MEASUREMENT OF ROTATIONAL MOLECULAR MOTION BY TIME-RESOLVED SATURATION TRANSFER ELECTRON PARAMAGNETIC RESONANCE

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ABSTRACT We have used saturation-recovery electron paramagnetic resonance (SR-EPR), a time-resolved saturation transfer EPR technique, to measure directly the microsecond rotational diffusion of spin-labeled proteins. SR-EPR uses an intense microwave pulse to saturate a spin population having narrow distribution of orientations with respect to the magnetic field. The time evolution of the signal is then observed. The signal increases in time as saturation is relieved by spin-lattice relaxation (T_1) as well as by saturation transfer due to spectral diffusion (τ_{sd}), which is a function of rotational diffusion (τ_r) and spectral position. In the presence of both events, the recovery is biphasic, with the initial phase related to both τ_r and T_1, and the second phase determined only by T_1. We have measured the saturation recoveries of spin-labeled hemoglobin tumbling in media of known viscosities as a function of rotational correlation time (τ_r) and pulse duration (t_p). The τ_r values estimated from the initial phase of recovery were in good agreement with theory. Variation of the pulse time can also be used to determine τ_r. For $t_p < \tau_{sd}$, the recoveries were observed to be biphasic, for $t_p > \tau_{sd}$ a single-exponential. T_1 values were determined from the recoveries after pulses quenching spectral diffusion or from the second phase of recovery after shorter pulses. These results demonstrate that SR-EPR is applicable to the study of motion of spin-labeled proteins. Its time resolution should provide a significant advantage over steady state techniques, particularly in the case of motional anisotropy or system heterogeneity.

INTRODUCTION

Measurement of microsecond molecular dynamics is of biophysical importance, since large-scale motions of proteins in membranes and other assemblies occur on this time scale, as do many enzymatic reactions (Robinson et al., 1985; Thomas, 1985). The most important techniques used in such studies are spectroscopic methods, including both optical and magnetic resonance. The principal magnetic resonance technique used is saturation transfer electron paramagnetic resonance (ST-EPR). While the optical methods are usually time-resolved, ST-EPR is a steady state technique that relies on competition between various relaxation mechanisms and the modulation frequency. Although the spectral lineshapes for isotropic motion have been shown to be a function of the rate of rotational diffusion, in the case of anisotropic motion they are sensitive to both the rate and the amplitude (Robinson and Dalton, 1980; Thomas et al., 1985). However, in recent years, there has been an ongoing effort to develop time-domain EPR techniques capable of studying molecular dynamics. Time-domain EPR should, in principle, have all the advantages of optical methods while retaining the orientational resolution of magnetic resonance. One approach is spin-echo EPR, in which the time evolution of the echo is sensitive to dynamic processes, such as rotational motion (Madden et al., 1980; Schwartz et al., 1982), and lateral diffusion (Stillman and Schwartz, 1979). Even more promising are time-resolved saturation transfer experiments. In this type of experiment, one orientational population is saturated by an intense microwave pulse, while some other population is observed. This can be achieved by using two microwave sources of different frequency, pulsed ELDOR (Nechtschein and Hyde, 1970; Hyde et al., 1984), or changing the magnetic field after a saturating pulse, (Rengan et al., 1979; Dzuba et al., 1984; Millhauser and Freed, 1984). The magnetization can be monitored by measuring absorption with a low intensity c.w. microwave, or by detecting the electron spin echo. Finally, the third class of experiments is pulse-induced EPR, where the magnetization at a given time after the saturating pulse is displayed as a function of magnetic field (Percival and Hyde, 1975; Brown, 1979). The spectra taken at different times will reflect the effects of spectral diffusion. Very comprehensive reviews of time-domain EPR were recently published (Thomann et al., 1984; Kevan and Schwartz, 1979).

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We report here the detection of microsecond rotational motions of spin-labeled hemoglobin (Hb) tumbling isotropically in media of known viscosity, using saturation-recovery EPR (SR-EPR). This is a simplified saturation transfer technique in which the saturated population is also the observed one. SR-EPR was previously used in measurements of spin-lattice relaxation times, its potential to measure rotational dynamics was previously noted (Smigel et al., 1974; Hyde, 1979).

THEORY

The response of magnetization M_0 to a saturating pulse in the absence of spectral diffusion was given by Percival and Hyde (Percival and Hyde, 1975)

$$\begin{aligned} m_y(t)/m_{z\infty} = {} & m_y(0)\exp(-t/T_2) \\ & + \gamma H_1 T_2 (m_z(0) - 1)/(1 + \gamma^2 H_1^2 T_1 T_2) \\ & \times \exp(-t/T_1 - \gamma^2 H_1^2 T_2 t) \\ & - \gamma H_1 T_2 m_z(0)\exp(-t/T_2) \\ & + \gamma H_1 T_2/(1 + \gamma^2 H_1^2 T_1 T_2), \end{aligned} \tag{1}$$

where $m_y(0)$, and $m_z(0)$ are projections of M_0 on the y- and z-axes, $m_{z\infty}$ is the equilibrium magnetization along the z-axis, γ is gyromagnetic ratio and H_1 is the observing field intensity. The bulk magnetization is the sum of all individual electron magnetic moments μ_e, $M_0 = n\mu_e$, where n is the net difference of the electron-spin population between the two spin states as determined by the Boltzmann distribution. The first term in Eq. 1 is free induction decay (FID), which can be supressed by modulation of the phase of the saturating pulse with respect to the phase in the observing arm (Huisjen and Hyde, 1974). The next term is saturation recovery. In the limit of small H_1, the recovery is characterized by the exponential constant T_1, the spin-lattice relaxation time. The third term is the response of the residual magnetization along the z-axis. To eliminate this term one can vary the intensity of the saturating pulse, resulting in different values of $m_z(0)$. Finally, the time-independent term, which does not affect the time evolution of magnetization, is a saturation factor.

The sensitivity of SR-EPR to motionally modulated spectral diffusion was first demonstrated theoretically and experimentally by Dalton and co-workers (Smigel et al., 1974; Hyde et al., 1975). The basis for this sensitivity can be explained qualitatively: a high-intensity saturating pulse perturbs the Boltzmann equilibrium of resonating spins, the deviation denoted by Δn_1. The equilibrium can be restored either by loss of energy to the lattice or by exchange with the nonresonant spin population Δn_2. The rate equations for the population deviations are

$$\begin{aligned} \frac{d\Delta n_1}{dt} &= -\frac{\Delta n_1}{T_1} + \frac{\Delta n_1 - \Delta n_2}{2\tau_{sd}} \\ \frac{d\Delta n_2}{dt} &= -\frac{\Delta n_2}{T_1} - \frac{\Delta n_1 - \Delta n_2}{2\tau_{sd}} \end{aligned} \tag{2}$$

where τ_{sd} is the exponential time constant for spectral diffusion from resonant to nonresonant field positions (in analogy with optical spectroscopy, this is the time required for the transfer of probes from a high to a low-absorption probability). This set of coupled differential equations can be solved to yield (Robinson et al., 1985)

$$\begin{aligned} \Delta n_1 &= a_1 \exp(-t/T_1) + a_2 \exp - t(\tau_{sd}^{-1} + T_1^{-1}), \\ \Delta n_2 &= a_1 \exp(-t/T_1) - a_2 \exp - t(\tau_{sd}^{-1} + T_1^{-1}). \end{aligned} \tag{3}$$

Thus, the approach to equilibrium in the presence of spectral diffusion is double-exponential, with the faster rate constant equal to the sum of T_1^{-1} and τ_{sd}^{-1}, and the slower rate constant equal to T_1^{-1}. Therefore, if $\tau_{sd} \leqq T_1$, saturation is transferred away from resonance. The saturation recovery experiments then become time-resolved saturation transfer experiments. The spectral diffusion time τ_{sd} can be estimated from the time it takes a molecule to diffuse away from resonance. To change the resonant field by more than the linewidth ΔH the molecule has to rotate by the angle

$$\theta_{sd} = \Delta H/(\partial H_{res}/\partial\theta), \tag{4}$$

where θ is the angle between the principal axis of the nitroxide and the external magnetic field. H_{res} is the resonant field given by $H_{res}(\theta) = h\nu - m_i A(\theta)/\beta g(\theta)$, where $g(\theta)$ and $A(\theta)$ are effective values of the g and nuclear hyperfine interaction tensors of the spin label at an angle θ. From the Debye theory of Brownian motion of a molecule rotating with correlation time τ_r, the time for the mean-square angular displacement θ_{sd}^2 is (Hyde and Dalton, 1981)

$$\tau_{sd}/\tau_r = (3\pi^2/8)\theta_{sd}^2. \tag{5}$$

$\partial H_{res}/\partial\theta$ varies significantly with spectral position; therefore, so do θ_{sd} and τ_{sd}. For the low- and central-field positions investigated here, $\partial H_{res}/\partial\theta = 20$ and 8.8 Gauss/rad, respectively (Thomas et al., 1976), which results in a τ_{sd}/τ_r ratio of 0.1 and 0.8 for these spectral positions. In transient optical anisotropy measurements, this ratio is always 1, due to much lower orientational resolution of optical anisotropy. (Thomas, 1986). This explains why optical techniques are usually limited to measuring $\tau_r \leqq$ the excited-state lifetime, whereas ST-EPR methods are sensitive to rotational correlation times considerably greater than T_1.

Of course, whether the spectral diffusion effects will be observed at all depends on the relative magnitude of the pre-exponential factors a_1 and a_2, which are determined by the initial conditions, i.e., on the distribution of saturation at the end of the pulse. This, in turn, is a function of pulse strength and duration, and of the rates of rotational diffusion and spin-lattice relaxation. As pointed out by Smigel et al. (1974), the maximum sensitivity to τ_{sd} is exhibited when the pulse is shorter than the relaxation time and spin-diffusion time, $t_p < T_1 \simeq \tau_{sd}$. In the case of very

slow diffusion, $\tau_{sd} \gg T_1$, there is no coupling between neighboring spin packets and the saturation decays with T_1. At the other extreme, when the diffusion is fast enough to equilibrate saturation among the spin packets during the pulse, i.e., $\tau_{sd} \ll t_p \simeq T_1$, the observed saturation is still relieved by the spin-lattice relaxation alone.

METHODS

Saturation Recovery

The apparatus used here has been described previously (Huisjen and Hyde, 1974; Percival and Hyde, 1975; Kusumi et al., 1982). Instead of using a bimodal cavity, a loop-gap resonator ($Q \simeq 500$) was employed, with the advantage of shorter ringing times ($t_{ring} = Q/2\pi\nu$) and a higher microwave power to magnetic field conversion ($H_1/P = 5$ Gauss/W) (Froncisz and Hyde, 1982). Reflected microwaves were amplified with a GAs-FET amplifier that was protected from the pumping microwaves by an additional PIN diode synchronized with the PIN diode in the pump arm of the bridge. The signal was recorded and averaged by a computer-controlled receiver processor (Forrer et al., 1980), with 512 apertures 40 ns in duration (80 ns for long recoveries). All data were obtained with 50 Hz pump-phase modulation for suppression of free-induction decay and 25 Hz magnetic field modulation for improvement of baseline stability (Huisjen and Hyde, 1974). Repetition rates were between 5 and 12.5 kHz.

Analysis

The curves were digitized using a digital plotter (model HP-7475A; Hewlett-Packard Co., Palo Alto, CA) interfaced to an HP-85 computer. The recoveries were subsequently analysed on a personal computer (model 150; Zenith Data Systems Corporation, St. Joseph, MI) using a nonlinear least-squares procedure capable of fitting to a sum of up to three exponential functions of the form $A_i \exp(-t/\tau_i)$ (Fig. 1). The number of exponential functions was increased until no further improvement could be obtained in the χ values and residuals. In all cases this was achieved with one or two exponential terms. Care was taken to ascertain that the fitted parameters were independent of the initial values.

In view of the very fast switching of PIN diodes (10 ns) no attempt was made to correct for the finite rise and decay times of the pumping pulse.

Sample Preparation

Human Hb was prepared from outdated blood (Squier and Thomas, 1986). Rotational correlation times for hemoglobin tumbling in glycerol-water mixtures at different temperatures were taken from Fajer and Marsh (1982). Samples were deoxygenated in their gas-permeable sample cells (TPX capillaries, 10-μl volume) for 30 min before the experiment (Popp and Hyde, 1981).

RESULTS

Typical magnetization recoveries for spin-labeled Hb tumbling in media of different viscosities are shown in Fig. 2. The two traces at each correlation time are the recoveries after short (0.5 μs) and long (>20 μs) saturating pulses. The initial recovery after the short saturating pulse is faster than in the case of the long pulse for the microsecond correlation times. After the first phase, the magnetization recovers with the same rate for both pulses, as confirmed by multiexponential data analysis. The short-pulse recovery can be described in terms of two exponentials, while the response to the long pulse is best simulated by a single-exponential function.

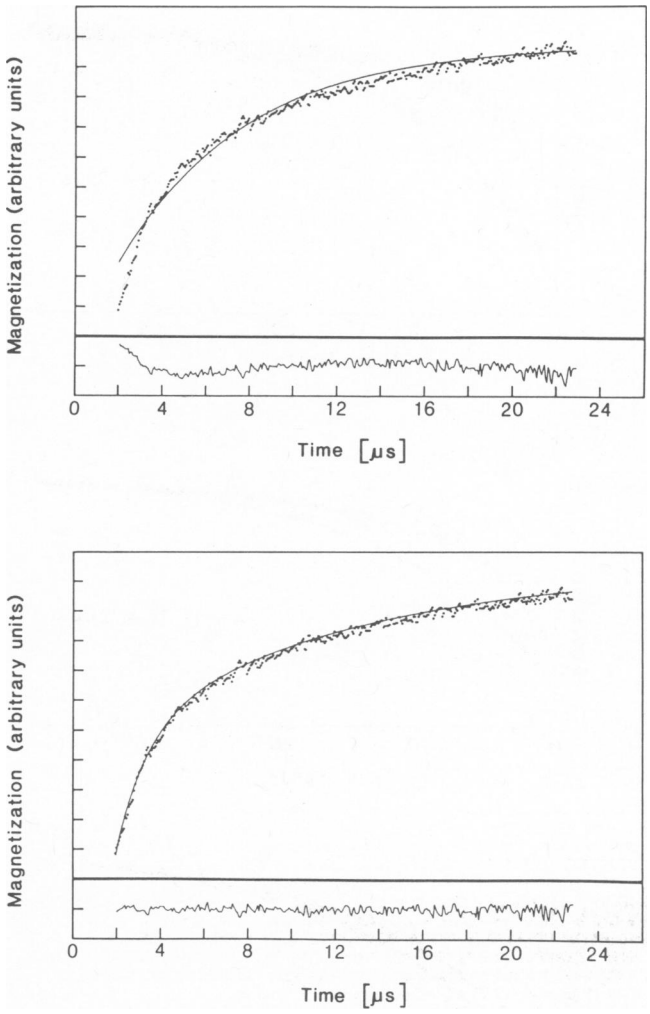

FIGURE 1 Exponential deconvolution of magnetization recoveries. (*Top*) Single-exponential fit with three variable parameters; τ, pre-exponential factor, and the baseline. (*Bottom*) Double-exponential fit with five variable parameters; τ_1, τ_2, pre-exponential factors, and the baseline. The bottom of each figure shows expanded (×5) residuals for each fit. The experimental recoveries (dotted line) in response to a 0.5-μs saturating pulse were obtained for the low-field spectral position of Hb diffusing with τ_r of 20 μs. The residuals indicate that the two-exponential fit is better.

Observing Power

The saturation-recovery signal decays as $\exp-(T_1^{-1} + \gamma^2 H_1^2 T_2)\, t$ in the absence of spectral diffusion. As the observing power approaches saturation, $\gamma^2 H_1^2 T_1 T_2 \simeq 1$, the recovery time becomes shorter than T_1. The true T_1 can be observed only in the limit of zero power (Atkins et al., 1973; Percival and Hyde, 1975). Fig. 3 displays observed recovery times (in response to long pulses) at two correlation times and at two resonant fields for $\tau_r = 20$ μs. Only in the case of recoveries at the central field for $\tau_r = 20$ μs was there a dependence on the observing power. All of the following experiments were performed at an observing field amplitude of 0.025 Gauss, where saturation is 13% (assuming $T_2 = 0.02$ μs).

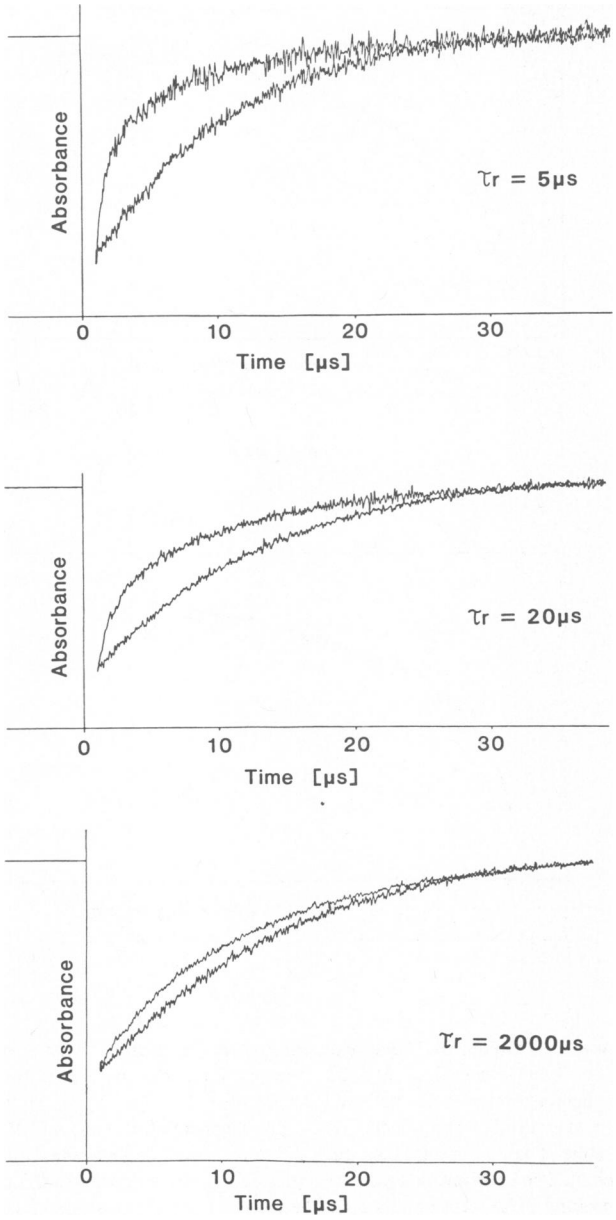

FIGURE 2 Magnetization recovery of spin-labeled haemoglobin in media of varying viscosity after short and long saturating pulses. (*Top*) Rotational correlation time τ_r = 5 μs; pulses 0.1 and 40 μs. (*Middle*) τ_r = 20 μs, pulses 0.5 and 40 μs. (*Bottom*) τ_r = 2,000 μs, pulses were 0.5 and 20 μs. The probed region was 8.4 Gauss upfield from the low-field peak of the first derivative absorption spectrum. The y-axis is in arbitrary absorbance units.

Pumping Power

Initial fast decays could, in principle, be due to residual magnetization along the z-axis remaining after the saturating pulse, which, in response to the observing field, yields the term $m_z(0)\exp(-t/T_2)$ (Percival and Hyde, 1975). If $m_z(0) \neq 0$ this term must be considered. To ascertain that the first phase of recovery is not a T_2 mechanism, experiments at various pumping powers were performed. The following observations argue against this possibility: (*a*)

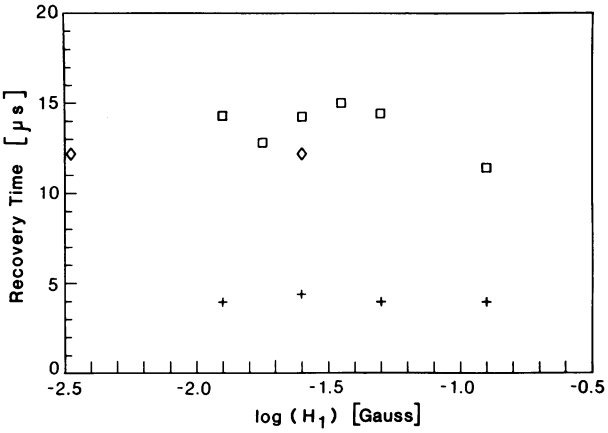

FIGURE 3 Recovery times after long saturating pulses as a function of observing field intensity. □, τ_r = 20 μs, central-field; ◇, τ_r = 20 μs, low-field; +, τ_r = 0.02 μs, central-field. Note that pulse fields smaller than 0.05 Gauss do not affect the recovery times.

For the shortest of employed pulses, 0.1 μs, a 30-fold decrease of the pumping field strength (corresponding to a change from $m_z(0) = -0.7M_0$ to $m_z(0) \simeq M_0$, where $m_z(0) = \cos(\gamma t_p H_p)$ did not affect the amplitude of the fast decay; (*b*) an increase of the pulse time to 7.5 μs with a concomitant decrease of pumping power resulting in a 148° rotation, $m_z(0) = -0.83\ M_0$, resulted in a single-exponential decay; and (*c*) the sign of the $m_z(0)$ term is opposite to the saturation-recovery signal, so this term should manifest itself as a decay rather than a recovery. However, all the observed fast initial recoveries had the same sign as that of the slow phase. These findings establish that even when $m_z(0) \simeq \pm M_0$, the T_2 decay is much too short to be observed.

Spin-Lattice Relaxation

The spin-lattice relaxation time (T_1) can be obtained from recoveries after long pulses, $t_p > \tau_{sd}$, which suppress spectral diffusion (Smigel et al., 1974; Freed, 1974). In the case of long correlation times, $\tau_{sd} \gg T_1$, the response to short pulses should also be characterized by T_1. The recovery times τ of single-exponential decays are shown in Fig. 4 as a function of rotational correlation times. τ_r for frozen or ammonium sulfate-precipitated Hb is taken as infinity. (The time of the second phase of recovery is taken for precipitated Hb.) The relaxation time increases monotonically from 4 to 16 μs with the correlation time increasing from 0.02 to 2,000 μs. It is a weak dependence; T_1 is proportional to $\tau_r^{0.12}$. Note, however, that the value of 7 μs obtained for both the frozen and precipitated sample are significantly lower than that for the Hb is 88% glycerol at −36°C. There was no temperature dependence of the recovery rate for the precipitated Hb over 24°C, implying that the difference is most likely to be due to the solvent, not to motional differences.

The single-exponential recoveries (long pulses) were not significantly affected by the resonant field position. Table I

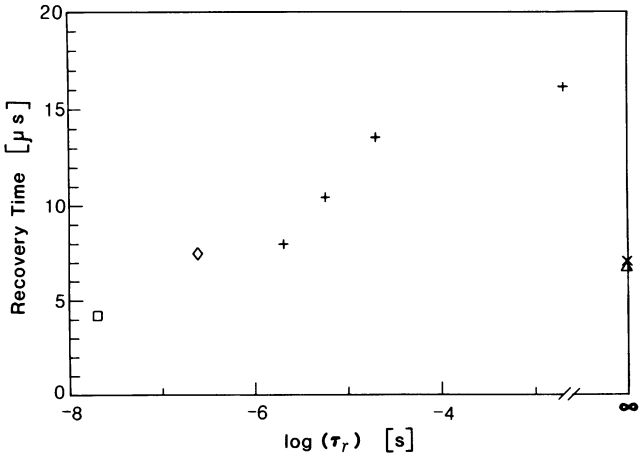

FIGURE 4 Spin-lattice relaxation time dependence on the rotational correlational time. The water–glycerol mixtures to obtain desired rates of diffusion were: □, 0% glycerol, 20°C; ◇, 37% glycerol, −4.0°C; +, 88% glycerol, 21°C, 10°C, −4°C, and −37°C; △, frozen H_2O, −20°C; ×, ammonium sulfate–precipitated hemoglobin, −5°C.

shows the average times of recovery at the low and central field for molecules with correlation times spanning five orders of magnitude. In all cases there is a good agreement between the values obtained at the two field positions; the same applies to ammonium sulfate precipitated Hb as observed previously for spin-labeled Hb (Huisjen and Hyde, 1974).

In addition to nuclear manifold invariance, we have found no orientational dependence of T_1. Spins contributing to the signal at the turning point in a powder spectrum (i.e., a spectrum of a randomly oriented sample) are oriented with their principal axes at angle $\theta = 0°$ with respect to the external field. The spins contributing to the spectrum 8.4 Gauss upfield from the low-field peak of the first harmonic absorption spectrum are oriented at $\theta = 75°$. The recoveries for these two positions were identical for both the fast and the slow phases of magnetization recovery of precipitated Hb.

TABLE I
SPECTRAL POSITION DEPENDENCE OF RECOVERY TIMES*

Rotational correlation time	Recovery time	
	Low-field‡	Central-field§
μs	μs	
0.02	4.6 (0.3)	4.1 (0.1)
2.00	7.7 (0.5)	8.2 (0.1)
20.00	13.8 (0.8)	13.5 (0.5)
2,000.00	16.1 (0.2)	16.2 (1.0)

*Average of single exponential recoveries.
‡Low field denotes resonant field 8.2 Gauss upfield from the low field peak of the V_1 powder spectrum.
§Central field denotes baseline crossing point of the V_1 powder spectrum.

Spectral Diffusion

Sensitivity of saturation recoveries to the molecular rotational rates is demonstrated in Fig. 2. For short correlation times and short pulses, the magnetization recoveries have two exponentials. As predicted by Eqs. 3 and 5, the time constant for the initial phase of recovery increases linearly with the rotational correlation time from 0.4 μs at $\tau_r = 5$ μs to 1.9 μs at $\tau_r = 20$ μs. For the very slow, millisecond motions, recoveries are characterized by single exponentials, irrespective of pulse duration. No initial fast decay was observed for this case. (The time constants of these one-exponential recoveries are, however, slightly dependent on the pulse length, probably reflecting small spectral diffusion effects.) For $\tau_r = 0.02$ μs and $\tau_r = 2{,}000$ μs the recoveries were single exponentials, as they were for Hb frozen in water, Table II.

Double-exponential decays were observed for the range of correlation times from 0.2 to 20 μs in response to short (0.1 μs) saturating pulses. The second, longer time constant, τ_2, agrees well with the single-exponential decay after long (40 μs) pulses, confirming that the slow phase of the short-pulse response is determined by T_1.

Unexpectedly, double-exponential recoveries were observed for ammonium sulfate-precipitated Hb. The time of the fast phase of recovery was between 0.4 and 1.8 μs, hence similar to the times observed in the presence of spectral diffusion due to rotational motion. However, there was an important difference: the short recovery time was independent of the pulse duration. The fast recovery was observed with pulses five times longer in duration than the recovery time. The recovery time of the first phase does not vary with the nuclear manifold, suggesting that the effect cannot be ascribed to nuclear spin flips.

Spectral diffusion can also be determined by the extent of pulse quenching. As shown in Fig. 5, for $\tau_r = 20$ μs, the decays are double exponentials with τ_1 constant at 2.0 ± 0.3 μs and τ_2 constant at 12.5 ± 1.5 μs for pulses not longer than 2.5 μs. For longer pulses, single-exponential recoveries were observed. The recovery time was slightly if significantly longer at 13 ± 1.6 μs. The poor signal-to-noise ratio prevented quantitative analysis of preexponential factors, which in principle should also be related to the ratio of spectral diffusion and spin-lattice relaxation (Robinson et al., 1985). At shorter correlation times, e.g., 0.24 μs, double exponentials were only observed with pulses no longer than 0.5 μs.

DISCUSSION

Spectral Diffusion

The behavior of magnetization observed in this study is consistent with the theoretical predictions for the competition between spectral diffusion and spin-lattice relaxation. As pointed out by Dalton and co-workers (Smigel et al., 1974), for pulses longer than the rotational correlation

TABLE II
MAGNETIZATION RECOVERY AT DIFFERENT ROTATIONAL CORRELATION TIMES*

Correlation time	Low field‡			Central field§		
	Short pulse‖		Long pulse¶	Short pulse		Long pulse
	τ_1	τ_2	τ	τ_1	τ_2	τ
μs	μs		μs	μs		μs
0.02			4.6 (0.3)			4.1(0.1)
0.24				0.6 (0.2)	8.2 (0.2)	7.5 (0.1)
2.00			6.9 (0.5)	1.0 (0.5)	9.0 (0.8)	8.1 (0.2)
5.00	0.6 (0.2)	6.8 (0.8)	9.7 (0.7)			
20.00	2.0 (0.5)	12.0 (0.5)	13.9 (0.9)	12.9 (0.3)		12.4 (0.2)
2,000.00	13.4 (0.2)		16.1 (0.2)	11.5 (1.4)		16.2 (1.0)
frozen						6.7 (0.5)

*Data digitized including noise.
‡Low field denotes resonant field 8.2 Gauss upfield from the low field peak of the V_1 powder spectrum.
§Central field denotes baseline crossing point of the V_1 powder spectrum.
‖Short pulse means pulses of 0.1 ~ 0.3 μs in duration.
¶Long pulse means pulses of 20 ~ 40 μs.

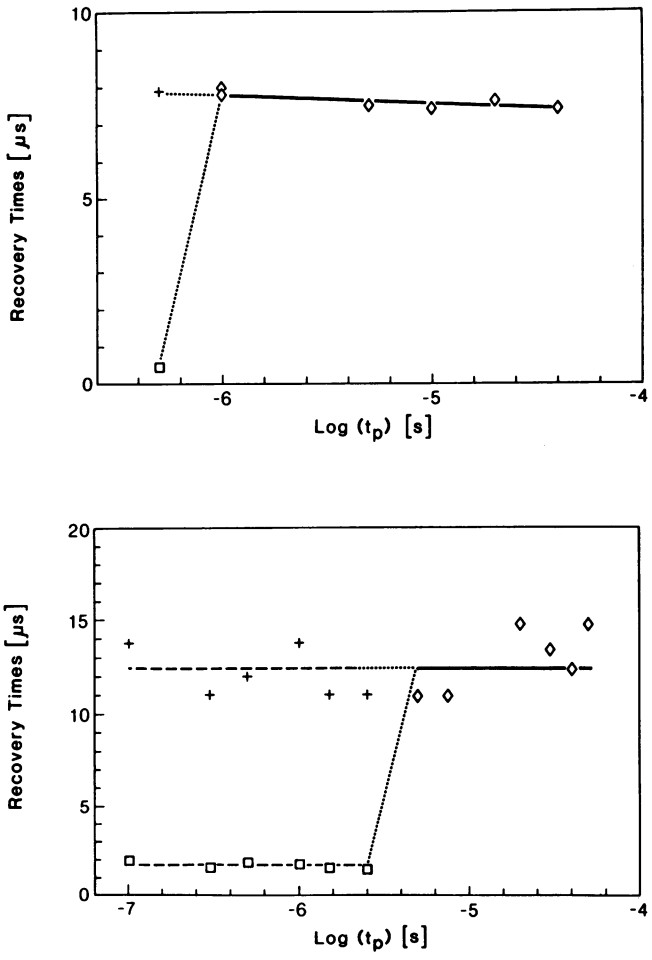

FIGURE 5 Dependence of the recovery rates on the saturating pulse duration. (*Top*) $\tau_r = 0.24$ μs central-field. (*Bottom*) $\tau_r = 20.0$ μs, low-field region. □, initial phase exponential time, (τ_1) of double-exponential recoveries; +, late phase recovery time (τ_2); ◇, τ of one-exponential curves.

time ($t_p > \tau_r$) spectral diffusion equilibrates the saturation within the pulse duration, and the recovery is characterized by a single exponential with a time constant equal to T_1. This is, however, not the case for pulses shorter than τ_r if $\tau_{sd} \simeq T_1$. In that case, the saturation is relieved by both the spectral diffusion and the relaxation, giving rise to double-exponential recoveries as in the two decays of Fig. 2. Finally, when $T_1 \ll \tau_{sd}$, the saturated spin packet is virtually isolated from nonresonant spins and the recovery time is T_1, as in the bottom of Fig. 2.

This immediately suggests two independent ways of measuring rotational motion. Firstly, the fast phase recovery is related to the spectral diffusion, $\tau_1^{-1} = \tau_{sd}^{-1} + T_1^{-1}$. The spectral diffusion time τ_{sd} is itself a function of spectral position and rotational correlation time. The other method of determining τ_{sd} is to vary the pulse length of magnetization recovery. The pulse length at which the number of exponentials changes from two to one should approximately define τ_{sd}.

Let us focus on the direct determination of τ_{sd} from the curve analysis (Table II). At short correlation times, the shortest available microwave pulse $t_p = 0.1$ μs is as much as an order of magnitude longer than τ_{sd}. Saturation is equilibrated during the pulse, and the recovery is solely determined by T_1. At long correlation times, the spectral diffusion rate is too small to be resolved from the other relaxation mechanisms, but it still has an effect on the rate of magnetization recovery.

Double-exponential decays were observed for the range of correlation times from 0.2 to 20 μs in response to 0.1-μs saturating pulses. The second, longer time constant (τ_2) agrees well with the single-exponential decay of long, 40 μs, pulses confirming that the slow phase of the short-pulse response is determined by T_1. The initial phase was determined with less accuracy, since the data collection

was delayed by 0.5 μs in most cases to avoid instrumental artifacts. Despite this problem, there is clearly a correlation between rotational motion and the rate of the initial decay. At any given spectral position, low or central field, the initial recovery was faster at shorter rotational correlation times. The observed times τ_{sd} at the central field are expected to be longer due to smaller angular dependence, and the failure to observe two-exponential decay at the central field for $\tau_r = 20$ μs is probably a direct consequence of the larger value of τ_{sd}/τ_r at this spectral position (see Eq. 5 and Table III).

In general, there is good agreement between initial decay time constants and the times predicted from the spectral diffusion times (Table III). Observed τ_1's were 0.6 (0.2) μs at $\tau_r = 5$ μs, the predicted time being 0.7 μs. At $\tau_r = 20$ μs τ_1 was 2.0 ± 0.7 μs, as compared to the predicted 2.5 μs. At the central field the times were 0.6 ± 0.3 and 0.9 ± 0.5 μs, comparing to 0.2 and 1.3 ms predicted from correlation times of 0.2 and 2.0 ms.

Fig. 5 illustrates the other approach, in which the recovery is studied as a function of pulse duration. For the pulses shorter than τ_{sd} one would predict double-exponential decays with times being independent of t_p, but with the relative proportion of the fast recovery decreasing as the pulse length approaches the spectral diffusion time (Robinson et al., 1985). For longer pulses, the recovery is a single exponential with the rate determined by spin-lattice relaxation, as discussed previously. The pulse length at which the transition between single and double-exponential recoveries takes place for $\tau_r = 20$ μs at the low-field position is $t_p = 2.5 \sim 5.0$ μs, which compares well with the τ_{sd} value of 3 μs (calculated from viscosity) and with the value of 2.3 μs obtained from the multiexponential analysis above. A similar agreement between the two experimental methods is observed at $\tau_r = 0.24$ μs, although both values were larger than that predicted from viscosity.

Spin-Lattice Relaxation

The strongest T_1-dependence on diffusion was registered for H_2O-glycerol solutions in which viscosity was varied by composition and temperature. T_1 for spin-labeled Hb was a very weak function of τ_r in the microsecond range in agreement with the results for TANOL (Hyde, 1978), and much weaker than for TANONE in sec-butyl benzene tumbling in the submicrosecond range. The difference reflects stronger coupling of spins to the lattice in the faster motional region. The rotational modulation of anisotropic g and hyperfine interactions contributes only 0.06% of the total relaxation rate (Popp and Hyde, 1981). The above change in T_1 represents probably an upper bound, since the frozen and precipitated samples exhibited less than twofold increase of T_1 over the value for Hb tumbling in water.

Importantly, the relaxation times were not dependent on the nuclear manifold nor on the spectral position within a manifold. The first observation is consistent with the data obtained for diffusing molecules and excludes nuclear relaxation as a possible mechanism determining the lifetime of the excited state of the nitroxide radical. The second finding establishes the scalar character of T_1 in the absence of rotational motion.

Whatever the mechanism is, the observation of a relatively small variation of T_1 confirms the tacit assumption in steady state saturation transfer EPR that the relaxation remains constant over the range of motions studied and hence, the lineshape is solely determined by τ_r. Nevertheless, the fourfold change in T_1 in the commonly used Hb model system should be taken into account when employing calibration curves for STEPR spectra analysis.

In summary, we have demonstrated that saturation recovery EPR can be applied as a time-resolved saturation transfer EPR and is useful for measuring quantitatively the rotational diffusion of spin-labeled biomolecules in the microsecond time range. The rotational rates can be measured in two ways, either by analysis of the fast recovery phase or by spectral diffusion quenching with pulses of varying duration. The method is most accurate in the measurement of τ_r values of the order of the spin-lattice relaxation time (~12 μs). Correlation times much shorter than T_1, down to the instrumental dead time (0.5 ~ 1.0 μs for the present instrument), are most accurately measured at spectral positions having minimum orientational resolution (e.g., the central region), while τ_r values an order of magnitude longer than T_1 can be measured most accurately at spectral positions having high orientational resolution (e.g., the low-field region). In fact, spectral diffusion effects are present even in the single-exponential decays at millisecond correlation times, and quantitative analysis should extend the application range of the method to this slow motional regime. It seems clear that this technique can be extended to the measurement of anisotropic or heterogenous motions in an analogy to time-resolved optical anisotropy measurements (Thomas, 1986). Although the optical methods should continue to offer superior sensitivity for the study of small amounts of material (Garland and Johnson, 1985), the greater orientational resolution of EPR, coupled with the time resolu-

TABLE III
EXPERIMENTAL AND PREDICTED CORRELATION TIMES

Spectral Position	τ_1*	τ‡	τ_{sd}§	τ_r (recovery)‖	τ_r (viscosity)
			μs		
Central field	0.6 (0.2)	7.5 (0.1)	0.65 (0.3)	0.7 (0.3)	0.24
Central field	1.0 (0.5)	8.1 (0.2)	1.14 (0.6)	1.2 (0.6)	2.00
Low field	0.6 (0.2)	9.7 (0.7)	0.64 (0.2)	4.3 (1.3)	5.00
Low field	2.0 (0.5)	13.9 (0.9)	2.34 (0.7)	15.6 (4.7)	20.00

*τ_1 from initial recovery after a short pulse.
‡τ from total recovery after a long pulse.
§τ_{sd} calculated from $(\tau_1^{-1} - T_1^{-1})^{-1}$.
‖τ_r (recovery) calculated from Eqs. 4 and 5, assuming 4 Gauss for ΔH, 20 and 8 Gauss/rad for $\partial H_{res}/\partial\theta$ for low- and central-field, respectively.

tion as reported in the present study, promises unequaled precision in the analysis of complex motions, particularly in oriented systems.

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