Solid-State NMR Studies of the Molecular Dynamics and Phase Behavior of Mixed-Chain Phosphatidylcholines†

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ABSTRACT: Solid-state ^{13}C, ^{31}P, and ^{2}H NMR experiments have been performed as a function of temperature on aqueous dispersions of two mixed-chain phosphatidylcholines, 1-myristoyl-2-palmitoylphosphatidylcholine (MPPC) and 1-myristoyl-2-stearoylphosphatidylcholine (MSPC), and of dipalmitoyl-PC (DPPC). The NMR spectra show that MPPC and MSPC differ from DPPC in their phase behavior in that they have no stable $L_{\beta'}$ phase. Instead, the results for these mixed-chain species show a transition directly from the subphase (L_c phase) to the $P_{\beta'}$ phase. For MSPC the ^{13}C spectra also show that the conformation at the *sn*-2 carbonyl group in the $P_{\beta'}$ and L_α phases differs from that of MPPC, DPPC, and other diacyl lipids previously examined. At low temperatures the full asymmetric ("rigid lattice") tensor is observed in the *sn*-2 $^{13}C{=}O$ spectra of all three lipids and in the ^{31}P spectra of MPPC and MSPC at temperatures above 0 °C when the samples have been stored at lower temperatures for sufficient periods. Thus, motion at the *sn*-2 carbonyl and, for MPPC and MSPC, of the phosphate group as well is in the slow limit on the NMR time scale. At higher temperatures the *sn*-2 $^{13}C{=}O$ spectra of both MPPC and MSPC show a superposition of two components as seen before in the $P_{\beta'}$ phase of diacyl PCs. The rigid component disappears only after the appearance of the narrow, isotropic component, indicating that these lipids go directly from the subphase to the $P_{\beta'}$ phase, with no intermediate $L_{\beta'}$ phase. In contrast the ^{13}C spectra of DPPC contain an intermediate region with only the broad, axially symmetric line shape observed before in the $L_{\beta'}$ phase of diacyl PCs and DPPE. Finally, the ^{2}H spectra of 2-[12,12-$^{2}H_2$]-MPPC show substantial motion even at 2 °C after annealing at low temperature. This motion presumably results from trans–gauche isomerization along the acyl chain, since overall molecular motion is ruled out by the ^{13}C and ^{31}P spectra.

Although most phospholipids whose phase behavior has been investigated are species with two identical acyl chains, the phospholipids found in most biological membranes are heterogeneous in their acyl chain composition. To approach a better understanding of natural membranes, we have undertaken solid-state NMR[1] studies of two mixed-chain phospholipids, 1-myristoyl-2-palmitoylphosphatidylcholine (MPPC) and 1-myristoyl-2-stearoylphosphatidylcholine (MSPC), with the aim of characterizing their phase behavior and obtaining a picture of the dynamic structure of these molecules.

As will be seen below, the phase behavior of both MPPC and MSPC differs from that of the well-studied diacylphosphatidylcholine DPPC, although they do share some common properties. Until recently, DPPC was believed to have three stable phases, $L_{\beta'}$ ("gel"), $P_{\beta'}$ ("ripple"), and L_α ("liquid crystalline"), and DSC studies showed two calorimetric transitions, the pretransition ($L_{\beta'} \rightarrow P_{\beta'}$) and the main transition ($P_{\beta'} \rightarrow L_\alpha$). Recently several groups have described a fourth, more highly ordered, phase in DPPC which forms upon annealing for long periods (tens of hours) at temperatures around 0 °C (Chen et al., 1980; Füldner, 1981; Ruocco & Shipley, 1982a). We will refer to this phase as the subphase, although it has also been called L_c (Ruocco & Shipley, 1982a), L_ϵ (Stümpel et al., 1981), and the subgel phase (Nagle & Wilkinson, 1982). The kinetics of formation of the subphase are highly dependent on the temperature of annealing, and hysteresis is often observed. Furthermore, the transition from this phase to $L_{\beta'}$ is commonly called the subtransition.

The earliest DSC investigations of MPPC and MSPC (Keough & Davis, 1979; Chen & Sturtevant, 1981; Stümpel et al., 1981) showed only two and one transitions, respectively, suggesting that these lipids have fewer phases than DPPC. However, more recent studies (Stümpel et al., 1983; this work) do show a second transition in MSPC after incubating at low temperature for at least 1 h. The requirement for low-temperature annealing suggests that this second transition in MSPC is a subtransition. The higher temperature transition is the usual main ($P_{\beta'} \rightarrow L_\alpha$) transition.

In the present work, ^{13}C, ^{31}P, and ^{2}H NMR have been used to examine the dynamic structure of MPPC and MSPC as a function of temperature. ^{13}C NMR is useful for studying lateral diffusion in bilayers and motion of lipid molecules about their long axes (Wittebort et al., 1981, 1982). The present ^{13}C results show that in some phases there is little or no motion at low temperatures, whereas at high temperatures both long axis and lateral diffusion are present. ^{31}P NMR is a convenient probe for examining head group motions, and we have found ^{31}P spectra characteristic of rigid and perhaps anhydrous head groups after sufficiently long annealing times (near 0 °C). Finally, the ^{2}H spectra of MPPC indicate the presence of substantial motion in the acyl chains at low temperatures, even when the ^{13}C and ^{31}P spectra show no axial or head group motion. The ^{2}H spectra resemble those observed in glycolipids by Huang et al. (1980), again under conditions of no axial motion.

Experimental Procedures

The synthesis of mixed acid glycerides and phospholipids requires some care because of the possibility of chain migration during acylation of the glycerol moiety. This type of rearrangement has been known to lipid chemists for a long time, and under equilibrium conditions the mixture consists of

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[1] Abbreviations: NMR, nuclear magnetic resonance; MPPC, 1-myristoyl-2-palmitoyl-*sn*-3-phosphatidylcholine; MSPC, 1-myristoyl-2-stearoyl-*sn*-3-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-*sn*-3-phosphatidylcholine; DPPE, 1,2-dipalmitoyl-*sn*-3-phosphatidylethanolamine; PGAC, *N*-palmitoyl galactosylcerebroside; DSC, differential scanning calorimetry; T_m, main-phase transition temperature; Me_4Si, tetramethylsilane.

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90–92% 1-acyl glyceride and 10–8% 2-acyl derivative (Martin, 1953). The mechanism of the acyl migration of monopalmitin (Doerschuk, 1952) proceeds through a five-membered transition state and does not involve any stereochemical change in the backbone of the glycerol molecule.

The rearrangement of di- and monoacyl glycerides was used earlier in synthetic work (Buchnea & Baer, 1960; Jackson et al., 1944); a review by Mattson and Volpenhein in 1962 emphasized its role in the synthesis of mixed-chain lipids and the chances of obtaining isomeric mixtures in the product. Later on, the danger of acyl chain migration was ignored, even by reviewers (Kates, 1977) when methods for the synthesis of mixed acylphosphatidylcholine were developed on the basis of attempted acylations of 2-lysolecithins. More recently, Gupta et al. (1977), Radhakrhishnan et al. (1981), and Mason et al. (1981) addressed themselves to this problem and successfully developed techniques which reduce chain migration to 5 and 1 mol % or better, respectively.

For the syntheses of mixed acyl 3-*sn*-phosphatidylcholines described in this paper we essentially followed the procedure of Gupta et al. (1977) with a few modifications. We have found the results to be reproducible, the extent of acyl chain migration being 3–4 mol % as determined by ^{13}C NMR spectroscopy (see below). Our choice of the methodology was based on (1) the easier availability of the catalyst in the synthesis, (2) the smaller requirement for fatty acid anhydride, which is important because of the cost and difficulty of synthesis of isotopically labeled fatty acids, and (3) the fact that all manipulations were done at room temperature (25 °C), thereby avoiding the extremely cold conditions required by the procedure of Mason et al.

Materials and Methods. 1,2-Dimyristoyl-*sn*-glycerophosphocholine and phospholipase A_2 were purchased from Sigma Chemical Co. [1-^{13}C]Palmitic acid and [1-^{13}C]stearic acid were synthesized by reaction of a suitable long-chain bromide with $Na^{13}CN$ followed by hydrolysis to the fatty acid or were purchased from Kor Isotopes (Cambridge, MA). The synthesis of [12,12-$^2H_2$]palmitic acid was done according to the method previously described (Das Gupta et al., 1982). Purity of the starting materials and the synthesized phospholipids was routinely determined by thin-layer chromatography (TLC) using Merck silica gel plates.

A synthesis of 1-myristoyl-2-[1-^{13}C]stearoyl-*sn*-glycero-3-phosphocholine is described below as a typical example. 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (1 g, 1.4 mmol) was converted to 1-myristoyl-2-lysolecithin with phospholipase A_2 in a borate buffer. After a complete conversion as determined by TLC, the product was extracted and isolated by chromatography over Sephadex LH-20, as described by Gupta et al. (1977). We found that the chromatographic purification was essential in order to purify the 2-lyso derivative from contaiminating fatty acids. The 2-lysolecithin was acylated with [1-^{13}C]stearic acid anhydride in dry, freshly distilled chloroform (35 mL) in the presence of 4-(dimethylamino)-pyridine in an atmosphere of nitrogen. Stirring the reaction mixture for 30–35 h resulted in a homogeneous solution (35 ml) which was diluted with a mixture of cold water (35 mL) and methanol (70 mL) and then extracted with a mixture of chloroform (35 mL) and 0.1 N hydrochloric acid (35 mL) to remove the 4-(dimethylamino)pyridine. The lower layer containing the phospholipid was separated out and carefully evaporated to dryness. The residue was chromatographed over silica gel. After the column was eluted with methylene chloride, methanol was increasingly added to the eluting mixture. After removal of the free fatty acid and its derivative, the mixed diacyl phospholipid was eluted with methylene chloride–methanol (50:50 v/v), as monitored by TLC. The dry product weighed 0.780 g; yield 78%. It should be mentioned here that when chloroform instead of methylene chloride was used in the elution mixture, the aqueous dispersions of the diacyl phospholipid were found to have an acidic pH, which leads to hydrolysis of the product.

Analysis of Labeled Samples. All samples were checked for purity by TLC and by differential scanning calorimetry (DSC) on a Perkin-Elmer DSC-2 at a heating rate of 5 °C/min. MSPC after annealing overnight at 0 °C gave transitions at 24 and 41 °C; MPPC after annealing for several minutes gave transitions at 27 and 34 °C. The main transition temperatures are consistent with previous reports (Keough & Davis, 1979; Stümpel et al., 1981, 1983; Chen & Sturtevant, 1980) and indicate reasonable sample purity. In order to monitor chain migration in the ^{13}C-labeled samples, we examined proton-decoupled ^{13}C NMR spectra of $CDCl_3$ solutions which were recorded on a Bruker 270 spectrometer. The *sn*-1 and *sn*-2 C=O resonances occur at 173.0 and 172.6 ppm, respectively, relative to Me_4Si. The integrated intensities of each peak were compared with those of natural abundance DPPC under identical conditions by using the $N-(CH_3)_3$ and chain terminal CH_3 peaks as intensity standards. In a typical spectrum the *sn*-1 peaks showed 3.3-fold (MPPC) and 4.5-fold (MSPC) enhancement over natural abundance, while the intensities of the *sn*-2 peaks were consistent with the enrichment of the ^{13}C-labeled fatty acids used. Chain migration was therefore less than 5% in both cases.

Sample Preparation. For both ^{13}C and ^2H samples, 50–70 mg of dry lipid was hydrated with an equal weight of ^2H-depleted H_2O, sealed under vacuum, and incubated at 45–50 °C for at least 30 min. The samples were then stored at 2–4 °C for 4 days to 2 months before beginning the NMR experiments. Samples were kept on ice during transfer to the precooled probe. ^{31}P spectra were taken on the ^{13}C-labeled samples. After synthesis the 2-[1-^{13}C]MSPC was found to be acidic. Therefore, it was neutralized with 0.2 M sodium phosphate buffer, pH 7.0, and then dialyzed against distilled deionized water with several changes to remove the buffer. Nevertheless, a trace amount of residual phosphate may have remained with the lipid (see Results).

NMR Methods. A home-built solid-state spectrometer with a 7.4-T magnet was used for all experiments. Most of the ^{13}C proton-decoupled data were taken with cross-polarization with a subsequent refocusing pulse to remove base-line distortions, although a Hahn echo (90°–τ–180°) sequence was used above the main transition temperature and also in the $P_{\beta'}$ phase of MSPC to compare line shapes. The ^{13}C 90° pulse was 5 μs, 2-ms mixing times were used for cross-polarization, and 5-s recycle delays were used to prevent sample heating from the proton decoupling pulses. ^{31}P spectra were taken with a Hahn echo with proton decoupling. The ^{31}P 90° pulse length was 4.1 μs. ^2H spectra were taken with a quadrupole echo ($90°_x$–τ–$90°_y$) sequence by using τ = 40 μs and 90° pulses of 2.1 μs. Phase cycling was used in all experiments.

Sample temperature was controlled with heated air or with cold N_2 gas. For collection of data as a function of time at low temperatures, the sample was cooled as quickly as possible and kept at constant temperature until the tuning had stabilized (20–30 min) before data acquisition was started.

Results

^{13}C Spectra. Figure 1 shows temperature-dependent spectra of (a) MPPC, (b) MSPC, and (c) DPPC, all ^{13}C labeled at the *sn*-2 carbonyl and kept at 2–4 °C for at least 11 days after

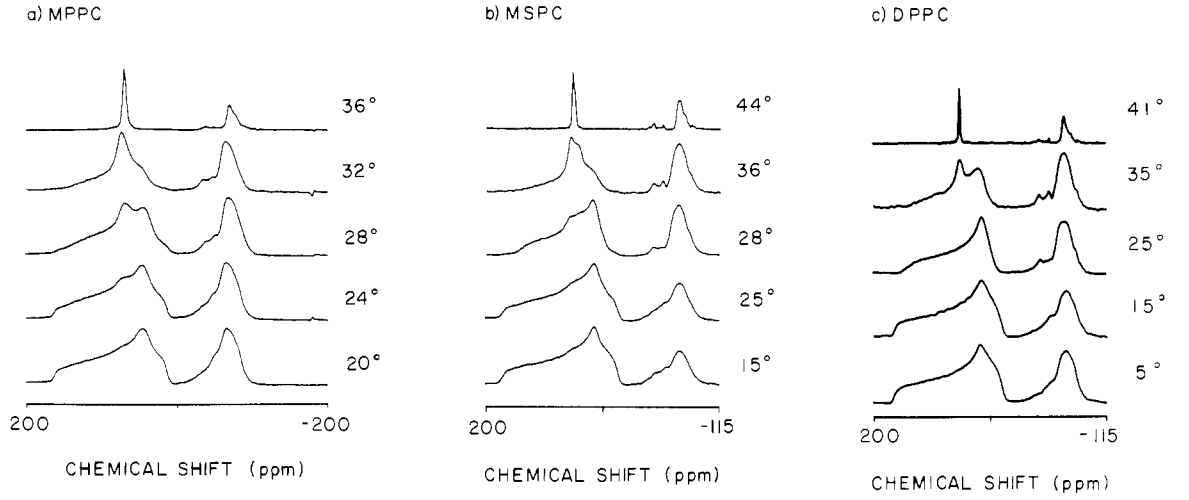

FIGURE 1: Proton-decoupled ^{13}C spectra of hydrated lipids (50 wt % water) with temperature increasing after annealing at 2 °C: (a) 1-myristoyl-2-[1-^{13}C]palmitoyl-PC (MPPC) after 11 days of annealing; (b) 1-myristoyl-2-[1-^{13}C]stearoyl-PC (MSPC) after 2 months of annealing; (c) 2-[1-^{13}C]dipalmitoyl-PC (DPPC) after several months of annealing. Chemical shift is referenced to [1-^{13}C]ethylene glycol as an external standard.

hydration. The carbonyl spectra are to the left; on the right side of the spectra are the contributions from the remainder of the molecule at natural abundance. At the lowest temperatures shown, the spectra of all three lipids clearly show axially asymmetric powder patterns at least 150 ppm wide, corresponding to the rigid lattice shift tensor. As the temperature is raised, the behavior of the two mixed-chain PCs differs from that of DPPC. At 25 °C, DPPC gives a single narrower (116 ppm wide) axially symmetric spectrum as seen before in the $L_{\beta'}$ phase (Wittebort et al., 1981, 1982), and then at higher temperatures the two-component spectra characteristic of the $P_{\beta'}$ phase are seen. For MPPC and MSPC, however, the rigid component is present in decreasing amounts up through at least 28 °C for MPPC and 25 °C for MSPC, at which temperatures there is substantial amount of the isotropic component.

At higher temperatures the isotropic component increases in intensity until above the main transition temperature (T_m), where the broad components have completely disappeared. The ^{13}C line shape for MSPC in the $P_{\beta'}$ phase (Figures 1b and 2b,c) differs from that of MPPC and DPPC in that the spectral contribution of the "L_α-like" component is no longer an isotropic-like line but rather looks either like two peaks or like a small axially symmetric powder pattern with a central dip and with sign opposite to that of the "$L_{\beta'}$-like" component. Cross-polarization can result in distorted line shapes since magnetization transfer is a function of the ^{13}C–^1H angles and distances as well as the motional rates; therefore, Hahn echo spectra were taken for MSPC in this temperature range (B. A. Lewis, unpublished results). The Hahn echo spectra clearly showed a powder pattern with no central dip rather than two peaks. Also, the Hahn echo spectra showed an apparently larger fraction of the L_α-like component relative to the $L_{\beta'}$-like component than in Figure 1b; these differences in apparent fraction are expected and result from the reduced efficiency of cross-polarization to the L_α-like component. Figure 2 shows spectra for MSPC taken with the temperature decreasing from above T_m. The spectra resemble those at the same temperatures in Figure 1 except that at 2 °C the rigid component was not seen even after 17 h; thus, the spectral features of the $P_{\beta'}$ phase are immediately reversible and show no hysteresis, while the reappearance of the full subphase spectra clearly requires days or months.

^{13}C spectra of MPPC and DPPC were also recorded as a function of time at low temperature after heating above T_m

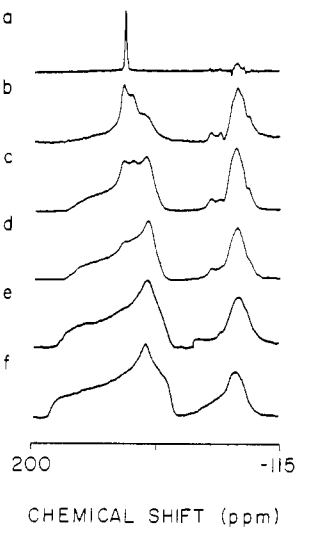

FIGURE 2: Proton-decoupled ^{13}C spectra of hydrated MSPC with temperature decreasing from above T_m: (a) 51 °C; (b) 36 °C; (c) 32 °C; (d) 26 °C; (e) kept at 4 °C for 17 h after cooling; (f) kept at 2 °C for 2 months.

(data not shown). While all three lipids showed spectra broader than the $L_{\beta'}$ spectra of DPPC (Figure 1c, 25 C) within an hour after cooling, significantly axially asymmetric spectra were also not observed for DPPC after 16 h at 2 °C. The ^{13}C spectrum of MPPC, on the other hand, showed nearly as much asymmetry after only 1 h at 11 °C as after 11 days at 2 °C.

^{31}P Spectra. Shown in Figure 3a are the temperature-dependent ^{31}P spectra of MPPC, starting at low temperature after annealing at 2 °C for 4 weeks. A spectrum identical with that of Figure 3a at 2 °C was obtained after 14 h. As in the ^{13}C spectra, the ^{31}P spectra of MPPC show a major rigid-lattice component, about 176 ppm wide, which persists at least up to 24 °C. At higher temperatures the MPPC spectra resemble those of diacyl-PCs in the $P_{\beta'}$ and L_α phase. Even at 2 °C a small amount of this higher temperature component is apparent. After annealing for 7 days (Figure 3b), the ^{31}P spectra of MSPC are strikingly different from those of Figure 3a. The broad but featureless spectrum at 2 °C is narrower than the rigid-lattice spectrum and gradually narrows until 20 °C, where the axially symmetric spectrum 65 ppm wide characteristic of the $L_{\beta'}$ or $P_{\beta'}$ phase is observed. This powder pattern then narrows further to 46 ppm as the temperature increases through T_m, as in MPPC and diacyl-PCs. However,

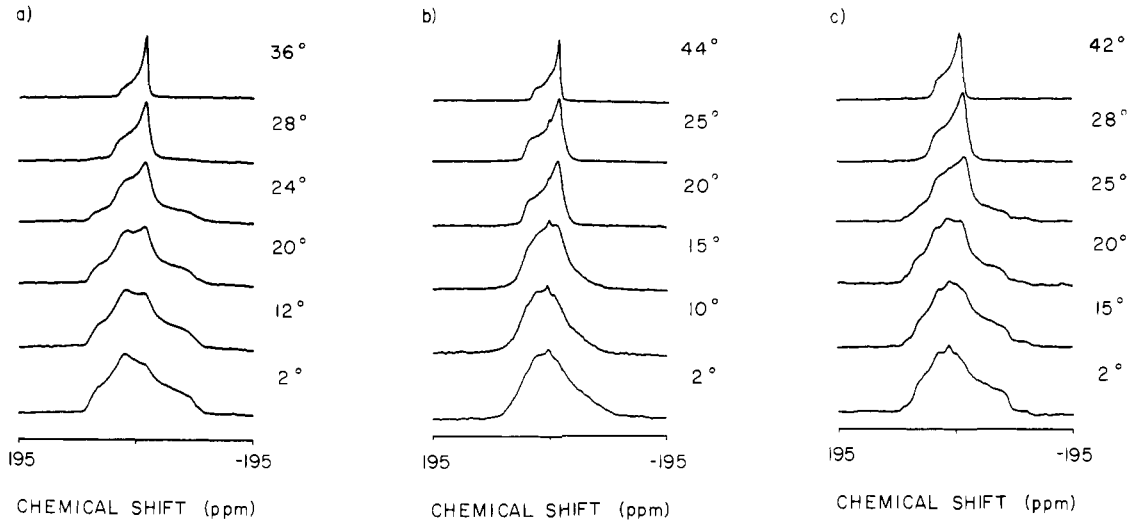

FIGURE 3: Proton-decoupled ^{31}P spectra of hydrated lipids with temperature increasing after annealing at 2 °C: (a) MPPC after 4 weeks of annealing; (b) MSPC after 7 days of annealing; (c) MSPC after 6 weeks of annealing. Chemical shift is referenced to 85% H_3PO_4 as an external standard.

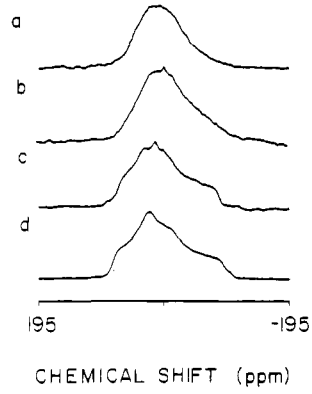

FIGURE 4: Proton-decoupled ^{31}P spectra of hydrated lipids at 2 °C after annealing at 2 °C: (a) DPPC after 2 months of annealing; (b) MSPC after 7 days of annealing; (c) MSPC after 6 weeks of annealing; (d) MPPC after 4 weeks of annealing.

as illustrated in Figure 3c, a longer annealing time (6 weeks) resulted in ^{31}P spectra for MSPC different from those of Figure 3b and similar to those shown in Figure 3a for MPPC. Again, the low-temperature spectra show axially asymmetric rigid-lattice powder patterns, and the rigid component persists up through 25 °C, giving way to the standard axially symmetric $P_{\beta'}$ (65 ppm) and L_α (50 ppm) spectra at higher temperatures. In the 2–25 °C spectra of Figure 3c, two axially asymmetric tensors of different breadths, 170 and 220 ppm, can be distinguished; the broader may represent anhydrous lipid (see Discussion). In addition, both sets of MSPC spectra, taken on separately prepared samples, show a small sharp component which remains constant with temperature. This peak may be due to residual phosphate buffer used for neutralization. Finally, Figure 4 presents four ^{31}P low-temperature spectra for comparison: DPPC after 2 months of annealing (a), MSPC after 7 days (b), MSPC after 6 weeks (c), and MPPC after 4 weeks (d). Spectra a and b of Figure 4 show the line shapes characteristic of the intermediate to slow motion regime (Herzfeld et al., 1978; Campbell et al., 1979), while the shapes in spectra c and d are characteristic of motion in the rigid-lattice limit.

^2H Spectra. Spectra of 2-[12,12-$^2H_2$]MPPC with temperature increasing after 4 days of annealing at 2 °C are presented in Figure 5. The 16 °C spectrum is flat topped and 121 kHz wide and resembles several spectra obtained by Huang et al. (Huang et al., 1980; T. H. Huang, R. P. Skarjune, R. J. Wittebort, E. Oldfield, and R. G. Griffin, unpublished results) and Blume et al. (1982a). Specifically, the 16 °C spectrum of [12,12-$^2H_2$]DPPC resembles that obtained for the 12-position of PGAC at −40 °C, the 6-position at 0 °C, and the 2-position at +18 °C and for 2-(4,4-$^2H_2$]DPPE at 1 °C. In the 16 °C spectrum of MPPC the shoulders (parallel edges) are clearly visible. As the temperature is raised, however, the spectra no longer resemble those of PGAC, which collapse to $\eta = 1$ spectra at higher temperatures below T_m. Rather, the higher MPPC temperatures have line shapes similar to those seen for the acyl chains of DPPE and DPPC in the gel phase (Blume et al., 1982a,b). At 34 °C two components are clearly visible. Finally, at 37 °C a single powder pattern with a quadrupole splitting of 23 kHz is observed.

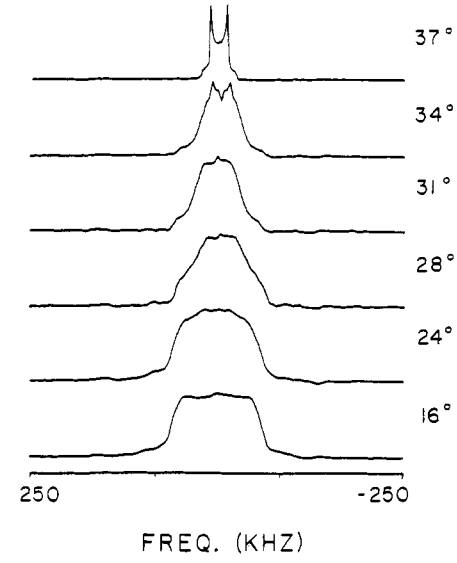

FIGURE 5: ^2H spectra of hydrated 1-myristoyl-2-[12,12-$^2H_2$]palmitoyl-PC with temperature increasing after annealing at 2 °C for 4 days.

When the temperature was rapidly decreased from 38 to 12 °C and maintained at 12 °C, within 2 h the original flat-topped spectrum was observed (data not shown). Thus, the motion of this part of the molecule also shows that the kinetics of formation of the subphase in MPPC are quite rapid.

Discussion

Previous solid-state NMR studies on diacyl-PCs ^{13}C labeled at the *sn*-2 carbonyl position (Wittebort et al., 1981, 1982)

have provided considerable information on molecular structure and dynamics in the $L_{\beta'}$, $P_{\beta'}$, and L_α phases. In all three phases, axially symmetric powder patterns were observed. Since dry powders of the labeled PCs exhibit broader, axially asymmetric rigid-lattice powder patterns 148 ppm wide, these axially symmetric spectra indicated fast motional averaging ($\tau_c < 10^{-4}$ s) of the ester carbonyl chemical shift tensor about a specific axis. Line-shape simulations of the $L_{\beta'}$ and L_α phase spectra, based on a model of the motion involving three-site axial hops, indicated angles of about 28° and 54°, respectively, between the principal axis of the chemical shift tensor and the diffusion axis. Furthermore, the $P_{\beta'}$ phase spectra could be simulated as a superposition of the $L_{\beta'}$ and L_α components with slow exchange (50–300 s^{-1}) between the two components.

The L_α phase *sn*-2 $^{13}C{=}O$ spectra of diacyl-PCs consist of a line about 1 ppm wide and could also be interpreted as arising from fast isotropic motion. However, the possibility of isotropic molecular motion in the L_α phase is ruled out by ^{31}P spectra, which show axially symmetric powder patterns about 45 ppm wide. The ^{31}P spectra broaden gradually through the $P_{\beta'}$ and $L_{\beta'}$ phases to about 65 ppm and, as with the $^{13}C{=}O$, remain axially symmetric in all phases (Seelig, 1978). ^{31}P spectra of many phospholipids in model and biological membranes all show very similar axially symmetric powder patterns in the $L_{\beta'}$, $P_{\beta'}$, and L_α phases.

The only published NMR spectra of a lipid in the subphase are from Füldner (1981); these ^{31}P spectra of DPPC in the subphase were approximately 180 ppm wide and had shapes characteristic of motion which is slow, in the range 10^4 s^{-1}, but not at the slow limit (Campbell et al., 1979). The rigid-lattice asymmetric tensor for hydrated phosphatidylcholines are about 185–205 ppm wide and for anhydrous phospholipids are around 230 ppm wide (Griffin, 1976; Herzfeld et al., 1978).

^2H magnetic resonance spectroscopy has been widely used to examine lipid structure and dynamics. Recently several studies have used specific ^2H labeling and line-shape analysis to examine acyl chain dynamics in the more ordered lipid phases—for example, in the L_β phase of DPPE (Blume et al., 1982a) and the lamellar crystalline phase of *N*-palmitoyl galactosylcerebroside (PGAC) (Huang et al., 1980; T. H. Huang et al., unpublished results). In the latter case, at temperatures below 82 °C, axial diffusion of the whole molecule does not occur; nevertheless, rigid-lattice ^2H spectra for acyl chain labeled positions are not observed down to temperatures of ~0 °C. Instead, the spectra are broad (~120 kHz) and are often flat topped. The spectra at low temperatures can be simulated by introducing a small amount of trans–gauche isomerization, and the degree of isomerization increases with position down the acyl chain (away from the glycerol backbone).

The ^{13}C NMR spectra presented in Figure 1 for MPPC, MSPC, and DPPC support three major conclusions. First, for all three lipids at the lowest temperatures shown, any motion of the *sn*-2 carbonyl group must be *much* slower than 10^4 s^{-1} because the full axially asymmetric rigid-lattice tensor is observed. This contrasts with the rapid axial diffusion in the $L_{\beta'}$ phase of DPPC (Figure 1c at 25 °C) which averages the tensor to give an axially symmetric powder pattern. The absence of rapid motion is consistent with the high degree of order observed by X-ray diffraction for DPPC (Ruocco & Shipley, 1982a,b) and MPPC (Serrallach et al., 1984; Stümpel et al., 1983) in the subphase. Second, for both mixed-chain lipids the low-temperature transition (at 27 °C for MPPC and 24 °C for MSPC) observed by DSC (Stümpel et al., 1983; Serrallach et al., 1984; this work) represents a transition directly from the subphase to the $P_{\beta'}$ phase, and there is probably no separate $L_{\beta'}$ phase in these species upon heating after annealing at low temperatures. Thus, this calorimetric transition represents a gradual melting of the subphase. The third major conclusion is that in the subphase and $P_{\beta'}$ phase of MPPC there is little lateral diffusion between the phases. This suggests that either the diffusion constants are small or that the domains are large. Figure 2 shows that the $P_{\beta'}$ phase spectra are reversible and do not depend on sample history, while as expected, the rigid subphase component does not immediately reappear upon cooling. For each species the rate of formation of the subphase apparently depends critically on temperature and probably on cooling rate, although we have not explored the full range of conditions. Since the rigid component appears more rapidly at a higher temperature for MPPC than for MSPC or DPPC, when all samples are held at low temperatures, the ^{13}C data also suggest that the subphase in MPPC is more stable than in MSPC or DPPC. Specifically, it persists until higher temperatures upon heating, and, although the main transition temperature is lower for MPPC than for the other species, the subphase reappears more quickly upon cooling.

A further conclusion from the ^{13}C spectra of MSPC in the $P_{\beta'}$ phase is that the conformation at the *sn*-2 carbonyl group in MSPC differs from that in MPPC or the diacyl-PCs. The small powder pattern of reversed sign seen in Figures 1b and 2b for the L_α-like component indicates that the angle between the principal axis of the carbonyl chemical shift tensor and the molecular diffusion axis is a few degrees greater than the "magic angle" (54.7°). In the L_α phase at 44° (Figure 1b), just above T_m, a small powder pattern is still observed, but when the temperature is raised further, to 51° (Figure 2a), the spectrum narrows to a sharp line. These results suggest either that the conformation at this position gradually changes with increasing temperature or that an additional type of motional averaging such as wobble about the long axis of the molecule increases in amplitude with temperature.

The ^{31}P NMR spectra in Figure 3a,c support the major conclusions above and show that the phosphate group as well as the *sn*-2 carbonyl is rigid on the NMR time scale for MPPC and MSPC in the subphase when the sample has been sufficiently annealed. It is clear from these ^{31}P spectra as well as from the ^{13}C spectra that as the temperature is raised, any exchange between the subphase and $P_{\beta'}$ ($L_{\beta'}$-like) populations is slow, again suggesting the presence of large, phase-separated domains.

For MSPC after 6 days of annealing (Figures 3b and 4b), and DPPC even after 2 months of annealing (Figure 4a), however, the ^{31}P spectra present a different picture. At temperatures even below those at which the ^{13}C spectra show rigid limit powder patterns, the ^{31}P spectra indicate motion at an intermediate rate. The DPPC spectrum presented here (Figure 4a) is in agreement with results of Füdner and its shape resembles those simulated for diffusion rates of $\sim 5 \times 10^4$ s^{-1} (Campbell et al., 1979). As the temperature is raised from 2 to 20 °C, the MSPC spectra show a gradual narrowing to typical $L_{\beta'}$ or $P_{\beta'}$ ^{31}P phospholipid spectra, with no evidence of two nonexchanging populations (phase separation). This behavior contrasts with that of MPPC after 4 weeks and MSPC after 6 weeks of annealing, as discussed above. Thus, both the rate of motion of the phosphate groups and the persistence of the subphase to higher temperatures depend on the length of annealing time at low temperature. In addition, the low-temperature MSPC spectra after 6 weeks of annealing show a very broad component about 220 ppm wide which may

be due to anhydrous phosphate groups. This observation suggests that dehydration of the head groups may result from sufficiently long annealing of the subphase and is consistent with observations by Ruocco & Shipley (1982b) of reduced hydration in the subphase of DPPC.

From the ^2H results for MPPC, it is apparent that the acyl chains are not as rigidly held in the subphase as are the carbonyl or phosphate groups. However, comparison of the spectra of the CD_2 group at the 12-position of the *sn*-2 chain of MPPC with those obtained for several positions along the chains in PGAC (T. H. Huang et al., unpublished results) suggests that acyl chain mobility in the MPPC subphase is much lower than that of PGAC in its lamellar crystalline phase. For example, the spectrum of 2-[12,12-$^2H_2$]MPPC at 16 °C closely resembles that of [6,6-$^2H_2$]PGAC at 0 °C. Thus, even though the labeled position is much farther down the acyl chain in the MPPC sample, less motion (presumably due to trans–gauche isomerization) is present at a given temperature than at positions closer to the head group in PGAC.

The breadth of the 2-[12,12-$^2H_2$]MPPC spectrum at 12 °C is also close to that of 2-[4,4-$^2H_2$]DPPE at 1 °C (Blume et al., 1982a), but the line shapes differ. In the DPPE spectrum, no parallel edges are visible, while they are clearly present in the MPPC spectra and in the PGAC spectra discussed above. These differences arise from the fact that DPPE in the gel phase undergoes fast axial diffusion, while the ^{13}C spectra both for MPPC in the subphase and PGAC in its low-temperature phase clearly show no overall molecular motion.

The NMR results presented here confirm recent suggestions (Stümpel et al., 1983; Serrallach et al., 1984) that the $L_{\beta'}$ phase in saturated mixed-chain phosphatidylcholines is not observed on heating an annealed sample and is present only transiently upon cooling, in contrast to the case for the PCs with two identical saturated chains. As yet, however, the molecular reasons for the dramatic difference in phase behavior induced by a two-carbon change in one or the other acyl chain have not been elucidated. In the case of MPPC, the ends of the two acyl chains in an untilted bilayer should be approximately even, since the staggering of the *sn*-2 chain reduces its effective length (Seelig, 1978); the subphase may be stabilized by this effect, as indicated by the rapid kinetics of its formation in MPPC. However, the fact that MSPC, PMPC, and other mixed-chain PCs with uneven chain ends also show stabilization of the subphase relative to the $L_{\beta'}$ phase requires a more sophisticated explanation. Since the $L_{\beta'}$ phase appears to be stabilized by the presence of two identical acyl chains, perhaps an important factor is the geometrical relationship of the ends of the two chains in the tilted conformation. For example, evenness of the chain ends in the direction perpendicular to the chain tilt might be a necessary feature of the $L_{\beta'}$ phase in phosphatidylcholines.

Thus, all the data consistently point to a general restriction of motion in the subphase of MPPC and MSPC, but it is also clear that the specific rate of molecular motion varies with position in the molecule and with the sample history as well as with temperature. At short annealing times the phosphorus spectra of MSPC and also of DPPC indicate considerably more motion than do the carbonyl spectra, while at longer annealing times in MSPC the phosphate motion decreases to less than the slow limit, and an anhydrous component is observed. We would predict from these results that DPPC might also exhibit a rigid-lattice ^{31}P spectrum if annealed for sufficiently extended periods.

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Registry No. MPPC, 69525-80-0; MSPC, 76343-22-1; DPPC, 63-89-8; 1-myristoyl-2-[1-^{13}C]stearoyl-*sn*-glycero-3-phosphocholine, 89088-44-8; 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine, 18194-24-6; 1-myristoyl-2-lysolecithin, 20559-16-4; [1-^{13}C]stearic acid anhydride, 89088-45-9.

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