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Paramagnetic Resonance Evidence for Phase Transitions in Bilayers of Pure Spin-Labeled Lipids

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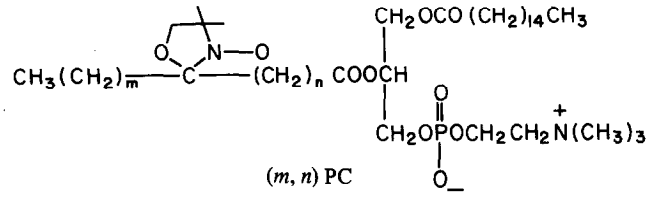
The paramagnetic resonance lineshapes for lipid bilayers composed of neat spin-labeled lipids have been studied as a function of temperature and structure of the lipid spin label. Evidence for phase transitions in the bilayers of two paramagnetic lipids is presented. Very stable hysteresis in the phase transition on heating and cooling of one of the lipids is observed and the effect of additives on the hysteresis is investigated. A plot of chemical potential versus temperature for lipid phases of different geometry is compatible with the observed hysteresis.

INTRODUCTION

When paramagnetic lipid components are present at high concentrations in phospholipid bilayers, the effects of spin–spin interactions on the paramagnetic resonance spectra of the bilayers may be employed to measure rates of lateral motion (*1–4*), and to demonstrate phase separations (*2, 5, 6*). In principle, rather detailed information about the composition and packing geometry for molecules in membranes may be obtained for mixtures of paramagnetic lipids and other diamagnetic membrane components. This is especially true for rigid phases where dipolar contributions to the lineshape predominate. These approaches differ from the more common use of low concentrations of paramagnetic lipids as spin-labeled probes in unlabeled lipid membranes (*7*) because, at high concentrations of spin label, the physical properties of the paramagnetic lipids themselves must be considered. The possibility of an order–disorder phase transition for the paramagnetic lipids in the temperature range being studied is particularly important when high concentrations of the paramagnetic lipids are employed. In this paper we describe the temperature dependence of the paramagnetic resonance spectra of a series of three synthetic, paramagnetic phosphatidyl cholines, (*m, n*) PC. Both lineshapes and phase transition temperatures are found to depend strongly on the position of the nitroxide ring on the 2-acyl chain of the



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lipids. For the lipid labeled near the methyl end of the fatty acid chains, marked and persistent hysteresis is noted in the phase transition. The stability of the hysteresis under a number of conditions will also be described.

EXPERIMENTAL PROCEDURES

Three paramagnetic lipids were prepared (*8*) from 1-palmitoyl-*sn*-glycero-3-phosphorylcholine by acylation with the anhydrides of spin-labeled fatty acids in which the label group was on C-5, C-12, or C-16 of an eighteen-carbon chain. Fatty acids were synthesized in this laboratory by established procedures (*9*). The spin-labeled fatty acids are composed of two enantiomers at the carbon bearing the nitroxide ring and therefore, the synthetic lipids are a mixture of two diasterioisomers. The positional specificity of palmitic and spin-labeled fatty acid substitutions on the glycerol backbone was verified by phospholipase A_2 treatment and preparative thin-layer chromatography to give diamagnetic lysolecithin and paramagnetic fatty acids.

For electron paramagnetic resonance measurements, 1 to 2 mg of lipid or lipid mixture in chloroform was coated on the wall of a 10-ml flask by evaporation under vacuum for 1 hr. The mixture was incubated for 30 min at a temperature above the lipid phase transition with 50 to 100 μl of buffer (0.1 *M* Tris–HCl, pH 7.4) and shaken vigorously with glass beads on a vortex mixer for several minutes.

X-Band EPR spectroscopy was carried out on a Varian E-12 EPR spectrometer equipped with a variable-temperature controller (Varian E257/WL257). The magnetic field was calibrated with Fremy's Salt, the scan rate was 100 G/8 min, and the microwave power was 10 mW. We monitored the temperature by reading the voltage of a type T thermocouple (copper–constantan, TFCP, TFCI-003, connected with cold junction MCJ-T, Omega Engineering) fixed in the sample tube holder. By careful adjustment of the orientation of the sample holder, the presence of the thermocouple junction in the cavity had a negligible effect on either temperature reading or EPR signal. To avoid errors due to temperature gradients in the cavity, the sample volume was kept at a minimum and the sample was placed beside the thermocouple hot junction. The temperature was changed at a rate of 6–8°C per hour. The sample tube was placed horizontally in the cavity to keep liposomes from settling to the bottom of sample tubes. Sealed 50-μl pipettes (Corning Glassware) were used as sample tubes.

TEMPERATURE DEPENDENCE OF RESONANCE SPECTRA

EPR spectra of each of the (*m,n* PC) dispersions were recorded at temperatures from 10 to 60°C. Some of the spectra are presented in Fig. 1. The single-line spectra result from strong spin–spin interactions through exchange and dipolar mechanisms. The high-temperature spectra show an exchange-narrowed nearly Lorentzian lineshape and at low temperature, the broader shape indicates a larger contribution from dipolar interactions.

In order to tabulate the spectral data, we have used several empirical parameters, in particular, the apparent peak-to-peak linewidth of the derivative curve (ΔH_{pp}) and the width at half-maximum height of the low-field half of the derivative curve ($\Delta H^{d}_{1/2}$). The parameter $\Delta H^{d}_{1/2}$ is effectively a measurement of the maximum differences between the pairs of spectra shown in Fig. 1. The peak-to-peak width, ΔH_{pp}, is plotted as a function of temperature for the three *m,n* PC spin labels in Fig. 2. The variations in ΔH_{pp} for 12,3

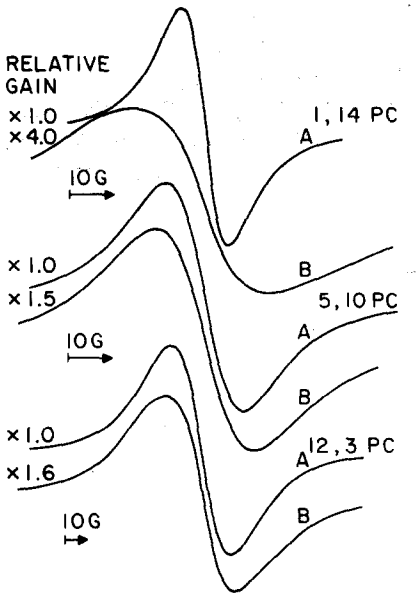

FIG. 1. EPR spectra of dispersions of spin-labeled lipids, *m,n* PC, at two different temperatures: (A) 40°C, and (B) 20°C. The relative receiver gains for the recordings are shown at the left of each line. Note that the 10-G marker is twice as large for 1,14 PC and 5,10 PC as for 12,3 PC.

and 5,10 PC are continuous over the temperature range. In contrast, when liposomes of 1,14 PC are heated from 15°C, the width decreases sharply from ~30 to 11 G at 30–32°C. This range of linewidth change is comparable to that of a similar spin-label lipid synthesized from egg yolk lecithin (*10*), and indicates the possibility of a phase transition. While the linewidth changes are reversible on heating and cooling for 12,3

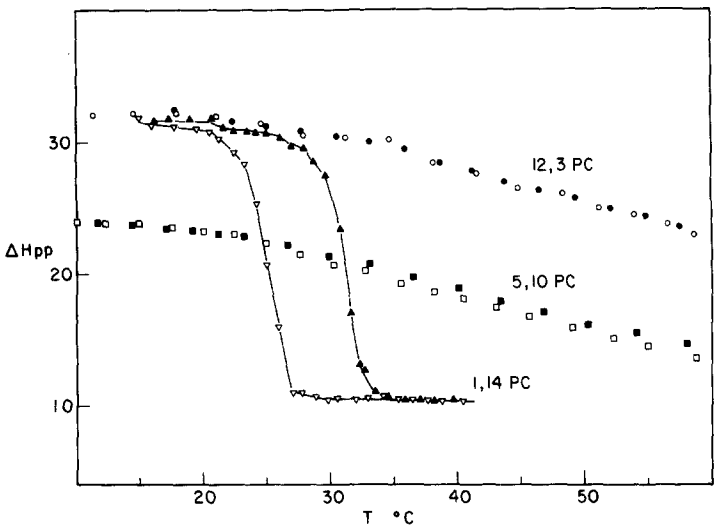

FIG. 2. The plots of peak-to-peak linewidth (ΔH_{pp}) vs temperature for *m,n* PC's (heating: closed symbols and cooling: open symbols).

and 5,10 PC, this is not true for 1,14 PC. As the temperature of the 1,14 PC dispersion is decreased from 40°C, no abrupt spectral changes are observed until 25°C. The plot of the second parameter, $\Delta H^{d}_{1/2}$, shown in Fig. 3, again shows the marked hysteresis in the spectral change for 1,14 PC. In addition, this parameter shows a change for 12,3 PC which may also be indicative of a phase transition. Preliminary investigations of these lipids by differential scanning calorimetry show that indeed phase transitions are observed for 12,3 and 1,14 PC (but not for 5,10 PC) at approximately the temperatures of the EPR changes and that the enthalpies of transitions are similar to that observed for the diamagnetic phosphatidyl choline, dipalmitoyl lecithin (S.-C. Chen, J. M. Sturtevant, and B. J. Gaffney, unpublished results).

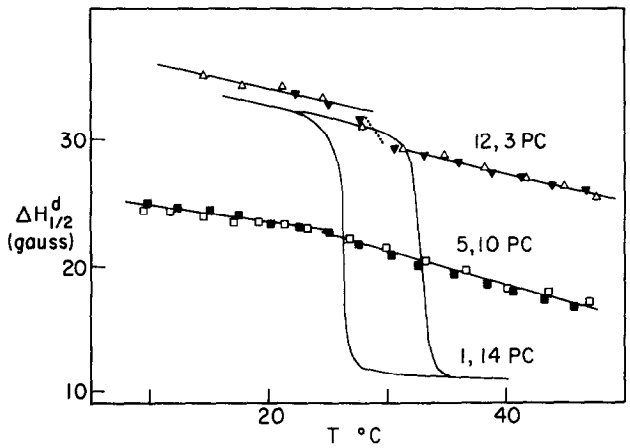

FIG. 3. The plots of width at half-height of derivative curve ($\Delta H^{d}_{1/2}$) vs temperature for 12,3; 5,10, and 1,14 PC (heating: closed symbols; and, cooling: open symbols; symbols not shown for 1,14 PC).

HYSTERESIS IN THE 1,14 PC PHASE TRANSITION

The hysteresis effect in the 1,14 PC phase transition (Figs. 2 and 3) was studied further for stability and reversibility of the heating and cooling curves. The results of kinetic experiments are shown in Fig. 4, where the peak-to-peak widths of the EPR spectra are given as a function of time at different temperatures along the heating (upper) and cooling (lower) branches of the hysteresis loop. Evidently, the hysteresis persists for very long times. However, as we show in the next section (see particularly Fig. 9), the fractional changes in the paramagnetic resonance signal parameters do not correspond directly to the change in the fraction of molecules which exist in states above and below the phase transition. Most of the EPR signal change occurs in the *first* 50% of the molecular transition for heating curve and in the *last* 50% of the transition on the cooling curve. Thus, in Fig. 4, the time dependence of the linewidth for the samples cooled to the temperatures shown indicates that the *cooling curve* is less stable at temperatures near the middle of the transition (27.5–26.5°C) than at temperatures at the beginning of the transition (28.5°C). However, for the *heating* curve, since the spectral change is almost complete when only 50% of the transition in molecular state has occurred, the time dependence of the linewidth change can only be obtained for

about the first 20% of the transition. Figure 4 indicates that the heating curve is very stable with time at the onset of the transition.

The reversibility with respect to temperature change of each branch of the hysteresis detected in the resonance spectra is shown in Fig. 5. With reference to the analysis of

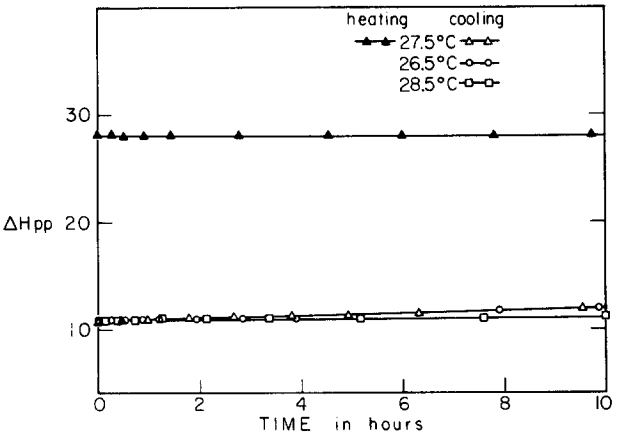

FIG. 4. The time dependence of ΔH_{pp} for points on the heating and cooling branches of the hysteresis loop for 1,14 PC. The sample remained in the EPR cavity at the temperature indicated for the entire period of the measurement.

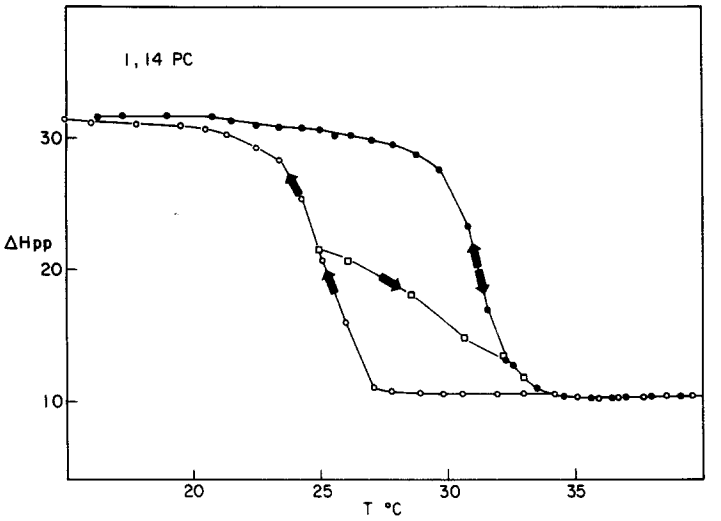

FIG. 5. Reversibility of the heating (closed symbols) and cooling (open symbols) curves for 1,14 PC, as indicated by the arrows. The heating curve is reversible up to 32°C.

the next section this figure indicates that the first ~15% of the change in molecular state on the heating curve is reversible while the last ~15% of the change on cooling is irreversible (see also Fig. 10).

One approach to discerning the molecular basis of hysteresis in the 1,14 PC transition is to search for conditions under which the transition may occur without

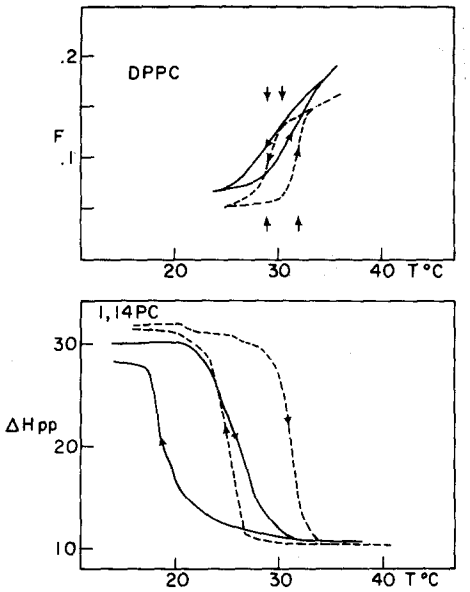

FIG. 6. The effects of 3 mole% of dicetyl phosphate on the hysteresis loop for two lipid transitions: the pretransition dipalmiotylphosphatidyl choline (DPPC, upper plot) and 1,14 PC (lower plot). For DPPC, the hysteresis loop was obtained from the partitioning of the spin label, TEMPO, between lipid and water (*20*). The ordinate gives *F*, the fraction of TEMPO in the membrane. The transitions for the pure lipid dispersions are shown in dashed lines, and, for the mixtures with 3 mole% dicetyl phosphate, by solid lines. For the DPPC pretransition, the change of the hysteresis magnitude is indicated by the vertical arrows.

hysteresis. We have prepared lipid dispersions containing 3–5 mole% of various additives including 3 mole% of dicetyl phosphate (to introduce a surface charge), 3 mole% of diphenyl hexatriene (to perturb the center of the bilayer), and 5 mole% of lysolecithin or fatty acid (components that would be present if partial hydrolysis of

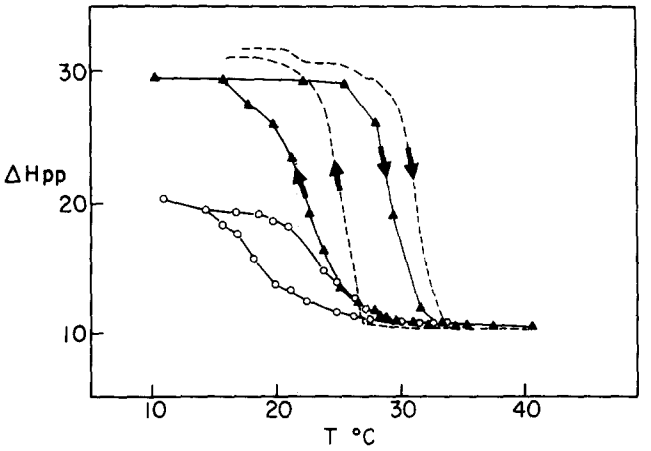

FIG. 7. The effects of 5 mole% of palmitic acid (▲——▲) and palmitoyl lysolecithin (O——O) on the hysteresis loop of the 1,14 PC phase transition (broken line for pure 1,14 PC). Three mole percent diphenyl hexatriene (not shown) has the same effect as that shown for 5 mole% palmitic acid. The directions of the loops are indicated by the arrows.

lipids had occurred). As shown in Figs. 6 and 7, none of these conditions abolishes the hysteresis. In Fig. 6, the effects of dicetyl phosphate on the pretransition of dipalmitoyl lecithin and on the transition of 1,14 PC appear to be slightly different: the DPPC transition is diminished in the width of the hysteresis but is shifted very little in temperature while the 1,14 PC transition is shifted in temperature by about 6°C but the width of the hysteresis loop is not diminished. Lysolecithin is the only additive that we have found to diminish the width of the hysteresis in 1,14 PC (see Fig. 7).

An attempt was made to study the 1,14 PC transition in oriented bilayers. At 40°C, the value of ΔH_{pp} was 9.5 G when the magnetic field was perpendicular to the bilayer normal, and 11 G for the normal parallel to the field. Unfortunately, the phase diagram for various mixtures of 1,14 PC and water appears to be so altered at the low degrees of hydration used in oriented bilayers that no transition is observed between 15 and 40°C.

Sonication of 1,14 PC and gel filtration on Sepharose 4B produced some small vesicles, as in the case of other lipids (*11*), but the vesicle suspension was found to aggregate very rapidly (within an hour) so results of magnetic resonance experiments on this sample are rather ambiguous. However, based on measurements for this sample of heterogeneous vesicle size, sonication does not seem to diminish the width of the hysteresis but does shift it to lower temperatures.

ANALYSIS OF PARAMAGNETIC RESONANCE SPECTRA

For samples containing high concentrations of nitroxide spin labels, the complete spin Hamiltonian including electron–electron interactions is, in conventional notation,

$$\mathscr{H} = |\beta|\,\mathbf{S}\cdot\mathsf{g}\cdot\mathbf{H}_0 + h\mathbf{S}\cdot\mathsf{T}\cdot\mathbf{I} - |\beta_n|\,\mathbf{I}\cdot\mathsf{g}_N\cdot\mathbf{H}_0 + \mathscr{H}\,(\text{exchange}) + \mathscr{H}\,(\text{dipole}). \quad [1]$$

The nuclear Zeeman contribution (third term) is nearly negligible for X-band EPR spectra and has been taken as such in our calculations. Several analyses, in terms of [1], of the EPR spectra of nitroxide lipid molecules at high concentrations in membranes have appeared previously (*2, 4*). For some molecules, such as 1,14 PC, where the spectra are nearly isotropic, the observed spectra may be simulated quite well without including anisotropy in the g and T tensors. A more precise calculation (*4*) has included anisotropy for calculations of the spectra of 1,14 PC over a range of temperatures.

Here we report only calculations for 1,14 PC where isotropy is a reasonable approximation. Thus, the effect of the exchange interaction is calculated by solving the modified Bloch equations in the rotating frame and assuming that one-third of the molecular collisions are effective in producing spin exchange (i.e., 100% efficiency in spin exchange between molecules of both unlike nuclear and unlike electron spin). The influence of dipolar interaction, ΔH_d, though undoubtedly highly anisotropic at low temperatures, is treated in the present approximation as a single contribution to the three linewidths, ΔH.

$$\Delta H = \Delta H^\circ + \Delta H_d. \quad [2]$$

The linewidths for 1,14 PC in the absence of spin–spin interactions, ΔH°, are estimated from the spectra of 0.5% 1,14 PC in bilayers of an unlabeled lipid (1-palmitoyl-2-(16-methyl)stearoyl lecithin) above and below its phase transition. Table 1 lists the parameters which produced a good fit of computed lineshapes to those of experiments

TABLE 1

PARAMETERS FOR CALCULATION OF SPECTRA OF 1,14 PC[a]

Temperature of experiment (°C)	Linewidths (MHz)			a_H (MHz)[b]	W_{ex} (MHz)[b]	ΔH_d (MHz)[b]
	$I=+1$	$I=0$	$I=-1$			
35.9	4.5	4.4	8.7	37.7	17	0
					20[a]	4[a]
					1000	25
20.6	14.1	9.2	32.0	37.7	5	37.5

[a] Results of this set of parameters are shown in Fig. 8.

[b] a_H = separation between Lorentzian lines; W_{ex} = exchange frequency; H_d = dipolar contribution to linewidth.

with bilayers of pure 1,14 PC. Clearly the high-temperature spectra can be simulated equally well by a wide range of combinations of spin exchange and dipolar interactions. Figure 8 shows a comparison of experimental and calculated lineshapes for low and high concentrations of 1,14 PC in bilayers at ~36°C (just one of the possibilities for the high-concentration calculation is presented). Although the spin-exchange frequency for high-temperature 1,14 PC bilayers cannot be uniquely determined, a minimum possible value is 17 MHz (if the dipolar broadening is zero). For the spectrum at 20°C (below the phase transition), the calculated dipolar broadening of 37.5 MHz corresponds to a separation of nitroxide radicals of 9.2 Å if the labels are in a rigid, triangular lattice (*2*) and if there are no dipolar interactions between labels on opposite sides of the bilayers (an unproved assumption).

A second point must be considered in analyzing the spectra of 1,14 PC. The EPR spectra of liposomes of this lipid at temperatures below the phase transition are reduced in amplitude compared to those above the transition. The area under the absorption curve remains constant so that an increase in linewidth is accompanied by a decrease in amplitude. Therefore, at the transition temperature, the overall lineshape is dominated by the contribution from the fluid region of the 1,14 PC bilayers. Assuming that no intermediate state with appreciable lifetime occurs during the transition from one state to the other, a proper spectral parameter at an intermediate point in the transition should reflect the fraction of lipid bilayer in either of the two phases. A calibration curve for the transition in the spectra was obtained by adding the curves at 20.6·C (below the transition) and 35.9°C (above the transition) in varying proportions (based on the double integral of each curve). Figure 9 shows the variation in the apparent linewidth for the summation curves as a function of the fraction of the integrated intensity contributed by the spectrum of the high-temperature state. This is equivalent, for a two-state transition, to linewidth (ΔH_{pp}) vs the fraction of molecules in the high-temperature, or fluid, state (X_F).

From the foregoing, it is clear that the midpoint of the spectral transition plotted in Fig. 5 will occur at a lower temperature than the midpoint of the transition in the fraction of molecules in either the low- or the high-temperature state. Figure 10 shows the result of applying the correction factors from Fig. 9 to the plot of spectral data in

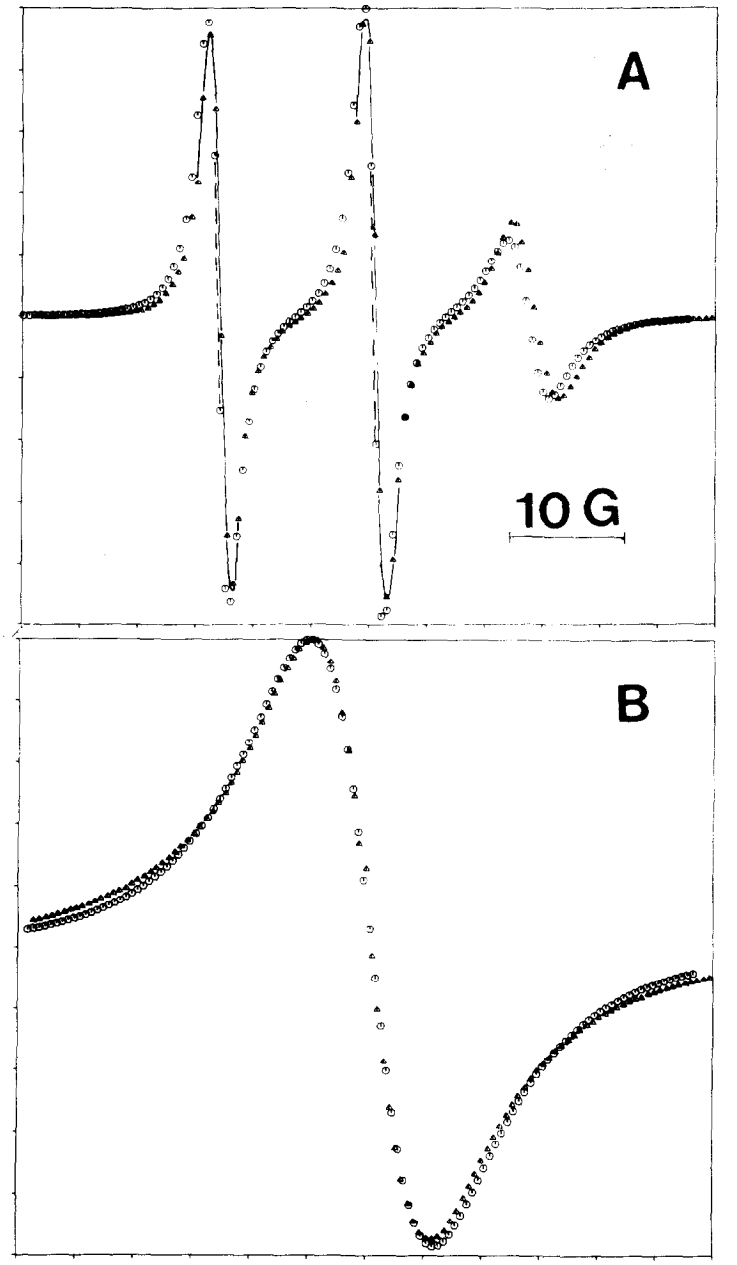

FIG. 8. Comparison of computer-simulated (O) and experimental (▲) spectra for 1,14 PC at two concentrations: (A) dilute, and (B) 100%. The parameters used in the calculation are given in Table 1. ($W_{ex} = 20$ MHz, $\Delta H_d = 4$ MHz.)

Fig. 5. The midpoints in the transition from the low-temperature to the high-temperature molecular state are at 27 and 34°C for the cooling and heating curves, respectively.

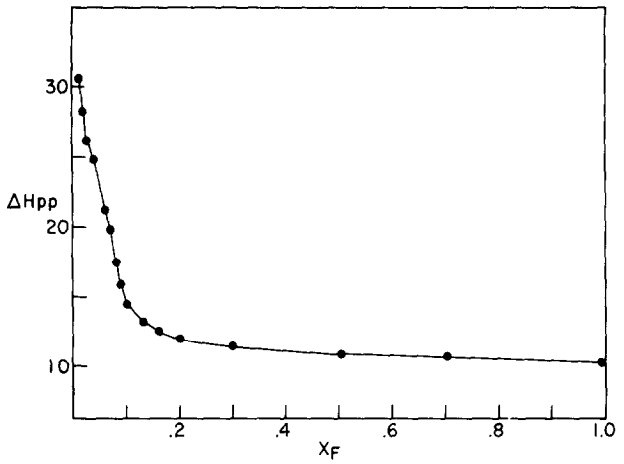

FIG. 9. The plot of peak-to-peak width vs X_F (the fraction of fluid phase), for various sums of high-temperature (35.9°C) and low-temperature (20.6°C) spectra for 1,14 PC.

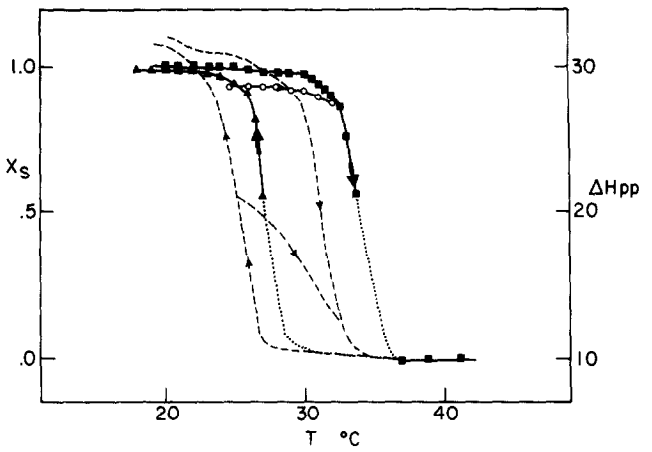

FIG. 10. The corrected transition curve for 1,14 PC from EPR data. The fractions of solid phase, X_s, at different temperatures are determined by the relationship of Fig. 9 (solid and dotted lines). For comparison, the uncorrected data of Fig. 5 is also shown (dashed lines).

DISCUSSION

In the high-temperature region (>35°C) of Figs. 2 and 3, linewidths of pure spin-labeled lipid bilayers decrease as the distance of the label group from the polar head group increases, undoubtedly another manifestation of the flexibility gradient (*12*) in lipid bilayers. Between 0 and 25°C, the linewidth of 1,14 PC is greater than that of 5,10 PC. This observation indicates that although 1,14 PC has passed through an "order–disorder" transition at the temperatures of the abrupt linewidth change, 5,10 PC is in the disordered, or liquid-crystalline, state at least at temperatures above 0°C. In addition, Fig. 3 provides evidence of a phase transition for 12,3 PC at ~30°C. Thus the variation in the transition temperatures of the *m,n* PC, with the position of the spin-labeled group (value of *n*) compares well with that observed in a series of lipids having one

unsaturated fatty acid with a double bond at different positions on the chain (*13*). The transition temperature for a lipid with a centrally located double bond is lower than that of a lipid with a double bond at either end of the hydrocarbon chain. These observations suggest that the transition temperature is to a large extent determined by the length of saturated chain segments that overlap to give stable close packing. This argument is also compatible with the fact that phospholipids with chain lengths of less than ten carbons do not form stable bilayers.

Earlier studies by Devaux and McConnell of interactions between 1,14 PC labels incorporated into egg lecithin–cholesterol (4 : 1) liposomes at 0.01–0.10 mole fractions led to an estimated rate of lateral diffusion in the range of 40–70°C (*3*). Collision frequencies estimated in that study were of the order of 10^7 sec^{-1}. In the experiments presented here, the lineshape for the 100% 1,14 PC bilayers at 40°C can be reasonably simulated by a spin-exchange frequency of 17 MHz (for zero dipolar contribution) or greater (varying dipolar contribution). This frequency is in the exchange-narrowing region where the linewidth should decrease with increasing temperature regardless of the precise combination of values of dipolar and exchange terms. In agreement with this expectation we find that between 40 and 75°C, the linewidth of 1,14 PC decreases from ~10.5 to 8 G. An exchange frequency of 17 MHz corresponds to a minimum frequency of molecular collisions of 3.2×10^8 sec^{-1}, which is clearly an order of magnitude higher than the frequency of motion involved in lateral diffusion. It thus appears that at the high concentrations of spin labels used in this study, collisions between adjacent molecules due to isomerization about carbon–carbon bonds are the predominant mechanism for spin exchange.

Hysteresis in phase transitions of lipid bilayers has previously been described for two cases: (1) in the pretransition of phosphatidyl cholines with identical fatty acid chains (*14*), and (2) in bilayers of negatively charged lipids (*15*). Because 1,14 PC has a net neutral charge in the head group, the hysteresis reported here would appear to be different from that reported for negatively charged lipids. It is possible, however, that the conformation of the phosphoryl choline head groups above and/or below the transition is such that the 1,14 PC bilayers do have a slight surface charge. The most obvious difference between the hysteresis in the transition of 1,14 PC and that in the pretransition of saturated phosphatidyl cholines is that the transition for the spin-labeled lipid is associated with the main order–disorder transition (based on our preliminary differential scanning calorimetry measurements with J. M. Sturtevant).

Each case of lipid transition with hysteresis must be associated with some barrier to attaining thermodynamic equilibrium. For the pretransition of saturated lecithins, a change in geometry from one solid phase to another (*16*) could easily be very slow. Slow transition from one solid phase to another has certainly been observed for mixtures of two substances which are partially miscible in the solid state (*17*). Transitions between phases of very different geometries may also be slow. The latter consideration leads to a possible explanation of the 1,14 PC hysteresis in the context of the known geometries of the saturated diacyl PC's. Figure 11 shows a hypothetical plot of chemical potential, μ, as a function of temperature. Each of the three curves on the plot is assigned to one of the three phases known for dipalmitoyl lecithin (DPPC) (*16*), L_α (a fluid phase), L'_β and P'_β (rigid phases of different geometry). For dipalmitoyl lecithin, the plot for the P'_β phase must cross that of the L'_β phase at a lower temperature

than that of the main transition point, T^*. The feature which distinguishes Fig. 11 from the diagram appropriate for DPPC is that the chemical potential of the P'_β phase is shifted above the intersection of the plots for the L_α and L'_β phases. At equilibrium, the P'_β phase would never be observed for Fig. 11. We suggest however, that the geometries of the phases (which may include bound water (*16*)) may be such that the transition from L_α to L'_β is very slow unless the P'_β phase intervenes. For the diagram of Fig. 11, this suggestion then leads to hysteresis in the main transition. It is of interest that the first 15% of the heating curve for 1,14 PC is reversible (Fig. 5). If the transitions occur as shown in Fig. 11, the reversibility may mean that the transition temperature of the

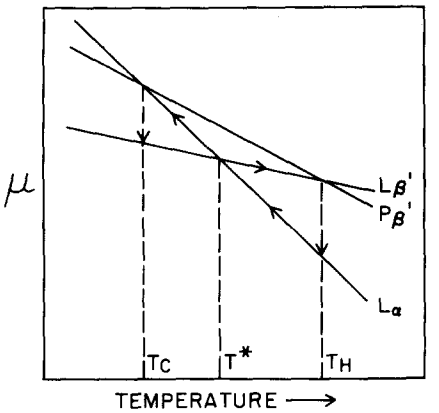

FIG. 11. A hypothetical plot of chemical potential (μ) vs temperature for a lipid which has two rigid, low-temperature states of different geometry (L'_β and P'_β) and one fluid, high-temperature phase (L_α). Term T^* is the normal order–disorder transition; T_H and T_C are the observed transitions for the heating and cooling branches, respectively, of the hysteresis loop.

heating curve (T_H) is slightly higher (due to superheating) than the intersection of the rigid L'_β- and P'_β-phase curves. If so, the hypothesis of Fig. 11 could be demonstrated by experiment if the presence of the P'_β phase could be detected by freeze-fracture (*18, 19*) or X-ray diffraction (*16, 19*). Of course, it is possible to describe the hysteresis phenomenon of 1,14 PC without including the P'_β phase (or some other specific phase) in Fig. 11 simply by invoking an energy barrier to the transition. An energy barrier due to charge repulsion in the phase transitions of negatively charged lipids has been proposed (*15*). The energy barrier for 1,14 PC is presumably steric. However, Fig. 11 has the added attraction for lipids with net zero charge in the head group of offering an explanation for the absence of a pretransition in mixed-chain phosphatidyl cholines and in phosphatidyl ethanolamines (*19*).

A study has been made to define the size and composition of domains in lipid monolayers containing high concentrations of a steroid spin label (*2*). Rigid phases where dipolar contributions to the lineshape predominate should be most amenable to this sort of analysis. The observation of phase transitions for 12,3 and 1,14 PC allows the choice of experimental conditions (e.g., temperature) to favor formation of rigid phases. The fact that one of the lipids, 5,10 PC, is in a fluid state throughout the temperature range may be useful for other types of experiment, where, for instance, the activity of an enzyme in a reconstituted membrane requires the presence of fluid lipids.

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