NEW EPR METHODS FOR THE STUDY OF
VERY SLOW MOTION: APPLICATION
TO SPIN-LABELED HEMOGLOBIN

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INTRODUCTION

Nitroxide-radical spin labels have been widely used to study motion in biological systems. Smith has recently reviewed the subject and provides access to the literature. The present work is concerned with the development of methodology that will permit studying slower motions than has previously been possible. Practically, the spin-label technique has been restricted to rotational correlation times \( T_\phi \) shorter than \( 3 \times 10^{-3} \) sec.\(^2\). Theoretically, any motion affects the ordinary linear epr spectrum, but several problems arise as the motion becomes slow. (1) The effect of motion on the spectrum becomes less and less as the spectra asymptotically approach the line shape expected from a rigid powder. Hence, the signal-to-noise ratio of the motional effects goes to zero. (2) Kreilick has observed temperature-dependent couplings of nitroxide radicals using nmr techniques. Thus, the inhomogeneous line width (i.e., the line width that would be observed in a single crystal, and arising from unresolved hyperfine couplings to protons of the radical) is temperature-dependent, and this affects the spectral shape. (3) It is necessary to know the theoretical line shape that is approached as the motion slows, but the expediency of freezing the sample fails because the dielectric constant of the solvent affects the hyperfine coupling and is itself generally temperature-dependent and changes markedly upon freezing.

It has occurred to us that methods involving the nonlinear response of the spin system in which intense irradiating fields are employed and the observed spectrum depends to a considerable degree on relaxation to the lattice might permit determination of very slow motion. If the spin-state lifetime limiting line width is \( 1/\gamma T_{1e} \), then the slowest motion that could in principle be measured by a nonlinear method would involve spectral diffusion by an amount \( 1/\gamma T_{1e} \) in a time \( T_{1e} \). Here, \( \gamma \) is the gyromagnetic ratio of the electron and \( T_{1e} \) is the electron longitudinal (or spin-lattice) relaxation time. Assuming that the anisotropy, \( \Delta \omega \), of the typical nitroxide radical is about \( 2\pi \times 10^3 \) rad (this being the spectral width swept out by the \( m_I = -1 \) nuclear spin configuration as the radical undergoes isotropic rotational diffusion) and that \( T_{1e} = 10^{-6} \) sec, we can estimate a limiting maximum \( \tau_2 \) for motion of spectral diffusion by \( \Delta \omega T_{1e} \), \( \Delta \omega T_{1e} )^2 = 0.4 \) sec. Success in extending the present range to times as long as \( 10^{-3} \) or \( 10^{-4} \) sec would be very useful. These slow times are relevant for many biological processes, and this is a time scale not readily accessible to other analytical methods.
The present work originated in an endor study of triphenylmethyl in supercooled toluene, where the averaging of electron-nuclear interactions was observed directly as the correlation time (i.e., temperature) was altered. A further development was a study of progressive-saturation characteristics of very slowly tumbling flavin radicals, both conjugated to the apoprotein and as free flavins again in supercooled toluene. It was observed that the saturation behavior depended strongly on motion even though the ordinary (unsaturated) spectrum showed no discernible indications of the motion.

This work is primarily a continuation, however, of a paper by Hyde and Dalton (HD, below) employing adiabatic rapid-passage methods to study the effects of motion. The experimental technique involved observation of the dispersion, setting the reference phase of the phase-sensitive detector 90° out-of-phase with respect to the field modulation and obtaining spectra at several field-modulation frequencies. As before, a model system was used: tanol in supercooled sec-butyl benzene.

The key idea of the experiment is that rotational diffusion causes individual spins to pass through resonance because of g and hyperfine anisotropy at varying degrees of adiabaticity, and that if the velocities of passage resulting from this motion are comparable to sweep rates from the field modulation, something ought to happen when the spectrometer is set to observe rapid-passage signals. Signals were obtained of 10–20% of the ordinary in-phase intensity, with a shape that depended largely on the product $\tau_2\omega_m$, where $\omega_m$ is the field-modulation frequency, and an argument was advanced that permitted extraction of approximate numerical values from the observed line shape. These out-of-phase signals depend totally on relaxation phenomena; nothing is detected at lower microwave powers. Thus, one is observing the pure effect rather than a small change in a larger signal as for the in-phase spectrum. The signals change shape markedly when varying the modulation frequency, which gives an added experimental parameter. The two relevant relaxation times in HD are $T_{1e}$ and $\tau_2$. $T_{1n}$ (the nuclear longitudinal relaxation time) is ten times $T_{1e}$, and it was shown that both $T_{1e}$ and $T_{1n}$ did not vary very much over the conditions of the experiment. A prime necessity for a successful methodology to measure $\tau_2$ is that it be possible to separate $\tau_2$ from other relaxation times. This was apparently the case in the method employed by HD.

The present work has two main themes. The first is to explore and extend the out-of-phase method for studying relaxation processes. Some further experiments using the $\chi'$ dispersion first harmonic out-of-phase method employed by HD have been performed. Two other techniques were investigated while detecting the absorption $\chi''$: setting the reference signal of the phase-sensitive detector 90° out-of-phase with respect to the field modulation; and operating the phase detector at twice the field-modulation frequency with the reference signal out-of-phase, where in-phase yields ordinary second-derivative epr spectra. With saturating microwave fields, signals are obtained in all three
experimental arrangements. To state at once a main conclusion: all displays, both in-phase and out-of-phase, depend to some degree on all relaxation parameters, but some displays are clearly more dependent on one parameter than another; they are not all equivalent. The second theme is to test these methods on a system of somewhat greater biological relevance. We have applied all methods, with apparent success, to spin-labeled hemoglobin in glycerol at +5°.

We attempt throughout to give a qualitative physical explanation of observed signals, depending heavily on Weger. In this thorough work, rapid-passage phenomena are classified as "fast" or "not fast." (Fast means $\omega_mT_{1e} \gg 1$, where $\omega_m$ is the field-modulation frequency. See Weger's Table II). It seems clear in the present work that we are neither Fast or Not Fast. This intermediate condition is not treated separately; therefore Weger can only be used in an approximate manner. And no author has yet considered motional effects on passage spectra from a theoretical point of view.

Passage phenomena are complex, and exhaustive theory and experiment are required for analysis of every system. It is the fact that all nitroxide radicals have essentially the same relaxation behavior, and that the spin-label method has wide and growing application that makes it worthwhile to attempt to understand spin-label passage spectra in detail.

**EXPERIMENTAL METHODS AND SAMPLE PREPARATION**

**Apparatus**

A Varian E-9 X-band spectrometer equipped with Dispersion Reference Arm was used in this work. Experiments involving modulation at $\omega_m$ and phase-sensitive detection at $2\omega_m$ required instrumental modifications of a straightforward nature. In order to obtain satisfactory out-of-phase nulls, the receivers must reject odd harmonics, and harmonic and subharmonic content of the field modulation must be low. The reference phase of the phase-sensitive detector was set using dpph (2,2-diphenyl-1-picrylhydrazyl) and also CuSO$_4$. These samples do not saturate with available powers in the temperature range of the experiments. In all out-of-phase spectra, the normal in-phase signals were rejected by at least a factor of 100. Temperature control and measurement were critical aspects of the experiments in supercooled sec-butyl benzene. The viscosity changes by a factor of ten between $-97.5°$ and $-106.5°$ and by another factor of ten between $-106.5°$ and $-114°$. The measurement temperatures employed here were the same as those used by Hyde and Dalton.

**Tanol Sample Preparation**

Tanol was dissolved in sec-butyl benzene at 10$^{-3}$ M concentration, and the sample was degassed by multiple freeze-thaw cycles under vacuum. Some preliminary experiments on nondegassed samples were performed. The effect of dissolved oxygen on $T_{1e}$ is minimal, if any. At $-97.5°$, $-106.5°$, and $-114°$, tanol in supercooled sec-butyl benzene was estimated to have rotational correlation times of $2 \times 10^{-7}$, $2 \times 10^{-6}$, and $2 \times 10^{-5}$ sec.
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Hemoglobin Sample Preparation

Human oxyhemoglobin was prepared in pH 7, 0.1 M phosphate buffer and labeled in the manner of McCalley and coworkers with a maleimide spin-label.

This label binds to each of two identical β-93 cysteine residues in each hemoglobin molecule, where it is rigidly bound and produces a strongly immobilized epr spectrum. The sample of labeled hemoglobin in glycerol was prepared by dissolving 1 ml of glycerol in 5 ml of hemoglobin solution and evaporating under vacuum at 0°C for 12 hours. After reoxygenation, the visible absorption spectrum was unchanged by the process, and the slight changes in the normal epr spectrum were the same as those observed when the viscosity was increased by adding sucrose. (There is a small shift in hyperfine coupling because of the difference in polarity between glycerol and water.) The hemoglobin in glycerol-water sample was prepared by adding 0.100 ml of a solution of labeled hemoglobin in aqueous buffer to 0.500 ml of the hemoglobin-glycerol sample. Both samples are 1.7 × 10⁻³ M in hemoglobin as determined by absorption at 576 nm, with 1.3 spin-labels per molecule as determined by double integration of the epr spectra.

Estimates of the macroscopic viscosities of the hemoglobin samples were made from published viscosities of glycerol and its aqueous solutions, neglecting the effect of the protein. At 5°C, these viscosities are 70 P and 4.0 P. The rotational correlation time for maleimide-labeled hemoglobin, assuming isotropic rotational diffusion, for viscosities less than one poise obeys the equation (Reference 2, Figure 4) \[ \tau_\alpha = \left( 7.6 \times 10^{-4} \right) \eta / T. \] The Debye expression \[ r_\alpha = 4 \pi r^3 \eta / (3kT) \] is equivalent to this equation using a radius, r, of 29 Å. This is a reasonable value for hemoglobin. Thus we estimate \[ \tau_\alpha = 1.9 \times 10^{-4} \] and \[ 1.1 \times 10^{-5} \] sec for the two samples at +5°C, where all hemoglobin spectra were recorded.

This is the method employed by Hyde and Dalton. FIGURE 1 may be used to understand the experiment. Adiabatic rapid passage means that in the rotating frame, the angle between the magnetization and the effective field remains constant during passage. Referring to the left half of the Figure, assume that \( \omega_m T_1 \alpha < 1 \), so that the spin system has come to equilibrium when the sinusoidal modulation field is at one end of its excursion with a sweep rate going to zero. The vectors \( \mathbf{M} \) and \( \mathbf{H}_{\text{eff}} \) are parallel, and during passage a dispersion signal is detected as the projection of \( \mathbf{M} \) on the +x-axis. If now the system can come to equilibrium again at the other end of the sweep, then \( \mathbf{M} \)
will initially be along the +z-axis, during passage $\mathbf{M}$ and $\mathbf{H}_{\text{eff}}$ will be antiparallel, and the signal will be the projection of $\mathbf{M}$ on the $-x$-axis. Thus the signal from a single spin packet will be $90^\circ$ out-of-phase with respect to the field modulation. Weger integrates this result from a single spin packet over the envelope $h(\omega - \omega_n)$ that is the distribution of spins, calling it Case 2. The result is a signal that has the shape $h(\omega - \omega_n)$ and is $90^\circ$ out-of-phase. Modulation phases are shown in Figure 2; compare traces a and c.

The effect of rotational diffusion is to reduce the intensity of passage signals from portions of the spectrum that involve greater anisotropy. Thus, since $T_{1n}$ is long, spectral fragments from the three nuclear spin configurations can be ordered in ascending anisotropies: 0, $+1$, $-1$. For a given modulation frequency, the 0 configuration gives more intense signals than the $+1$, on a percentage basis compared with $h(e^{j\omega - \omega_n})$. Similarly, $+1$ yields greater signals than $-1$, normalized to $h(\omega - \omega_n)$. Within a given configuration, the turning points yield larger signals than the region between turning points. As the product $\omega_m T_{1n}$ increases, the rapid-passage spectra approach $h(\omega - \omega_n)$ in shape.

An extreme case in HD was the observation of tanol at $-114^\circ$ ($\eta$ of sec-butyl benzene = 1100 P) and 100 kHz field-modulation frequency. If no motion were present, the line shape should be $h(\omega - \omega_n)$. To check this, a single integration of the ordinary, unsaturated derivative-like absorption spectrum was performed and superimposed on the passage spectrum (Figure 3). Motional effects are obviously still present. Greatest percentage deviation is for the $-1$ (right side) nuclear spin configuration, where motion is likely to have the greatest effect.

Passage line shapes are only slightly dependent on microwave power. (See for example Figure 4, obtained at $\eta = 110$ P and a modulation frequency of

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**Figure 1.** Schematic representation in the rotating frame of rapid, adiabatic, “not fast” passage for a single spin packet. During passage $\mathbf{H}_{\text{eff}}$ and $\mathbf{M}$ remain parallel, with the ends of these vectors tracing paths indicated by the lines labeled with open arrows.
FIGURE 2. Phase relationships of the three out-of-phase experiments discussed here. Ticks indicate switching times for the phase-sensitive detector. Thus $a$ is 90° out-of-phase with respect to $c$ and $d$, while $h$ defines the second harmonic in-phase condition and is 90° out-of-phase with respect to $c$.

FIGURE 3. Rapid-passage $\chi'$ first harmonic out-of-phase spectrum of tanol measured at 100 kHz and $-114^\circ$ C in sec-butyl benzene ($\eta=1,100$ P) superimposed on an integrated unsaturated absorption spectrum recorded at $-114^\circ$ C.
FIGURE 4. Rapid-passage $\chi'$ first harmonic out-of-phase spectrum of tanol in sec-butyl benzene recorded at $-106.5^\circ$ ($\eta=110$ P) at two microwave powers using 1 kHz field-modulation frequency.

1 kHz. Motion has nearly eliminated signal intensity between turning points.) Experiments of this nature confirm the thesis that the shape is much more dependent on $T_s$ than $T_{ie}$.

Rapid-passage spectra from hemoglobin are shown in FIGURE 5. The two spectra on the left are in-phase dispersion signals obtained at the saturating microwave field (20 mW incident on the cavity) that was employed for the passage spectra. Out-of-phase signals are on the right. (Spin-labeled hemoglobin at $+5^\circ$ saturates with somewhat greater difficulty than tanol in sec-butyl benzene at $-114^\circ$. A power of five to ten milliwatts was used for tanol.) The $\eta=4.0$ spectrum in the figure ($\tau_2 = 1.1 \times 10^{-5}$ sec) corresponds closely in shape to that measured by HD for tanol in sec-butyl benzene at $-106.5^\circ$ ($\eta=110$ P) and 100 kHz. HD assigned $\tau_2 = 2 \times 10^{-6}$ sec to this spectrum. The spectrum of the $\eta=70$ P sample has progressed toward the shape expected from a rigid powder, but not as much as expected from the HD experiment. Thus, while there is qualitative consistency between experiments on hemoglobin and on tanol, there are quantitative discrepancies. The passage spectra from hemoglobin suggest that more motion is present than was expected. Perhaps the microscopic viscosity is much less than the macroscopic viscosity, or the estimated macroscopic viscosities are in error, or librational motions of the label as well as the tumbling of the protein are affecting the passage.

The out-of-phase spectrum for $\eta=4.0$ P is 10% of the intensity of the in-phase spectrum measured at 20 mW. The corresponding percentage for $\eta=70$ P is 25%. The signals are of good intensity, but high noise when observing the dispersion that arises from demodulation of klystron FM noise.
degrades the quality of the spectra. (There are several experimental approaches toward overcoming this type of noise that are presently being considered.)

$\chi''$ SECOND HARMONIC OUT-OF-PHASE

To our knowledge, this is the first time that experiments of this class have been reported. The motivation follows directly from Weger, however, who mentions the possibility of detecting such signals on theoretical grounds. Figure 6 may be used to understand the situation. The concept of adiabaticity is always an approximation. Inversion of magnetization involves extraction of energy from the radiation field by the spin system and so there must always be some absorption. Assume that $\omega_m T_{1e} \sim 1$, so that relatively little relaxation occurs during, for example, any 10% of the cycle, but in general the system has no memory of previous cycles. Consider a spin packet that resonates near one end of a field-modulation swing (left side, Figure 6). The effective field approaches from the top, comes to the end of the sweep, and turns around. The magnetization tends to follow the effective field. There is a small projection on the absorption (+y-axis) coming down, and (since it is assumed that no relaxation occurs) a small projection on the $-y$-axis (corresponding to emission) going up. The line with open arrows is the locus of the end of the magnetization vector during the passage. In Figure 3, the signal has the form of trace e. If now one considers a sweep through a spin packet resonating at the other end of the field-modulation sweep (right side, Figure 6), projections are first on the +y-axis and then on the $-y$-axis. From the switching indicated on Figure 3e, one can see that the signals from the ends of the sweep add rather than subtract, and thus a signal of the shape of $h(\omega - \omega_0)$ is expected.

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**Figure 5.** $\chi'$ first harmonic in- and out-of-phase spectra of hemoglobin in glycerol, using 5 G 100 kHz field modulation and 20 mW of incident microwave power. Ticks are at 20 G intervals.
Referring to Weger's paper; the small crossing through the baseline of Figure 18, iii, c, treated as a subcase of Case 3, has been emphasized here. Second harmonic out-of-phase absorption traces for tanol in sec-butyl benzene are shown in Figure 7. The lower left spectrum resembles $h'(\omega - \omega_0)$ in shape and corresponds to the case just discussed. This signal is 40% of the in-phase second harmonic signal. At ten times lower viscosity and 50 kHz field modulation (upper left), considerable evidence of motion is present. Thus the spectral fragment associated with $-1$ nuclear spin configuration is less pronounced than that of $+1$, which is in turn of lower amplitude than 0, all relative to $h'(\omega - \omega_0)$, and the turning points are more intense than the region between turning points. This upper left spectrum corresponds nearly to that obtained when detecting $\chi'$ 1st harmonic out-of-phase at this viscosity but with 1 kHz field-modulation frequency, a factor of 50 lower frequency. Some of this difference in shape arises because the $\chi'$ first harmonic out-of-phase signal arises from that portion of the sinusoidal sweep where the sweep rate is greatest, while the $\chi''$ 2nd harmonic out-of-phase signal arises from the extrema of the sweep where the sweep rate goes to zero.

The spectra on the right side of Figure 7 show effects of motion, but they are approaching, in shape, the second derivative of the absorption. Spectra on the right are one third as intense as those on the left. The in-phase second harmonic spectra obtained at both viscosities and modulation frequencies are substantially identical. The $\chi''$ second harmonic out-of-phase spectra from hemoglobin are shown in Figure 8. Again, the corresponding in-phase spectra are of little interest, being independent of viscosity and modulation frequency.

In comparing Figures 7 and 8, one is immediately struck by the similarity of line shapes. It would appear, however, that somewhat more motion is present in the hemoglobin than in the tanol sample. This is the same con-
Conclusion reached when tanol and hemoglobin were compared in the preceding section using the $\chi'$ first harmonic out-of-phase technique. In addition, the ratio of outside peak intensity to center peak intensity is greater in the tanol than in the hemoglobin sample. This might be the consequence of a torsional oscillation about the C-N bond of the maleimide radical in hemoglobin. The intensities of the out-of-phase signals were about 20% of the in-phase signals.
at 50 kHz and 10% at 5 kHz. In magnitude, these second harmonic out-of-phase absorption signals are about three times less than the first harmonic out-of-phase dispersion signals, but their quality is better because of low sensitivity of the spectrometer to demodulation of klystron FM noise when tuned to the absorption.

**χ'' First Harmonic Out-of-Phase**

This experimental arrangement has been employed in extensive investigations of the tanol in supercooled sec-butyl benzene sample and of the hemoglobin samples. To state the conclusion at the outset: The spectra appear insensitive to motion and sensitive to $T_{1e}$. Figure 9 illustrates the spectra obtained from hemoglobin. The shapes are quite independent of $\tau_z$ and change somewhat with $\omega_m$. The intensities of the out-of-phase signals at both frequencies are 10% of the in-phase intensities. The in- and out-of-phase shapes are quite similar, and it is possible to find an intermediate angle that nulls the signal to about 3–4%.

Since the shapes do not change very much with microwave power, relaxation information apparently must be extracted by plots of signal intensity versus microwave power. The out-of-phase signal varies approximately as $H_1^2$ at intermediate saturation. (As usual, the unsaturated ordinary epr intensity varies as $H_1$.) Both in- and out-of-phase signals level off at higher powers in somewhat different ways, and there are some differences in the saturation behaviors of different portions of the spectrum. Thus the $-1$ (high field) peak saturates at higher powers than the $+1$ (low field) peak. We have attempted a variety of methods to process the data in such a manner that a number dependent only on the product $\tau_z\omega_m$ and not on the individual factors could be obtained, without success.

![Figure 9](image-url)
When using 1 kHz field modulation, the out-of-phase signal from tanol is about 2.5% of the in-phase signal at 10 mW incident power, and this percentage is nearly independent of temperature. When using 10 or 100 kHz, this ratio is about 10–20%, and is again temperature-independent. In HD it was argued that $T_{1e}$ does not change very much for tanol over the temperature range of $-97.5$ to $-114^\circ$ in sec-butyl benzene, and one would not expect a difference in $T_{1e}$ between the two hemoglobin samples. The fact that $\chi''$ first harmonic out-of-phase spectra from both hemoglobin and tanol do not change suggests that this display might be a useful one in observing $T_{1e}$. It does not appear satisfactory for the present purpose of studying slow motion.

**CONCLUSIONS**

This paper is a summary of work-in-progress to date. The following conclusions are somewhat tentative. (1) The $\chi'$ first harmonic out-of-phase technique appears the most accessible of the methods to theoretical treatment, and if the problem of demodulation of klystron FM noise can be overcome it should be a very practical method for studying slow motion. All experiments are consistent with the assumption that the shapes depend on $\tau_2$ to a much greater extent than on $T_{1e}$. (2) The $\chi''$ second harmonic out-of-phase technique yields the greatest line shape changes upon change in $\tau_2$ of any method. It therefore appears maximally sensitive to observation of very slow motion, perhaps slower than possible with the $\chi'$ out-of-phase method. (3) The $\chi''$ first harmonic out-of-phase method is probably useful only for obtaining information concerning $T_{1e}$. (4) A most important result is the demonstration of feasibility of these methods in the study of motion of a protein in a viscous environment. (5) Qualitative consistency between the two very different model systems, tanol in supercooled sec-butyl benzene and maleimide-labeled hemoglobin in glycerol, yields confidence in the viability of both model systems. Empirical correlations in a series of similar samples appear quite feasible, permitting ordering of the samples as to degree of motion. (6) There are quantitative discrepancies between the two models of about a factor of five in apparent $\tau_2$ compared with theoretical correlation times, and this remains a problem for further investigation.

**REFERENCES**

11. Handbook of Chemistry and Physics. 1961. 43rd edit.: 2227. The Chemical Rubber Publishing Co. Cleveland, Ohio. (This Table does not appear in recent editions.)

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**DISCUSSION**

**DR. MAILER** *(Medical College of Wisconsin, Milwaukee, Wisc.)*: If you have no rotational correlation time to worry about, you can measure $T_{1e}$ with this phase method down to $10^{-1}$ sec. I did this on cytochrome $c$ at low temperatures. Did your phase stay constant at 90 degrees of the actual signal?

**DR. HYDE:** When looking at the first harmonic absorption, the signal that you see out-of-phase has a shape similar to that seen in-phase. In this case a null at an intermediate reference phase angle can be obtained of about 3–4%. On the other hand, the dispersion and the second harmonic absorption out-of-phase line shapes are quite different from in-phase line shapes. In this case good nulls at intermediate reference phase angles cannot be obtained.

**DR. MAILER:** What about going from $\omega_m T_{1e} > 1$ to $\omega_m T_{1e} < 1$, and sweeping the reference phase angle?

**DR. HYDE:** There is certainly information to be obtained in varying the reference phase, but the line shapes are very complex. I would like to emphasize the value of the out-of-phase method. Everything you see arises from relaxation probabilities that are comparable to $\omega_m$. 