Interactions of ^{14}N:^{15}N Stearic Acid Spin-Label Pairs: Effects of Host Lipid Alkyl Chain Length and Unsaturation[†]

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ABSTRACT: Electron–electron double resonance (ELDOR) and saturation recovery electron paramagnetic resonance (EPR) spectroscopy have been employed to examine the interactions of ^{14}N:^{15}N stearic acid spin-label pairs in fluid-phase model membrane bilayers composed of a variety of phospholipids. The [^{14}N]-16-doxylstearate:[^{15}N]-16-doxylstearate (16:16) pair was utilized to measure lateral diffusion of the spin-labels, while the [^{14}N]-16-doxylstearate:[^{15}N]-5-doxylstearate (16:5) pair provided information on vertical fluctuations of the 16-doxylstearate nitroxide moiety toward the membrane surface. Three saturated host lipids of varying alkyl chain length [dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine (DSPC)], an α-saturated, β-unsaturated lipid [1-palmitoyl-2-oleoylphosphatidylcholine (POPC)], and phosphatidylcholine from a natural source [egg yolk phosphatidylcholine (egg PC)] were utilized as host lipids. Lateral diffusion of the stearic acid spin-labels was only slightly affected by alkyl chain length at a given reduced temperature (T_r) in the saturated host lipids but was significantly decreased in POPC at the same T_r. Lateral diffusion in DMPC, POPC, and egg PC was quite similar at 37 °C. A strong correlation was noted between lateral diffusion constants and rotational mobility of [^{14}N]-16-doxylstearate. Vertical fluctuations were likewise only slightly influenced by alkyl chain length but were strongly diminished in POPC and egg PC relative to the saturated systems. This diminution of the 16:5 interaction was observed even under conditions where no differences were discernible by conventional EPR. These studies indicate that vertical fluctuation of 16-doxylstearate is quite sensitive to host lipid unsaturation and that ELDOR studies of interactions between ^{14}N:^{15}N spin-label pairs can provide information on spin-label motion beyond that given by conventional EPR.

There are several factors known to be involved in the interrelationship between the molecular composition and physical state of biological membranes. These include the composition and content of integral membrane proteins, cholesterol content, variations in the relative amounts of the major classes of phospholipids, and lipid alkyl chain unsaturation. The influence of each of these factors on membrane structure has been assessed by several different biophysical techniques, and a number of studies have established the correlation of physical changes with membrane function.

Recently, we have introduced the use of electron–electron double-resonance (ELDOR)[1] and saturation recovery EPR spectroscopy to examine interactions of stearic acid spin-labeles in model membranes (Popp & Hyde, 1982; Feix et al., 1984). The ELDOR methodology provides a direct measure of the bimolecular collision rate between probe molecules and is therefore applicable to problems concerning short-range diffusion where molecules are not displaced over relatively large (i.e., micrometer) distances. This may be particularly important for the study of diffusion within regions of lateral phase separation and the examination of interactions between motionally restricted membrane components.

In our prior work utilizing ^{14}N:^{15}N spin-label pairs (Feix et al., 1984), we examined interactions between various isomers of nitroxide-labeled stearic acid in model membranes of dimyristoylphosphatidylcholine (DMPC). Interaction between the various spin-label pairs paralleled their separation along the stearic acid alkyl chain, and lateral diffusion constants calculated from the experimental data were found to be in excellent agreement with those reported previously for lipid analogue probes by a variety of biophysical methods. Of particular interest was our observation of interaction between nitroxide moieties located at carbon 16, near the terminal methyl group of stearic acid, and carbon 5 located near the bilayer surface. The strong temperature and pH dependencies suggested that this 16:5 interaction occurred due to excursions of the terminal methyl region of the stearic acid spin-labels toward the membrane surface, and we proposed that the degree to which the host membrane allowed such vertical fluctuations might be a significant parameter for describing the physical state of the bilayer.

In the present work we extend our ELDOR studies of interactions between ^{14}N:^{15}N spin-label pairs to model membranes other than DMPC. The $^{14}NC16$:$^{15}NC16$ spin-label pair is utilized to obtain a measure of the lateral diffusion constant (D) for stearic acid spin-labels, while the $^{14}NC16$:$^{15}NC5$ pair—in which interaction requires a vertical component of the motion as well as lateral diffusion—is used to examine vertical fluctuations (Figure 1). In particular, we examine the effects of host lipid alkyl chain length and unsaturation on lateral diffusion and the rate of vertical fluctuations. Such

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[1] Abbreviations: D, lateral diffusion constant; diO-C_{18}(3), 3,3′-dioctadecyloxadicarbocyanine; DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DSPC, distearoylphosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; egg PC, egg yolk phosphatidylcholine; ELDOR, electron–electron double resonance; EPR, electron paramagnetic resonance; Hex, Heisenberg spin exchange; C5, 2-(3-carboxypropyl)-4,4-dimethyl-2-tridecyl-3-oxazolidineoxyl; C16, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidineoxyl; NBD, 4-nitrobenz-2-oxa-1,3-diazole; NMR, nuclear magnetic resonance; T_m, main gel to liquid crystalline phase transition temperature; T_r, reduced temperature; τ_c, rotational correlation time.

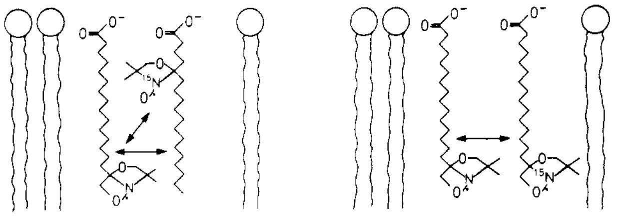

FIGURE 1: Schematic of ^{14}N:^{15}N spin-label pair interactions. Interactions of ^{14}NC16 with ^{15}NC5 (left) and between ^{14}NC16 and ^{15}NC16 (right). The 16:16 interaction is used to measure the lateral diffusion limited rate of encounter, while the 16:5 interaction is dependent on vertical fluctuation of the C16 spin-label as well as the lateral diffusion.

studies provide insights into the relative sensitivity of the ELDOR methodology to specific changes in membrane composition and should help form a basis for understanding future studies of more complex membrane systems.

MATERIALS AND METHODS

The [^{15}N]nitroxide spin-labels employed in this study, 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidineoxyl (C16 stearic acid spin-label or C16) 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). The [^{14}N]nitroxide derivative of C16 was obtained from Sigma. Dimyristoylphosphatidylcholine (DMPC), dipalmitoylphosphatidylcholine (DPPC), and distearoylphosphatidylcholine (DSPC) were from Sigma. Egg yolk phosphatidylcholine (>99% pure) and 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) were from Avanti Polar Lipids, Inc. Stock solutions of the spin-labels (ca. 0.5 mM) and lipids (0.15) M were prepared in chloroform and stored at −20 °C. Multilamellar liposomes (40% lipid by weight) were prepared by directly hydrating the dried lipids and spin-labels with an appropriate amount of buffer as described previously (Feix et al., 1984). All samples were run in 0.1 M borate, pH 9.5, to ensure that the stearic acid spin-labels would be in the ionized state (Ptak et al., 1980; Feix et al., 1984).

ELDOR measurements were made as described in our previous study of ^{14}N:^{15}N spin-label pairs (Feix et al., 1984). All samples were run in TPX capillaries (0.8 cm i.d.). Samples were thoroughly deoxygenated under nitrogen gas to avoid inaccuracies due to O_2-dependent shortening of the nitroxide spin–lattice relaxation time (Popp & Hyde, 1981). ELDOR reductions were measured for the (^{14}N) $M_I = 0$ lines as the (^{15}N) $M_I = -{}^1/_2$ transition was pumped (Figure 2). The low-field (^{14}N $M_I = 1$) line was used for normalization.

The ELDOR reduction factor R is defined as

$$R = 1 - (I_p/I_o) \tag{1}$$

where I_p and I_o are the intensities of the observed transition with the pumping field on and off, respectively (Hyde et al., 1968; Eastman et al., 1970; Feix et al., 1984). Reductions were determined at a series of 8–10 pumping field intensities, and R^{-1} was plotted against the inverse of the pump power, P^{-1} (Popp & Hyde, 1982; Feix et al., 1984). Extrapolation to infinite pumping power gives a parameter, R_∞^{-1}, that is related to the relaxation processes occurring in the spin system (Eastman et al., 1970).

In the present system, bimolecular collision rates may be calculated from experimental data via the relationships (Hyde et al., 1968):

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

and

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

where W_{Hex} is the Heisenberg exchange frequency and W_e ($=2T_{1e}^{-1}$) the probability of electron spin–lattice relaxation for the observed transition. As defined in eq 3, W_{Hex} represents the bimolecular collision frequency of a ^{14}N spin-label with ^{15}N probes. It is assumed that reduction occurs due to Heisenberg exchange and that the nuclear spin states of the pumped species are strongly coupled by nuclear relaxation (Feix et al., 1984). Lateral diffusion constants (D) were calculated according to the model of of Traüble and Sackmann (1972):

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

where F (60 Å^2), d_c (20 Å), and λ (8 Å) are the area per lipid molecule, the critical interaction distance, and the diffusional step length, respectively. P is the probability of exchange upon collision, θ a geometric factor related to the structure of the bilayer, and c the mol fraction of the pumped species. For comparative purposes, we have used the constant values given previously (Traüble & Sackmann, 1972).

Saturation recovery measurements of spin–lattice relaxation times (T_{1e}) were maded on a pulse EPR spectrometer by using equipment (Huisjen & Hyde, 1974; Percival & Hyde, 1975) and methods (Hyde, 1979) described previously. In the present context the pertinent recovery times are those of the transition observed (i.e., the ^{14}N $M_I = 0$ line) in the corresponding ELDOR experiments (Yoshida et al., 1973; Yin et al., 1987a) and all T_{1e} measurements were made on that line.

Recent work has shown that saturation recovery decays from samples containing ^{14}N:^{15}N spin-label pairs are multiexponential (Yin et al., 1987b). This is particularly true in the initial phase of the recovery, where information on exchange rates may be obtained. We have minimized this complication by confining our analysis to the tail of the recovery curve, which is characteristic of T_{1e} for the observed species, and by using a long ($>3T_{1e}$) saturating pulse. All experiments were done by using a loop-gap resonator (Froncisz & Hyde, 1982), enabling saturation of the spin system with relatively low microwave powers. Typically, an 8-μs pulse of 50 mW ($H_1 \simeq 1.2$ G) was used for saturation, followed by detection with an observer field of 0.25 mW ($H_1 \simeq 0.08$ G). The observed T_{1e} taken from the tail of the recovery curve was independent of pulse length for times longer than 2 μs and did not change with observed power below 1.0 mW. The pump field was phase modulated to suppress free-induction decay (Percival & Hyde, 1975), and the recovery signal was detected by using low frequency (25-Hz) field modulation. Recovery times were independent of the 25-Hz modulation amplitude below 2.5 G, and a 1.0-G modulation was routinely employed.

Conventional EPR spectra were obtained on a Varian E-109 X-band spectrometer with Varian temperature control accessories. Spectra were obtained with 5-mW incident microwave power and a 100-kHz field modulation of 0.5 G. Rotational correlation times (τ_c) were calculated by using an average of the linear and quadratic line width formalisms (Stone et al., 1965; Keith et al., 1970):

$$\tau_c = (6.51 \times 10^{-10})\ \Delta H(0)\ [(h_0/h_{-1})^{1/2} - 1]\ (\mathrm{s}) \tag{5}$$

where $\Delta H(0)$ is the peak-to-peak width of the center line and h_0 and h_{-1} are the amplitudes of the $M_I = 0$ and -1 lines, respectively.

RESULTS

ELDOR spectra of the ^{14}NC16:^{15}NC16 and ^{14}NC16:^{15}NC5 spin-label pairs in DMPC are shown in Figure 2. In each

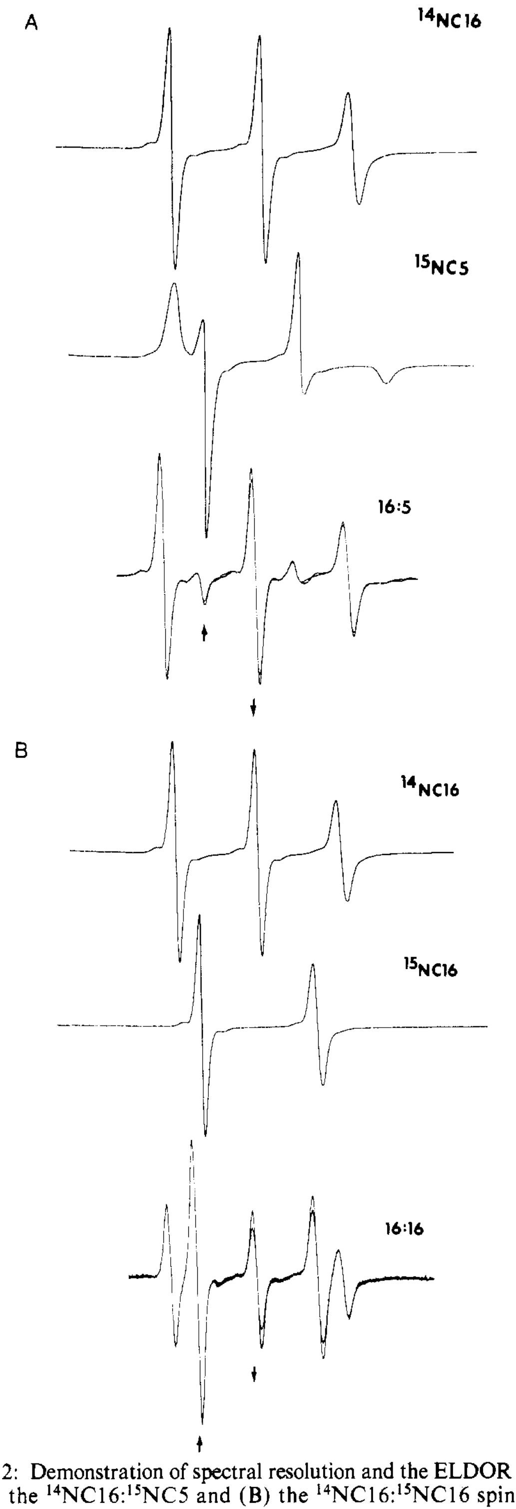

FIGURE 2: Demonstration of spectral resolution and the ELDOR effect for (A) the ^{14}NC16:^{15}NC5 and (B) the ^{14}NC16:^{15}NC16 spin-label pairs. The upper spectra show relative positions of lines arising from the respective ^{14}N and ^{15}N spin-labels. At bottom are superimposed spectra of the ^{14}N:^{15}N pairs recorded with the pump field off and at 400 mW. Samples contain 0.5 mol % ^{14}N spin-label and 0.5 mol % ^{15}N spin-label in DMPC, equilibrated with 0.1 M sodium borate, pH 9.5. The temperature is 37 °C. Arrows define the position of the pumping field (↑) when the observer field (↓) is centered on the ^{14}N $M_I = 0$ line. Frequency separations of the pumping and observing microwave fields are 24 MHz in (A) and 26 MHz in (B).

case, part of the low-field ($M_I = -^1/_2$) ^{15}N transition lying between the low-field ($M_I = -1$) and center-field ($M_I = 0$) ^{14}N lines is resolved. This was true for all of the lipid systems studied in the fluid phase. Quantitative ELDOR measurements were therefore made by pumping the ^{15}N $M_I = -^1/_2$ line while simultaneously observing the ^{14}N $M_I = 0$ transition. At the frequency differences (pump–observe) employed, no observable ELDOR effects occur with samples containing either ^{15}N or ^{14}N spin-label alone.

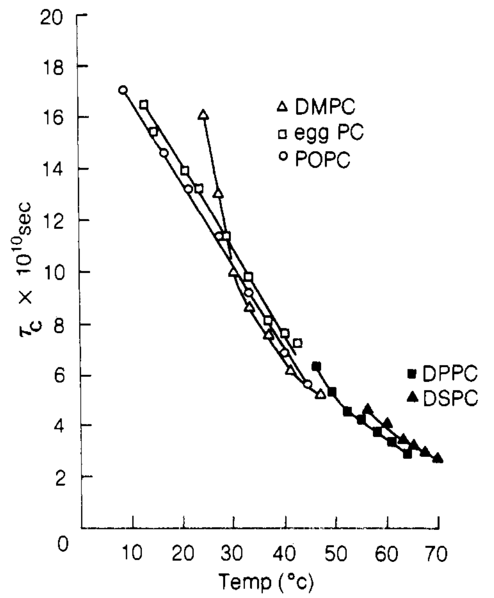

FIGURE 3: Rotational correlation times (τ_c) of ^{14}NC16 (0.5 mol %) in the various host liquids as a function of temperature. Values of τ_c are an average of the linear and quadratic solutions obtained from line width analysis (Stone et al., 1965; Keith et al., 1970).

Table I: ELDOR and Saturation Recovery Data for the ^{14}NC16:^{15}NC5 Spin-Label Pair

host lipid	temp (°C)	$R_\infty{}^a$ (%)	T_{1e} ($\times 10^6$ s)	$W_{\text{Hex}}{}^b$ (MHz)
DMPC	37	31.3	2.81	0.162
DPPC	55	29.2	2.54	0.162
DSPC	68	26.4	2.34	0.153
POPC	37	24.2	2.76	0.116
egg PC	37	23.7	2.72	0.114

[a] R_∞ is the inverse of $R_\infty{}^{-1}$, determined experimentally. [b] W_{Hex} is calculated from experimental data according to eq 2 and 3 (see text). Samples contained 0.5 mol % ^{14}NC16 and 0.5 mol % ^{15}NC5. Liposomes were equilibrated with 0.1 M borate, pH 9.5, to give 40% lipid by weight (see text). Mean standard deviations are ±0.9% for R_∞, ±0.14 μs for T_{1e}, and ±0.018 MHz for W_{Hex}.

Figure 3 shows the apparent rotational correlation time (τ_c) for ^{14}NC16 in membranes composed of the various host lipids as a function of temperature. τ_c is inversely proportional to the rate of rotational motion of the nitroxide moiety, so that τ_c decreases as rotational motion increases. τ_c for ^{14}NC16 was very similar in POPC and egg PC throughout the temperature range explored. The apparent rotational correlation time of ^{14}NC16 in DMPC was also quite similar to that observed in POPC and egg PC at temperatures above 30 °C, while showing a more rapid increase as the main phase transition temperature[2] was approached. τ_c was much shorter for DPPC and DSPC in a reduced temperature[3] range similar to that examined for DMPC. ELDOR studies were conducted at 37 °C for DMPC, POPC, and egg PC on the basis of similarity

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[2] Main phase transition temperatures (T_m) are 24 (DMPC), 41 (DPPC), and 55 °C (DSPC) (Ladbrooke & Chapman, 1969; Silvius, 1982). T_m values of −5 to 3 °C have been reported for POPC (Silvius, 1982). Egg PC exhibits a broad phase change by differential scanning calorimetry from −15 to −7 °C (Ladbrooke & Chapman, 1969).

[3] The reduced temperature (T_r) is defined as $(T - T_m)/T_m$, where all temperatures are in Kelvin. T_r is thus a measure of the displacement away from the main phase transition temperature for any given host lipid. Acyl chains of phospholipids at the same T_r are subject to similar average molecular forces (Seelig & Seelig, 1977).

Table II: ELDOR and Saturation Recovery Data and Lateral Diffusion Constants for the ^{14}NC16:^{15}NC16 Spin-Label Pair

host lipid [temp (°C)]	R_∞ (%)	T_{1e} ($\times 10^6$ s)	W_{Hex}[a] (MHz)	D[b] ($\times 10^8$ cm^2/s)
DMPC (37)	32.3	2.40	0.198	4.61
DPPC (55)	31.3	2.32	0.205	4.77
DSPC (68)	29.5	2.12	0.216	5.03
POPC (37)	32.3	2.30	0.207	4.82
egg PC (37)	30.3	2.28	0.190	4.42

[a] From eq 2 and 3. [b] From eq 4. Samples contained 0.5 mol % ^{14}NC16 and 0.25 mol % ^{15}NC16. Liposomes were hydrated in 0.1 M borate, pH 9.5, to contain 40% lipid by weight (see text). Mean standard deviations were ±0.7% for R_∞, ±0.14 μs for T_{1e}, ±0.016 MHz for W_{Hex}, and $\pm 0.37 \times 10^{-8}$ cm^2/s for D. Note that, for comparison with exchange rates for the 16:16 pair (Table I), W_{Hex} values should be multiplied by a factor of 2 to normalize the concentration of ^{15}N probe.

of the rotational motion of ^{14}NC16 at that temperature as measured by conventional EPR spectroscopy. Studies in DPPC and DSPC were conducted at temperatures (55 and 68 °C, respectively) corresponding to 37 °C in DMPC on a reduced temperature scale.

ELDOR reductions (R_∞), electron spin-lattice relaxation times (T_{1e}), and calculated Heisenberg exchange frequencies (W_{Hex}) for the ^{14}NC16:^{15}NC5 spin-label pair in the various lipid systems are given in Table I. The ELDOR reduction for 16:5 in DMPC, DPPC, and DSPC decreases slightly with increasing alkyl chain length. This is due at least in part to faster relaxation of the observed ^{14}N spin system, indicated by progressively shorter T_{1e} values (Table I). The trend toward slightly faster T_{1e}'s with increasing chain length in the saturated lipids is consistent with the faster τ_c of ^{14}NC16 shown in Figure 3 and was expected on theoretical basis (e.g., Popp and Hyde (1982) and references cited therein). Heisenberg exchange rates in DMPC, DPPC, and DSPC are not significantly different, indicating little dependence of vertical fluctuations on alkyl chain length.

The bimolecular collision rate of 16:5 in egg PC and POPC, as given by W_{Hex}, is significantly less than that observed in the saturated phospholipid systems. W_{Hex} for 16:5 in egg PC and POPC is approximately 30% less than in DMPC under conditions where τ_c and T_{1e} are virtually the same. This result provides strong indication that the double bond in the β-alkyl chain of egg PC and POPC interferes with the vertical fluctuation of ^{14}NC16.

ELDOR and saturation recovery data for the ^{14}NC16:^{15}NC16 pair, along with calculated lateral diffusion constants, are given in Table II. The concentration of ^{15}N spin-label (the pumped species) for the 16:16 pair was half that used for the 16:5 pair (Table I). This maintained the interaction frequency in the range for optimal ELDOR sensitivity (i.e., where $W_{\text{Hex}} \simeq W_e$) to facilitate comparison of different lipid systems. W_{Hex} increases linearly with the concentration of the pumped species, in agreement with eq 4 (for $c \ll 1$). Manipulation of label concentration in this fashion extends the applicability of ELDOR experiments to an even broader range of diffusion rates.

Although a trend toward faster lateral diffusion with increasing alkyl chain length is noted for the saturated host lipids, these differences were not statistically significant. The ELDOR and saturation recovery results obtained for the 16:16 pair were also similar in those systems containing unsaturated alkyl chains (egg PC and POPC). These results indicate that alkyl chain unsaturation and chain length matching have relatively little effect on the lateral diffusion of stearic acid spin-labels when compared under the temperature conditions described. However, we also examined interaction of the 16:16 pair in POPC at a reduced temperature corresponding to that of the saturated systems. We found D at 8 °C (based on T_m of −5 °C) to be 2.12×10^{-8} cm$^-$/s—less than half that of the saturated host phospholipids.

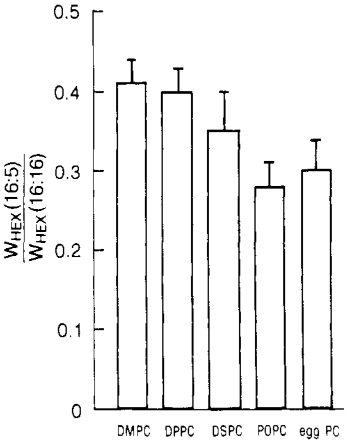

FIGURE 4: Relative probability of vertical fluctuation as a function of host lipid composition. Shown are the Heisenberg exchange rates for 16:5 pairs normalized to the 16:16 exchange frequency for the various host lipids. Label concentrations were 0.5 mol % ^{14}NC16 and 0.25 mol % ^{15}N spin-label for both pairs. The data are for the temperatures given in Tables I and II. Error bars represent the maximum combined standard deviations of W_{Hex}(16:16) and W_{Hex}(16:5).

The relative probability of Heisenberg exchange for the 16:5 spin-label pair normalized to the 16:16 exchange probability at the same probe concentration is depicted in Figure 4 for each of the host phospholipids. Normalization of W_{Hex} for 16:5 to the 16:16 exchange frequency in this fashion takes into account that the interaction between nitroxide probes is limited by the lateral diffusion rate of their carrier molecules, regardless of their position along the stearic acid alkyl chain. Thus in DMPC the probability of 16:5 interaction is approximately 0.41 for each diffusion-limited encounter of spin-labels. The relative probability of 16:5 interaction falls to 0.40 for DPPC, to 0.36 for DSPC, and to 0.30 and 0.28 for egg PC and POPC, respectively (Figure 4).

DISCUSSION

The current work examines the influence of host lipid unsaturation and alkyl chain length on the interaction of ^{14}N:^{15}N stearic acid spin-label pairs. Our previous ELDOR studies indicated that the bimolecular collision rate for the 16:16 spin-label pair could be utilized to determine lateral diffusion constants for stearic acid spin-labels and that strong interactions occurred between C16 and C5 spin-labels in fluid phase DMPC (Feix et al., 1984). We suggested that the 16:5 interaction was due to excursions of the terminal methyl region of the C16 spin-label toward the membrane surface, and we termed this motion vertical fluctuation. Because vertical fluctuation is dependent on the rotational mobility of the spin probe as well as its lateral diffusion, we reasoned that the 16:5 interaction should be sensitive to changes in the physical state of the bilayer such as those that accompany alterations in lipid composition.

The present experiments indicate that vertical fluctuations of the terminal methyl group of stearic acid spin-labels toward the membrane surface, as observed by interaction of ^{14}NC16 with ^{15}NC5, are only moderately influenced by alkyl chain length. Heisenberg exchange rates (i.e., bimolecular collision frequencies) for the 16:5 pair through the series of saturated host lipids (DMPC, DPPC, DSPC) were not significantly different (Table I). A decrease in the relative probability of the 16:5 interaction (normalized to the 16:16 collision frequency) was observed in DSPC; however, the change was relatively small. These results indicate that mismatch in alkyl chain length between the stearic acid spin-label and the host phospholipid is not a major factor in the occurrence of vertical fluctuation.

A much more striking decrease in 16:5 interaction was observed with host lipid unsaturation. W_{Hex} for the 16:5 pair was decreased by approximately 30% in egg PC and POPC, which contain cis double bonds in the β-alkyl chain, relative to the saturated host lipids (Table I). This was observed under conditions where egg PC and POPC were well above the reduced temperature corresponding to that of the saturated phospholipids, and where the conventional EPR parameters of the stearic acid spin-labels in egg PC and POPC were virtually identical with those in DMPC (Figure 3). The results indicate that under these conditions the rates of rotational reorientation of the C16 nitroxide in egg PC, POPC, and DMPC are similar while the extent of vertical fluctuation in the unsaturated systems is decreased. This suggests that the cis double bonds in the β-alkyl chains of POPC and egg PC act as a barrier to vertical fluctuations.

Comparable effects of phospholipid alkyl chain unsaturation on the physical properties of membrane bilayers have been found in studies utilizing other biophysical methods. In a deuterium NMR study Seelig and Seelig (1977) found that POPC was significantly more ordered than DPPC in the middle region of the alkyl chain (fourth through tenth methylene carbons) at corresponding reduced temperatures in the fluid phase. Using resonance Raman spectroscopy, Lavialle and Levin (1980) similarly concluded that the introduction of a cis double bond—although decreasing intramolecular order along the unsaturated alkyl chain—increased the lateral, intermolecular order in POPC bilayers relative to DMPC in the fluid phase. These findings of increased intermolecular order in the fluid phase upon incorporation of the relatively rigid carbon–carbon double bond are consistent with our results indicating that host lipid unsaturation restricts vertical fluctuation of the intercalated stearic acid spin-labels.

An intermolecular ordering imparted by alkyl chain unsaturation is also suggested by the lateral diffusion rates of the stearic acid spin-labels if systems are compared at corresponding reduced temperatures. We find D for the C16 spin-label in POPC at 8 °C to be less than half that of the saturated systems at the corresponding reduced temperature. Others have also reported that lateral diffusion in both egg PC (Kuo & Wade, 1979; Derzko & Jacobson, 1980) and POPC (Vaz et al., 1985) is much slower than in saturated systems (e.g., DMPC) when compared at corresponding reduced temperatures.

In contrast, we observed little difference in D for C16 in egg PC, POPC, and DMPC with all systems at 37 °C—under conditions where the rotational motion of ^{14}NC16 was similar. Consequently, variation in alkyl chain unsaturation in a natural biological membrane may influence the rate of vertical fluctuations but would not be expected to markedly affect lateral diffusion.

In our studies the 16:16 exchange frequency (and corresponding calculated values of D) were only slightly influenced by alkyl chain length in saturated host lipids at corresponding reduced temperatures. A number of fluorescence recovery after photobleaching (FRAP) experiments have found that lateral diffusion of fluorescent probes in saturated host lipids increases with increasing alkyl chain length when compared at corresponding reduced temperatures in the fluid phase (Wu et al., 1977; Derzko & Jacobson, 1980; Vaz et al., 1985). Reported differences for D in DMPC and DPPC vary from approximately a factor of 2 (Derzko & Jacobson, 1980) to 25% (Wu et al., 1977). Kuo and Wade (1979), using a pulsed-gradient NMR technique, also found diffusion of lipids in DPPC to be greater than in DMPC at corresponding T_r [from data in Figure 4 of Kuo and Wade (1979)]. While our results are in general agreement with this trend, the differences we observe are somewhat smaller.

The basis for the above discrepancy is somewhat uncertain but could arise from a variety of sources. The probe molecules employed in the FRAP experiments, NBD derivatives of phosphatidylethanolamine and long-chain carbocyanine dyes [e.g., diO-C$_{18}$(3)], are considerably bulkier than the spin-label probes used in this study. The larger fluorescent molecules may well be more strongly influenced by small differences in bilayer structure [see, for example, the discussion on mean free volume in Vaz et al. (1985)] than the smaller and more flexible stearic acid spin-labels. Additionally, both the FRAP and pulsed-gradient NMR experiments measure long-range, macroscopic diffusion whereas the ELDOR experiment is based on the kinetics of probe–probe interaction. For $D = 5 \times 10^{-8}$ cm^2/s, the root mean square displacement on the time scale of the ELDOR experiment (10^{-6} s) is approximately 50 Å, as opposed to the micrometer displacement that occurs on the time scale of the other methods. We conclude that while structural differences may exist in saturated host lipids of differing alkyl chain length which can influence the long-range lateral diffusion of lipids, these differences are not likely to produce (or be responsible for) significant changes in the ELDOR detection of interaction for the 16:16 spin-label pair.

In spite of the above differences, the lateral diffusion constants determined in our dual-label ELDOR studies are in excellent agreement (within a factor of 2–4) with those reported by numerous photobleaching recovery studies [e.g., Wu et al. (1977), Reubenstein et al. (1979), Derzko and Jacobson (1980), and Vaz et al. (1985)], pulsed-gradient NMR studies (Kuo & Wade, 1979), and EPR studies based on spin-exchange line broadening (Scandella et al., 1972; Traüble & Sackmann, 1972; Davoust et al., 1983). The relative sensitivity to changes in temperature (Feix et al., 1984) and host lipid unsaturation (this work) is also in good agreement with other methods. Thus the viability of ELDOR methodology utilizing the ^{14}NC16:^{15}NC16 spin-label pair to measure lipid lateral diffusion would seem to be well established.

Results throughout these studies were consistent with the physical interdependence of lateral and rotational diffusion processes [e.g., Saffman and Delbrück (1975)]. The slight increases in D for DPPC and DSPC relative to the other systems corresponded well with the slightly faster rotational correlation times observed in those lipids (Figure 3). As mentioned previously, lateral diffusion constants in DMPC, POPC, and egg PC were similar under conditions where τ_c for ^{14}NC16 was the same. Most strikingly, the decrease for D for POPC at 8 °C relative to 37 °C [$(2.12 \times 10^{-8}$ cm^2/s$)/(4.76 \times 10^{-8}$ cm^2/s$) = 0.45$] was virtually identical with the accompanying decrease in rotational mobility [$\tau_c(8\ ^\circ\text{C})/\tau_c(37\ ^\circ\text{C}) = 0.44$]. A similar consistency was also noted in the temperature dependence of D and τ_c in DMPC (data not shown).

We have also in this work introduced the concept of normalizing exchange frequencies between probes on dissimilar carrier molecules (e.g., the 16:5 pair) by the exchange probability due to lateral diffusion (W_{Hex} for 16:16, Figure 4). Although this had little impact on the present data (since lateral diffusion rates in the various systems did not greatly vary), such an approach does provide a means to compare the propensity for vertical fluctuations in systems where the lateral diffusion limited rate of encounter differs. Assuming that probe–probe interaction is lateral diffusion limited (i.e., that only one collision between nitroxide moieties occurs per encounter; Traüble & Sackmann, 1972; Davoust et al., 1983), it may also provide insight into separating the intramolecular component of motion (e.g., the vertical excursions) from lateral diffusion processes. Such normalization has proven useful in evaluating the effects of cholesterol on the 16:5 interaction (Yin et al., 1987a).

Given the above considerations, we believe this normalization procedure to be generally applicable for examining relative interaction rates between pairs of lipid analogue probes. Because Heisenberg exchange frequencies are used, potential difficulties due to differences in T_{1e} have been eliminated. (Simple normalization of R_∞'s would not be valid for systems having different T_{1e}'s.) This procedure may be particularly appropriate in the given case (i.e., for 16:16 and 16:5), since the observed species is the same in both pairs. However, we feel this need not be the case as long as exchange probabilities are used for comparison.

The studies reported herein utilize stearic acid spin-labels to probe the physical characteristics of membrane bilayers and reflect the degree of motional freedom (lateral diffusion or vertical fluctuation) allowed these probes by the various host lipids. Further insight into the occurrence of vertical fluctuations in biological membranes could no doubt be gained from the use of phospholipid spin-labels. Indeed, the degree to which the alkyl chains of phospholipids undergo vertical fluctuation (if at all) is an interesting question in itself. Such studies await the synthesis (in progress) of the appropriate ^{15}N analogues. The present work does show, however, that the relative resistance imposed upon vertical fluctuation of stearic acid spin-labels is sensitive to changes in membrane phospholipid composition. Studies employing stearic acid spin-label pairs do therefore appear to be capable of providing insights into the physical properties of biological membranes.

In conclusion, we have examined the sensitivity of the dual-label ELDOR methodology to certain types of changes in membrane composition. We find that lateral diffusion of stearic acid spin-labels is only slightly influenced by either alkyl chain length or unsaturation of the host phospholipid under conditions where the rotational mobility of the spin probe is similar. Vertical fluctuations, as evidenced by interaction of C16 and C5 stearic acid spin-labels, are only moderately affected by alkyl chain length but are significantly diminished by host lipid unsaturation. This is observed even under conditions where conventional EPR spectra are not different, indicating that the ELDOR approach can indeed give information regarding spin-label motion additional to that obtained by usual spin-labeling methods. Nonetheless, the interaction of C16 and C5 in bilayers composed of *sn*-2 unsaturated phospholipids, which predominate in natural biological membranes, does occur to an appreciable degree. Further insight into the physiological and biochemical significance of these results, and of vertical fluctuations in general, may be gained by the extension of these studies to other model systems and to native biological membranes.

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Registry No. DMPC, 13699-48-4; DPPC, 2644-64-6; DSPC, 4539-70-2; POPC, 6753-55-5; [^{14}N]-16-doxylstearate, 53034-38-1; [^{15}N]-16-doxylstearate, 69633-15-4; [^{15}N]-5-doxylstearate, 88292-51-7.

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