Biochimica et Biophysica Acta, 406 (1975) 197–205

BBA 77074

ROTATIONAL CORRELATION TIMES AND PARTITION COEFFICIENTS OF A SPIN LABEL SOLUTE IN LECITHIN VESICLES

M. TOMKIEWICZ and G. A. CORKER

IBM Thomas J. Watson Research Center, Yorktown Heights, N. Y. 10598 (U.S.A.)

(Received April 1st, 1975)

SUMMARY

Di-*tert*-butylnitroxide dissolved in an aqueous suspension of egg yolk lecithin vesicles is distributed between the two phases. Partition coefficients of the nitroxide between the lipid and the water, calculated from the nitroxide electron paramagnetic resonance (EPR) spectra, decrease with decreasing temperature until approximately the freezing point of the solvent. Below this temperature the nitroxide is detected only in the lecithin. The rotational correlation times of the spin label present in the lecithin were calculated for the temperature range from +45 to −60 °C. At low temperatures, the EPR spectra are characteristic of a superposition of two spectra resulting from the nitroxide dissolved in the lipid in two environments with different rotational correlation times.

INTRODUCTION

Nitroxide spin label studies have become a very convenient method for studying dynamic phenomena in membrane model systems [1]. We have extended the temperature range of such a study to subfreezing temperatures. When one studies dynamic processes, either in biological or artificial systems, which involve short lived intermediates, it is sometimes advantageous to monitor the processes at subfreezing temperatures where the dynamic events are generally slower or stopped completely. A better understanding of the motional properties of a solute dissolved in an artificial membrane, above and below the freezing point of the solvent, might help to clarify some aspects of these kinds of studies.

EXPERIMENTAL

The nitroxide was synthesized according to Hoffman et al. [2] and purified by spinning band distillation and gas chromatography. Chromatographically pure egg yolk lecithin was obtained from the Grand Island Biological Co.

A 10 % by weight aqueous suspension of lecithin in 10 mM Tris buffer, at pH 7 with 0.15 M NaCl and 10^{-5} M EDTA was sonicated for 15 min at 0 °C under N_2 to form a mixture of single wall and multilamellar vesicles. We did not attempt to

separate the two. The exact spatial arrangement of the lipid had no influence on the results that will be present here. Identical results were obtained with unsonicated suspensions of the lipid and with 30-min sonicated suspensions as with the 15-min sonicated samples. The nitroxide in water solution was added after sonication of the lipid. The results obtained were the same whether the nitroxide was added prior or after sonication. However, the high vapor pressure of the nitroxide and the nitrogen purge caused decreased concentrations of the nitroxide.

Two concentrations of the nitroxide (0.66 and 3.3 mM) were investigated. The EPR spectra were obtained with a Varian E-3 spectrometer equipped with a V4500 variable temperature control unit. Temperatures were measured with a copper constantan thermocouple. These are accurate within 4 °C. Single scan EPR spectra were digitized using a TMC Cat 1000. Data were analyzed on an IBM virtual machine facility/370 model 168. The temperature range investigated was from +45 to −150 °C. We arbitrarily divide this range into two regions with some overlap: the first region from +45 to −20 °C; the second region from the freezing point of the particular sample to −150 °C. Measurements in region 1 were made using the variable temperature, aqueous cell which allowed measurements continuously through the freezing point. Measurements in region 2 were conducted using a 3-mm cylindrical EPR tube. This enabled us to detect the freezing point of the solvent by the rapid change in the dielectric loss in the EPR cavity upon the formation of ice.

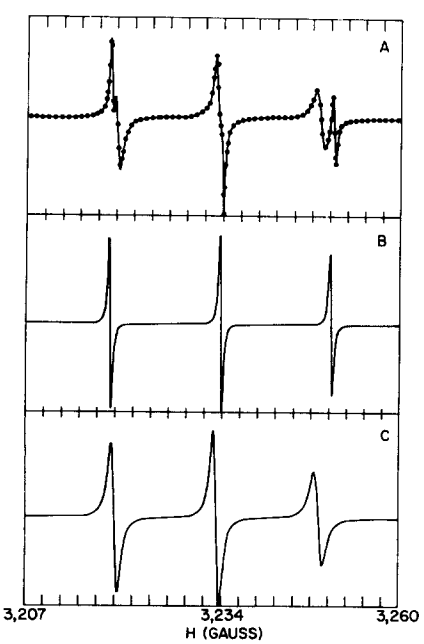

Fig. 1. EPR spectra of 3.3 mM di-*tert*-butylnitroxide in a suspension of 10 % egg yolk lecithin in 10 mM Tris buffer at 45 °C, pH 7. (A) The solid line is experimental, the points represent the spectrum obtained by the superposition of B and C. (B) Calculated spectrum (see text) of the nitroxide in the aqueous phase. (C) Calculated spectrum of the nitroxide in the lecithin.

Analysis of the spectra

The observed spectra can be divided into two main groups. The first consists of a series of overlapping spectra of the spin label partitioned between the phospholipid membrane and the aqueous phase. These spectra were analyzed [3] by the least mean square fitting of the experimental spectra to a superposition of two sets of three first derivative Lorentzian curves. An example of such a fit is given in Fig. 1. The second group consists of spectra of the spin label in one component only. Whenever possible, the required fitting parameters were taken directly from the experimental plot.

We did not observe qualitative differences between spectra recorded with freshly sonicated samples and those recorded after freezing and thawing of the samples. The slight changes that are observed can be explained as resulting from slight differences of the temperature. Although the rate of cooling influences the extent of aggregation, freeze-thawing of suspended vesicles causes them to aggregate and to precipitate. This also indicates that the spin label is not very sensitive to the geometrical arrangement of the phospholipid bilayers.

RESULTS

Nitroxide in vesicles in region 1

(*A*) *Partition coefficients and interphase transit.* In this section we followed closely the procedure developed by Dix et al. [3]. As mentioned by these workers, the observation of two distinct spectra implies that the rate of exchange of the radical between water and lecithin is slow on the EPR time scale. Using the same nomenclature as they used, the partition coefficient of the nitroxide between lecithin and water is given by:

$$K = I_{EL} V_{H_2O} / I_{H_2O} V_{EL} \tag{1}$$

where I_{EL} and I_{H_2O} are the integrated EPR intensities of the nitroxide lines in lecithin and water, respectively, and V_{H_2O} and V_{EL} are the volumes of lecithin and water in the solution. We used a value of $V_{H_2O}/V_{EL} = 9$ (the densities are about the same [4]). The plot of log K versus $1/T$ is given in Fig. 2. Dix et al. [3] reported large discontinuities in the slope and the magnitude of log K near the transition temperature ($\approx$ 41 °C) of the phospholipid (dipalmitoyl phosphatidylcholine in their study). In our system, discontinuities in these values were observed near the freezing point of the solvent, at approx. −9 °C.

At approx. −9 °C there is a sharp discontinuity in the partition coefficient and a complete expulsion of the nitroxide from the water into the lipid. The freezing of the solvent occurs also around the same temperature. As will be mentioned below, parallel experiments with the nitroxide in buffer show that below approx. −25 °C, the nitroxide crystallizes out of solution. This is approx. 25 °C below the freezing point of the buffer. We do not know whether the mechanism operating in the buffer-nitroxide system at −25 °C operates at −9 °C in the lecithin buffer-nitroxide system. In the lecithin-containing system, the integrated sum of the EPR line intensities remained constant within 10 % accuracy when measured above, at, or below −9 °C. This indicates that the discontinuity in K is not due to immobilization of the nitroxide with concomitant, complete broadening of the spectra in the frozen aqueous solvent.

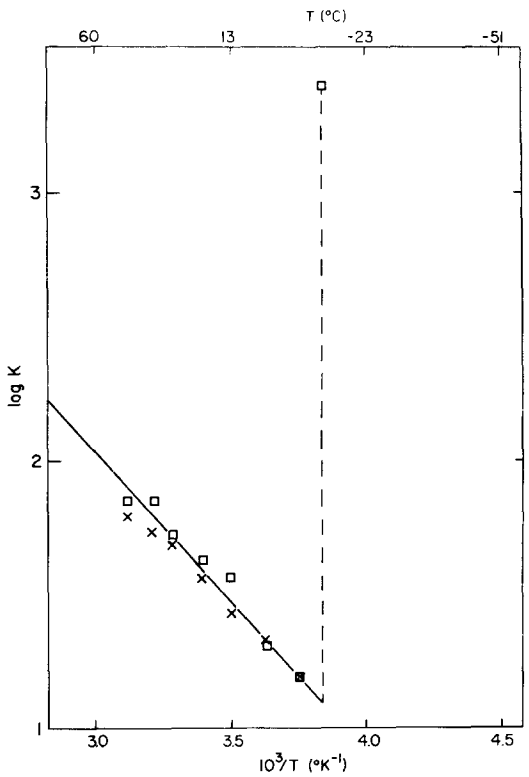

Fig. 2. The partition coefficient of the nitroxide between the aqueous and the lecithin phases as a function of temperature at two different nitroxide concentrations; ×, 0.66 mM; □, 3.3 mM. The dashed line represents the discontinuity in the partition coefficient at −9 °C.

The experiments with the nitroxide in the solvent itself, also, support this conclusion.

(*B*) *Correlation times.* The correlation times (τ_c) of the spin label in the first temperature range were calculated according to the theory developed by Kivelson [5] and applied to spin label studies by Stone et al. [6]. Kivelson's treatment is valid only for a set of well defined conditions (see refs 5 and 6). These validity conditions require, of course, a priori knowledge of the correlation times. However, a reasonably good test is that the spectrum can be analyzed in terms of three non overlapping Lorentzians with linewidths dependent upon the nuclear quantum number. All the spectra of the nitroxide in the lipid which are discussed in this section satisfy this requirement. The correlation times for the nitroxide in buffer are too fast for the theory to apply and they will not be treated here. The parameters required for Kivelson's theory were obtained by the following.

The g values were measured relative to aqueous nitroxide solution for which the isotropic hyperfine interaction is 17.16 G and $g_0 = 2.0056$ [7]. The hyperfine constants vary only slightly with temperature. The value of A_{zz} was taken as the distance between the extreme peaks of the spectrum of the "immobilized" radical. For the nitroxide in the lipid membrane we find: $A_{zz} = 35.4$ G; the isotropic hyperfine interaction, a, was taken from the high temperature spectrum to be $a = 15.8$ G.

From these values we get $A_{xx} = A_{yy} = 6$ G. The g values were calculated so that the ratio $g_{\parallel}/g_{\perp}$ would agree with the single crystal values [8] and the averaged sum would agree with our measured value of $g_0 = 2.0053$. The values thus found were: $g_x = 2.0082$, $g_y = 2.0056$ and $g_z = 2.0021$. The error in the g values is $\pm$ 0.0005 and in the hyperfine constant, $\pm$ 0.5 G. With these values, the linewidth of the radical EPR lines as a function of the rotational correlation time (τ_c) and nuclear spin quantum number (M) is given by the following:

$$(T_2(M))^{-1} = \tau_c [(1.95 - 1.23 M + 1.49 M^2) \times 10^{16}] + X \quad (2)$$

where $(T_2(M))^{-1}$ is expressed in MHz and X represents contributions to the linewidth from other broadening mechanisms which are independent of nuclear spin quantum number.

This gives three equations for τ_c and X, one for each quantum state ($M = 1, 0, -1$). Due to the lack of knowledge about the functional form or physical origin of X, Stone et al. [6] subtracted $(T_2(0))^{-1}$ from $(T_2(\pm 1))^{-1}$ and obtained two independent equations for τ_c. The agreement of τ_c using these two equations is an approximate estimate of the applicability of Eqn 2.

The spectra of the nitroxide in the lipid membrane for the temperature range discussed here is characterized by the similarity of the widths of $M = 0$ and $M = 1$ lines while the $M = -1$ line is considerably broader. In this case, the procedure of Stone et al. [6] gives unequal weight to the experimental error when calculating the

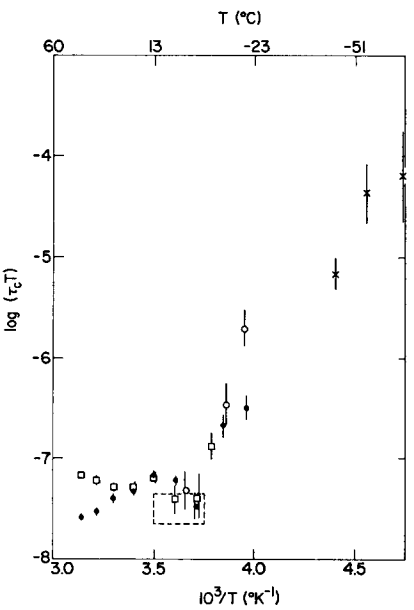

Fig. 3. Correlation times of the nitroxide as a function of temperature under different experimental conditions as follows: ○, 0.66 mM nitroxide in buffer (τ_c calculated by Eqn 2); ×, 0.66 mM nitroxide in a 10 % lecithin suspension (τ_c calculated according to ref. 13); □, 3.3 mM nitroxide in 10 % lecithin suspension (τ_c calculated by Eqn 2), and ●, 0.66 mM nitroxide dissolved in 10 % lecithin (τ_c calculated according to Eqn 2). The correlation times in the lecithin suspensions are of the nitroxide in the lecithin phase.

two values of τ_c. We felt it to be more appropriate to subtract $(T_2(-1))^{-1}$ from $(T_2(+1))^{-1}$ and $(T_2(0))^{-1}$. This procedure gives more equal weight to the errors in the data. These results are presented in Fig. 3, where the error marks represent the spread in τ_c obtained from the $M = 0$ and $M = 1$ peaks. It should be mentioned that this procedure assumes only that X is independent of the nuclear quantum number.

In order to check the percentage contribution of X to the total linewidth and the dependence of X on τ_c, we solved the set of three equations (Eqn 2) by least mean square and got X and τ_c. The results for τ_c using this procedure are within the error boundaries of τ_c obtained by the previous procedure, indicating that X is independent, at least to first order, on τ_c. The X, thus found, over the temperature range studied, range from 20 to almost 90 % of the observed peak width. This indicates that a significant portion of the linewidth is independent of the nuclear spin. The large uncertainties, and the deviation of the points inside the marked square in Fig. 3, are due in part to the lower K value and the resulting reduction in intensity of the lines in the membrane relative to those in water.

The particular presentation in Fig. 3 was chosen to show the deviation from simple Stokes relation [9]:

$$\tau_c = \frac{4\pi\mu r^3}{3\,kT}, \text{ or, } \ln\,[T\tau_c] = \ln\left[\frac{4\pi r^3\mu_0}{3k}\right] + \frac{\Delta E}{RT} \tag{3}$$

since μ, the viscosity of the membrane, is given by $\mu = \mu_0 \exp(\Delta E/RT)$; r is the molecular radius. The deviations in the correlation times at high temperatures between the two concentrations used, cannot be explained on the basis of spin exchange interaction because this interaction is nuclear spin independent [5] and will enter into X in Eqn 2.

Di-tert-butylnitroxide in ice in temperature region 2

The EPR spectrum of the nitroxide in Tris buffer was measured from the melting point to $T = -150$ °C. The spectra at temperatures $0 > T > -26$ °C fall within the limits of Kivelson's [5] theory and the correlation times at those temperatures were calculated. The results are included in Fig. 3. The τ_c at the freezing temperature was found to be: $\tau_c = 1.8 \pm 1 \cdot 10^{-10}$ s while τ_c for the same temperature, using Eqn 3 with $r = 4$Å [10] and $\mu = 1.787$ cP [11], was found to be $1.3 \cdot 10^{-10}$ s.

At approx. -25 °C there is a very sharp phase transition. The three sharp lines collapse to one broad line. The width of this line first increases upon decreasing the temperature but then starts to decrease with further decreases in temperature. This behavior is typical of high concentrations of free radicals when the spin-spin exchange interaction is the dominant relaxation mode. There are no traces of solubilized, immobilized nitroxide, the total absorption is under the one line. The simplest explanation for this observation which correlates with what was observed with samples containing lecithin is that at $T = -25$ °C, the nitroxide was suddenly expelled from the buffer and formed microcrystals.

Di-tert-butylnitroxide in vesicles in region 2

As was mentioned above, when the temperature is less than -9 °C, all of the nitroxide is concentrated in the vesicles. Several spectra obtained in this temperature range are shown in Fig. 4.

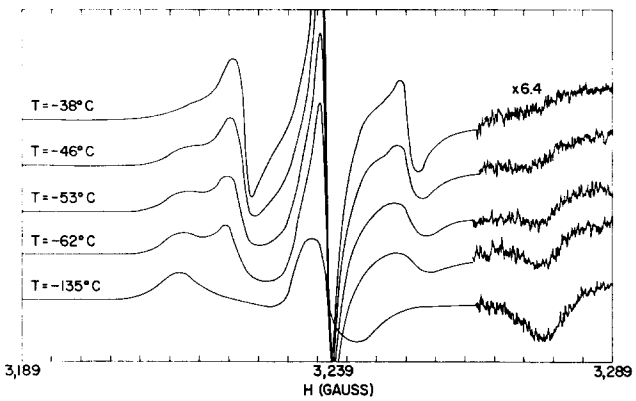

Fig. 4. EPR spectra of 0.66 mM nitroxide in 10 % lecithin vesicles at subfreezing temperatures.

It is clear that at these temperatures the analysis of the spectra according to Kivelson's [5] theory is no longer valid. Recently a number of experimental and theoretical studies [12] attempted to analyze EPR data for slowly rotating spin systems which cannot be treated by Kivelson's [5] theory. Comparing the spectra in Fig. 4 with published experimental spectra of small spin labels with slow molecular reorientation [1, 13] and with simulated spectra [12, 14] show clearly that the spectra of the nitroxide in the vesicles in this temperature range are more complicated than just to be caused by changes in correlation times.

DISCUSSION

Dix et al. [3] measured the partition coefficient of di-*tert*-butylnitroxide between dipalmitoyl lecithin and water and observed that the enthalpy (ΔH) and entrophy (ΔS) of partition both increased by an order of magnitude when the lipid became ordered (the phase transition for dipalmitoyl lecithin is approx. 41 °C). For egg yolk lecithin, we do not observe a discontinuity in the temperature dependence of the partition coefficient except at approx. −9 °C when the nitroxide is expelled from the ice phase. The magnitudes of the enthalpy ΔH and entrophy ΔS, 5.8 ± 0.3 kcal/mole and 27 ± 3 cal/mol degree, respectively, calculated from Fig. 2, compared to the values obtained by Dix et al. [3], suggest that the egg yolk lecithin is in a "fluid" state at least to −9 °C. The nitroxide is expelled from pure buffer at approx. −25 °C. Although this transition occurs approx. 15 degrees below the temperature at which we observe the discontinuity in the partition coefficient between egg yolk lecithin and water, an ice-nitroxide interaction is probably responsible for both effects.

Although broadened because of the mixture of fatty acid chains, the reported transition temperature of egg yolk lecithin occurs near 0 °C [15]. Thus the lecithin should be essentially ordered at the temperatures at which the spectra shown in Fig. 4 were obtained. The nitroxide is present only in the lecithin at these temperatures. However, we still observed spectra indicative of the nitroxide in two environments. Although one could postulate that these two environments are due to the differences in phospholipid vesicle dimensions, we observe the same spectra in unsonicated lipid suspensions which contain large aggregates of lipid bilayers and in lipid suspensions

which were sonicated for times greater than those used to obtain the data in Fig. 4. The two environments appear to be a property of the lipid itself and do not arise because of the geometry of the bilayers formed in water. The spectra in Fig. 4 can be best described by a slow (on the EPR time scale) equilibrium of the nitroxide between two environments, in which the nitroxide samples the environments at two different rates. The observation of both slow and fast rotating nitroxide in the phospholipids constitute an extreme example of a "flexibility gradient" in phospholipid bilayers [16]. The slowly reorienting component is most likely near the polar head groups while the faster moving one is concentrated mainly at the tails of the hydrocarbon chains of the fatty acids. The temperature-dependent equilibrium concentrations of di-*tert*-butylnitroxide in these two environments demonstrates a continuous translational movement in the bilayers even at low temperatures.

Assuming that the outer peaks in Fig. 4 arise due to a fraction of the radical which is "immobilized", we can use the theory of McCalley et al. [14] and calculate the correlation times of the immobilized part. The correlation times obtained this way are shown also in Fig. 3. From the shape and width of the lines at plus or minus 15 G from the center field, it is clear that the correlation times of molecules in the less restrictive environment are at least an order of magnitude faster than those in the more restrictive environment, the "immobilized part". The exact values of the correlation times and the equilibrium coefficients between those two forms require full simulation of the spectra. A sufficiently good theory which describes this situation is not yet available in order to perform this task.

Griffith et al. [17] have observed spectra similar to those in Fig. 4 for a system containing nitroxide, phospholipid and cytochrome oxidase.

Our experiments with di-*tert*-butylnitroxide in buffer also confirm the results of Goldman et al. [13] obtained with peroxylamine disulfonate. We also observe very fast rotation of a spin label just below the solvent freezing point. However, the correlation time that we find for the nitroxide is an order of magnitude slower than the one reported by Goldman et al. [13] for the disulfonate. The rotation time that we obtain agrees with one calculated from Eqn 3 using the macroscopic viscosity at the appropriate temperature. As mentioned by Goldman et al. [13], it is possible that the fast rotation involves formation of clatherates [18] around molecules of the spin label. The "freezing out" of the nitroxide from the ice phase might also involve changes in clatherate structures.

None of our observations confirm or deny the occurrence of bound water to egg yolk lecithin vesicles [19].

REFERENCES

1 Jost, P., Waggoner, A. S. and Griffith, O. H. (1971) in Structure and Function of Biological Membranes (Rothfield, L. I., ed.), Chapt. 3, Academic Press, New York
2 Hoffman, A. K., Feldman, A. M., Gelblum, E. and Hodgson, W. G. (1964) J. Am. Chem. Soc. 86, 639–646
3 Dix, J. A., Diamond, J. M. and Kivelson, D. (1973) Proc. Natl. Acad. Sci. U.S. 71, 474–478
4 Lecuyer, H. and Dervichian, D. G. (1969) J. Mol. Biol. 45, 39–57
5 Kivelson, D. (1960) J. Chem. Phys. 33, 1094–1106
6 Stone, T. J., Buckman, T., Nordio, P. L. and McConnell, H. M. (1965) Proc. Natl. Acad. Sci. U.S. 54, 1010–1017
7 Birrel, G. B., Van, S. P. and Griffith, O. H. (1973) J. Am. Chem. Soc. 95, 2451–2458

8 Griffith, O. H., Libertini, L. J. and Birrel, G. B. (1971) J. Phys. Chem. 75, 3417–3425
9 Pake, G. E. and Estle, T. L. (1973) in The Physical Principles of Electron Paramagnetic Resonance, Chapt. 9, W. A. Benjamin, Inc.
10 Griffith, O. H., Dehlinger, P. J. and Van, S. P. (1973) J. Membrane Biol. 15, 159–192
11 Handbook of Chemistry and Physics (Weast, R. C., ed.)
12 Freed, J. H. (1972) Ann. Rev. Phys. Chem. 23, 265–310
13 Goldman, S. A., Bruno, G. V., Polnaszek, C. F. and Freed, J. H. (1972) J. Chem. Phys. 56, 716–735
14 McCalley, R. C., Shimshick, E. J. and McConnell, H. M. (1972) Chem. Phys. Lett. 13, 115–119
15 Phillips, M. C., (1972) in Progress in Surface and Membrane Science (Danielli, J. F., Rosenberg, M. D. and Cadenhead, D. A., eds), Vol. 5, p. 167, Academic Press, New York
16 Hubbel, W. L. and McConnell, H. M. (1971) J. Am. Chem. Soc. 93, 314–326
17 Griffith, O. H., Jost, P., Capaloi, R. A. and Vanderkooi, G. (1973) Ann. N.Y. Acad. Sci. 222, 561
18 Davidson, D. W. (1973) in Water, a Comprehensive Treatise (Franks, F., ed.), Vol. 2, Chapt. 3, Plenum Press, New York
19 Chapman, D. and Dodd, G. H. (1971) in Structure and Function of Biological Membrane (Rothfield, L. I., ed.), Chapt. 2, Academic Press, New York