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Kinetics of Phase Equilibrium in a Binary Mixture of Phospholipids

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Abstract: A 50:50 mol % binary mixture of dipalmitoylphosphatidylcholine (DPPC) and dielaidoylphosphatidylcholine (DEPC) has been studied using ^{13}C nuclear magnetic resonance at 25.2 and 90.5 MHz. Each phospholipid was enriched in the choline methyl groups with ^{13}C. The line width of the ^{13}C resonance of the higher melting lipid (DPPC) in this binary mixture increases rapidly at temperatures below ~32 °C, the same temperature as determined earlier by spin label paramagnetic resonance and by freeze-fracture electron microscopy to mark the onset of a lateral phase separation in the plane of the membrane. The temperature dependence of the observed ^{13}C line widths differs quantitatively but not qualitatively from theoretically calculated line widths based on the previously reported phase diagram for this mixture of lipids. The discrepancy may be due to density and composition fluctuations (nucleation) in the fluid phase of the lipids. Such fluctuations are suspected to be of importance for the transport of certain molecules through cell membranes.

A number of membrane-related biological functions are strongly correlated with the physical properties of the membrane lipids. These functions include active transport,[1] enzymatic activity,[2] membrane assembly,[3] and complement-mediated immune attack.[4] Some of these correlations are relatively trivial; when all the membrane lipids are frozen or highly viscous, transport is inhibited or stopped. Other correlations are certainly not trivial, and their explanation should lead to a better understanding of the biophysical events associated with membrane function. A change in the uptake of β-galactosides and β-glucosides into cells of *Escherichia coli* associated with the *onset* of lateral phase separations in the plane of the membrane is one example, as discussed later.[5]

All of the membrane functions enumerated above are kinetic processes, and it is therefore of interest to investigate the kinetic properties of lipids, and lipid mixtures. In this paper we (a) describe the ^{13}C nuclear magnetic resonance spectra of a binary mixture of two phospholipids, dielaidoylphosphatidylcholine (DEPC) and dipalmitoylphosphatidylcholine (DPPC), in which the two lipids are enriched with ^{13}C in the choline methyl groups, (b) give a theoretical calculation of resonance line widths expected for a two-phase bilayer system in which the lipids undergo rapid lateral diffusion, and (c) show that the experimental and theoretical results indicate an unexpectedly rapid phase equilibration in this system, which may be due to rapid density and composition fluctuations (nucleation) in the fluid lipid phase. This study is relevant to the effects of phase separations on membrane functions, such as transport.[1]

Experimental Section

Reagents. The following materials were obtained from commercial sources and used without further purification: 2-aminoethanol (Sigma), dipalmitoylphosphatidylcholine (DPPC), dipalmitoylphosphatidylethanolamine (DPPE) (Calbiochem), 90% ^{13}C-enriched methyl iodide (Merck), and elaidic acid (Analabs).

^{13}C Choline Enriched Dipalmitoylphosphatidylcholine (DPPC*). DPPE was methylated under basic conditions in freshly distilled methanol at 45 °C. The methylation was followed by thin-layer chromatography and the reaction usually stopped after 4 h. The various amines were separated by silica plate chromatography (Analtech) or silica gel column chromatography (Bio Sil-A, 200–325 mesh), eluted with a chloroform–methanol gradient. The product showed the same R_f as unlabeled DPPC on silica gel thin-layer chromatography, developed in chloroform:methanol:water 65:35:4, and stained with sulfuric acid. The concentration of the solution was measured by method of McClare.[6] The product was identified using ^1H and ^{13}C nuclear resonance, and thin-layer chromatography. An aqueous dispersion of the lipid showed the same transition temperature by the spin label TEMPO method (42 °C) as did the unlabeled material.

Dielaidoylphosphatidylcholine (DEPC). DEPC was prepared by the method of Cubero Robles and Van den Berg[7] from *o*-(*syn*-glycero-3-phosphoryl)choline (available as the $CdCl_2$ adduct from Sigma) and the sodium salt of elaidic acid. The product was purified by silicic acid column chromatography as for DPPC* above.

^{13}C Enriched Choline. This was prepared by direct methylation of ethanolamine under basic conditions, and was partially purified using an ion exchange column (Bio-Rad, AG-50W-x8, NH_4^+ form). Thin-layer chromatography was performed on cellulose plates (Analtech) and developed in propanol–NH_4OH(14.8 M)–H_2O, 6:3:1. The spots were visualized in an iodine chamber.

Phosphatidic Acid of DEPC. This phosphatidic acid was prepared by the action of phospholipase D, freshly extracted from cabbage according to Davidson and Long.[8] The purification followed the method of Kornberg and McConnell.[9]

^{13}C Enriched Dielaidoylphosphatidylcholine (DEPC*). The condensation of the phosphatidic acid with the ^{13}C enriched choline was carried out in freshly distilled pyridine and catalyzed by

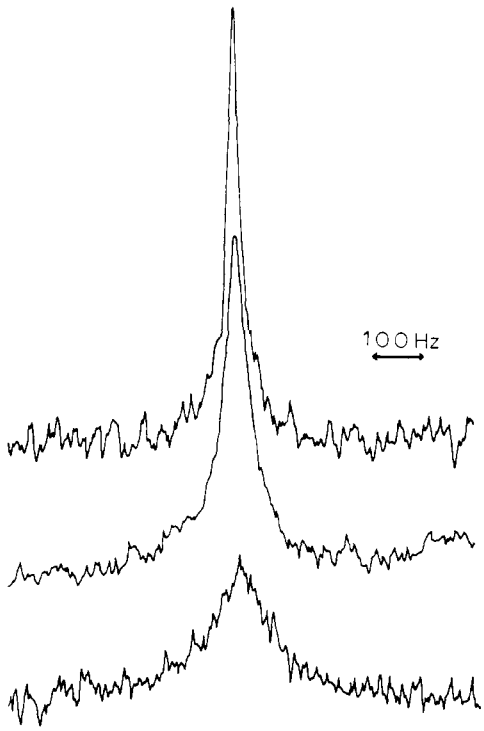

Figure 1. ^{13}C nuclear magnetic resonance signal from ^{13}C-enriched choline methyl groups in dipalmitoylphosphatidylcholine at 33, 23, and 13 °C (upper, middle, and lower spectra). Spectra were taken at 25.2 MHz using different samples, each containing 5 mg of enriched dipalmitoylphosphatidylcholine, and 5 mg of nonenriched dielaidoylphosphatidylcholine.

2,4,6-triisopropylbenzylsulfonyl chloride.[9,10] The choline was previously dried by distillation of the pyridine from the reaction vessel. Purification was accomplished by column chromatography. The various lipids were stored as stock solutions in methanol at −20 °C. They showed only one spot by thin-layer chromatography.

Sample Preparation. Lipids were mixed in a chloroform–methanol solution and coated on the inside of a flask by removal of the solvent under vacuum. The concentrations of the various solutions were checked prior to an experiment by the method of McClare.[6] Usually 5 mg of a ^{13}C-labeled lipid was mixed with the other unlabeled lipid so as to made a 50:50 molar ratio. After overnight drying on a vacuum line, the mixture was dispersed in 300 μl of a weakly buffered D_2O solution, pH 6.6, and put into a 5-mm NMR tube under a nitrogen atmosphere. All experiments were performed on unsonicated dispersions. After a nuclear magnetic resonance experiment, the binding of the spin label TEMPO (2,2,6,6-tetramethylpiperidin-1-oxyl) was determined as a function of the temperature by first drying out the lipids, adding TEMPO, and then monitoring the electron paramagnetic resonance spectrum. See Shimshick and McConnell for a description of this method.[11]

Measurements. The EPR measurements were performed on a Varian E-12 Spectrometer operating at X band. The temperature was measured with a copper–constantan thermocouple, connected to a microvoltmeter. The nuclear magnetic experiments were performed at two different frequencies, at 25.2 MHz using a Varian XL-100 interfaced with a Varian 620i Computer, and at 90.5 MHz using a superconducting Bruker 360 interfaced with a Nicolet 1080 Computer. Both spectrometers were operated in the pulse mode. Temperature was measured with a copper–constantan thermocouple connected to a potentiometer. Free-induction decays were accumulated for 15 to 30 min and multiplied by a smoothing exponential for signal-to-noise improvement. Usually 4K of memory was used to give a 2K frequency spectrum. Protons were decoupled using 5 to 10 W of power.

^{13}C Resonance Spectra. Resonance spectra observed at 25.2 MHz are illustrated in Figure 1. Resonance spectra obtained at 90.5 MHz are similar, except that they are broader and show somewhat anisotropic line shapes at the lower temperatures where

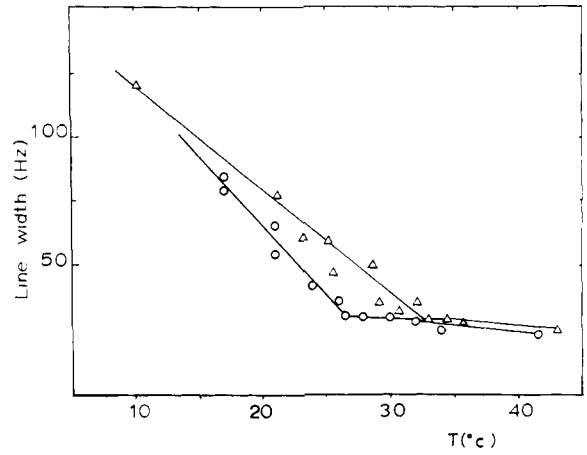

Figure 2. ^{13}C nuclear resonance line widths of ^{13}C-enriched choline methyl groups in dipalmitoylphosphatidylcholine (△) and dielaidoylphosphatidylcholine (○) at 25.2 MHz. Spectra were obtained from 50:50 mol % binary mixtures in which only one of the component lipids was enriched with ^{13}C. Line widths are measured at half the peak height.

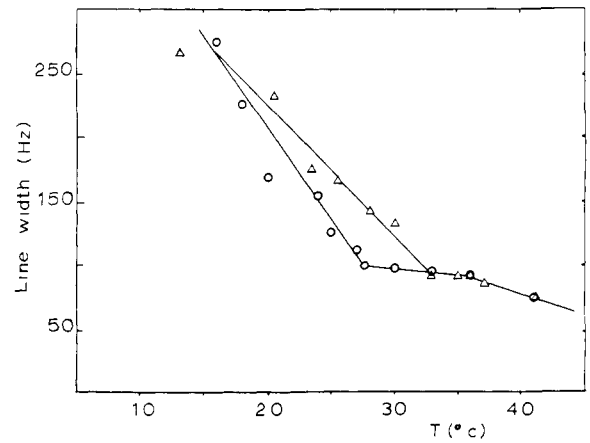

Figure 3. ^{13}C nuclear resonance line widths of ^{13}C-enriched choline methyl groups in dipalmitoylphosphatidylcholine (△) and dielaidoylphosphatidylcholine (○) at 90.5 MHz. Spectra were obtained from a 50:50 mol % binary mixture in which only one of the component lipids was enriched with ^{13}C. Line widths are measured at half the peak height.

the proportion of solid phase lipids is higher. At the higher field there is a small shift of the resonance spectrum to higher fields, of the order of 30 Hz on lowering the temperature from 32 to 15 °C. Observed line widths (line width, at half the peak height) are given in Figures 2 and 3 for spectra recorded at 25.2 and 90.5 MHz. The line widths of DPPC* broaden rapidly at ~32 °C, the temperature corresponding to the onset of the lateral phase separation in a 50:50 mol % mixture of DEPC and DPPC (see later). At all temperatures below 32 °C, the line widths of DPPC* are always greater than those of DEPC*, corresponding to the preferential inclusion of DPPC* in the solid phase lipids. The line widths of DPPC* and DEPC* become essentially equal to one another as the temperatures approach ~12 °C, corresponding to the completion of the lateral phase separation.

The DEPC–DPPC Phase Diagram and Domain Structure. The phase diagram representing lateral phase separations in binary mixtures of DEPC and DPPC reported by Wu and McConnell[12] is given in Figure 4. The phase diagram was determined by the spin-label TEMPO binding method, discussed extensively elsewhere.[11,12] Previous studies of this phase diagram with freeze-fracture electron microscopy[13] have an important bearing on the present work, and are briefly summarized here.

For a number of binary mixtures of phospholipids, including DEPC–DPPC, rapid temperature quenching (~100–1000 °C/s) followed by freeze-fracture allows electron microscopic visualiza-

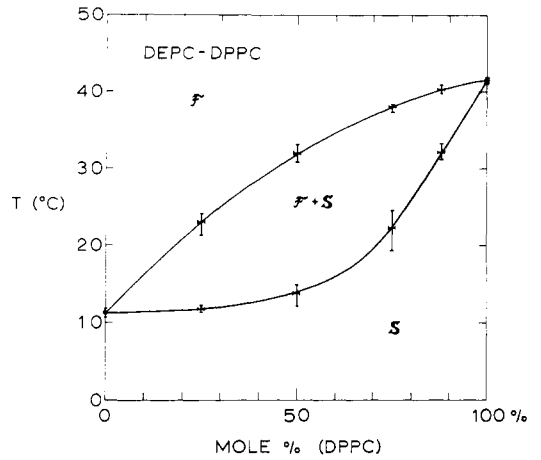

Figure 4. The phase diagram for binary mixtures of dipalmitoylphosphatidylcholine (DPPC) and dielaidoylphosphatidylcholine (DEPC). The symbols F and S represent "fluid" and "solid" temperature-composition regions, and F + S represents a region of lateral phase separation in the plane of the domain where fluid and solid phases coexist. The phase diagram is taken from Wu and McConnell.[12]

tion of coexisting fluid and solid (crystalline) domains in the plane of the membrane. Solid domains are recognized by regular band patterns, and fluid domains are recognized by the total absence of these bands, or by a very irregular band pattern.[13] Fluid domains can also be recognized by the inclusion of low concentrations of membrane proteins that are preferentially soluble in the fluid domains.[14,15] Grant, Wu, and McConnell[13] have carried out freeze-fracture studies of DEPC-DPPC mixtures, so as to verify the phase diagram shown in Figure 4. As expected, samples containing 50:50 mol % DPPC and DEPC that were quenched from 36, 30, 17, and 10 °C showed all fluid, mostly fluid and some solid, mostly solid and some fluid, and all solid domains, respectively. Experiments such as these verify the interpretation of this phase diagram as representing lateral phase separations and, at least semiquantitatively, the location of the solidus and fluidus curves.

A crucial question for the present work is whether or not the *solidus* curve represents a phase of composition equilibrium, especially in view of the fact that nonequilibrium conditions tend to "bow" apparent solidus curves to lower temperatures.[16] Evidence for composition equilibrium is provided by the quenching experiment at 25 °C where a statistical survey of the freeze-fracture replicas shows roughly equal areas of fluid and solid. This result, coupled with the fact that the spin-label studies showed complete temperature reversibility and no time dependence, gives us confidence that the solidus curve does represent an equilibrium composition.

The present studies employ liposomes, large extended multilamellar phospholipid bilayers. In these systems the solid and fluid domain size are large, of the order of microns. In vesicles of DEPC-DPPC having diameters of microns, there are relatively few domain boundaries, indicating that the boundary free energy is large enough that the solid phase does not tend to break up into a large number of separate, macroscopic solid phase domains.

Simulation of Resonance Spectra. In order to simulate the resonance spectra, we employ a relatively crude model calculation, and then later discuss how the various approximations employed are, or are not, significant for the interpretation of the observed resonance spectra. The essential idea is that the ^{13}C resonance spectra in fluid phase lipids are relatively sharp, and the ^{13}C resonance spectra in solid phase lipids are relatively broad. When both fluid and solid phase lipids coexist in equilibrium, we must take into account the possibility that any given ^{13}C nucleus may spend part of its time in the fluid phase, and part of its time in the solid phase. The present calculation differs from the usual treatment of the effects of chemical exchange on nuclear resonance spectra in one important respect. For typical sizes of solid and fluid domains revealed by freeze-fracture electron microscopy ($\sim 1\ \mu$) some ^{13}C nuclei in a fluid domain are able to diffuse to a solid domain sufficiently rapidly so as to affect their resonance line shapes, but other ^{13}C nuclei are too far away from the solid domains for their resonance spectra to be affected. Similar considerations apply to ^{13}C nuclei in the solid phase domains.

We utilize a simple rectangular membrane having a length L and an infinite width. The membrane is divided into two domains, a solid domain S and a fluid domain F. This picture is similar to DPPC-DEPC domains seen in freeze-fracture in that the domain boundary is linear, and the distance L is of the order of microns.[13-15] Although this picture does not faithfully represent all details of domains seen in freeze-fracture it is less restrictive than it would appear at first glance, as discussed later.

The membrane is also divided into N zones of length L/N. At temperatures where solid and fluid domains coexist, zones 1, 2, 3, . . . , k are solid, and zones $k + 1$, $k + 2$, . . . , N are fluid. The value of k is determined from the phase diagram in Figure 1 for each temperature. We consider a 90° pulse experiment such that at time $t = 0$ the applied radiofrequency field is turned off, and the entire ^{13}C nuclear magnetization v is perpendicular to the direction of the strong applied field $\mathbf{H}_0$. The nuclear magnetization in each zone i, v_i, is assumed to follow the Bloch equations, modified to include the transfer of ^{13}C magnetization between zones, by lateral diffusion, and by adsorption and desorption between F and S domains, at the domain boundary. The component of magnetization v_i changes in time due to (a) its intrinsic decay rate, $v_i/T_2(i)$, (b) transfer of magnetization out of the ith zone due to diffusion $-\pi_{i\to i+1}v_i - \pi_{i\to i-1}v_i$, and (c) transfer of magnetization into the ith zone due to diffusion from neighboring zones, $\pi_{i+1\to i}v_{i+1} + \pi_{i-1\to i}v_{i-1}$.

After the $\pi/2$ pulse, the equation of motion of $v_i(t)$ is,

$$\dot{v}_i(t) = v_i/T_2(i) - (\pi_{i\to i+1} + \pi_{i\to i-1})v_i + \pi_{i+1\to i}v_{i+1} + \pi_{i-1\to i}v_{i-1} \quad (1)$$

All the $T_2(i)$ in the fluid domain are set equal to T_{2F} which in turn is set equal to $1/\pi\Delta\nu_F$, where $\Delta\nu_F$ is the half-width at half-height of the ^{13}C nuclear resonance absorption in the fluid phase. A similar approximation is used for obtaining T_{2S} appropriate for the solid phase.

If the kth zone is solid and borders the fluid phase then $\pi_{k\to k-1}$ represents lateral diffusion in the solid phase, and $\pi_{k\to k+1}$ represents desorption from the solid phase to the fluid phase. Similarly, $\pi_{k+1\to k+2}$ represents diffusion in the fluid phase, and $\pi_{k+1\to k}$ represents absorption onto the solid from the fluid.

The equations of motion for the $v_i(t)$ can be written in matrix form,

$$\dot{v} = \mathbf{A}v \quad (2)$$

where $\mathbf{A}$ is the matrix of coefficients.

$$\mathbf{A} = \begin{bmatrix} \left(\frac{1}{T_{2S}} + \pi_S\right) & -\pi_S & & & \\ -\pi_S & \left(\frac{1}{T_{2S}} + 2\pi_S\right) & -\pi_S & & \\ & \ddots & & & \\ & & -\pi_F & \left(\frac{1}{T_{2F}} + 2\pi_F\right) & -\pi_F \\ & & & -\pi_F & \left(\frac{1}{T_{2F}} + \pi_F\right) \end{bmatrix} \quad (3)$$

Let Λ and $\mathbf{U}$ be the matrix of eigenvalues and associated eigenvectors of the matrix $\mathbf{A}$.

$$\mathbf{U}^{-1}\mathbf{A}\mathbf{U}^{+1} = \Lambda \quad (4)$$

The solution of (2) is, in matrix form,

$$v(t) = \mathbf{U}^{+1}[\exp(-\Lambda t)]\mathbf{U}^{-1}v(0) \quad (5)$$

In our experiment, the recorded signal is the Fourier transform of the free-induction decay. Thus,

$$\text{signal}\ (\nu) = F\mathbf{U}^{+1}\left[\frac{|\lambda|}{\lambda^2 + 4\pi^2\nu^2}\right]\mathbf{U}^{-1}v(0) \quad (6)$$

$$F = (1, 1, 1, \ldots, 1) \quad (7)$$

In eq 5 and 6 $[\exp(-\Lambda t)]$ and $[\lambda/(\lambda^2 + 4\pi^2\nu^2)]$ represent diagonal matrices, the elements of which are $\exp(-\lambda t)$ and $\lambda/(\lambda^2 + 4\pi^2\nu^2)$; the λ are the diagonal elements of Λ. The frequency ν (in Hz) is the frequency from the center of the resonance. The collected sig-

nal is then the sum of N Lorentzian signals, corresponding to the N "normal modes of diffusion".

In the present calculation, the relative proportions of solid and fluid domains are determined by the temperature, according to the phase diagram in Figure 4, for a 50:50 mole ratio of DPPC and DEPC. The number of fluid (or solid) zones is proportional to the relative proportion of fluid (or solid) in equilibrium. At the boundary between the fluid and solid, equilibrium requires

$$X_S\pi_{S\to F} = X_F\pi_{F\to S} \tag{8}$$

where X_S and X_F are the mole fractions of ^{13}C present either as labeled DPPC or DPPE in the S and F domains, as given by the phase diagram.

We estimate the fluid phase transition probabilities π_F to be approximately t^{-1}, where t is the time required for a ^{13}C labeled molecule to move a distance (L/N), by random diffusion.

$$\pi_F = t^{-1} = 2D_F(N/L)^2 \tag{9}$$

Here D_F is the previously determined diffusion coefficient for random, two-dimensional diffusion; $D_F \sim 10^{-8}$ cm^2/s.[17,18] The temperature dependence of D_F is neglected, since previous experiments suggest that this temperature dependence is not large for the temperature range of interest here.[19] There is no experimental information available for lateral diffusion coefficients for solid phase liquids, D_S.

Resonance spectra have been calculated using a PDP8 computer. Calculated spectra depend on the parameters $T_2(F)$, $T_2(S)$, π_S, π_F, $\pi_{S\to F}$, $\pi_{F\to S}$, and L. As indicated earlier $T_2(F)$ and $T_2(S)$ are obtained from experimental line widths. L is required to be of the order of a micron, to within a factor of 2. The order of magnitude of π_F is set by the experimental value of D_F and the assumed value of L, according to eq 9. The relative values of $\pi_{S\to F}$ and $\pi_{F\to S}$ are determined by equilibrium considerations (eq 8). It is very unlikely that $\pi_{F\to S}$ can exceed π_F. Nothing is known concerning the magnitude of π_S, except that it is very likely that $\pi_S \ll \pi_F$.

We have calculated a large number of resonance spectra, limited only by the restraints listed above. Figure 5 gives the results of an illustrative calculation, using $\pi_F = 3000$ s^{-1}, $\pi_S = 30$ s^{-1}, $\pi_{F\to S} = 300$ s^{-1}, and $L = 10^{-4}$ cm. It will be seen that the calculated line widths for ^{13}C-labeled DPPC and DEPC are in *qualitative* accord with the observed results. For example, the observed and calculated line widths of DPPC* exceed those of DEPC*, due to the preferential incorporation of DPPC* in the solid phase. However, there is evidently a significant quantitative discrepancy. Experimentally, the resonance spectrum of DPPC* "senses" the onset of the phase separation much more clearly than is indicated by the theoretical calculation. This discrepancy is even more pronounced in the case of DEPC*. The discrepancy between the calculated and observed line broadening for DPPC* can be removed if the diffusion constant in the fluid phase is increased by a factor of 10; this does not remove a significant discrepancy between observed and calculated line broadening for DEPC*.

Approximations Used for the Calculated Spectra. The calculations leading to the results given in Figure 5 clearly underestimate the observed line broadenings given in Figures 2 and 3, particularly in the temperature region at the *beginning* of the lateral phase separation. In this section we consider briefly the question as to whether this discrepancy might arise from inadequate approximations used in the theory, especially the possible improper choice of values for the various numerical parameters.

(a) Line Width Parameters. There is as yet no quantitative theory for the ^{13}C line widths observed in either the fluid phase lipids or the solid phase lipids. We have attempted to include the line width effects in our model calculation by equating the observed line widths in the pure fluid phase, or the solid phase, to the Lorentzian line width parameters, $1/\pi T_{2F}$ and $1/\pi T_{2S}$. The largest possible source of error introduced by this approximation arises from inhomogeneous contributions to the line widths, from chemical shift anisotropies, as well as from macroscopic shielding effects. In the former case, different orientations of the bilayer planes relative to the applied field direction must give different resonance line positions, and thus inhomogeneous line shapes. In principle inhomogeneous contributions to the line widths can also arise from diamagnetic screening effects in multilamellar phospholipid vesicles (macroscopic shielding effect). Both contributions to the line widths are field dependent. The observed line widths are field dependent although not so strongly as to be proportional to the field strength. The line shapes are also somewhat anisotropic, especially when the proportion of solid phase lipids is large. In principle all of these effects could be included in the model calculation. The contribution of chemical shift anisotropies to calculated line widths should be similar in magnitude and in their temperature dependence to those calculated in the preceding section; in particular it is safe to assume that the inclusion of a chemical shift anisotropy in the calculation would also lead to line broadenings similar to those seen in Figure 5. The macroscopic shielding effects provide a mechanism whereby the onset of solid phase formation in one region of the membrane could affect ^{13}C line widths in the fluid phases, at considerable distances. However, near the onset of the phase separation such effects should act equally on ^{13}C nuclei in DPPC* and DEPC*, and this is evidently not the case. We conclude that inhomogeneous contributions to the line widths do not account for the quantitative discrepancy between the model calculations and the experimental results at least in the temperature region of the onset of the lateral phase separation. On the other hand, we cannot rule out the possibility that macroscopic shielding effects make a significant contribution to the "premature" broadening of the DEPC* resonance (at ~25 °C), since at this temperature a relatively large proportion (~50%) of the lipids are in solid phase domains.

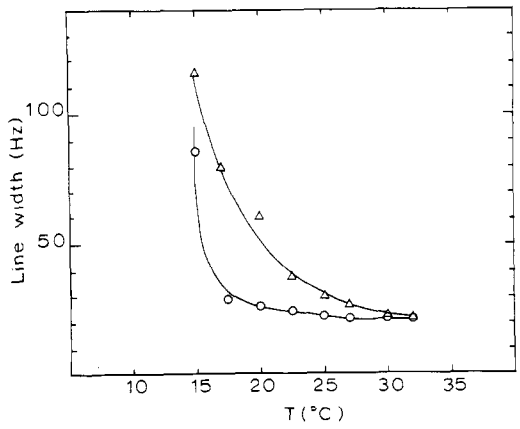

Figure 5. Theoretically calculated line widths for the ^{13}C-choline nuclear magnetic resonance signals for dipalmitoylphosphatidylcholine (△) and dielaidoylphosphatidylcholine (○) in a 50 mol % binary mixture of the two lipids. Calculation appropriate for a ^{13}C resonance frequency of 25.2 MHz. See text for details.

(b) Domain Structure. The domain size used for our calculations has been observed in many preparations of DEPC and DPPC, as well as in other binary mixtures of phosphatidylcholines. Thus it is unlikely that this is a source of the discrepancy. It is entirely possible that freeze-fracture electron microscopy does not reveal small regions of solid phase lipids that are present in the fluid domains. Such domains could then be the nucleation centers referred to in the following Discussion.

(c) The Diffusion Constant. It was pointed out in the preceding section that increasing the lateral diffusion constant and adsorption rates for lipids by a factor of 10 over that reported previously largely removes the discrepancy between observed and calculated line width for DPPC*. More recent studies of the lateral diffusion constant using spin-label enhanced nuclear relaxation are also consistent with the previously reported diffusion constant ($D \sim 2 \times 10^{-8}$ s^{-1}) but do not conclusively rule out a diffusion constant a factor of 10 larger.[20] Thus, it is conceivable, but unlikely, that the larger diffusion constant is accurate.

Discussion

The overall features of the ^{13}C nuclear resonance spectra reported in the present work are in accord with previous studies of lateral phase separations in binary mixtures of DEPC and DPPC, using spin labels and freeze-fracture electron microscopy. Specifically, the temperature measured for the onset of the phase separation (~32 °C) agrees with the earlier work.[12,13] Also, as expected,[12] DPPC is

clearly preferentially incorporated into the solid phase as the temperature is lowered below 32 °C. Perhaps the most interesting feature of the present results is the quantitative discrepancy between the observed and calculated temperature dependence of the ^{13}C line broadening. In essence, the observed DPPC* resonance senses the onset of the lateral phase separation more strongly than expected on the basis of the theoretical calculations. The calculation assumes that ^{13}C nuclei in fluid domains are only broadened by lateral diffusion to solid domains. The calculated line broadening at the onset of the phase separation is small since the typical distance a molecule must diffuse ($\sim 1\ \mu$) is too far for this motion to be an efficient broadening mechanism for all ^{13}C nuclei in fluid domains.

A possible source of this "early" line broadening is nucleation throughout the fluid phase. If this process is sufficiently rapid, and extensive, then a large proportion of ^{13}C nuclei in the fluid phase may sense a relatively rigid environment, for a short period of time, without the need for long-range lateral diffusion. Unfortunately, it is difficult to give any theoretical estimate of the nucleation rate, since it depends critically on the number of molecules required for a nucleation center. Random composition fluctuations of the magnitude of those related by tie lines connecting the fluidus and solidus curves are very probable for groups of molecules of the order of ten, and become rapidly less probable for larger groups of molecules. Freeze-fracture electron microphotographic studies of lipid mixtures such as DEPC and DPPC as yet show no evidence of such nucleation centers; however, when membrane proteins are included in these mixtures one often observes patterns of solid phase crystallinity that indicate that such proteins can serve as nucleation centers for the growth of large solid phase domains (W. Kleemann and H. M. McConnell, unpublished).

In previous work, studies have been made of the temperature dependence of the active uptake of β-galactosides and β-glucosides into *E. coli* cells having simple fatty acid compositions.[1] In one case (E. coli 30Eβox$^-$ grown on elaidic acid) there is a virtually discontinuous marked *increase* in the transport rate with *decreasing* temperature, at the same temperature shown by the spin-label TEMPO method to mark the onset of a lateral phase separation.[1] It has been suggested that density fluctuations (nucleation) in the immediate vicinity of the transport proteins may be responsible for this enhancement of transport.[1] The possible density fluctuations reported in the present work fall in a frequency domain that is consistent with this earlier proposal, and the present study provides the only physical evidence yet available for such fluctuations.

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π-Spin Distribution in the Radical Anions of [2.2]Paracyclophane and Some of Its Symmetric Benzo Derivatives

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Abstract: The radical anions of [2.2]paracyclophane (**1**), *syn*- and *anti*-[2.2](1,4)naphthalenophanes (**2** and **3**, respectively), and [2.2](9,10)anthracenophane (**4**) have been studied by ESR and ENDOR spectroscopy. The results confirm the expectation that the spin distribution between the two equivalent π systems is governed by the association of the radical anion with its positively charged counterion. Thus, a non-uniform spin distribution is favored by decreasing solvation of the cation and by an increasing π-charge localization in the sequence $\mathbf{4}\cdot^- < \mathbf{2}\cdot^- \approx \mathbf{3}\cdot^- < \mathbf{1}\cdot^-$.

We would like to report on ESR and ENDOR studies of the radical anions of [2.2]paracyclophane (**1**), *anti*- and *syn*-[2.2](1,4)naphthalenophanes (**2** and **3**, respectively), and [2.2](9,10)anthracenophane (**4**). Table I lists the pertinent coupling constants observed with three different solvents: 1,2-dimethoxyethane (DME), tetrahydrofuran (THF), and 2-methyltetrahydrofuran (MTHF); the counterion, K^+, and the temperature, −90 °C, are the same