_R = 2.0$  and 5.2 ns. Within experimental uncertainty, the values of  $T_{1e}$  were unchanged by solvent deuteration, which indicates that the nuclear spin diffusion contribution to electron spin relaxation proposed in ref 1 is not significant in the tumbling correlation regime studied here, so the contribution described by eq 5 was not included in the simulations. (iii) Deuteration of the nitroxyl caused a decrease in  $1/T_{1e}$  at each of the frequencies studied (Figure 1–3), which indicates that protons play a significant role in spin lattice relaxation in this motional regime. (iv) Replacement of  $^{14}\text{N}$  by  $^{15}\text{N}$  in the deuterated nitroxyl caused a further decrease in  $1/T_{1e}$  consistent with a contribution from modulation of nitrogen nuclear hyperfine anisotropy. (v) For all of the samples studied, the relaxation rates for the three  $^{14}\text{N}$  (or two  $^{15}\text{N}$ ) nitrogen hyperfine lines were the same, within experimental uncertainty, which is consistent with ref 17 and rules out the model described by eq 2.

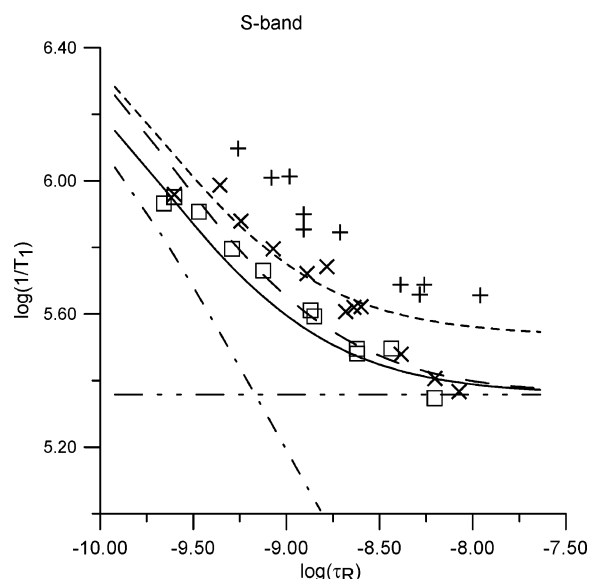
**Relaxation Processes.** As in our prior studies of spin lattice relaxation in solids,<sup>6,41,42</sup> we seek the minimal set of processes required to produce a reasonable simulation of the data at the three microwave frequencies. Some processes can be eliminated as unlikely, based on prior results.<sup>6</sup> For example, there is no



**Figure 1.** Dependence of nitroxyl electron spin relaxation rates ( $1/T_1$ ) at  $20 \pm 1$  °C on tumbling correlation time at X-band. For  $\tau_R < 2$  ns ( $\log > -8.7$ ), the nitroxyl is tempol in water:glycerol mixtures. For  $\tau > 2$  ns, the nitroxyl is EDTA-SL in water:glycerol mixtures or tempol in water:sorbitol mixtures. Experimental data are (+) natural isotope abundance tempol, (x) tempol- $d_{17}$ , (□)  $^{15}\text{N}$ -tempol- $d_{17}$ . Relaxation rates including spin rotation (eq 1), modulation of  $g$  and  $A$  anisotropy (eq 4), and a thermally activated process (eq 8) with parameters as given in the text were calculated for (—) natural isotope abundance tempol, (---) tempol- $d_{17}$ , and (- - -)  $^{15}\text{N}$ -tempol- $d_{17}$ . For  $^{15}\text{N}$ -tempol- $d_{17}$  the contributions from modulation of nitrogen hyperfine anisotropy (-·-) and the thermally activated process (-·-·-) are shown.

need to include a direct process, nor is there any evidence for low-lying excited states that would suggest invoking an Orbach process. Analysis of  $1/T_{1e}$  for tempol doped into the solid diamagnetic analogue at room temperature indicated that a thermally activated process made a much larger contribution than the Raman process at X- and S-band,<sup>24</sup> so the Raman process was not included in the analysis of the fluid solution data.

Predicted values of  $1/T_{1e}$  as a function of  $\tau_R$  were calculated initially for all of the data sets including contributions from spin rotation (eq 1) and the modulation of  $g$  and  $A$  anisotropy as described by either eq 3, 4 + 6, or 7. At L-band for  $\tau_R < \sim 1$  ns, the predicted relaxation rates that were calculated using eq 3 to describe the modulation of  $g$  and  $A$  anisotropy (including the cross term) were significantly faster than the experimental values, so this model was not pursued further. The small difference in coefficients between eq 4 and 7 had little impact on the calculated value of  $1/T_{1e}$ , so eq 4 was used in subsequent calculations. Calculations based on spin rotation (eq 1), modulation of  $A$  anisotropy by molecular tumbling (eq 4), and modulation of  $g$  anisotropy (eq 6) predicted much stronger dependence of  $1/T_{1e}$  on  $\tau_R$  than was observed experimentally and theoretically predicted relaxation rates were slower than the experimental rates for most values of  $\tau_R$ . These observations indicated that one or more additional processes are required to match the experimental data. The dependence of the relaxation rates on microwave frequency implies that the additional process or processes are described by a spectral density function. By analogy with the analysis of the variable temperature data for tempol doped into a solid host,<sup>24</sup> a contribution from a thermally activated process (eq 8) was added. The activation energy ( $E_a$ ) and  $\tau_c^0$  for this process were fixed at the values observed in the

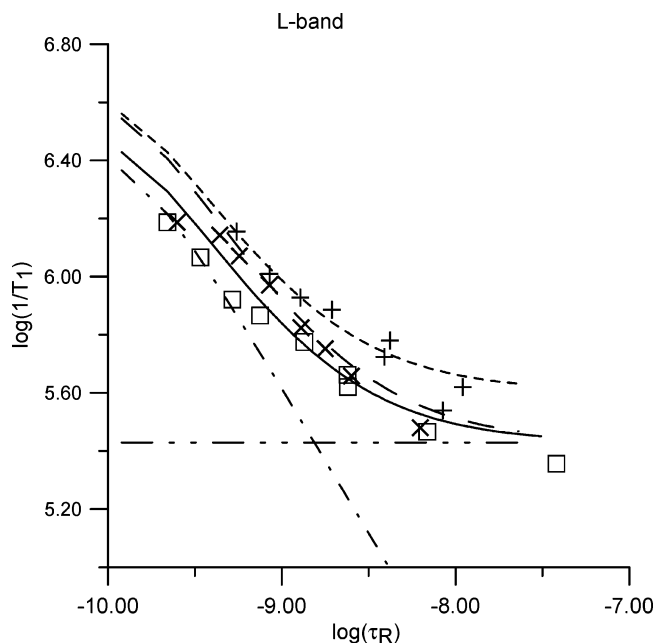


**Figure 2.** Dependence of nitroxyl electron spin relaxation rates ( $1/T_1$ ) at  $20 \pm 1$  °C on tumbling correlation time at S-band. For  $\tau < 2$  ns, the nitroxyl is tempol in water:glycerol mixtures. For  $\tau > 2$  ns, the nitroxyl is EDTA-SL in water:glycerol mixtures or tempol in water:sorbitol mixtures. Experimental data are (+) natural isotope abundance tempol, (x) tempol- $d_{17}$ , (□)  $^{15}\text{N}$ -tempol- $d_{17}$ . Relaxation rates including spin rotation (eq 1), modulation of  $g$  and  $A$  anisotropy (eq 4), and a thermally activated process (eq 8) with parameters as given in the text were calculated for (—) natural isotope abundance tempol, (---) tempol- $d_{17}$ , and (- - -)  $^{15}\text{N}$ -tempol- $d_{17}$ . For  $^{15}\text{N}$ -tempol- $d_{17}$  the contributions from modulation of nitrogen hyperfine anisotropy (-·-) and the thermally activated process (-·-·-) are shown.

doped solid ( $E_a = 1100$  K and  $\tau_c^0 = 2.5 \times 10^{-12}$  s). The assumption was made that  $E_a$  was independent of  $\tau_R$ , which is reasonable as a first approximation for intramolecular processes. Further experiments will be required to determine whether there is a weak dependence of  $E_a$  on  $\tau_R$ .

The fit lines calculated including contributions from spin rotation (eq 1), modulation of  $g$  and  $A$  anisotropy (eq 4 and 6), and a thermal process (eq 8) for the three isotope combinations are shown in Figures 1–3 for data at X-band, S-band, and L-band, respectively. The calculated contribution for  $^{15}\text{N}$ -tempol- $d_{17}$  from the dominant processes, modulation of  $A$  anisotropy and the thermally activated process, also are shown in the figures. The small calculated isotope effect for  $^{14}\text{N}$  vs  $^{15}\text{N}$  is consistent with the experimental data. The effect of the larger magnetic moment for  $^{15}\text{N}$  than for  $^{14}\text{N}$  is largely offset by the decrease in  $I(I + 1)$ . Modulation of  $A$  anisotropy makes a substantial contribution to  $T_{1e}$  at L-band and S-band, particularly at the shorter tumbling correlation times. The contribution from modulation of  $A$  anisotropy increases in the order X-band < S-band < L-band because as  $\omega$  decreases the denominator of eq 4 decreases. The contribution from spin rotation is very small at these frequencies for the motional regime examined. Extrapolation of the fit lines indicates that spin rotation makes a larger contribution at shorter tumbling correlation times than were included in the present study.

At X-band and S-band the agreement between calculation and experiment is better for tempol- $d_{17}$  and  $^{15}\text{N}$ -tempol- $d_{17}$  than for the natural isotope abundance tempol. The discrepancy is particularly large at small values of  $\tau_R$  where the calculated relaxation rate is dominated by modulation of nitrogen hyperfine interaction, for which there is no predicted proton/deuteron isotope effect. The discrepancy suggests that an additional contribution involving proton nuclear spins may be required to fit the data.



**Figure 3.** Dependence of L-band nitroxyl electron spin relaxation rates ( $1/T_1$ ) at  $20 \pm 1$  °C on tumbling correlation time. For  $\tau < 2$  ns, the nitroxyl is tempol in water:glycerol mixtures. For  $\tau > 2$  ns, the nitroxyl is EDTA-SL in water:glycerol mixtures or tempol in water:sorbitol mixtures. Experimental data are (+) natural isotope abundance tempol, (x) tempol- $d_{17}$ , (□)  $^{15}\text{N}$ -tempol- $d_{17}$ . Relaxation rates including spin rotation (eq 1), modulation of  $g$  and  $A$  anisotropy (eq 4), and a thermally activated process (eq 8) with parameters as given in the text were calculated for (---) natural isotope abundance tempol, (- - -) tempol- $d_{17}$ , and (—)  $^{15}\text{N}$ -tempol- $d_{17}$ . For  $^{15}\text{N}$ -tempol- $d_{17}$  the contributions from modulation of nitrogen hyperfine anisotropy (---) and the thermally activated process (---) are shown.

At both X- and S-band for  $\tau_R > \sim 3$  ns ( $\log \tau_R \sim -8.5$ ), the weak dependence of relaxation rate on tumbling correlation time and the dependence on  $\omega$  indicate that a thermally activated process or processes dominates the spin lattice relaxation. The curves shown in Figures 1–3 were calculated with  $\tau_{\text{therm}} = 1.1 \times 10^{-10}$  s. The contribution to the spin lattice relaxation from a dynamic process is largest when  $\tau_{\text{therm}} \sim 1/\omega$ . For  $\tau_{\text{therm}} = 1.1 \times 10^{-10}$  s, this condition is closer to being fulfilled at L-band ( $1/\omega = 8.4 \times 10^{-11}$  s) or S-band ( $1/\omega = 5.9 \times 10^{-11}$  s) than at X-band ( $1/\omega = 1.7 \times 10^{-11}$  s), which makes the contribution to  $1/T_{1e}$  from the dynamic process larger at the lower frequencies than at X-band. The fits to the experimental data were obtained with  $C_{\text{therm}}(\text{H}) = 2.8 \times 10^{16} \text{ s}^{-2}$  and  $C_{\text{therm}}(\text{D}) = 1.9 \times 10^{16} \text{ s}^{-2}$  at X-band. The ratio of these two values (1.5) reflects the proton/deuteron isotope effect on the relaxation. When the values of  $C_{\text{therm}}$  were assumed to be independent of microwave frequency, the calculated relaxation rates at frequencies lower than X-band were too fast. To fit the experimental data, a linear increase of  $C_{\text{therm}}$  with increasing microwave frequency was used. This dependence of the coefficient of the contribution from the thermally activated process on microwave frequency is similar to what was observed in our previous studies of the frequency dependence of the contribution to  $1/T_{1e}$  from methyl group rotation in radicals in irradiated organic solids.<sup>42,43</sup>

On going from S-band to L-band, in the motional regime studied, the calculated contribution from modulation of  $A$  anisotropy increases more than the contribution from the thermally activated process, so the thermally activated process is a smaller fraction of the rate at L-band than at S-band.

**Proposed Contributions to the Thermally Activated Process(es).** A model for the thermally activated process or

processes will need to take into account the following experimental observations. (i) Deuteration of the solvent has no impact on  $T_{1e}$  so fluctuating dipolar interactions with solvent nuclei is not a significant contribution. (ii) Deuteration of protons in the spin label decreases  $1/T_{1e}$  by about a factor of 1.5, which is much smaller than the ratio of dipole moments, and suggests that fluctuation of intramolecular dipolar interactions is not the sole contribution to the mechanism. However, 1.5 is approximately the calculated isotope effect for a C–H(D) vibration, which may be the fortuitous result of the sum of multiple contributions or may indicate that molecular vibrations coupled to the thermally activated motion contribute to the relaxation. (iii) To fit the experimental data it was necessary to make  $C_{\text{therm}}$  dependent on microwave frequency, which implies that the contribution from the thermally activated process is less frequency dependent than predicted by the spectral density function in eq 8.

Thermally activated processes that average inequivalent environments for nuclei that are coupled to the unpaired electron can absorb energy from the electron spin system when the frequency of the process is comparable to the electron Larmor frequency.<sup>6,44–51</sup> Examples of processes that can have these effects include methyl group rotation, amino group rotation, motion of a hydrogen-bonded proton, and ring motions that interconvert axial and equatorial methyls or ring protons. Dramatic effects on  $T_{1e}$  of methyl rotation at a rate comparable to the electron Larmor frequency have been observed for methyl-containing radicals in irradiated solids.<sup>42,43</sup> The  $E_a$  for rotation of the nitroxyl ring methyl groups was found to be in the range of 1030 to 1150 K based on spin-echo dephasing data in glassy solutions<sup>52–54</sup> and about 1100 K (9 kJ/mol) based on ENDOR data for tempone in a solid host.<sup>25</sup> Analysis of the temperature dependence of  $1/T_{1e}$  at S- and X-bands for tempol doped into its diamagnetic analogue found  $E_a = 1100$  K for the thermally activated process that contributed to the relaxation.<sup>24</sup> The thermally activated process was tentatively assigned to methyl rotation. In the present study, the deuterium isotope effect on the nitroxyl spin lattice relaxation rates is about a factor of 1.5, which is much smaller than predicted for dipolar interaction between a proton or deuteron and the unpaired electron. It therefore appears that modulation of electron–nuclear dipolar coupling via methyl rotation cannot be the sole contributor to the thermally activated process.

Although much of the discussion in the EPR literature of the effects of dynamic processes focuses on averaging of dipolar interactions, it should also be noted that averaging of inequivalent isotropic couplings also causes fluctuations in the energy of the electron spin that could effect spin lattice relaxation. These processes are analogous to what is called “scalar” relaxation in NMR.<sup>15</sup> Isotropic couplings to individual methyl protons are inequivalent<sup>55,56</sup> so averaging of these couplings by rotation of the methyl groups could contribute to relaxation that would be described by a spectral density function. The predicted H/D isotope effect on this contribution is larger than the experimental value (1.5) so this process also cannot be the dominant contribution.

Intramolecular ring motions and inversions at the nitroxyl nitrogen may contribute to the thermally activated relaxation process. Since nitroxyl  $g$  values vary with geometry,<sup>57</sup> modulation of geometry<sup>58–60</sup> modulates  $g$  values and may provide a relaxation mechanism. The contribution from this mechanism would decrease with decreasing microwave frequency and could contribute to a frequency dependence of  $C_{\text{therm}}$ . Ring motions could modulate inequivalent couplings to methyl protons as well



as modulating  $g$  values so these motions could result in a spectral density function with a coefficient that combined a frequency-dependent term arising from modulation of  $g$  values and a frequency independent term arising from modulation of  $A$  values.

Further studies, with nitroxyls of different structure and with other radicals are needed to identify the relevant motions. However, the limited data available suggest that relaxation rates at room temperature are similar for a range of nitroxyls, and therefore, the relevant motions must be common to a variety of nitroxyls. The range of accessible motions for nitroxyl radicals raises the possibility that there is more than one thermally activated process that contributes to the relaxation. A sum of several contributions could lead to the experimentally observed weaker dependence on frequency than is predicted for a single spectral density function of the form shown in eq 8.

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