Hubbard, R., Brown, P. K., & Yoshizawa, T. (1965) *Cold Spring Harbor Symp. Quant. Biol. 30*, 301–315.
Hubbard, R., Brown, P. K., & Bownds, D. (1971) *Methods Enzymol. 33*, 615–653.
Kawamura, S., Wakabayashi, S., Maeda, A., & Yoshizawa, T. (1978) *Vision Res. 18*, 457–462.
Kropf, A., Brown, P. K., & Hubbard, R. (1959) *Nature (London) 183*, 446–448.
Maeda, A., Iwasa, T., & Yoshizawa, T. (1977) *J. Biochem. (Tokyo) 82*, 1599–1604.
Maeda, A., Ogurusu, T., Shichida, Y., Tokunaga, F., & Yoshizawa, T. (1978a) *FEBS Lett. 92*, 77–80.
Maeda, A., Shichida, Y., & Yoshizawa, T. (1978b) *J. Biochem. (Tokyo) 83*, 661–663.
Oesterhelt, D., & Schuhmann, L. (1974) *FEBS Lett. 44*, 262–265.
Pilkiewicz, F. G., Pettei, M. J., Yudd, A. P., & Nakanishi, K. (1977) *Exp. Eye Res. 24*, 421–423.
Rotmans, J. P., & Kropf, A. (1975) *Vision Res. 15*, 1301–1302.
Shichida, Y., Tokunaga, F., & Yoshizawa, T. (1978) *Biochim. Biophys. Acta 504*, 413–430.
Shichida, Y., Tokunaga, F., & Yoshizawa, T. (1979) *Photochem. Photobiol. 29*, 343–351.
Suzuki, T., Uji, K., & Kito, Y. (1976) *Biochim. Biophys. Acta 428*, 321-338.
Tokunaga, F., Shichida, Y., & Yoshizawa, T. (1975) *FEBS Lett. 55*, 229–232.
Yoshizawa, T. (1972) in *Handbook of Sensory Physiology VII/1, Photochemistry of Vision* (Dartnall, H. J. A., Ed.) pp 146–179, Springer-Verlag, West Berlin.
Yoshizawa, T., & Wald, G. (1964) *Nature (London) 201*, 340–345.

Gel to Liquid-Crystalline Phase Transitions in Water Dispersions of Saturated Mixed-Acid Phosphatidylcholines†

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ABSTRACT: Mixed-acid saturated lecithins containing myristate, palmitate, and stearate chains have been synthesized by phospholipase A_2 digestion of the appropriate single-acid lecithin, followed by reacylation of the lysolecithin with the desired fatty acid anhydride. Variable amounts of acyl migration were found to occur during the syntheses. The positional isomers 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphorylcholine and 2-myristoyl-1-palmitoyl-*sn*-glycero-3-phosphorylcholine as well as 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphorylcholine and 2-palmitoyl-1-stearoyl-*sn*-glycero-3-phosphorylcholine have been made. The thermotropic behavior of these lipids dispersed in excess water has been studied by differential scanning calorimetry. Positional isomers of mixed-acid saturated lecithins containing acids whose chain lengths differ by two methylene units are found to have different gel to liquid-crystalline transition temperatures and enthalpies. For each isomeric pair of mixed-acid lecithins, the one which had the longer chain in the sn-1 position of glycerol was found to have the lower transition temperature of the two. A partial phase diagram has been prepared by use of the palmitoyl and myristoyl mixed-acid lecithins so as to obtain extrapolated transition temperatures for the pure lecithins without acyl migration. The gel to liquid-crystalline transition temperatures of water dispersions of 2-myristoyl-1-palmitoyl-*sn*-glycero-3-phosphorylcholine and 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphorylcholine were found to be 27.2 and 35.5 °C, respectively. A model which proposes slight differences in chain packing in the bilayer in the gel state is presented as a possible explanation for the observed differences in the thermotropic behavior of the positional isomers.

Water dispersions of phosphatidylcholines containing a single type of saturated fatty acyl residue have been studied extensively as models for biological membranes. A considerable body of information is now available from a number of physical techniques on the thermotropic phase changes which occur in aqueous suspensions of these simple lipids alone and of their binary mixtures (Chapman, 1975; Lee, 1977a,b). On the other hand, mixed-acid lecithins as single species have received only scant study in model membranes (Phillips et al., 1972; DeKruyff et al., 1973; Barton & Gunstone, 1975; Seelig & Waespe-Šarčevič, 1978), although they comprise by far the majority of naturally occurring lecithins.

Here we report on the thermal characteristics of lecithins in which myristoyl, palmitoyl, and stearoyl chains have been mixed. Although thermal data is available on dispersions of mixtures of single-acid lecithins containing these chains (Ladbrooke et al., 1968; Phillips et al., 1970; Shimshick & McConnell, 1973; Chapman et al., 1974; Mabrey & Sturtevant, 1976), no systematic study of mixtures of these chains in the same phosphatidylcholine molecule has been carried out previously. Small modifications within a given molecule—in the position of double bonds, in the methylation of the head-group nitrogen, and in the types of glycerol–hydrocarbon chain links—have been observed to change the gel to liquid-crystalline transition temperature of phospholipids in water dispersions (Abramson, 1970; Vaughan & Keough, 1974; Barton & Gunstone, 1975). Here we report another intramolecular structural change which has a significant effect on the thermal phase transition of a phospholipid, but in this case no new types of bonds or chemical groups are introduced. We observe that in a mixed-acid saturated lecithin, when dispersed in water, the gel to liquid-crystalline phase transition temperature is determined by the distribution of the two acyl

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chains between the sn-1 and -2 positions of the glycerol backbone.

Materials and Methods

Egg Yolk Lecithin. Egg yolk lecithin was prepared by a method based on that of Singleton et al. (1965). The yolks of a dozen fresh eggs were blended in a Waring Blendor in 500 mL of acetone for approximately 5 min. The extract was filtered under vacuum through a coarse filter (four layers of Kimwipes optical wipers; Kimberley-Clark Corp.), and the filtrate was discarded. The filter cake was reextracted three more times with 500-mL portions of acetone. The resulting filter cake was blended with 150 mL of 95% ethanol and vacuum-filtered by use of Whatman 1 filter paper. The residue was extracted twice more with 95% ethanol, and the filtrates were added together. The combined filtrate was evaporated under vacuum to a thick, yellow syrup to which 150 mL of absolute ethanol was added. Next, 150 mL of acetone was added and insoluble material removed by filtration. The clear liquid was placed in a separatory funnel, and 150 mL of water was added to precipitate the lipid. After the solvent was removed, the solid was dissolved in 100 mL of anhydrous diethyl ether, and this solution was evaporated to dryness under vacuum. The residue was dissolved in 50 mL of diethyl ether, the lecithin fraction was precipitated with 125 mL of acetone, and the supernatant was decanted. This acetone precipitation from ether solution was repeated until the yellow colored material was removed (usually two to three times). The crude egg yolk lecithin so obtained was dissolved in a minimum of chloroform and applied to an alumina column, activity grade I [25 g of Woelm neutral alumina (ICN Pharmaceuticals) per g of crude lecithin]. The column was eluted with five bed volumes of chloroform, five bed volumes of chloroform–methanol (9:1 v/v), followed by chloroform–methanol (8:2 v/v), until the lecithin was eluted. This was monitored by thin-layer chromatography on silica gel G by use of chloroform–methanol–water (65:25:4 v/v/v) as the developing solvent (Wagner et al., 1961). Generally, 500-mL fractions were collected, and those containing lecithin were pooled, concentrated, and stored under N_2.

Single-Acid Lecithins. Some single-acid saturated lecithins were obtained from Calbiochem or Sigma Chemical Co., but most were made in our own laboratory.

The preparations of DMPC,[1] DPPC, and DSPC were carried out essentially as described by Cubero-Robles & van den Berg (1969) for the acylation of free glycerophosphorylcholine with the exception that acylation times of 6–16 h and 6 mol of acyl anhydride and 0.5 mol of Na_2O per mol of original lecithin were employed. Glycerophosphorylcholine was prepared from purified egg yolk lecithin by the procedure of Brockerhoff & Yurkowski (1965), and fatty acid anhydrides were prepared by the method of Selinger & Lapidot (1966). Purification of the lecithins is described below. All were found to be essentially pure as judged by GLC and by the DSC thermograms of their water dispersions. On TLC of heavily loaded samples, a small band running slightly ahead of the main PC spot was observed in some preparations, perhaps due to the presence of small amounts of 1,3-diacyllecithin.

Mixed-Acid Lecithins. Mixed-acid phosphatidylcholines were synthesized according to the following procedure. Lysolecithins were prepared by incubating a diethyl ether–methanol (95:5 v/v) solution of DMPC or DPPC or an ether–methanol–chloroform (85:10:5 v/v/v) solution of DSPC with *Crotalus adamanteus* venom (Miami Serpentarium) dispersed in 10 mM $CaCl_2$ (Wells & Hanahan, 1969). Lipid concentrations were 15 mg/mL, and enough enzyme suspension (12 mg/mL in 10 mM $CaCl_2$ solution) was added so that the enzyme/lipid ratio was 24 μg/mg. With small amounts (250 mg) of DMPC or DPPC, after 5 min of incubation with gentle shaking at room temperature, we added another equal amount of enzyme suspension. The reaction was stopped after 10 min by addition of an equal volume of ethanol. With larger amounts (1–1.5 g of starting material) incubations of 15–20 min were required. These conditions yielded complete hydrolysis of DMPC and DPPC, but to achieve complete hydrolysis of larger amounts of DSPC an incubation period of 90 min with extra additions of enzyme suspension at 10, 30, and 60 min were employed. After the addition of ethanol, the mixture was taken to dryness under vacuum, and the residue was resuspended in the original volume of ether. This was cooled to −20 °C and centrifuged in a clinical centrifuge. The resulting pellet was resuspended in ether, cooled, and centrifuged again. The pellet of lysolecithin was transferred to a round-bottom flask in methanol, the methanol was evaporated under reduced pressure to spread the lysolecithin onto the flask, and then the flask was dried under vacuum for 3 h at 40 °C. Reacylation with the appropriate anhydride was carried out as described by Cubero-Robles & van den Berg (1969) by use of 6 mol of anhydride and 0.5 mol of Na_2O per mol of lysolecithin. In general, reaction times of 6–8 h and temperatures of 75 °C were sufficient (80 °C and 6–8 h for stearoyl anhydride). Progress of the reaction was monitored by TLC.

The crude mixed-acid lecithin was suspended in boiling chloroform, cooled to −20 °C, and filtered through Whatman 1 paper under vacuum. The volume of the filtrate was reduced to a minimum, and the filtrate was treated with four volumes of acetone at 0 °C to precipitate lecithin. The precipitate, when redissolved in a small volume of chloroform, was applied to a silicic acid–Hyflo Super Cel (Fisher) (1:1 w/w) column (40 g of combined adsorbent/g of lipid). The column was eluted with five bed volumes of chloroform, five bed volumes of chloroform–methanol (9:1 v/v), and 10–15 bed volumes of chloroform–methanol (8:2 v/v), until all the lecithin was eluted as detected by TLC. The eluent was monitored by TLC, and fractions containing lecithin were pooled, evaporated, and redissolved in a small volume of chloroform. They were filtered through a double Millipore filter (0.47 and 1.2 μM pore sizes) to remove silicic acid fines. Lecithin was obtained from this solution by addition of acetone until no further precipitation occurred. Usually this precipitation was repeated once or twice to obtain the final product. (In one case excess fatty acid was obtained in the product, and it was finally purified by TLC on a preparative level on silica gel G by use of the solvent system described below. The material scraped from the plate was eluted in a column by use of chloroform–methanol (1:1 v/v), followed by methanol.)

Lipid Analyses. Thin-layer chromatographic analyses of lipids were carried out by use of silica gel G and the solvent systems chloroform–methanol–water (65:25:4 v/v/v) for phospholipids and hexane–diethyl ether–glacial acetic acid (90:10:1 v/v/v) or heptane–isopropyl ether–glacial acetic acid

[1] Abbreviations used: TLC, thin-layer chromatography; GLC, gas–liquid chromatography; DSC, differential scanning calorimetry; lc, liquid crystal(line); DMPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphorylcholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphorylcholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphorylcholine; MPPC, 1-myristoyl-2-palmitoyl-*sn*-glycero-3-phosphorylcholine; PMPC, 2-myristoyl-1-palmitoyl-*sn*-glycero-3-phosphorylcholine; PSPC, 1-palmitoyl-2-stearoyl-*sn*-glycero-3-phosphorylcholine; SPPC, 2-palmitoyl-1-stearoyl-*sn*-glycero-3-phosphorylcholine; T_c, gel to lc transition temperature.

Table I: Analytical Data for Mixed-Acid Lecithin Preparations[a]

lipid	batch no.	amt of single-acid lecithin at start (g)	amt of mixed-acid lecithin obtained (g)	% acyl[b] migration
PMPC	1	0.25	0.025	6.2
	2	0.25	0.067	13.0
	3	0.25	0.085	4.8
	4	2.0	ND[c]	19.0
	5	1.0	0.20	5.5
	6	2.0	1.1	22.0
MPPC	1	1.0	0.32	7.9
	2	0.50	0.13	6.0
	3	1.5	0.41	18.0
SPPC	1	1.0	0.25	4.2
PSPC	1	1.0	ND[c]	19.0

[a] Only batches in which the two acids were present in equimolar proportions were tested. [b] Obtained from the average of the concentration of the "wrong" acid found in free fatty acids and lysolecithins released by phospholipase A_2 hydrolysis of the products. Small variations between both estimates were usually obtained, differences being within one percentage point. [c] Not determined.

(60:40:4 v/v/v) for neutral lipids (Wagner et al., 1961; Mangold & Malins, 1960; Breckenridge & Kuksis, 1968).

Fatty acid analysis was carried out on a Packard Model 7400 gas chromatograph as described before (Dunphy et al., 1977). Positional analyses were carried out on small portions of lipid products by use of the hydrolytic procedure described above. The fatty acid content of lysolecithin and free fatty acids released by phospholipase A_2 digestion was determined after separation of the products by TLC as described above.

Differential Scanning Calorimetry. Differential scanning calorimetry was performed by use of a Perkin-Elmer DSC-2 calorimeter. The instrument was calibrated by use of pure indium (Perkin-Elmer) and hexadecane (Aldrich). Scanning rates of 5°/min and a sensitivity of 5 mcal/s full scale were generally employed. All samples were heated and cooled at least twice from −20 to 50–60 °C to ensure that excess water was present and that the transitions were reproducible. We determined transition temperatures by taking the point of intersection of the tangent to the leading edge of the transition and the extrapolated base line (Eliasz et al., 1976).

Samples were prepared by removal of solvent from the lipids under N_2, followed by evacuation for 1 h over P_2O_5. Water was added to give a lipid/water weight ratio of 1/2, and the materials were dispersed by use of a vortex mixer at temperatures approximately 10–15 °C above the T_c.

After DSC analysis was complete sample pans were opened in 3 mL of $CHCl_3$–CH_3OH (1:1 v/v), and the lipid suspension was dissolved. The pans were washed twice more with solvent, and the combined solvents were made up to 10.0 mL. Aliquots (2.0 mL) were taken in duplicate for phosphorus determination (Fiske & Subbarow, 1925; Dawson, 1960). Enthalpies were based upon the lipid P content of the samples.

Results

Conditions for Synthesis and Characterization of Products. Since the products produced from these syntheses were to be used to investigate thermotropic phase behavior, an attempt was made to obtain samples of as high purity as was feasible, consistent with an aim that these procedures could eventually be scaled up for large-scale preparations. During column chromatography, in particular, yield has been sacrificed by rejection of early and late lecithin-containing fractions which might contain small amounts of undetected contaminants. The yields for a number of preparations of mixed-acid lecithins are given in Table I. No systematic variation in yield with scale was found when 0.25–2.0 g of starting single-acid lecithin was used. Yields varied from 10–55% in the preparation of mixed-acid lecithins from single-acid lecithins, although most preparations gave yields in the range of 20–30%.

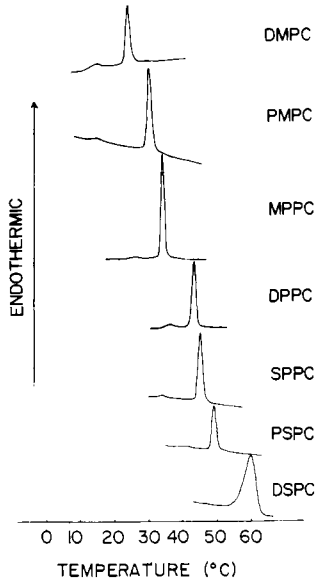

FIGURE 1: Tracings of heating thermograms of saturated single- and mixed-acid lecithins.

Only batches of mixed-acid lecithins which showed 50 ± 1 mol % of each acid on GLC analysis are included in Table I and were used for subsequent analyses. Using equal mixtures of the appropriate fatty acids and quantitative GLC standards, we determined that 50 ± 1 mol % was equimolar within the limits of accuracy of our GLC determination. We have obtained lecithins which contained excess amounts of the fatty acid at the 1 position (52–55 mol %). This indicates that some "parent" single-acid lecithin was present in the preparations. It is impossible to remove this material after synthesis when both acids are saturated, and thus such preparations have been discarded. Purification of the intermediate lysolecithins by column chromatography was not employed because of the possibility of enhancing acyl migration in the lysolecithins. Early attempts to achieve complete phospholipase-catalyzed hydrolysis of single-acid lecithins by use of long incubations always led to products with parent lecithins. We have observed that there is an optimal time for complete hydrolysis, after which parent lecithins begin to appear in the products. This hydrolysis time depended not only on the ratio of enzyme to lipid but also on the type of lecithin being hydrolyzed. Distearoyl- and egg yolk lecithins required different times than did dipalmitoyl- and dimyristoyllecithins.

Acyl migration did occur during these syntheses, however, despite our efforts to keep it to a minimum. The extent of migration can be easily determined by phospholipid A_2 redigestion of the products. The migrations obtained in our various preparations are shown in Table I where it can be seen that over the 11 preparations migration varied from 4.2 to 22.0%. While no definite correlation was observed, preparations starting with 1 g or more of single-acid lecithin generally resulted in larger amounts of migration and, thus, the wrong positional isomers in the product.

Phase Transitions in Mixed-Acid Lecithins. Figure 1 shows tracings of the DSC heating thermograms of the gel to lc transitional endotherms of a number of mixed-acid lecithins having myristic, palmitic, and stearic acid residues, along with those of the parent single-acid lecithins. The mixed-acid

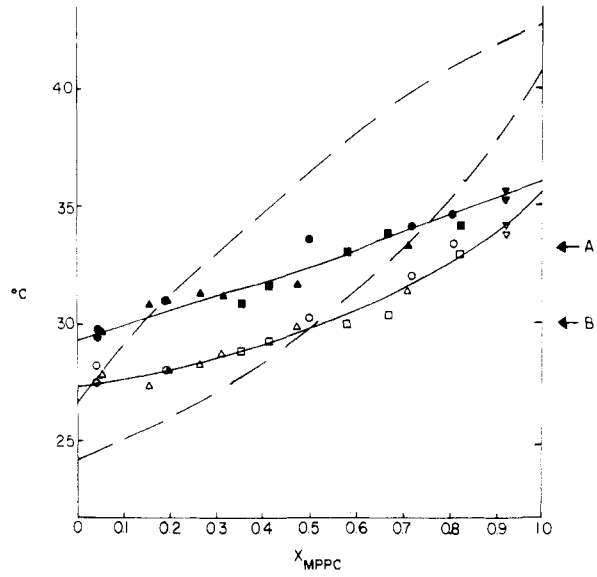

FIGURE 2: Phase diagrams of PMPC–MPPC (—) and DMPC–DPPC (---) (Chapman et al., 1974). (□,■) PMPC (batch 4)–MPPC (batch 3); (△,▲) PMPC (batch 5)–MPPC (batch 1); (○,●) PMPC (batch 3)–MPPC (batch 1); (▽,▼) MPPC (batch 2). (A) Midpoint between T_c of DMPC and DPPC. (B) Onset temperatures of equimolar mix of DMPC and DPPC obtained by Maybrey & Sturtevant (1976).

Table II: Best Estimates of Parameters for the Gel to Liquid-Crystalline Transitions of Pure PMPC and MPPC in Excess Water

lipid	T_c (°C)	ΔH kcal mol^{-1}	ΔH kJ mol^{-1}
PMPC	27.2 ± 0.2[a]	6.5 ± 0.6	27.2 ± 2.5
MPPC	35.3 ± 1.2	7.9 ± 0.6	33.1 ± 2.5

[a] Errors are values obtained as standard errors of these estimates from the least-squares approximation.

lecithins show cooperative transitions, and the transition temperatures are substantially different for each positional isomer. In most of our preparations small endotherms or pretransitions occurring at temperatures below the main endotherms and which are characteristic at saturated single-acid lecithins were observed.

Since, as can be seen from the data in Table I, each preparation of mixed-acid lecithin contained some of its reversed positional isomer, we have attempted to estimate the effect of these "contaminants" on the measured transition temperature by constructing a partial phase diagram of the two mixed-acid lecithins having palmitate and myristate chains. The diagram, constructed from a number of preparations, is shown in Figure 2. The solid lines shown in the diagram are obtained by an iterative least-squares approximation of the observed data to the theoretical equations for solidus and liquidus curves as used by Mabrey & Sturtevant (1976) and Lee (1977b). The extrapolated values for the transition temperatures of pure PMPC (27.2 °C) and MPPC (35.5 °C) are given in Table II, and these indicate a difference of 8.3 °C. We obtained the liquidus points by using onsets from cooling curves as opposed to taking the completion of the heating-run transitions. The pure compound "freezing points" have not been constrained to fit the onset temperatures from heating runs but are obtained also by extrapolation of the data from the fluidus curve. Corrections have not been applied to this data to attempt to account for the finite transition width (Maybrey & Sturtevant, 1976; Lee, 1978). The fact that the freezing and melting points do not coincide

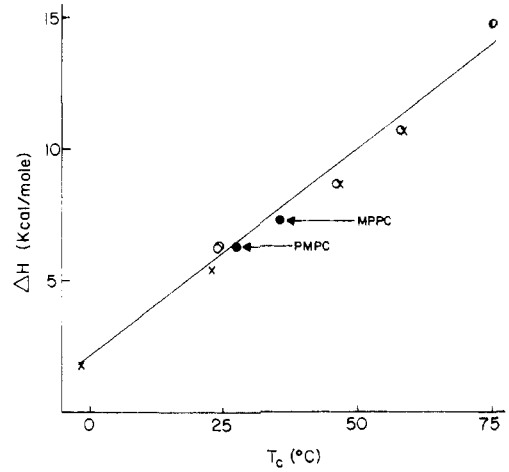

FIGURE 3: Linear relationship between enthalpy of transition and transition temperature for saturated lecithins. (×) From Mabrey & Sturtevant (1976). (○) From this laboratory and Chapman et al. (1974). (◑) From Ladbrooke & Chapman (1969). (●) PMPC and MPPC.

under these circumstances is in keeping with what we have observed before with single acid lecithins (Chapman et al., 1974), although the gel to liquid-crystalline transition of very pure DPPC has recently been found to be very close to an isothermal first-order transition on heating in a very sensitive calorimeter (Albon & Sturtevant, 1978). If the solidus data are fit to a third-order polynomial, values of 27.9 and 35.2 °C are obtained for transition temperatures of PMPC and MPPC, respectively.

The dashed line in Figure 2 is obtained from the data for mixtures of DMPC and DPPC presented before (Chapman et al., 1974). The arrows in Figure 2 indicate (A) the midpoint between the T_c for DMPC and DPPC and (B) the T_c found for an equimolar mix of DMPC and DPPC by Mabrey & Sturtevant (1976). It is noteworthy that the transition temperature of MPPC is close to the midpoint between those of DMPC and DPPC, while that of PMPC is significantly lower. It is also of interest that equimolar mixtures of DMPC and DPPC have onset temperatures which are the same as those from equimolar mixtures of PMPC and MPPC.

Half-height widths of our best PMPC and MPPC mixes have given values of 1 to 1.1 °C for each lipid. Pure DMPC and DPPC run under the same conditions (5 °C/min) have given similar half-height widths. Thus, the cooperativity of the transitions is similar, at least as measured by this criterion.

The most reliable estimates we obtained for enthalpies of PMPC and MPPC were 29.5 kJ mol^{-1} (7.04 kcal mol^{-1}) and 30.9 kJ mol^{-1} (7.38 kcal mol^{-1}), respectively, from our least migrated products. The approximation to the solidus and liquidus curves did not help improve those estimates. However, it has been noted by Mabrey & Sturtevant (1976) that there is a linear relationship between the T_c of single-acid saturated lecithins and their respective enthalpies. PMPC and MPPC are shown on such a plot in Figure 3 where the values for single-acid lecithins are those obtained in a number of laboratories and the straight line is obtained by a least-squares fit. The values for PMPC and MPPC fall within the level of 95% confidence for the fit of the line, and, thus, we have obtained our best estimates of the enthalpies for the pure compounds from this line. These enthalpy estimates are shown in Table II along with T_c values for PMPC MPPC.

Values of the transition temperatures for water dispersions of the samples of SPPC and PSPC shown in Table I have been observed to be 44.0 and 47.4 °C, respectively. This difference

is not as great as it is in the case of PMPC and MPPC; however, neither PSPC nor SPPC is pure (Table I), and the transition temperatures of the pure compounds would be expected to have been overestimated in one case (PSPC) and underestimated in the other (SPPC). It is worthy of note that, as it was with the PM–MP pair, it is the lipid of the PS–SP pair which has the configuration 1-short-2-long which also has the higher transition temperature of the two.

Discussion

Product Preparation. Since the final products of these syntheses were to be used for thermal measurements, we have been acutely aware of the necessity to avoid potential contaminants which could alter transition temperature and enthalpy values. In the development of the partial synthesis for the mixed-acid lecithins, we have slightly modified a number of existing procedures to suit our laboratory and to be consistent with a procedure which was rapid and simple but gave reasonable yields of high-purity product. To avoid any possible contamination of product with lipids of head groups other than choline, we have purified egg yolk lecithin as the starting material for synthesis. Fairly large amounts (50–100 g) of egg yolk lecithin can be prepared and glycerophosphorylcholine can be obtained in 3–4 days. The procedure recently given by Oldfield et al. (1978) for obtaining glycerophosphorylcholine might provide for some time saving over the procedure outlined above. Our reaction conditions for reacylation of glycerophosphorylcholine are slightly different from those of Cubero-Robles & van den Berg (1969), but we have been able to achieve shorter reaction times. We have routinely isolated the single-acid products of lecithin synthesis because they have had other uses in the laboratory, but as noted by Oldfield et al. (1978) this is not a necessary step in the preparation of mixed-acid species.

In establishing conditions for enzymatic degradation, we were concerned with achieving complete hydrolysis as quickly as possible in the presence of as little water as possible to reduce acyl migration in the lysolecithin. It was important that hydrolysis be complete so as to avoid the necessity for chromatographic purification of the lysolecithin during which acyl migration could occur. As long as hydrolysis is essentially complete, the simple ether precipitation to purify lysolecithin is adequate. However, some care is necessary as we have found that even small amounts of unhydrolyzed, saturated lecithins can precipitate with the lysolecithins, leading to reacylated products which have some parent single-acid lecithin contamination. This results in a molar excess of the putative 1-position acid in the final mixed-acid lecithin on GLC analysis.

In our early preparations we had attempted to hydrolyze DPPC and DMPC under conditions established for egg yolk lecithin using small enzyme amounts and long (16–24 h) incubation conditions. We had observed that, in the hydrolysis of egg yolk using 8 μg of enzyme/mg of lipid, deacylation was complete in 8 h. To ensure complete hydrolysis of the disaturated compounds, we had continued hydrolysis to 16–24 h. This procedure inevitably resulted in some single-acid lecithin in our final product. A more detailed examination of hydrolytic conditions of egg yolk lecithin and DPPC indicated that there was an optimum time for complete hydrolysis after which some diacyllecithin was again observed. So our initial preparations using long hydrolysis times had contaminating parent single-acid lecithins as indicated by the presence of a molar excess of the putative 1-position acid in the total acids of the mixed-acid lecithin. Thus, conditions requiring long hydrolysis times are to be avoided. Presumably, during prolonged incubation (hours) the enzyme denatures and conditions are sufficiently anhydrous for reacylation to occur. We have not studied the effect systematically but have adjusted our incubation conditions to avoid it. The conditions described above are those which yield final mixed-acid lecithins that do not show any parent lecithins as evidenced by their having equimolar amounts of both fatty acids present. In large scale preparations (>1 g), we now check the extent of hydrolysis using TLC while the reaction vessel is held at −70 °C. If any residual lecithin is present, we can easily restart the reaction by warming and by adding another aliquot of enzyme. If hydrolysis is complete, the normal procedure with the addition of ethanol is followed.

Distearoyllecithin is insoluble (or partially insoluble) in the simple ether–methanol solutions used for dimyristoyl- and dipalmitoyllecithins. If DSPC is suspended in ether–methanol, we observe little or no hydrolysis. The addition of 5% $CHCl_3$ and an extra 5% CH_3OH allows for appropriate dispersion of the substrate so that hydrolysis does proceed at a reasonable rate, although more slowly than DMPC and DPPC hydrolyses. Also, more enzyme additions appear to be necessary to achieve complete hydrolysis. This is presumably either because the presence of $CHCl_3$ denatures the enzyme and/or because the physical form of the substrate does not allow for efficient attack. It has been known for some time that snake venom phospholipase is not active in large amounts of $CHCl_3$ (Hanahan, 1952).

We have analyzed our preparative records to see if the extent of acyl migration in a sample had any relationship to scale, reaction temperature or time, or fatty acid or lysolecithin type. We obtain different amounts of migration in different preparations of the same materials as is evident in Table I, but there does seem to be some correlation of the extent of migration with large-scale preparations. It is perhaps not surprising that acyl migration is difficult to control under the basic reaction conditions used. Even though our reaction times are substantially shorter than those of Cubero-Robles & van den Berg (1969), we have obtained in our best preparation acyl migrations which are only marginally better than those reported by the previous authors and, unfortunately, have obtained lipids with much more migration. Our experience indicates that, if acyl migration is an important parameter in the mixed-acid lecithin to be studied, then it will need to be determined on each individual preparation made by this procedure. One cannot depend upon previously obtained performance to indicate the degree of contamination with positional isomers. As is shown above, the presence of the positional isomers in some lecithins can affect the values obtained for some physical–chemical parameters.

Phase Behavior. The mixed-acid lecithins which have been synthesized all contain saturated fatty acids, and thus the different transition temperatures and enthalpies for the positional isomers presumably reflect subtle variations in the hydrocarbon region of the bilayer formed by these compounds and not in the hydrophilic surfaces of the bilayer. Since the enthalpy and T_c data from the mixed-acid lecithins, PMPC and MPPC, fall on the straight line for the saturated single-acid lecithins, it is reasonable to expect that these, fundamentally, are arranged as are the single-acid lecithins. The transition temperature and enthalpies for single-acid lecithins depend upon chain length and thus upon thickness of the hydrocarbon region of the bilayers. Such a dependence of T_c and of enthalpy on bilayer thickness in these positional isomers can be used to account for the values observed above for PMPC and MPPC with a small variation in chain packing in

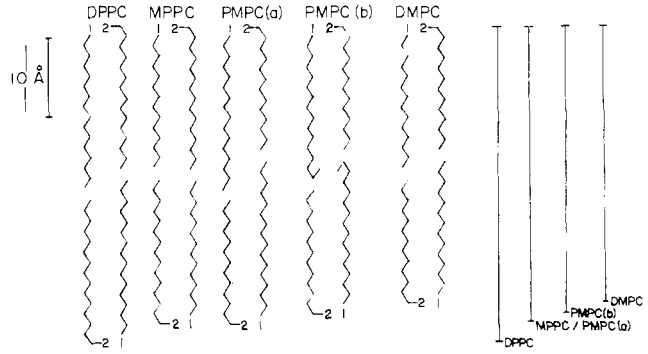

FIGURE 4: Proposed models for packing of the hydrocarbon chains in the gel state in saturated lecithins.

one of these as is discussed below.

It has been observed in deuteron magnetic resonance (Oldfield et al., 1978; Seelig & Seelig, 1975; Haberkorn et al., 1977) and neutron diffraction (Büldt et al., 1978) studies of selectively deuterated DPPC and DMPC that there are distinct differences between the chains at positions 1 and 2 of the glycerol backbone of the lecithin molecules. A difference in orientation of the first segments of the 1 and 2 chains together with a difference in the bilayer depth at which the first segments start [based upon the X-ray data for crystalline dimyristoylphosphatidylethanolamine (Hitchcock et al., 1974)] results in a difference in the distance to which the two chains penetrate the bilayer. Neutron diffraction analysis on selectively deuterated DPPC in the gel state at low water content (5–6 wt % water) has shown that the two chains are out of step by about 1.8–1.9 Å or 1.5 carbon–carbon bonds (Büldt et al., 1978). Since the differences in the chains at the 1 and 2 positions of glycerol have been shown for DPPC and DMPC at high water content in the liquid-crystalline state (Seelig & Seelig, 1975; Haberkorn et al., 1977) and for DMPC together with 30 mol % cholesterol below the pure lipid phase transition (Oldfield et al., 1978; Haberkorn et al., 1977), it is not unreasonable to assume that the chains of the mixed-acid lecithins at high water content are also out of step by about 1.5 carbon–carbon bonds. In Figure 4 we have attempted to portray the hydrocarbon interiors of the bilayers formed by the disaturated lecithins DPPC, MPPC, PMPC, and DMPC. The chains are in an all-trans configuration except for PMPC(b), and the difference in orientation of the first carbon–carbon bonds together with the difference in the penetration of the two chains is shown. It can be seen that, if a similar disposition of hydrocarbon chains exists in the mixed-acid as well as the single-acid lecithins, then, in the all-trans forms of the mixed-acid lecithins, one gets a large difference in the depths to which the two chains of each positional isomer penetrate the bilayer. In PMPC the palmitate chain (sn-1 position) extends about four carbon–carbon bonds past the myristate chain [Figure 4, PMPC(a)]. MPPC, on the other hand, has chains that would be expected to terminate with the methyl groups of both chains in register (Figure 4, MPPC). As can be seen in Figure 4 one can expect to get equivalent bilayer thickness (and presumably equal van der Waals interaction) in the all-trans state for PMPC and MPPC only if a considerable overlap occurs in the center of the bilayer of PMPC [Figure 4, PMPC(a)]. The slight overlap shown for DPPC and DMPC in the bilayer is consistent with the neutron diffraction data on DPPC deuterated at C-15 in the fatty acid chain (Büldt et al., 1978). For material with 25% water at 38 °C which most closely corresponds to the situation just before the main endotherm, the average distance of carbon-15 from the center of the bilayer was 2.0 ± 1 Å. MPPC is, of course, presumed to have no overlap.

We suggest that PMPC, because of the effective chain-length difference in the interior, packs less efficiently to give a state intermediate between MPPC and DMPC as is shown in Figure 4, PMPC(b). Here the overlap is reduced by the introduction of a gauche bond near the end of the 1 chain so as to effectively shorten it. The outcome of the packing arrangement suggested for the gel state of PMPC shown in Figure 4, PMPC(b), is to obtain a hydrocarbon thickness for PMPC which is intermediate between those of MPPC and DMPC but in which there is only a minimal difference in packing arrangement. This is consistent with the fact that the T_c and the enthalpy of the PMPC gel to lc transition are intermediate between those of MPPC and DMPC. It is noted that in the case of the positional isomers containing stearic and palmitic acids it is SPPC, the one with the longer claim in the sn-1 position, which has a lower temperature than PSPC. This is consistent with the observations on PMPC and MPPC and with the explanation given above for these differences in positional isomers.

In Figure 4 the chains are all shown without tilt for the sake of ease of presentation. The same arguments would hold if all the lipids had chains tilted to the same extent as do DPPC and DMPC (Tardieu et al., 1973). It is possible, however, that differences in chain tilt between MPPC and PMPC in the gel state could contribute to their different thermotropic behavior. Tardieu et al. (1973) have observed that mixed-acid lecithins with greatly different chains have their chains oriented perpendicular to the plane of the bilayer in the gel state. Whether or not the effective chain-length difference exhibited by PMPC in the all-trans state shown in Figure 4, PMPC(a), would be enough to cause it to have a different chain tilt than does MPPC is unknown at this time. Tardieu et al. (1973) made their observations on a saturated mixed-acid lecithin (2-lauroyl-1-stearoyllecithin) where the effective difference in penetration between the two chains would be about 8 carbon–carbon bonds. Also, whether a change in tilt alone could account for the different T_c values and enthalpies is a moot point. It would seem, however, that the total van der Waals forces would be the same irrespective of the tilt angle of the chains.

The differences in transition temperatures for positional isomers of mixed-acid lecithins must reflect a variation in the way in which these lecithins pack in a bilayer, albeit small, and whatever its origin. Such a variation in packing in the bilayer could lead to different kinds of interactions between other components in biological membranes and two positional isomers, based not only upon stereochemistry, but also upon differences in physical properties. The next steps in this study will be to characterize the thermal properties of bilayers of mixed-acid lecithins of greater chain-length difference and of different saturation, with a view to establishing whether or not the phenomenon described here could have special biological significance in view of the unique positional distribution found in most biological phospholipids. Such a positional isomersion has been suggested to be a potential mechanism of homeoviscous adaptation (Smith, 1976).

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References

Abramson, M. B. (1970) *Adv. Exp. Med. Biol. 7*, 37–53.
Albon, N., & Sturtevant, J. M. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2258–2260.

Barton, P. G., & Gunstone, F. D. (1975) *J. Biol. Chem. 250*, 4470–4476.
Breckenridge, W. C., & Kuksis, A. (1968) *Lipids 3*, 291–300.
Brockerhoff, H., & Yurkowski, M. (1965) *Can. J. Biochem. 43*, 1777.
Büldt, G., Gally, H. U., Seelig, A., Seelig, J., & Zaccai, G. (1978) *Nature (London) 271*, 182–184.
Chapman, D. (1975) *Q. Rev. Biophys. 8*, 185–235.
Chapman, D., Urbina, J., & Keough, K. M. (1974) *J. Biol. Chem. 249*, 2512–2521.
Cubero-Robles, E., & van den Berg, D. (1969) *Biochim. Biophys. Acta 187*, 520–526.
Dawson, R. M. C. (1960) *Biochem. J. 75*, 45–53.
DeKruyff, B., Demel, R. A., Slotboom, A. J., van Deenan, L. L. M., & Rosenthal, A. F. (1973) *Biochim. Biophys. Acta 307*, 1–19.
Dunphy, G. B., Keough, K. M. W., & Nolan, R. A. (1977) *Can. Entomol. 109*, 347–350.
Eliasz, A. W., Chapman, D., & Ewing, D. F. (1976) *Biochim. Biophys. Acta 448*, 220–230.
Fiske, C. H., & Subbarow, Y. (1925) *J. Biol. Chem. 66*, 375–400.
Haberkorn, R. A., Griffin, R. G., Meadows, M. D., & Oldfield, E. (1977) *J. Am. Chem. Soc. 99*, 7353–7355.
Hanahan, D. J. (1952) *J. Biol. Chem. 195*, 199–206.
Hitchcock, P. B., Mason, R., Thomas, K. M., & Shipley, G. G. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3036–3040.
Ladbrooke, B. D., & Chapman, D. (1969) *Chem. Phys. Lipids, 3*, 304–367.
Ladbrooke, B. D., Williams, R. M., & Chapman, D. (1968) *Biochim. Biophys. Acta 150*, 333–340.
Lee, A. G. (1977a) *Biochim. Biophys. Acta 472*, 237–281.
Lee, A. G. (1977b) *Biochim. Biophys. Acta 472*, 285–344.
Lee, A. G. (1978) *Biochim. Biophys. Acta 507*, 433–444.
Mangold, H. K., & Malins, D. C. (1960) *J. Am. Oil Chem. Soc. 37*, 383–385.
Mabrey, S., & Sturtevant, J. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3862–3866.
Oldfield, E., Meadows, M., Rice, D., & Jacobs, R. (1978) *Biochemistry 17*, 2727–2740.
Phillips, M. C., Ladbrooke, B. D., & Chapman, D. (1970) *Biochim. Biophys. Acta 196*, 35–44.
Phillips, M. C., Hauser, H., & Paltauf, F. (1972) *Chem. Phys. Lipids 8*, 127–133.
Seelig, A., & Seelig, J. (1975) *Biochim. Biophys. Acta 406*, 1–5.
Seelig, J., & Waespe-Šarčevič, N. (1978) *Biochemistry 17*, 3310–3315.
Selinger, Z., & Lapidot, Y. (1966) *J. Lipid Res. 7*, 174–175.
Shimshick, E. J., & McConnell, H. M. (1973) *Biochemistry 12*, 2351–2360.
Singleton, W. S., Gray, M. S., Brown, M. L., & White, J. L. (1965) *J. Am. Oil Chem. Soc. 42*, 53–56.
Smith, M. W. (1976) *Biochem. Soc. Symp. No. 41*, 43–60.
Tardieu, A., Luzzati, V., & Reman, F. C. (1973) *J. Mol. Biol. 75*, 711–733.
Vaughan, D. J., & Keough, K. M. (1974) *FEBS Lett. 47*, 158–161.
Wagner, H., Hörhammer, L., & Wolff, P. (1961) *Biochem. Z. 334*, 175–184.
Wells, M. A., & Hanahan, D. J. (1969) *Methods Enzymol. 14*, 178–184.

Electrostatic Interactions at Charged Lipid Membranes. Electrostatically Induced Tilt†

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ABSTRACT: The changes in bilayer structure induced by surface charges in the case of an ionizable lipid were studied by X-ray diffraction, Raman spectroscopy, and film-balance measurements. With increasing surface charge in the ordered phase, the X-ray results show a decrease in bilayer thickness, whereas the hydrocarbon chain packing stays essentially constant, the Raman data signify that the internal chain ordering does not change, and the monolayer studies show a lateral expansion of the bilayer. These results are interpreted in terms of a tilt of the chains caused by the surface charges on the polar heads. The tilt angle between the direction of the chains and the bilayer normal is obtained by a detailed theoretical evaluation. The tilt allows for a better understanding of the electrostatically induced shift of the phase transition temperature and of the shift induced by the binding of water in the case of lecithin in contrast to ethanolamine.

Since the first studies of lipid membranes it has been known that their properties vary with temperature. There are gradual variations and also an abrupt change is observed, the so-called ordered–fluid transition. In most biological systems, however, temperature is kept constant. Therefore one is interested in other external parameters by which membrane properties can be regulated. Biological membranes may contain up to 20% ionizable lipids. So one is tempted to expect electrostatic surface charges to represent a relevant regulation mechanism. They can be switched on and off when we vary the concentrations of ions in the electrolyte environment and, on the other hand, change the lateral packing of the lipid molecules, i.e., the membrane structure. Electrostatic effects at charged membranes have been studied extensively by use of pure lipids (for a review see Träuble, 1976). It was shown that the ordered–fluid phase transition can be triggered electrostatically, e.g., by varying the external pH at constant temperature. So the electrostatic regulation of membrane properties can be

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