Progressive Saturation and Saturation Transfer ESR for Measuring Exchange Processes of Spin-Labelled Lipids and Proteins in Membranes

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1 Introduction

The conventional electron spin resonance (ESR) spectra from spin-labelled biomolecules are sensitive to rotational motions on the timescale of 10^{-11} to 10^{-8} s, which is determined essentially by spin–spin (*i.e.* T_2) relaxation processes arising from modulation of the nitroxide hyperfine and *g*-value anisotropies (see *e.g.* ref. 1). The success of the technique depends in part on the versatile chemistry of the stable nitroxide free radical as a reporter group (see *e.g.* ref. 2). Typical spin-labelled biological lipids and reagents for covalent modification of protein side chains are shown in Figure 1. Conventional spin label ESR spectroscopy has therefore found wide application as a probe method for studying lipid chain motions in membranes and conformational changes in proteins, as well as in other areas of biophysical chemistry (see *e.g.* ref. 3). Extension of the motional sensitivity to slower time scales is possible by determining the microwave saturation properties of the ESR spectrum that are governed by the nitroxide spin–lattice relaxation time, T_1, which for the relevant cases lies in the microsecond time regime. Such methods have already been exploited in the determination of slow rotational diffusion rates by using saturation transfer ESR spectroscopy in which the non-linear ESR signal out-of-phase with respect to the field modulation is detected under conditions of partial microwave saturation.[4] One of the most fruitful examples of the standard use of spin label saturation transfer ESR spectroscopy is in the study of the rotational mobility and aggregation states of proteins in membranes (see *e.g.* refs. 1 and 5).

In the present article, attention is concentrated on a different set of continuous wave saturation experiments which are aimed at determining slow exchange processes. Such applications are new and therefore deserve a detailed presentation of the methods of analysis. Both two-site exchange and Heisenberg spin exchange are considered, since these offer the possibility of determining not only apparent unimolecular, but also true bimolecular rate constants. The motivation for such studies lies principally in determining slow exchange and translational diffusion rates involving membrane proteins, since these are not readily accessible by the more usual methods of spin label ESR spectroscopy. For Heisenberg exchange, the spin exchange frequency not only of a single labelled species but also of two labelled species may be determined, hence yielding information on the relative location and mutual accessibility of the labelled groups. The methods employed are either the progressive saturation of the conventional ESR spectrum with increasing microwave power, or the determination of the integrated intensity of the saturation transfer ESR spectrum, which is an unconventional but more sensitive technique. Because of the inhomogeneous broadening associated with spin label powder patterns, both sets of experiments are best analysed in terms of the integrated spectral intensity. Applications range from studies of the lateral diffusion of membrane proteins, *via* the exchange of lipids associated with membrane proteins, to the insertion of proteins in membranes, and many further applications in biophysical chemistry are to be anticipated. The methods for analysing such experiments and the associated rate equations are presented here.

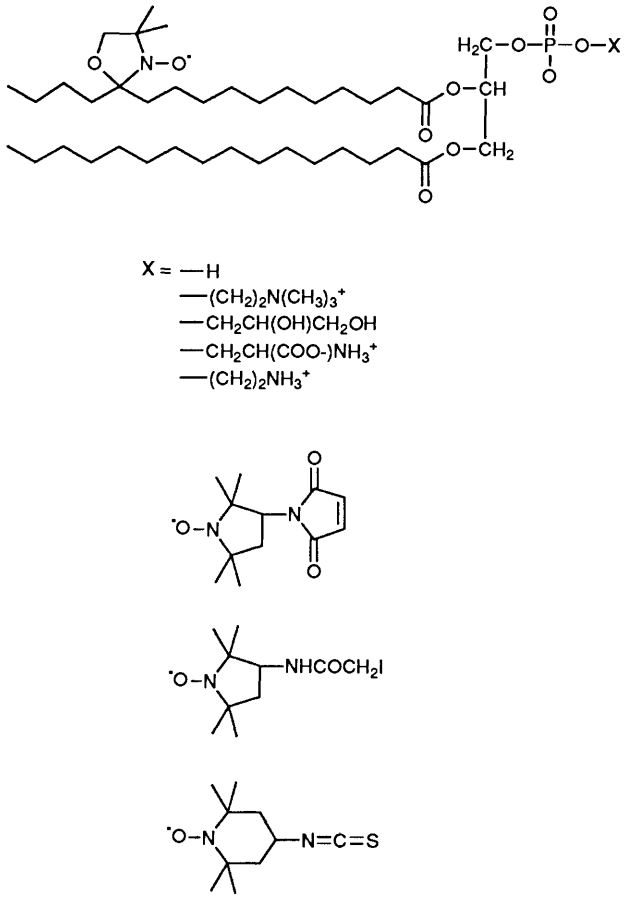

Figure 1 Typical spin-labelled phospholipids (upper structure) and reagents for reaction at nucleophilic centres in amino acid side chains of proteins (lower three structures).

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Dr. Marsh's current research interests centre around studies of the structure and dynamics of biological membranes and of lipid bilayer model membranes, using different biophysical techniques, the principal being spin label electron spin resonance spectroscopy.

2 Continuous Wave Saturation

Classically, the saturation of the intensity of the conventional ESR spectrum can be described in terms of the population differences, n_i, between the $M_{s_i} = \pm\frac{1}{2}$ levels of the ith spin system, where $n_i = N_i^- - N_i^+$ with $N_i^\pm$ being the spin populations of the two levels (see Figure 2). The standard expression for the steady-state population difference under continuous wave (CW) irradiation of the kth spin system is:[6]

$$n_k = n_k^0/(1 + 2WT_{1,k}^{eff}) \quad (1)$$

where n_k^0 is the Boltzmann equilibrium population difference and W is the rate at which transitions are induced by the microwave H_1 field. The latter is given from standard radiation theory by:[7]

$$W = \pi/2.\gamma^2 H_1^2 g(\omega - \omega_k) \quad (2)$$

where γ is the electron gyromagnetic ratio, ω is the angular microwave frequency and $g(\omega - \omega_k)$ is the line shape function for the spin packet centred at ω_k. Equation 1 serves to define the effective spin–lattice relaxation time, $T_{1,k}^{eff}$, in a CW saturation ESR experiment.

Because the ESR absorption is proportional both to W and to n (*i.e.* to the microwave susceptibility), the line height of the absorption spectrum is given by:

$$a(\omega - \omega_k) = a_0.g(\omega - \omega_k)/[1 + \pi\gamma^2 H_1^2 g(\omega - \omega_k)T_{1,k}^{eff}] \quad (3)$$

where the absorption line height in the absence of saturation is obtained by putting $T_{1,k} = 0$. For Lorentzian spin packets, as is the usual case, the normalized line shape is given by:

$$g(\omega - \omega_k) = (T_{2,k}/\pi)/[1 + (\omega - \omega_k)^2 T_{2,k}^2] \quad (4)$$

where $T_{2,k}$ is transverse (spin–spin) relaxation time. From equations 3 and 4 the saturation of the integrated intensity of the ESR absorption line shape, $S_k = \int a(\omega - \omega_k).d\omega$, is given by:

$$S_k = S_{0,k}/(1 + \sigma_k^{eff})^{\frac{1}{2}} \quad (5)$$

where $S_{o,k}$ is the integrated intensity of the ESR absorption in the absence of saturation, and $\sigma_k^{eff} = \gamma^2 H_1^2 T_{1,k}^{eff} T_{2,k}$ is the effective saturation parameter. Since the individual spin packets saturate independently and contribute additively to the total intensity, equation 5 holds good irrespective of the degree of inhomogeneous broadening, and also for anisotropic powder patterns if anisotropy in T_1 and T_2 may be neglected.[8] This is not true, however, for the saturation curves normally deduced from the absorption line heights, because these depend strongly on the degree of inhomogeneous broadening relative to that of the saturation broadening.[9] Thus in complex spin systems, the CW saturation is best analysed in terms of the second integral of the conventional first-derivative absorption spectrum. Typical microwave power saturation curves for a spin-labelled lipid in different membrane systems are given in Figure 3. In treating multicomponent systems, the integrated intensity of the absorption spectrum has the further advantage of being directly additive, the saturation of the total integral in such multicomponent systems being described by:

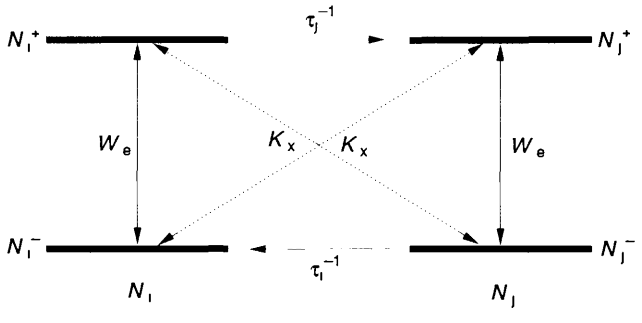

Figure 2 Energy levels, spin populations ($N_i^\pm$) and transitions for two spin environments, i and j, in the ESR line shape. The spin population difference is given by: $n_i = N_i^- - N_i^+$ The transition rate for spin-lattice relaxation is $2W_e = 1/T_1^0$. The rate of Heisenberg spin exchange between spins i and j is $2K_x N_i^\pm N_j^\mp$, and that for chemical (or physical) exchange is $N_i\tau_i^{-1} = N_j\tau_j^{-1}$.

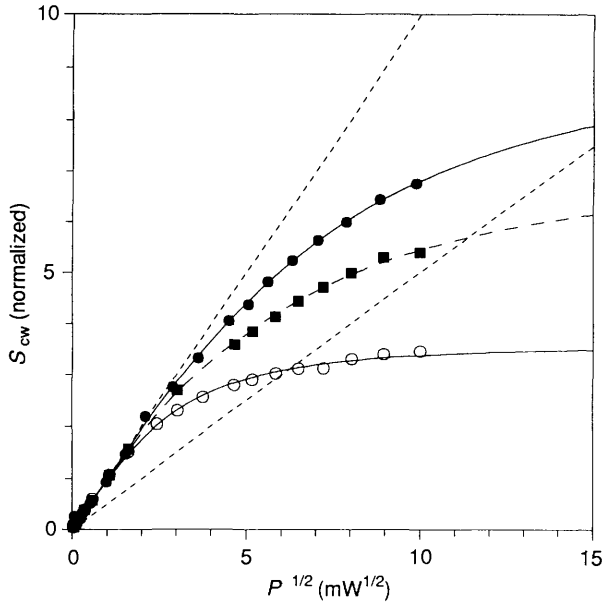

Figure 3 Continuous wave ESR power saturation curves for a spin-labelled lipid in different membrane systems. Data are given for the lipid membrane alone (●), the delipidated protein alone (○), and the lipid–protein membrane complex (■). The straight dashed lines correspond to the predicted dependences for no saturation and for half saturation (upper and lower, respectively). The full lines are non-linear least squares fits of the saturation data to equation 5, and the dashed curved line is the dependence predicted for the lipid–protein complex according to equation 6 from the fits to the two single-component data sets. The data were recorded at a low temperature in the gel phase, for which the lipid exchange between the two environments is very slow. (Data from ref. 14).

$$S_{tot} = S_{0,tot}\sum_i f_i/(1 + \sigma_i^{eff})^{\frac{1}{2}} \quad (6)$$

where f_i and $S_{o,tot}$ are the fractional intensity of component i and the total integrated absorption intensity, respectively, in the absence of saturation. An alternative expression in terms of relaxation enhancements, which are of principal interest, is:

$$S_{tot} = S_{0,tot}\sum_i f_i/[1 + \sigma_i^0(T_{1,i}^{eff}/T_{1,i}^0)]^{\frac{1}{2}} \quad (7)$$

where σ_i^0 and $T_{1,i}^0$ are the effective saturation parameter and T_1, respectively, in the absence of additional relaxation enhancement. In this case, it is assumed, appropriate to the cases to be considered, that T_2 is unchanged because it is far less sensitive to the additional relaxation processes than is T_1. An example of the saturation behaviour of a two-component system, in comparison with that of the individual single components is given in Figure 3.

3 Saturation Transfer ESR Intensities

The normalized integrated intensity of the phase-quadrature, second harmonic, absorption (V_2') saturation transfer ESR spectrum is defined by:[10]

$$I_{ST} = \int V_2'(H).dH/\iint V_1(H).d^2H \quad (8)$$

where V_1 represents the conventional in-phase, first harmonic, absorption ESR signal. Theoretical simulations of the V_2' saturation transfer spectra[4] have shown that the intensity is approximately proportional to the effective spin–lattice relaxation time, T_1^{eff}. Experimental support for this relation has come also from the linear dependence of $1/I_{ST}$ for the spin label on paramagnetic ion concentration, confirming that $1/I_{ST}$ corresponds to the spin–lattice relaxation rate of the spin label that is enhanced by paramagnetic relaxation.[11] The dependence of the saturation transfer integral intensity on the effective spin label T_1 can therefore be expressed as:

$$I_{ST,k} = (I^0_{ST,k}/T^0_{1,k})T^{eff}_{1,k} \tag{9}$$

where $I^0_{ST,k}$ and $T^0_{1,k}$ are the intrinsic saturation transfer integral and spin label T_1, respectively, in the absence of additional relaxation enhancement. Comparison of equations 5 and 9 thus indicates that the integrated intensity of the V_2' saturation transfer spectrum is more sensitive to changes in T_1 than is the saturation of the conventional V_1 ESR spectrum. The direct linear dependence on T_1 also allows simplifications in the analysis of more complex systems, since the total integrated STESR spectral intensity for a multicomponent system (*cf.* equation 7) is given simply by:

$$I_{ST,tot} = I^0_{ST,tot}\sum_i f_i(T_{1,i}^{eff}/T^0_{1,i}) \tag{10}$$

where f_i and $I^0_{ST,tot}$ are the fractional intensity in the conventional V_1 spectrum of component i and the total saturation transfer integral, respectively, in the absence of additional relaxation processes. The usefulness of this method has been demonstrated experimentally both for two-component lipid membranes[10] and for reconstituted lipid–protein membranes.[10,12]

4 Two-site Exchange and Heterospecies Heisenberg Spin Exchange

Kinetically, physical exchange between two inequivalent sites and the Heisenberg spin exchange between spin labels in two inequivalent sites are found to be indistinguishable. Physical exchange between two sites, b and f, is considered first:

$$\mathrm{b} \underset{\tau_f^{-1}}{\overset{\tau_b^{-1}}{\rightleftharpoons}} \mathrm{f}$$

where τ_i^{-1} (i = b or f) is the pseudo-unimolecular rate of transfer per unit time from site i (*cf.* Figure 2). If f_i is the fractional population at site i, then the rate constants are related by detailed balance:

$$f_b\tau_b^{-1} = f_f\tau_f^{-1} \tag{11}$$

where $f_b + f_f = 1$. Because physical exchange occurs with conservation of spin orientation, the rate equation for the population difference at site b is given simply by:

$$dn_b/dt = -n_b\tau_b^{-1} + n_f\tau_f^{-1} \tag{12}$$

and similarly for the population difference at site f.

The situation for Heisenberg spin exchange is somewhat more complicated. Heisenberg exchange between spin labels at the two sites takes place between spins of opposite orientations (*i.e.* cross relaxation):

$$\mathrm{b}^{\pm} \underset{}{\overset{K_x}{\rightleftharpoons}} \mathrm{f}^{\mp}$$

where K_x is the bimolecular rate constant for spin exchange (*cf.* Figure 2). The net rate equation for the population difference at site b is therefore:

$$dn_b/dt = -2K_x(N_b^- N_f^+ - N_b^+ N_f^-) \tag{13}$$

This may be rewritten as:[13]

$$dn_b/dt = -K_x(N_f n_b - N_b n_f) \tag{14}$$

where the notation $N_i = N_i^+ + N_i^-$, with i = b or f, is used, and a similar rate equation can be derived for the population difference at site f. The spin exchange frequency is defined in the usual bimolecular formalism by: $\tau_{ex}^{-1} = K_x(N_b + N_f)$. It can then be seen that equations 12 and 14 are formally equivalent, with the identities $\tau_b^{-1} = f_f\tau_{ex}^{-1}$ and $\tau_f^{-1} = f_b\tau_{ex}^{-1}$, which are fully consistent with equation 11. Thus only two-site physical exchange need be considered to cover both cases.

5 Saturation with Two-site Exchange

If transitions of the spins at site b are induced by the H_1 microwave field, the net steady state condition for the spin population difference at this site is:

$$dn_b/dt = -2Wn_b + (n_b^0 - n_b)/T^0_{1,b} - n_b\tau_b^{-1} + n_f\tau_f^{-1} = 0 \tag{15}$$

where the first term on the right represents the transitions induced by the microwave field, the second is the spin-lattice relaxation and the final terms represent the exchange between sites (*cf.* Figure 2). The corresponding steady state condition for spins at site f which is not irradiated by the microwave field is:

$$dn_f/dt = (n_f^0 - n_f)/T^0_{1,f} - n_f\tau_f^{-1} + n_b\tau_b^{-1} = 0 \tag{16}$$

Solution of equations 15 and 16, together with equation 11, yields the standard expression for the saturation of the spin system at site b (*i.e.* equation 1), where the effective spin–lattice relaxation time, $T^{eff}_{1,b}$, at site b is given by (*cf.* ref. 13):

$$T^0_{1,b}/T^{eff}_{1,b} = 1 + T^0_{1,b}\tau_b^{-1}/(1 + T^0_{1,f}\tau_f^{-1}) \tag{17}$$

The corresponding result for two-site Heisenberg spin exchange is:

$$T^0_{1,b}/T^{eff}_{1,b} = 1 + f_fT^0_{1,b}\tau_{ex}^{-1}/(1 + f_bT^0_{1,f}\tau_{ex}^{-1}) \tag{18}$$

which is obtained from the identities between the exchange rates in the two cases. Equivalent expressions can be derived for site f by permutation of the indices.

Equations 17 and 18 are exactly of the form required for analysis of progressive saturation or saturation transfer ESR measurements by using equation 7 or 10, respectively. Equation 17 may be expressed in terms of a single exchange rate, τ_b^{-1} or τ_f^{-1} as required, by means of the relation given in equation 11. Equation 18 is already in this form. Additionally, if the ratio $T^0_{1,f}/T^0_{1,b}$ is known, *e.g.* from the ratio of the saturation transfer ESR intensities in the absence of exchange (*cf.* equation 9), the effective T_1 may be expressed solely in terms of a dimensionless exchange rate, *e.g.* $T^0_{1,b}\tau_b^{-1}$. As an illustration, representative dependences of the saturation transfer ESR integral on the rate of two-site exchange are given in Figure 4. Depending on conditions, useful sensitivity can be obtained for exchange rates up to five times the intrinsic spin–lattice relaxation rate. The largest sensitivity is found at low exchange rates, less than the intrinsic spin–lattice relaxation rate.

The method has been applied successfully to detecting the physical exchange of spin-labelled lipids at the hydrophobic interface with integral proteins in membranes,[14] as is illlustrated diagrammatically in Figure 5A. The lipid exchange rates are found to lie in the range of the intrinsic spin–lattice relaxation rate ($\approx 10^6\ s^{-1}$) and to reflect the thermodynamic selectivity of different lipid species for the protein. In gel phase membranes, where the lipid chain mobility is drastically reduced, the lipid exchange rates are found to be vanishingly small, or at least are

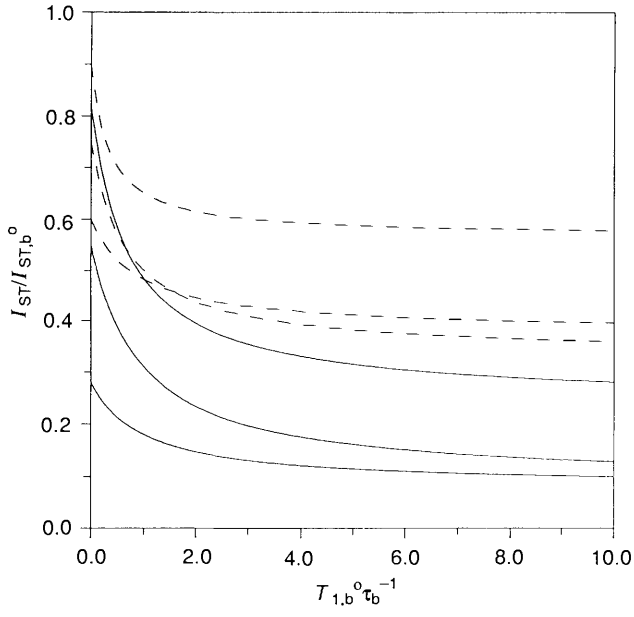

Figure 4 Dependence of the normalized saturation transfer integral intensity, I_{ST}, for a two-component spin label system (b and f) on the scaled exchange rate, $T^{o}_{1,b}\tau_b^{-1}$, of component b. Dependences are given for ratios of the saturation transfer integrals of the two components in the absence of exchange of $I^{o}_{ST,f}/I^{o}_{ST,b} = 0.1$ (full lines) and 0.5 (dashed lines), where from upper to lower curves the fraction of component b is $f_b = 0.8$, 0.5, 0.2. Calculated from equations 10 and 17.

very strongly reduced relative to those in normal fluid lipid membranes. Heisenberg spin exchange between different sites has also been studied recently in the binding of a spin-labelled precursor protein to negatively charged lipid membranes containing spin-labelled lipids, as is illustrated diagrammatically in Figure 5B.[15] The spin exchange frequency of the spin label on the protein was found to depend sensitively on the position of the spin label in the lipid molecule with which spin exchange was taking place (*cf.* Figure 5B). In this way, it was possible to define the degree of penetration of the protein into the membrane, which is likely to be a determining parameter in the translocation of the precursor protein across the membrane. The mature holoprotein was found to be located at the membrane surface and not to penetrate the membrane significantly.

6 Saturation with Heisenberg Spin Exchange

The effects of Heisenberg spin exchange between spin labels of a single species on their ESR saturation properties are now considered. In contrast to the two-site case, many different distinct transitions are coupled by spin exchange. These may correspond to different hyperfine states, or to different orientations of the spin labels relative to the external magnetic field direction in a powder sample (see Figure 6). Effectively, equations 15 and 16 must then be summed over all these states undergoing mutual spin exchange. Using the spin exchange formalism, the general steady rate equation for the population difference of the ith transition is:[13]

$$dn_i/dt = -2Wn_k\delta_{i,k} + (n_i^0 - n_i)/T_1^0 - K_x(Nn_i - N_in) \quad (19)$$

where $N = \sum N_i$ and $n = \sum n_i$. In this equation, transitions are assumed to be induced by the microwave field in the kth spin system, *i.e.* $\delta_{i,k} = 1$ for i = k and is zero otherwise. Summing equation 19 over all i gives the following relation:

$$-2Wn_k + (\sum n_i^0 - n)/T_1^0 = 0 \quad (20)$$

Solution of equations 19 and 20 for i = k yields the standard expression for the saturation of the kth spin system (*i.e.* equation 1), with the effective spin-lattice relaxation time being given by:[13]

$$T_{1,k}^{eff} = T_1^0(1 + Z_kT_1^0\tau_{ex}^{-1})/(1 + T_1^0\tau_{ex}^{-1}) \quad (21)$$

where the exchange frequency is: $\tau_{ex}^{-1} = K_xN$, and $Z_k = N_k/N$ is the fractional population (or degeneracy) of the transition being saturated. For powder saturation transfer ESR spectra from unoriented systems (*e.g.* membranes), the effect of this relaxation enhancement is to reduce the intensity of the spectrum without appreciable changes in line shape (see Figure 6 and ref. 16).

Representative dependences of the effective spin-lattice relaxation rate, and hence of the saturation transfer ESR integral (*cf.* equation 10), on the Heisenberg spin exchange frequency are given in Figure 7. When the degree of degeneracy is low ($Z_k \approx 0$) a linear relation between the effective relaxation rate and spin

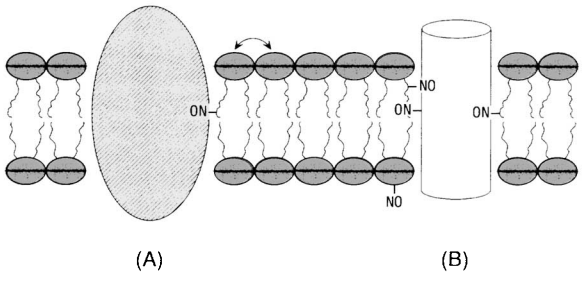

Figure 5 Diagrammatic illustration of spin-label exchange processes in biological membranes. (A) Two-site exchange for a spin-labelled (−NO) lipid molecule between sites at the lipid–protein interface and in the lipid bilayer regions of the membrane. The spin label has different relaxation times in the two environments. (B) Heisenberg spin exchange between spin-labelled lipids and a spin-labelled membrane-penetrating protein. The highest (collisional) spin exchange frequency is obtained for the spin-label group on the lipid that is positioned at the same depth in the membrane as that on the protein.

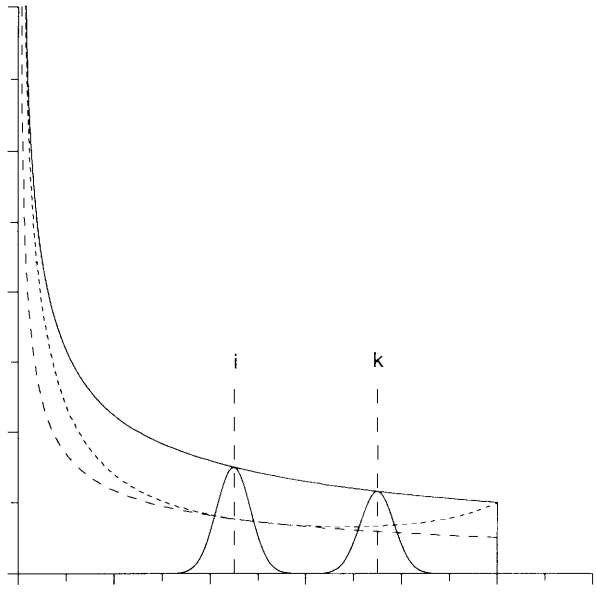

Figure 6 Exchange between spin packets, i and k, in a powder spectral line shape from randomly oriented spin labels. A given spin packet, k, undergoes Heisenberg spin exchange with all other spin packets, i. This results in a powder line shape given by the long-dashed line (*cf.* ref. 16). For rotational diffusion, a spin packet at position k undergoes an angular displacement to position i, where the size of the displacement depends on the diffusion mechanism. For Brownian diffusion, this produces a powder line shape given by the short-dashed line (*cf.* ref. 16).

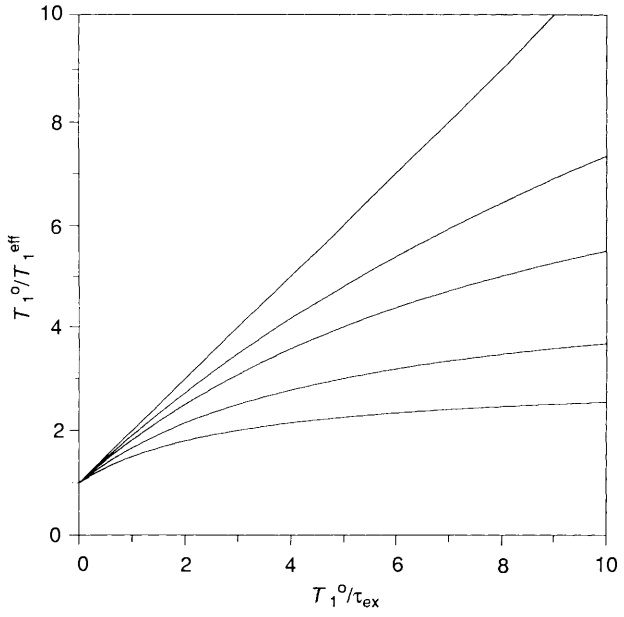

Figure 7 Dependence of the normalized effective spin-lattice relaxation rate, T_1^0/T_1^{eff}, on the scaled Heisenberg spin exchange frequency, $T_1^0\tau_{ex}^{-1}$, obtained from equation 21. From upper to lower, the values of the degeneracy factor are: $Z_k = 0$, 0.05, 0.1, 0.2, and 1/3.

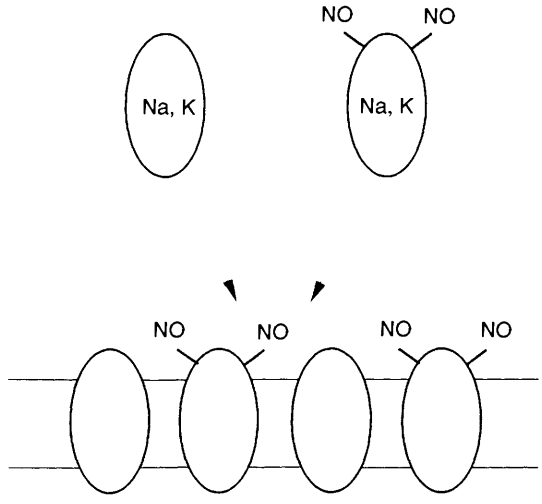

Figure 8 Scheme for measurement of the translational diffusion rate of the Na,K-ATPase in reconstituted membranes of the same protein/lipid ratio, but different fractions of spin-labelled protein. Solubilized spin-labelled protein is mixed with solubilized unlabelled protein at different proportions and the membranes then are reconstituted by precipitation from the detergent solution. The Heisenberg spin exchange frequency is determined by the relative concentration of spin-labelled protein and the collision rate between proteins in the membrane.

exchange frequency is obtained, as would be expected for a true relaxation enhancement, rather than a cross relaxation, because the redistribution of saturation between the different exchange-coupled states does not limit the effective relaxation. In this limiting case, the saturation transfer integral intensity is given by the following expression:

$$1/I_{ST} = 1/I_{ST}^0 + T_1^0\tau_{ex}^{-1} \qquad (22)$$

which is found to be applicable in many cases of interest where the spin exchange frequency is low. At high exchange frequencies, on the other hand, $T_{1,k}^{eff}$ tends to a limiting value of $Z_kT_1^0$ that represents the maximum redistribution of saturation throughout the various distinct spin label states. Beyond this limit, the spin–spin relaxation time, T_2, then becomes sensitive to the spin exchange process and the exchange frequency can be determined conventionally from the line widths and line shapes of the in-phase ESR spectra.[17]

The principal application of this saturation transfer ESR method is in the determination of slow bimolecular collision rates between spin-labelled molecules, for which the exchange frequency is too low to affect T_2 and hence is not amenable to the usual lineshape analysis. Whereas the latter is suitable for determination of translational diffusion rates of spin-labelled lipids in membranes (*cf.* ref. 17), it cannot be applied to the slower translational diffusion of spin-labelled integral membrane proteins which are more dilute and have broader ESR lines. The viability of the saturation transfer ESR method for such measurements has been verified from the viscosity and temperature dependence of the translational diffusion coefficients of a spin-labelled protein in homogeneous solution.[18] A strategy for measurement with integral membrane proteins is indicated in Figure 8. Solubilized spin-labelled protein is combined with unlabelled membrane protein at different relative proportions and membranes are then reconstituted at the same total protein/lipid ratio in order to ensure a constant effective intramembrane viscosity. The dependence of the saturation transfer ESR integral intensity on relative spin concentration in the reconstituted Na,K-ATPase membranes was found to conform to that given in equation 22.[19] The local translational diffusion coefficients deduced from the resulting bimolecular collision rate constants were in the expected range ($\approx 2 \times 10^{-8}$ cm^2s^{-1}), but were much larger than corresponding values found for the long-range diffusion coefficients in whole cell systems, indicating considerable barriers to long-range diffusion in the latter case. A further application is the use of equation 22, with suitable calibrations, to determine the local concentrations of spin-labelled probe molecules in heterogeneous systems. This approach has been employed successfully to a study of the kinetics of the nucleation and growth of lipid domains in a model membrane system.[20]

7 Slow Rotational Diffusion and Saturation Studies

It was shown above that two-site physical exchange and Heisenberg spin exchange are kinetically equivalent. Rotational diffusion of a spin labelled molecule can be approximated as physical exchange in small steps. This results in a spectral diffusion of saturation which is analogous to that of the Heisenberg spin exchange between distinct transitions in an anisotropic powder spectrum of a single spin-labelled species (*cf.* Figure 6), which was treated in the immediately preceding section. This equivalence has led to a simple description of the standard saturation transfer ESR experiment used for the determination of slow rotational diffusion rates, particularly of membrane proteins and supramolecular aggregates, that is outlined below.[21]

The spectral diffusion rate arising from spin label rotation is determined by parameters governing the angular dependence of the resonance positions and the width of a spin packet, and is inversely proportional to the rotational correlation time, τ_R, which is defined by $\tau_R = 1/(6D_R)$ where D_R is the rotational diffusion coefficient.[22] This spectral diffusion rate ($\propto \tau_R^{-1}$) is equivalent to the spin exchange frequency, τ_{ex}^{-1}, introduced above. However, because the spectral diffusion rate depends on the angular dependence of the resonance positions, the extent of the resulting reduction in intensity varies throughout the spectrum, producing a large change in the saturation transfer ESR line shape (see Figure 6). Thus the effects of rotational diffusion on the saturation transfer ESR spectrum can be distinguished readily from those of Heisenberg exchange when both spectral line shapes and intensities are taken into account.

A standard method of extracting rotational correlation times from the saturation transfer ESR spectrum is *via* the ratios of the

line heights at the most sensitive part of the spectrum to those at the extreme turning points in the spectrum that are insensitive to rotational diffusion (*cf.* Figure 6). Since the line heights and intensities of the saturation transfer ESR spectra are directly proportional to the effective T_1 (*cf.* equation 9), the spectral line height ratios and integrated intensities, R, that are normally used for establishing rotational correlation time calibrations (*cf.* refs. 4 and 10), therefore may be expressed from equation 21 in the following general form:

$$R = R_0(1 + a/\tau_R)/(1 + b/\tau_R) \quad (23)$$

where R_0 is the value of the measured parameter R in the absence of rotational diffusion, a and b are constants to be fitted that depend only on intrinsic spectral parameters, and the ratio a/b is equal to the orientational degeneracy parameter ($\approx \sin\theta$). Equation 23 describes very well the dependence on rotational correlation time of the diagnostic line height ratios and intensities of the saturation transfer ESR spectra from spin-labelled haemoglobin in glycerol–water mixtures, as is seen from Figure 9.[21] It therefore can be used to give the following simple expression for the correlation time calibrations of the experimental STESR spectra:

$$\tau_R = k/(R_0 - R) - b \quad (24)$$

where values of the calibration constants, k, R_0, and b, for the different spectral parameters can be found in ref. 23. This is a much more readily accessible form than hitherto has been presented and has the additional advantage of reflecting directly the underlying spectral diffusion process.

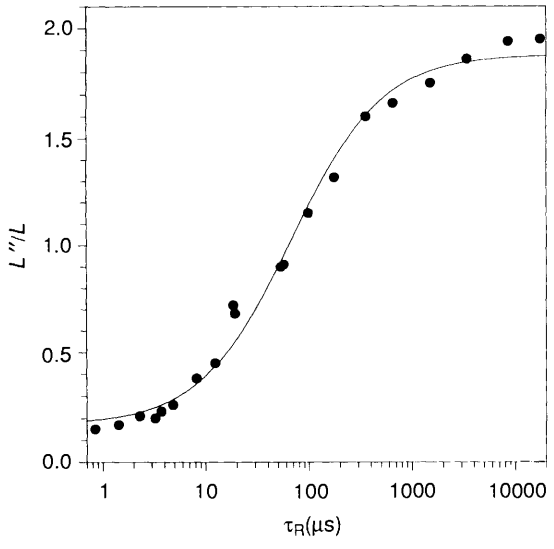

Figure 9 Rotational correlation time (τ_R) calibration obtained with spin-labelled haemoglobin for the low-field diagnostic line height ratio, L''/L, of the second-harmonic, 90°-out-of-phase, absorption saturation transfer ESR spectrum (V'_2-display). The full line is a non-linear least-squares fit of the data to equation 23, with the fitting parameters: $(L''/L)_0 = 1.88$, $a = 6.18$ μs and $b = 67.9$ μs ($m_I = +1$ hyperfine manifold). From ref. 21.

Slow rotational diffusion of spin-labelled molecules also may be studied from the power saturation of the conventional ESR spectra. Squier and Thomas[24,25] have demonstrated the feasibility of this approach and have made calibrations of the rotational correlation time by using spin-labelled haemoglobin. The parameter measured, R', is the ratio of the second integrals of the conventional first-derivative ESR spectra recorded at low (subsaturating) and high (partially saturating) microwave powers, where both effectively are normalized to the values in the absence of saturation. The low and high microwave powers were chosen to correspond to values of H_1 at the sample of 0.032 and 0.25 gauss, respectively. From equation 5, the low/high power second integral ratio is therefore given by:

$$R' = (1 + \sigma_{eff})^{\frac{1}{2}} \quad (25)$$

where σ_{eff} is the saturation parameter at the high (partially saturating) microwave power. It is found that such conventional ESR saturation measurements are also amenable to the analysis presented above for the effects of slow rotational diffusion on saturation transfer ESR spectra.[26] As before, equation 25 can be written in terms of the relaxation enhancement as:

$$R' = [1 + \sigma^0(T_1^{eff}/T_1^0)]^{\frac{1}{2}} \quad (26)$$

where σ^0 and T_1^0 are the saturation parameter at the high (partially saturating) microwave power and the value of T_1, respectively, in the absence of rotational diffusion. For slow rotational diffusion, T_2 remains essentially unchanged in equation 26. Making the same substitution as was done immediately above for the value of T_1^{eff} in the presence of rotational diffusion, then yields:

$$R' = [1 + \sigma^0(1 + a/\tau_R)/(1 + b/\tau_R)]^{\frac{1}{2}} \quad (27)$$

where the parameters to be fitted are a and b, as previously, and σ^0. As for the saturation transfer ESR spectra, this equation is capable of describing the correlation time calibration for the saturation of the conventional ESR spectrum with a reasonably high degree of accuracy. The fitted values for a and b are reasonably close to those obtained for the saturation transfer ESR integral intensity,[26] as for consistency they should be, hence giving further support to the method used in the analysis. The corresponding rotational correlation time calibration for the saturation of the conventional ESR spectrum is then given by:

$$\tau_R = k'/(1 + \sigma^0 - R'^2) - b \quad (28)$$

where $k' = 116.6$ μs, $\sigma^0 = 3.12$ and $b = 46.8$ μs. It is to be anticipated that this readily accessible formulation will facilitate further applications of this alternative new form of saturation transfer ESR spectroscopy.

Conclusions

Continuous wave saturation of spin label ESR spectra is exquisitely sensitive to slow physical exchange and weak Heisenberg spin exchange processes which lie in the characteristic frequency range of the spin–lattice relaxation rate. Such situations occur widely in the biological applications of spin label ESR methodology. Measurements are made of the integrated spectral intensity, either with progressive saturation of the conventional in-phase ESR spectrum, or from the out-of-phase saturation transfer ESR spectrum. The effects of (two-site) physical exchange and Heisenberg spin exchange on the effective spin–lattice relaxation have equivalent formulations, which lead also to a simplified description of the response of the saturation transfer ESR spectra to slow rotational diffusion. There is thus a very direct connection between the spin dynamics that are reflected by the ESR saturation properties and the molecular dynamics of the spin-labelled system of interest. The applications are likely to be many and currently include studies of lipid–protein interactions, protein translational and rotational diffusion, and lipid domain formation in biological membranes.

9 References

1 D. Marsh and L. I. Horváth in 'Advanced EPR. Applications in Biology and Biochemistry', ed. A. J. Hoff, Elsevier, Amsterdam, 1989, pp. 707—752.
2 B. J. Gaffney in 'Spin Labeling. Theory and Applications', ed. L. J.

Berliner, Academic Press, New York, 1976, pp. 183—238.
3 D. Marsh in 'Membrane Spectroscopy', ed. E. Grell, Springer-Verlag, Berlin–Heidelberg–New York, 1981, pp. 51—142.
4 D. D. Thomas, L. R. Dalton, and J. S. Hyde, *J. Chem. Phys.*, 1976, **65**, 3006.
5 P. F. Knowles and D. Marsh, *Biochem. J.*, 1991, **274**, 625.
6 C. P. Slichter, 'Principles of Magnetic Resonance', 2nd. Edn., Springer-Verlag, Berlin–Heidelberg–New York, 1978.
7 A. M. Portis, *Phys. Rev.*, 1953, **91**, 1071.
8 T. Páli, L. I. Horváth, and D. Marsh, *J. Magn. Reson.*, 1993, **A101**, 215.
9 T. G. Castner, Jr., *Phys. Rev.*, 1959, **115**, 1506.
10 L. I. Horváth and D. Marsh, *J. Magn. Reson.*, 1983, **54**, 363.
11 T. Páli, R. Bartucci, L. I. Horváth, and D. Marsh, *Biophys. J.*, 1992, **61**, 1595.
12 L. I. Horváth, P. J. Brophy, and D. Marsh, *Biochim. Biophys. Acta*, 1993, **1147**, 277.
13 D. Marsh, *J. Magn. Reson.*, 1992, **99**, 332.
14 L. I. Horváth, P. J. Brophy, and D. Marsh, *Biophys. J.*, 1993, **64**, 622.
15 M. M. E. Snel, B. De Kruijff, and D. Marsh, *Biophys. J.*, 1993, **64**, A16.
16 D. Marsh and L. I. Horváth, *J. Magn. Reson.*, 1992, **97**, 13.
17 D. Marsh in 'Biological Magnetic Resonance. Spin Labelling Theory and Applications', ed. L. J. Berliner and J. Reuben, Vol. 8, Plenum, New York, 1989, pp. 255—303.
18 V. V. Khramtsov and D. Marsh, *Biochim. Biophys. Acta*, 1991, **1068**, 257.
19 M. Esmann and D. Marsh, *Proc. Natl. Acad. Sci. USA*, 1992, **89**, 7606.
20 T. Páli, R. Bartucci, L. I. Horváth, and D. Marsh, *Biophys. J.*, 1993, **64**, 1781.
21 D. Marsh and L. I. Horváth, *J. Magn. Reson.*, 1992, **99**, 323.
22 P. Fajer, D. D. Thomas, J. B. Feix, and J. S. Hyde, *Biophys. J.*, 1986, **50**, 1195.
23 D. Marsh, *Appl. Magn. Reson.*, 1992, **3**, 53.
24 T. C. Squier and D. D. Thomas, *Biophys. J.*, 1986, **49**, 921.
25 T. C. Squier and D. D. Thomas, *Biophys. J.*, 1989, **56**, 735.
26 D. Marsh, 1993, to be published.