Saturation Transfer EPR Studies of Microsecond Rotational Motions in Biological Membranes

David D. Thomas

1. INTRODUCTION

In order to elucidate the molecular details of dynamic processes in biological membranes, it is necessary to make direct measurements of molecular motions and to correlate these motions with biological function. It has been largely in response to this need that nitroxide spin-labels and electron paramagnetic resonance (EPR) techniques have been developed. It was precisely in the study of membranes, however, that a crucial shortcoming of EPR became evident. The conventional EPR experiment on a nitroxide radical is sensitive to rotational motion only if it occurs in the nsec time range or faster. This time range is appropriate for the rapid rotational motions of fluid lipid hydrocarbon chains and protein side chains, and for the rapid tumbling of small proteins in aqueous solution. However, the relatively high effective viscosity of membranes (usually at least 100 times that of water) results in many motions in the μsec range or slower, particularly those of integral membrane proteins and gel-phase lipids. Partly as a result of this limitation of conventional EPR to the detection of nsec motions, an unbalanced description of membrane dynamics emerged, weighted heavily toward the lipids, despite the primary importance of proteins in determining membrane function.

The need for sensitivity to the slow rotational motions of membrane proteins was
a primary motivation for the development of saturation transfer EPR (ST-EPR), a method with maximum sensitivity to μsec rotational motions of nitroxide spin-labels. In the summer of 1972, Hyde and Dalton (1972) published the seminal paper in the saturation transfer literature, in which they described an EPR experiment (dispersion, detected under conditions of moderate saturation, with the 100 kHz phase-sensitive detector set 90° out-of-phase with respect to the applied 100 kHz field modulation, later referred to as $U_1$) that showed marked line-shape sensitivity to the μsec rotational motions of nitroxide radicals in a supercooled organic glass. H. McConnell, who had led the development of conventional EPR for membrane studies, became interested in using ST-EPR to study slow motions in membranes. Accordingly, he encouraged me, his graduate student, to choose ST-EPR as my thesis topic. I worked with Hyde on the experimental methodology, seeking procedures useful in biological studies (Hyde and Thomas, 1973), and with McConnell on the theory, seeking verification that the changes observed in experimental ST-EPR spectra of model systems were those predicted for rotational diffusion (Thomas and McConnell, 1974). The methodological developments were summarized in a paper in 1976 (Thomas et al., 1976), and applications of ST-EPR to membranes began to appear in print in 1977 (discussed below).

The primary purpose of the present review is to discuss the published applications of ST-EPR to membranes (as of December 1982), focusing on studies of membrane-bound enzymes. Because the technology of ST-EPR is new and developing, the first section is a condensed review of saturation transfer methodology, focusing on recent developments. In the sections that follow, the applications to membrane enzymes are discussed, followed by applications to other membranes.

ST-EPR has been reviewed comprehensively by Hyde and Thomas (1980), and in other papers mentioned in that article. Applications of ST-EPR to biological membranes have been briefly reviewed (Thomas, 1982). Applications of conventional EPR to biological membranes have been reviewed in the first edition of this volume (Gaffney and Lin, 1976; Vignais and Devaux, 1976). EPR studies of spin-labels have been reviewed comprehensively in two volumes edited by Berliner (1976, 1979), and were recently reviewed by Marsh (1981).

II. ST-EPR METHODOLOGY

A. General Principles of ST-EPR

ST-EPR experiments are performed on the same types of nitroxide spin-labels used in conventional EPR experiments (Figure 1). For both conventional and saturation transfer EPR, the sensitivity of the experiment to rotational motion arises from the high degree of orientational resolution in the nitroxide EPR spectrum. Due to anisotropic magnetic interactions, each position in the spectrum of a nitroxide radical corresponds to a narrow range of orientations of the spin-label relative to the applied D.C. magnetic field. Thus, scanning the spectrum is equivalent to scanning probe orientation, and rotational motion transfers spins from one part of the spectrum to another. If the minimum time required for this transfer (roughly equal to 0.1 τ, where
Figure 1. Typical nitroxide spin-labels used in ST-EPR studies. The probe most often used to monitor protein rotational motion is the maleimide derivative of TEMPO, usually designated MSL (N-[1-oxyl]-2,2,6,6-tetramethyl-4-pipeli-danyl]-maleimide). The five-membered pyrroldinyl derivative (designated 5MSL in this article) is also used. At or below pH 7, MSL reacts selectively with protein SH groups, and selectivity can be enhanced by blocking fast-reacting groups with diamagnetic maleimide derivatives (Hidalgo and Thomas, 1977) or by treating with ferricyanide (Graceffa and Seidel, 1980; Swanson et al., 1980). Selective reaction with a particular protein is not sufficient; the probe must be strongly immobilized, so that its spectra depend on large-scale protein motions, not on local probe motions. Lipid hydrocarbon chain motions are monitored by using fatty acid spin-labels like the one pictured. FASL (1,14) is a derivative of stearic acid with the nitroso attached at position 16, so it is often designated 16-SASL or 16-NS. The chain length and position of the nitroxide can be varied, and either the free fatty acid or its phospholipid derivative can be used. Maleimide derivatives, such as MSL (1,14), have been synthesized by Devaux and co-workers, for the purpose of probing the mobility of lipid chains adjacent to protein ( boundary lipid). Except for samples containing gel-phase lipids, these lipid probes usually undergo nsec motions in lipids, making spectral analysis more appropriate with conventional EPR than with ST-EPR.

\[ \tau \] is the rotational correlation time) is comparable to (or less than) the inverse of the frequency resolution in the spectrum (roughly equal to \( T_2 \), the transverse relaxation time), the spectrum will be narrowed by the rotational motion. For a typical nitroxide radical, \( T_2 \) is about 20 nsec, so motional narrowing occurs only when the rotational correlation time is less than 1 \( \mu \)sec. The term "conventional" EPR usually refers to an experiment that probes the linear response of a spin system, i.e., an experiment in which the microwave field intensity is low enough that saturation is negligible. Under these conditions, the spectrum of a nitroxide is determined almost exclusively by motional narrowing, and is thus sensitive only to rotational correlation times less than 1 \( \mu \)sec.

However, if the microwave radiation is intense enough to cause saturation, so that the response of the spin system is no longer linear, a rotation-dependent transfer of spins that occurs within a time comparable to the longitudinal relaxation time \( (T_1) \) will cause a transfer of saturation from one part of the spectrum to another. A rotation-dependent transfer of spins occurs within about 0.1 \( \tau \) (just as in conventional EPR), and \( T_1 \) is about 10 \( \mu \)sec for a slowly rotating nitroxide. Therefore, saturation transfer occurs whenever the rotational correlation time becomes less than 1 nsec; optimum sensitivity to \( \tau \) occurs in the range of 10-100 \( \mu \)sec, and saturation transfer becomes maximal when \( \tau \) is less than 1 \( \mu \)sec. The development of ST-EPR technology has been a process of searching for an EPR experiment that is optimally sensitive to saturation, hence sensitive to saturation transfer, and hence sensitive to \( \mu \)sec rotational motions.

B. Methodology Used in Most Published Applications

The early development of the instrumentation, experimental methodology, and theory of saturation transfer EPR was summarized in a paper by Thomas et al. (1976), and virtually all applications of ST-EPR have been based closely on the techniques
Figure 2. ST-EPR ($V'_2$) reference spectra (A) and parameter plot (B) obtained from MSL-labeled hemoglobin in aqueous glycerol solutions. These data, or equivalent data, have been used to analyze most $V'_2$ spectra to date. The rotational correlation time ($\tau_2$) was determined from the measured viscosity, temperature, and the known molecular volume (Thomas et al., 1976). The microwave power was set at 63 mW, resulting in an amplitude ($H_\text{rms}$) of 0.25 gauss for an aqueous sample at 20°C. Spectra are plotted so that their intensities are approximately equal. Adapted from Thomas et al. (1976) and Thomas (1978).
described in that paper. Those techniques are sometimes designated "passage" ST-EPR, because they are based on the use of high-frequency (50 kHz or 100 kHz) field modulation to pass the saturated spin system rapidly through resonance. In the absence of saturation (in conventional EPR), the spin system follows the modulation faithfully, but in the presence of saturation, the spin system lags behind the field modulation (if the modulation is rapid relative to the relaxation rate $1/T_1$), and a signal is observed 90° out of phase with respect to the modulation. By observing this out-of-phase signal, the investigator observes selectively a signal that depends on saturation, and hence on saturation transfer. In general, saturation transfer will decrease the saturation at the resonance position, and hence will decrease the amplitude of the out-of-phase signal. In addition, because rotation causes more saturation transfer in some regions of the spectrum than others, rotational motion changes the shape of the spectrum (Thomas et al., 1976). In order to assess the utility of different instrumental approaches for detecting saturation transfer, aqueous glycerol solutions of spin-labeled hemoglobin (for which the rotational correlation times were known) were used to generate reference spectra. Two modes of detection and display were found to be particularly sensitive, in both amplitude and shape, to rotational motion. One was $U_1'$ (dispersion, first harmonic, out-of-phase), using 100 kHz field modulation and detection, the method introduced in the first ST-EPR paper by Hyde and Dalton (1972). The $U_1'$ display, however, was not easily applied to biological systems, because its low signal-to-noise
prevented the study of spin-label concentrations below about 1 mM. The second display discussed in detail by Thomas et al. (1976) was \( V'_2 \) (absorption, second harmonic, out-of-phase), using 50 kHz field modulation and 100 kHz detection, the method introduced in the first biological application of ST-EPR by Hyde and Thomas (1973). Although the \( V'_2 \) spectrum was found to be more complex in shape and more difficult to analyze theoretically, it offered a much higher signal-to-noise ratio than \( U'_1 \), permitting experiments on relatively dilute (as low as 10 \( \mu \)M in nitroxide) aqueous samples. To permit a quantitative analysis of \( V'_2 \) spectra, Thomas et al. (1976) plotted selected spectral peak ratios against rotational correlation time for the hemoglobin model system. These plots were verified by computer simulation of the spectra, using the diffusion-coupled Bloch equations of Thomas and McConnell (1974). These reference spectra and plots (Figure 2) have been used to analyze most of the published ST-EPR spectra of spin-labeled membranes. The remainder of this section will briefly outline some of the recently proposed advances in ST-EPR technology that will probably be used in future applications.

C. Recent Developments in ST-EPR Methodology

1. The \( V'_2 \) Experiment

Most methodological developments have focused on refining the \( V'_2 \) method, attempting to improve the precision and accuracy of measurements and to avoid ambiguities in interpretation of data. The \( V'_2 \) experiment is performed under conditions (saturation, high modulation amplitude, out-of-phase detection) that make it more sensitive than \( V'_1 \) to slow rotational motion, but also increase the sensitivity to other experimental variables, such as microwave power, \( T_1 \), modulation amplitude, modulation phase, and the size, shape, and dielectric constant of the sample. Changes in these variables can cause spectral changes similar to those caused by motional changes, so these experimental conditions must be carefully controlled.

One of the most important parameters to be controlled and monitored is the amplitude of the microwave field (\( H_1 \)) at the sample, since an increase in \( H_1 \) can cause spectral effects similar to those of a shorter correlation time (Thomas et al., 1976). It is crucial, therefore, to perform experiments at the same value of \( H_1 \) if their spectra are to be compared. The reference spectra of Thomas et al. (1976) were recorded at a microwave power of 63 mW, chosen so that \( H_1 \) was 0.25 gauss for an aqueous sample at room temperature, as determined from saturation studies on a sample with known saturation properties. Subsequently, some workers have used the same power setting (63 mW) without calibrating the instrument to determine \( H_1 \) accurately. While the \( V'_2 \) spectra are still sensitive to \( \mu \)sec rotational motions, correlation times determined with the reference spectra of Thomas et al. (1976) are not likely to be accurate unless the same \( H_1 \) values are used. A more subtle problem arises from the variation of dielectric constant (and hence \( H_1 \)) caused by the variation in temperature and glycerol concentration in the hemoglobin solutions used to obtain the reference spectra, or caused by the variation in lipid and protein content in membrane samples. Kusumi et al. (1980) and Fajer and Marsh (1982) developed procedures for monitoring \( H_1 \) and
adjusting the microwave power to compensate for changes in dielectric constant, and published reference spectra obtained at constant $H_1$. Fajer and Marsh (1982) also pointed out that the use of very small samples (5 mm long or less) reduces problems due to field inhomogeneities within the EPR cavity. Considering the high sensitivity of $V_2'$ spectra to instrumental parameters, it is advisable in each laboratory to prepare spin-labeled hemoglobin as a standard and to produce reference spectra (Figures 1 and 3).

A number of investigators have explored other ways of parameterizing $V_2'$ spectra, besides those suggested by Thomas et al. (1976). One of the most striking features of the $V_2'$ spectrum is that a correlation time of 10 $\mu$sec or greater results in a spectrum that is entirely above the baseline, while a correlation time of 100 nsec results in a spectrum that is nearly symmetrical about the baseline (having equal areas above and below the baseline). Evans (1981) used a digital computer to quantitate this observation, showing that the integral of the $V_2'$ spectrum, appropriately normalized, can be used to estimate the effective rotational correlation time (illustrated in Figure 3). Although integration might obscure some features of the line shape that are sensitive to the details of the motion, Evans (1981) showed that it suppresses spectral components corresponding to weakly immobilized spin-labels ($\tau < 10^{-8}$ sec), thus permitting the selective study of strongly immobilized labels ($\tau > 10^{-7}$ sec).

Although most methods of estimating correlation times from $V_2'$ spectra have

Figure 3. Conventional ($V_1'$) and ST-EPR ($V_2'$) reference spectra obtained from MSL-labeled hemoglobin in aqueous glycerol solutions. The spectra on the left are from a sample in 50% glycerol at $-12^\circ$C, corresponding to a $\tau$ value of $10^{-8}$ sec (Thomas et al., 1976). The spectra on the right are from a sample in 90% glycerol at $-32^\circ$C, corresponding to a $\tau$ value of $10^{-4}$ sec (Thomas et al., 1976). Power was adjusted so that $H_1$ was 0.25 G, using the method of Fajer and Marsh (1982). $V_2'$ (out-of-phase) spectra are normalized to correspond to the same number of spins (by dividing by the double integral of $V_2$) so that the effects of motion on both shape and absolute amplitude are illustrated. $V_2$ (in-phase) spectra are shown at a gain ten times less than $V_2'$. The most commonly used parameter is $L(V_2')/L(V_2)$, which measures the change in shape of the spectrum (Thomas et al., 1976). However, both $L(V_2')$ and $L(V_2)$ increase with $\tau$. It is clear that greater sensitivity to $\tau$ is achieved if the parameter $L(V_2')/L(V_2)$ is used, since the $V_2$ spectrum is relatively insensitive to motion (and has higher S/N). Plots of this parameter vs. $\tau$ are currently used in the author's laboratory to determine effective $\tau$ values with greater precision than previously possible (Squier and Thomas, to be published). Maximum precision can be obtained by measuring the normalized integral of $V_2'$ (bottom), as proposed by Evans (1981), although information about possible anisotropic motion is lost.
been based on changes in the shape of the spectrum, the overall intensity of the spectrum is affected as well. All points in the \( V_2' \) spectrum decrease in intensity as rotational motion increases, while \( V_2 \) (in-phase) changes very little with motion (Figure 3). Thus, the ratio of two peaks in \( V_2' \) is less sensitive to motion than the ratio of a peak in \( V_2' \) to a peak in \( V_2 \). This principle is illustrated in Figure 3, using spectra from spin-labeled hemoglobin.

In addition to spectral integration, several other digital manipulations of \( V_2' \) spectra have been proposed. Hemmingsa and De Jager (1981) showed that a “magnitude” ST-EPR spectrum, equal to the root-mean-square of the digitized \( V_2 \) and \( V_2' \) spectra, is sensitive in shape to \( \tau \) and has the advantage of being insensitive to errors in setting the reference phase on the phase-sensitive detector. Another group (Watanabe et al., 1980, 1982; Sasaki et al., 1980) has used a transient recorder and a Fourier transform to detect \( V_2 \) and \( V_2' \) digitally, without the need for the standard analog phase-sensitive detector.

Several methods have been used recently to obtain increased resolution in ST-EPR studies, including the use of \( ^{15}\text{N} \)- and \( ^{2}\text{H} \)-substituted spin-labels (Beth et al., 1981b; Johnson et al., 1982b) and a higher microwave frequency (Johnson and Hyde, 1981).

2. Other ST-EPR Methods

As mentioned above, the \( U_1' \) (out-of-phase dispersion) experiment has not been used much in biological experiments, because of the high noise levels observed when using a conventional resonant cavity. Recent studies using alternative cavity structures have reduced these noise levels significantly (Maier et al., 1980; Francisz and Hyde, 1982). The most promising of these devices is the “loop-gap resonator” (Francisz and Hyde, 1982), which yields \( U_1' \) spectra having better signal-to-noise ratios than can be achieved with \( V_2' \) spectra. Considering the simpler data analysis possible with \( U_1' \), these devices could make \( U_1' \) the ST-EPR experiment of choice in the near future. As discussed below, steady-state ST-EPR experiments (\( V_2' \) and \( U_1' \)) suffer from some ambiguity in interpretation, especially when motions are anisotropic. Time-resolved measurements, such as saturation recovery or pulsed electron double resonance, should remove much of this ambiguity, as in the case of time-resolved fluorescence.

3. Theoretical Analysis

Although the dependence of ST-EPR line shapes on rotational motion can be qualitatively understood by making physical arguments as discussed above and in other papers (Thomas et al., 1976; Thomas, 1978; Hyde and Thomas, 1980), the quantitative analysis of spectra requires spectral simulation by computer. Recent work has focused on extending the theory beyond the model of isotropic Brownian motion (Thomas and McConnell, 1974; Thomas et al., 1976) to consider the types of anisotropic motions more likely to occur in biological systems, particularly in membranes. Simulations are even more important for anisotropic motion than for isotropic, since it is much more difficult to find experimental model systems for generating reliable reference spectra (Gaffney, 1979). In general, as in the case of conventional EPR, spectra depend not
only on the rates (diffusion coefficients) and amplitudes of motions, but also on the orientation of the nitroxide relative to the axes of diffusion. If, for example, the principal nitroxide axis is approximately parallel to the axis of diffusion, the effect on the spectrum will be much less (the effective \( \tau \) value, using isotropic motion reference spectra, will be greater) than if it is nearly perpendicular. Robinson and Dalton (1980, 1981) have simulated ST-EPR spectra (mainly \( U_1' \)) corresponding to the unrestricted motion of rigid ellipsoids of revolution, tumbling in solution. Lindahl and Thomas (1982) have considered the effects of restricted-amplitude motions on \( V_2' \) spectra, reporting that restriction of the angular amplitude of motion, without a change in the rate, can cause a substantial increase in the effective \( \tau \) (obtained from isotropic reference spectra). As in the case of conventional EPR, extremely anisotropic motions can sometimes be recognized directly from the ST-EPR line shape, that is, there is no isotropic motion that could give rise to the same shape. This is equivalent to the observation that different spectral parameters (L/L, C/C, H/H) yield different effective correlation times when analyzed with parameter plots obtained with isotropic motion reference spectra. However, because of the lack of motional narrowing effects in the \( \mu \)sec time range, line-shape changes are usually more subtle and less uniquely interpretable for ST-EPR than for conventional EPR, so the interpretation of spectra is more ambiguous. In most cases, it remains necessary to compare the observed spectrum with reference spectra corresponding to isotropic motion (or some other model) and to refer to the resulting \( \tau \) value as an effective correlation time. Caution must be used when comparing these effective \( \tau \) values from different systems or in the same system under different conditions, since changes in \( \tau \) can be due to changes in the rate of motion, angular amplitude of motion, or probe orientation. Spectra obtained from oriented membranes can help reduce this ambiguity by defining the orientation of the spin-label relative to the membrane (Sasaki et al., 1982). Time-resolved ST-EPR, e.g., pulsed saturation-recovery experiments, should also be important in reducing the ambiguity of data interpretation.

III. MEMBRANE-BOUND ENZYMES

A. Sarcoplasmic Reticulum Calcium Transport ATPase

The first membrane enzyme to be studied by ST-EPR was the Ca-ATPase of sarcoplasmic reticulum (SR). This 100,000-dalton integral membrane protein pumps \( \text{Ca}^{2+} \) across the membrane (into the SR) to relax the muscle, in a reaction that is coupled to ATP hydrolysis and requires \( \text{Mg}^{2+} \). As in the case of most other membrane systems, previous physical studies (including EPR) had focused almost exclusively on the lipids, resulting in proposals that enzyme activity depends on a fluid lipid environment (Warren et al., 1974; Hidalgo et al., 1976). A series of ST-EPR studies have been carried out to investigate the rotational dynamics of both proteins and lipids, and to determine whether these motions are correlated with enzymatic activity in this system (Thomas and Hidalgo, 1978; Hidalgo et al., 1978; Thomas et al., 1982; Squier, 1982), and independent studies have been carried out by others (Kirino et al., 1978; Kaizu et al., 1980; Pringle and Hidalgo, 1982). As in most other ST-EPR studies, the probe
chosen to study protein motion was a maleimide spin-label (MSL). Hidalgo and Thomas (1977) developed a procedure for labeling the Ca-ATPase in SR specifically with this probe, with only a slight effect on enzymatic activity (Thomas et al., 1982). The next step was to determine whether the spin-label was rigidly bound to the protein, enough so that the $V_2'$ spectrum could be used to characterize the protein's overall rotational motion. This question often arises in ST-EPR studies, and Figure 4 shows how it was addressed (Thomas and Hidalgo, 1978). The conventional ($V_1$) spectrum of SR vesicles (top left) is characteristic of "strongly immobilized" probes, implying that there are no significant nsec rotational motions. The $V_2'$ spectrum (Figure 4A, right) has a shape characteristic of μsec rotational motion. The value of $L'/L$ is 0.75, corresponding to an effective rotational correlation time of 60 μsec (using Figure 2). This was the first measurement of the rotational motion of this protein, and it was later confirmed using

![Figure 4](image)

Figure 4. Conventional ($V_1$) and ST-EPR ($V_2'$) spectra of MSL attached to the Ca-ATPase of SR at 4°C. (A) A suspension of membrane vesicles ($L'/L = 0.75$, $\tau = 60$ μsec). (B) A membrane pellet ($L'/L = 0.75$). (C) Vesicles treated with 80 mM glutaraldehyde for 10 min ($L'/L = 0.91$). Gels showed that the proteins were partially crosslinked, with about half of the Ca-ATPase remaining monomeric. (D) SR vesicles treated with 80 mM glutaraldehyde for 12 hr ($L'/L = 1.1$). Gels showed that the proteins were completely crosslinked. The $V_1$ spectra were unchanged by crosslinking, implying that the inhibited motions are in the μsec time range. From Thomas and Hidalgo (1978).
optical spectroscopy. The spectra are unaffected by pellet formation (Figure 4B), implying that the observed motion is not that of the membrane vesicles themselves. Protein–protein crosslinking with glutaraldehyde does inhibit the motion (Figure 4C and D). The most likely interpretation of these observations is that the effective correlation time of 60 µsec characterizes the overall rotational motion of the protein within the membrane at 4°C. However, since glutaraldehyde can cause intramolecular as well as intermolecular crosslinking, the glutaraldehyde-sensitive motions could also be slow internal protein motions.

Kirino et al. (1978) labeled SR by a similar procedure and published a $V_2$ spectrum taken at 2°C (Figure 5) that was similar to that obtained by Thomas and Hidalgo (1978) at 4°C (Figure 4A). However, other data reported by Kirino et al. (1978) indicated somewhat slower motion than reported by Thomas and Hidalgo (1978), and it is not clear whether this was due to differences in instrumentation, sample preparation, or data interpretation. One clear difference was the appearance of a weakly immobilized component in the spectra of Kirino et al. (1978) that was not present in the spectra of Thomas and Hidalgo (1978). This component tends to cause errors in the direction of longer effective correlation times.

Having established a method for detecting the µsec rotational motions of the Ca-ATPase, the investigators in these two groups proceeded to vary conditions and to correlate the protein motions with lipid motions and enzymatic activity. The motion of the protein probe (MSL) was shown to increase with temperature, having a temperature dependence qualitatively similar to that of Ca-ATPase enzymatic activity (Thomas and Hidalgo, 1978; Kirino et al., 1978; Kaiu et al., 1980). However, the presence of weakly immobilized probes in some preparations (Kirino et al., 1978; Kaiu et al., 1980) appeared to cause artifactual increases in the effective $\tau$ as the temperature increased above 20°C.

Hidalgo et al. (1978) studied the effect of gel-phase lipid (dipalmitoyl phosphatidyl choline at 4°C) on the enzyme's activity and rotational mobility. When the endogenous SR lipids were replaced by DPPC (yielding a preparation designated DPL-ATPase), conventional EPR spectra of a stearic acid spin-label (Figure 5, left) showed that lipid hydrocarbon chain mobility was greatly decreased, to the point where motion was

Figure 5. Conventional ($V_1$) and ST-EPR ($V_2$) spectra of a stearic acid spin-label (having the doxyl group attached to C5) in membranes containing the purified Ca-ATPase and either endogenous SR lipids (top) or DPPC (bottom), at 4°C. Spectra are 100 G wide. Note that $V_1$ is affected by the lipid change, implying that micro motions are decreased. However, since the $V_1$ spectrum of DPL-ATPase has the characteristic shape of "strongly immobilized" spin-labels, the motion in this case is too slow to be detected by $V_1$ ($\tau > 10^8$ sec), so $V_2$ must be used to determine the effective $\tau$ (10^8 sec, using Figure 1). From Hidalgo et al. (1978).
detectable only by saturation transfer EPR (Figure 5, right). The effective rotational correlation time (using Figure 2) is 100 μsec. These were the first published ST-EPR spectra of lipid spin-labels, and they demonstrate the utility of this technique for studying strongly restricted lipid motions, particularly in the gel phase.

Figure 6 shows that the rigid lipid environment in DPL-ATPase strongly inhibits protein rotational mobility. L*/L increases from 0.75 (in SR lipid, top left) to 1.13 (in DPPC, center left), corresponding to at least a ten-fold increase in the effective rotational correlation time (using Figure 2). It is not clear whether this decrease in protein mobility is due directly to the decrease in microviscosity or to protein aggregation that might accompany lipid gel formation. Note that the protein motion (Figure 6) is considerably slower than the lipid motion (Figure 5) in each preparation. The decreased protein mobility in DPL-ATPase is accompanied by a strong inhibition of enzymatic activity. The addition of the detergent Triton X-100 to DPL-ATPase fluidizes the lipid environment (Hidalgo et al., 1978), reversing the inhibition of both protein mobility (Figure 6, bottom left) and enzymatic activity. Similarly, lipid fluidity, enzymatic activity, and protein mobility are all restored in DPL-ATPase above the lipid phase transition temperature (Hidalgo et al., 1978). Finally, enzymatic activity and protein mobility are both inhibited by the addition of high (10 mM) Ca²⁺ to SR (Figure 6, top), or to Triton-treated DPL-ATPase (Figure 6, bottom), even though high Ca²⁺ has no detectable effect on lipid chain mobility (Hidalgo et al., 1978). In summary, enzymatic activity shows a remarkable correlation with protein rotational mobility, and the requirements of the enzyme for a fluid lipid environment and for submillimolar Ca²⁺ may arise from the requirement for protein mobility.

In recent ST-EPR studies on SR, reduction of the lipid–protein ratio, achieved by extraction with deoxycholate, has been used as a means of reducing the enzyme's rotational mobility (Thomas et al., 1982; Squier, 1982). In the first study (Thomas et al., 1982), it was found that reducing the lipid–protein ratio by 55% results in a substantial reduction of the protein mobility (monitored by ST-EPR of MSL attached

![Figure 6](image-url)
to the Ca-ATPase), an increase in the fraction of restricted lipid chains (monitored by conventional EPR on a freely diffusing fatty acid spin-label; see Figure 1), and a decrease in the mobility of the lipid chains adjacent to the protein (monitored by conventional EPR of MSL (1,14); see Figure 1). The reduced protein mobility most likely arises from increased protein–protein interactions in the crowded membrane, which in turn may play a role in the increased restriction of lipid chain mobility. In another study (Squier, 1982), the effect of this decreased protein mobility on enzymatic activity was studied over a wide range of lipid–protein ratios. ST-EPR spectra showed that reducing the lipid–protein ratio below a critical level of about 40 lipids per protein reduces protein rotational mobility, and that the fractional decrease in protein mobility (1/r) correlates well with the fractional decrease in Ca-ATPase activity. Since it had been previously shown that delipidation inhibits phosphoenzyme decomposition (Warren et al., 1974), the step most likely to be associated with transport, this result provided further evidence that protein mobility (or at least protein disaggregation) is required for a crucial step in the Ca\(^{2+}\) transport process.

B. Mitochondrial Electron Transport Chain

Although the difficult study of protein motions in mitochondrial membranes is in its early stages, questions about protein motion and function seem especially important in the electron transport chain, the function of which involves the dynamic interaction of several integral enzyme complexes. While most probe studies of membrane proteins have been done on systems having one predominant protein, facilitating selective labeling of the protein in native membranes, the complex composition of mitochondrial membranes has required the purification of the enzymes before spin-labeling with MSL.

Swanson et al. (1980) carried out an ST-SPR study of purified cytochrome c oxidase (complex IV, ferrocytochrome c : oxygen oxidoreductase, EC 1.9.3.1), the terminal component of the electron transport chain, from beef heart mitochondria. These workers purified the enzyme using detergent (either deoxycholate or Triton), spin-labeled the protein with MSL (using ferricyanide and cysteine to eliminate signal from weakly immobilized spin-labels, as suggested by Graceffa and Seidel, 1980), and recombined the purified enzyme with lipid to increase the lipid–protein ratio. Spin-labeling did not affect enzyme activity, and the lipid addition reconstituted the full enzyme function (including respiratory control). \(V_2'\) spectra of MSL were used to assess protein mobility, \(V_1\) spectra of fatty acid spin-labels were used to monitor lipid chain mobility, and \(V_1\) spectra of MSL (1,14) were used to monitor the mobility of lipid chains adjacent to the protein. The \(V_2'\) spectrum of the monomeric enzyme in detergent solution and of glutaraldehyde-crosslinked membranes both indicated that the spin-label was rigidly bound, reporting the overall motion of the protein. Membranes with a low lipid–protein ratio showed low protein mobility and low lipid mobility, as in the case of the Ca-ATPase (discussed above). However, in contrast with the results of the studies on Ca-ATPase motion, it was found that a high fluid lipid–protein ratio was not always sufficient to produce \(\mu\)sec protein rotational mobility. One method of preparation resulted in high protein mobility (\(L''/L = 0.65\), effective
$\tau = 40 \mu\text{sec}$, in agreement with an independent study by Ariano and Azzi, 1980), while another resulted in much lower mobility ($L^2/L > 1$, implying little or no submillisecond motion; Figure 7). Thus, cytochrome oxidase appears to have a greater tendency to aggregate than does the Ca-ATPase. Since the two cytochrome oxidase preparations were both found to have high enzyme activity, the activity of this enzyme does not appear to require submillisecond rotational mobility, in contrast with the Ca-ATPase (discussed above). The nsec mobility of MSL (1,14) did correlate with $\mu\text{sec}$ protein mobility, suggesting that the protein aggregation that decreases protein mobility also decreases boundary lipid mobility.

Cytochrome $c$ reductase (complex III of the electron transport chain in beef heart mitochondria, ubiquinol : cytochrome $c$ oxidoreductase, EC 1.10.2.2) has been studied by ST-EPR (Quintanilha et al., 1982). Spin-labeling with MSL resulted in a $V_1$ spectrum that indicated only strongly immobilized probes, so treatment with ferricyanide was unnecessary. The $V_2$ spectra showed relatively high protein mobility in solution ($\tau = 400 \text{nsec}$) and in membranes containing a high lipid–protein ratio ($\tau = 70 \mu\text{sec}$). Since this preparation was treated essentially as the cytochrome oxidase preparation that showed much slower protein motion, this result suggests that cytochrome reductase

*Figure 7. ST-EPR ($V_1$) spectra of purified (left) and reconstituted (right) cytochrome $c$ oxidase, at $4^\circ\text{C}$. Top and bottom rows show the results of two different preparation procedures, starting from the same MSL-labeled enzyme preparation. From Swanson et al. (1980).*
has a lower tendency to self-aggregate. The boundary lipid probe MSL (1,14) was also mobile, consistent with unaggregated protein.

An ST-EPR study on both cytochrome c reductase (complex III) and NADH-ubiquinone oxidoreductase (complex I) has been performed by Poore et al. (1982). They recombined both enzymes in the same membrane with varying lipid content, and then measured protein mobility (with ST-EPR), lipid chain mobility (with conventional EPR), and enzymatic activity. They found μsec protein mobility at high levels of fluid lipid, and much slower motion when the lipid–protein ratio was low or when the lipid was in the gel state. Enzyme kinetics showed an interesting correlation with molecular dynamics, providing insight into models for electron transport function.

C. Cytochrome P-450

Cytochrome P-450 (EC 1.14.14.1) from rabbit liver microsomes has been studied by ST-EPR (Schwarz et al., 1982a). As in the case of mitochondrial membranes, this is an interesting system in which to study the interaction of different membrane-bound enzymes. In these microsomes, the two major enzymes are P-450 and its reductase, and alternative structural models have been proposed in which the enzymes diffuse freely or are in a specific and rigid cluster. These workers used SMSE to label either intact microsomes or a water soluble purified enzyme designated P-450 LM2. The labeling reaction in microsomes was 70% specific for P-450, and NADPH-dependent reduction of the enzyme was still possible after labeling. Conventional (V′) EPR spectra indicated that the probe was strongly immobilized at 20°C, ST-EPR (V′) spectra of the microsomes (Figure 8) were unaffected by pelleting, and glutaraldehyde produced a V′ spectrum in which L''/L = 1.2 (τ > 1 μsec). Thus the V′ spectra appeared to reflect protein motion within the membrane. The V′ spectrum of the soluble enzyme indicated an effective τ of about 220 nsec (C''/C = −0.7), much longer than predicted for a monomer, and consistent with previous studies showing the soluble enzyme to be a hexamer. The effective τ in microsomes was 480 μsec (L''/L = 1.0), much slower than has been observed for most other integral membrane proteins in fluid lipids.

![Figure 8](image_url)

**Figure 8.** ST-EPR (V′) spectra of SMSE-labeled cytochrome P-450 at 20°C. (a) The water-soluble P-450 LM2 in buffer solution. (b) Suspension of microsomes. (c) Pellet of microsomes, obtained by centrifugation at 100,000 g for 1 hr. (d) Glutaraldehyde-treated microsomes. From Schwarz et al. (1982a).
authors concluded that the enzyme is probably in an aggregated state in the membrane. Although this conclusion seems plausible, it was based on assuming an effective microviscosity of ten poise and using the Stokes–Einstein equation (which applies to an isotropic fluid) to estimate the predicted $\tau$ for a monomer (21 $\mu$sec). Besides the large uncertainty in predicting the rotational diffusion coefficient of a membrane protein from theory, there is the added uncertainty in the relative orientation of the probe’s principle axis and the axis of diffusion, which determines the relationship between the diffusion coefficient and the effective correlation time. In short, it is difficult to attach much quantitative significance to the absolute value of an effective correlation time determined from a $V_2'$ spectrum of a membrane protein. The conclusion that proteins are aggregated would be much more convincing if spectra could be obtained for comparison under conditions where the proteins are more mobile at the same microviscosity, as was done for cytochrome oxidase (Swanson et al., 1980) and for acetyl choline receptor (discussed below).

Schwarz et al. (1982b) have demonstrated that K$_3$Fe(CN)$_6$ may be used to selectively eliminate spectral components due to weakly immobilized spin-labels in P-450, for purposes of simplifying the interpretation of $V_2'$ spectra. This is a reversible line-broadening effect, and should not be confused with the chemical destruction of the nitroxide group in the presence of ferricyanide and nearby SH groups (Graceffa and Seidel, 1980).

D. GAPDH

The only membrane-bound enzyme studied by ST-EPR that is a peripheral membrane protein has been glyceraldehyde-3-phosphate dehydrogenase (GAPDHase, D-glyceraldehyde-3-phosphate : NAD$^+$ oxidoreductase, EC 1.2.1.12). Beth et al. (1981a,b) labeled the enzyme with $[^{15}N,^{2}H]$-MSL at a level of one spin-label per tetramer, resulting in a 28% decrease in enzymatic activity. Thus, it appears that the activity of the labeled subunit is abolished by labeling, but the remaining three subunits have normal activity. ST-EPR ($V_2'$) spectra were obtained at 2°C from the enzyme free in aqueous solution (Figure 9, top) and bound to red blood cell membranes (Figure 9, center), where it is known to bind to the cytoplasmic segment of band 3, an integral membrane protein. Although these spectra have different features from those of $^{16}$N spin-labels, they can be analyzed by a similar method, i.e., by comparing them with reference spectra obtained from GAPDHase in glycerol (Figure 9, bottom), or from computer simulations (Figure 9, dashed curves). The result is that the rotational mobility of the probe is substantially slowed by this binding; the effective $\tau$ increases from 160 nsec to about 20 $\mu$sec. Nevertheless, this motion is much faster than that of band 3 (measured by optical methods), indicating motion of the probe with respect to the membrane. It is not clear whether this motion is due to motion within GAPDHase, motion of GAPDHase with respect to band 3, or motion of a flexible segment of band 3. Spectra were found to be indistinguishable from reference spectra corresponding to isotropic motion.
Figure 9. ST-EPR ($V_2^*$) spectra of [5N,3H]-MSL-labeled GAPDHase at 2° in solution (top), bound to the cytoplasmic segment of band 3 in red blood cell membranes (center), and in 78% glycerol (bottom). Dashed curves show the results of computer simulations for isotropic rotational diffusion ($\tau = 20 \mu$sec). From Beth et al (1981a).
IV. OTHER MEMBRANE PROTEINS

A. Rhodopsin

Rhodopsin, the photoreceptor protein of retinal rod disk membranes, has been studied by ST-EPR in the laboratories of Devaux and Ohnishi. This work has been reviewed recently by Devaux (1982). Disk membranes, like SR membranes, are easily isolated and contain one predominant protein, making them an excellent system in which to study the rotational motion of spin-labeled membrane proteins, either in native or recombinant membranes. Furthermore, recent models of visual transduction involve light-induced protein–protein interactions within these membranes. Workers in Devaux's laboratory (Baroin et al., 1977) labeled rhodopsin with 5MSL in native disk membranes. AT 20°C, considerable rotational motion was observed, corresponding to an effective correlation time of 20 μsec (Figure 10, left). Most of the submillisecond motion was stopped when the proteins were crosslinked with glutaraldehyde, indicating that the observed motion was probably that of the protein as a whole (Figure 10, right). These results were in agreement with previous time-resolved optical measurements, and were thus important in establishing the utility of ST-EPR in studying membrane protein motions. The EPR experiments went beyond the optical experiments, because they could be performed in both the presence and the absence of light. A brief exposure to light had no effect on the motion of rhodopsin, indicating that light-induced aggregation of rhodopsin (which would be expected to decrease rotational mobility) is not involved in visual transduction. Ohnishi's group (Kusumi et al., 1978) obtained similar results with ST-EPR, except that they used MSL and observed much less weakly-immobilized signal. They observed some residual motion after glutaraldehyde crosslinking, suggesting some segmental motion within rhodopsin. They also observed a slight decrease in mobility after prolonged (and presumably irrelevant physiologically) exposure to light, as later confirmed by Devaux's group (Baroin et al., 1979).

Devaux and co-workers (Baroin et al., 1979; Favre et al., 1979; Davoust et al., 1980) have carried out studies to correlate the rotational mobility of the protein with that of lipid chains. In disk membranes of normal composition, in which the protein is quite mobile (presumably, therefore, not aggregated), they found that the boundary-lipid probe [5MSL(1,14)] was nearly as mobile as a probe in the bulk of the lipid (and much more mobile than the protein probe). However, when the lipid content was reduced by phospholipase treatment, the protein (5MSL) and boundary-lipid [5MSL(1,14)] probes were both immobilized, probably due to protein–protein interactions. These workers concluded that the concept of a strongly immobilized boundary layer of lipid coating an intrinsic membrane protein is not valid for rhodopsin, and that the evidence for strongly immobilized lipid in the presence of integral membrane proteins may often be an indication that lipid has been trapped by protein aggregation. As in the case of subsequent studies on cytochrome oxidase and Ca-ATPase, this finding does not contradict models in which slight restriction of lipid chain motions must occur at protein surfaces.

Several ST-EPR studies have been done on recombinant membranes containing
Figure 10. Conventional [V,(a)] and ST-EPR [V,(b)] EPR spectra of SML attached to rhodopsin in retinal rod outer segment disk membranes before (1) and after (2) crosslinking with glutaraldehyde. From Baroin et al. (1977).
purified rhodopsin and exogenous lipid (Davoust et al., 1980; Kusumi et al., 1980; Kusumi and Hyde, 1982). Protein mobility was found to increase with temperature, but the transition was much more gradual than that of the lipid chain mobility, and significant submillisecond protein mobility was still observed below the transition temperature. The immediate cause of decreased protein mobility at low temperature may be protein aggregation, not increased lipid microviscosity around the protein (Devaux, 1982). As is the case of native disk membranes, protein mobility decreased when the lipid–protein ratio was decreased, presumably due to protein–protein interactions (Kusumi et al., 1980; Kusumi and Hyde, 1982). It was suggested that phospholipid chain length is important in determining protein aggregation (Davoust et al., 1980; Kusumi and Hyde, 1982).

B. Acetyl Choline Receptor

The rotational motion of acetylcholine receptors, from Torpedo marmorata membranes, has been studied by ST-EPR using either 5MSL (Rousselet and Devaux, 1977; Rousselet et al., 1981) or a spin-labeled α-toxin (Rousselet et al., 1982). In native membrane fragments, the labeled receptor was found to have little or no µsec rotational mobility (Rousselet and Devaux, 1977), suggesting protein aggregation, and thus immobilization, was not affected much by temperature variation or fusion of exogenous lipids (Rousselet et al., 1981). These results suggest the presence of persistent receptor clusters, as verified by electron microscopy. Detectable µsec rotational motion (L"/L = 0.65, τ = 40 µsec) was observed upon alkaline extraction, which removes a 43,000-dalton peripheral protein, and this increased mobility was reversed when this protein was added back (Rousselet et al., 1981). The mobility of receptors in the alkaline-extracted membranes, in contrast with the native membranes, was sensitive to temperature and lipid changes. Thus, protein mobility in native acetylcholine receptor membranes appears to be controlled primarily by protein–protein interactions, not by lipid–protein interactions.

C. Red Blood Cell Membranes

ST-EPR has been used to study the motions of both peripheral and integral proteins of the red blood cell membrane, as well as the lipids. Fung et al. (1979) have used MSL to study both intact red cell membranes and the isolated spectrin–actin complex, which forms part of the peripheral cytoskeleton on the inside surface of red cells. Although weakly immobilized spin-labels (nsec motions detected by conventional EPR) made spectral analysis difficult at neutral pH, the probes were strongly immobilized at pH 4.5 (where the proteins precipitate) making it possible to use ST-EPR to study µsec rotational motions. Lemaigre-Dubreil et al. (1980) purified spectrin, labeled it with MSL, and found no weakly immobilized labels even at pH 7 (where spectrin is not highly aggregated). They observed rapid µsec motions that were only slightly restricted upon binding to red cell membranes, suggesting that spectrin is flexible and that its flexibility is relatively independent of membrane attachment.

Workers in Ohnishi's laboratory have isolated band 3, an integral membrane
protein, from red blood cells, recombined it with DMPC, labeled it with MSL, and studied its rotational motion with ST-EPR. They found that the effective correlation time (from $V_2'$ spectra) was about 100 µsec at 30° (above the lipid phase transition temperature) and increased to about 300 µsec at 10° (below the transition temperature). The response of the protein mobility to the phase transition was significant but less pronounced than that of the lipid, as also observed for the Ca-ATPase (Hidalgo et al., 1978) and for rhodopsin (Davoust et al., 1980; Kusumi et al., 1980). The addition of peripheral ("cytoskeletal") membrane proteins resulted in decreased protein mobility, consistent with protein–protein crosslinking. These workers prepared partially oriented membranes, permitting them to estimate the orientation of the probe relative to the membrane, and hence to estimate the rotational diffusion coefficient ($3.9 \times 10^4$ s$^{-1}$ at 37°).

Fung (1981) studied both protein motions (using MSL) and lipid motions using a fatty acid spin-label [FASL(10,3) or 5NS] and both conventional EPR ($V_1$) and saturation transfer EPR (both $V_2'$ and $U_1'$), in red blood cell membranes (ghosts). $U_1'$ spectra appeared more sensitive than $V_2'$ to lipid motions (probably in the range around $10^{-7}$ sec), while $V_2'$ spectra appeared more sensitive than $U_1'$ to the slower protein motions. Other peripheral membrane proteins that have been studied by ST-EPR are GAPDHase (Beth et al., 1981a, discussed above), hemoglobin (Cassoly, 1982), and ankyrin (Cassoly, 1982).

V. LIPOID PROBES

In several of the above-mentioned saturation transfer studies on membrane proteins, spin-labels have also been used to probe lipid chain motions. In most cases, it has been found that lipid motions are sufficiently rapid (in the nsec range) that conventional EPR suffices to detect them. However, ST-EPR was needed when lipids were in the gel-phase (Hidalgo et al., 1978) and when the membranes were so highly delipated that the remaining lipid probes were immobilized by protein (Favre et al., 1979). It is interesting to note that, in both of these cases, the ST-EPR spectra indicated that the lipid probes, although immobile on the nsec time scale, were more mobile than the protein probes.

ST-EPR has been used recently to study lipid model membranes in the gel phase, i.e., at temperatures below the gel-to-liquid crystal phase transition. These studies indicate the presence of substantial millisecond rotational mobility of hydrocarbon chains and steroids in gel-phase lipid. Spectra of a phosphatidylcholine spin-label (labeled at the C5 position, designated 5-PCS) in DPPC dispersions showed a marked increase in rotational motion as the temperature increased above 25°C, a temperature that is well below the main phase transition and that probably corresponds to the calorimetric "pretransition" (Marsh, 1980; Marsh and Watts, 1980; Watts and Marsh, 1981). Due to the well-known orientation of the nitroxide group relative to the membrane, the pronounced spectral changes in the center of the spectrum were assigned to long-axis rotation of the lipid probe (Figure 11). Phosphatidyl ethanolamine, which does not display a pretransition, showed much smaller $V_2'$ effects than PC below the
transition temperature (Figure 11). Similarly, DMPG showed much larger $V_2'$ effects at pH 8, where it has a pretransition, than at pH 1.5, where it does not. Delmelle et al. (1980) also studied DPPC in the gel phase, including studies on the effect of cholesterol, using both fatty acid and steroid spin-labels, and recording $V_1$ and $V_2'$ spectra on oriented membranes as well as dispersions. Koole et al. (1981) reported $V_2'$ spectra of a steroid spin-label in oriented membranes containing DMPC and cholesterol. Johnson et al. (1982a) showed that $V_2'$ spectra obtained at a higher frequency (35 GHz) should have increased sensitivity to distinguish among different anisotropic motional models. In most of these $V_2'$ studies on lipids in the gel phase, these systems have been discussed as useful model systems for understanding the effects of anisotropic motions on ST-EPR spectra. The orientation of the nitroxide principal axis relative to the likely diffusion axes are well known, thus greatly reducing the ambiguity of spectral
interpretation. However, the interpretation of spectra in terms of slow (μsec), anisotropic rotations is complicated by the fact that conventional (V₁) EPR spectra show some evidence of rapid (nsec) anisotropic motions in almost all of these systems, even in the gel phase (Delmelle et al., 1980). Until the effects of such motions on ST-EPR spectra are understood, it remains true that the unambiguous interpretation of V₂* spectra in terms of anisotropic motion requires that V₁ spectra show no motional narrowing effects.

Although the quantitative interpretation of lipid V₂* spectra is difficult, it is still true that V₂* spectra are often more sensitive to changes than are V₁ spectra, and this increased sensitivity can be exploited in qualitative studies where precise physical interpretation is not crucial (Wilkerson et al., 1978; Swift et al., 1980; Fung, 1981).

VI. SUMMARY

A little more than 10 years after the origination of saturation transfer EPR, this technique has become established as one of the most important tools in the study of molecular dynamics in membranes, particularly in the study of μsec protein motions. Although the majority of ST-EPR studies have been done on the development of methodology using simple model systems, an increasing number of applications are focusing on the relationship between molecular dynamics and enzyme function. The required equipment is widely available in commercial spectrometers, and many investigators have learned to take the extra care required to obtain reliable data. The major limitations of the technique, as in any molecular probe method, is the labeling problem—specificity, rigidity, and preservation of function. More controls are required than in conventional EPR, the theory is more complex, and the temptation to overinterpret data is not always resisted. Future refinements in theory and instrumentation (especially time-resolved EPR) along with increasingly detailed information about the protein structures that are undergoing the detected motions, will continue to reduce the ambiguity of data interpretation and make ST-EPR a more powerful technique in future studies of membrane molecular dynamics.

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REFERENCES


