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An Electron–Electron Double-Resonance Study of Interactions between [^{14}N]- and [^{15}N]Stearic Acid Spin-Label Pairs: Lateral Diffusion and Vertical Fluctuations in Dimyristoylphosphatidylcholine†

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ABSTRACT: Vertical fluctuations of the terminal methyl groups of stearic acid acyl chains toward the surface of dimyristoylphosphatidylcholine (DMPC) bilayers have been investigated by using spin-label electron–electron double-resonance (ELDOR) methodology. Spin-label pairs consisting of two populations of stearic acid spin-labels were employed, each at 0.25 mol % concentration, where the nitroxides of the first population were ^{15}N substituted and the nitroxides of the second contained ^{14}N. Various combinations of labels with the nitroxide moieties located at carbons 5, 12, or 16 (C5, C12, C16) were used. ELDOR permits measurement of collision frequencies between the two constituents of the pair, for example, between ^{15}N spin-labels at C5 and ^{14}N labels at C16. Intramolecular contributions to the ELDOR effect including nitrogen nuclear relaxation are eliminated by the use of spin-label pairs. Above the main phase transition temperature, bimolecular collisions between C5 and C16 occur with about half the frequency of C16:C16 collisions. It is concluded that vertical fluctuations are very pronounced. A dependence of these fluctuations on temperature and pH has been observed. Lateral diffusion constants calculated from the bimolecular collision frequencies of C16:C16 pairs are 4.56×10^{-8}, 5.77×10^{-8}, and 8.09×10^{-8} cm^2/s at 27, 37, and 47 °C. These values are in good agreement with previous measurements of lipid diffusion in DMPC.

Current investigations of the structure of biological membranes often concern the extent to which various constituents can diffuse laterally and interact. A number of biophysical methods have been developed to examine the lateral mobility of integral membrane components, including photobleaching recovery and redistribution techniques, pulsed gradient nuclear magnetic resonance, eximer formation, electron paramagnetic resonance (EPR)[1] techniques employing line-width analysis, and, more recently, observation of electron–electron double resonance (ELDOR). Of these, only eximer formation and the EPR methods are rigorously capable of examining mo-

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; ELDOR, electron–electron double resonance; END, electron–nuclear dipolar; EPR, electron paramagnetic resonance; Hex, Heisenberg spin exchange; psEED, pseudosecular electron–electron dipolar; C5, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidineoxyl; C12, 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidineoxyl; C16, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidineoxyl; tanone, 4-oxy-2,2,6,6-tetramethyl-1-piperidineoxyl.

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lecular diffusion within a restricted region, because they rely on probe–probe interaction and not displacement over relatively large (i.e., micron) distances. Since it has now been shown in many systems [see Cherry (1979) and Edidin (1981) for reviews] that unrestricted long-range diffusion of integral membrane components generally does not occur, the development of methods that are sensitive to constituent interactions within restricted domains is essential for a more complete understanding of biological membranes.

Popp & Hyde (1982) approached the problem of measuring the bimolecular collision frequency between stearic acid spin-labels in model membranes by using ELDOR spectroscopy[2] to observe the rate of saturation transfer between [^{14}N]nitroxide hyperfine transitions relative to the electron spin–lattice relaxation probability (W_e) and by independently measuring W_e by saturation recovery. This method allowed determination of the Heisenberg exchange rate (W_{Hex}), which is effectively the bimolecular collision frequency between probes. These workers, using the theoretical model of Traüble & Sackmann (1972), which relates collision frequencies to diffusion constants, were able to obtain lateral diffusion constants for the spin-labels in multibilayers of dimyristoylphosphatidylcholine (DMPC) above 37 °C. These values were in excellent agreement with previous fluorescence (Wu et al., 1977) and magnetic resonance (Kuo & Wade, 1979) studies. However, as the main phase transition temperature was approached, complications arose due to interference from electron–nuclear dipole (END) induced nuclear relaxation, a competing intramolecular mechanism for the transfer of saturation between nuclear spin states. This is expected on theoretical grounds: END-induced nuclear relaxation increases with decreasing rotational mobility of the spin-label. Indeed, at 27 °C the END-induced nuclear relaxation dominated intermolecular spin–spin interactions, and the method could not be used to determine molecular collision frequencies. Popp & Hyde (1982) suggested that the difficulties posed by fast intramolecular relaxation might be overcome by the use of ^{14}N:^{15}N spin-label pairs.

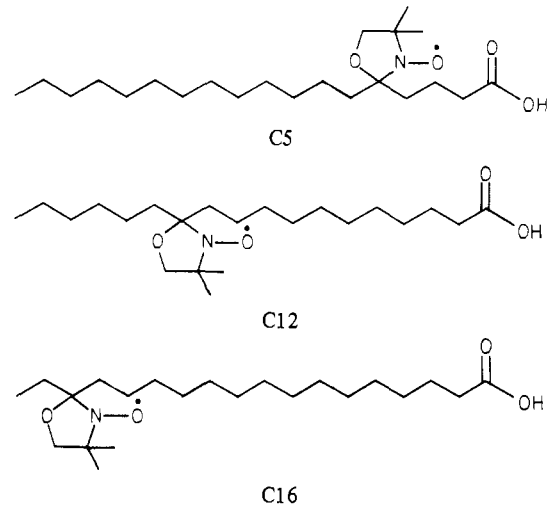
[^{15}N]Nitroxide spin-labels have been utilized as a means to enhance EPR sensitivity and to facilitate quantitative computerized simulation of EPR and saturation-transfer spectra (Beth et al., 1981). The use of ^{15}N spin-labels in double-labeled systems had been suggested as a means to study lipid–protein interactions (Bienvenue et al., 1978; Devaux et al., 1981). Seigneuret et al. (1981) have reported the use of ^{14}N:^{15}N spin-label pairs in conventional (i.e., first harmonic absorption) EPR studies of lipid–lipid and lipid–protein interactions on the basis of empirical observation of spin–spin broadening. ELDOR studies of [^{14}N]- and [^{15}N]tanone in solution have been reported by Stetter et al. (1976).

In the present work, we introduce the use of ^{14}N:^{15}N spin-label pairs to the ELDOR/saturation-recovery methodology. Utilization of the dual-label methodology eliminates END-induced nuclear relaxation as an ELDOR-active process, thereby extending the applicability of this approach to systems in which the rotational diffusion of the spin-labels is in the slow tumbling regime. Additionally, our techniques should permit future investigations of interactions between populations of probes with dissimilar carrier molecules (such as proteins and lipids), enabling the study of dynamic processes within membranes that have not heretofore been accessible. A preliminary account of this work has been published (Feix et al., 1983).

[2] For an excellent textbook discussion of electron–electron double-resonance spectroscopy, see Kevan & Kispert (1976).

Experimental Procedures

Materials and Sample Preparation. Three ^{15}N spin-labels, namely, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidineoxyl (C16 stearic acid spin-label or C16), 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidineoxyl (C12 stearic acid spin-label or C12), and 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidineoxyl (C5 stearic acid spin-label or C5), were synthesized according to the procedure of Venkataramu et al. (1982). [^{14}N]Nitroxide derivatives of C5, C12, and C16 were obtained from Syva (Palo Alto, CA). Dimyristoylphosphatidylcholine (DMPC) was from Sigma. Stock solutions of the spin-labels (ca. 0.5 mM) and DMPC (0.10 g/mL) were prepared in chloroform and stored at −20 °C.



C5

C12

C16

To prepare liposomes, the spin-label and DMPC stock solutions were mixed to give 0.25 mol % (each) spin-label, and all traces of solvent were evaporated with a stream of dry nitrogen gas. Samples were further dried under vacuum ($P < 10^{-1}$ torr) for at least 8 h. The dried samples were dispersed in the appropriate buffer by vortex mixing at 47 °C, well above the phase transition temperature. The resulting multilamellar dispersions (40% lipid by weight) were transferred to a capillary made from the methylpentene polymer TPX and placed in the microwave cavity under a stream of N_2 gas at 37 °C. Deoxygenation of the samples via gaseous exchange through the TPX capillary (Popp & Hyde, 1981) was allowed to proceed for at least 45 min prior to ELDOR measurements. The same TPX capillary was used for all spectra of individual spin-labels and spin-label ^{14}N:^{15}N pairs. Removal of oxygen is essential to avoid inaccuracies arising from O_2-dependent shortening of the nitroxide spin–lattice relaxation time (Popp & Hyde, 1981).

Experimental Methodology. ELDOR instrumentation was as described in the previous study on lateral diffusion of C16 stearic acid spin-label in DMPC (Popp & Hyde, 1982). A Varian E800 ELDOR bridge with a Varian E101 microwave bridge was used to acquire ELDOR spectra. The cavity supported orthogonal rectangular TE_{103} and TE_{102} modes [Figure 2 of Hyde et al. (1968)], with the TE_{103} mode employed as the pump mode and the TE_{102} as the observing mode.

The frequency difference between pumping and observing fields was set with a frequency counter and varied slightly according to the spectral resolution of the selected ^{14}N:^{15}N pair (see Results). Although any of a number of combinations of pumped and observed transitions could be used in these experiments, we always employed frequency differences such that the ^{15}N ($M_I = -1/2$) transition would be pumped as the ^{14}N

($M_I = 0$) midfield line was observed. All tabulated ELDOR data are from this combination of pumped and observed transitions.

The ELDOR reduction factor, R, is defined (Hyde et al., 1968; Eastman et al., 1970; Popp & Hyde, 1982) as [(signal with pump off) − (signal with pump on)]/(signal with pump off) or, equivalently

$$R = 1 - (I_p/I_o) \quad (1)$$

where I_p and I_o are the peak to peak amplitudes of the observed transition with the pumping field turned on and off, respectively. As has been stated previously (Eastman et al., 1970; Popp & Hyde, 1982), the ELDOR reduction observed at any given power is a function of a number of experimental variables, including the modulation field amplitude, pumping power, and positioning of the pump and observer fields relative to the transition frequencies, but becomes independent of such considerations upon extrapolation to infinite pumping power. Reductions were determined for each sample at several (usually 8–10) pumping field intensities from 120 to 400 mW. A Hewlett-Packard power meter connected to the pump arm with a cross-guide coupler was used to monitor the incident power of the pumping microwave field. The reduction at infinite power (R_∞) was obtained from a linear regression least-squares extrapolation of R^{-1} vs. P^{-1} (see Figure 6), where P is the measured incident pump power.

ELDOR reductions were the average of three spectra. The spectra were normalized to the peak-to-peak height of the low-field $M_I = 1$ transition. Since no ELDOR transfer of saturation is induced with this transition, it is possible to use this line as an internal control for experimental variables such as temperature and sample position. The spectral intensity of this low-field peak did not vary by more than 2% during an experiment. The temperature was controlled within ±1 °C with the Varian variable-temperature accessory equipment and was measured with a copper/constantan thermocouple immersed in the sample and a Fluke 2190A digital thermometer.

In the fast motional range for magnetically dilute samples, saturation transfer between ^{14}N and ^{15}N populations should occur predominantly by Heisenberg spin exchange (Sackmann & Traüble, 1972; Scandella et al., 1972; Hyde & Sarna, 1979). However, it has been noted that pseudosecular ($S_1^{\pm}$, $S_2^{\mp}$) dipolar interactions—which are not easily distinguished from Heisenberg exchange—could be significant (Freed, 1979). We have not attempted to dissect the relative contributions of dipolar and exchange mechanisms to the observed effect but rather have chosen simply to report the extrapolated reduction at infinite power (R_∞) as a semiquantitative measure of the strength of interaction between a given spin-label pair. This matter will be considered further under Discussion.

Results

Spectral Resolution. Figure 1 demonstrates the ELDOR effect observed with the ^{14}NC16:^{15}NC16 pair (0.25:0.25 mol %) in DMPC at 47 °C, pH 9.5. The frequency difference between pumping and observing fields was 26 MHz. When the magnetic field was swept through an observed resonance condition for the $M_I = 0$ transition, transfer of saturation occurred from the simultaneously pumped $M_I = -1/2$ transition. This results in a decrease in signal intensity of the observed $M_I = 0$ transition. Similarly, an ELDOR reduction is also observed in the $M_I = 1/2$ transition, as the magnetic field simultaneously sweeps the observing field through $M_I = 1/2$ and the pumping field through $M_I = 0$. No transition is coincidentally saturated when the $M_I = 1, -1/2$, or -1 is induced by the observing (rf) field, no transfer of saturation occurs, and no ELDOR reduction is seen. Pumping and observing field frequency differences for dual-label ELDOR experiments were always such that no reduction was observed at maximum pump power with either label alone.

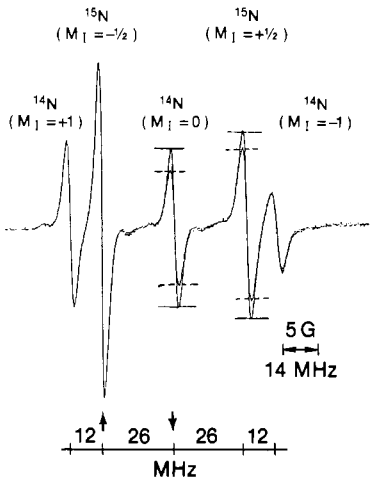


FIGURE 1: Demonstration of the ELDOR effect for ^{14}NC16:^{15}NC16 (0.25:0.25 mol %) in multibilayer liposomes of DMPC equilibrated with 0.1 M borate buffer, pH 9.5. Temperature was 47 °C. Superimposed spectra were recorded with pump field off and at 400-mW incident power. Horizontal lines indicate reduction in peak to peak amplitude used to quantitate ELDOR transfer of saturation (see Experimental Methodology). Arrows define frequency separation between pumping and observing fields, (↑) indicating position of pump field when observer field (↓) is centered on the ^{14}N ($M_I = 0$) resonance (pump minus observe frequency difference 26 MHz). Field calibration in megahertz is given.

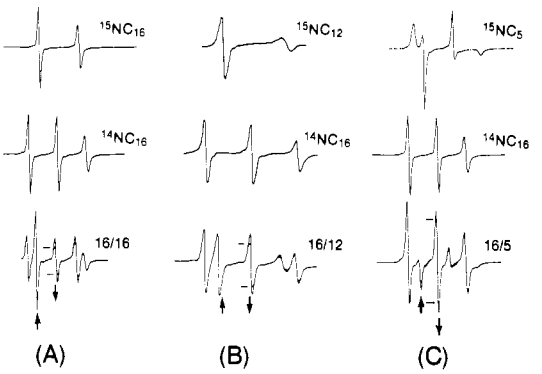


FIGURE 2: Spectra of (A) ^{15}NC16, (B) ^{15}NC12, and (C) ^{15}NC5 (each at 0.25 mol %) paired with 0.25 mol % ^{14}NC16 in DMPC liposomes equilibrated with 0.1 M sodium borate, pH 9.5. All spectra were recorded at 47 °C. Arrows indicate the relative position of the pump (↑) and observer (↓) fields employed in ELDOR experiments. Spectra are aligned for comparison. Bottom row shows superimposed spectra taken with the pump field off and at 400-mW incident power, demonstrating the ELDOR effect. Horizontal lines denote reductions in the peak to peak amplitude of the $M_I = 0$ transition in the spectra obtained at 400-mW pump power.

The foremost requirement for dual-label ELDOR experiments is the resolution of features from the ^{14}N and ^{15}N spectra. Such resolution is easily achieved when both members of the pair are mobile, as shown in Figure 1. Satisfaction of this resolution requirement ensures unambiguous interpretation of results. It may well be the case that useful ELDOR spectra can be obtained even when overlap occurs, but this complication was avoided in the present work. The spectra in Figures 2 and 3 demonstrate that for all three of the [^{15}N]stearic acid spin-labels paired with either ^{14}NC16 or ^{14}NC12, the ^{14}N $M_I = 0$ transition and at least some part of the ^{15}N $M_I = -1/2$ (low-field) transition are well resolved in liquid-crystalline DMPC.

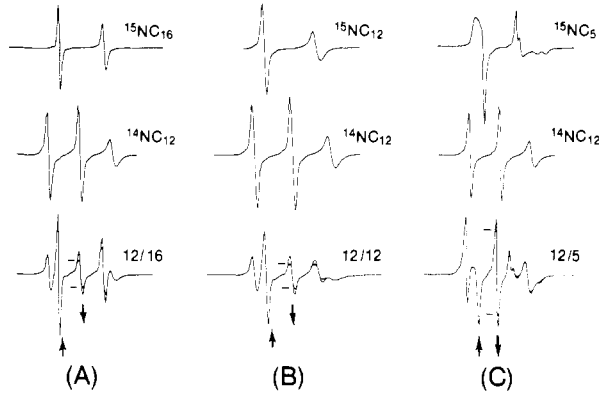


FIGURE 3: Spectra for (A) ^{15}NC16, (B) ^{15}NC12, and (C) ^{15}NC5 (0.25 mol % each) paired with 0.25 mol % ^{14}NC12. Conditions were as in Figure 2 except for (C), in which the buffer was 0.1 M phosphate, pH 6.5. Top row again contains spectra of the individual ^{15}N spin-labels, aligned with (middle row) the spectrum of ^{14}NC12. Bottom row is superimposed spectra taken with the pump field off and at 400-mW incident power. Horizontal lines denote reductions in the peak to peak amplitude of the $M_I = 0$ transition in the pumped spectra.

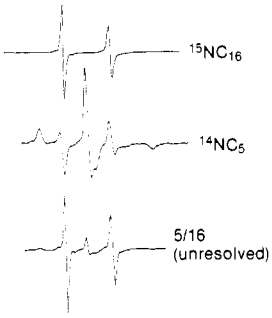


FIGURE 4: Superposition spectra demonstrating lack of spectral resolution for ^{14}NC5:^{15}NC16 (0.25:0.25 mol %) in DMPC (47 °C, pH 9.5). Both components of the ^{15}N spectrum overlap with the $T_\perp$ features of ^{14}NC5.

For the 16:16[3] and 16:12 pairs, the frequency difference between pumping and observing fields (26 MHz) was such that the pumping field was centered on the low-field ^{15}N line when the observing field was at the midpoint of the ^{14}N ($M_I = 0$) transition (Figure 2A,B). The 12:16 and 12:12 pairs have well-resolved ^{14}N midfield transitions but show some overlap in the low-field region (Figure 3A,B). Consequently, the relative positionings of the pump and observer fields were adjusted so that the pump field was slightly skewed to the high-field side of the ^{15}N low-field ($M_I = -1/2$) line when the observer field was centered on the ^{14}N ($M_I = 0$) midfield line.

For the 16:5 and 12:5 pairs, the pump field was set at the low-field, $T_\perp$ component of ^{15}NC5 when the observer was at ^{14}N ($M_I = 0$), as shown in Figures 2C and 3C. Samples containing ^{14}NC5 were resolved only at low- and high-field ^{14}N turning points and were therefore not suitable for field swept, CW ELDOR (e.g., Figure 4). Lack of spectral resolution also precluded studies below the DMPC phase transition temperature of 24 °C. Careful examination of control spectra, which contained only one member of a spin-label pair (e.g., a sample of 0.25 mol % ^{14}NC16 in DMPC), demonstrated no reduction under conditions used for dual-label ELDOR experiments.

pH Dependence of Interaction between ^{14}NC16 and ^{15}NC5. Examination of ^{15}NC5 spectra at various pHs revealed that this probe was capable of occupying at least two distinct environments in the bilayer. As seen in Figure 5, there is a shift in the positions of the low- and high-field turning points between pH 4.5 and 9.5, and two-component spectra are readily apparent at both pH 6.0 and 7.0. This is in agreement with similar observations reported for ^{14}NC5 (Ptak et al., 1978; Sanson et al., 1976).

The intensity of the ELDOR effect for the ^{14}NC16:^{15}NC5 pair also showed a dependence on pH that was not seen with the 16:16 pair. R_∞ values for the 16:5 pair at pH 4.5, 7.0, and 9.5 were 25, 19.4, and 18%, respectively. R_∞ values for the 16:16 pair at these pHs were 34, 31, and 33%. Investigations not specifically concerning pH dependence were carried out at pH 9.5 so that the ^{14}N and ^{15}N fatty acid spin-labels would each exist in single, carboxyl-ionized populations. Although interactions were somewhat larger between protonated spin-labels (at pH 4.5), positioning of the ionized species (at pH 9.5) along the bilayer normal is better defined and therefore

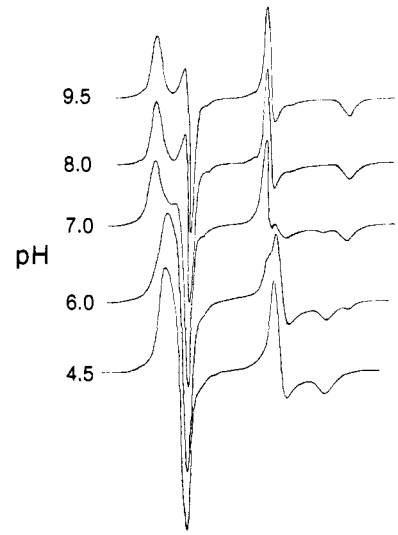


FIGURE 5: Variation of the ESR spectrum of ^{15}NC5 with pH. Samples containing 0.25 mol % ^{15}NC5 in DMPC were prepared as described in the text and equilibrated with (A) 0.1 M sodium borate, pH 9.5, (B) 0.1 M sodium phosphate, pH 8.0, (C) 0.1 M sodium phosphate, pH 7.0, (D) 0.1 M sodium phosphate, pH 6.0, and (E) 0.1 M sodium acetate, pH 4.5. Conventional, first harmonic absorption X-band spectra were recorded at 57 °C.

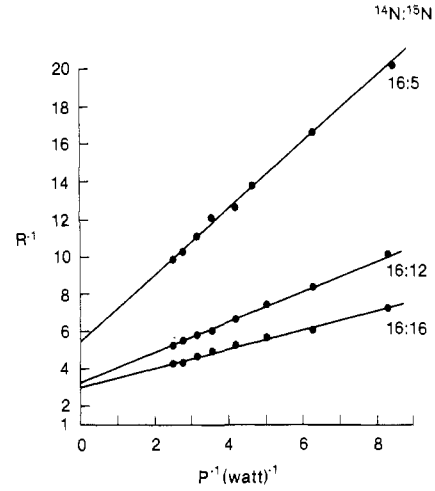


FIGURE 6: Determination of R_∞^{-1}. Plot of $1/R$ (see eq 1) vs. the inverse of the measured pump power for 0.25:0.25 mol % ^{14}NC16:^{15}NC16, ^{14}NC16:^{15}NC12, and ^{14}NC16:^{14}NC5 in DMPC at 47 °C, pH 9.5. Intercepts (R_∞^{-1}) are 3.06 ± 0.10 for 16:16, 3.25 ± 0.13 for 16:12, and 5.47 ± 0.17 for 16:5. Each point represents the average of three scans.

[3] For brevity, spin-label pairs will be designated by the fatty acid carbon to which the nitroxide moiety is conjugated in the order ^{14}N spin-label:^{15}N spin-label, i.e., ^{14}NC16:^{15}NC5 is referred to as simply 16:5.

Table I: Calculation of Lateral Diffusion Constants[a]

T (°C)	$R_\infty^{-1} \pm$ SD[b]	b''[c]	W_{Hex} (MHz)[d]	D (cm²/s)[e]	D (cm²/s)[f]
27	3.32 ± 0.08	0.431	0.196		4.56×10^{-8}
37	3.20 ± 0.07	0.455	0.253	5.8×10^{-8}	5.77×10^{-8}
47	3.06 ± 0.10	0.486	0.348	10.0×10^{-8}	8.09×10^{-8}

[a] Based on the interaction between ^{14}NC16 and ^{15}NC16. [b] Standard deviations are from least-squares linear regression analysis. [c] From eq 2. [d] From eq 3 using W_e values given by Popp & Hyde (1982). [e] Data of Popp & Hyde (1982). [f] From eq 4 where, for comparative purposes, the constants have been assigned the values originally given by Traüble & Sackmann (1972).

Table II: R_∞ Values (%) of Stearic Acid Spin-Label Pairs in DMPC at 47 °C, pH 9.5[a]

	^{15}NC16	^{15}NC12	^{15}NC5
^{14}NC16	32.7	30.8	18.4
^{14}NC12	30.9	32.2	26.2

[a] R_∞ values were obtained from the intercepts of R^{-1} vs. P^{-1} plots as shown in Figure 6. Reductions are expressed as percent of the observed intensity with pump power off (see eq 1 in text). Standard deviations are approximately ±1%.

Table III: R_∞ Values of Stearic Acid Spin-Label Pairs as a Function of Temperature[a]

temp (°C)	^{14}NC16:^{15}NC16 (%)	^{14}NC16:^{15}NC5 (%)
27	30.1	14.0
37	31.3	15.6
47	32.7	18.4

[a] R_∞ values were obtained from the intercepts of R^{-1} vs. P^{-1} plots as shown in Figure 6. Reductions are expressed as percent of the observed intensity with pump power off (see eq 1 in text). Standard deviations are approximately ±1%.

more suitable for studies concerning the interaction of probes located at different regions of the bilayer.

Interactions between Spin-Labels at Different Positions in the Alkyl Chain. In Figure 6, the inverse of the reduction in the observed ^{14}N $M_I = 0$ peak height, R^{-1} (see eq 1), is plotted against the inverse of the pumping power, P^{-1}, for ^{14}NC16 paired with the various ^{15}N spin-labels in DMPC. The ELDOR reductions demonstrate a linear dependence on power with correlation coefficients > 0.99, providing good accuracy in intercept determinations of R_∞^{-1} (Table I). Intercept values are expressed in Table II as percent reductions and demonstrate that the interaction of ^{14}NC16 is greatest (i.e., has the smallest R_∞^{-1}) with ^{15}NC16, slightly weaker with ^{15}NC12, and much weaker with ^{15}NC5. Similar results were obtained when the various ^{15}N spin-labels were paired with ^{14}NC12 (Table II), the 12:12 pair having a slightly greater reduction than 12:16 and 12:5 showing a much weaker interaction.

R_∞ values for the 16:16 and 16:5 pairs at various temperatures above the gel to liquid-crystalline phase transition of DMPC are given in Table III. The 16:5 interaction shows a stronger variation with temperature than that observed for 16:16. R_∞ for 16:5 increased by approximately 27% between 27 and 47 °C, while the corresponding change in R_∞ for the 16:16 pair was 8%.

Estimation of Bimolecular Collision Rates and Lateral Diffusion Constants. To determine the bimolecular collision frequencies from our experimental data, we employ the relationships (Hyde et al., 1968)

$$b'' = (R_\infty^{-1} - 1)^{-1} \tag{2}$$

and

$$W_{Hex} = 2b''W_e \tag{3}$$

where we have assumed that reduction occurs due to Heisenberg exchange only and that the nuclear spin states of each isotopic system are strongly coupled by END relaxation,[4] as shown by the data of Popp & Hyde (1982). As defined by eq 3, W_{Hex} represents the true bimolecular collision frequency of a ^{14}N spin-label with ^{15}N probes: the factor of 2—which corresponds to the total number of independent spin eigenstates available to the pumped population (Eastman et al., 1969)—takes into account the requirement that colliding radicals must be in opposite spin states for exchange to occur.

For comparison with previous work concerning lateral diffusion in lipid bilayers, the lateral diffusion constant, D, may be calculated from W_{Hex} by using the relationship developed by Traüble & Sackmann (1972):

$$W_{Hex} = 2d_c \frac{c}{1+c} \frac{1}{F} \frac{D}{\lambda} \frac{P}{\theta} \tag{4}$$

where F is the area occupied by a single phospholipid, λ is the step length of random Brownian diffusion, d_c is the critical interaction distance, θ is a geometrical factor related to the lattice structure of the bilayer, P is the probability that spin exchange will occur upon collision, and c is the mole fraction of the pumped species. Preliminary studies with the 16:16 pair indicate that W_{Hex} does vary with $c/(1+c)$ in a linear fashion. Results of these calculations (Table I) are in good agreement with the data of Popp & Hyde (1982), as well as with values obtained for the rate of lipid diffusion in DMPC by a wide variety of other methods [discussed in Kuo & Wade (1979), Rubenstein et al., (1979), Chang et al. (1981), and Criado et al. (1982)].

Discussion

In the present work, we have introduced the use of ^{14}N:^{15}N spin-label pairs to ELDOR and saturation-recovery EPR spectroscopy for the study of molecular interactions in biological membranes. In addition to eliminating interference from intramolecular relaxation processes, this approach allows direct examination of molecular dynamics in systems where diffusion is restricted or nonuniform and permits the study of interactions between probes bound to differing carrier molecules or located in diverse environments. Thus, introduction of the dual-label methodology is felt to be a significant advance in the application of ELDOR spectroscopy to the study of intermolecular interactions in biological systems.

The basic requirement for application of the dual-label approach is that of spectral resolution. Clear resolution of ^{14}N and ^{15}N spectral features allows assessment of interaction between the different isotopic spin systems without interference

[4] This latter assumption effectively reduces the present system to the equivalent of a single nucleus, spin $I = {}^1/_2$, as treated by Hyde et al. (1968). The data of Popp & Hyde (1982) indicate that this assumption is good at 27 and 37 °C and begins to break down at higher temperatures (cf. values for W_n/W_e in their Table I). Failure of this assumption results in the statistical factor of 2 in eq 3 becoming larger, producing larger values of W_{Hex} and leading to larger estimates of D. Thus, nuclear relaxation processes do present a complication in ^{14}N:^{15}N ELDOR methodology when W_n, the nuclear relaxation probability, is similar to W_e even though these processes cannot directly couple the two isotopic spin systems. We note that agreement of our data with that of Popp and Hyde is excellent at 37 °C and that they give a slightly larger value of W_{Hex} at 47 °C than that which we report in Table I.

from interaction among probes within each population. As illustrated by the superposition spectra presented in Figure 2 and 3, this resolution requirement is satisfied in liquid-crystalline DMPC for all combinations of C16, C12, and C5 fatty acid spin-labels, with the exception of those involving ^{14}NC5 (e.g., Figure 4). As experience with interpretation of ELDOR spectra from spin-label pairs increases, it is possible that this resolution requirement can be relaxed.

The present experiments demonstrate that most spectral features are resolved when both ^{14}N and ^{15}N probes are in the fast motional region (e.g., the 16:16 pair) and that $T_\perp$ features of the ^{15}NC5 spectrum—representative of a rotationally immobile spin-label—are resolved from the spectral components of a rapidly tumbling ^{14}N probe (e.g., the 16:5 pair). Such resolution allows examination of probe–probe interactions between spin-labels with equilibrium positions in distinctly different regions of the lipid bilayer. Dual-label ELDOR studies appear possible on any sample in which both spin-label populations are rotationally mobile (e.g., the 16:16 pair) and in systems where the ^{14}N probe is mobile and the ^{15}N population is rotationally immobile (e.g., 12:5 and 16:5). Thus, a wide variety of dual-spin-label or spin-label–spin probe experiments are indeed accessible by this methodology.

Perhaps the most significant advance presented in this work is the elimination of END-induced nuclear relaxation as an ELDOR-active process. The END mechanism, an intramolecular process that very effectively couples the hyperfine transitions within each isotopic spin system, limited the usefulness of this methodology for studying lipid diffusion in previous studies employing a single (^{14}N) population of spin-labels. Introduction of the dual-label methodology allowed us to work at all temperatures above the main phase transition of the host lipid, and we have reported data for the 16:16 pair at 27 °C that was inaccessible in the previous ELDOR studies of lipid diffusion (Popp & Hyde, 1982).

In the present experiments, the only potential mechanisms for transfer of saturation between pumped and observed transitions are Heisenberg spin exchange (Hex) and pseudosecular electron–electron dipolar (psEED) relaxation. Comparison of the relative interactions between the various spin-label pairs, although certainly dependent on the spatial separation of the nitroxide moieties along the fatty acid alkyl chain, does not appear to exhibit the $1/a^3$ dependence (where a is the distance of closest approach between interacting radicals) that is expected if psEED relaxation dominates (Hyde & Rao, 1978). Furthermore, the 16:16 interaction does not follow the η/T (η being viscosity, estimated from rotational correlation times) dependence indicated by Freed (1979) for the psEED interaction but rather increases approximately as T/η—as would be expected for the diffusion-limited exchange interaction. Although we cannot as yet make a definitive statement regarding the occurrence of psEED relaxation, the available information supports Heisenberg exchange as the dominant ELDOR-active mechanism of interaction between ^{14}N:^{15}N spin-label pairs.

Although further theoretical development is required before the relative contributions of these processes can be fully separated, both psEED and Hex are intermolecular phenomena, and the reductions observed must therefore be due to physical interaction between the spin-label pairs. If psEED relaxation were significant, the effects would be difficult to distinguish from the case of dominant Heisenberg exchange interaction with a somewhat altered interaction distance. Thus, under conditions of like temperature and concentration, the ELDOR reduction extrapolated to infinite pumping power is a quantitative measure of the interaction between members of a spin-label pair. Furthermore, R_∞ may be qualitatively utilized in comparing the molecular dynamics of different systems or in assessing the response of a particular system to some given perturbation.

Examination of the relative degrees of interaction between the various spin-label pairs (Table II) provides further support for the reliability of these methods in assessing intermolecular (probe–probe) interactions. The R_∞ values do indeed parallel the spatial separation of the spin-labels along the fatty acid alkyl chain, i.e., the interaction of 16:16 = 12:12 > 16:12 = 12:16 >> 12:5 > 16:5.

The similarity of the 16:16 and 12:12 reductions suggests that the degree of interaction between fatty acid spin-labels is not significantly affected by the size of the "cone" inside of which angular fluctuations are allowed but is limited rather by the lateral diffusion limited rate of encounter. This is also supported by the close agreement between the temperature dependence of the estimated lateral diffusion constants for the 16:16 pair (Table I) and that reported previously for lipid diffusion in DMPC by photobleaching recovery [e.g., Criado et al. (1982)].

If the magnitude of the 16:16 (or 12:12) interaction, as measured by R_∞, does in fact represent the lateral diffusion limit of the encounter frequency, then deviation from the R_∞ value of the 16:16 pair (especially as seen with 12:5 and 16:5) implies a dependence of R_∞ on the vertical flexibility of the deeper probe, i.e., the propensity of that probe to approach the level of the bilayer occupied by the other member of the spin-label pair, namely, C5. This is borne out experimentally by the strong temperature dependence of the 16:5 interaction noted above. Such considerations provide a new means to examine the motional fluctuations undergone by a fatty acid spin-label intercalated into a lipid bilayer.

The occurrence of a strong interaction between ^{14}NC16 and ^{15}NC5 is our most surprising experimental result. In addition to the above-mentioned temperature dependence, the 16:5 interaction was markedly enhanced at low pH, where protonation of the stearic acid carboxyl group presumably allows displacement of the nitroxide moiety at C5 deeper into the membrane bilayer (Sanson et al., 1976). These observations are all consistent with the suggestion that the C16 nitroxide makes high-frequency excursions toward the bilayer surface, entering the region of the membrane occupied by the spin-label located at C5.

Godici & Landsberger (1974), who observed the effect of stearic acid spin-labels on ^{13}C nuclear relaxation rates in egg phosphatidylcholine, concluded that the C16 nitroxide was localized primarily near the center of the bilayer. Such observations would however be sensitive principally to the equilibrium position of the spin-label and may be reconciled with our present findings if the proposed fluctuations occur on a time scale that is fast relative to the NMR measurement.

More recently, Seigneuret et al. (1981) observed spin–spin interactions between [^{14}N]- and [^{15}N]nitroxide lipid–analogue spin-labels in lecithin bilayers by conventional EPR methods. They concluded that the interaction observed between ^{15}NC16 and an ^{14}N spin-label near the membrane surface was not significant. The reason for this discrepancy is as yet unknown but could arise from differences in either the systems (i.e., the host lipid, the nature of the spin-labels) or the spectroscopic methods employed.

In our experiments, the C16 nitroxide interacts with the spin-label at C5 with a probability approximately half that of the 16:16 interaction. This reflects a remarkable flexibility

in the fatty acid alkyl chain [see also Barclay & Ingold (1981)] and suggests that the relative degree of resistance imposed on vertical fluctuations—or transverse order—may be an important physical characteristic of a membrane bilayer.

In summary, the utilization of electron–electron double-resonance spectroscopy is a promising method for the examination of interactions between dual ^{14}N:^{15}N spin-labels in biological membranes. We have established that, under a wide range of conditions, spectral resolution between the signals arising from the ^{14}N and ^{15}N systems is achieved. Additionally, the magnitude of the ELDOR reduction appears to reflect the degree of interaction between spin-label pairs, and estimates of lateral diffusion constants obtained from these experiments are in good agreement with those found for lipid diffusion by other methods. Because this methodology is potentially capable of detecting short-range, local diffusion in systems where displacement over relatively large distances does not occur, it may provide a useful approach to the investigation of phenomena such as lipid domain formation and the lateral diffusion of integral membrane proteins.

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Registry No. C5, 29545-48-0; C12, 29545-47-9; C16, 53034-38-1; DMPC, 18194-24-6.

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