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Chemistry

A photochemical technique for measuring lateral diffusion of spin-labeled phospholipids in membranes

(nitroxides/photobleaching/lipid bilayers/translational motion/alkylcobaltate)

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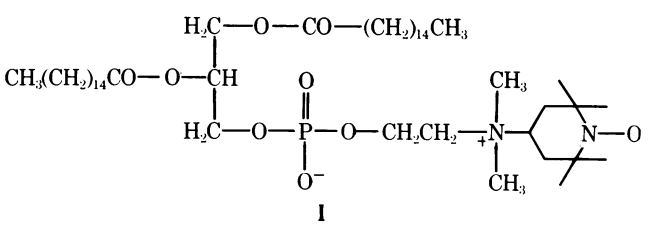
ABSTRACT **A technique is described for the measurement of lateral diffusion of any spin-labeled molecule in planar phospholipid multilayers, using low probe concentrations and a photochemical reaction to generate the initial concentration gradient. Features of the technique include simplicity of data analysis, versatility, and applicability over a wide range of motional rates. Measurements have been made on dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine in the fluid phase (using a phospholipid with head-group spin label), and excellent agreement is found with recent nuclear magnetic resonance spin-echo measurements, as well as with other spin-label and fluorescence photobleaching results.**

The importance of lateral motion in cell surface immunochemistry has been extensively discussed (1–6). However, the several methods available for measuring lateral diffusion coefficients in phospholipid bilayer membranes, which include spin exchange line broadening (7, 8), spin label-enhanced nuclear relaxation (9), NMR field-gradient spin echo (C. G. Wade and A.-L. Kuo, personal communication), and fluorescence recovery after photobleaching (10, 11) have all suffered from various limitations that prevent them from being suitable for the systematic study of the temperature and composition dependence over wide ranges that is needed in order to interpret properly the biological measurements. In particular, all of the foregoing methods except the pattern photobleaching method of Smith and McConnell (11) are restricted to relatively fast motion at high temperatures. We have therefore developed a method, using a low concentration of spin labels in conjunction with a photosensitive alkylcobalt compound, that overcomes many of these difficulties. Measurements on the fluid phases of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine are reported; preliminary measurements on solid phases are also mentioned.

MATERIALS AND METHODS

Chemicals. Phospholipids were obtained from Calbiochem or Sigma; they were found by thin-layer chromatography and gas chromatography of fatty acid methyl esters (done by B. Copeland) to be >99% pure. Cholesterol was recrystallized twice from ethanol and stored under argon, protected from light.

Spin-labeled compound **I** was provided by P. Brûlet (5, 6).



I

Carboxymethylpentacyanocobaltate was synthesized according to the method of Halpern and Maher (12). Chemical details have been given elsewhere (13, 14).

Chloroform was washed with water, dried, distilled, and stored frozen in a liquid nitrogen refrigerator until just before use.

Apparatus. A schematic diagram of the experiment is shown in Fig. 1. The collimated beam of a Spectra-Physics 164-05 argon ion laser (3511 and 3534 Å), expanded to a diameter ($1/e^2$ points) of about 40 mm, is incident on a multilayer sample between optically flat quartz plates with a metal mask in front. The plates are 14 × 25 mm, 1/16th inch (1.6 mm) thick (Suprasil 1, from Esco Products). The samples are held with the bilayer plane perpendicular to the table, inside a thermostated box whose temperature can be maintained ±0.02°C by a Forma Scientific model 70 water bath. Details of construction are given in ref. 14.

For measurements in "fluid" phases, with $D \sim 10^{-7}$–10^{-8} cm^2/sec, a mechanically machined mask was used, consisting of a row of evenly spaced bars of width 1.2 mm (equal slots and bars). For smaller diffusion coefficients a vacuum-deposited thin film was used (deposited directly on one of the quartz plates): 0.05 μm of chromium followed by ~1 μm of gold [which is better for use with electron paramagnetic resonance (EPR) due to the ease of contamination of Cr by Cr^{3+} but does not adhere well to quartz]. The pattern is an array of metal squares on a square lattice. These masks were made by Buckbee-Meers Co. (St. Paul, MN).

Sample Preparation. The phospholipid (in ethanol) was mixed with the desired amount of spin label (0.1–1%) in a 10-ml flask and the solvent was removed by a rotary evaporator at 45°C. The alkylcobalt complex (in water) was then added (3–10 times the amount of spin label), and the mixture was dispersed by Vortex mixing and then lyophilized. $CHCl_3$ (200 μl per μmol of lipid) was added to the solid, and the resulting solution was spotted onto the quartz plates, 1 μmol of lipid per plate, in such a way as to get an even film over the whole plate. (The plate rests on a flat, level stage heated to 45°C by circulating water, and a gentle stream of argon is blown down onto it the whole time.) After complete evaporation of solvent the plates were put into a chamber, under argon, at 100% humidity and left at 45°C for ~12 hr. This produced sticky, semihydrated lipid. The top plate was then put onto each plate and moved gently but firmly back and forth several times; the plates were then returned to the hydration chamber for another 12 hr, now with a flat weight (~600 g) on top. It was sometimes necessary to leave the samples at room temperature in the chamber (with or without weight) for several hr more to get fully hydrated, oriented samples. Orientation was checked on a microscope with crossed Nicol prisms; samples were used only if they were either completely black or had only a few discrete white lines.

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Abbreviation: EPR, electron paramagnetic resonance.

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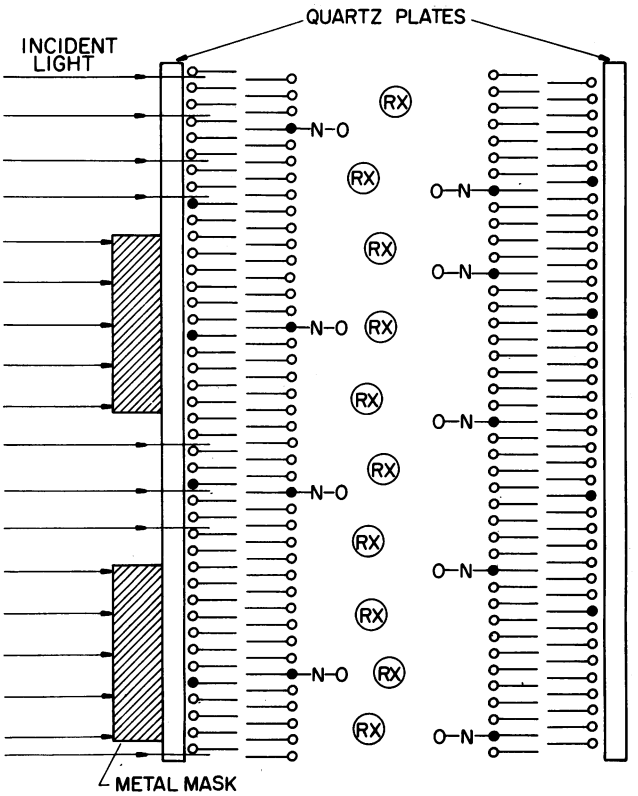

FIG. 1. Diagram of experiment. —N—O represents the spin label I; RX represents the alkylcobalt complex $[Co(CN)_5CH_2CO_2^-]^{4-}$.

(The results were not different when such lines were present.)

The samples were kept at all times in an atmosphere of argon at 100% relative humidity.

EPR Spectroscopy. A Varian E-4 with an E-238 (TM_{110}) cavity was used, interfaced with a PDP-8E computer for double integration of spectra when needed. Spectra were recorded at a constant temperature in the solid phase of the lipid in question.

Analysis. When a multilayer sample prepared as described is exposed to the laser beam, the paramagnetic resonance signal intensity is reduced to <5% of its initial value in ~1 min, due to reaction of photochemically produced carboxymethyl radicals with the nitroxide (13). The procedure by which this process is used to measure lipid diffusion is as follows.

The equation of diffusion is

$$\frac{\partial U(x,t)}{\partial t} = D\nabla^2 U(x,t) \qquad [1]$$

The photolysis for a brief time t_p sets up the initial condition shown in Fig. 2. The solution of Eq. 1 is then

$$U(x,t) = \frac{1}{2}\left[\operatorname{erf}\frac{x_1 + x}{2\sqrt{Dt}} + \operatorname{erf}\frac{x_1 - x}{2\sqrt{Dt}}\right]$$

$$\operatorname{erf} z = \frac{2}{\sqrt{\pi}}\int_0^z e^{-p^2}dp \qquad [2]$$

Letting I_1, I_2, and I_3 represent the EPR signal intensities before the first exposure, after the first exposure, and after the second exposure, respectively, we have

$$F = \frac{I_2 - I_3}{I_2} = 1 - \int_{-x_1}^{x_1} U(x,t_1)dx \qquad [3]$$

Eqs. 2 and 3 are iteratively solved for D.

Only I_1 and I_3 are measured; the sample is sealed in the thermostated chamber undisturbed between the first and second exposure. I_2 is obtained from $I_2 = \alpha I_1$, in which α is the fraction of plate area protected by the mask.

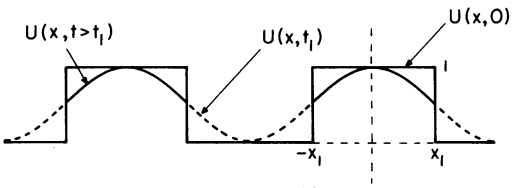

FIG. 2. Concentration of spin label, U, as a function of position, x. Before photolysis there is a uniform concentration normalized to unity, with EPR intensity I_1. After a brief exposure to light, there is the distribution $U(x,0)$, with EPR intensity I_2. After a time t_1, diffusion results in the distribution $U(x,t_1)$, given by Eq. 2. After a second brief exposure, there is the distribution $U(x,t > t_1)$ [the same as $U(x,t_1)$ except zero where the line is broken], with EPR intensity I_3.

In the case of the thin-film masks, which set up a two-dimensional pattern, rectangular symmetry is retained so the solution is just the product of one-dimensional solutions.

RESULTS

Detailed studies of the chemical kinetics are given in ref. 14. The reaction in multilayer samples proceeds in the same way as in bulk solution (13). The absence of any reaction with phospholipids is evidenced by three observations. (*i*) No new spots appear in thin-layer chromatograms after photolysis. (*ii*) No new paramagnetic resonance signals (of other radicals) have been seen. (*iii*) When the nitroxide is in excess the reaction is always stoichiometric. [The rate of reaction of alkyl radicals with nitroxides is essentially diffusion controlled (15), while hydrogen abstraction reactions have activation energies of at least several kcal/mol; hence the selectivity is not unexpected.] No change in the phase transition curve measured by 2,2,6,6-tetramethylpiperidinyl-*N*-oxyl for dimyristoylphosphatidylcholine (16) was seen in the presence of 10 mM alkylcobalt complex.

When the photolysis is carried out with the large mask (1.2-mm spacing) in place, at a temperature such that spin label diffusion is negligible, the EPR signal decays to a constant value expected from the amount of exposed area, confirming that no alkyl radicals diffuse behind the mask. This is to be expected from the high efficiency of capture in diffusion-controlled reactions, especially in two dimensions (17), and the presence of the $Co^{II}(CN)_5^{3-}$ fragment, the concentration of which increases as the spin label decreases, so that highly reactive traps for the alkyl radicals are always present.

Diffusion coefficients measured in the fluid phases of dimyristoyl and dipalmitoyl phosphatidylcholine are given in Table 1. The values listed are each obtained from a separate sample, giving some idea of the current degree of reproducibility. Error is estimated to be around 50%, the bulk of which is due to technical problems that can be eliminated. With the most recent version of the apparatus, agreement within 5–10% on different samples treated identically has been obtained.

When a small mask is used, with squares of 460 μm and open spaces of 160 μm, the fraction of spin label reduced in a single exposure is about 10% greater than the exposed area. Calculations of diffraction patterns indicate that this is the probable source of discrepancy, and a modified design will allow the mask to be within ~1 μm of the bilayers, thus effectively eliminating diffraction. Estimates using the currently available data are in general agreement with Smith and McConnell (11) for dimyristoyl phosphatidylcholine at 15–17°C.

Table 1. Diffusion constants of spin-labeled phospholipids in lipid membranes*

Dimyristoyl phosphatidylcholine†		Dipalmitoyl phosphatidylcholine	
Temp., °C	D, cm²/sec × 10^8	Temp., °C	D, cm²/sec × 10^8
29.4	13	48.1	9.9
29.4	12	48.0	12
29.4	18	47.5	8.5
28.8	16	47.8	9.9
28.7	13		
26.7	12		
24.9	8.0		
23.5	2.8		
23.5	2.7		
23.3	1.5		
22.8	1.0		
22.3	<0.1		

* Each entry in the table is the result of a separate measurement.
† Chain-melting transition temperature = 23.2°C for dimyristoyl phosphatidylcholine (16).

DISCUSSION

The order of magnitude of these numbers is similar to that of previous results obtained using various methods (7–11). An important comparison can be made to the recent results of Kuo and Wade (personal communication), who used the spin-echo NMR technique. At 48°C, 40 wt % ^2H_2O in multilayers of dipalmitoyl phosphatidylcholine they found $D = 9.1 \times 10^{-8}$ cm²/sec, in excellent agreement with the present work (well within the error limits of both measurements). This proves two important points: (*i*) D measured on a short distance scale (~0.2–0.7 μm for NMR) is the same as that measured on a large distance scale (~500 μm for the present method); (*ii*) D for a dilute solution (≲1 mol %) of a spin label phospholipid probe is identical to the true self-diffusion coefficient of the solvent lipid. These results are of particular importance in that the NMR method is unlikely to be extended to solid phases or to protein diffusion, and the foregoing comparison enhances our confidence in the spin label technique.

There are a number of aspects of this method that we believe will be advantageous compared to the conceptually similar fluorescence photobleaching method (10, 11).

(*i*) Although the comparison with the NMR data (C. G. Wade and A.-L. Kuo, personal communication) indicates that the motion of the present probe is not significantly different from that of the unmodified lipid, there are certainly cases in which such differences will occur. Rey and McConnell (18) observed the formation of long-lived hexamers of one particular spin-labeled membrane-bound molecule; the diffusion coefficient of that probe will certainly not be identical to that of the present one. The method described here allows measurement of D for *any* nitroxide probe. Such possible structural dependence is significant not only for its intrinsic physical content but because of the use of such different spin labels as tools in immunochemistry (5, 18).

(*ii*) The method described here is especially well suited to very precise temperature control (being carried out in an isolated box rather than a microscope stage or magnetic resonance cavity), and should be particularly useful for studies near transition temperatures. (It may be noted also that because the alkylcobalt photolysis has a vastly higher quantum yield than that for fluorescence photobleaching, much less heat is introduced into the sample by the light beam.)

(*iii*) The nitroxide allows one to see immediately motional characteristics and orientation, as well as probe–probe interactions (clustering, phase segregation). This is complementary to the ability to see fluorescent probe distribution visually.

(*iv*) Both translational diffusion and rotational diffusion of the same molecule can be measured [using saturation transfer spectroscopy (19, 20) for the latter]; this may allow an accurate test of the Saffman–Delbrück hydrodynamic equation (21).

The photobleaching method promises to yield a great deal of information on the motion of molecules on cell surfaces; however this will be interpretable only if accompanied by a thorough knowledge of the nature of diffusion in simpler membranes. The technique described here should contribute substantially to that goal.

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