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THE DIFFUSION-CONCENTRATION PRODUCT OF OXYGEN IN LIPID BILAYERS USING THE SPIN-LABEL T_1 METHOD

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Summary

A method is described to measure the oxygen diffusion-concentration product, $D_O[O_2]$, at any locus that can be probed or labeled using nitroxide radicals. The method is based on the dependence of the spin-lattice relaxation time T_1 of the spin label on the bimolecular collision rate with oxygen. Strong Heisenberg exchange between spin label and oxygen contributes directly to T_1 of the spin label, while dipolar interactions are negligible. Both time-domain and continuous wave saturation methods for studying T_1 are considered. The method has been applied to phospholipid liposomes using fatty acid spin labels. A discontinuity in $D_O[O_2]$ at the main phase transition was observed.

Introduction

Three molecular probe techniques have been advanced to measure the frequency of collisions between the probe and molecular oxygen: fluorescence quenching [1,2], ESR spin-label line-broadening [3—7], and NMR proton spin-lattice relaxation [8—10]. There seems to be no need to write a lengthy rationale for the development of such techniques. Concentration and diffusion of oxygen are critical matters in molecular biology.

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Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine, ELDOR, electron-electron double resonance; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

All three methods have been applied to the study of oxygen in synthetic phospholipid bilayers. Windrem and Plachy [7] report that the diffusion-concentration product $D_O[O_2]$ is greater at the center of the bilayer than near the polar head groups, a result consistent with the NMR experiment of McDonald et al. [10]. Fischkoff and Vanderkooi [2] observed a discontinuity in $D_O[O_2]$ at the main phase-transition temperature, which was not reported in the other papers. All methods yielded similar numerical values for $D_O[O_2]$.

In all methods the frequency of collisions ω_E with the molecular probe is measured and interpreted in terms of the Smoluchowski equation:

$$\omega_E = 4\pi R(D_O + D_p)[O_2] \tag{1}$$

R is the interaction distance, D_O and D_p are the oxygen and probe diffusion constants, and $[O_2]$ is expressed in molecules per unit volume. Experimentally the diffusion-concentration product $D_O[O_2]$ is determined by measuring ω_E and making a reasonable guess about R. D_p is usually negligible compared with D_O. Separation of D_O and $[O_2]$ requires an independent experiment.

A difficulty with fluorescence quenching is that the theory of quenching by oxygen is very imperfectly developed. The process appears to be of a 'strong encounter' type — the probability of quenching is unity for each collision.

The NMR method is of a 'weak encounter' type — the probability of an observable event per collision is less than unity. Quantitative measurements are difficult, and this method also may be too slow if one wishes to observe the time evolution of $D_O[O_2]$.

Consider the ESR method in more detail. ESR spin-label line-broadening arises from Heisenberg exchange between probe and oxygen. The process also appears to be of a 'strong encounter' type, although the equations of Salikhov et al. [11] show that it becomes weak if either the translational correlation time or the spin-lattice relaxation time of molecular oxygen is too short. If the nitroxide radical is small and tumbling freely, resolved hyperfine couplings from methyl protons are observed. These spectral features are quite sharp in the absence of oxygen and become broadened at normal oxygen concentrations [5—7]. The changes occur over a range of oxygen concentrations that happens to coincide with oxygen concentrations of biological relevance. However, for spin-labeled macromolecules or for probes in viscous environments, proton couplings are not observed, and the other spectral features are relatively broad. As a consequence, the changes in lineshape because of collision with O_2 are difficult to measure. For example, Windrem and Plachy [7] used 1 atm of oxygen in order to see effects of oxygen on spin-labeled stearic acid in synthetic membranes.

There is a consensus in the ESR literature that dipolar interactions between spin label and oxygen make a negligible contribution to the linewidth. See, for example, the discussion of Windrem and Plachy [7].

Hyde and Sarna [12] pointed out that Heisenberg exchange between a fast relaxing species such as O_2 and a slower relaxing species such as a spin label is an effective spin-lattice relaxation mechanism for the spin label. This process has not been described in the theoretical literature. It involves the non-secular transitions arising from the exchange Hamiltonian. It is the exchange of electron quantum numbers that results in a transfer of Boltzmann population dif-

ferences from the fast-relaxing species to the slow-relaxing species. There is a statistical factor involved. Consider collisions between a spin-1 system such as O_2 and a spin-$\frac{1}{2}$ system. The effective encounters involve +1, $-\frac{1}{2}$, and -1, $+\frac{1}{2}$ collisions. Collisions of the type +1, $+\frac{1}{2}$ and -1, $-\frac{1}{2}$ do not result in non-secular transitions. Collisions described by 0, $-\frac{1}{2}$ and 0, $+\frac{1}{2}$ result in non-secular transitions but no transfer of Boltzmann population differences between the two spin systems. Thus we can write for the electron relaxation probability:

$$W_e(\text{with } O_2) = W_e(\text{no } O_2) + \tfrac{1}{3} p \omega_E \tag{2a}$$

or

$$\frac{1}{T_1(\text{with } O_2)} = \frac{1}{T_1(\text{no } O_2)} + \tfrac{2}{3} p \omega_E \tag{2b}$$

A factor p has been introduced to allow for the possibility that some collisions might be ineffective either for steric reasons or because the collisions are not of the strong encounter type. The spin-lattice relaxation time of nitroxide radical spin labels in the absence of oxygen lies between 10^{-5} and 10^{-6} s. For example, we have recently measured T_1 (no O_2) for stearic acid spin-labeled near the terminal methyl group (I(1, 14)) in membranes above the main transition and obtained a value of $2 \cdot 10^{-6}$ s (Popp, C.A. and Hyde, J.S., unpublished data). (Note that this is the true T_1 rather than the effective T_1. We will discuss the difference in more detail later in the paper.) In the present paper, the measurement of T_1 as mediated by Heisenberg exchange with oxygen is utilized to determine the $D_O[O_2]$ product.

The rationale for this approach in comparison with the more customary measurement of linebroadening is straightforward. ESR linewidths of nitroxide-radical spin labels are typically of the order of 3 G, which corresponds to a T_2^* of $2 \cdot 10^{-8}$ s. (The asterisk indicates that the linewidth is determined in a complicated way from motion, unresolved proton couplings and anisotropic magnetic interactions.) Thus, T_1 is two orders of magnitude longer. A method that measures changes in T_1 would therefore be sensitive to two orders of magnitude lower values of $D_O[O_2]$ than a method that measures changes in T_2^*. These two orders of magnitude effectively encompass the entire range of biological relevance and would make it possible to measure the oxygen diffusion-concentration product at any locus that can be spin-labeled or investigated with spin probes.

The effect of oxygen on T_1 of spin-labeled systems can be measured directly using the technique of saturation recovery [13], or indirectly using the simple and widely available technique of continuous wave saturation. In the present paper, a hybrid method involving both techniques is utilized to determine the $D_O[O_2]$ product in synthetic phospholipid liposomes.

It is appropriate in evaluating the usefulness of T_1 measurements in the determination of the diffusion-concentration product that possible effects arising from dipole-dipole interaction be considered. Several lines of evidence indicate that these effects make negligible contributions to T_1 of the spin label. These are summarized in the following two paragraphs.

McDonald et al. [10] measured the contribution ΔT_1 of molecular oxygen in air to the nuclear spin-lattice relaxation time of the terminal methyl group in

DMPC at 37°C: $\Delta T_1 = 7.3$ s. The theory [14,15] of relaxation induced by oxygen predicts that the dipolar contribution of oxygen to the spin-lattice relaxation time of a spin label will be ΔT_1 (spin label) $\geqslant 7.3[\gamma(\text{proton})/\gamma(\text{electron})]^2 = 1.6 \cdot 10^{-5}$ s. The equal sign is valid if $\omega\tau << 1$ where τ is the translational correlation time. For oxygen diffusion past a spin label, it is likely that $\omega\tau \approx 1$, which will increase ΔT_1 (spin label) by about a factor of 2. Thus from NMR at 37°C it is estimated that the effect of dipolar interactions of oxygen on the spin-lattice relaxation time of spin labels will be at least 15-times less than was measured by Popp and Hyde (unpublished data) for the intrinsic T_1 of the spin label near the terminal methyl group in DMPC at 40°C.

The only ESR experimental measurements of dipolar interactions in fluids are those of Hyde and Sarna [12], who studied the interaction of lanthanides with spin labels. Heisenberg exchange is reduced in lanthanides because the paramagnetism involves 4f electrons that are protected in collisions by 5s and 5p electrons. Dipolar interactions are enhanced because of the high effective magnetic moments. Dy^{3+} may have a spin-lattice relaxation time that is similar to that of O_2. Changes in spin-label T_1 because of collisions with 10^{-2} M Dy^{3+} were about $2 \cdot 10^{-6}$ s over a wide range of viscosities (see Fig. 5 of Ref. 12). This is the same value as the intrinsic T_1 measured by Popp and Hyde (unpublished data) for the I(1, 14) label in DMPC at 40°C. Since both the effective magnetic moment and the concentration of oxygen are lower, it would seem by analogy that dipolar interactions between O_2 and spin-labels in membranes will make a negligible contribution to the T_1 of the spin label.

Theory

One does not have a good model for the structure of the spectrum of a slowly tumbling spin label in the anisotropic environment of a membrane. The spectral features are inhomogeneously broadened by interactions with protons, and the transverse relaxation time varies across the spectrum. Experimentally in the continuous wave saturation method one measures $P_{1/2}$, which is the incident power at which the signal is half as great as it would be in the absence of saturation. The quantity can be measured, reported, and duplicated in other laboratories without knowledge of the motional model and is a convenient empirical parameter.

For any model of the structure of the spectrum,

$$P_{1/2}(\text{no } O_2)T_1(\text{eff., no } O_2) = k \tag{3}$$

where k is a constant that depends on the model and the spectral position. This equation states that a proportionality exists between the induced transition rate and the relaxation probability. In the presence of oxygen,

$$P_{1/2}(\text{with } O_2)T_1(\text{eff., with } O_2) = k \tag{4}$$

where k is the same in Eqns. 3 and 4. Here, 'eff.' stands for effective relaxation times that arise from all possible longitudinal and transverse relaxation pathways. Eqn. 4 is valid in the slow and very slow tumbling domain of a spin label where contributions to the transverse relaxation time by Heisenberg exchange between the spin label and oxygen are negligible compared with the intrinsic

phase memory time. Measurements reported here were made on the center ($m_I = 0$) line where the signal intensity is highest. However, the assumption of negligible contribution by O_2 to the transverse relaxation time is better for the $m_I = \pm 1$ transitions. For spin labels undergoing either fast or extremely slow rotational diffusion, it might be desirable to make measurements on these latter transitions.

It is possible to calculate the effective relaxation time of Eqns. 3 and 4 in terms of the true relaxation times of Eqn. 2 using the saturation factors given by Hyde et al. [16] in their Table V:

$$T_1^{-1}(\text{eff.}) = T_1^{-1}\left(\frac{(b + 3b'' + 1)(3b + 3b'' + 1)}{(1 + 3b'')(1 + b'') + b(b + 4b'' + 2)}\right) \tag{5a}$$

Here $b = W_n/W_e$, $b'' = \omega_{ex}/6W_e$, where the electron and nitrogen nuclear relaxation probabilities are given by W_e and W_n and ω_{ex} is the exchange frequency between spin labels. The spin-label concentration can always be made low enough that b'' can be neglected. Then

$$T_1^{-1}(\text{eff.}) = T_1^{-1}\left(\frac{1 + 3b}{1 + b}\right) \tag{5b}$$

For spin labels, b is at a maximum at a rotational correlation time of 10^{-8} s, and for times between 10^{-7} and 10^{-9} s it will be greater than 1. This leads to strong coupling of the three transitions and reduces $T_1(\text{eff.})$ by a factor that can be as great as 3.

Let us define:

$$b''' = \frac{p\omega_E}{3W_e(\text{no } O_2)} \tag{6}$$

With some straightforward algebraic manipulation, Eqns. 2—6 can be combined, yielding

$$\frac{P_{1/2}(\text{with } O_2)}{P_{1/2}(\text{no } O_2)} = (1 + b''')\left(\frac{1 + b'''/(1 + 3b)}{1 + b'''/(1 + b)}\right) \tag{7}$$

The term in large brackets cannot be greater than 1 nor less than 1/3.

Eqn. 7 is used to interpret the data of this paper. Popp and Hyde (unpublished results) have measured b using ELDOR [16] and W_e using the saturation-recovery technique [13]. In the present work $P_{1/2}$ ratios are measured, Eqn. 7 is solved for b''', and the diffusion concentration product calculated according to

$$D_0[O_2] = \frac{3b'''W_e(\text{no } O_2)}{p\ 4\pi R} \tag{8}$$

where it is assumed that D_p is negligible.

Materials and Methods

Materials

Doxylstearic acid spin labels (I(12, 3), I(5, 10), I(1, 14)) were purchased from Syva Corp., Palo Alto, CA. Lipids were obtained from Sigma Chemical Co., St. Louis, MO, and were used without further purification.

Sample preparation

Phospholipids and spin labels were dissolved in chloroform and mixed at a molar ratio of 200 : 1. A solution containing 27.2 mg of DMPC or 29.4 mg of DPPC was first dried under nitrogen and then under vacuum for 3—4 h. A 0.1 ml solution of 65 mM NaCl and 10 mM Hepes buffer, pH 7.6, was added and the sample then vortexed at 50—60°C. The resulting suspension of vesicles was pelleted by centrifugation for 15 min at 12 800 × g at 4°C. The excess buffer was removed and the resulting sample contained about 40% lipid by weight.

ESR measurements

ESR spectra were obtained with a Varian E-109 spectrometer at X-band using 100 kHz field modulation and a modulation amplitude of 1 G. All measurements were made on the center ($m_I = 0$) line. Samples were placed in gas-permeable sample tubes (see below) and positioned in a Varian variable temperature dewar insert in the ESR cavity. A Varian temperature accessory using either nitrogen or dry air was employed. (The reader is cautioned that dry air leads to damage of the heater sensor assembly of this unit after prolonged usage.) Microwave power as high as 500 mW was used in some measurements, which resulted in considerable heating of the sample. The actual sample temperature was measured with a Fluke 2190A digital thermometer with a copper-constantan thermocouple located in the sample at the center of the microwave cavity.

A gradient of temperature along the sample because of microwave heating was observed. For example, if a power of 200 mW was used and a temperature of 25°C established at the center of the cavity, a temperature of 24.3°C was measured at approx. 6 mm from the center, 22.5°C at approx. 10 mm, and 21.6°C at approx. 13 mm. At 500 mW the corresponding temperatures were found to be 23°C at approx. 6 mm, 18.8°C at approx. 10 mm, and 16.3°C at approx. 13 mm. Since in the Varian multipurpose cavity about 90% of the ESR signal intensity arises from the approx. 5 mm region with respect to the center, this gradient is not a serious source of error.

The sample tubes were capillaries machined from a methylpentene polymer known as TPX [17]. Dimensions are: 1.0 mm i.d., 0.2 mm wall thickness and 35 mm length. This plastic is permeable to oxygen, nitrogen and carbon dioxide, and it is substantially impermeable to water. The capillary was fixed inside the quartz dewar insert with a special plastic holder. The arrangement allowed convenient and rapid equilibration of the sample with air or nitrogen over a wide range of temperatures. Equilibration typically required 15—30 min [17].

Results

Continuous wave measurements of $P_{1/2}$ were made using the three stearic acid spin labels incorporated into DMPC vesicles saturated with air and with nitrogen over a wide range of temperature. The results are shown in Fig. 1.

The temperature corresponding to the main phase transition is indicated in Fig. 1 by vertical dashed lines. It is noted that there is a discontinuity in $P_{1/2}$ at this temperature in the presence of air, and that $P_{1/2}$ varies continuously in the absence of air. It is not yet known whether T_1(eff., no O_2) varies continuously

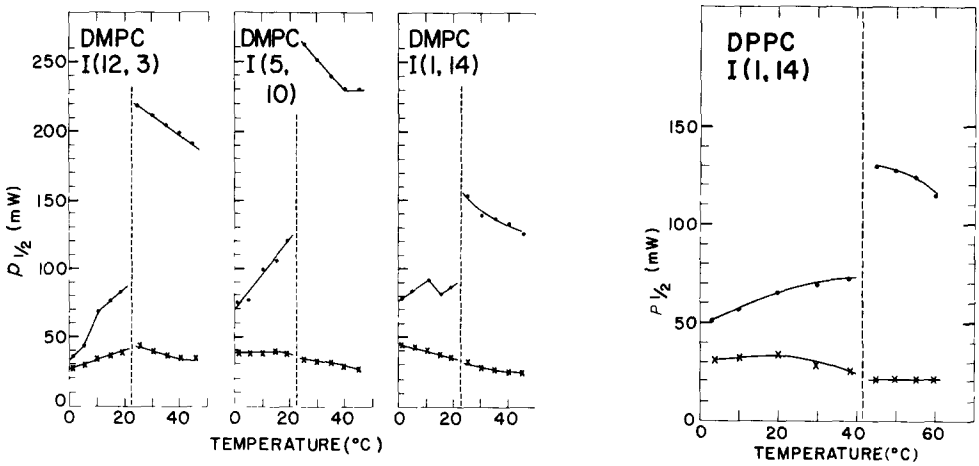

Fig. 1. $P_{1/2}$ data with (dots) and without (crosses) oxygen as a function of temperature for the three fatty acid spin labels in DMPC. The main phase transition temperature is indicated by the vertical dashed lines.

Fig. 2. $P_{1/2}$ data with (dots) and without (crosses) oxygen as a function of temperature for a fatty acid spin label with the nitroxide moiety near the center of the DPPC bilayer. The main phase-transition temperature is indicated by the vertical dashed line.

across this temperature. It is possible that equal and opposite changes in T_1(eff.) and T_2 at the transition temperature result in a continuity of $P_{1/2}$(no O_2).

The discontinuity in the ratio $P_{1/2}$(with O_2)/$P_{1/2}$(no O_2) at the transition temperatures requires, according to Eqn. 7, a discontinuity in b'''. Since it is possible that there is a discontinuity in W_e(no O_2) as well as ω_E, a quantitative interpretation of Fig. 1 in terms of the magnitude of the $D_O[O_2]$ discontinuity is not yet possible. Direct measurements of W_e(no O_2) on both sides of the transition temperature are required. Nevertheless, since all three labels show similar behavior, a conservative interpretation of Fig. 1 is that there is indeed a discontinuity in $D_O[O_2]$, by about a factor of 2 as was observed by Fischkoff and Vanderkooi [2] using fluorescence quenching. Uncertainty in the differences of W_e for the three labels does not permit a conclusion to be drawn concerning the variation of $D_O[O_2]$ across the bilayer as reported by Windrem and Plachy [7]. Again, direct measurements of W_e(no O_2) for the three labels are required before this question can be addressed.

There is, in addition, some indication in Fig. 1 of an inflection in $P_{1/2}$ near the pre-transition temperature.

Fig. 2 shows an equivalent experiment using I(1, 14) in DPPC. As in DMPC, a discontinuity is observed in $P_{1/2}$ at the main transition temperature in samples equilibrated with air. Again, this discontinuity disappears under nitrogen.

Calculation of $D_O[O_2]$

In this section Eqns. 7 and 8 are utilized to determine $D_O[O_2]$ at 40°C in DMPC, and the result is compared with the values obtained by Fischkoff and Vanderkooi [2] and by Windrem and Plachy [7].

Popp and Hyde (unpublished data) have measured T_1(no O_2) for I(1, 14) in

DMPC at 39°C by using a long saturating pulse and then observing the saturation-recovery signal [13]. A value of $2 \cdot 10^{-6}$ s was obtained. These same workers, using ELDOR, have measured the effects of Heisenberg exchange and nuclear spin-lattice relaxation on transfer of saturation, obtaining values of $b = W_n/W_e = 3.26$ and $b'' = \omega_{ex}/6W_e = 0.3$ for 0.005 molar ratio of spin label to lipid. See Ref. 16 for a discussion of ELDOR. In the present work, $P_{1/2}$(with O_2)/$P_{1/2}$(no O_2) = 5.28 for I(1, 14) at 40°C in DMPC. Neglecting b'', since it is small, solution of the quadratic Eqn. 7 yields $b''' = 7.60$. Using the measured value of W_e, $\omega_E = 4.7 \cdot 10^6$ s^{-1}.

Windrem and Plachy [7] estimate the interaction distance R to be $4.5 \cdot 10^{-8}$ cm. Assuming $p = 1$, Eqn. 8 leads to $D_O[O_2] = 1.0 \cdot 10^{13}$ cm$^{-1} \cdot$ s^{-1}. It is often assumed, as did Fischkoff and Vanderkooi [2], that the oxygen concentration in the membrane is 4.4-times that in water at 40°C, based on the data of Battino et al. [18]. Direct support for this value in membranes is lacking, but assuming it to be correct, $[O_2] = 1.06 \cdot 10^{18}$ molecules/cm^3 and $D_O = 9.5 \cdot 10^{-6}$ cm$^2 \cdot$ s^{-1}. Corresponding values obtained by Windrem and Plachy (as estimated from their Fig. 2) and by Fischkoff and Vanderkooi were $1.8 \cdot 10^{-5}$ and $1.5 \cdot 10^{-5}$ cm$^2 \cdot$ s^{-1}, respectively.

This is considered very good agreement and the consistency gives support to the validity of all three methods.

Discussion

A method has been developed here that permits measurement of the diffusion-concentration product of oxygen in any environment that can be spin-labeled. It uses, as the basic 'clock' in measuring the bimolecular collision rate, the spin-lattice relaxation time of the spin label. It is now apparent that the range of variation of this time lies within 10^{-5}—10^{-6} s. More detailed data are beginning to accumulate [12,14,19—22] on this time for a variety of labels in a variety of environments. It seems likely, by using this data and making $P_{1/2}$ measurements as described here, that many useful qualitative conclusions concerning oxygen transport can be drawn. However, as we work on the problem of whether or not oxygen transport is mediated in a biologically relevant manner by membrane constituents in specific systems, there seems to be no truly satisfactory alternative to the direct measurements of relaxation times by time domain techniques.

The complication arising from nuclear relaxation induced by modulation of electron-nuclear dipolar interactions through rotational diffusion (the term b in Eqn. 7) does not appear serious. If ELDOR equipment is not available, the rotational correlation times can be determined from the ESR spectrum and W_n easily calculated. Although an explicit experimental study of W_n for spin labels has not been published, sufficient data are accumulating to suggest that there will be no surprises. The term in large brackets in Eqn. 7 is rather insensitive to b in any event, so that errors in b do not result in large errors in b'''.

An optimum methodology may well be to combine $P_{1/2}$ measurements with direct measurements of the spin-lattice relaxation time in the absence of oxygen, using Eqns. 7 and 8 to interpret the data. Direct measurement of T_1 of spin labels in the presence of oxygen presents considerable technical problems because the times become very short.

In the present work the discontinuity in the diffusion-solubility product first reported by Fischkoff and Vanderkooi [2] at the main phase transition of pure phospholipid liposomes has been confirmed. Whether this discontinuity involves D_O, $[O_2]$, or both is an interesting question.

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