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Translational Diffusion Coefficient and Partition Coefficient of a Spin-Labeled Solute in Lecithin Bilayer Membranes

(electron spin resonance/permeability/liposomes/phospholipid/biological membranes)

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ABSTRACT **The translational diffusion coefficient and the partition coefficient of a spin-labeled solute, di-*t*-butyl nitroxide, in an aqueous suspension of dipalmitoyl lecithin vesicles have been studied by electron spin resonance spectroscopy. When the lecithin is cooled through its phase transition temperature near 41°C, some solute is "frozen out" of the bilayer, and the standard partial molar enthalpy and entropy of partition go more positive by a factor of 8 and 6, respectively. However, the apparent diffusion constant in the lecithin phase is only slightly smaller than that in water, both above and below the transition temperature. The fraction of bilayer volume within which solute is distributed may increase with temperature, contributing to the positive enthalpy of partition. Comparison of time constants suggests that there is a permeability barrier to this solute in the periphery of the bilayer.**

A central problem of membrane biology is the understanding of solute permeation through biological membranes and lipid bilayers. The structure and dynamics of the membranes themselves play an important role in permeation, but in this study we concentrate on the behavior of the solute molecules, in particular, on the distribution and translational motions of a relatively small nonelectrolyte within the membrane. Although we have studied only one such system, and this in a preliminary manner, the general features observed may be reflected in other systems as well. Specifically, we have used electron spin resonance (ESR) techniques to determine the partition coefficient (K), self-diffusion coefficient (D), and a lower bound on the inter-phase transit time of the paramagnetic solute di-*t*-butyl nitroxide (DTBN) in an aqueous suspension of sonicated vesicles of the phospholipid, dipalmitoyl lecithin (DPL).

Knowledge of K, D, and Membrane:Water Interfacial Resistances Is Essential for Understanding Permeability. Membrane permeability to solutes is usually described in terms of a permeability coefficient $P \equiv (A^{-1}\, dn/dt)/\Delta c$, where $A^{-1}\, dn/dt$ is the solute's molar flux across unit area of membrane, and Δc is the concentration difference of solute between the solutions on opposite sides of the membrane. P depends upon K (the equilibrium ratio of the solute's concentration in the membrane to its aqueous concentration), D (the solute's diffusion coefficient in the membrane), x_0 (membrane thickness), and r' and r'' (resistances of the two membrane:water interfaces to solute flow). A relation (1) among these variables is: $P = [r' + r'' + \int_0^{x_0} dx/K(x)D(x)]^{-1}$, where the x-axis is taken perpendicular to the plane of the membrane. K and D are virtually certain to vary with position in a biological membrane or bilayer. Thus, a detailed analysis of permeability requires knowledge of K, D, and r. Few attempts (e.g., refs. 2–5) have been made to determine these quantities experimentally in biological membranes or bilayers.

K and D Can Be Measured by Means of ESR Spectroscopy. Previous ESR studies have elucidated various motional characteristics of membranes, by incorporating a nitroxide spin label in the membrane structure (6–8), or by studying the line widths of nitroxide solutes in membrane:water systems (9–12). We used the solute DTBN, whose ESR spectrum at low concentration in low-viscosity liquids consists of three nitrogen hyperfine lines of equal integrated intensity and very nearly equal line widths, together with ^{13}C satellites. The position of the central line is largely determined by the isotropic g-value (g), and the nearly equal separation of the hyperfine lines is determined by the isotropic coupling parameter (a). Both g and a vary slightly with solvent polarity (13) and very slightly with temperature. In the lecithin:water system we used, the different polarities of the aqueous phase and the hydrocarbon-like membrane phase let one distinguish the ESR spectra arising from DTBN in the two phases by their different g and a values. The integrated intensity of the spectrum in each phase (the double integral of the observed derivative spectrum) is proportional to the number of DTBN free radicals in that phase. Thus, K is measured as the ratio of the integrated ESR intensities for DTBN in the two phases, divided by the volume ratio of the two phases.

In dilute solutions the ESR line widths are determined by anisotropic intramolecular magnetic interactions, which are modulated by molecular tumbling, and by variations in molecular angular momentum (14). Unresolved hyperfine splittings due to the magnetic interaction of the unpaired electron in DTBN with the various protons also contribute to the line widths. In our experiments at low DTBN concentrations, the unresolved hyperfine lines and the spin-rotational interaction modulated by changes in molecular angular momentum probably dominate. Thus, we can estimate only an upper limit to the molecular tumbling rate from the line width.

In more concentrated DTBN solutions, radical–radical encounters give rise to spin exchange; this causes line broadening that is proportional to DTBN concentration and to the frequency of radical–radical encounters. The encounter frequency depends in turn on diffusion, so that concentration-

Abbreviations: DPL, dipalmitoyl lecithin; DTBN, di-*t*-butyl nitroxide; ESR, electron spin resonance.

dependent line-broadening can be used to estimate self-diffusion constants (15). At high concentrations the hyperfine components become broad and no longer well resolved, and the line shapes become difficult to describe.

If DTBN molecules are held rigidly, the lines widen due to the slow modulation of the anisotropic magnetic interactions. Very broad lines with low peak heights would be difficult to observe. Thus, if there were some very immobile DTBN radicals in the membrane (e.g., near the polar head groups of lecithin), we might not observe these molecules, and they would be excluded from our measurements of partition coefficients.

Finally, inter-phase transit times can be estimated for DTBN. If DTBN travels slowly (on an ESR time scale) between two phases, the ESR spectra will consist of two distinct spectra, one from each phase, and a lower limit on the transit time can be calculated from the minimum frequency separation of the nitrogen hyperfine components arising from each phase (16). If DTBN travels quickly between phases, it will see an "average" environment and yield a single spectrum, thereby setting an upper limit on the inter-phase transit time. Inter-phase transit times comparable to the separation of the hyperfine components from each phase can be well estimated, since the spectra from the two phases are "blended" into a characteristic spectrum.

MATERIALS AND METHODS

DTBN and DPL. DTBN was prepared by Dr. K. Ogan (17) by the method of Hoffman *et al.* (18). The fraction used was 90% pure. A 10%-by-weight aqueous suspension of DPL vesicles in 2.125 mM K_2HPO_4 + 0.375 mM KH_2PO_4 was prepared as described elsewhere (1), and sonicated. In water, DPL forms multilamellar vesicles. The molecules of each lamella are arranged in bilayers about 30 Å thick, and lamellae are separated by about 36 Å (5, 19–21). DTBN was added at 0.25, 1.0, 2.5, 11, and 25 mM. Samples at each concentration were drawn into a pyrex capillary tube without degassing, and both ends were sealed.

ESR Spectra. Spectra at each concentration were measured at five temperatures (33, 36, 40, 50, and 63°C) with a Varian 100 kHz X-Band V-4507 spectrometer system, equipped with a V-4540 temperature control unit. At higher temperatures there was progressive loss of signal intensity (5% in 1 hr at 50°C, 20% in 1 hr at 63°C), probably due to decomposition of DTBN.

Analysis of Spectra at Low Concentration. Low-concentration (0.25, 1.0, and 2.5 mM) spectra were analyzed as two sets of three first-derivative Lorentzian curves; each hyperfine line from the DPL phase was separated from, but overlapped considerably with, the corresponding water line. Spectra, including ^{13}C satellites, were fitted by the least squares program of Bevington (22). The integrated intensity of an ESR line is proportional to the peak-to-peak width squared times the height.

Analysis of Spectra at High Concentrations. For the two higher concentrations, the spectra of the water-phase DTBN maintained their well-resolved character, but the DPL-phase lines did not, due to spin exchange. On the assumption that in these concentrated solutions the line broadening for DTBN in DPL is due solely to spin exchange, the rather complex ESR spectrum can be obtained as described previously (17, 23). The parameters for the ESR spectrum of DTBN in DPL are the exchange frequency, the nitrogen hyperfine coupling constant for DTBN in DPL, and an overall intensity factor. Bevington's least-squares program was used to fit the experimental spectra to three first-derivative Lorentzian curves and to the more complex, exchange-broadened line shapes corresponding to DTBN in water and in DPL, respectively. Line widths and exchange frequencies were determined with an accuracy of better than 10%. Integrated intensities were obtained numerically by Simpson's formula (24).

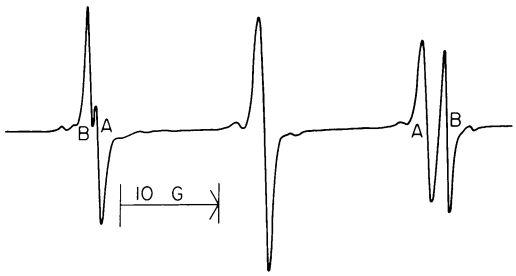

FIG. 1. ESR spectrum of DTBN (0.25 mM at 51.2°C) in an aqueous suspension of dipalmitoyl lecithin vesicles. The centers of the high-field lines (*right*) and low-field lines (*left*) are well enough separated for the two phases (*A*, lecithin; *B*, water) that two distinct spectra are observed. However, only one spectrum is observed for the middle-field lines (*center*), since the centers of the lines for the two phases nearly coincide.

At the lower three temperatures this method did not give good agreement with spectra for the higher two concentrations, possibly because the only relaxation mechanism considered for the spectra of DTBN in DPL was spin exchange. In concentrated DTBN solutions at low temperatures, where the "DPL lines" are broad, weak, and overlapping, the "water lines" dominate, and the "DPL lines" are poorly described. Comparison of ESR lines from the water and lecithin phases with those from deoxygenated water and hexane, respectively, suggests that broadening due to O_2 is small.

RESULTS

Spectra. The spectra obtained (Fig. 1) are similar to those expected for rapidly tumbling DTBN partitioned between an aqueous and a hydrocarbon phase (12). At low DTBN concentration, if the ESR line widths were predominantly due to anisotropic magnetic interactions modulated by molecular tumbling, the rotational correlation time would be in the range 3–30 psec, with DTBN tumbling about twice as fast in water as in DPL. However, as already mentioned, these times are only upper limits. The nitrogen isotropic hyperfine coupling constants in the two phases are $a^N_{H_2O}$ = 18.2 ± 0.9, a^N_{DPL} = 16.8 ± 1.0 G. Based on an assumed g-value for DTBN in water of 2.0053 (12), the g-value for DTBN in DPL is 2.0056 ± 0.0002, independent of temperature variation within experimental uncertainty.

DTBN Travels Slowly Between Phases. The observation of two distinct spectra (compare also refs. 9–12) implies that the rate of travel of DTBN between water and DPL is slow on an ESR time scale. The minimum separation of the hyperfine lines corresponding to DTBN in water and in DPL is 0.6 G, which corresponds to a lower limit τ_{min} = 100 nsec for the inter-phase transit time of DTBN.

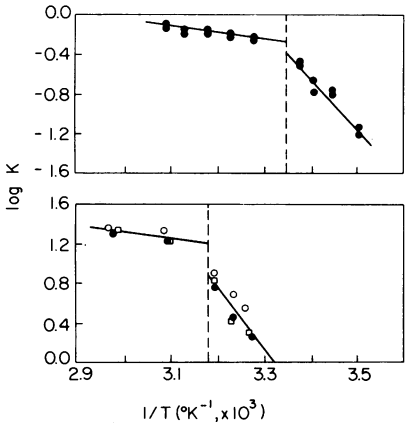

FIG. 2. Temperature dependence of nonelectrolyte partition between lecithin bilayers and water. *Above:* K of *n*-butyramide in dimyristoyl lecithin, measured by radioactive tracers (from ref. 1). *Below:* K of di-*t*-butyl nitroxide (○, 0.25 mM; ●, 1.0 mM; □, 2.5 mM) in dipalmitoyl lecithin (from present study). The dashed vertical line indicates for each lecithin the phase transition temperature below which the hydrocarbon tails "freeze." The straight lines are fitted by least mean squares through the points above or below the transition temperature. Here and in Fig. 3, the factors in the axis labels indicate that the numbers on the axes are the product of that factor and the experimental values.

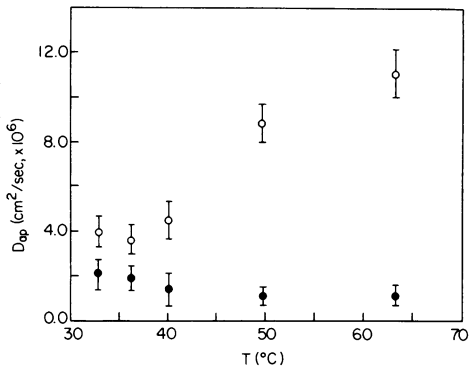

FIG. 3. Apparent diffusion coefficient of DTBN in an aqueous suspension of dipalmitoyl lecithin vesicles, as a function of temperature. ○, DTBN in water; ●, DTBN in lecithin. Correction of the latter values for the temperature-dependent distributional volume of DTBN in lecithin would tend to yield *D* values that increased with temperature.

The Partition Coefficient Can Be Determined. Since DTBN travels slowly between phases, *K* can be determined as

$$K = I_{DPL}V_{H_2O}/I_{H_2O}V_{DPL} \quad [1]$$

where I_{DPL} and I_{H_2O} are the integrated ESR intensities of the DTBN lines in DPL and H_2O respectively, and V_{DPL} and V_{H_2O} are the volumes of DPL and H_2O in the solution. In our system V_{H_2O}/V_{DPL} is 9.0 ± 0.9; we have assumed that partial molar volumes are constant on mixing, and that water and DPL have equal densities [the density of egg yolk lecithin is 1.01 (25)]. We have also assumed that DTBN has the entire volume of DPL in which to move (see below).

In Fig. 2, log *K* is plotted versus $1/T$, where *T* is absolute temperature. The slope of this graph is $-2.303R\Delta H$, where *R* is the gas constant and ΔH is the standard partial molar enthalpy of transfer for DTBN from water to DPL (1). Fig. 2 (*below*) reveals a striking discontinuity in log *K* and in the slope of log *K* near the transition temperature of 41°C, below which maximally hydrated DPL passes from a liquid crystalline to a crystalline state, associated with an ordering or "freezing" of the hydrocarbon chains in the bilayer interior (21). Fig. 2 (*above*) illustrates for comparison the same striking effects which Katz and Diamond (1, 5) measured by radioactive tracers for the solute *n*-butyramide in a closely related system, dimyristoyl lecithin vesicles and water. This lipid differs from dipalmitoyl lecithin in having shorter hydrocarbon tails and a lower transition temperature.

As calculated from Fig. 2, the enthalpy ΔH and entropy ΔS of partition both increase by an order of magnitude on freezing these two systems. For DTBN in DPL, the values (accurate to about ± 20%) are: above 41°C, ΔH = 3.3 kcal/mol, ΔS = 16 cal/mol·°K; below 41°C, ΔH = 27 kcal/mol, ΔS = 89 cal/mol·°K. The large uncertainties at temperatures below 41°C are due, in part, to the lower *K* values and consequent reduction in intensity of the lines in DPL relative to those in water. At high DTBN concentrations some structural change might occur in the bilayer. These values are in the same range as corresponding values for three solutes in dimyristoyl lecithin, both above and below its transition temperature near 25°C (1, 5).

The other effect illustrated in Fig. 2, the abrupt decrease in *K* on cooling through the transition temperature, as seen by extrapolating *K* from above and from below, suggests a "freezing out" of solute from the membrane. This jump in *K* is about 55% for DTBN in DPL, and from 5 to 20% for the three solutes studied in dimyristoyl lecithin. The jump is modest compared to the enormous enthalpy and entropy changes, since these affect *K* in opposite directions. Similar effects on freezing have been deduced for the solute malonyl gramicidin A′ in glyceryl dipalmitate-distearate membranes from conductance measurements (26)

Below the transition temperature the hydrocarbon tails of the bilayer interior pack more closely with fewer "kinks." The increased positive enthalpy of partition on "freezing" may mean that insertion of a solute into the membrane disrupts stronger non-bonding forces between hydrocarbon tails when the tails are "frozen" than when they are "melted." The increased entropy changes below the transition temperature may be attributed to disruption of the more orderly, crystalline array by the inserted solute (5).

The Diffusion Coefficient Can Be Determined by Studying Spin Exchange As a Function of Concentration. It is not the concentration of DTBN in the mixture (c_{mix}) but that in water (c_{H_2O}) and in DPL (c_{DPL}) that are important. From Eq. 1 we obtain

$$c_{DPL} = c_{mix}\,(1 + V_{H_2O}/V_{DPL})/(1 + V_{H_2O}/KV_{DPL}) \quad [2a]$$

$$c_{H_2O} = c_{mix}\,(1 + V_{DPL}/V_{H_2O})/(1 + KV_{DPL}/V_{H_2O}). \quad [2b]$$

The exchange rate for the well-resolved Lorentzians of the lower three concentrations was determined by assuming that

$$\omega_{ex} = (3/2)(\Gamma - \Gamma_0)\gamma_e \quad [3]$$

where ω_{ex} is the spin exchange frequency, γ_e the electron gyromagnetic ratio, and Γ and Γ_0 the line half-width at the given concentration and at very low concentrations, respectively. The factor 3/2 arises because there is one chance out of three that two molecules of like spin will exchange and thus have no effect on Γ. For the broad, more complex line

shape arising from DTBN in DPL at the two higher concentrations, we assumed that Γ is due only to spin exchange. A Γ_0 correction could also be included at high concentrations, but Γ_0 is modified by spin exchange and decreases with increasing concentration (27). In any case, this correction is probably within our experimental uncertainty.

The concentration dependence of ω_{ex} is related to an "apparent" translational diffusion coefficient, D_{ap}, by

$$D_{ap} = \omega_{ex}/8\pi dcN \quad [4]$$

where N is Avogadro's number, $2d$ the distance of minimum radical-radical approach required for spin exchange to occur, and c the concentration of DTBN in the appropriate phase. The distance d is assumed to be 5 Å. The diffusion coefficient in Eq. **4** is an apparent one, because we have assumed in calculating c that DTBN is distributed throughout the entire volume of DPL (see below). Calculated D_{ap}'s in DPL are somewhat lower than those in water, by up to an order of magnitude (Fig. 3). This lowering of D in the bilayer may be analogous to, though far less marked than, low D values in long-chain polymers like rubber (28).

DTBN is Mobile in the Bilayer, Both Above and Below the Transition Temperature. D_{ap} values for DTBN in the DPL phase indicate the existence of a fluid region in the bilayer. This has been demonstrated previously, most notably by the spin label experiments of Hubbell and McConnell (7). What is interesting about the present study is that there is no abrupt change in the apparent diffusion coefficient in the bilayer (Fig. 3) as the system is cooled through the transition temperature, although DTBN is "frozen out" of the bilayer and ΔH and ΔS of partition change greatly. The lack of change in D_{ap} is compatible with the line widths of the low concentration spectra, which do not vary appreciably through the transition temperature. This suggests little variation in solute rotational motion through the transition, although, as discussed above, other line-broadening effects obscure the interpretation. We have not yet studied unsonicated vesicles, in which the transition may occur over a smaller temperature range.

A Model for the Motion of DTBN in DPL. The similarity of the DTBN spectrum in DPL to that in hydrocarbon liquids, and consideration of DTBN's structure in the light of nonelectrolyte K's studied in other model systems (29), suggest that most of the DTBN in DPL is in the bilayer's hydrocarbon interior rather than among the polar head groups. Our K and D measurements then suggest the following model. DTBN is distributed throughout much of the hydrocarbon interior above the transition temperature, but below it DTBN is concentrated in the center of the bilayer near the terminal methyl groups, which remain relatively fluid. The fraction of bilayer volume occupied by DTBN would thus increase with increasing temperature, perhaps abruptly at the transition, but also more gradually over a wide temperature range, as the periphery of the hydrocarbon region became increasingly fluid and available to the solute. These changes in solute distributional volume, and the possibly two-dimensional diffusional motion (parallel to the plane of the membrane), would explain why there is more change in K than in D for DTBN at the transition, and would contribute to the temperature dependence of K and the positive ΔH of partition. In accordance with this interpretation are studies by Hubbell and McConnell (ref. 7; see also 6), who showed that fluidity increases towards the center of the bilayer, both above and below the transition temperature.

If this model is correct, then the total DPL volume V_{DPL} in Eqs. **1** and **2** should be replaced by a temperature-dependent effective volume V_{eff}, where $V_{eff} \leq V_{DPL}$. For this reason, D_{ap} calculated above for DTBN in DPL is termed an apparent diffusion coefficient. If the graph of D against T (Fig. 3) were corrected, with V_{eff} replacing V_{DPL}, D might increase with T, as observed in most systems. Our results could alternatively mean that at the transition there is little or no change either in the total amount of DTBN in the membrane or in its distribution profile, but that the peripheral DTBN molecules become immobile and do not contribute to the ESR spectrum. However, the fact that our plot of log K versus $1/T$ agrees with that obtained by other techniques (1, 5) suggests that our first interpretation is reasonable.

There is a Permeability Barrier to DTBN Towards the Bilayer Periphery. From D_{ap} and the bilayer thickness x_0 (= 30 Å), one can estimate the characteristic time $\tau_D = x_0^2/8\ D_{ap}$. This is the time that would be required for a DTBN molecule to diffuse from the center of the bilayer to the water phase, if the interior or the bilayer were homogeneous and isotropic: i.e., if the value D_{ap} were valid in the direction perpendicular to the bilayer surfaces, and if there were no interfacial resistance between the bilayer and water. The range of calculated τ_D values is 5–10 nsec. However, we have found a lower limit τ_{min} of 100 nsec for transit of DTBN between DPL and water. Since τ_{min} is at least an order of magnitude larger than τ_D, there must be a permeability barrier towards the bilayer periphery. This barrier could be due to a region of low K or low D or both, or to an interfacial resistance. Additional support for the existence of a permeability barrier comes from comparisons of measured K's with permeability coefficients in other lecithin bilayers. These comparisons would require postulating D's 5 or 6 orders of magnitude below free-solution values if the bilayers were homogeneous and without interfacial resistances (5). Even if the correction factor (V_{eff}/V_{DPL}) were 0.1, such postulated D's would be so far below the D's measured by us as to make the existence of a permeability barrier likely for other solutes in other lecithin bilayers as well.

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