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LETTERS

Effects of Defect Annealing on Concentration-Dependent ESR Spectra from Hydrated Dimyristoylphosphatidylcholine

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This work reports the dramatic ESR spectral changes which occur for fully hydrated multilayers of dimyristoylphosphatidylcholine (DMPC) having significant concentrations of spin-label, which have been allowed to anneal over a period of 30 days. The ESR spectra initially show concentration-dependent line broadening but then gradually become characteristic of concentration-independent spectra. Spin concentration measurements show no significant loss of radical concentration. These observations may be related to recent work of Chan and Webb showing that intially there are extensive structural defects, which gradually anneal out in the same time period.

The study of concentration-dependent ESR spectra of spin-labeled phospholipids has been shown by McConnell and co-workers[1-3] as well as by Sackmann and Träuble[4,5] to allow the estimate of diffusion coefficients D for lateral diffusion in membranes. The method of fluorescence recovery has been used for the same purpose.[6-8] Recently, Chan and Webb[9] have developed a technique for carefully annealing fully hydrated multilayers to remove the defects in their multilayer structure. They find that after initial annealing the remaining defects are elementary edge dislocations, which are abrupt terminations of single bilayers. They have obtained fluorescent intensity results on these annealed samples that imply a great slowing down of interbilayer permeation by fluorescent probes in the liquid crystalline L_α phase, and, using fluorescence bleaching, they find that in the gel phase, intrabilayer diffusion is greatly slowed down. These results are to be expected if the primary diffusion of the phospholipids in nonannealed multilayers is along defect channels due to edge (and/or screw) dislocations. Chan and Webb find that the annealing procedure for fully hydrated multilayers is very slow, taking about 2–4 weeks for virtual completion.

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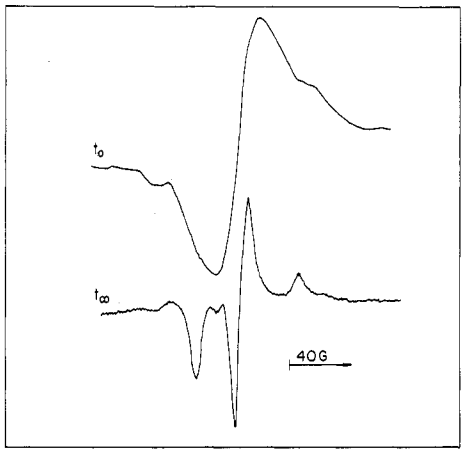

Figure 1. Spectra of 0.1 mole fraction 10,3-PC spin label in fully hydrated DMPC at 25 °C. The t_0 spectrum was obtained after hydration, while the t_∞ spectrum was obtained 30 days later, during which the sample was allowed to anneal in a humid atmosphere at 35–45 °C. All spectra are independent of orientation of the plates relative to the magnetic field.

We have undertaken the study of the effects of protracted annealing of fully hydrated phospholipids on concentration-dependent ESR spectra and its possible implications for measuring rates of lateral diffusion. A preliminary report of this work is presented here. We do indeed find dramatic changes in the ESR spectra, and attribute them to the annealing of defects. The changes are consistent with a decrease in concentration-dependent spin–spin interactions (dipolar and/or spin exchange).

In our work we studied dimyristoylphosphatidylcholine (DMPC) with the spin-labels (a) 10,3-PC [NO-labeled DPPC (dipalmitoylphophatidylcholine) with the oxazolidine ring at position 5 down the S2 chain (from the carbonyl carbon)] and (b) 10,5 stearic acid as well as 1,14 stearic acid (i.e., stearic acid labeled with the oxazolidine ring at positions 7 and 16, respectively, down the chain).

The following experiments were performed:

(1) A homogeneous solution of 0.1 mole fraction 10,3-PC in DMPC (obtained by first preparing a homogeneous solution of 10,3-PC and DMPC in ethanol and then evaporating the solvent) that is kept between two parallel glass plates spaced 50 μm apart was fully hydrated according to the procedure of Chan and Webb[9] and then kept for several hours at 35–45 °C in an Ar atmosphere saturated with water vapor in order to guarantee full hydration. After this treatment the initial or t_0 ESR spectrum was recorded (at 25 °C). The sample was subsequently kept at 35–45 °C for 30 days in the saturated atmosphere, and the final or t_∞ spectrum was recorded. The results of this experiment are presented in Figure 1. The spectra show no variation due to reorientation of the external field from perpendicular to the bilayers to parallel to the bilayer plane. This experiment indicates that the sample is macroscopically isotropic. However, there is a dramatic difference in the spectra for t_0 and t_∞ due to greatly increased spectral resolution for the latter which would be expected to result from reduced intermolecular spin interactions between spin-labels (see below).

(2) An experiment analogous to (1) was run with 0.1 mole fraction stearic acid spin labels in DMPC (10,5 stearic acid and 1,14 stearic acid gave *identical* spectra), and ESR spectra were recorded daily between t_0 and t_∞, for $t_0 - t_\infty \approx 30$ days. The results were basically similar to those obtained in experiment 1 as illustrated in Figure 2. Here, one sees the evolution of the dramatic spectral changes over this period.

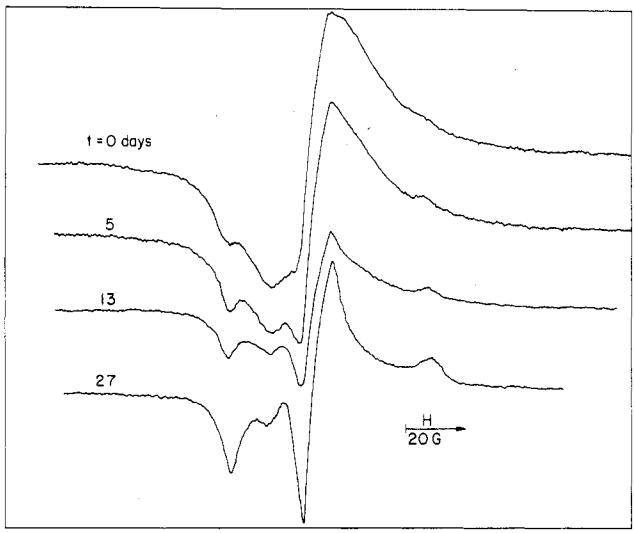

Figure 2. Spectra of 0.1 mole fraction 10,5 stearic acid in fully hydrated DMPC at 25 °C. The spectra are labeled with the time lapse after initial preparation. Between ESR measurements the sample was allowed to anneal in a humid atmosphere at 35–45 °C. All spectra are independent of orientation of the plates relative to the magnetic field.

(3) A *patch* of oily 10,5 stearic acid label of about 1 mm in diameter was deposited on an exposed end of the plate sample using (a) a nonannealed DMPC multilayer sample (i.e., a t_0 preparation) and (b) an *annealed* DMPC multilayer sample (i.e., kept for 30 days at 35–45 °C). In both cases the samples were then returned to the annealing atmosphere.

The results obtained in experiment 3a were found to be identical with those found in experiment 2. That is, the t_∞ spectrum developed after about 30 days. With (3b) similar changes occurred in the ESR spectrum as with (2) and (3a) but the t_∞ spectrum was observed after only about 10 days.

(4) Fully hydrated DMPC multilayers in tube samples containing 10,3-PC, 7,6-PC, and 1,12-PC (about 0.01 mole fraction relative to total lipid) were prepared. Above 25 °C, concentration-independent ESR spectra characteristic of the liquid crystalline phase were obtained and showed the difference in the local mobility at positions 5, 8, and 14, respectively, which one expects.[10] Similar higher concentration (0.1 mole fraction 7,6-PC) samples showed significant decrease in the concentration-dependent broadening with increased temperature, which is characteristic of either intermolecular spin dipolar broadening and/or *weak* spin exchange.[11-13]

(5) We have carefully examined the possibility of spin decay occurring during the experiments described above, which, should it take place, could explain our observations. Equal intensities (obtained by double integration of the first derivative ESR spectra) of the t_0 through t_∞ spectra would prove that no spin decay has occurred. Unfortunately, we found that the signal-to-noise ratio obtained from the plate samples depends critically on the amount of bulk water droplets trapped between the glass surfaces during the hydration process. This is difficult to monitor

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and therefore interferes with precise intensity measurements. Thus the following experiments were performed to get around this difficulty.

(a) A homogeneous solution of 10^{-3} mole fraction 10,5 stearic acid in nonannealed fully hydrated DMPC bilayers was deposited between parallel glass plates. This gave the concentration-independent pattern of isolated spins (cf. t_∞ results of Figure 2). A small amount of DPPH powder or, alternatively, a small tube containing PD-Tempone in toluene was glued to the plates so that superimosed 10,5 stearic acid and DPPH (or PDT) spectra were observed. This sample was treated in the manner of experiments 1–3, and spectra were recorded daily. We found no change in the stearic acid spectral features relative to the DPPH (or PD-Tempone) signals. We checked separately that no spin decay of DPPH or PD-Tempone occurs under these experimental conditions. This experiment rules out any observable spin decay in initially nonannealed bilayers at a low level of spin concentration.

(b) For nonannealed DMPC bilayers at a high level of spin concentration (~0.1 mole fraction 10,5 stearic acid spin probe) we prepared a homogeneous solution and sealed the plates so as to keep the bulk water content constant. (We did not seal the plates routinely, since, as described above, in experiments 3a and 3b material was added during the experiment. Also, sealing presents several technical problems.)

We repeated experiment 2 for three different samples and double integrated the t_0 through t_∞ spectra, yielding a constant integrated intensity to within 20% over this time period. A calibration sample (in a separate tube) of a standard solution of 10^{-4} M PD-Tempone in toluene was used. Its integrated intensity was found to be reproducible as a function of time to within 5–10%. Thus, there is no appreciable loss of concentration of spin label, so we cannot attribute the drastic changes observed in experiments 1 and 2 to any such "chemical" loss.

Also, several of the above samples were checked for their water content by means of gravimetric measurements, and it was found that at room temperature all samples considered were fully hydrated.

Interpretation

We first introduce the following definitions:

τ_{2D} the correlation time corresponding to two-dimensional diffusion through the hydrophobic region of the phospholipid bilayer

τ_{1D} the correlation time corresponding to quasi-one-dimensional diffusion along defects

t_{da} the time required to anneal defects in bilayer structure

We now note that since experiments 2 and 3a gave identical results, we are obviously *not* measuring τ_{2D} but t_{da} in these experiments. If the spatial randomization of the spin-labels by translational diffusion (τ_{2D}) were the main effect dominating the ESR spectrum, the time t required for the initial exchange-narrowed line to evolve into a regular powder pattern would have depended on the radius R_0 of the spin-label clusters present at the beginning, according to[1] $t \propto R_0^2$. But while $R_0 \approx 0.5$ mm in experiment 3a, experiment 2 was on a homogeneous sample (which at worst contains submicroscopic clusters). Thus, close to t_0, most regions are nonannealed and the probe molecules must be diffusing *rapidly* along defect lines or else they must be concentrated in the defect regions, thus giving rise to a concentration-broadened ESR spectrum. Intermediate between t_0 and t_∞, spectra are superimposed patterns arising from annealed and nonannealed regions. In the course of time, more regions become annealed, and, in these, the spin dipolar (or exchange) interactions are reduced giving rise to a pattern characteristic of a collection of isolated spins.[14] Our results show that the time for defect annealing is about 30 days; in any case, in experiment 3a the patch quickly disappears.

In experiment 3b one might expect to measure τ_{2D} for the 35–40 °C temperature range,[15] since the bilayers have already been annealed. One must, however, consider the possibility of (slow) permeation of the oily patch of labeled stearic acid probes into the multilayer structure (and/or local disturbance of the annealed structure near the oily patch). Also, in order to perform an accurate measurement, it would be necessary to calibrate the homogeneous spectra for a range of concentrations from a series of *annealed* samples. If, nevertheless, we attempt a crude estimate[1] based upon an annealed dilution of 0.1 mole fraction required for good spectral resolution (cf. Figure 2), then we obtain $D \sim (2R_0)^2/(4t) \sim 3 \times 10^{-9}$ cm^2/s or somewhat slower than usually obtained,[1-7] but subject to all the above uncertainties.

We plan, in future work, to accurately examine the role of structural defects and of annealing on diffusion measured by time-evolved ESR spot techniques and by concentration-dependent line broadening of annealed samples with homogeneously distributed spin-labels. One may also expect to be able to study the molecular structural properties of the defects as well as the extent of clustering of probe molecules[5,6] in the defect regions by ESR spin-label and spin-probe methods. The role of variations in sample preparation upon initial defect formation is also relevant. These matters are currently under investigation.

Acknowledgment. We thank Dr. W. Chan and Professor W. Webb for their considerable advice and for calling to our attention their results on defect annealing of phospholipid multilayers. We also thank Dr. Eva Igner, Mr. Martin Gafney, and Ms. Leslie Schwartz for their help on aspects of this work and Professor G. Feigensen for the spin-labeled phospholipids. This work was supported by NIH Grant GM-25862-03.

(14) The macroscopically isotropic spectra in Figures 1 and 2 can be interpreted in terms of either high microscopic ordering of the spin-label chains and fast motion about the axis of ordering [here the z' ordering axis is also the z''' magnetic-tensor axis, cf. E. Meirovitch and J. H. Freed, *J. Phys. Chem.*, 84, 3281 (1980)], or else of immobilized spin labels (cf. *ibid*).

(15) The room temperature spectra of Figures 1 and 2 should also be of the liquid crystalline phase, although (nearly) pure DMPC has its phase transition at about 23 °C.[3,6] Chen and Gaffney [*J. Mag. Reson.*, 29, 341 (1978)] have shown that pure label 5,10PC does not even exhibit the order–disorder phase transition, and their work is consistent with a centrally located nitroxide moiety causing the maximum lowering of the phase transition.