Differential Thermal Analysis of Dipalmitoylphosphatidylcholine–Fatty Acid Mixtures†

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ABSTRACT: Mixtures of dipalmitoylphosphatidylcholine (DPPC) with palmitic, stearic, and myristic acids and the sodium salts of these acids were analyzed by differential thermal analysis (DTA) over a wide range of lipid compositions, all in excess water. All three fatty acids raise the liquid-crystal phase transition temperature and form sharp-melting complexes, with 1:2 DPPC–fatty acid stoichiometry observed for palmitic and stearic acids and suggested for myristic acid. Phase diagrams of the peritectic type, indicating nonideal mixing, were fitted to the DPPC–palmitic acid and DPPC–stearic acid data. In contrast, DPPC forms nearly ideal mixtures with the putative DPPC–myristic acid complex. At levels of only a few mole percent, both sodium stearate and myristate remove the pretransition and main transition and produce new peaks at ~30 and ~48 °C; the relative areas of the new peaks were unreproducible for the DPPC–myristate system. Sodium palmitate is the least disruptive of any of the sodium soaps or fatty acids; up to 80 mol % palmitate, the transition is lowered 3 °C and approximately doubled in width. The pretransition is detectable up to 36 mol %, and the main transition persists up to 88 mol % palmitate. The apparent pK of palmitic acid (12 mol %) in DPPC bilayers was determined to be 10.2 by direct pH measurement of ternary DPPC mixtures with known palmitic acid/sodium palmitate ratios; the intrinsic pK is estimated to be ≲8.5.

Although free fatty acids comprise only a minor component of biological membranes, the interaction of fatty acids with membranes and particularly the lipid bilayer is important in several respects. Effects of free fatty acids on biological and lipid bilayer membranes include modification of membrane fluidity and enzyme activity (Orly & Schramm, 1975), promotion of fusion of lipid bilayers (Kantor & Prestegard, 1978) and erythrocytes (Ahkong et al., 1973), stimulation of phospholipid release (Massari et al., 1980) and intermembrane lipid exchange (Papahadjopoulos et al., 1976), and modification of permeability in various membranes (Andreasen & McNamee, 1980; Wojtczak, 1976; Schramm et al., 1967). Specific polyunsaturated fatty acids induce brain edema characteristic of meningitis and brain abscess (Chan & Fishman, 1978); fatty acids and related compounds can stabilize erythrocytes against hypotonic hemolysis (Raz & Livne, 1973). Arachidonate as well as other fatty acids have been implicated in thrombogenesis (Furlow & Bass, 1975) and as both elicitors of disease–defense metabolites and growth regulators in plants (Bostock et al., 1981, and references therein). The lymphocyte plasma membranes of leukemic mice and men have abnormally high free fatty acid and glyceride levels (Johnson & Robinson, 1979). Furthermore, fatty acids and their derivatives have been widely used as physical probes of membrane structure, including the techniques of NMR (Seelig & Seelig, 1977; Stockton et al., 1974; Birdsall et al., 1971; Gent, 1978; Godici & Landsberger, 1975), ESR (Andreasen & McNamme, 1980; Sackmann et al., 1973; Marsh, 1980; Delmelle et al., 1980), fluorescence (Thulborn et al., 1979; Sklar et al., 1975; Cadenhead et al., 1977; Kimelman et al., 1979; Fraley et al., 1978) Raman spectroscopy (Verma et al., 1980), dielectric measurements (Ashcroft et al., 1980), and condensed phase radioluminescence (von Tscharner & Radda, 1980). An understanding of fatty acid behavior in bilayer membranes is therefore desirable both to explain numerous physiological consequences and to evaluate the reliability of fatty acid probes as nonperturbing reporters of membrane structure.

Biological activity, perturbation of lipid bilayer structure (e.g., Usher et al., 1978; Ashcroft et al., 1980), and probe–probe interactions (e.g., Hauser et al., 1979) have all been indicated with less than 10% of total lipid as free fatty acid. [The highest natural level of membrane free fatty acid we have seen reported is 8% of total lipid in rat liver plasma membranes (Ray et al., 1969).] Studies from several laboratories permit the following generalizations to be stated about the effects of free fatty acids on lecithin bilayer membranes: (1) long-chain saturated or trans unsaturated fatty acids increase and broaden the main liquid-crystal phase transition, (2) fatty acids of 10 or less carbons, cis unsaturated long-chain fatty acids, and fatty acid derivative probes lower and broaden the transition, and (3) all fatty acids eliminate the pretransition (Verma et al., 1980; Usher et al., 1978; Mabrey & Sturtevant, 1977; Eliasz et al., 1976; Ladbrooke & Chapman, 1969). Conflicting reports have been published on the clustered vs. the randomly dispersed state of fatty acids in lipid bilayers. Phase diagrams showing nearly ideal mixing in DMPC[1]–myristic acid (Kantor & Prestegard, 1978) and DPPC–palmitic acid mixtures (Mabrey & Sturtevant, 1977) have been inferred from scanning calorimetry; however, the mixing is between lecithin and 1:2 mole ratio lecithin–fatty acid complex. Formation of the 1:2 complex seems characteristic of saturated fatty acid–lecithin mixtures of the same carbon chain lengths and was not observed for other alkyl derivatives (Mabrey & Sturtevant,

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[1] Abbreviations used: DMPC, *O*-(1,2-dimyristoyl-*sn*-glycero-3-phosphoryl)choline; DPPC, *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)choline; DLPC, *O*-(1,2-dilauroyl-*sn*-glycero-3-phosphoryl)-choline; HP, palmitic acid (hexadecanoic acid); HS, stearic acid (octadecanoic acid); HM, myristic acid (tetradecanoic acid); NaP, sodium palmitate (sodium hexadecanoate); NaS, sodium stearate (sodium octadecanoate); NaM, sodium myristate (sodium tetradecanoate); DTA, differential thermal analysis.

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1977). There is a surprisingly large shift in the carboxyl pK of fatty acids both in pure fatty acid monolayers and when fatty acids are incorporated into lecithin bilayers. The monolayer pK is $\gtrsim$9 (Hauser et al., 1976) compared to a normal carboxyl solution pK of about 4. ζ potential measurements after Ca^{2+} binding to unsonicated mixed dispersions of lecithins with ~10 mol % fatty acids and fatty acid spin-labels indicate a pK of about 10; these results were attributed to clustering of protonated fatty acids but not of the ionized fatty acids or of the fatty acid spin-labels (Hauser et al., 1979). Raman analysis of various lecithin–fatty acid mixtures (1:0.6 mole ratio) indicated multiple transitions consistent with phase separation (Verma et al., 1980). Ptak et al. (1980) using titration by NMR of ^{13}C-enriched fatty acids (and other derivatives) mixed with lecithin in sonicated vesicles found an ionic strength and buffer-dependent apparent pK of 7.2–7.4. They saw no evidence on the NMR time scale of lecithin–fatty acid segregation and attributed the pK shift to a change in polarity of the carboxyl environment. Another ^{13}C NMR study found a pK of 8.4 for 16 mol % myristic acid in egg lecithin vesicles (Kantor & Prestegard, 1978). Thus, at physiological pH, free fatty acids in a membrane are expected to be protonated and uncharged. Relatively little has been published on the behavior of ionized fatty acids (soaps) in lipid bilayer membranes. Exchange of a fluorescent fatty acid derivative between DMPC vesicles proceeds by aqueous transfer of the fatty acid anion (Doody et al., 1980). The diffusion rates within the bilayer are lower for anionic than for uncharged fatty acids (Rigaud et al., 1977).

We have investigated binary mixtures of DPPC with myristic, palmitic, and stearic acids and the sodium salts of these acids. Coarse suspensions (multilamellar) in excess water were examined by DTA over as wide a composition range as possible. Phase diagrams are proposed for some of the systems; the phase behavior of the DPPC–palmitic acid system is seen to be of the peritectic type rather than the simple-mixing type previously published (Marbrey & Sturtevant, 1977). The protonated fatty acids behaved much differently than their corresponding sodium salts, and the behavior of both forms was sensitive to variations in carbon chain length. The pK of palmitic acid in DPPC bilayers was determined by direct pH measurement of ternary mixtures with known acid/salt ratios to be 10.2.

Materials and Methods

Materials. DPPC was purchased from Calbiochem (San Diego, CA) and was shown by thin-layer chromatography and densitometry after H_2SO_4 charring to be >99.6% pure. Myristic, palmitic, and stearic acids and their sodium salts were purchased from Applied Science (State College, PA) and were stated to be >99% pure. The alkyl group purity of DPPC and of the fatty acids and salts was verified by gas–liquid chromatography.

DTA was performed by using a Du Pont 900 differential thermal analyzer with a Standard DTA (0–500 °C) module. Samples were prepared in Du Pont's so-called macrotubes (4 × 25 mm), with the ceramic support collar used to center the thermocouples. pH measurements were made of DTA samples in the macrotubes with a microcombination pH electrode (Microelectronics, Inc., Londonderry, NH).

Sample Preparation. Careful sample preparation was essential for reproducible results. Known weights of DPPC (~5 mg) and fatty acid or salt were mixed in a macrotube by addition of appropriate aliquots of methanolic stock solutions. The methanol was evaporated by gentle heating of the macrotube in a sand bath followed by drying in a vacuum oven at 50 °C for at least 3 h. Exact lipid composition was established by weighing on a semimicrobalance after solvent removal; typically gravimetric and volumetric lipid masses agreed to within 1%. The dried lipid mixture was dispersed in 30 μL of hot distilled water by repeated heating in a jet of steam, tapping on the tabletop, and centrifugation (1000g) until the sample appeared homogeneous to the eye. After a final centrifugation to pellet the lipid, the thermocouple was carefully positioned in the center of the sample with the support of a shortened ceramic collar which was itself gently bedded on top of the lipid pellet. It is essential that there be no occluded air bubbles and that no sample be squeezed up between the collar and the walls of the tube.

Thermal Analysis. DTA thermograms were obtained under an N_2 atmosphere at a heating rate of 5 °C/min at instrument sensitivity and range settings of ΔT = 0.2 °C/in. and T = 10 °C/in. Triplicate scans were run of each sample after cooling to ~10 °C with N_2.

Results

DPPC–Palmitic Acid Mixtures. DTA thermograms for mixtures of DPPC and palmitic acid are shown in Figure 1A. As the mole fraction of palmitic acid is raised several changes occur: (1) The pretransition first shifts to higher temperature and then is lost; (2) a new peak appears above the main transition, and simultaneously shifts to higher temperatures, and grows at the expense of the original main transition (which remains nearly unshifted); (3) after loss of the original main transition, the peak continues to shift to higher temperatures and becomes sharper until X_{HP} = 0.68; (4) from X_{HP} = 0.68 to X_{HP} = 1, the thermal behavior is similar to pure palmitic acid.

DPPC–Stearic Acid Mixtures. As seen in Figure 1B, stearic acid behaved in DPPC bilayers very similarly to palmitic acid. In particular, note the new high temperature transition which grows at the expense of the main transition. Although it was difficult to achieve acceptable sample preparation for X_{HS} > 0.6, as with palmitic acid, compound formation at $X_{HS} \simeq 0.66$ was suggested. The size of the X_{HS} = 0.66 peak was surprisingly large.

DPPC–Myristic Acid Mixtures. Figure 1C shows that myristic acid behaves much differently than palmitic or stearic acids in that the main transition remains a single peak. The peak first broadens and then sharpens. Sample preparation with X_{HM} > 0.55 was even more difficult than with stearic acid, but there was a definite trend to a sharp peak indicative of compound formation, perhaps at X_{HM} = 0.67.

DPPC–Sodium Palmitate Mixtures. Sodium palmitate behaved completely differently than palmitic acid, as seen in Figure 2A. The pretransition was not eliminated until X_{NaP} = 0.33. The main transition was slightly broadened and shifted to lower temperatures, from 41.5 °C for pure DPPC to 38.5 °C for X_{NaP} = 0.50. Above X_{NaP} = 0.5, a broad high temperature peak appeared, but the original peak remained for X_{NaP} up to at least 0.88 (not shown).

DPPC–Sodium Stearate Mixtures. Figure 2B shows that sodium stearate was dramatically different than either sodium palmitate or stearic acid. Even at X_{NaS} < 0.06, the original main transition is not observed. A rather sharp 30 °C transition grows with increasing X_{NaS}, while a broad irregular transition is maintained around 48 °C. A third broad transition, at 68 °C, first appears at X_{NaS} = 0.3.

DPPC–Sodium Myristate Mixtures. This system proved totally intractable. Despite repeated attempts to rigorously control the variables of sample preparation, reproducible thermograms could not be obtained. As with the sodium

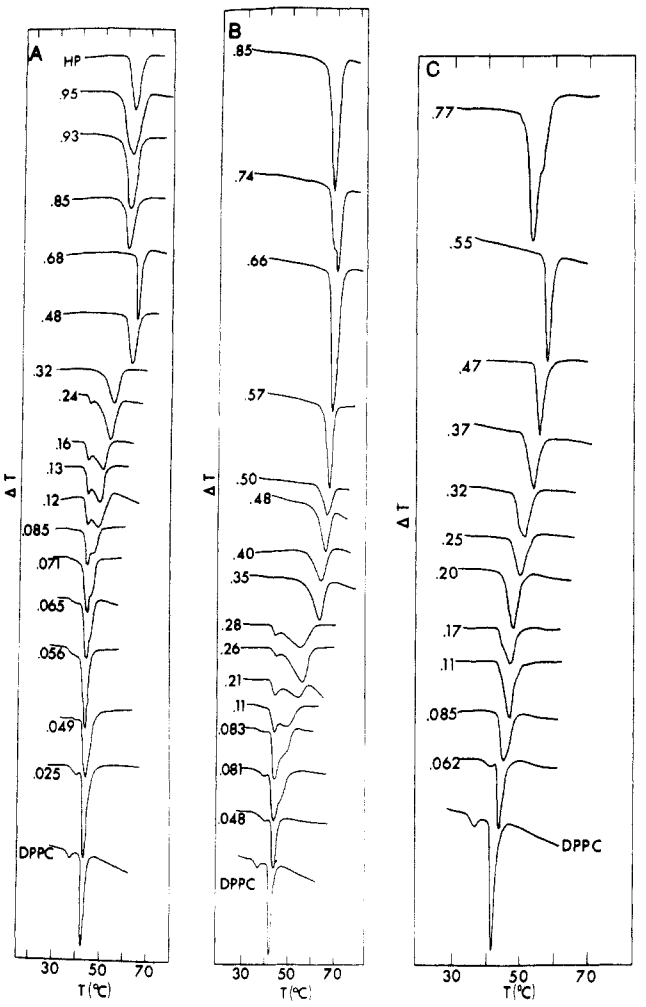

FIGURE 1: (A) DTA thermograms for mixtures of DPPC and palmitic acid in excess water. Mole fraction of palmitic acid is indicated beside each thermogram. (B) DTA thermograms for mixtures of DPPC and stearic acid in excess water. Mole fraction of stearic acid is indicated beside each thermogram. (C) DTA thermograms for mixtures of DPPC and myristic acid in excess water. Mole fraction of myristic acid is indicated beside each thermogram.

stearate, a few mole percent of sodium myristate replaced the original main transition with a lower and a higher peak; however, as seen in Figure 2C, the relative sizes of the new peaks varied greatly. Repetitive thermal scans were reproducible within a given sample tube preparation.

Phase Diagram Construction. The method of Etter et al. (1969) was used to construct phase diagrams for the DPPC–palmitic acid (Figure 3) and DPPC–stearic acid (Figure 4) systems on the basis of the thermograms of Figure 1A,B. These thermograms are consistent with peritectic phase behavior in the following respects. The main transition initially shifts to higher temperature, ruling out monotectic and eutectic behaviors. There is a composition range over which two transitions are seen. The lower transition is constant at the peritectic temperature; three phases are in equilibrium during the solid A → solid B + liquid transformation. The upper transition grows at the expense of the lower transition and shifts to higher temperatures as fatty acid is increased. After fatty acid is increased beyond the high fatty acid end of the peritectic line, a single broad transition is seen which increases in temperature and sharpens as the pure component is approached; the pure "component" in these cases seems to be a 1:2 DPPC–fatty acid complex (Mabrey & Sturtevant, 1977).

The thermograms in Figure 1A,B were fitted to the peritectic model as follows. Once the double transition appeared,

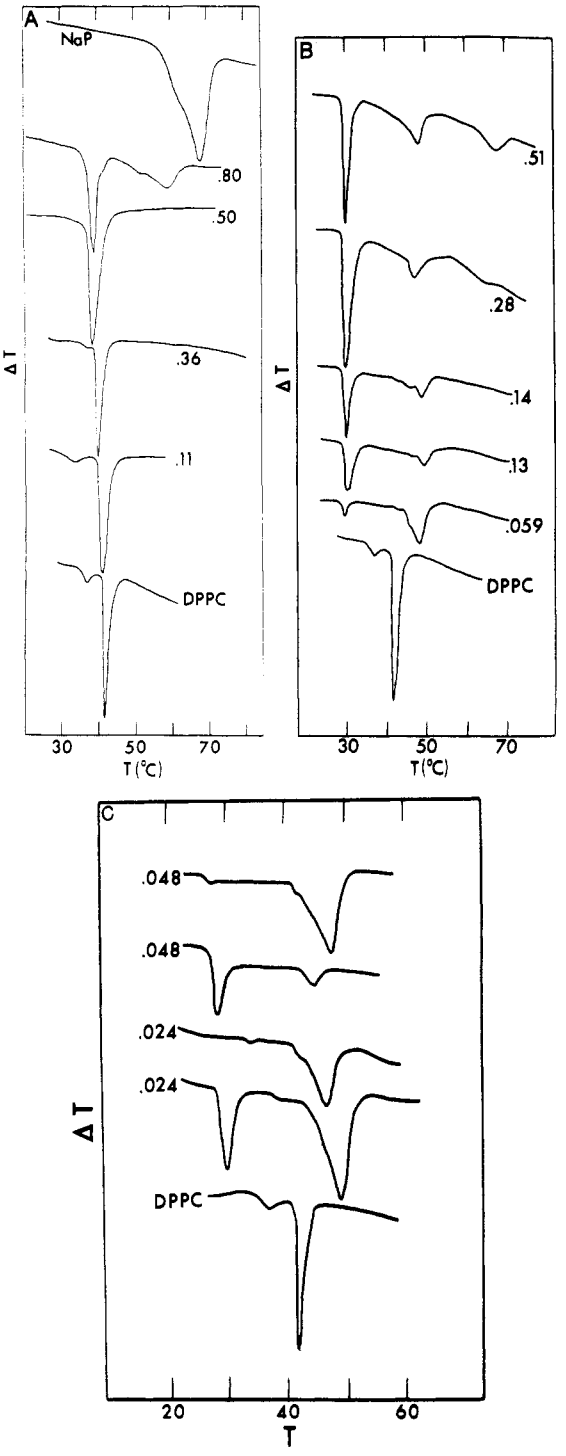

FIGURE 2: (A) DTA thermograms for mixtures of DPPC and sodium palmitate in excess water. Mole fraction of palmitate is indicated beside each thermogram. (B) DTA thermograms for mixtures of DPPC and sodium stearate in excess water. Mole fraction of stearate is indicated beside each thermogram. (C) DTA thermograms for mixtures of DPPC and sodium myristate in excess water. Mole fraction of myristate is indicated beside each thermogram.

the peak of the lower transition was taken as the isothermal peritectic temperature, and the liquidus curve was defined by the return to base line of the higher temperature transition. At fatty acid compositions above the peritectic range, the solidus and liquidus were defined as liftoff and return to base line, respectively. The liftoff and return to base line points were determined as the intersections of the tangents to the peak sides with the interpolated base line. An approximate correction for the "instrumental" peak width was made by assuming that the transition of pure DPPC in excess water is

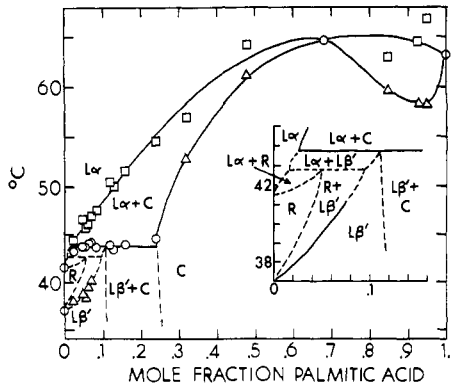

FIGURE 3: Phase diagram for DPPC–palmitic acid system in excess water constructed from DTA data of Figure 1A as outlined under Results. Circles represent DTA peaks presumed to be isothermal. Squares and triangles correspond to liquidus and solidus data points, respectively. Dashed lines have been positioned arbitrarily. The inset shows an enlargement of the low palmitic acid region. Single-phase regions are indicated as follows: Lβ', a gel solid solution of palmitic acid in DPPC with the Lβ' structure of pure DPPC in water; R, a gel solid solution of palmitic acid in DPPC with the rippled structure found between the pretransition and main transition of pure DPPC in water; Lα, a liquid-crystal solution of palmitic acid in DPPC with the Lα structure of pure DPPC in water; C, a gel solid solution of DPPC in a 1:2 complex of DPPC–palmitic acid The two-phase regions, in which pairs of the above phases coexist, are all indicated in the inset. The major peritectic line at 43.7 °C corresponds to the Lβ' $\rightleftarrows$ Lα + C equilibrium. The minor peritectic line, speculatively located at 42.8 °C, corresponds to the R $\rightleftarrows$ Lα + Lβ' equilibrium. The situation at greater than 66% palmitic acid is uncertain, partly because pure palmitic acid in water does not appear to melt isothermally. The simplest possibility is as follows: at low temperatures a solid solution of 1:2 complex C and palmitic acid; above the solidus curve, a two-phase region where, first, two compositions of the solid solution coexist, and then, above the melting point of pure palmitic acid, solid solution and liquid-crystal coexist.

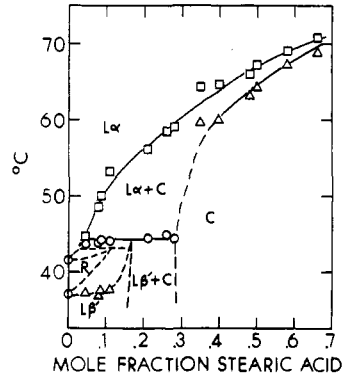

FIGURE 4: Phase diagram for DPPC–stearic acid system in excess water constructed from DTA data in Figure 4 as outlined under Results. See legend to Figure 3 for description of phase regions.

isothermal (Albon & Sturtevant, 1978) and by subtracting the pure DPPC width-at-base line from the apparent width of other peaks. Thus, 1.9 °C was added to liftoff and subtracted from return to base line temperatures.

In order to accommodate the pretransition without violating the Gibbs phase rule or the boundary rule for phase diagram construction (Yeh, 1970), it was necessary to postulate a second peritectic transition beneath the low fatty acid portion of the main peritectic line. (Since excess bulk water comprises a constant phase, a maximum of three lipid phases are permitted to coexist, and then only at a fixed temperature.) The two peritectic lines would be too close to each other and to the pretransition to be individually resolved by DTA. The speculative lines are indicated by dashed lines on the phase diagrams; although the dashed lines are positioned arbitrarily, their angles of intersection are drawn in accord with the

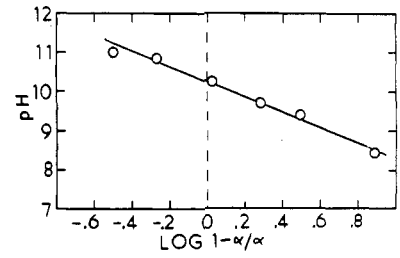

FIGURE 5: Determination of pK of palmitic acid in DPPC bilayers as intercept of plot of pH vs. log $[(1 - \alpha)/\alpha]$. The degree of dissociation α was taken as the mole ratio of NaP/(NaP + HP) in the original ternary lipid mixtures with DPPC.

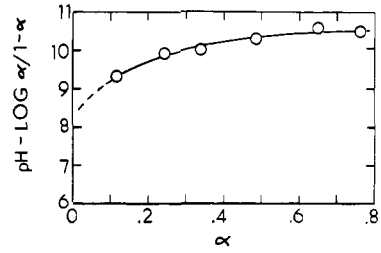

FIGURE 6: Plot of $[\text{pH} - \log \alpha/(1 - \alpha)]$ vs. α for estimation of intrinsic pK by extrapolation to $\alpha = 0$. The same samples were used as in Figure 5.

boundary curvature rule (Yeh, 1970). In labeling the phase diagram regions, where appropriate we use the notation of Luzzati and co-workers (e.g., Luzzati & Tardieu, 1974); we assume structures very similar to the pure DPPC persist in the liquid and solid solution regions at the low fatty acid side of the phase diagram. The nature of the structure between the pretransition and the main transition has been analyzed by several groups (Janiak et al., 1976; Copeland & McConnell, 1980; Stumpel et al., 1980; Ulmius et al., 1977; Rand et al., 1975) and is still unsettled; we indicate this region with R since the structure is generally believed to be rippled. The region of the putative 1:2 complex is indicated C.

DPPC–Palmitic Acid–Sodium Palmitate Mixtures and pK Determination. Ternary mixtures of DPPC–palmitic acid–sodium palmitate were prepared with a constant mole fraction of 0.12 of the total acid and soap. As the ratio of acid to soap was varied, the DTA thermograms (not shown) smoothly changed between those shown in Figures 1A and 2A for the corresponding binary mixtures. The pH of these acid–soap buffer samples was measured by using a micro combination pH electrode directly in the sample tube. The pH data were plotted following a modified Henderson–Hasselbalch equation [see reference to Katchalsky & Spitnik (1947) in Hendrickson & Fullington (1965)]

$$\text{pH} = \text{p}K - n \log [(1 - \alpha)/\alpha]$$

where α is the degree of dissociation (here taken from the initial sample composition) and n is a constant equal to 1 for monomeric acids and greater than 1 for polymeric acids. K, the acid ionization constant, is expected to increase, and n is expected to decrease with increasing ionic strength. The intercept, seen in Figure 5, indicates an apparent pK of 10.2 for 12% palmitic acid in DPPC bilayers at very low ionic strength and 21 °C; the slope of 1.9 is reasonable since the bilayer surface would resemble a polymeric acid. The same data are plotted Figure 6 according to Tanford (1961) such that the $\alpha = 0$ intercept is the intrinsic pK in the absence of electrostatic interaction between neighboring carboxyl groups. Since the slope of the extrapolation to $\alpha = 0$ is ionic strength dependent

and the ionic strength was low but not controlled (varied with salt/soap ratio), we can only estimate the intrinsic pK; an approximate upper limit to the intrinsic pK is 8.5.

Discussion

The peritectic behavior we report for the DPPC–palmitic acid system disagrees with the nearly ideal mixing behavior reported by Mabrey & Sturtevant (1977) for the same system. It is unclear whether they did not see or merely did not interpret the double peaks that lead us to postulate peritectic behavior. According to their phase diagram (their Figure 3), three samples with compositions in our peritectic range were analyzed, but those thermograms were not presented. Unresolved discrepancies when theoretical predictions and experimental results were compared for both transition curves and enthalpies (Mabrey & Sturtevant, 1977) suggest that their model of ideal phase behavior may be incorrect. The apparent formation of a 1:2 DPPC–fatty acid complex which they reported for palmitic and myristic acids is consistent with our results; however, we also saw a similar sharp-melting complex with stearic acid whereas they saw a broad complex peak. Although DTA data cannot provide quantitative enthalpy data, it is apparent from peak size (Figure 1B) that the 1:2 DPPC–stearic acid complex had an unexpectedly high heat of melting; a similar effect was reported by Mabrey & Sturtevant (1977) for the 1:2 DPPC–lauric acid mixture. Possible experimental sources of disagreement between our results and those of Mabrey & Sturtevant (1977) include the following: (1) They used a high-sensitivity differential scanning calorimeter with a scanning rate one-tenth of our rate and therefore presumably closer to equilibrium; however, our lack of hysteresis effects after rapid cooling and the agreement of much of our work suggest that we have no serious irreversibility problems. (2) They used a phosphate buffer (0.01 M, pH 7.0) whereas our samples were without any added source of buffering or ionic strength. (3) Their lipid concentrations were at least $^1/_{50}$ of ours, although occasional variations in sample water content indicated we had excess water.

In contrast to the peritectic phase behavior of DPPC–palmitic acid and DPPC–stearic acid, the DTA of DPPC–myristic acid mixtures is consistent with nearly ideal mixing phase behavior, such as reported for DMPC–myristic acid mixtures (Kantor & Prestegard, 1978), for DMPC–oleic acid mixtures (Finer & Phillips, 1973), and for mixtures of similar phospholipids (e.g., van Dijck et al., 1977; Shimshick & McConnell, 1973; Mabrey & Sturtevant, 1976). Actually, all three fatty acids which we studied behave nonideally with DPPC in the sense that a complex is formed. The apparent ideal mixing of DPPC and myristic acid is properly thought of as ideal mixing of DPPC with a putative 1:2 DPPC–myristic acid complex.

A structure of the 1:2 DPPC–fatty acid complexes that is plausible on the basis of examination of space-filling models involves two carboxyls serving as donors in hydrogen bonds with a single DPPC phosphate moiety. Hydrogen binding has also been implicated in the interaction of various long-chain alcohols with DMPC and DPPC (Pringle & Miller, 1979). Eliasz et al. (1976) suggest 1:1 complexes for DPPC with both 12- and 14-carbon acids and alcohols and a 1:4 complex for DPPC with octadecanol. Complexes have also been reported for several other amphipath pairs (Hui & Barton, 1973, and references therein).

Although the several boundaries indicated by dashed lines in the peritectic phase diagrams (Figures 3 and 4) are arbitrarily positioned, the qualitative arrangement is the simplest that does not violate the rules of phase diagram construction (Yeh, 1970). The existence and exact positions of these boundaries could be determined by methods such as fluorescence probe and rapid freeze–fracture electron microscopy, which were used to establish the DPPC–cholesterol phase diagram (Lentz et al., 1980), or deuteron NMR, as used by Ulmius et al. (1977) for the DPPC–D_2O phase diagram. The complexity of behavior predicted by our phase diagrams can be illustrated by considering the transformations along the isopleth for 0.03 mole fraction palmitic acid in DPPC (use inset of Figure 3). At low temperature, the system is a solid solution of palmitic acid in DPPC of the Lβ' structure. From 38.4 to 40 °C (the vestige of the pretransition), the Lβ' solid solution melts and is in equilibrium with solid solution of palmitic acid in the DPPC ripple structure R. From 40 to 42 °C, R alone exists. From 42 to 42.8 °C, R melts and coexists with Lα, the liquid-crystal solution of palmitic acid in DPPC. At 42.8 °C, there is a peritectic halt while the remaining R converts to Lα and Lβ' (the original, low temperature form). Lα and Lβ' coexist to 43.7 °C, where another peritectic halt converts the Lβ' to Lα and C, the solid solution of DPPC in the 1:2 DPPC–palmitic acid complex. From 43.7 to 44.5 °C, Lα and C coexist; at 44.5 °C the melting of C to Lα is complete. Thus, over a 6 °C temperature interval, five separate thermal events including two isothermal peritectic transformations are predicted; as seen in Figure 1A, these transitions were not resolvable since the base-line peak width of an isothermal process was ~4 °C on our DTA apparatus.

The effect of fatty acid sodium salts (soaps) on DPPC bilayers is dramatically different than the corresponding fatty acids. Sodium palmitate has surprisingly little effect: evidence of the pretransition remains with up to 36 mol % palmitate; evidence of the main transition persists to at least 80 mol % palmitate; up to 80 mol % palmitate, the main transition temperature changes by only −3 °C, and the half-width is increased by less than a factor of 2. The DPPC bilayer accommodates up to equimolar palmitate with little disruption. At approximately 50 mol % palmitate, a second high temperature phase, presumably rich in palmitate, begins to accumulate.

The stearate and myristate soaps behave very differently than the palmitate. This contrasts to the nearly identical behavior of palmitic and stearic acids. Unless there is a perfect match of alkyl chain lengths, the soaps effectively disrupt both the pretransition and the main transition of DPPC bilayers at soap levels of only a few mole percent. The relationship between the structural transitions of pure DPPC and of DPPC–soap mixtures is unknown. In general, when multiple peaks are observed, DTA cannot distinguish between phase separation with different domains melting independently and the entire sample remaining homogeneous and passing through a sequence of transformations. In the case of stearate, the continuous development of the sharp 30 °C peak at X_{NaS} increases, without much change in the 48 °C peak, suggests formation of separate domains of a stearate-rich complex with DPPC. The subsequent appearance and development of a new peak at ~67 °C, with little change in the other peaks, suggests accumulation of excess stearate.

Although intersample reproducibility was poor with the DPPC–myristate system, the results were similar to the stearate results in that at only a few mole percent soap, the original transitions are replaced by a ~30 °C transition and a broader 45–49 °C transition. Repeated attempts to discover the origin of the intersample irreproducibiltiy failed; no trend with variation of sample preparation details could be discovered. The fact that all tubes gave good intrasample repro-

ducibility upon repetitive scans indicates a particularly stubborn metastability.

Our measured pK of 10.2 for 12 mol % palmitic acid in DPPC at 21 °C is reasonably consistent with others' results for similar systems. It is apparent from the complex phase behavior described here that understanding this high pK will require additional studies at various compositions and temperatures. If no differences are found, the suggestion of Ptak et al. (1980) that the pK shift is simply due to the polarity of the bilayer environment will be supported, although subtle differences in carboxyl positioning in the various phases might significantly affect the polarity around the carboxyl group. Formation of a 1:2 DPPC–fatty acid complex seems inconsistent with the fatty acid clustering explanation of the pK shift reported by Hauser et al. (1979). Perhaps formation of the the 1:2 complex shifts the pK, since the ionized fatty acids could not participate in the complex. The phase diagram, since it indicates conditions under which complex does and does not exist, permits testing of this hypothesis. Further studies on this phenomenon are in progress.

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Molecular Details of Anesthetic–Lipid Interaction As Seen by Deuterium and Phosphorus-31 Nuclear Magnetic Resonance†

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ABSTRACT: Phosphatidylcholines (PC) deuterated in the fatty acyl chain (*sn*-2 chain, carbons 2, 6, 12, and 16) and in the head-group methyl (PC-d_9) and methylene (PC-d_4) moieties have been synthesized. Addition of the local anesthetic tetracaine hydrochloride (TTC) to the lipid dispersions produced different effects, depending on whether the anesthetic was positively charged (pH 5.5) or uncharged (pH 9.0), as monitored by the deuterium and phosphorus-31 nuclear magnetic resonance (^{2}H and ^{31}P NMR) of the phospholipid. A decrease in the ^{2}H NMR quadrupole splittings of the PC fatty acyl chains was observed at both pH values, the relative disordering effect becoming larger with the depth in the bilayer of the carbon segment. The decrease in order was more pronounced at low pH. At pH 5.5, the effect of TTC on the head group deuterated PC dispersions was to increase the quadrupole splittings for the two most peripheral segments (*N*-methyl and methylene groups) but to decrease that of the *O*-methylene segment. At pH 9.0, TTC decreased slightly the quadrupole splittings for all three head-group segments. The ^{31}P residual chemical shift anisotropy of the PC phosphate group increased upon addition of TTC at low pH and remained unchanged at high pH. The results are interpreted in terms of a model that depicts the charged dimethylammonium moiety of the anesthetic as being located in the head-group region, while the uncharged form penetrates more deeply into the membrane. The changes observed at low pH in the ^{2}H and ^{31}P NMR spectra of the phospholipid head group resemble those caused by ions and suggest that the head-group portion of the molecule undergoes a conformational change upon interaction with the anesthetic. Similar results were obtained for a 1:1 phosphatidylcholine:phosphatidylserine mixture. The anesthetic-induced disordering leads to an increase in phospholipid cross-sectional area and a decrease in membrane thickness, which could be related to the membrane expansion of one proposed mechanism of anesthesia. The results corroborate the conclusions obtained in previous work using deuterated local anesthetics [Boulanger, Y., Schreier, S., Leitch, L. C., & Smith, I. C. P. (1980) *Can. J. Biochem. 58*, 986–995].

The lipid structure of natural and model membranes has been investigated with techniques such as X-ray diffraction (Janiak et al., 1979), electron microscopy (Branton, 1969), neutron diffraction (Büldt et al., 1978), electron spin resonance (ESR)[1] (Schreier et al., 1978), and proton (Sheetz & Chan, 1972), carbon-13 (Lee et al., 1974), deuterium (Smith et al., 1978; Seelig, 1977), and phosphorus-31 (Cullis & de Kruijff, 1979) nuclear magnetic resonance (NMR). Broad-band ^{31}P NMR has been mostly used to distinguish between bilayer, hexagonal, and isotropic phases (Seelig, 1978; Cullis & de Kruijff, 1979), whereas ^{2}H NMR has been applied to several phospholipid dispersion systems and biological membranes and constitutes an excellent probe of the degree of molecular order (Seelig & Browning, 1978; Smith et al., 1979). Phosphatidylcholine (PC), in particular, has been deuterated in every position and the extent of order experienced by each deuterated group monitored by ^{2}H NMR under varying conditions such as temperature (Ulmius et al., 1977; Davis, 1979), ionic strength (Brown & Seelig, 1977), degree of unsaturation of the fatty acyl chains (Seelig & Waespe-Šarčevič, 1978), protein content (Rice et al., 1979), and cholesterol addition (Stockton & Smith, 1976). However, except in the case of the local anesthetic benzyl alcohol (Turner & Oldfield, 1979) and of the tertiary amine local anesthetics procaine and tetracaine (Boulanger et al., 1980), no ^{2}H NMR study has been reported on the influence of anesthetics.

Several theories have been proposed for the mechanism of action of local anesthetics. They have been suggested to act at the membrane level by interaction with either proteins or lipids. Theories involving proteins have focused on the interaction with a specific receptor (Hille, 1980) or on nonspecific binding to hydrophobic sites (Richards et al., 1980). As for interaction with lipids, changes in overall organization of the bilayer (Butler et al., 1973) as well as effects on lipid phase transition (Hill, 1974; Jain & Wu, 1977) and lateral phase separations (Trudell, 1977) have been postulated to play a role. The phenomenon of membrane expansion caused by anesthetics (whether related to the lipid and/or the protein membrane components) has also been proposed to be involved with the mechanism of anesthesia (Seeman, 1975). As pointed out by Ritchie (1975), many experiments do indicate that more than one of these theories could apply. Most likely, the

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[1] Abbreviations used: PC, phosphatidylcholine; PC-d_9, phosphatidylcholine deuterated in the head-group methyl groups; PC-d_4, phosphatidylcholine deuterated in the head-group methylene moieties; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; PS, phosphatidylserine; TTC, tetracaine hydrochloride; BPC buffer, borate–phosphate–citrate buffer; NMR, nuclear magnetic resonance; ESR, electron spin resonance; CSA, chemical shift anisotropy.

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