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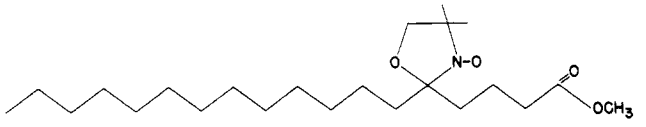
Complement-Induced Decrease in Membrane Mobility: Introducing a More Sensitive Index of Spin-Label Motion†

Ronald P. Mason,* Elda B. Giavedoni, and Agustin P. Dalmasso

ABSTRACT: We have used spin-labeling to investigate complement-induced changes in lipid organization of antibody-sensitized sheep erythrocyte membranes. The spectrum of methyl 5-doxylstearate incorporated into the lipid component of sheep erythrocyte membranes is typical of a membrane bilayer. The membranes from complement-lysed erythrocytes have a small, but statistically significant, reduction in fluidity when compared to membranes from osmotically-lysed erythrocytes, as indicated by a small increase in $T_{\parallel}'$. In theory, measurements of the widths of the outer hyperfine extrema should be more sensitive to motion than the separation of the outer hyperfine extrema ($2T_{\parallel}'$). Our results indicate that the half-width at half-height of the outer hyperfine extrema show a severalfold greater percentage change than $T_{\parallel}'$. The sign and magnitude of these changes are in general agreement with previous predictions. Our results imply that motional corrections to the S formalism of Hubbell, Gaffney, and McConnell are necessary because spin-label motion appears to be explicitly represented in this type of electron spin resonance spectra.

The cytotoxic action of complement is due to the effect of the late-acting components on the cell membrane. Membrane-bound C5b,6,7 complex[1] constitutes the receptor site for C8 (Kolb et al., 1972). C8 is directly responsible for the production of membrane damage (Stolfi, 1968; Tamura et al., 1972) and C9 acts as a cofactor that enhances the activity of bound C8 (Hadding and Müller-Eberhard, 1969). Although the mechanism by which C8 and C9 cause membrane damage is not understood, efforts to demonstrate an enzymatic mechanism, such as the degradation of phosphoglycerides to lysophosphoglycerides, have met with failure (Kinsky, 1972; Lachmann et al., 1973). Giavedoni and Dalmasso (1976) demonstrated that (a) complement lysis of sheep red blood cells is accompanied by a substantial loss of membrane phospholipids into the fluid phase, and (b) cholesterol and phospholipids from complement-lysed red blood cell membranes are not dissociable by treatment with 2.4 M KSCN, in contrast to membrane lipids from cells lysed osmotically or by freeze-thawing. The latter finding suggested that the membrane lipids might undergo rearrangements as the result of the action of complement (Giavedoni and Dalmasso, 1976). Therefore, we investigated the effect of complement on the organization of membrane lipids by means of spin-labeling with methyl 5-doxylstearate, that is, methyl stearate substituted with a nitroxide-containing oxazolidine ring at the 5 position.



Our results indicate that membranes from complement-lysed red blood cells have a small, but statistically significant, reduction in fluidity when compared to membranes from osmotically-lysed cells (Giavedoni et al., 1976).

The use of stearic acid spin-labels to detect very small changes in the fluidity of erythrocyte membranes has been reported by Kury and McConnell (1975) and Butterfield et al. (1974). The interpretation of the ESR spectra of these labels in erythrocyte membranes is usually based on the S formalism of Hubbell, Gaffney, and McConnell. Kury and McConnell detected prostagtandin E induced changes in S of less than 1%. Butterfield et al. found that the value of S for erythrocytes from patients with myotonic muscular dystrophy was on the average a few percent less than that of normal controls. These small changes in S or equivalently in $T_{\parallel}'$ and $T_{\perp}'$ are near the limit of precision to which the measurements can be made. In particular, the measurement of the separation of the low- and high-field extrema ($2T_{\parallel}'$) is clearly the limiting factor in the determination of small changes in S.

A new, more sensitive, method of monitoring the fluidity of spin-labels by measuring the widths of the outer ESR hyperfine extrema has been proposed. In theory, calculations showed that the measurement of the widths of the outer hyperfine extrema are more sensitive to motion than the separation measurements usually employed, such as $T_{\parallel}'$ and S. For example, the extrema widths are sensitive to rotational correlation times, τ_R's, as long as 3×10^{-6} s, whereas the measurement of the extrema separation is insensitive to τ_R's $\geq 3 \times 10^{-7}$ s (Mason and Freed, 1974). While investigating complement-induced changes in lipid organization of antibody-sensitized sheep erythrocyte membranes, we found results which are consistent with the predictions of Mason and Freed (1974). Our results indicate that the motion of the methyl 5-doxylstearate in the membrane bilayer of sheep erythrocytes is explicitly represented in the ESR spectra and also imply a breakdown in the assumption of the S formalism of Hubbell, Gaffney, and McConnell that motion transverse to the rotational symmetry axis is too slow on the ESR time scale to affect the position of the outer hyperfine extrema.

Experimental Procedure

Sheep erythrocytes were washed three times with Veronal buffer, resuspended at 5×10^8 cells/mL, and sensitized with

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[1] Abbreviations and terminology for complement components and intermediary complexes conforms to recommendation of Committee on Complement Nomenclature of the World Health Organization ((1968), *Bull. W.H.O. 39*, 935-938).

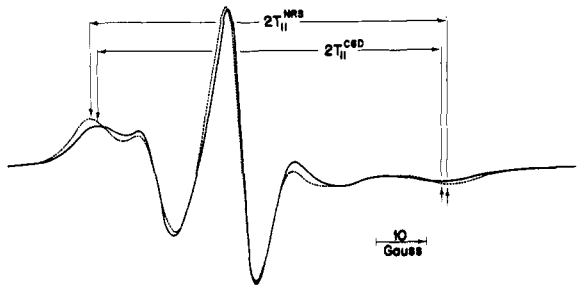

FIGURE 1: A comparison of the ESR spectra of methyl 5-doxylstearate in membranes after complement lysis with normal rabbit serum (NRS) and in membranes after treatment with C6-deficient serum (C6D) and hypotonic lysis.

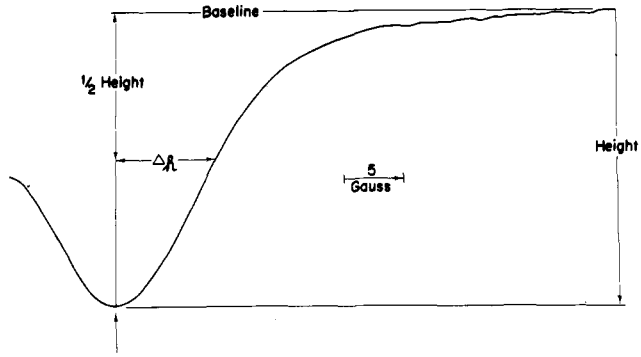

FIGURE 2: An example of a scan used in the measurement of the outer half-width at half-height of the high-field extremum. The scan range was 40 G. An 8-min scan with a 10-s time constant was used. The baseline was determined from the region upfield of the extremum.

2 hemolytic units of rabbit anti-sheep red blood cell antiserum (Giavedoni and Dalmasso, 1976). After 30 min at 37 °C, the sensitized cells were washed once. Washes and cell suspension were carried out in Veronal buffer composed of 145 mM NaCl, 5 mM Veronal, 0.5 mM $MgCl_2$, and 0.15 mM $CaCl_2$, pH 7.4. Hypotonic lysis was performed with 5 mM Veronal buffer in which the NaCl had been omitted (Giavedoni and Dalmasso, 1976). Treatment of antibody-sensitized erythrocytes with complement was carried out by incubations of 50 mL of sensitized cells (5×10^8 cells/mL) with 7 mL of rabbit serum for 60 min at 37 °C. Under these conditions, complete lysis was obtained. Controls consisted of antibody-sensitized erythrocytes exposed to equivalent amounts of C6-deficient rabbit serum (Rother et al., 1966). After 60 min at 37 °C, the cells were separated by centrifugation at 1800*g* for 5 min, washed once with Veronal buffer, and lysed with hypotonic Veronal buffer for 15 min at 37 °C. Membranes from erythrocytes and antibody-sensitized erythrocytes were prepared by hypotonic lysis for 15 min at 37 °C. These membranes as well as those lysed by complement were centrifuged at 30 000*g* for 15 min and washed once with Veronal buffer. Membranes were resuspended at 40–50% in Veronal buffer and methyl 5-doxylstearate (Syva Associates) introduced according to Butterfield et al. (1974).

The addition of reconstituted guinea pig serum to sheep erythrocytes labeled with methyl 5-doxylstearate results in the hydrolysis of the methyl ester to form 5-doxylstearic acid (Whisnant, 1975). In our experiments, the membranes were washed after incubation with serum and then spin-labeled. In addition, sheep erythrocyte membranes, which have been labeled with 5-doxylstearic acid, have a contribution from a narrow three-line spectrum superimposed on the anisotropically immobilized spectrum (Whisnant, 1975). In no instance have we observed such a spectrum during our investigations.

Bieri et al. (1974) have reported spin-label-induced changes in erythrocyte morphology. When we examined by dark-field microscopy erythrocytes in which 10^6 molecules of the spin-label/cell had been incorporated, we observed small protuberances on the erythrocytes. Butterfield et al. (1976) have recently reported that, in spite of these morphological changes, the ESR spectra are apparently representative of the unperturbed erythrocyte membrane.

All ESR spectra were recorded under identical conditions at room temperature with a Varian E-4 spectrometer so changes in our parameters do not reflect changes in temperature, power saturation, or magnetic-field modulation. The widths of the low- and high-field hyperfine extrema were determined from 20 and 40 G scans, respectively. The SEM of the widths of the extrema is comparable to that of the $2T_{\parallel}'$ measurement and was equal within a factor of two to 0.1 G. In both cases, the determination of the true maximum of the extrema limits the precision of the measurement.

Results

Our ESR spectra of erythrocyte cells and ghosts labeled with methyl 5-doxylstearate are typical of anisotropically immobilized spectra obtained from artificial lipid bilayer vesicles (Hubbell and McConnell, 1971; Mason et al., 1974) and biological membranes, including erythrocyte membranes (Butterfield et al., 1974; and Landsberger et al., 1972). In our experiments, the separation of the outer extrema ($2T_{\parallel}'$) and the outer half-widths at half-height of the low- (Δ_l) and high-field (Δ_h) extrema were determined. The parallel component of the nitrogen hyperfine tensor, $T_{\parallel}'$, is measured as one-half the separation of the outer hyperfine extrema (Figure 1). In spectra where rotational motion is explicitly represented in the spectra, this separation is referred to as $2T_z'$ (Goldman et al., 1972a). In the *S* formalism of Hubbell, Gaffney, and McConnell, $T_{\parallel}'$ is closely related to the order parameter *S*, $S = (T_{\parallel}' - T_{\perp}')/(T_z - T_x)$, where T_z and T_x are components of the hyperfine tensor (Hubbell and McConnell, 1971). The width parameters, Δ_l and Δ_h, are the outer half-widths at half-height of the low- and high-field extrema, respectively. The measurement of Δ_h is illustrated in Figure 2, where the height of the extrema is measured from the true baseline upfield from the high-field extrema.

The width parameters, Δ_l and Δ_h, were described by Mason and Freed (1974) who proposed that these measurements should be more sensitive than the separation measurement T_z' (i.e., $T_{\parallel}'$ in anisotropically immobilized spectra). The rationale for this expectation is based on an analogy to the well-known case of chemical exchange where magnetic resonance absorption lines are known to broaden before they shift as the exchange rate increases (i.e., Δ_l and Δ_h should be a more sensitive index of motion than T_z'). Rigorous calculations confirmed the intuitive idea that the extrema widths should be several times more sensitive to motion than T_z'.

In the case of anisotropically immobilized spectra, where $\tau_{R\parallel}$ is short enough to fulfill the assumption of Hubbell and McConnell (1971) that the *g*- and hyperfine tensors are completely averaged by the motion about the rotational symmetry axis, motion transverse to the rotational symmetry axis will broaden and shift the outer hyperfine extrema. This situation was considered by Goldman et al. (1972a) for the special case of fast rotation about the 2p-π orbital of a nitroxide

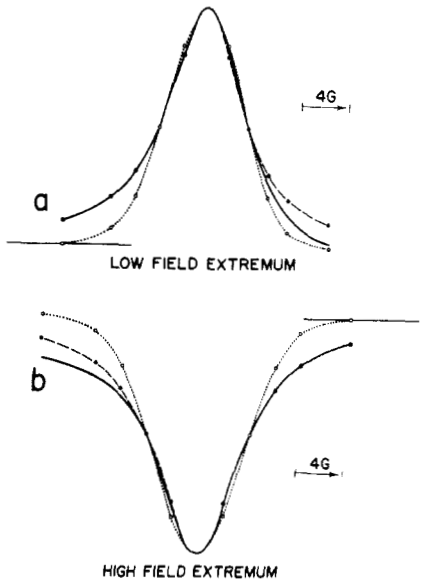

FIGURE 3: (a) The computed low-field extremum of the ESR spectrum (—) is superimposed on Gaussian (·· O ··) and Lorentzian (- - ● - -) absorption curves normalized to have the same width at half-height for the respective wings of the computed extremum. (b) As in a, except that the computed high-field extremum is shown. The outer wings of both computed extrema were indistinguishable from a Lorentzian absorption curve. The magnetic parameters used for these calculations are $\delta = 3.0$ G, $g_x = g_y = 2.0075$, $g_z = 2.0027$, $A_x = A_y = 6.0$ G, $A_z = 32.0$ G, $B_0 = 3300$ G.

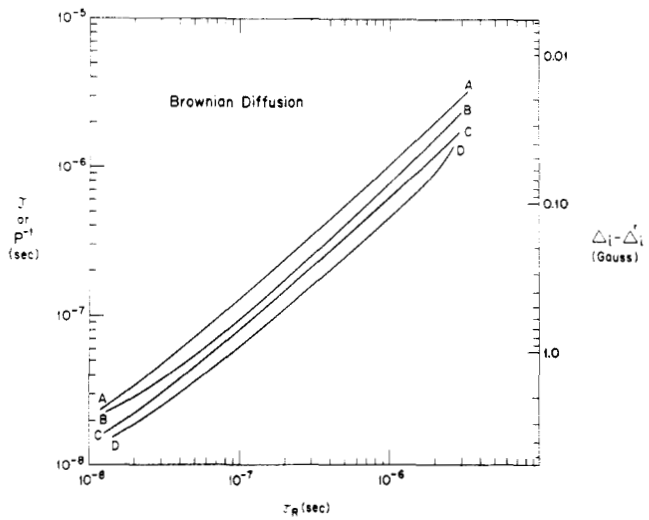

FIGURE 4: Graph of the lifetime broadening vs. the excess width ($\Delta_i - \Delta_i^r$) in gauss vs. τ_R for nitroxide isotropic rotational reorientation or $\tau_{R_\perp}$ for very anisotropic rotational motion from computer simulations of Brownian diffusion. Curves A and B are for $\delta = 3.0$ G, and the low- and high-field extrema, respectively, and curves C and D are the same as A and B, respectively, except $\delta = 1.0$ G. The magnetic parameters are otherwise the same as in Figure 3.

spin-label. This type of rotation merely averages the already approximately axial magnetic parameters, and any change in $\mathbf{T}_\parallel'$ is necessarily due to motion transverse to the rotational symmetry axis (i.e., $\tau_{R_\perp} < 3 \times 10^{-7}$ s). In the earlier work of Mason and Freed (1974), the extrema widths, Δ's, were defined as one-half the full widths at half-height. In the case of anisotropically immobilized spectra, the measurement of the full width at half-height is not possible because of the distortion of the inner wings of the outer hyperfine extrema. Although the outer wings may also be distorted to some extent by overlap, the definition of the Δ's, as illustrated in Figure 2 for the high-field extrema, enables us to use the concepts developed by Mason and Freed (1974).

Hubbell and McConnell (1971) showed that the derivative line shape of the outer hyperfine extrema for an isotropic distribution of spin-labels corresponds to the absorption curve of those spin-labels which have their rotational symmetry axes or equivalently their z' axes of the effective Hamiltonian parallel to the magnetic field. In order to test their analysis, we have compared the Δ's measured from the outer wings of computed spectra (Goldman et al., 1972b) with the theoretical value of $(\sqrt{3}/2)\,\delta = 0.866\delta$, where δ is the Lorentzian peak-to-trough derivative line width used in the computation of rigid limit spectra. We found that $\Delta_l^r = 0.845\delta$ and $\Delta_h^r = 0.885\delta$, where Δ^r's are rigid limit extrema widths. This result is independent of δ over the range $1.0 \leq \delta \leq 4.0$ G and of T_z over the range $27 \leq T_z \leq 40$ G, and is in agreement with the theoretical value of 0.866δ. In Mason and Freed (1974), the ratios of the Δ's calculated from the full widths at half-height to the δ's were determined and found to be in poorer agreement with the theoretical value than that obtained above with half-widths. Apparently, overlap of the central part of the spectrum with the inner wings of the outer hyperfine extrema caused the poor agreement in the previous analysis. Figure 3 illustrates the high- and low-field extrema of a computer rigid limit spectrum (i.e., a superposition of first derivative Lorentzians) along with one pure Lorentzian and Gaussian absorption line shape normalized to have the same width at half-height as the computed extrema. As predicted by Hubbell and McConnell (1971), the outer wings of the computed extrema are indistinguishable from a pure Lorentzian absorption line, whereas the inner wings deviated from the Lorentzian line shape apparently as a result of overlap. This excellent agreement between the first derivative computed spectra and the absorption line shape directly verifies the correctness of the analysis of McConnell and co-workers.

Mason and Freed (1974) rigorously computed incipient motional effects of the outer hyperfine extrema in near rigid limit spectra. The basic idea for these calculations resulted from an analogy to the concept of lifetime broadening as developed in chemical-exchange theory. Two-site chemical-exchange theory considers the effects of random jumps between two sites with different resonance frequencies. A rigid limit nitroxide spectrum can be thought of as representing two sites corresponding to different orientations of the nitroxide 2p-π orbital in the magnetic field. One site is either the low- or the high-field extrema which results from those nitroxides oriented such that their 2p-π orbitals lie within a cone of angle Ω about the magnetic field direction where the component of the nitrogen nuclear spin is either $+1$ or -1, respectively, for the low- and high-field extrema (McConnell and McFarland, 1970). Reorientation of a 2p-π orbital out of the cone into any other orientation results in an ESR spectrum which only contributes to the central region of the rigid limit ESR spectra (i.e., all orientations of the 2p-π orbital outside of the cone represent the second site). As in the well known case of chemical exchange, the outer hyperfine extrema are broadened by P^{-1}, where P is the time proportional transition probability.

$$(1/T_2) = (1/T_2)^{\text{rigid}}_{\text{limit}} + P$$

where

$$(1/T_2) = |\gamma_e|\Delta_i \quad i = i \text{ or } h$$

and

TABLE I: Effect of Complement Lysis on Spin-Label Mobility.

Treatment of Erythrocytes	Av Magnetic Resonance Parameters (G)		
	$T_{\parallel}'$	Δ_l	Δ_h
Hypotonic lysis of erythrocytes	26.70 (0.14)[a]	4.59 (0.06)	6.71 (0.22)
Hypotonic lysis of antibody-sensitized cells	26.55 (0.13) $P^b > 0.5$	4.78 (0.04) $P < 0.05$	6.90 (0.11) $P > 0.5$
Hypotonic lysis of antibody-sensitized cells after treatment with C6-deficient rabbit serum	26.48 (0.10) $P > 0.3$	4.60 (0.04) $P > 0.9$	6.75 (0.16) $P > 0.9$
Complement lysis of antibody-sensitized cells with normal rabbit serum	27.62 (0.13) $P < 0.01$	3.93 (0.07) $P < 0.001$	5.99 (0.12) $P < 0.02$

[a] Mean (SEM) of five experiments. [b] Compared to membranes from the hypotonic lysis of erythrocytes.

$$(1/T_2)\,\underset{\text{limit}}{\overset{\text{rigid}}{=}}\,|\gamma_e|\sqrt{3}/2\delta$$

The extrema, therefore, have lifetime broadening because P^{-1} is the average time, τ, a nitroxide 2p-π orbital stays within the cone before a reorientation out of the cone occurs,

$$\tau = P^{-1} = \frac{1}{|\gamma_e|\,(\Delta_i - \sqrt{3}/2\delta)}$$

This excess width expressed as a lifetime, τ, correlates very well with the isotropic rotational correlation time used in the rigorous computation, as is shown in Figures 4 and 5.

In Mason and Freed (1974), the excess width ($\Delta_i - \Delta_i^r$) was determined from the full width at half-height and was compared with τ_R's for Brownian rotational diffusion, which is appropriate for spin-labels rigidly attached to macromolecules (McCalley et al., 1972). The correlation between calculated values of $\Delta_i - \Delta_i^r$ and τ_R in an approximate free diffusion model was also examined. This non-Brownian model has given the best results for the unattached spin-label (Goldman et al., 1972b; and Hwang et al., 1975). The Brownian diffusion model was found to give a highly nonlinear correlation between the excess width and τ_R, whereas the correlation with the approximate free diffusion model was more linear. The linearity of these correlations, especially in the case of Brownian diffusion (Figure 4), was improved by using the outer half-widths of the extrema, implying again that overlap of the inner wings of the outer hyperfine extrema with the central region of the spectra had distorted the earlier results. The mean time required to reorient by an angle Ω is for Brownian diffusion given by $1/\tau_R\Omega^2$, whereas for the approximate free diffusion model the mean time is $1/\sqrt{7}\tau_R\Omega^2$ (Mason and Freed, 1974). So very near the rigid limit, where the inhomogeneous broadening is responsible for almost all of the extrema widths, the lifetime broadening phenomenon should be $\sqrt{7}$ times larger for the free diffusion model than for Brownian diffusion. In fact, near the rigid limit where the cone of angle Ω is well defined (i.e., τ_R's longer than 10^{-6}), the free diffusion model does give about twice the excess width for a given τ_R, as can be seen by comparing Figures 4 and 5.

In Table I and Figure 1, the effect of complement lysis of erythrocytes on $T_{\parallel}'$, Δ_l, and Δ_h is shown and compared with a variety of controls. Each of the parameters is similar in membranes from hypotonically lysed erythrocytes, antibody-sensitive erythrocytes, and antibody-sensitized erythrocytes treated with C6-deficient rabbit serum. In contrast, membranes from antibody-sensitized erythrocytes lysed with normal rabbit serum and then exposed to hypotonic buffer had an increase of 3.4% in $T_{\parallel}'$ and a decrease of 14% in Δ_l and of 11% in Δ_h compared to hypotonically lysed erythrocytes. All

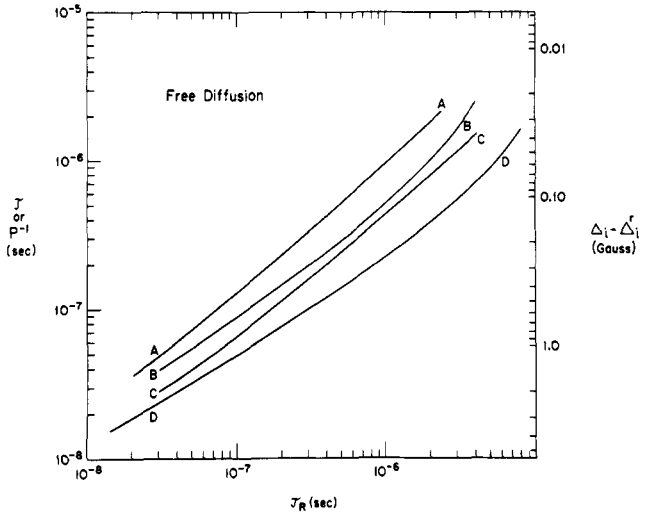

FIGURE 5: As in Figure 4, except that an approximate free-diffusion model for rotational reorientation was used.

of these changes indicate that complement lysis causes a decrease in the mobility of the spin-label. This decrease in spin-label mobility is complement specific and not simply the result of lysis. Preliminary results show that membranes from sensitized ghosts with bound Cl-7 complex and then lysed with C8 or C8 and C9 also have decreased spin-label mobility, but membranes from sensitized ghosts with bound Cl-7 complex and lysed hypotonically do not.

As expected from theory, the percentage in Δ_l and Δ_h is severalfold greater and of the opposite sign than the change observed in $T_{\parallel}'$. The percentage change in Δ_l is greater than that observed in Δ_h. This result is not consistent with the interpretation of these changes being due solely to a change in $\tau_{R\perp}$. Concomitant with any change in $\tau_{R\perp}$ changes in $\tau_{R\parallel}$ and the true S are certainly to be expected (Mason et al., 1974) and may account for the result that Δ_l changed by a larger percentage than Δ_h. Computed spectral simulations would be necessary to quantitate the interpretation of these spectra.

Discussion

The use of stearic acid and phospholipid spin-labels to detect changes in membranes, especially erythrocyte membranes, has been widely used. The interpretation of the ESR spectra in stearic acid spin-labeled erythrocyte membranes is usually based on the S formalism of Hubbell, Gaffney, and McConnell in which the time-dependent spin Hamiltonian for a nitroxide, $H(t)$, is assumed to be decomposable into a time-averaged time-dependent effective Hamiltonian, H', and a time-dependent perturbation, $H(t) - H'$, characterized by short

correlation times for nitroxide motion. The utilization of an effective time-independent Hamiltonian enables results to be expressed in terms of the axially-symmetric components of the nitrogen hyperfine tensor, $\mathbf{T}_{\parallel}'$ and $\mathbf{T}_{\perp}'$, or equivalently in terms of the order parameter, S, and the nitrogen isotropic coupling constant A_0'. In order for this formalism to be valid, motion about the symmetry axis of rotation, $R_{\parallel}$, must be fast enough and, simultaneously, motion transverse to the symmetry axis of rotation, $R_{\perp}$, must be slow enough so that the symmetry axis of rotation becomes the symmetry axis of a time-invariant cylindrical rigid-limit type Hamiltonian. Rigorous calculations of Mason et al. (1974) showed that rotational correlation times, $\tau_{R_{\parallel}} = \frac{1}{6} R_{\parallel}$, as short as 2×10^{-10} s can be too long, and the assumption of Hubbell and McConnell that rotational motion about the symmetry axis is fast enough is not fulfilled in many spectra which apparently reflect cylindrical symmetry. Variations in A_0' have usually been attributed to changes in the polarity of the local environment of the nitroxide group. Recent work based on the theory of Freed et al. has found that the increase in A_0' may be only apparent and is really due to a change in the rotational rate about the symmetry axis of rotation, and not to changes in the polarity of the local environment (Mason et al., 1974). Even if motion about the symmetry axis of rotation is fast enough to become the symmetry axis of an effective Hamiltonian with cylindrical symmetry, motion transverse to the symmetry axis of rotation must still be slow on the ESR time scales. It can be determined from the equations in Goldman et al. (1972a) that if $\mathbf{T}_{\parallel}'$ is 32 G then $\tau_{R_{\perp}}$'s shorter than 3×10^{-7} s will decrease the separation of the outer hyperfine extrema by as much as 1%. As $\mathbf{T}_{\parallel}'$ becomes smaller due to a decrease in S, even longer $\tau_{R_{\perp}}$'s will cause a decrease in the measured $\mathbf{T}_{\parallel}'$. For instance, if $\mathbf{T}_{\parallel}'$ is only 20 G, then $\tau_{R_{\perp}}$'s shorter than 5×10^{-7} s will decrease the apparent $\mathbf{T}_{\parallel}'$ by 1%.

In summary, the S formalism predicts that an increase (decrease) in S can only arise from a decrease (increase) in the root mean square angle between the symmetry axis of rotation and molecular z axis, which lies along the 2p-π orbital of the nitrogen atom, whereas Mason et al. (1974) have shown an increase (decrease) in S can also arise from a decrease (increase) in the rate of rotation either about or transverse to the rotational symmetry axis.

Our interest in the measurement of Δ_l and Δ_h in these systems arose from the realization that $\mathbf{T}_{\parallel}'$ could be an explicit function of the rotational orientation of the spin-label, as is the case for the isotropic motion of spherical spin-labels in viscous solvents (Goldman et al., 1972b) or spin-labeled hemoglobin (McCalley et al., 1972).

The measurement of Δ_l has been found to have greater statistical significance than $\mathbf{T}_{\parallel}'$ for a given change in the mobility of methyl 5-doxylstearate within the erythrocyte membrane, whereas the measurements of Δ_h are hampered by a poorer signal-to-noise ratio and the observed changes are of comparable statistical significance to $\mathbf{T}_{\parallel}'$. The lifetime broadening model of Mason and Freed predicted that as $\mathbf{T}_{\parallel}'$ approaches the rigid limit value T_z the outer hyperfine extrema will sharpen and that the percentage change in the line widths will be as much as an order of magnitude more sensitive to changes in τ_R than $\mathbf{T}_{\parallel}'$. This prediction is at least partially fulfilled in this case because an increase in $\mathbf{T}_{\parallel}'$ (3.4%) is accompanied by a decrease in Δ_l (14%) and Δ_h (11%).

In the rigid limit where the spectrum is not influenced by motion, proton nuclear hyperfine structure and the distribution of magnetic parameters due to the heterogeneity of the spin-label environment dominate the line widths. These inhomogeneous contributions to the widths Δ_l and Δ_h will be quickly averaged with the onset of molecular motion (Mason and Freed, 1974). So the lifetime broadening contribution to Δ_l and Δ_h always competes with the motional averaging of the inhomogeneous or rigid limit widths. In fact, Bullock et al. (1975) found that Δ_l and Δ_h actually decreased as motions increased in a spin-labeled polymer. They reported unusually broad rigid limit Δ^r's of about 5.0 G, whereas other workers have reported rigid limit Δ^r's of 1.2 to 3.5 G (Gaffney and McConnell, 1974; Hwang et al., 1975). Apparently, when the inhomogeneous contribution to the width is as large as 5.0 G, the lifetime broadening contribution to the Δ's is not as important as the averaging of the very inhomogeneous environment.

The magnitude of the inhomogeneous contribution to Δ_l and Δ_h in the spectrum of fatty acid spin-labels in erythrocyte membranes appears to be less important, because in this system we have observed a decrease in Δ_l and Δ_h associated with an increase in $\mathbf{T}_{\parallel}'$. In support of this interpretation, McFarland and McConnell (1971) have found through deuteration that the line-width contribution of the unresolved proton hyperfine structure is negligible in lecithin bilayers. The effect of the heterogeneity in egg lecithin acyl chains also appeared insignificant because the line widths are nearly identical in dioleoyllecithin (Gaffney and McNamee, 1974). In this context, it should be noted that $\mathbf{T}_{\parallel}'$ is also affected by the inhomogeneous contribution to the line width (Goldman et al., 1972a), although the estimates of τ_R from the Δ's are more dependent on the inhomogeneous width contribution than $\mathbf{T}_{\parallel}'$ is.

One marked advantage of the Δ index of fluidity over $\mathbf{T}_{\parallel}'$ or S is that the Δ's are independent of changes in the polarity of the environment about the nitroxide, because Mason and Freed (1974) showed that the Δ's were independent of T_z over the range 27–40 G. Therefore, the width parameter need not be corrected for changes in polarity of the environment in contrast to S (Hubbell and McConnell, 1971).

As in any interpretation of experimental data, other explanations are possible. For instance, spin exchange of the nitroxide labels will broaden the outer hyperfine extrema (Butterfield et al., 1976). In our work, we have carefully verified that our results are independent of the spin-label concentration, but it is possible that differential segregation of the spin-label could lead to line-width differences. At present, changes in extrema widths which do not correlate with changes in $\mathbf{T}_{\parallel}'$ must be suspected until more experience with Δ_l and Δ_h has been acquired.

The small decrease in fluidity specific to complement lysis is probably the result of the late-binding components of complement because C6-deficient serum did not affect the membrane fluidity. This small decrease in erythrocyte membrane fluidity could be produced in a number of ways. The additional lipid–protein interactions due to the C7-9 components would be expected to decrease the fluidity of the membrane, as has been observed for the insertion of rhodopsin into membranes (Hong and Hubbell, 1972). If complement does have a lipase activity, then a decrease in erythrocyte membrane fluidity is also expected, because spin-labeling experiments by Simpkins et al. (1971) found that phospholipase A_2 treatment caused erythrocyte membranes to become less fluid. The third and most likely explanation for the observed decrease in the spin-label's mobility is the substantial loss of phospholipids (or a phospholipid byproduct) relative to cholesterol accompanying complement lysis (Giavedoni and Dalmasso, 1976). In a wide variety of systems, the addition of cholesterol has been found

to restrict the motion of spin-labels. For instance, increasing the cholesterol:phospholipid ratio of guinea pig erythrocytes with a high cholesterol diet decreased the mobility of the stearate spin-labels (Kroes et al., 1972); therefore, the decreased mobility we have observed as a result of complement lysis is most likely due to an increase in the cholesterol:phospholipid ratio of the membrane relative to the hypotonic lysis control.

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