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Molecular Motion of Small Nonelectrolyte Molecules in Lecithin Bilayers

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Summary. We have used magnetic resonance spectroscopy, both ESR and ^{13}C spin relaxation, to measure translational and rotational mobilities and partition coefficients of small nitroxide solutes in dipalmitoyl lecithin liposomes. Above the bilayer transition temperature, T_c, the bilayer interior is quite fluid, as determined from the solutes' rapid rotational and moderately rapid translational motion; the rotational and translational viscosities within the bilayer are $\eta_R < 1$ cP and $\eta_T = 6$–10 cP, respectively. η_T and η_R are independent of molecular size for all solutes studied, but all were small compared to the size of the phospholipids. η_T, and probably η_R, are relatively independent of temperature above T_c, but both increase very sharply as temperature is lowered below T_c; at 32 °C, η_R increases to 6 cP and η_T is greater than 1000 cP. Anisotropy of rotational motion increases gradually as temperature is lowered to T_c, and changes little below T_c; anisotropy of translational motion was not investigated. ^{13}C nuclear spin relaxation measurements indicate that translational motion of nitroxide solutes is more rapid in the center of the bilayer than near the polar interface. It takes at least 100 nsec for a solute molecule to cross the bilayer/water interface. We estimate a lower limit of 2 sec/cm for the interfacial resistance to solute diffusion; this result indicates that interfacial resistance dominates permeation across the membrane. The relative solubility, or partition coefficient, is a strong function of solute structure, and decreases abruptly on cooling through the transition temperature. From the partition coefficient and its temperature dependence we calculate the free energy, enthalpy, and entropy of partition. Effects of cholesterol on partition and diffusion coefficients are compatible with the interpretation that bilayers containing cholesterol consist of two phases.

Phospholipids not only constitute the supporting matrix of biological membranes, but they also represent an interesting quasi-fluid state of matter whose properties have been studied extensively in recent years (Chapman, 1975; Melchion & Steim, 1976). The interior of the bilayer, the hydrophobic region, is fluid. The polar headgroups of the phospho-

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lipid molecules form the bilayer surfaces (the lipid-bilayer interfaces), and the hydrocarbon segments extend into the bilayer interior, exhibiting less order and greater flexibility as one moves from the polar heads toward the interior (Hubbell & McConnell, 1971; Seelig & Seelig, 1974; Stockton *et al.*, 1976). The fact that the bilayer surfaces are a mere 30–60 Å apart plays a major role in determining the properties of the hydrophobic "fluid" constituting the bilayer interior. In view of the small distances involved and the highly anisotropic structure and motion of phospholipid bilayers, bilayer solvent properties might well be different from those of bulk hydrocarbon solvents.

The phospholipid molecules appear to diffuse slowly along the bilayer surface (Devaux & McConnell, 1972; Webb, 1976) and to "flip-flop" across the bilayer at an even slower rate (Kornberg & McConnell, 1971). If well-sonicated, the phospholipids form spherical vesicles (enclosed by a bilayer membrane), with a diameter of about 250 Å; there is water both within and external to the vesicles (Atwood & Saunders, 1965; Huang, 1969). A critical temperature, T_c, exists below which a first-order "freezing" of the hydrocarbon tails apparently takes place (Chapman, Williams & Ladbrooke, 1967), i.e., a transition from a liquid crystalline to a crystalline gel phase. Although both the amplitude (Seelig & Seelig, 1974) and frequency (Lee *et al.*, 1972) of the hydrocarbon chain motion decrease abruptly below T_c, the end segments of the phospholipids near the center of the bilayer retain considerable flexibility even below T_c (Hubbell & McConnell, 1971; Seelig & Seelig, 1974; Stockton *et al.*, 1976). Above T_c the motion of the hydrocarbon chains in sonicated samples yields sufficient motional narrowing for the observation of high resolution proton NMR spectra (Levine *et al.*, 1972; Horowitz, 1972; Finer, Flook & Hauser, 1972; Bloom *et al.*, 1975). T_c increases with increasing hydrocarbon chain length and with increasing saturation of the hydrocarbon chain, and decreases slightly with addition of cholesterol (Ladbrooke, Williams & Chapman, 1968; Rothman & Engleman, 1972; Hinz & Sturtevant, 1972; Shimshick & McConnell, 1973).

We report magnetic resonance studies of the rotational and translational mobilities, and of the partition coefficients as well, of relatively small paramagnetic nitroxide molecules (shown in Fig. 1) dissolved in unsonicated dipalmitoyl lecithin (DPL) bilayers. The partition coefficient can be obtained from the ratio of the integrated intensity of those electron spin resonance (ESR) lines corresponding to nitroxide solute in the bilayer to the line intensity corresponding to solute in the aqueous

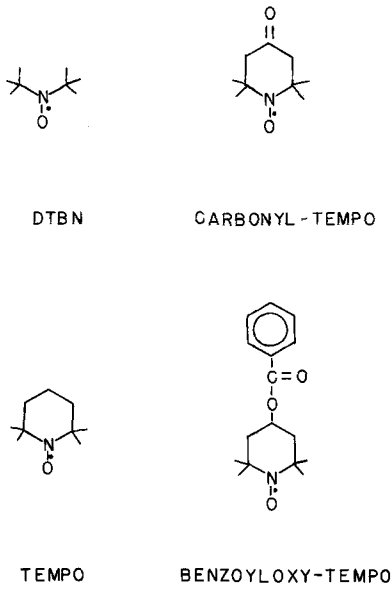

Fig. 1. The nitroxide solutes used in this study

phase. The rotational correlation frequencies as well as rotational anisotropy of the solutes are obtained from the low concentration ESR linewidths. The translational mobilities of the solutes within the bilayer are obtained from the concentration-dependent ESR linewidths. These linewidths arise from spin-exchange and dipolar intermolecular interactions that take place only when the paramagnetic molecules "collide"; thus, these measurements yield information concerning the diffusion of the paramagnetic species. An indication of how solute mobility varies with position in the bilayer can be obtained by studying the effect of the paramagnetic solute on the spin-lattice relaxation frequency ($1/T_1^N$) of the various ^{13}C nuclei along the phospholipid hydrocarbon chain.

"Fluidity" may refer to the flexibility of the hydrocarbon chains or the self-diffusion of the phospholipids along the bilayer; or it may refer to the "viscosity" determined by either the translational diffusion or the rotational diffusion of probe molecules within the bilayer. In a bulk phase, the viscosities determined by all these approaches agree, but this is not the case for bilayers. Furthermore, probes of different sizes in the bilayer may yield different effective viscosities, and because of the bilayer's ordered structure (Lieb & Stein, 1971) the "fluidity" may vary with position from the interface to the center of the bilayer and may be anisotropic. Whereas others have studied the behavior of large molecules

in membranes (e.g., Sackman & Traüble, 1972*a*; Devaux & McConnell, 1972), we have focused on the behavior of small probes of different sizes in the bilayer.

Our results are also relevant to understanding passive permeation of small nonelectrolytes through bilayers, a process that depends sensitively on bilayer structure. Classical permeation studies (Overton, 1899) treated the membrane as a "black box". In a more detailed picture, the permeability coefficient can be resolved into its dependence on rate constants for the jumps of solute across the two water/bilayer interfaces, on a partition coefficient, K, which is the equilibrium ratio of the solute's concentration in the membrane to its concentration in water, and on a translational diffusion coefficient, D, for solute in the membrane (Zwolinski, Eyring & Reese, 1949; Wartiovaara & Collander, 1960; Lieb & Stein, 1971; Diamond & Katz, 1974). In still more detail, the positional dependence of K and D within the bilayer must be considered. These parameters have been studied far more extensively for ion permeation than for nonelectrolyte permeation. Our experiments do not completely specify these parameters for the nitroxide solutes studied, but do provide some limiting values. In particular, we believe our estimates of nonelectrolyte interfacial resistance to be the most reliable to date.

Our experiments differ from most previous experiments with small nitroxide molecules in lipid bilayers in that we have made quantitative measurements of linewidth to obtain mobility data without having to reduce the intensity of the water signal by adding high concentrations of paramagnetic ions (Morse *et al.*, 1975), and also in that we have not merely reported a ratio of the membrane ESR line peak-height to the water line peak-height (Shimshick & McConnell, 1973; Lee *et al.*, 1974), but have determined the ratios of ESR integrated intensities in determining partition coefficients. Work that overlaps part of our study was carried out by Galla & Sackmann (1974), who measured the lateral diffusion of pyrene by a fluorescence technique, and by Hubbell & McConnell (1968), who looked in less detail at the motion of TEMPO in membranes.

Materials and Methods

Materials

4-keto-2,2,6,6-tetramethylpiperidine-1-oxyl (carbonyl-TEMPO, Fig. 1) was obtained from Aldrich Chemical Co. Di-*t*-butyl nitroxide (DTBN, Fig. 1) was synthesized by Dr. Ken Ogan according to the method of Hoffman, Feldman, Gelblum, and Hodgson (1964).

2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO, Fig. 1) and 4-benzoyloxy-2,2,6,6-tetramethylpiperidine-1-oxyl (benzoyloxy-TEMPO, Fig. 1) were synthesized by the method of Rozantsev and Neiman (1964) and Rozantzev (1971). β-γ-dipalmitoyl-DL-α-lecithin (DPL) was obtained from Nutritional Biochemical Co. of ICN Pharmaceuticals, Inc.

Sample Preparation

Aqueous lecithin dispersions were prepared by a slight modification of procedures described previously (Bangham, Standish & Watkins, 1965; Katz & Diamond, 1974*a*). Generally a 10% chloroform solution of DPL was rotary evaporated to dryness, then transferred to a high vacuum line for several hours. Phosphate buffer was added to form a 10 to 15% (w/w) lecithin solution. The solution was shaken in a water bath at 45 °C for 36 to 48 hr. The spin-labelled solutes were added as concentrated water solutions; the water-insoluble benzoyloxy-TEMPO or cholesterol was added as a chloroform solution to the DPL chloroform solution before rotary evaporation. The benzoyloxy-TEMPO samples showed no "clustering" of spin-label in the aqueous phase, as reported previously when hydrophobic spin-labelled lecithin samples were prepared in the above manner (Barratt, Green & Chapman, 1969). For linewidth measurements, the final range of solute concentration (c) in the dispersion was adjusted so that the concentration (c_b) *in the membrane* (*see below*) was from 1 to 25 mM (1 to 50 mM for benzoyloxy-TEMPO). The nondegassed spin-labelled lecithin solutions were drawn into a 100 μl pipette, and both ends of the pipette were sealed. These samples showed little "settling out" of the liposomes, and the ESR signal was stable over the several days the samples were used.

Sonicated deoxygenated samples in D_2O were prepared for the NMR experiments by the method described above, except that the buffer was deoxygenated by bubbling nitrogen through it, the samples were prepared in a nitrogen atmosphere, and the lecithin liposome solution was sonicated under nitrogen at low power. DTBN was added after sonication so that the concentration in the bilayer was 15 mM. A comparison of the ESR spectra of these samples before and after the NMR experiment showed that DTBN underwent little or no degradation over the course of the experiment.

A comparison of ESR linewidths of the nondegassed samples with those of the deoxygenated samples showed that oxygen broadening was small, about 0.1 G in water and 0.2 G in membrane. We found no difference in the partition coefficient or in the concentration dependence of the linewidth of DTBN between the unsonicated and sonicated systems.

ESR spectra were obtained at 9.2 GHz with a Varian E-12 spectrometer interfaced to a DEC PDP 11/45 computer. The temperature was regulated by a Varian V-4540 temperature control unit and measured to ±0.5 °C with a thermocouple inserted into the center of the ESR cavity before and after recording a spectrum. From one to four spectra at each concentration and temperature were recorded and digitized and the results averaged. Digitized spectra were analyzed with the nonlinear least squares program of Bevington (1969) by fitting the spectra to an appropriate combination of first derivative Lorentzian curves. Most linewidth measurements were made with the high field set of lines, since the resolution was greatest for these lines. Linewidths at twelve concentrations were measured at each temperature, and the least squares slopes determined.

Above the bilayer transition temperature T_c (at which DPL passes from a crystalline gel phase to a liquid crystalline phase), plots of linewidth *vs.* solute concentration in the membrane were linear for the four solutes. Below T_c, plots were linear for the two solutes studied, DTBN and TEMPO. In contrast, Sackmann and Traüble (1972*b*) found that, below T_c, the linewidth of the rather large, hydrophobic androstane spin label was not linear with concentration, presumably due to "clustering" of solute below T_c.

13-C NMR spectra were obtained at 57 °C with a Varian CFT-20 Fourier transform spectrometer operating at 18.7 kG and 20 MHz with an internal deuterium lock. Spin-lattice relaxation times were obtained by the inversion-recovery technique (Farrar & Becker, 1971).

At high solute concentrations in the membrane one would expect membrane structural changes that would alter both the solute's partition coefficient (K) and its mobility and would also shift the membrane's T_c. Effects of high solute concentrations on solute permeability (anesthetic effects) are presumably mediated by changes in membrane structure. Three observations indicate that such effects were either negligible in our experiments or else did not affect the properties we measured: (i) Within the accuracy of our experiments, K's did not vary with solute membrane concentration over the range 0.5 to 25 mM. (ii) Over this same range the plots of linewidth against concentration were linear, a result that suggests constancy of solute diffusion coefficients with membrane concentration. (iii) Rotational mobilities, partition coefficients, and concentration dependent line broadening of solutes showed abrupt changes near 41 °C (e.g., Figs. 2, 3, 4, 6), the phase transition temperature for DPL bilayers without added solutes. Since this phase transition is a sensitive indicator of membrane structure, the solutes used were evidently not causing significant alterations in membrane structure. This is consistent with the observation, based on differential scanning calorimetry, that TEMPO does not affect the phase transition behavior of DPL (Jain & Wu, 1977).

Results and Discussion

The ESR spectra: Hyperfine Splittings

DTBN, TEMPO, and carbonyl-TEMPO in DPL dispersions gave spectra that were characteristic of rapidly tumbling spin labels partitioned between an aqueous phase and a less polar hydrocarbon phase (Bales & Baur, 1970). An example of our DTBN spectra has been published previously (Dix, Diamond & Kivelson, 1974). The ESR spectrum of the hydrophobic benzoyloxy-TEMPO molecule, however, was dominated by the membrane signal above the crystalline gel-liquid crystalline phase transition temperature, T_c, while below T_c the spectrum consisted of a very much broadened membrane signal and a small aqueous signal.

The nitrogen hyperfine coupling constants (a_N), defined as one half the separation of the outermost hyperfine lines, are given in Table 1 for solutes in the aqueous phase, the membrane phase, and, for comparison, in degassed hexane. Since a_N correlates with solvent polarity (Griffith, Dehlinger & Van, 1974), these values suggest that the average environment seen by solutes in the bilayer is not strictly hexane-like, but intermediate in polarity between water and hexane.[1] The hyperfine

1 One must be careful to substract out shifts in apparent hyperfine splittings due to increased mobility of the spin label as one moves further from the polar head groups (*see* Rottem *et al.*, 1970).

Table 1. Summary of the hyperfine splitting constants, mobility data, bilayer viscosities determined from the mobility data, and thermodynamic data for the four nitroxide solutes in DPL[a]

	DTBN	TEMPO	Carbonyl-TEMPO	Benzoyloxy-TEMPO
a_N in H_2O (G)	17.2±0.1	17.1± 0.15	15.9±0.15	16.9±0.2
a_N in DPL (G)	15.8±0.1	15.7± 0.15	14.9±0.15	15.7±0.15
a_N in degassed hexane (G)	15.0±0.15	15.2± 0.15	14.2±0.15	15.1±0.1
$\Delta B_c/c_b$ at 50 °C (G/M)	26 ±4	24 ± 4	19 ±3	30 ±4
$\Delta B_c/c_b$ at 35 °C (G/M)	50 ±13	100 ±12	–	–
$r_{hy}=r_{ex}$ (estimated) (A°)	3.2	3.2	3.2	6
$10^6 D$ at 50 °C (cm^2/sec)	1.0±0.2	0.9± 0.2	0.7±0.3	0.6±0.1
τ_R at 50 °C (psec)	20		–	200
τ_R at 35 °C (psec)	175	–	–	–
ε (anisotropy) at 50 °C	0.1	–	–	–
ε (anisotropy) at 35 °C	0.4	–	–	–
η_R at 50 °C (cP)	0.6	–	–	1.0
η_R at 35 °C (cP)	5	–	–	–
η_T at 50 °C (cP)	7	8	10	6
η_T at 35 °C (cP)	>1000	>1000	–	–
K at 50 °C	18 ±2	20 ±2	2.1 ±0.2	>2000
K at 35 °C	4.6 ±0.7	3.7 ±0.5	<0.15	> 200
ΔG at 50 °C (kcal/m)	−1.8 ±0.2	−1.9 ±0.2	−.48±0.05	< −4.9
ΔG at 35 °C (kcal/m)	−0.94±0.14	−0.84±0.11	> −1.2	< −3.2
ΔH at 50 °C (kcal/m)	4.5 ±0.8	7.5 ±0.8	4.9 ±0.8	–
ΔH at 35 °C (kcal/m)	12 ±4	16 ±3	–	–
ΔS at 50 °C (cal/m)	20 ±3	30 ±3	17 ±2	–
ΔS at 35 °C (cal/m)	43 ±14	55 ±4	–	–

[a] The transition temperature of DPL is 41 °C.

coupling constant did not vary with temperature in the range studied, 32 to 65 °C, which indicates that there is no major difference in the average polarity of the environment seen by solute above and below the transition temperature.

As all solutes studied, except benzoyloxy-TEMPO above T_c, gave separate water and membrane signals at all temperatures, the rate of travel of solute from membrane to water must be slow on an ESR time scale. The minimum separation of hyperfine lines corresponding to solute in water and in membrane is 0.1 G; this implies that the interphase transit time is slower than 100 nsec (Wertz & Boulton, 1972; Dix *et al.*, 1974).

The Rotational Motion

We can determine a rotational correlation time, τ_R, for a nitroxide radical in the bilayer by studying the three linewidths in an ESR spectrum arising from a very dilute spectrum of the radical in the bilayer. From the theory of ESR linewidths for nitroxide radicals we can obtain two separate expressions for τ_R (Kivelson, 1960; Stone *et al.*, 1965):

$$\tau_R = (15/8\, b B \Delta\gamma)(\sqrt{3}/2\,\gamma_e)\,\Delta B(-1)\{1-[h(-1)/h(+1)]^{1/2}\} \quad (1)$$

$$\tau_R = [120/(15\, b^2 + 32\, b B \Delta\gamma)](\sqrt{3}/2\,\gamma_e)\,\Delta B(-1)\{1-[h(-1)/h(0)]^{1/2}\}. \quad (2)$$

In these equations, $\Delta B(M)$ and $h(M)$ are the low concentration peak-to-peak widths and heights, respectively, of the M^{th} hyperfine line; $M = \pm 1$ or 0 ($M = -1$ is the high field line); B is the applied magnetic field (3200 G in our experiments); γ_e is the electronic magneto-gyric factor; b and $\Delta\gamma$ are the anisotropic parts of the hyperfine coupling constant and magneto-gyric factor, respectively (Kivelson, 1960). For DTBN the factors preceding $\Delta B(-1)$ in Eqs. (1) and (2) are 5.35×10^{-10} sec/G-rad and 3.69×10^{-10} sec/G-rad, respectively (Libertini & Griffith, 1970). For DTBN these equations are valid only in the range $20\,\text{psec} < \tau_R < 3{,}000\,\text{psec}$ (Kivelson, 1960); the τ_R's for DTBN in DPL below T_c fall within this range, while above T_c, the τ_R's are less than 20 psec. Furthermore, Eqs. (1) and (2) hold if the magnetic parameters are axially symmetric and the molecule tumbles isotropically. As discussed below, these conditions are not strictly met in our experiments.

In addition to the shortcomings, discussed above, in the determination of τ_R, the low concentration linewidths $\Delta B(-1)$ contain contributions from unresolved proton extra-hyperfine splittings (Plachy & Kivelson, 1967). Thus, the τ_R's calculated from Eqs. (1) and (2) are merely upper limits to the rotational correlation time. The good Lorentzian character of the DTBN spectrum suggests that proton hyperfine splitting does not dominate the linewidth; the less good Lorentzian character of the benzoyloxy-TEMPO spectrum introduces uncertainty into the analysis of the linewidth. Furthermore, we have computer simulated, with typical values of a_H and of the "true" low concentration width (ΔB_o) of each hyperfine line for DTBN in hydrocarbons, the spectrum arising from a line split by 18 equivalent protons. The resulting linewidth, though larger than ΔB_o, is less than 5% larger if $\Delta B_o > 1$ G and less than $2\,\Delta B_o$ for the linewidths studied that were less than 1 G wide; the importance of hyperfine splitting should, therefore, be more important above than below T_c. For more precise measurements of τ_R, one should use per-deuterated nitroxides; because the magnetic moment of the deuteron is considerably less than that of the proton, deuteron hyperfine splittings are considerably smaller (Polnaszek & Freed, 1975; Plachy & Windrem, 1977). Alternatively, the proton hyperfine broadening can be eliminated by extrapolating the high concentration ESR linewidths to zero nitroxide concentration; at high nitroxide concentrations the proton hyperfine splittings are exchange-narrowed, and the ESR linewidths are linear in nitroxide concentration (Plachy & Kivelson, 1967). However, because of the experimental uncertainties in determining the zero concentration intercept, this approach is of dubious value.

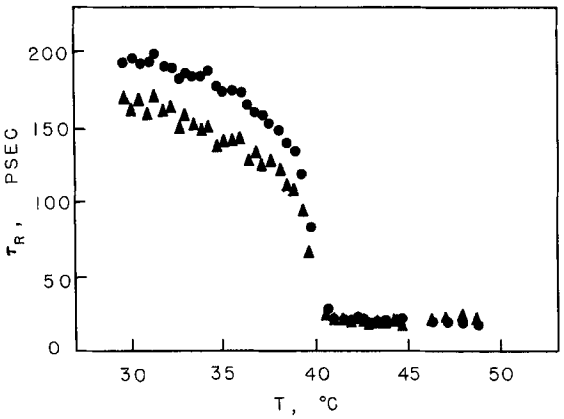

Fig. 2. The rotational correlation time, τ_R, for DTBN in DPL as a function of temperature. ●: calculated with Eq. (1) of the text; ▲: calculated with Eq. (2) of the text. The transition temperature of DPL is 41 °C

Correlation times for DTBN, determined with each of the two equations above, are plotted against temperature in Fig. 2. These two τ_R values at temperatures below T_c differ by 10–20%. This discrepancy is probably due to the fact that DTBN does not have axially symmetric magnetic parameters (Libertini & Griffith, 1970), and is probably undergoing anisotropic tumbling as well *(see below)*. The two calculated values of τ_R agree much more closely above T_c, which suggests that the rotational motion is more isotropic above T_c than below.

The striking effect seen in Fig. 2 is the sharp increase in τ_R on cooling through the phase transition; this implies a much smaller degree of rotational mobility below T_c than above. (The increase is probably even more striking than indicated because of proton hyperfine splitting as discussed above.) This effect has been seen by others and has been used to correlate phase changes in natural membranes with biological factors (Hoffman, Schofield & Rich, 1969; Sinesky, 1974; Wisnieski, Huang & Fox, 1974). Although one cannot say much about the detailed nature of the phase change on the basis of τ_R values alone, one can infer that the medium becomes much more rigid below T_c; this interpretation is compatible with the ordering and stiffening of the hydrocarbon chains below T_c suggested by other experiments (Lee *et al.*, 1972; Seelig & Seelig, 1974; Stockton *et al.*, 1976), and by the studies of translational motion discussed in the next section.

We have also measured the rotational correlation time for benzoyloxy-TEMPO in DPL above T_c, assuming that it has the same magnetic parameters as DTBN. At equivalent temperatures, τ_R for

benzoyloxy-TEMPO was an order of magnitude greater than τ_R for DTBN. This is to be expected since τ_R varies as the molecular volume. τ_R for benzoyloxy-TEMPO is considerably more temperature dependent than τ_R for DTBN, decreasing from 300 psec at 42 °C to 150 psec at 57 °C. Perhaps this is because temperature dependence at the relatively small τ_R for DTBN is largely obscured by line broadening due to proton hyperfine splitting, whereas the broad benzoyloxy-TEMPO lines are unaffected by this effect.

Tomkiewicz and Corker (1975) have studied the rotational mobility of DTBN in egg yolk lecithin bilayers. Their results for τ_R in the temperature range of 0–45 °C (over which egg yolk lecithin is above its transition temperature) are in the range 20–60 psec, in agreement with our results for DTBN in DPL above T_c.

The anisotropy of the rotational motion can also be considered. Nordio (1970) and Vasserman, Kuznetsov, Kovarskii and Bychachenko (1971) have shown that the parameter ε

$$\varepsilon = [\Delta B(+1) - \Delta B(0)]/[\Delta B(-1) - \Delta B(0)] \tag{3}$$

is independent of correlation time for molecules carrying out isotropic rotation or for molecules carrying out anisotropic rotations but with axially symmetric magnetic parameters. However, ε depends on the ratio $(\tau_{\|}/\tau_{\perp})$ for axially symmetric rotation, where $\tau_{\|}$ is the correlation time for rotation about the symmetry axis and $\tau_{\perp}$ the correlation time for rotation about an axis perpendicular to the symmetry axis. For DTBN molecules, the magnetic parameters are not axially symmetric (Libertini & Griffith, 1970), and the rotational motion is probably not isotropic; therefore ε can be regarded only as an empirical indication of the rotational anisotropy. Fig. 3 shows that ε decreases with temperature above T_c. The most likely interpretation of these results is that below T_c the "tight" bilayer structure preferentially impedes solute rotations in certain directions and that above T_c the preferential barriers to rotations decrease gradually with temperature. Whereas τ_R changes sharply at T_c (Fig. 2), ε undergoes a gradual change (Fig. 3); the reasons for this difference are unclear.

The Translational Motion (ESR Studies)

We can estimate the translational mobility of solute in the bilayer by studying the concentration dependence of the ESR linewidth arising

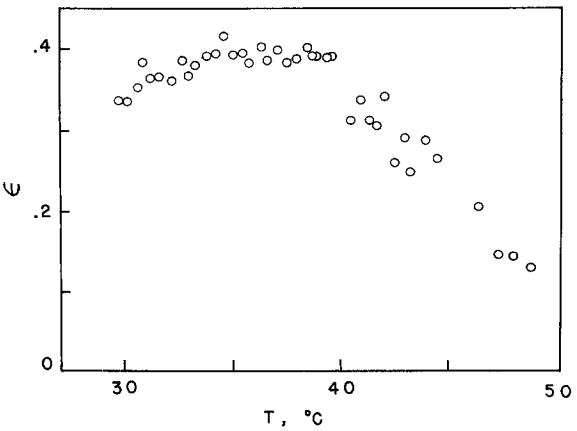

Fig. 3. The rotational anisotropy factor, ε, of DTBN in DPL liposomes as a function of temperature

from solute in the bilayer. The relevant concentration of the nitroxide solute is the concentration c_b in the bilayer; this concentration can readily be obtained from ESR intensities together with the partial volume of lipid in the system (Dix *et al.*, 1974). We can only determine an average value of c_b for the bilayer; the actual solute concentration probably varies with position in the part of the bilayer. We wish to study $\Delta B_c/c_b$ where ΔB_c is the concentration dependent ESR linewidth for the nitroxide in the bilayer. Except at very low concentrations, where proton extra-hyperfine splittings may contribute significantly, or at very high concentrations, where the nitrogen hyperfine lines begin to exchange narrow, ΔB_c is linear in c_b.

Both spin exchange and dipolar broadening can contribute to ΔB_c. In the spin exchange interaction, the two radicals can "exchange" their electronic spin states upon collision. This leads to a contribution $\Delta B_c'$ which is proportional to $D r_{ex} c_b$ where D is the translational self-diffusion coefficient for the nitroxide and r_{ex} is an interaction radius (approximately the molecular radius) for spin exchange to occur (Kivelson, 1960; Eastman *et al.*, 1969):

$$\Delta B_c'/c_b = (0.032 \pi N/3 \gamma_e \sqrt{3}) D r_{ex} \tag{4}$$

where N is Avagadro's number and γ_e is the electron magneto-gyric ratio. The factor preceding D in Eq. (4) is 6.61×10^{14} (G/M) (sec/cm^3). The spin exchange method of determining translational mobilities has been used with success for solutes in single component solvents of low

viscosity (Danner & Tuttle, 1963; Miller & Adams, 1966; Plachy & Kivelson, 1967; Eastman *et al.*, 1969).

The second interaction contributing to ΔB_c is a magnetic dipole-dipole coupling of two unpaired electronic spin states. This interaction leads to a contribution $\Delta B_c''$ which is proportional to $c_b/r_{ap} D$, where r_{ap} is one-half the minimum radical-radical approach distance (Abragam, 1961; Eastman *et al.*, 1969):

$$\Delta B_c''/c_b = (0.019\,\pi N\hbar^2/225\,\gamma_e\sqrt{3})(1/D r_{ap}). \tag{5}$$

The observed ΔB_c is presumably

$$\Delta B_c = \Delta B_c' + \Delta B_c''. \tag{6}$$

As the translational motion of the radicals becomes slower and the diffusion coefficient decreases, the spin exchange interaction becomes smaller, but the dipolar interaction becomes larger. If both interactions contribute significantly to the linewidth, then large changes in D, r_{ex} or r_{ap} will yield only small changes in the linewidth, and in this case $\Delta B_c/c_b$ is not a sensitive measure of these parameters.

The quantity $\Delta B_c/c_b$ is plotted against temperature in Fig. 4. The most interesting features about these data are: (i) above T_c, $\Delta B_c/c_b$ is approximately equal for all four solutes; (ii) above T_c, $\Delta B_c/c_b$ exhibits only weak or no temperature dependence; and (iii) below T_c, $\Delta B_c/c_b$ for DTBN and TEMPO increases sharply with decreasing temperature.

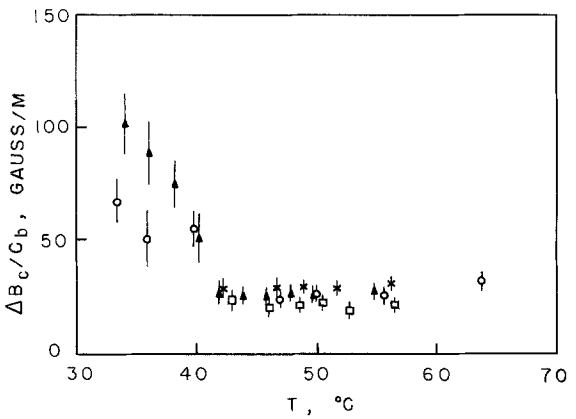

Fig. 4. $\Delta B_c/c_b$ *vs.* temperature in DPL (where ΔB_c is the concentration-dependent ESR linewidth and c_b is the concentration of solute in the bilayer) for DTBN (○), TEMPO (▲), carbonyl-TEMPO (□), and benzoyloxy-TEMPO (*)

The first feature can be explained if one assumes, quite reasonably, that r_{ex} and r_{ap} are probably both close to the hydrodynamic radius, r_{hy}, of the nitroxide solute; $\Delta B_c/c_b$ is thus a function of r_{hy}. For hydrodynamic Brownian motion, the diffusion coefficient is inversely related to molecular size; i.e., Dr_{hy} is independent of solute size. Thus the product of Dr_{ex} or Dr_{ap} in Eqs. (4) and (5), and hence $\Delta B_c/c_b$, should be approximately independent of molecular size in the hydrodynamic limit. Of course, the hydrodynamic limit holds for solute molecules which are far larger than the solvent molecules, and our nitroxides are small compared to the solvent phospholipids.

The second feature, the weak temperature dependence of $\Delta B_c/c_b$ above T_c, may mean that the diffusion coefficient is not strongly temperature dependent, in agreement with observations for other viscous media (Berner, Holcek & Kivelson, *unpublished observations*). At low viscosities, the diffusion coefficient is frequently well-described by the Stokes-Einstein relation

$$D = kT/6\pi\eta_T r_{hy} \tag{7}$$

where r_{hy} is the hydrodynamic radius of the diffusing particle and η_T is the coefficient of shear viscosity of the solvent. (The subscript T emphasizes that we are discussing translational motions). Berner, Holecek and Kivelson *(unpublished observations)* have been investigating the concentration dependent ESR linewidths of DTBN in solvents of various viscosities; they have also been studying, by spin-echo techniques, the translational diffusion coefficient of dimethylsulfoxide-d_6 in ethylene glycol. Their preliminary results indicate that although at low viscosity the Stokes-Einstein relation appears to be valid, at high viscosity the diffusion is not well described by the relation and is, in fact, nearly independent of η and hence of T. We conclude, therefore, that the relative temperature independence of $\Delta B_c/c_b$ within the bilayer above T_c probably represents the temperature independence of D in the highly viscous bilayer. The temperature independence of D above T_c is analogous to the temperature independence of τ_R discussed above. However, it should be noted that, for relatively high effective viscosities such as those encountered within the bilayer, the dipolar broadening with its D^{-1} dependence competes with the exchange broadening which is linear in D, so that $\Delta B_c/c_b$ might be relatively insensitive to T even if D exhibited a moderate variation with T.

The third feature of Fig. 4, the increase in $\Delta B_c/c_b$ below T_c, most likely means that the system is in the dipolar broadening regime, and, there-

fore, that the solute's diffusion coefficient has actually decreased considerably [*cf.* Eq. (5)] from its value above T_c. This conclusion is supported by the fact that $\Delta B_c/c_b$ increases with decreasing T. Similar anomalous temperature dependences of $\Delta B_c/c_b$ for large spin-labelled molecules in bilayers have been observed by Traüble and Sackmann (1972), who interpreted their results in terms of a "clustering" of solute below T_c, and by Devaux, Scandella and McConnell (1973), who gave a dipolar broadening interpretation. Our line shapes do not suggest "clustering".

One could, in principle, calculate a value for D from the measured value of $\Delta B_c/c_b$ together with Eqs. (4) through (6), but the dipolar calculation involves too many approximations. Berner, Holecek and Kivelson *(unpublished observations)* have measured $\Delta B_c/c$ for DTBN in various solvents over a wide range of temperatures and viscosities, and their results suggest that our values of $\Delta B_c/c_b$ for solute in the bilayer above T_c contain about 20% dipolar contribution. We thus calculate from the spin exchange interaction [Eq. (4)], corrected for a 20% dipolar contribution, diffusion coefficients for all four nitroxides in the bilayer above T_c (Table 1). We have used a value of $r_{ex} = 3.2$ Å, for DTBN (Plachy & Kivelson, 1967), TEMPO and carbonyl-TEMPO, and 6 Å for benzoyloxy-TEMPO. The Berner, Holecek and Kivelson results for very high viscosities, which give values of $\Delta B_c/c_b$ similar to ours for solute in the bilayer below T_c, appear to be dominated by dipolar contributions; at present all we can say about this problem is that D appears to be at least two orders of magnitude smaller below T_c than above T_c.

We have assumed that both above and below T_c the solute is distributed uniformly throughout the entire bilayer volume. As we show in our NMR studies, above T_c the deviations from the uniform distribution are probably not sufficiently great to alter the qualitative conclusions of this section. In order to explain the observed increase in $\Delta B_c/c_b$ below T_c (Fig. 4), one could postulate an "effective bilayer volume" as we did previously (Dix *et al.*, 1974) instead of the dipolar broadening explanation given above. The integrated intensity of the ESR lines of the nitroxide in the membrane, relative to that for the nitroxide in water, decreases abruptly as the temperature is lowered to T_c. We assumed above that this result was due to a uniform decrease of nitroxide concentration throughout the bilayer. However, it could also be explained by assuming that below T_c part of the lipid "freezes" and excludes nitroxide, whereas part exists as "liquid puddles" with the same solubility as above T_c (Dix *et al.*, 1974).

We assume in the puddle model that a fraction, f, of the bilayer

volume is fluid below T_c, that the solute is concentrated in this smaller fluid volume, and that the partition coefficient between water and fluid bilayer is approximately the same above and below T_c. (The fraction of volume that is fluid below T_c could not depend upon c_b because, as discussed below, the partition coefficient does not depend upon concentration.) With these assumptions, the bilayer concentration is corrected upward by $c_b' = c_b/f$ (where c_b' is the concentration of solute in the fluid bilayer), and the change in f at T_c is given by the change in the integrated intensity of the ESR lines of the nitroxide in the membrane relative to those of the nitroxide in water. If these corrections are made, we find that $(\Delta B_c/c_b)$ below T_c is approximately equal to that above T_c; this is consistent with the model, since diffusion would be taking place only within the effective bilayer volume whose properties by assumption are approximately the same below and above T_c.

However, this alternative explanation based on puddles is difficult to reconcile with the rotational mobility discussed above: the abrupt decrease in the rotational motion below T_c indicates that the nitroxide is probably not located in fluid puddles. Similar behavior of $\Delta B_c/c_b$ has been observed for DTBN in single-component viscous nematic solvents near and across the melting temperature (Berner & Kivelson, *unpublished observations*). Hence, we believe that the increase in $\Delta B_c/c_b$ with decreasing temperature below T_c is not merely an artifact related to neglect of reduced effective volume, but is probably due to dipolar contributions to the ESR linewidth.

In order to determine whether appreciable spin-exchange or dipole-dipole interactions occur between radicals in the bilayer and water phases, we have studied the effect of Ni^+ ions on the ESR linewidths of DTBN in both the aqueous and bilayer phases. Ni^{2+} is paramagnetic and broadens the ESR lines of free-radicals that are within the spin exchange interaction distance of Ni^{2+} (on the order of 5 Å), but it does not enter the bilayer (Morse *et al.*, 1975). Therefore, we would expect Ni^{2+} to broaden the ESR lines of radicals in water and of those in the membrane that are within 5 Å of the water/membrane interface, but not of radicals in the membrane interior. If there were a nitroxide concentration build-up in the membrane's interfacial region, we would expect Ni^{2+} to have an appreciable effect on the membrane linewidth. We found a very slight increase in the ESR linewidth of DTBN in the bilayer, but this could be accounted for by the increased concentration of DTBN in the bilayer due to the presence of Ni^{2+} ion in the water. These results suggest that spin exchange between radicals in the bilayer and those in the water is not important, and that there is no appreciable concentration build-up of solute at the interface.

There is evidence of "bound" water associated with lipid bilayers (Chapman *et al.*, 1967; Katz & Diamond, 1974*b*; Keith, Snipes & Chapman, 1977). For a 15% (w/w) aqueous lecithin dispersion, 4.7% (g H_2O/g DPL) of the water is bound. We have not tried to correct for this effect, which would reduce the apparent K and increase D by 25%, but is opposed by effects of reduced bilayer volume.

Rotational and Translational Viscosities

In discussing Brownian spheres of radius r_{hy} diffusing in a continuous, homogeneous fluid, one can relate the diffusion constant D to the "translational" shear viscosity η_T by means of the Stokes-Einstein relation [Eq. (7)]. In addition, the rotational correlation time τ_R can be related to a "rotational" viscosity η_R by means of the Debye relation

$$\tau_R = 4\pi r_T^3 \eta_R / 3kT. \tag{8}$$

For Brownian particles, $\eta = \eta_R = \eta_T$ where η is the shear viscosity. For small particles diffusing in a bilayer, the viscosity is not well specified, but we can *define* mean translational (η_T) and rotational (η_R) viscosities by means of Eqs. (7) and (8), respectively. These viscosities, given in Table 1, are in the range $\eta_T = 6$–10 cP and $\eta_R = 0.6$–1 cP for bilayer above T_c, and $\eta_R \approx 5$ cP and $\eta_T > 1{,}000$ cP for bilayer below T_c. η_R for DTBN and η_T for all four solutes are insensitive to temperature, but, it should be remembered, the ESR line broadening from which the apparent η_R is determined depends in part upon temperature-independent proton hyperfine splitting. On the other hand, τ_R, and hence η_R, for benzoyloxy-TEMPO in DPL are rather temperature sensitive. A comparison of η_R and η_T at equivalent temperatures suggest that small solutes in the bilayer are able to rotate more freely than they translate. Of course, both η_R and η_T may be anisotropic and vary across the bilayer.[2]

Keith, Snipes, Melhorn and Gunter (1977) have recently studied the rotational and translational motion of perdeuterated carbonyl-TEMPO in aqueous suspensions of polymer beads of different pore sizes by a method similar to ours. In this system, carbonyl-TEMPO presumably diffuses in the narrow aqueous channels of the polymer beads as well as the bulk phase water. Keith *et al.* (1977) found that while the translational motion decreased rapidly as the pore size of the bead decreased, the rotational motion changed little. In other words, the translational "viscosity" of small aqueous channels in polymer beads is greater than the rotational "viscosity". These results are similar to our results obtained for DTBN in DPL.

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2 In bulk liquids where the macroscopic viscosity η is known, one often finds that $(\eta_T/\eta) \approx 1$, but $(\eta_R/\eta) \ll 1$ (Kowert & Kivelson, 1976). For example, at 40 °C $\eta_T \approx \eta$ and $(\eta_R/\eta) = 0.3$ and 0.1 for DTBN in nondegassed water and hexane, respectively. In DPL we find that above T_c, $(\eta_R/\eta_T) \approx 0.1$. For DTBN the ESR data is dependable only if $\tau_R > 20$ psec; although $\tau_R \approx 20$ psec for DTBN in DPL, we see that the results are consistent with those in neat liquids.

Translational viscosities calculated from the permeability coefficients of small nonelectrolytes under the assumption of negligible interfacial resistance (e.g., Solomon, 1974) are much larger than our viscosities, probably because interfacial resistances dominate the permeability coefficient, and because the permeability coefficient yields an average viscosity for the whole bilayer while our probes are probably concentrated in the low-viscosity central part of the bilayer. Edidin (1974) has summarized previous estimates of bilayer viscosity from rotational and translational motions of solute probes, mostly of large molecules. These viscosity estimates are also generally much larger than ours, probably because large molecules probe the viscous interfacial region as well as the relatively fluid central part of the bilayer probed by our small permeants; because large probes more nearly obey the Brownian motion conditions; and, possibly, because the inverse size dependence of solute diffusion coefficients may be steeper in a bilayer than in free solution (Lieb & Stein, 1971).

Translational Motion (NMR Studies)

Solute diffusion in a highly structured and ordered system, such as a bilayer, is probably anisotropic. Furthermore, the diffusion tensor $D(x)$ is likely to be a function of position, (x), along the hydrocarbon chains. Since our ESR experiments yield only a diffusion constant averaged over all positions and directions, we have carried out ^{13}C nuclear magnetic spin relaxation experiments to measure gradients in $D(x)$ across the bilayer.

We measured the spin-lattice relaxation rate of various ^{13}C nuclei of DPL, with and without added DTBN. The unpaired electron of DTBN can interact via electron nuclear magnetic dipolar coupling with the spins of the carbon nuclei, and this interaction increases the rates at which the nuclei relax (the relaxation enhancement). Consequently, if the concentration of DTBN were great in a particular part of the bilayer, the relaxation enhancement of carbon nuclei in that part of the bilayer would be large. However, the relaxation enhancement depends not only on a local concentration at the N^{th} carbon, c_N, but also on a distance of closest approach, r_N, of DTBN and the N^{th} carbon nucleus, and on the mobility of DTBN at the N^{th} carbon, D_N. The appropriate expression is (Abragam, 1961):

$$1/T_1^N = (0.002\,\pi N \gamma_e^2 \gamma_C^2 \hbar^2/25)(c_N/D_N r_N) \tag{9}$$

where γ_C is the ^{13}C nuclear magnetogyric ratio, and $1/T_1^N$ is the difference in the relaxation rate with DTBN, i.e., $(1/T_1^N)_{DTBN}$, and without DTBN, i.e., $(1/T_1^N)_0$, present:

$$1/T_1^N = (1/T_1^N)_{DTBN} - (1/T_1^N)_0. \tag{10}$$

Equation (9) was obtained from Redfield relaxation theory, with the assumption that the motion of DTBN is isotropic and diffusional and that the dipolar interaction is negligible. Previous studies of relaxation enhancement have used lipid or lipid-like molecules with a nitroxide group attached to a particular carbon (Kornberg & McConnell, 1971; Levine *et al.*, 1972; Levine *et al.*, 1973; Godici & Landsberger, 1974; Godici & Landsberger, 1975; Brûlet & McConnell, 1975). In these studies, it was found that the relaxation was greatest for carbon nuclei nearest the nitroxide group.

The relaxation enhancement for DTBN in DPL at 57 °C is given in the second column of Table 2 and is plotted in Fig. 5 against chain position. The relaxation enhancement is not uniform along the chain. Instead, the effect is greater for carbon nuclei near the polar head than for carbon nuclei near the terminal methyl group. By means of Eq. (9), we can interpret this gradient in terms of the variation in c_N, r_N and D_N at carbon position. We shall assume that the distance of closest approach, r_N, does not vary significantly for the different carbon nuclei; since DTBN is rather large compared with the width of the hydrocarbon tail, r_N is probably not very different at different positions along the chain. We can conclude, therefore, that $[c_N/D_N]$ is greater near the polar heads than near the terminal methyls of the lipid.

Table 2. The relaxation enhancement $(1/T_1^N)$ of various ^{13}C nuclei of DPL at 57 °C due to 15 mM added DTBN, and the corresponding translational diffusion coefficient of DTBN obtained from $(1/T_1^N)$ under the assumption of uniform DTBN distribution across the bilayer and minimum DTBN-^{13}C nucleus approach distance of 5 Å[a]

Carbon	$1/T_1^N$ (sec^{-1})	$10^6 D_N$ (cm^2/sec)
$\overset{+}{N}(CH_3)_3$	1.07 ± 0.2	0.64 ± 0.13
C=O	1.26 ± 0.09	0.54 ± 0.04
C_{4-13}	1.13	0.60
C_{14}	0.41 ± 0.22	1.7 ± 0.9
C_{15}	0.52 ± 0.05	1.3 ± 0.1
C_{16}	0.35 ± 0.09	1.9 ± 0.5

[a] The carbon nuclei are numbered from the carbonyl carbon.

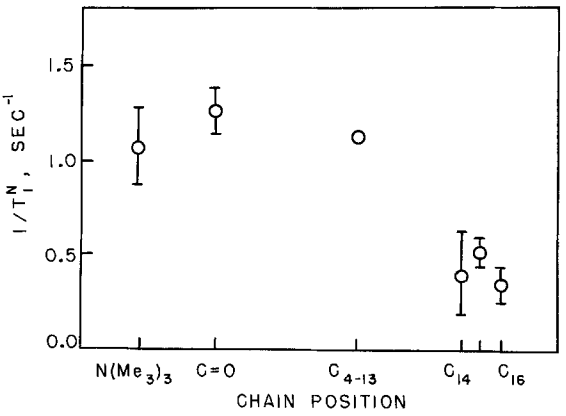

Fig. 5. The relaxation enhancement, $1/T_1^N$, of ^{13}C nuclei of DPL due to added DTBN, as a function of chain position. The carbon nuclei are numbered from the carbonyl carbon. The signals from carbons 4 through 13 were not resolved. DTBN concentration in the membrane is 15 mM and the temperature is 57 °C

We have no sure way of separating c_N from D_N; however, we suggest that DTBN is less mobile near the polar head than near the terminal methyls, since several lines of evidence indicate that DTBN should not exhibit a concentration build-up near the polar head. In the first place, DTBN is more soluble in hydrocarbon solvents than in water, an observation which suggests greater DTBN concentration in the hydrophobic region than near the polar heads. Secondly, Griffith *et al.* (1974) have measured a "polarity gradient" across the bilayer and they find the central region of the bilayer to be less polar than the interfacial region; DTBN would be expected to concentrate in the less polar, central region of the bilayer. Thirdly, the Ni^{2+} experiments described above suggest that there is not an appreciable DTBN concentration build-up at the interface. Finally, the ESR hyperfine measurements, also reported above, appear to argue against a DTBN build-up near the polar heads, since the hyperfine values reported in Table 1 suggest a mean hydrocarbon environment rather than an aqueous environment for the solutes in the bilayer. Since all indications argue against a nitroxide build-up near the polar heads and since (c_N/D_N) is largest near the polar heads, we conclude that the diffusional motion of DTBN is less near the interface than near the center of the bilayer. Similarly, the motion of the membrane hydrocarbon chains themselves is less near the interface than near the center (Hubbell & McConnell, 1971; Seelig & Seelig, 1974; Lee *et al.*, 1976).

Our measured D_N's depend upon the relative motions of the ^{13}C nuclei and the DTBN molecules; we have assumed that the motions of the ^{13}C nuclei contribute little to $(1/T_1^N)$. If we assume that the concentration of DTBN is uniform over the bilayer, then Eq. (9) and the measured $(1/T_1^N)$ can be used to determine $D_N r_N$ at each carbon nucleus; if we assume $r_N \approx 5$ Å, we can then calculate the D_N's in Table 2. The calculated D_N's for DTBN in the center of the bilayer are 2–3 times greater than that near the interface. An expected build-up of DTBN gradient near the hydrocarbon center of the bilayer would increase still further the ratio of calculated D_N near the center to that near the periphery of the bilayer over that indicated in Table 2. If the diffusional motion is not locally isotropic, the theory is more complex than that used above (Brûlet & McConnell, 1975).

Under the assumptions used to calculate the D_N's in Table 2, we can average the D_N's appropriately to obtain the mean value 0.8×10^{-6} cm^2/sec. This compares favorably with the value 1.0×10^{-6} cm^2/sec for the diffusion coefficient of DTBN obtained from the ESR experiments.

Sillerud and Barnett (1977) have done an NMR experiment similar to ours. They found that the TEMPO-induced line broadening (or enhancement of $1/T_2$) of the proton NMR signal from the head group methyl protons of egg lecithin was 24% less than the broadening of the signal from the chain methylene protons. This is in apparent contradiction with our results. The discrepancy may be due to the fact that the main methylene proton resonance measured by Sillerud and Barnett represents an unspecified average of all $-CH_2$-resonances in the hydrocarbon chain, whereas we were able to resolve the three end ^{13}C resonances of the chain (Fig. 5).

The Partition Coefficient

K is the ratio of the radical concentration within the bilayer to that in the aqueous medium. Operationally we specify K by the relation

$$K = [(h_{\mathrm{DPL}}(\Delta B)^2_{\mathrm{DPL}}/W_{\mathrm{DPL}})][(h_{\mathrm{H_2O}}(\Delta B)^2_{\mathrm{H_2O}}/W_{\mathrm{H_2O}})]^{-1} \tag{11}$$

where the subscripts DPL and H_2O indicate the phase in which the nitroxide is located, h and ΔB are the computer-fitted height and peak-to-peak ESR linewidths of the first derivative Lorentzian curve arising from the nitroxide, and W is the weight of each phase in the solution. The width squared times the height of a first derivative Lorentzian ESR

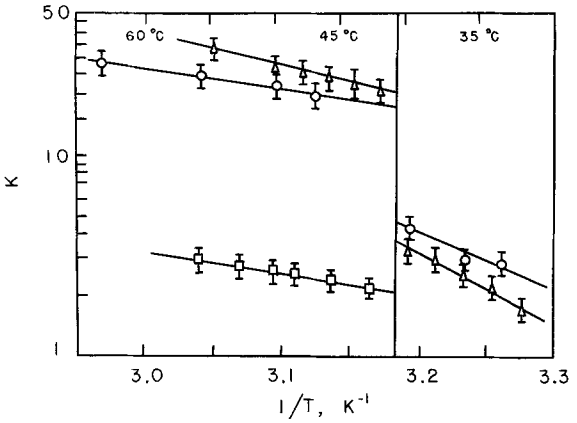

Fig. 6. Partition coefficients of nitroxide solutes in DPL liposomes as a function of reciprocal absolute temperature. ○: DTBN; △: TEMPO; □: carbonyl-TEMPO. The lines represent the linear least-squares fit to the logarithmic-reciprocal plot of the data

line is proportional to the number of molecules giving rise to the signal; as defined in Eq. (11), then, K is the ratio of the molal concentrations. However, since the densities of water and lecithin are about equal (Lecuyer & Derivichian, 1969) and the solute concentration is low, K is approximately equal to the molar concentration ratio. (We emphasize again that K is an average value for the whole bilayer.)

The partition coefficients are plotted logarithmically *vs.* reciprocal absolute temperature in Fig. 6. The ESR signal from carbonyl-TEMPO in the bilayer below T_c was undetectable; we estimate the upper limit for K for this molecule to be 0.15. For benzoyloxy-TEMPO in DPL above T_c the ESR signal in the water phase was not detectable and a lower limit for K of 2,000 is estimated; below T_c, the membrane line is poorly defined because of slow and/or restricted motion of the probe, and a lower limit of 200 is estimated for K.

On cooling through the transition temperature, K decreases abruptly by 75% for DTBN, 80% for TEMPO, and greater than 90% for carbonyl-TEMPO; this represents a "freezing out" of solute from the membrane as the hydrocarbon chains in the interior of the bilayer become more ordered. In order to determine if any solute was immobilized in the bilayer below T_c, and thus not contributing to the ESR spectrum, we measured the total integrated intensity of the DTBN membrane plus water lines, both above and below T_c. We found that, within 10%, the total intensity remained constant, suggesting that the decrease in K on cooling through the transition does indeed represent a

removal of solute from membrane. On the other hand, we found that even below T_c the hydrophobic benzoyloxy-TEMPO was "frozen in" due to its low solubility in water; the membrane ESR signal of this rather large solute in the membrane below T_c was characteristic of a system undergoing slow and/or restricted motion. A similar "freezing in" of hydrophobic solutes has been observed by other workers (Sackmann & Traüble, 1972*a*; Galla & Sackmann, 1974). The sharp decrease in the apparent value of K below T_c could arise from the "puddling" effect discussed above though, as explained, such puddles seem incompatible with the rotational data. If the "puddles" were maintained because of the presence of solute, the fraction of the bilayer remaining liquid below T_c would be proportional to overall nitroxide concentration, and K would then be concentration dependent, which it is not.

The partition coefficient is related to the partial molar standard free energy change on transfer of solute from water to bilayer (ΔG) by $K = \exp(-\Delta G/RT)$, where R is the gas constant. The water-solute intermolecular interactions usually dominate ΔG (Diamond & Wright, 1969). With TEMPO as a standard, the changes in K for DTBN, carbonyl-TEMPO, and benzoyloxy-TEMPO represent the effect of removing a $-CH_2-$ group in the ring of TEMPO, of adding a carbonyl group, and of adding a benzoyloxy group, respectively. The main effect of the addition of a carbonyl group to TEMPO is to lower the free energy of solute in water via hydrogen bond formation; K therefore decreases with the addition of a carbonyl group. The large increase in K seen with the addition of a benzoyloxy group results mainly from the increase in free energy of solute in water due to formation of entropic "hydrophobic bonds" (Frank & Evans, 1945; Tanford, 1973), and also due to the decrease in free energy in the membrane because of van der Waals forces between solute and the hydrocarbon chain of the membrane. The loss of a $-CH_2-$ group in going from TEMPO to DTBN should decrease the free energy of DTBN in water for similar reasons.

The temperature dependence of the partition coefficient is related to the partial molar enthalpy (ΔH) and entropy (ΔS) of partition by $\ln(K) = -\Delta H/RT + \Delta S/R$. The ΔH and ΔS values we calculated from Fig. 6 are given in Table 1. In all cases, both the enthalpy and entropy are large and positive, and change abruptly at T_c. However, since ΔH and ΔS affect ΔG in opposite directions, the change in ΔG (and in K) at the transition is relatively modest. The increased positive enthalpy observed upon freezing the membrane can be attributed to the increase in the energy necessary to disrupt the nonbonding forces between hydrocarbon chains

when they are frozen. The increased positive entropy can be attributed to the greater disorder introduced when solute is inserted into the ordered crystalline array of the hydrocarbon chains below T_c. These results agree with conclusions derived from a radioactive tracer method of measuring K's in bilayers (Diamond & Katz, 1974).

The Effect of Cholesterol

We have also determined the partition coefficient and the concentration dependence of the ESR linewidth of DTBN in DPL bilayers containing 0.24 mole ratio cholesterol to lipid. In the temperature range 45–63 °C, K increased with temperature from 4.8 to 12.7 (compared with 18 to 28 without cholesterol), and $\Delta B_c/c_b$ increased from 45 to 55 G/M (compared with 23–32 G/M without cholesterol). Below T_c, the membrane signal was barely detectable. Above T_c, cholesterol causes a decrease in the apparent K to a value intermediate between that of fluid and frozen bilayer, in agreement with the "intermediate fluid" condition discussed by Edidin (1974).

Shimshik and McConnell (1973) have suggested that bilayers containing cholesterol can be considered as two phases, a fluid phase of pure lipid, and a solid phase of lipid plus cholesterol. Assume that the composition of the solid bilayer is 2 parts DPL to 1 part cholesterol (Rothman & Engleman, 1972), that DTBN is excluded from solid bilayer, and that DPL and cholesterol have equal densities. If we use the phase diagram of Shimshick and McConnell (1973), we correct K and $\Delta B_c/c_b$ to give values for DTBN located only in the fluid bilayer. When corrected, K is in the range 16 to 18, and $\Delta B_c/c_b$ is in the range 15–30 G/M. These corrected values are close to those for bilayers containing no cholesterol, and are consistent with the hypothesis that cholesterol addition to bilayers results in formation of two phases.

Barriers to Permeation

There has been a long-standing debate in the physiological literature as to how a membrane's resistance to nonelectrolyte permeation is divided between the interfacial resistances and the diffusional resistance of the membrane interior. Several attempts have been made to solve this problem indirectly (e.g., Zwolinski *et al.*, 1949; Lieb & Stein, 1971), but

these attempts rested on arbitrary assumptions (Diamond & Wright, 1969). Our results permit us to make at least a crude comparison of these resistance terms.

If the diffusion and concentration of the permeant within a bilayer are uniform and the diffusion is isotropic, the permeability of a nonelectrolyte may be described by the following equation (Zwolinski *et al.*, 1949; Wartiovaara & Collander, 1960; Lieb & Stein, 1971; Diamond & Katz, 1974):

$$1/P = \frac{x_0}{KD} + 2\tau_i/y \tag{12}$$

where P is the permeability coefficient, x_o is the membrane "interior" thickness, K is the partition coefficient and D the solute diffusion constant within the "fluid" part of the membrane, y is the width of the interfacial (polar) region of the membrane, and τ_i is the time necessary to cross this interfacial region. (x_o/KD) and (τ_i/y) are the diffusional resistances of the membrane interior and of the interfacial region, respectively. (Properly, to account for inhomogeneity of the bilayer interior, x_o/KD should be replaced by an integral $\int_{x=0}^{x=x_o} dx/K(x)D(x)$ (Diamond & Katz, 1974). D and K are given in Table 1, and $x_o \approx 30$ Å for DPL. We assume $y = 5$ Å, and since the membrane thickness is about 40 Å, $x_o = 30$ Å.

If the mean time, τ_m, which a permeant remains in the membrane is known, it can be used to estimate the interfacial transit time τ_i. τ_m is roughly the time required for the permeant to diffuse from the center of the membrane to the interfacial region plus the interfacial transit time. Thus

$$\tau_m = \frac{x_o^2}{8D} + \tau_i. \tag{13}$$

Although we have not actually measured τ_m, we know (from the fact that the ESR lines of solute in membrane are resolvable from those in water) that τ_m exceeds 100 nsec, which means that $\tau_i \approx \tau_m$. Estimates of the solute diffusional resistances within the bilayer (Table 3) are many orders of magnitude below the estimated lower limit for interfacial resistance. Although numerous approximations enter into these calculations, they are unlikely to affect the conclusion that the interfacial resistance dominates the diffusional resistance for our solutes in DPL bilayers.

Table 3. The diffusional resistances of the bilayer interior (x_o/KD) and interface (τ_i/y) for the four nitroxide solutes in DPL above T_c. $x_o = 30$ Å, $\tau_i > 100$ nsec, $y = 5$ Å

	DTBN	TEMPO	Carbonyl-TEMPO	Benzoloxy-TEMPO
x_o/KD (sec/cm)	2×10^{-2}	2×10^{-2}	2×10^{-1}	$< 3 \times 10^{-4}$
τ_i/y (sec/cm)	> 2	> 2	> 2	> 2

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