

New Developments in Pulsed Electron Paramagnetic Resonance: Direct Measurement of Rotational Correlation Times from Decay Curves

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Perdeuterated ^{15}N TEMPOL in glycerol-water mixtures and spin-labeled hemoglobin are studied using the time domain technique of saturation recovery electron double resonance (SR-ELDOR), in the ultra-slow motional time regime. The electron spin lattice relaxation rate (T_{1e}^{-1}) and the nitrogen spin-lattice relaxation rate (T_{1n}^{-1}) are determined. Moreover, the characteristic rotational correlation times (τ_R) are measured **directly**. It is **not** possible, in general, to separate uniquely these rates from one another with Saturation Recovery Electron Paramagnetic Resonance (SR-EPR) experiments alone. The SR-ELDOR technique of pumping one spin manifold or orientation and observing at another changes the sign of the amplitudes of selected components contributing to the overall signal. Pooling the SR-EPR and various SR-ELDOR spectra enables all rates to be uniquely determined.

1 Review of Isotropic motion

Perdeuterated ^{15}N -Tanol [1] and spin-labeled hemoglobin (sl-Hb) [2] have been used as model systems undergoing Brownian isotropic rotational motion in the micro to millisecond time range as described by the characteristic rotational correlation time, (τ_R). The continuous wave technique of Saturation Transfer EPR (ST-EPR), which is very sensitive to motion on this time scale [3], relies on the competition among the different magnetization transfer mechanism of T_{1e} , T_{1n} , τ_R and the Zeeman modulation frequency, ω_m . There are many applications of ST-EPR to slow motion, primarily using ^{14}N spin labels. However, the relaxation and rotational rates can only be indirectly inferred from ST-EPR [4]. We will show that these rates may be directly obtained from SR-ELDOR experiments.

Hyde and co-workers invented the technique of Continuous Wave Electron-Electron Double Resonance (CW-ELDOR) [5]. They demonstrated that, by pumping one point in a spectrum and observing at another point, energy transfer could be detected. However, CW-ELDOR is an extremely complex technique and the results can be influenced by instrumental artefacts [6]. The technique has been applied by Stetter *et al.* [7] to nitroxides moving with sub-nanosecond times and by Smigel *et al.* [8] to nitroxides with microsecond correlation times. From CW-ELDOR, one cannot estimate either T_{1e} or T_{1n} independently, only their ratio. The Stetter [7] work used different isotopes of nitrogen to separate exchange due to lateral diffusion from nitrogen relaxation. (There can be no nuclear spin lattice relaxation between different isotopes so any interaction between them must come from collisions.)

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- 7 E. Stetter, H.-M. Vieth, and K.H. Hauser, *J. Mag. Res.*, **23**, 493-504, 1976.
- 8 M.D. Smigel, L.R. Dalton, J.S. Hyde, and L.A. Dalton, *Proc. Nat. Acad. Sci. USA*, **71**, 1925-1929, 1974.

Time Domain EPR: In a time domain EPR experiment, the spins are subjected to a short pulse of microwave power; the time dependence of the transient relaxation after the pulsing is monitored. The rates of relaxation are functions of the motional process. Two types of time domain experiments exist: one monitors spin echoes (a coherent detection of the x- and y-components of the magnetization) and the other monitors Saturation Recovery (as a polarization detection of the z-magnetization).

Freed [9] has applied spin-echo methodology to study motion in liquids. A (90° - τ - 180° - τ -Observe) sequence measures echo height as a function of the delay time τ to give the phase memory decay time T_M . Other workers have studied spin-labeled membranes and vesicles – showing changes in T_M with temperature, but do not relate results to any theory [10]. Freed [9] has also developed the technique of Fourier Transform EPR using multiple pulse techniques, originally developed for NMR. The variation of T_M across the spectrum can be directly obtained and related to motional models. Cross-relaxation from one manifold to another can also be measured. Rates are measured from the volumes of the main and cross-peaks in the 2-D display, and not from actual decay curves.

Huisjen and Hyde [11] pioneered the use of the SR-EPR technique in liquids and applied it to a number of systems [12], [13]. The Hyde group [14] has used SR-EPR, in conjunction with CW-ELDOR, to measure lateral diffusion of ^{14}N labelled lipids in bilayers. These important papers on translational motion set the stage for our work in rotational motion.

Measurement of rotational diffusion directly in the time domain with SR-EPR alone has proved im-

possible. Typical of an SR-EPR experiment is that of Fajer *et al.* on sl-Hb [15] which produced bi-exponential decays. The slower rate was (correctly) identified with T_{1e}^{-1} and the faster decay rate was associated with the rotational motion of the hemoglobin. The faster decay rate remained approximately constant despite a two order of magnitude change of correlation time. These authors then used a qualitative theory of spin diffusion to extract an effective correlation time. The data agreed only approximately with the known τ_R values. The problem with this type of experiment is the strong influence of the nitrogen nuclear spin-lattice relaxation, on the decays. As explained below, the observed decays were a mixture of all three relaxation processes. This makes the protocol for data collection and for analysis of the various decays much more complex than originally expected. Attempts in our laboratory using Saturation Recovery alone were similarly unsuccessful (unpublished experiments with Dr. P. Fajer). We believe we have now solved these fundamental problems using pulsed SR-ELDOR and SR-EPR together.

We will demonstrate that Pulsed EPR techniques, such as SR-EPR and SR-ELDOR, can be used to obtain both the nuclear and electronic spin-lattice relaxation rates directly and monitor changes in exchange rates. Moreover, the correlation time connecting two spectral positions can be directly measured as well. Relaxation time measurements, especially nuclear spin-lattice relaxation (T_{1n}), can also characterize the motion in the ultra-slow motional time range.

2 Theory

Figure 1 shows an absorption CW-EPR signal when the correlation time is longer than 0.1 microseconds. The figure illustrates the dependence of the signal on molecular orientation. The reorientation, characterized by a rotational correlation time, τ_R , moves the magnetization around within a given manifold. Nuclear spin flips, occurring at rate T_{1n}^{-1} , move the magnetization from one manifold to the other without causing molecular reorientation. Regardless of orientation or manifold, the magnetization relaxes to the lattice at rate T_{1e}^{-1} .

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- 10 K. Madden, L. Kevan, P.D. Morse and R.N. Schwartz, *J. Am. Chem. Soc.*, **104**, 10, 1982.
- 11 M. Huisjen and J.S. Hyde, *Rev. Sci. Inst.*, **45**, 669-675, 1974.
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- 13 A. Kusumi, W.K. Subczynski and J.S. Hyde, *Proc. Nat. Acad. Sci. USA*, **79**, 1854-1858, 1982.
- 14 J.-J. Yin, M. Pasenkiewicz-Gierula, and J.S. Hyde, *Proc. Nat. Acad. Sci. USA*, **84**, 964-968, 1987.
- 15 P. Fajer, D.D. Thomas, J.B. Feix, and J.S. Hyde, *Biophys. J.*, **50**, 1195-1202, 1986.

Theory [4], [16] predicts for the SR-ELDOR experiment that the τ_R of a molecule should appear as an exponential decay with a rate $(\tau_R^{-1} + T_{1e}^{-1})$ and that T_{1n} should appear as a decay of rate $(T_{1n}^{-1} + T_{1e}^{-1})$. These two processes have very similar rates, which is why motional rates have been so difficult to determine. However, suitable positions of pump and observer can be chosen so that the signs and the amplitudes of both T_{1n} and the motional decay curves can be individually changed, enabling analysis to disentangle the rates. The SR-ELDOR experiment measures the decay of the transient component of the z magnetization, $\langle M_z(t; \nu, \theta) \rangle$, where ν is the nuclear manifold (which is $\pm 1/2$ for ^{15}N), and θ is the orientation, as shown in Figure 1. The evolution of the magnetization can be understood, qualitatively at least, by a simple population analysis treatment.

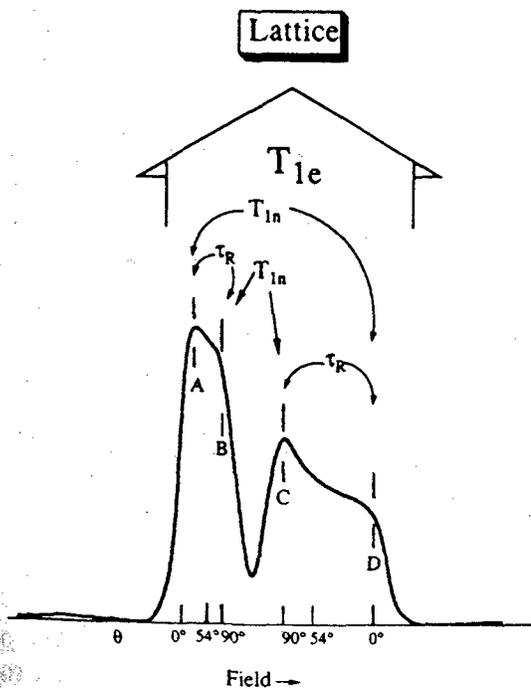


Figure 1: Linear Absorption EPR spectrum of sl-Hb illustrating the positions where SR-ELDOR experiments are performed and the dependence of the resonance positions of the magnetization on spin-label orientation. The figure further illustrates how τ_R , and T_{1n} cause spectral diffusion, whereas T_{1e} induces true spin-lattice relation.

Therefore the master equation is:

$$\frac{d}{dt} \langle M_z(t; \nu, \theta) \rangle = -\frac{1}{T_{1e}} \langle M_z(t; \nu, \theta) \rangle - \frac{1}{2T_{1n}} \{ \langle M_z(t; \nu, \theta) \rangle - \langle M_z(t; -\nu, \theta) \rangle \} - D \nabla_{\theta}^2 \langle M_z(t; \nu, \theta) \rangle \quad (1)$$

where D is the Einstein Rotational Diffusion Coefficient ($D = 1/6 \tau_R$) and ∇_{θ}^2 is the angular Laplacian operator. The system is pumped with a weak selective pulse so that the spin system is excited in manifold ν_p and at orientation θ_p . The observer frequency is set to manifold ν_o and at orientation θ_o . The recovery signal then has the approximate form:

$$\langle M_z(t; \nu_o, \theta_o | \nu_p, \theta_p) \rangle = \langle M_z(0; \nu_p, \theta_p) \rangle * e^{-t/T_{1e}} (1 + f_{\nu_o, \nu_p} e^{-t/T_{1n}}) * (1 + P_2(\cos(\theta_o)) \cdot P_2(\cos(\theta_p)) e^{-t/\tau_R}) \quad (2)$$

where $\langle M_z(t; \nu_o, \theta_o | \nu_p, \theta_p) \rangle$ is the magnetization at the observer position subject to the condition that all of the magnetization was at the pump position at time zero; and $\langle M_z(0; \nu_p, \theta_p) \rangle$ is the initial magnetization at time zero (when the pumping is completed). $P_2(\cos(\theta)) = (3 \cos^2(\theta) - 1)/2$ is the $\ell = 2$ component of the Legendre polynomials $P_{\ell}(x)$, and $f_{\nu, \nu'} = +1$ if $\nu = \nu'$ and $f_{\nu, \nu'} = -1$ if $\nu = -\nu'$ (which is the case of going from one manifold to the other). This result assumes a short duration pump time, a low observer amplitude and that the higher rotational functions are not significant [17]. Equation 2 predicts that one will observe 4 exponentially decaying components, wherein the rate of each component is a linear combination of T_{1e}^{-1} , T_{1n}^{-1} , and τ_R^{-1} . The amplitude of the components containing T_{1n}^{-1} , may be switched in sign by setting the pump and observer positions to different spin manifolds. The amplitude of the components containing τ_R^{-1} may be switched in sign by setting the pump and the observer to different ends of the same manifold.

17 T. Sugano, "A Study of Very Slow Rotational Diffusion by SR-EPR", Ph.D. Thesis, University of Washington, 1987.

3 Experiment

The details of the spectrometer and its performance are discussed more fully in another paper in these Proceedings [18]. SR-EPR and SR-ELDOR experiments are both polarization experiments which measure the recovery of the system to equilibrium. A selective pulse is applied to the spins which alters the polarization and burns a partial hole in the line. Under conditions of SR-EPR the frequency of observation is the same as the pump and therefore the signal is that of a pure recovery as the polarization spreads throughout the spin system and out to the lattice. Under conditions of SR-ELDOR, the frequency of the observer is different from that of the pump and the arrival of magnetization, as well as relaxation to equilibrium, are both detected. It is neither necessary, nor desirable, for the pump to be coherent with the observer. The experiments obtain decay curves taken with various pump times and pump-observer frequency differences; the rotational correlation time is determined by the solvent viscosity and temperature. The pump time controls the relative amplitudes of the various components which contribute to the overall relaxation of the magnetization; and the pump-observer frequency difference controls the signs of the amplitudes of the exponentially decaying components in ways predicted by equation 2.

The experiment proceeds as follows: for one set of experimental conditions, a number of decay curves over different time scales are obtained. These decays are linked by the fact that the exponentials which comprise them are all identical and only the time range of data collection is altered. For a different set of conditions e.g. in pulse length or in observer field position, the relative amplitudes and/or signs of the individual decays are altered and another set of linked spectra is collected. Both sets of linked data are then pooled with the constraint that the rates of the exponentials which make up the spectra are common to all and the amplitudes of each component within a linked set are the same. This technique [19], as an

application of Global Analysis, is well recognized in optical spectroscopy as being extremely effective in determining relaxation rates.

Once one has obtained a unique set of decay rates that minimizes the global χ^2 one must identify the processes which gives rise to each individual decay rate i.e. whether it is due to T_{1e}^{-1} , T_{1n}^{-1} or rotation. Suitable choice of experimental conditions for acquisition of the decays enable the rates to be distinguished. The strategies we have found useful are:

(i) Pumping and observing at the magic angle will reduce the amplitude of the motional rate, leaving just the T_{1e}^{-1} and T_{1n}^{-1} .

(ii) Pumping in one spin manifold and observing in the other will always invert the amplitude of a T_{1n} containing term.

(iii) Pumping at one extreme turning point of a spin manifold and observing the other will invert the amplitudes of the motionally dependent components.

(iv) Suppression of free induction decay (FID) is always important. In the nanosecond range of motion the FID has approximately the same time as T_{1e} and T_{1n} .

(v) A pump time that is long compared to the relaxation time of a particular component will tend to suppress the amplitude of that component.

(vi) Pooling the Saturation Recovery EPR and pulsed ELDOR decay curves for analysis is essential for all rates to be found – no single experiment alone is sufficient.

4 Results

We have obtained correlation times from perdeuterated ^{15}N TEMPOL in glycerol-water mixtures over the τ_R range from 0.1 to 100 microseconds: Bi-exponential recovery curves were obtained when using the SR-ELDOR protocol described above, when the "magic angle" ($\theta = 54^\circ$) was chosen for the pumping and observing positions (as shown in Figure 1). In Figure 2 are plotted the T_{1e}^{-1} and T_{1n}^{-1} rates versus correlation time in the slow motion range. The very weak dependence of T_{1e}^{-1} on correlation time suggests that the more traditional relaxation mechanisms

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- 19 J.M. Beecham, E. Gratton, M. Ameloot, J.R. Knutson, and L. Brand. in *Fluorescence Spectroscopy: Principles* J.R. Lakowicz (ed.) Plenum Press NY Volume 2 Chapter 5, 1991.

such as spin rotation and Electron-Nuclear-Dipolar coupling (END) are not the dominant ones. The power law dependence of T_{1e}^{-1} is on the order of $1/\tau_R^{1/8}$. The NMR literature suggests that a model of spin diffusion in liquids will have the same power law dependence as that experimentally observed [20]. This same power law dependence was also observed in similar measurements using SR-EPR [15]. The solid line superimposed on the T_{1e}^{-1} data is defined as:

$$T_{1e}^{-1} = \frac{\beta_e}{\tau_R^{1/8}} + \frac{\alpha^2 \tau_R}{1 + (\omega_e \tau_R)^p} + \frac{\Delta g^2}{9\tau_R} \quad (3)$$

where β_e is an adjustable parameter, and the second term (r.h.s.) is the END term and the third term

is the spin rotation term. The value of p in the second term is set to 2 when the motion is that of a simple isotropic Brownian diffusion. In this motional region only the first term is significant [18]. The values of T_{1n}^{-1} were also determined from the SR-ELDOR experiments. The amplitudes of the components with these relaxation rates clearly changed sign when the experiments were performed in such a way that the pump and observe positions were in different spin manifolds. The values are compared with the theory of the END mechanisms described previously [18] and a term added for the spin diffusion from the nucleus

$$T_{1n}^{-1} = \frac{\beta_n}{\tau_R^{1/8}} + \frac{\alpha^2 \tau_R}{1 + (\bar{A} \tau_R / 2)^p} \quad (4)$$

where β_n is an adjustable constant.

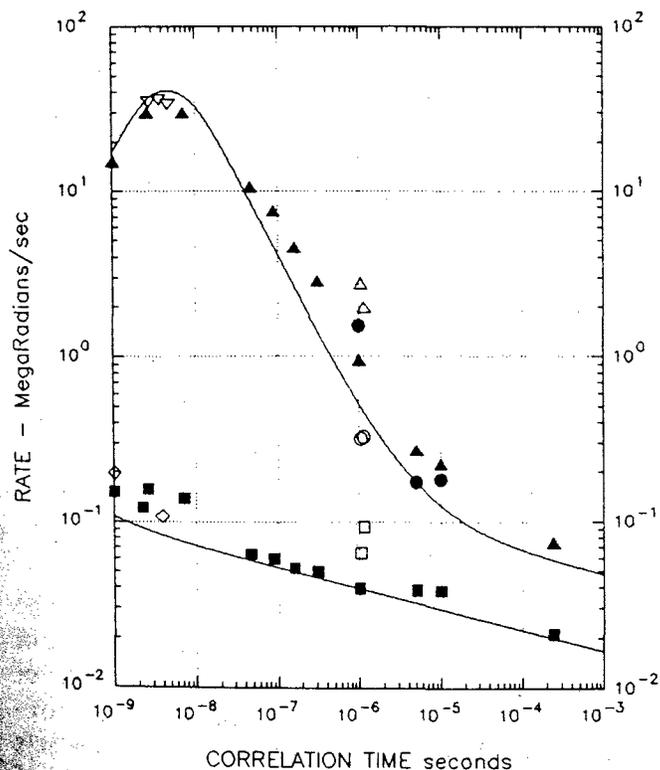


Figure 2: The spin lattice relaxation rates for Tanol (filled squares) and Hb (open squares); the nuclear spin-lattice relaxation rates for Tanol (filled triangles) and sl-HB (open triangles), and τ_R^{-1} estimated from the recovery rates (see Figure 3) for sl-Hb (open circles) as a function of the rotational correlation time τ_R estimated from the solvent viscosity and hydrodynamic volume. Superimposed on the curve are the theoretical fits from equations 3 and 4 (assuming $p = 2$, $\beta_e = 0.0063$, and $\beta_n = 0.032$).

Within the motional range from 10^{-8} to 10^{-6} seconds the rates T_{1n}^{-1} are a very strong function of the motion and uniquely determine the correlation time. This nicely fills in the "hole" in CW-EPR and ST-EPR in the microsecond range where neither technique is particularly sensitive to the correlation time alone. The fit using equation 4 assumed that $p = 2$. However, a closer examination of the relation of the data to the theory suggest that $p = 1.8$ may provide better agreement. There is a large literature on stretched exponential, which are described in the time domain by the Williams-Watts function and in the CW domain by the Cole-Davidson function [21]. The Cole-Davidson function modifies the $p = 2$ spectral density function (in equation 4) and motivates a $p < 2$ for cases where there is not a single exponential but there are several exponentials clustered about an average rate. It may well be the case that $p \sim 1.8$ to 1.9 is more appropriate than $p = 2$ because a detailed analysis of the relaxation parameters from SR-EPR and SR-ELDOR experiments shows [17] that there should be 6 exponentially decaying components around the central rate and this may well be an argument that $p < 2$ may occur. Other alternative explanations for the slight deviation of the experimental data from the theory may lie in the presence of oxygen or additional paramagnetic interactions (such as concentration effects).

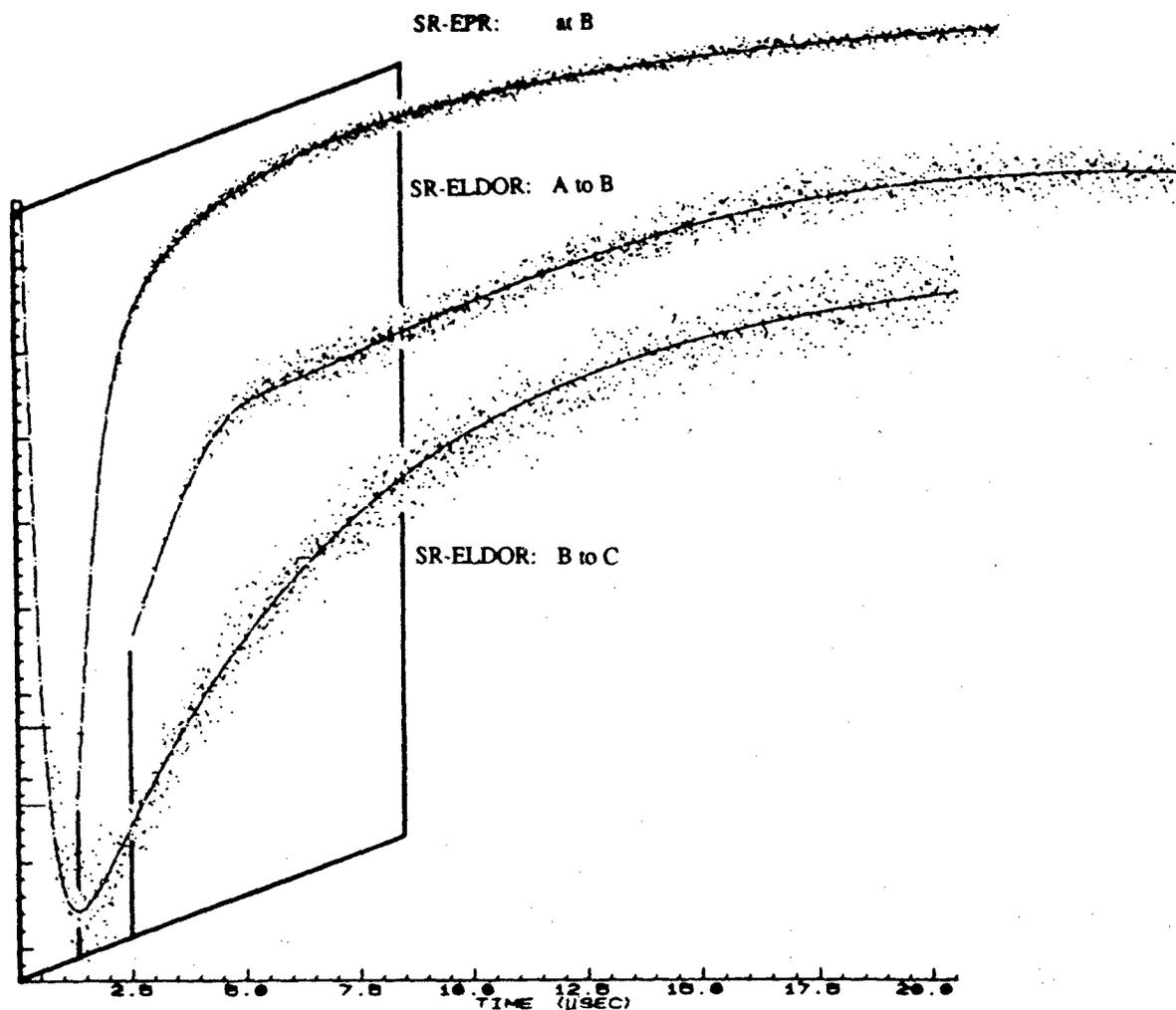


Figure 3: Three different SR-ELDOR spectra, taken at different pump and observation positions (see Figure 1). The top curve is the SR-EPR spectrum at position B (in Figure 1), the middle curve is the SR-ELDOR spectrum pumping at position A and observing at position B, and the bottom spectrum is the SR-ELDOR spectrum pumping at position B and observing at position C. Superimposed on each spectra is the least-squares best fit consisting of an adjustable baseline and three components each of which is a single exponential decay (see equation 2), the rates are given in Figure 2.

Figure 3 shows an example of the SR-EPR and SR-ELDOR data for sl-Hb tumbling with τ_R in the 1 to 5 microsecond time range. The top curve, (SR-EPR at B) is the SR-EPR data acquired at point B (defined in Figure 1). This curve is a simple recovery and all components have the same sign. The bottom curve (SR-ELDOR from B to C) is the data when the pump is set on B and the observer frequency is set to C. This represents a jump from one manifold to the other and the amplitude of the components, containing a T_{1n}^{-1} in the rate, change sign. The middle trace of Figure 3 (SR-ELDOR from A to B) is the data for the pump on A and the observer on B. This corresponds to the pump-observe case within a manifold. According to equation 2, the amplitude of the

components containing τ_R in the rate will change sign. Clearly there is a peculiar dip in the shape of this SR-ELDOR curve. The dip is characteristic of the component that depends directly on the correlation time. Superimposed on each of the data sets is a least-squares best fit simulation composed of three exponentials and a baseline. The three rates of the exponentials are the same in all three data sets. The rates are interpreted, according to equation 2, as T_{1e}^{-1} , T_{1n}^{-1} and τ_R^{-1} as 0.0937, 1.983 and 0.329 MRad/sec respectively with around a 10% error. Notice that the motional rate is in between the spin-lattice relaxation rate and the nuclear relaxation rate. These data are also shown in Figure 2. The nominal correlation time, determined from solvent viscosity and hydrodynamic

radius of sl-Hb, is 1.5 μsec . From the L''/L ratio calibration curve of the ST-EPR [4] one may estimate the correlation time to be 4.0 μsec and the rotational correlation time measured by SR-ELDOR is 3.3 μsec , which is in excellent agreement with the ST-EPR calibration data. This work, demonstrates that the different components of the experimental curve can be uniquely identified and their rates quantitatively measure, and hence T_{1e} , T_{1n} and τ_R may be directly measured. **This is the first time that τ_R has been directly measured in EPR and represents a fundamental advance in time domain methodology.**

5 Conclusions

We have measured the values of the electron and nuclear spin lattice relaxation times in the ultra-slow motion region using SR-ELDOR and SR-EPR and find that the measured values of T_{1e}^{-1} depend on $1/\tau_R^{1/8}$, or a power law dependence, which is consistent with a model of spin diffusion in liquids. The measured values of T_{1n} are well described by the electron-nuclear dipolar relaxation, (which has no adjustable parameters in it) and only the rates at motional times longer than 100 μsec suggest the need for a spin diffusion mechanism for T_{1n} as well as T_{1e} . Notice that for the case of spin labels the nuclear relaxation is much faster than the electron relaxation (an unusual situation). This is primarily a result of the ability of the electron, at this particular rotational rate, to efficiently relax the nucleus and the fact that the electron has very few other spins to relax it. By using SR-ELDOR and by pooling the data at different orientations the rotational correlation time can be measured directly, and is seen experimentally as a single exponential relaxation. This is, we believe, the first time rotational motion has been quantitatively measured as a single exponential decay in this type of experiment. These experimental results suggest that it may be possible to directly measure rotational reorientation by SR-ELDOR even for systems characterized by anisotropic motion.

These type of time domain experiments are a necessary underpinning and improvement on traditional CW techniques. Quantitative simulation of ST-EPR spectra requires knowledge of the relax-

ation times of the spin system. When performing progressive saturation studies using CW-EPR, the experiments can only be used to detect relative changes in relaxation times when motion is longer than a nanosecond [22]. (Absolute values are difficult to obtain accurately because the effective relaxation time for the CW experiment is a complex mixture of competing relaxation processes.) Direct measurement of T_{1e}^{-1} and T_{1n}^{-1} with pulse techniques is clearly superior.

Prior to the time domain experiments it was found in this laboratory that the T_{1n}^{-1} value in calculations of ST-EPR spectra in the microsecond motional range had to be set artificially high for good agreement between simulation and experiment [23]. The simulation assumes that the electron-nuclear dipolar (END) interaction is the sole mechanism for nuclear spin-lattice relaxation. The data in Figure 2 clearly show that other mechanisms add to the END rate (e.g. from proton spin diffusion) justifying the *ad hoc* addition of an extra rate into the calculations.

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