SPIN LABEL MOTION IN FATTY ACIDS

ROLF MEHLHORN, WALLACE SNIPES, *and* ALEC KEITH

From the Department of Physiology and Anatomy, University of California, Berkeley, California 94720, and the Department of Biophysics, The Pennsylvania State University, University Park, Pennsylvania 16802

ABSTRACT Spin labels dissolved in highly purified fatty acid systems exhibit nearly identical tumbling rates in liquid and solid phases. Even though the spin labels do not have the same molecular geometry as the lipid matrix the melting point of the matrix can be inferred by measurements of the temperature dependency of molecular motion.

INTRODUCTION

The solvent bulk viscosity depends primarily on solvent-solvent interactions and cannot be expected to describe the motion of small quantities of structurally different impurities dispersed in the solvent matrix. Furthermore, a parameter such as Stokes' equation for viscosity, $\tau_c = 4\pi\eta r^3/3kT$, will not necessarily yield numerical values for rotational correlation times (τ_c) that have any accuracy with respect to the freedom of molecular motion of the impurity molecule. The solvent-impurity interaction depends on the structure of both and is expected to be variable and difficult to predict.

In the temperature range of a melting point where a crystalline solid goes to an isotropic liquid there is little ambiguity that the bulk phase has changed physical states. Liquid crystals may present more subtle state changes where, for example, a temperature change leads to the crystalline solid going to a smectic liquid crystalline state which, at a still higher temperature, goes to a nematic liquid crystal state, and finally, as the temperature continues to increase, an isotropic liquid comes about. Some of these mesophases may not be visually perceptible. Melts which occur in biological structures over small domains may be still more difficult to detect or characterize. We chose purified fatty acids as model systems to interact with spin label impurities because they are easy to obtain in high purity and because the alkyl chains have the same chemical structure as the hydrocarbon domains of biological membranes.

The use of spin labels to probe biological membranes has yielded important information (Jost et al., 1971). The information available to spin labels is limited to the local environment of the spin label; therefore, it is important to have informa-

tion on how this local environment compares with matrix environments which are relatively unperturbed. We report the results of experiments which show that a variety of spin labels have molecular motion nearly as fast 10°C below the visual melting point as 10°C above. Data from these experiments also allow the inference of melting points from plots of molecular motion vs. temperature. The sample used here allowed the comparison of spin label detectable "melts" and visual melts. This observation can be extended to allow the inference of physical state changes in the local environment of spin labels localized in the hydrocarbon zones of biological membranes.

MATERIALS AND METHODS

The spin labels 2,2,6,6-tetramethyl-4-piperidinol-*N*-oxyl (TEMPOL) and 2,2,6,6-tetramethyl-4-piperidone-*N*-oxyl (TEMPONE) were prepared from 2,2,6,6-tetramethyl-4-piperidinol and 2,2,6,6-tetramethyl-4-piperidone essentially by the method of Briére et al. (1965). Both were purified by preparative thin-layer chromatography (TLC) employing silica gel-G with diethyl ether as a moving phase. The precursors to TEMPOL and TEMPONE were obtained from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). The four oxazolidine spin-labeled derivatives were prepared by the method of Keana et al. (1967). These spin labels, 5-nitroxide decane (5ND) (expected: C, 69.4; H, 11.7; N, 5.8; O, 13.2; found: C, 69.4; H, 11.7; N, 5.9; O, 13.0), 2-nitroxide tetradecane (2NT) (expected: C, 72.4; H, 12.2; N, 4.7; O, 10.7; found: C, 72.5; H, 12.0; N, 4.6; O, 10.9), 12-nitroxide stearate (12NS) (expected: C, 75.3; H, 12.1; N, 3.8; O, 8.7; found: C, 75.3; H, 12.3; N, 3.6; O, 8.8) and 6-nitroxide cholestane (6NC) (expected: C, 75.9; H, 11.0; N, 3.0; O, 10.1; found: C, 76.1; H, 11.0; N, 2.7; O, 10.2) were all purified by preparative thin-layer chromatography. The synthesis of 12NS is described in greater detail elsewhere (Waggoner et al., 1969). The spin-labeled phospholipid (PLN) shown in Fig. 1 represents the phosphatidyl choline and phosphatidyl ethanolamine fractions isolated from yeast after 24 h of growth on 12NS. In the preparation of PLN, yeast total lipids were prepared by the procedure of Folch et al. (1957), and this total lipid extract was streaked onto preparative TLC plates of silica gel-G. The plates were developed in diethyl ether twice and the fluorescent fraction located at the top inch of the plate was etched off. The plate was then developed in chloroform:methanol:water (65:25:4). The location of the phosphatidyl ethanolamine and phosphatidyl choline fractions were determined by the use of purified phospholipid standards. The two phospholipid fractions were scraped off together, eluted from the silica gel with the developing solvent, dried under vacuum, and resuspended in 95% ethanol to an approximate spin concentration of 10^{-1} M (determined by comparison of signal intensities). All fatty acids used were obtained from the Hormel Institute and were reported as better than 99% pure. These fatty acids were in sealed ampoules and were not further purified. Dipalmitoylphosphatidyl choline was obtained from the Sigma Chemical Co. (St. Louis, Mo.) and gave only one band on TLC. Spin labels were used at a concentration of 2×10^{-4} M, added to aliquots of the fatty acids, placed in electron spin resonance (ESR) tubes, degassed under vacuum, and sealed with a torch. Dipalmitoylphosphatidyl choline (15 mg) was dissolved in chloroform, 10^{-5} mmol of 5ND was added, the preparation was dried under a stream of air and allowed to come to equilibrium with atmospheric moisture. It is estimated that this sample was approximately 20% hydrated. The other dipalmitoylphosphatidyl choline fraction was prepared by sonicating 15 mg of the phospholipid in 1.0 ml of distilled water and adding 5ND at 2×10^{-4} M concentration.

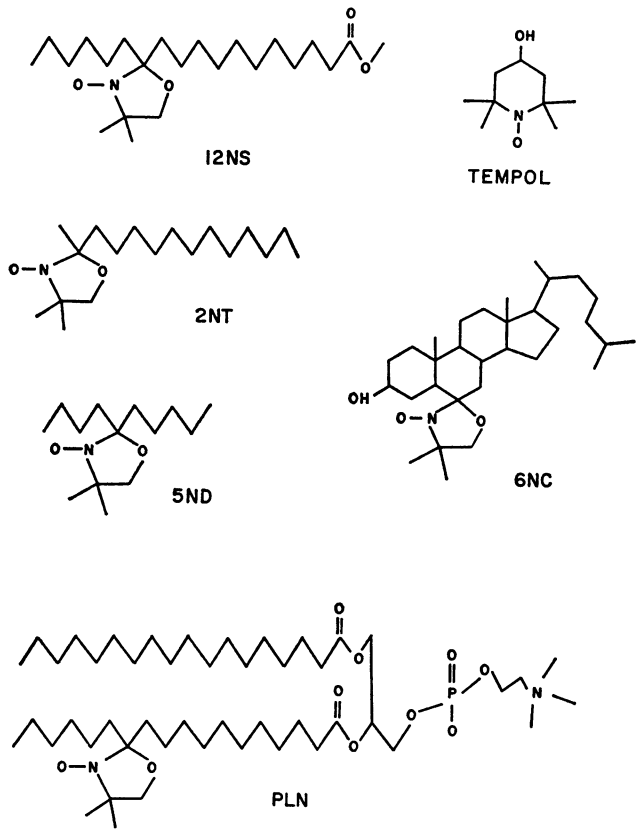

FIGURE 1 Nitroxide free-radicals used to study molecular tumbling in fatty acid environments.

Calculation of τ_c

The values of τ_c were calculated using the spectral parameters for b and $\Delta\gamma$ given by Waggoner et al. (1967). The form and use of equations derived from Kivelson (1960) have been presented earlier (Keith et al., 1970). The exact numerical values of b and $\Delta\gamma$ is unimportant for relative motion values as the values for b and $\Delta\gamma$ lead to a constant (K) in the expression,

$$\tau_0 = KW_0[(h_0/h_{-1})^{1/2} - 1]. \quad (1)$$

This expression, in its simplest form, is a line width difference and

$$\tau_0 = K(W_{-1} - W_0) \quad (2)$$

is altered into Eq. 1 by assuming lorentzian line shape in the vicinity between inflection points and because line heights are easy to measure with accuracy. W_0 is the width of the central line, h_0 and h_{-1} are the heights of the first derivative mid- and high field lines. The equation

used, Eq. 1, assumes isotropic motion and is relatively valid in the fast tumbling range ($\leq 10^{-9}$ s).

We use an approximation of rotational correlation time (τ_c) which we denote as τ_0 to emphasize that on a real time base the numerical values have varying degrees of error depending on the spin label, departure from true isotropic motion, and subtle effects of nonrandom distribution of spin labels. The Itzkowitz approximation (1967) is based on Monte Carlo spectral simulations and is valid only for isotropic motion.

Of the various spin labels used, all but 6NC and TEMPOL are expected to have some degree of enhanced motion about the nitroxide's z principal axis (Vasserman et al., 1971; Williams et al., 1971). Presently known spectral positions of tensor elements reveal that at X band, the true shapes are more nearly isotropic in appearance than for preferred motion about either the x or y principal axes (Williams et al., 1971). Still, the numerical values are not expected to be accurate. 6NC, in a matrix containing polar sites such as carboxyl groups, is expected to have its hydroxyl group associated with the other polar groups; therefore, this would result in enhanced motion about the sterols' long axes. An examination of the molecular geometry of 6NC shows that its motion is angular to all three principal axes and should give an isotropic signal. Freely tumbling 6NC would be roughly equivalent. TEMPOL is nearly spherical and should approximate isotropic motion in the fast tumbling range.

ESR Measurements

A Varian X band spectrometer (Varian Associated, Instrument Div., Palo Alto, Calif.) was used to obtain ESR spectra. The Varian variable temperature accessory was calibrated with an iron constantan thermocouple. Estimated accuracy for the temperatures is ±1.5°C. For all experiments reported here, the mobility changes (line shape changes) observed at different temperatures were completely reversible.

RESULTS

Fig. 2 shows ESR spectra of 2NT and 6NC in stearic acid at two different temperatures. At 80°C, stearic acid is a liquid whereas at 60°C it is a solid. Bulk viscosity measurements indicate that solid stearic acid has a viscosity several orders of magnitude larger than that of the liquid. If the mobility of the nitroxides were a true indication of the mobility of stearic acid in a polycrystalline solid, the nitroxide spectra in solid stearic acid should be typical of a sample with rigid, randomly oriented spins. Instead, the spectra for the nitroxides are almost identical at the two temperatures. Approximations of rotational correlation times (τ_0) calculated from the spectra show that the freedom of motion of each nitroxide in solid stearic acid is about half that in liquid stearic acid.

A variety of nitroxides in several fatty acids and fatty acid derivatives gave similar results. Table I presents data for some of these. For each fatty acid environment, the two temperatures chosen are about 10°C above and 10°C below its melting point. The ratios shown in the right-hand column of Table I give the calculated values of τ_0 at the higher temperature relative to that at the lower temperature. In all these cases there is only a factor of about 1.5–3 difference in the mobility of the nitroxides in the solid vs. the liquid state. No spectra were observed that showed complete immobilization of the nitroxide in solid fatty acids at 10°C below the melting point.

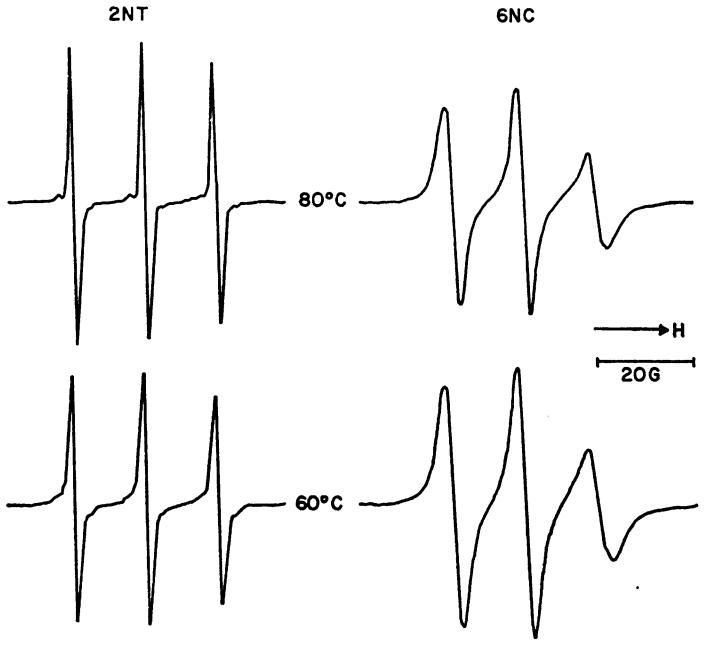

FIGURE 2 ESR spectra of the free radicals 2NT and 6NC in liquid and solid stearic acid.

Although there is no discontinuity in spin mobility at the fatty acid melting point, a difference in the activation energy (E_a) for motion in the solid and liquid environments was observed on a logarithmic plot of τ_0 vs. $1/RT$ for 6NC in stearic acid. In the solid, the motion is characterized by an activation energy of $E_a^s = 5.9$ kcal/mol, whereas the value in liquid stearic acid is $E_a^L = 10.1$ kcal/mol. Even though the meaning of these activation energies is not clear, the interactions which restrict the nitroxide motion are different for the two physical states.

In a similar plot for 12NS in elaidic acid, the *trans* isomer of oleic acid, the E_a for the solid state is greater than that for the liquid, in contrast to the behavior of 6NC in stearic acid. Table II gives values of E_a^s and E_a^L for several nitroxides in fatty acids and fatty acid derivatives.

Glycerol ordinarily exists as a supercooled liquid below its melting point, although crystals can be grown under certain conditions. For another comparison of bulk and microscopic viscosities the rotational motion of a nitroxide was examined in glycerol. For this purpose the spin label TEMPONE, the ketone analogue of TEMPOL, was used. The resulting Arrhenius plot (Fig. 3) shows a melting point of 21°C.[1] Handbook (Hodgman, 1961 *a*) viscosities are used to display η/T on the

[1] Due to uncertainties in τ_0 estimated at high viscosities by the method of Itzkowitz (1967), this number could be in error by a few degrees.

TABLE I

VALUES OF τ_0 FOR NITROXIDE PROBES IN SEVERAL FATTY ACID ENVIRONMENTS*

Fatty acid	Probe	Temperature	τ_0	Ratio
		°C	10^{-10}s	
Stearic	2NT	60	0.93	1.79
		80	0.52	
	6NC	60	7.20	2.18
		80	3.30	
Oleic	2NT	2	3.78	2.55
		22	1.48	
Elaidic	2NT	35	1.27	2.44
		55	0.52	
	12NS	35	7.12	2.25
		55	3.16	
	TEMPOL	35	1.44	2.88
		55	0.50	
Ethyl oleate	2NT	−20	2.43	2.61
		0	0.93	
	12NS	−20	15.11	1.56
		0	9.69	
	TEMPOL	−20	1.04	2.89
		0	0.36	
Linoelaidic	5ND	19	4.50	2.76
		39	1.63	

* Fatty acids are at temperatures 10°C above and 10°C below the melting point of each fatty acid. The ratio gives the relative values of τ_0 at the two temperatures.

same plot. No melting point is apparent, although a bulk phase change can be observed by other methods at 17.9°C (Hodgman, 1961 *b*).

In environments that are capable of partitioning into polar and nonpolar regions more abrupt spectral changes can occur during a phase transition. The spectra of Fig. 4 were obtained with the spin label 5ND dissolved in aqueous dispersions of dipalmitoylphosphatidyl choline. On the left-hand side of the figure spectra of 5ND in sonicated phospholipid vesicles are reproduced. For all these spectra, two environments can be discerned. At 20°C and lower temperatures the high, narrow peaks arise from water, as indicated by the hyperfine coupling constant of 16.5G. On the other hand, at 40°C the signal arises primarily from a nonpolar hydrocarbon environment, characterized by a coupling constant of 14.6G. It can be stated that a change in the effective partition coefficient of the nitroxide between the two environments occurs in this temperature range.

An analogous change of spectral features is seen on the right-hand side of Fig. 4. These spectra were observed in a dehydrated sample in which the water content is estimated to be about 20% of the total weight. At 40°C a composite spectrum

TABLE II

ACTIVATION ENERGIES CALCULATED FROM AN ARRHENIUS PLOT OF τ_0 VS. $1/RT$ FOR NITROXIDES IN FATTY ACID ENVIRONMENTS*

Fatty acid	Probe	E_a^L	E_a^s
		kcal/mol	*kcal/mol*
Stearic	2NT	7.2	3.8
Stearic	6NC	10.1	5.9
Tripalmitin	12NS	7.8	9.4
Elaidic	12NS	6.4	9.6
Oleic	PLN	7.1	9.3

* The value for the liquid state is given as E_a^L, that for the solid state as E_a^s.

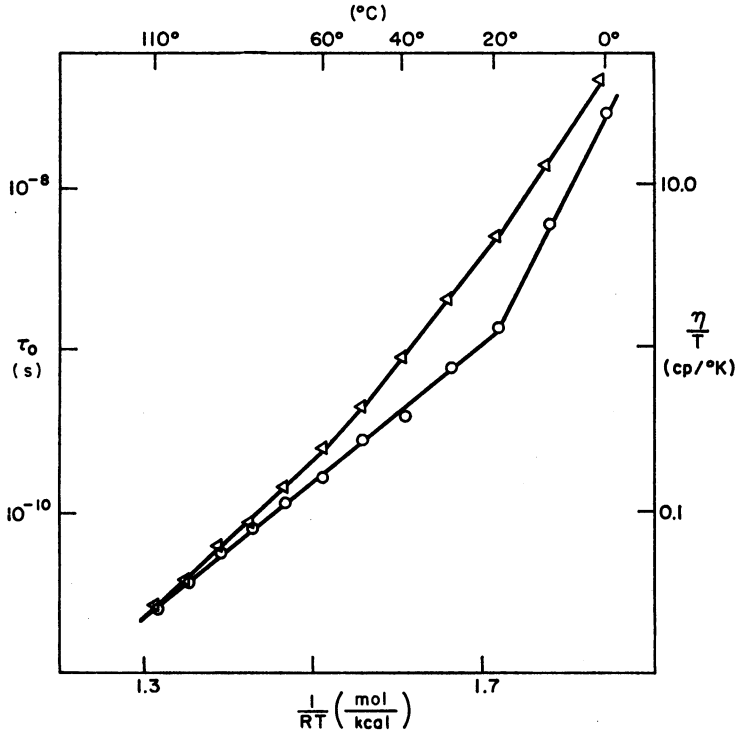

FIGURE 3 Arrhenius plots of the bulk parameter η/T (denoted by △) and the ESR parameter τ_0 (denoted by ○) in glycerol.

analogous to the 30°C spectrum in water is observed. At 50°C the spectrum arises primarily from a hydrophobic environment, as judged by the coupling constant (14.6G). The transitions seen in Fig. 4 can be correlated with the results of differential scanning calorimetry (Chapman et al., 1967). A small endothermic peak

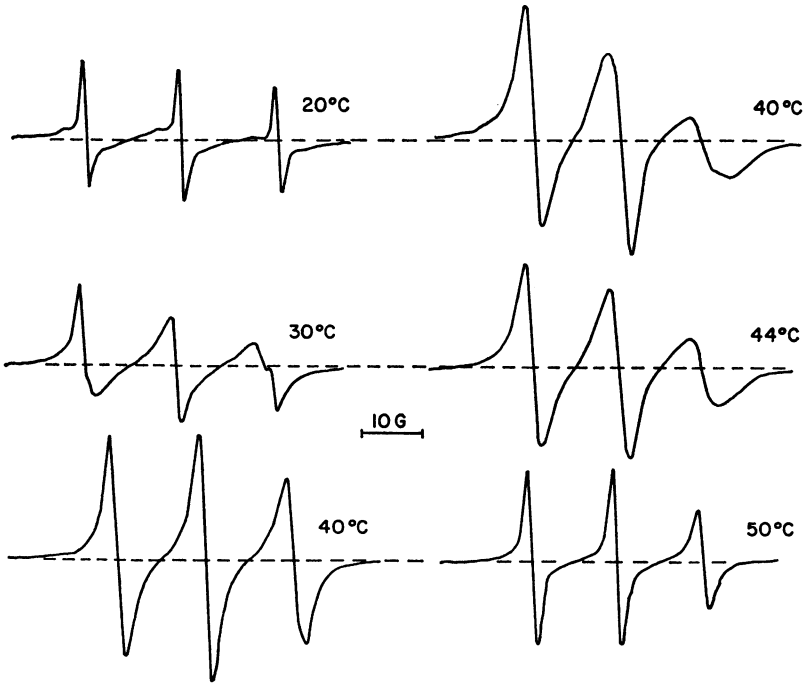

FIGURE 4 ESR spectra of 5ND in aqueous phospholipid systems. Left-hand side: aqueous dispersion of dipalmitoylphosphatidyl choline. Right-hand side: dehydrated to 20% water.

observed by thermal measurements at 35°C appears to correspond to the transition seen by ESR at about 30°C.

DISCUSSION

The failure to observe any striking phase transition with dilute solutions of nitroxide free-radicals in fatty acids indicates that visual appearance of physical state is inadequate for the description of microdomains. The glycerol data show Arrhenius plots of τ_0 obtained from ESR measurements and of η/T obtained from bulk measurements are quite similar in that the low and high temperature activation energies do not differ appreciably. In recent years, Arrhenius plots have been used extensively to infer membrane-lipid phase transitions (Mehlhorn and Keith, 1972). This body of literature has become important and is recognized as a valid way of relating the biological function to the physical properties of a given preparation. Both the Arrhenius plots of physical measurements such as spin label motion and the temperature dependence of biological function are phenomenological. Arrhenius E_a values of the fatty acid systems have the same general features as those of glycerol suggesting that in all these solvents the nitroxide probe is imbedded in local regions of supercooled liquid, i.e., a crystal cannot form in the neighborhood of the impurity.

The various τ_0 values arrived at for different spin labels do not necessarily reflect the motion of the total molecule. In the cases of TEMPOL and 6NC the structures

are rigid and the motion should be that of the entire molecule. All the oxazolidines attached to aliphatic chains probably have independent motion of the nitroxide such that rotations about methylene groups and intrachain flexing contribute to faster molecular motion than is expected from molecular weight considerations. These considerations are important in relation to the τ_c values of the different spin labels.

The specificity of interactions between unlike molecules can be seen in the data of Table I. For example, 2NT and TEMPOL have nearly identical τ_c values in elaidic acid at 55°C, whereas in ethyl oleate at 0°C the values of τ_0 for these two nitroxides differ by a factor of almost three. As another example, 12NS has a τ_c value about 15 times as great as TEMPOL in ethyl oleate at −20°C, whereas the ratio in elaidic acid at 35°C is only 5.

Since membranes are of molecular dimensions, the "local viscosity" experienced by the membrane components may be quite different from the bulk viscosity of the isolated components. In particular, the mobility of a spin label appears to reflect a fluid environment whether dissolved in a bulk liquid or solid.

The phospholipid results presented in Fig. 4 illustrate the complexity of spectra arising out of composite environments similar to those one might expect to find in membranes. Failure to recognize the presence of several environments could easily lead to an erroneous interpretation of these results. For example, line width measurement assuming a homogenous environment would indicate a slower tumbling rate at 40°C than at 20°C for the sonicated PL dispersion in water.

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