Weeds, A. G., & Taylor, R. S. (1975) *Nature (London) 257*, 54–56.
Werber, M. M., Szent-Györgyi, A. G., & Fasman, G. D. (1972) *Biochemistry 11*, 2872–2883.
Woessner, D. E., Snowenden, B. S., Jr., & Meyer, G. H. (1969) *J. Chem. Phys. 50*, 719–721.
Yazawa, M., Morita, F., & Yagi, K. (1972) *J. Biochem. (Tokyo) 71*, 301–310.

Dilatometric Study of Binary Mixtures of Phosphatidylcholines†

D. Allan Wilkinson* and John F. Nagle

ABSTRACT: Volumes of lipid dispersions as a function of temperature have been measured for two different kinds of binary mixtures of lecithins, (1) DMPC and DSPC and (2) DMPC and $DC_{20}PC$. Emphasis was placed on DMPC-rich compositions so as to resolve ambiguities regarding solid-phase immiscibility in DMPC–DSPC mixtures. Special attention has been paid to problems of equilibration in the low-temperature phase and to methods of mixing the lipids. We find that there is no solid–solid immiscibility in DMPC–DSPC mixtures, although this system is close to exhibiting such immiscibility, and that DMPC–$DC_{20}PC$ mixtures exhibit pronounced solid immiscibility.

The miscibility properties of phospholipids are of current interest, and, in particular, for certain binary mixtures of phosphatidylcholines, the nature of the gel phase is still an unsettled question. That is, at temperatures below those where solid and fluid domains coexist, is there a single-phase region or is there solid–solid immiscibility? An answer to this question is provided by the type of phase diagram constructed for the lipid pair under study. For example, a horizontal solidus line is indicative of monotectic phase behavior, i.e., immiscibility in the solidlike phase.

In practice, it has been somewhat difficult to determine immiscibility on this basis. A good example is the lipid pair DMPC–DSPC. A horizontal solidus line up to at least a 50:50 molar mixture has been reported by some (Phillips et al., 1970; Lentz et al., 1976; Van Dijck et al., 1977), whereas other experimenters have found a solidus line with a nonzero slope (Shimshick & McConnell, 1973; Mabrey & Sturtevant, 1976; Gent & Ho, 1978), although Shimshick and McConnell noted that their data indicated an incipient solid–solid immiscibility.

It would therefore seem useful to determine this phase diagram by another method, especially one that neither perturbs the lipid structure with a probe molecule nor departs significantly from thermal equilibrium. Since dilatometry is such a method, we feel that the present study is a worthwhile addition to the work cited above and, indeed, decides between the two types of phase behavior.

To provide comparison with the DMPC–DSPC system, we have also studied mixtures of DMPC and $DC_{20}PC$. Such mixtures, where the hydrocarbon chain length difference is six, should, we felt, display solid-phase separation and so corrobrate the analysis of the DMPC–DSPC phase diagram.

Materials and Methods

Dimyristoylphosphatidylcholine (DMPC) and distearoylphosphatidylcholine (DSPC) were obtained from Calbiochem and used without further purification. The DMPC reported on here gave the sharpest phase transition of any lipid we have ever studied. The DSPC displayed essentially identical density properties to those of earlier samples (Nagle & Wilkinson, 1978). Dieicosanoylphosphatidylcholine ($DC_{20}PC$) was purchased from Avanti Biochemical Co.

Two methods were used for mixing the lipids. A comparison of them is presented under Results. One method, currently standard practice, consisted of dissolving the dry lipid powders in chloroform, stirring for approximately 0.5 h, removing the solvent under vacuum, and suspending the mixture in water. The other method consisted of suspending the lipids in 3 mL of water above the phase-transition temperature and then sonicating the mixture until it became nearly transparent. A Branson Model 185 sonifier operating at approximately one-third power for 5–10 min was found adequate for this. The sonicated mixture was then placed under vacuum, the water was removed, and the dried lipids were resuspended in water. The mixture then once again had the typical appearance of a multilamellar suspension.

The mixed lipid samples were degassed, and their temperature–volume relationships were measured with a differential dilatometer described previously (Wilkinson & Nagle, 1978). Scan rates of 2–5 °C/h were used. Approximately 100–150 mg of lipid in 10 mL of H_2O was used for each experiment.

The absolute values of specific volume were obtained by the neutral buoyancy in D_2O–H_2O mixtures described in a previous paper (Nagle & Wilkinson, 1978).

Results

Evaluation of the Cosonication Technique. Since cosonication of lipids as a method of mixing at the molecular level has not been reported on previously, we first tried this method on a mixture where separate phase transitions for each component if present could be readily observed: 29 mol % DSPC in DMPC with a total lipid mass of 131 mg. Dilatometric scans from 18 to over 56 °C revealed only a single, broad transition whose onset and end point were close to published data on phase diagrams for these two lipids. We therefore concluded that cosonication did produce intimately mixed lipids.

In order to evaluate the cosonication method, we compared the results that it produced with those obtained using chloroform on both DMPC alone and DMPC with a small amount (4 mol %) of DSPC, a mixture where the phase transition was still expected to be quite narrow in temperature. Pure DMPC,

† From the Departments of Physics and Biological Sciences, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213. *Received March 27, 1979.* The research was supported by National Institutes of Health Grant 2 R01 GM21128-05.

0006-2960/79/0418-4244$01.00/0

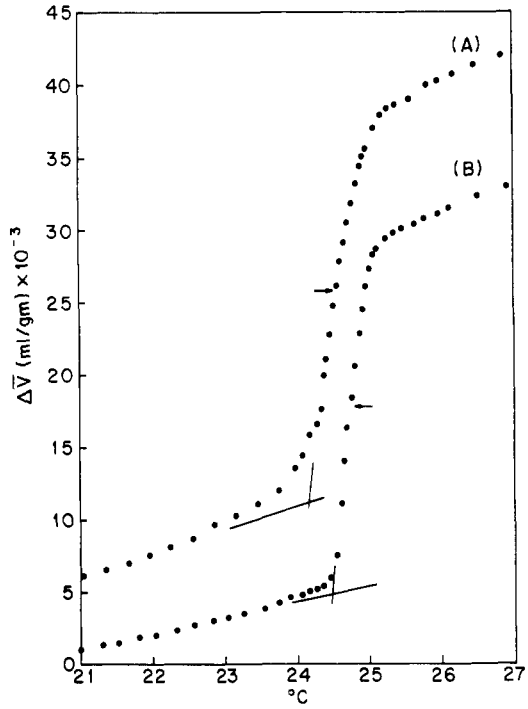

FIGURE 1: Change in specific volume as a function of temperature for (A) 4.3 mol % DSPC in DMPC mixed in chloroform and (B) 4.2 mol % DSPC in DMPC mixed by cosonication. The arrows indicate the midpoints of the transitions. The onset of the transition is indicated by the intersection of the two solid lines.

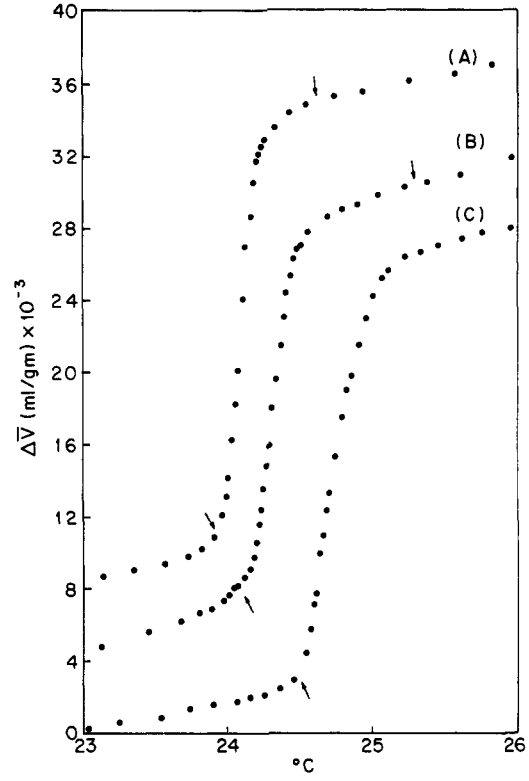

FIGURE 2: Change in specific volume as a function of temperature for (A) pure DMPC, (B) 1.8 mol % DSPC in DMPC, and (C) 4.2 mol % DSPC in DMPC. The ordinates have been shifted for each successive curve.

untreated by either method, had a transition half-width of 0.08 °C (i.e., the smallest temperature interval in which half the overall volume change occurs). Sonication of DMPC, followed by drying and resuspending, broadened the transition half-width by approximately 0.03 °C. Treatment in chloroform, however, caused a broadening of 0.10 °C. [A very similar change after chloroform application has been seen in calorimetric scans (J. M. Sturtevant, private communication).] Both cases of increase in transition width presumably are the result of impurities introduced by the method of dispersal. The numbers quoted above represent our best efforts to reduce contamination (e.g., minimizing sonication time, volume of chloroform, etc.). It would appear then that cosonication is the "cleaner" technique, at least in our hands.

Of more relevance to this study is a comparison of dilatometric scans on mixtures produced in the two ways. Figure 1 illustrates the differences observed in the region of the phase transition. The mixture formed in chlorofrom displays a transition which is both broader and less distinct at the low-temperature end compared to the cosonicated sample. In addition, there is a difference of 0.25 °C in the transition midpoint, the chloroform-treated mixture undergoing its phase change at the lower temperature. The sharper transitions and their more easily defined starting points in the case of cosonicated lipids have caused us to prefer this method for mixing, and the results presented below are on mixtures produced by cosonication.

DMPC–DSPC Mixtures. As we were interested in the question of monotectic behavior for these two phospholipids, it was clear that an investigation of the region of the phase diagram where DMPC is in high concentration was in order. Figure 2 shows detailed portions of dilatometric heating scans for pure DMPC and two mixtures containing small amounts of DSPC. The arrows indicate the start and end of the transition, the end of the transition for curve C not being in the range of this graph. It is clear that increasing the concentration of DSPC causes the onset, the midpoint, and the end of the phase transition to move to higher temperatures. A summary of the principal results of our dilatometric scans on mixtures of DMPC and DSPC is presented in Table I. The solidus temperatures (T_s) and the liquidus temperatures (T_l) have been corrected for the fact that each pure component does not display an isothermal transition. The corrections were made in the way indicated by Mabrey & Sturtevant (1976) and amount to less than 0.4 °C for T_s and 0.7 °C for T_l.

Table I: Dilatometric Data for Various DMPC–DSPC Mixtures[a]

mole fraction DSPC	T_s (°C)	T_l (°C)	$\Delta\bar{V}$ (mL/g) $\times 10^5$ measured	calcd
0.018	24.3	24.7	2400	2540
0.042	24.6	26.2	2600	2600
0.156	25.8	31.8	2700	2840
0.290	26.7	39.2	3000	3100

[a] Error limits for T_s are ±0.2, for T_l, ±0.5, and for $\Delta\bar{V}$, ±100.

The calculated values of $\Delta\bar{V}$ in the last column of Table I come from the simple additivity formula

$$\Delta\bar{V} = X_{\mathrm{DMPC}}\Delta\bar{V}_{\mathrm{DMPC}} + X_{\mathrm{DSPC}}\Delta\bar{V}_{\mathrm{DSPC}} \qquad (1)$$

where X denotes the mole fraction and $\Delta\bar{V}$ denotes the volume change of the pure material. The measured values are all within experimental error of these calculated values. It should be noted that the enthalpies measured for various mixtures of this lipid pair were 22% greater than the sum of the individual enthalpies; this was interpreted as due to the nonideality of this system (Mabrey & Sturtevant, 1976). Apparently, the nonideality is not manifest in the density changes at the transition.

Figure 3 shows a portion of the phase diagram for the system DMPC–DSPC. It includes data taken from several recent papers as well as our own data which concentrate on

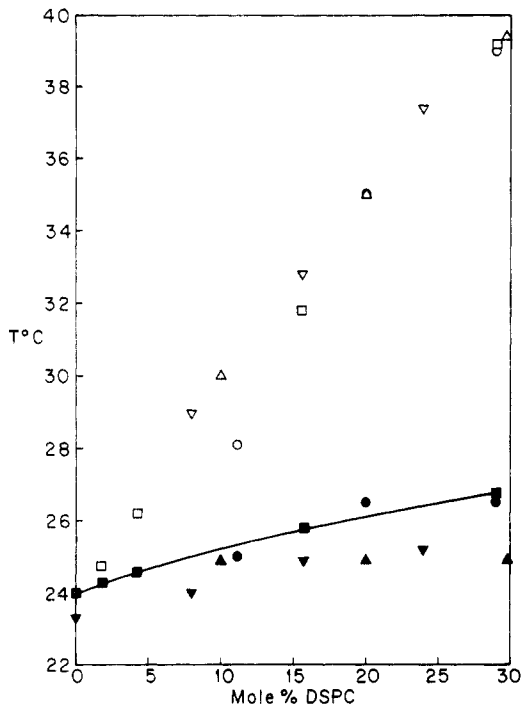

FIGURE 3: Phase diagram for the system DMPC–DSPC. Our data (■); data of Mabrey & Sturtevant (1976) (●); data of Shimshick & McConnell (1973) (▼); data of Van Dijck et al. (1977) (▲). The solid symbols represent solidus points and the open symbols represent fluidus points.

the low DSPC mole fraction end of the diagram in order to resolve the solid–solid miscibility question. There appears to be very good agreement on the liquidus curve, with what seems to be random scatter among the points rather than any consistent differences attributable to the experimental method of observation. It is in the slope of the solidus curve in this region where differences do show up. Our results, those from the calorimetry of Mabrey & Sturtevant (1976), and those from the ^{19}F NMR spectroscopy of Gent & Ho (1978) all nicely fit a line whose slope is nonzero and steeper than that of the solidus line reported by others. Also, for concentrations of DSPC higher than those shown in Figure 3, it is unlikely, based on our measurements and those of Mabrey & Sturtevant (1976), that the solidus curve becomes flat in any region of the phase diagram. Since the problem of measuring equilibrium values has been recently tackled with a view of resolving differences in this phase diagram (Van Dijck et al., 1977), we also made a considerable effort to eliminate kinetic effects resulting in spuriously high values for T_s.

Equilibration for These Mixtures. Since equilibration times for solid-phase separation to occur are not known (see Appendix for an estimate), several experimental methods were employed to ensure that the dilatometric scans would be free of significant kinetic problems. Heating rates were kept very low—2 °C/h in the region of the phase transition—and the samples were continuously stirred. A period of at least 24 h between successive experiments was allowed for equilibration in the low-temperature region (i.e., below T_s). In further efforts to equilibrate the mixtures, we incubated samples at 0.5 °C above and 0.5 °C below T_s for 36–48 h and then slowly cooled them to a low temperature before the beginning of a new heating scan. In this way, it was hoped that the formation of a metastable state, possible upon too rapid cooling through the transition, would be prevented and that phase separation in the "solid" region would occur. Dilatometric scans on samples "annealed" in this manner were not significantly different in any way from scans on nonannealed samples.

As a test of how close to equilibrium the heating scans were, we performed cooling scans at comparable rates (2–3 °C/h) on mixtures having low concentrations of DSPC. Any hysteresis present was within the error limits placed on the values given for T_l and T_s. Thus, we are confident that the data presented here represent equilibrium values for the transition properties of these mixtures.

Absolute Values of the Specific Volume. The specific volume of a lipid mixture when compared to the corresponding values for its components could in principle provide information about the nature of the mixture. For example, if the lipids in question are virtually immiscible, then one would expect to find large single-component regions in the multilamellar structure, and the measured specific volume, $\bar{V}_{mix}$, would be close to the appropriate average, viz.

$$\bar{V}_{mix} = X\bar{V}_1 + (1 - X)\bar{V}_2 \quad (2)$$

Departures from this relationship might occur because of imperfections in the boundary regions between different phases. Defects in the lipid packing would tend to increase the specific volume above what is predicted by eq 2. As long as the number of boundary molecules is much less than the number of interior molecules in a given phase, then eq 2 would be close to the measured value. But, as the miscibility of the lipids increases to the point where separate single-component regions (or even regions which are preponderant in one component) can no longer be said to exist, the effect of defects might become significant in determining the density. For example, in a bilayer composed of two types of phosphatidylcholine molecules differing in hydrocarbon chain length, were all the head groups restricted to the same plane, then there could be "holes" in the hydrophobic region created by having a shorter PC molecule next to a longer one, and the presence of such "holes" would result in a larger specific volume. Of course, such holes would tend to be filled in by interdigitation of the monolayers and by disordering of the ends of the longer chains; the latter mechanism would also increase $\bar{V}_{mix}$ compared to eq 2.

Specific volumes were measured for pure phosphatidylcholines and 1:1 molar mixtures of them. The results are presented in Table II. The specific volumes of DMPC and of DSPC quoted here are new determinations, but they are within experimental error of the values reported in our earlier work (Nagle & Wilkinson, 1978).

At 2.5 °C, which is in the range of the gel phase of both DMPC and DSPC (i.e., below the lower transition of each lipid), the equimolar mixture of these two lipids has a specific volume that is significantly higher than the mean value given by eq 2. The difference between the mean value and the measured value is even larger when DMPC is in the intermediate phase between its two transitions (at 21 °C). It is clear that the interaction between the two components of the mixture is important enough to invalidate eq 2 for this pair.

Table II: Specific Volumes[a]

lipid	$\bar{V}$ (mL/g) 2.5 °C	$\bar{V}$ (mL/g) 21 °C
DMPC	0.919	0.939
DSPC	0.927	0.943
$DC_{20}PC$	0.931	
DMPC + DSPC	0.926	0.944
DMPC + $DC_{20}PC$	0.925	

[a] The error limits are ±0.001 mL/g except for the DMPC + DSPC at 21 °C where the error is ±0.002 mL/g.

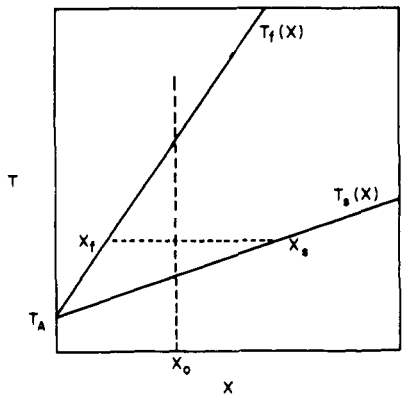

FIGURE 4: Model phase diagram for a binary mixture with temperature as a function of mole fraction, X. One tie line is shown, X_f–X_s, for a mixture of composition X_0.

An example of where this equation seems to hold will be given later.

Phenomenological Modeling. One way to evaluate our phase diagram for the DMPC–DSPC mixture is to try to reproduce in detail an experimental melting curve by the use of a simple model whose parameters are in fact derived from the phase diagram. Let us consider a phase diagram (Figure 4) with fluidus curve $T_f(X)$ and solidus curve $T_s(X)$. T_A is the transition temperature of component A and X_0 is the composition of the mixture under consideration. In the case where $T_f(X)$ and $T_s(X)$ are both linear functions of composition, we can write

$$T - T_A = SX_s \quad (3)$$

$$T - T_A = FX_f \quad (4)$$

where S and F are the slopes of $T_s(X)$ and $T_f(X)$, respectively, and X_s and X_f the compositions of the solid and fluid phases. Now, the relative amount of mixture in the fluid phase is

$$N_f = (X_s - X_0)/(X_s - X_f)$$

$$= [(1/S)(T - T_A) - X_0]/[(1/S - 1/F)(T - T_A)] \quad (5)$$

where $FX_0 > T - T_A > SX_0$.

Of course, $T_f(X)$ and $T_s(X)$ are not straight lines over the entire phase diagram for this or any other binary mixture. However, the solidus curve is linear over enough of the diagram that we may use eq 3 for modeling transitions of mixtures with high concentrations of DMPC. The fluidus curve may in fact not be linear even in that region of the phase diagram, and so a second-order term can be added to eq 4, viz.

$$T - T_A = F_1X_f^2 + F_2X_f \quad (6)$$

The coefficients S, F, F_1, and F_2 can all be evaluated directly from the experimental phase diagram.

The quantity N_f is a ratio of masses of material, and, in order to have it represent a volume ratio, we must use appropriate densities. The simplest case is where the density is independent of both composition and temperature. This is clearly incorrect, but the error is very small, less than 3% since the melting of DMPC leads to a density change of 2.6% and the error due to neglecting changes in the DSPC density is 1 order of magnitude smaller as we are concerned only with the DMPC end of the phase diagram. Thus, N_f represents with little error the volume fraction in the fluid phase and, hence, is directly proportional to our measurements of volume change.

A special case of eq 5 should be considered. If there is a horizontal solidus line, then $S = 0$, and the expression for N_f becomes

$$N_f = (1 - X_0)/[1 - (T - T_A)/F] \quad (7)$$

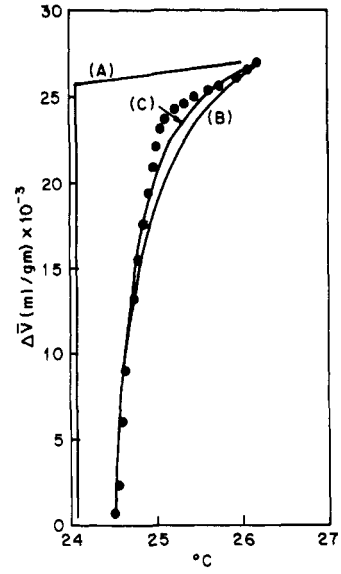

FIGURE 5: Modeling of the phase transition of the 4.2 mol % mixture. (A) Calculated curve for $S = 0$; (B) calculated curve for nonzero S and linear F; (C) calculated curve for nonzero S and curvilinear F. The dots are the experimental points.

The results of applying these models to the melting of a 4.2 mol % mixture of DSPC and DMPC are shown in Figure 5. The melting curve that is derived for the case $S = 0$ is clearly very different from the experimental results not only in temperature but also in sharpness of volume change. The other two model curves are for a solidus curve with a nonzero slope: $S = 0.1$ °C/mol %. Curve B is fitted to a linear $T_f(X)$ and curve C is fitted to a curvilinear $T_f(X)$. Both give reasonable approximations of the experimental data. Thus, the observed shape of the transition is consistent with the phase diagram we have constructed in Figure 3 with a solidus line having a nonzero slope.

DMPC–$DC_{20}PC$. Cosonicated mixtures of these two phospholipids with a wide composition range (1.7–87 mol % $DC_{20}PC$) were studied. In all cases, the onset of the transition was quite distinct so that the solidus line is well-defined. However, because of the broadness of the transition, the fluidus was impossible to distinguish with any certainty for more than 2 mol % $DC_{20}PC$. Because of the limitations of our apparatus at high temperatures, we do not have phase-transition data for pure $DC_{20}PC$. However, a value of 63 °C for the midpoint can be extrapolated from data on other saturated phosphatidylcholines (Nagle & Wilkinson, 1978). Although we therefore cannot correct T_s values of the mixture for inherent broadness due to nonisothermal melting of the components, the corrections would be expected to be similar in size to those applied to the DMPC–DSPC data. (Also, the asymmetry we have observed in transitions of PC's is such that the corrections to T_s are considerably smaller than those to T_f.) Figure 6 shows our data for this system. The solidus line appears to be horizontal but is displaced above T_m for DMPC by approximately 0.2–0.4 °C (see the inset in Figure 6). The specific volumes of $DC_{20}PC$ and of an equimolar mixture of DMPC and $DC_{20}PC$ at 2.5 °C appear in Table II. The value for $DC_{20}PC$ is consistent with those of other members of this homologous series. The mixture seems to display the additive property described by eq 2. This is then consistent with a phase diagram having a horizontal solidus and solid-phase immiscibility.

Lower Transition of DMPC. The density change at the lower transition of phosphatidylcholines is rather small [(250–300) × 10^{-5} mL/g], but we were able to determine its

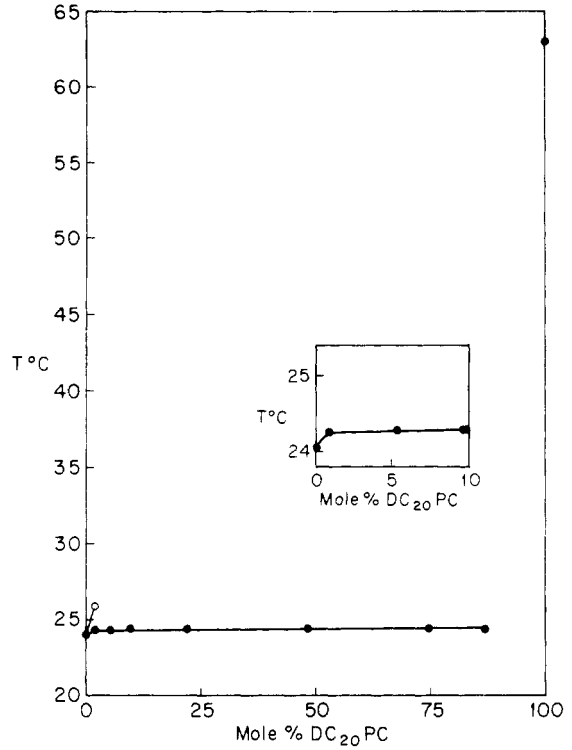

FIGURE 6: Phase diagram for the system DMPC–$DC_{20}PC$. The low $DC_{20}PC$ concentration end is magnified in the inset.

presence and estimate its size and temperature range for certain mixtures (those having at least 100 mg of DMPC in them). In the DMPC–DSPC system, the lower transition was observable in three mixtures (0.018, 0.043, and 0.156 mol % DSPC). The change in specific volume per mole of DMPC was approximately 0.8 that of the pure material alone; the transition-temperature range was unchanged from that seen with only DMPC. In the DMPC–$DC_{20}PC$ system, the lower transition was measurable in mixtures having up to 22 mol % of the longer chained PC. Once again, the density change was about 0.8 that of pure DMPC, and the transition-temperature range was close to what is measured for the single component.

Discussion

Two types of evidence were presented in the previous section regarding solid-phase miscibility in the DMPC–DSPC system. That is, the nature of the phase diagram (no observable monotectic behavior) and the magnitude of the specific volume of the equimolar mixture relative to the components both preclude solid–solid immiscibility. A similar conclusion based on consideration of the experimental phase diagram was reached by Mabrey & Sturtevant (1976) and by Gent & Ho (1978). A much different way of studying the gel-phase miscibility of these two lipids has recently been used (Mendelsohn & Maisano, 1978). They interpreted their Raman spectroscopic data on mixtures of deuterated DMPC with DSPC as indicative of significant intermixing of the two lipids in the low-temperature phase.

This conclusion about the DMPC–DSPC system is further strengthened by contrasting the phase behavior of this pair with that of DMPC–$DC_{20}PC$. Both the phase diagram and the measured specific volume in the gel phase of this latter mixture are consistent with immiscibility in the solidlike phase. Previously, Mabrey & Sturtevant (1976) found that, when there was a difference of six carbons in the fatty acid chains of the two components of a binary mixture of saturated phosphatidylcholines (DLPC and DSPC in their case), the phase diagram displays a horizontal solidus line. This result for mixtures with differences of six carbons is now even more firmly established because the thermal behavior of DMPC–$DC_{20}PC$ mixtures found in this paper is not complicated by the anomalous behavior exhibited by pure DLPC and DLPC-rich mixtures about 6 °C above the solidus temperature.

One feature of the solidus curve that we did not anticipate is the concave downward curvature near the pure DMPC end of the phase diagram. For DMPC–DSPC mixtures this curvature extends up to about 30 mol % DSPC in Figure 3. This behavior is in very good agreement with the statistical, mechanical calculations performed by Jacobs et al. (1977). In the case of the phase diagram of DMPC–$DC_{20}PC$ shown in Figure 6, the solidus curve is flat for less than 90 mol % $DC_{20}PC$ *except* for the pure DMPC transition temperature. Since the samples of pure DMPC were prepared in exactly the same way (cosonicaton followed by drying and redispersal) as the mixtures, we believe that the slightly lower transition temperature for pure DMPC is significant. After this work was performed, we became aware of a recent statistical mechanical calculation performed by Priest (Priest & Sheridan, 1979) which is consistent with this behavior for mixtures of lipids with differences of six carbons.

Finally, some comments concerning our method of preparing mixtures is in order. Since there seems to be almost no lipid exchange between different bilayers in multilamellar liposomes, formation of true mixtures requires the breaking up of these large structures. Cosonication, like the presence of an organic solvent, is a means of greatly reducing the size of such structures. While sonicated vesicles of different lipids may fuse to some extent, significant mixing likely occurs only after the multilamellar structures have been re-formed. In them there would exist patches of different lipids in the same bilayer, and intimate mixing would occur as a result of lateral diffusion.

Our preliminary experiments on the DMPC–DSPC system, using chloroform mixing, yielded results that would have produced a phase diagram having a solidus line with a smaller slope than the one we have presented in the previous section. This would have made it rather difficult to draw conclusions about solid-phase immiscibility. Another difficulty with chloroform-produced mixtures lay in the impossibility of modeling the transition behavior in any simple way because the onset of the transition was too far removed from the steepest part of the transition. That is, there appeared to be significant volume changes occurring just before the main melting of the DMPC in the mixture. Such changes, we felt, represented the presence of impurities rather than the consequence of mixing two different phosphatidylcholine molecules.

On the other hand, the cosonication technique not only produced mixtures whose transition properties were better defined but also permitted us to distinguish unambiguously between the phase behavior of the different mixture systems studied. How applicable our results with chloroform-produced mixtures are to other published data is difficult to ascertain. However, we would like to identify this as a possible source of the differences seen to date in the literature on the DMPC–DSPC system.

Appendix

A simple approach to estimating equilibration times for solid-phase separation is provided by solving the one-dimensional diffusion equation for the initial conditions U_0 =

1 for $X > 0$ and 0 for $X \leq 0$. The solution is $U = {}^1/_2[1 + \text{erf}\ [X/2(Dt)^{1/2}]]$ (Margenau & Murphy, 1956). That is, we can watch how the concentration, U, changes at a point X units away from the boundary $X = 0$. Obviously, equilibrium in this model requires an infinite amount of time, but we can see how long it takes to reach 90 or 99% of the final value for U. For X, we use the size of a domain in the solid phase, 300 nm (Hui & Parsons, 1975). The self-diffusion coefficient, D, has been measured for phospholipids in the low-temperature phase to be 10^{-10} cm^2/s (Fahey & Webb, 1978). Using these values, we find that it takes several minutes to reach 90% of the equilibrium value and several hours to attain 99% of it. We therefore feel that in waiting 24 h in the low-temperature phase we are allowing enough time for solid-phase immiscibility to appear.

References

Fahey, P. F., & Webb, W. W. (1978) *Biochemistry 17*, 3046.
Gent, M. P. N., & Ho, C. (1978) *Biochemistry 17*, 3023.
Hui, S. W., & Parsons, D. F. (1975) *Science 190*, 383.
Jacobs, R. E., Hudson, B. S., & Anderson, H. C. (1977) *Biochemistry 16*, 4349.
Lentz, R., Barenholz, Y., & Thompson, T. E. (1976) *Biochemistry 15*, 4529.
Mabrey, S., & Sturtevant, J. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 3862.
Margenau, H., & Murphy, G. (1956) *The Mathematics of Physics and Chemistry*, Van Nostrand, Princeton, NJ.
Mendelsohn, R., & Maisano, J. (1978) *Biochim. Biophys. Acta 506*, 192.
Nagle, J. F., & Wilkinson, D. A. (1978) *Biophys. J. 23*, 159.
Phillips, M. C., Ladbrooke, B. D., & Chapman, D. (1970) *Biochim. Biophys. Acta 196*, 35.
Priest, R., & Sheridan, J. (1979) *Biophys. J. 25*, 173a.
Shimshick, E. J., & McConnell, H. M. (1973) *Biochemistry 12*, 2351.
Van Dijck, P. W. M., Kaper, A. J., Oonk, H. A. J., & DeGier, J. (1977) *Biochim. Biophys. Acta 470*, 58.
Wilkinson, D. A., & Nagle, J. F. (1978) *Anal. Biochem. 84*, 263.

Electronic Transitions in the Isoalloxazine Ring and Orientation of Flavins in Model Membranes Studied by Polarized Light Spectroscopy†

Lennart B.-Å. Johansson, Åke Davidsson,* Göran Lindblom, and K. Razi Naqvi

ABSTRACT: The orientation of flavin mononucleotide (FMN) in model membranes and the directions of the transition moments of the first three bands in the electronic absorption spectrum of the oxidized form of the isoalloxazine ring have been determined by means of linear dichroism and polarized fluorescence spectroscopy. Measured counterclockwise relative to the axis connecting the two nitrogens in the central ring (considered positive when going in the direction from –CN< to ⩾N), these angles are 58 ± 4° (450-nm band), 97 ± 3° (350-nm band), and 119 ± 2° (260-nm band).

Biological membranes, being well organized structures, owe many, if not all, of their functions to the spatial arrangement of their constituents. Information about molecular orientation is therefore necessary for unravelling the functional mechanisms of a membrane. This pertains in particular to the enzymes (flavoproteins and cytochromes) in the respiratory chain. Here we have studied the flavin mononucleotide (FMN), which is a prosthetic group of many flavoproteins.

A technique that has long been used for studying the orientation of chromophores in biological membranes is linear dichroism (LD) in which one studies the absorption of plane-polarized light by an ensemble of oriented chromophores (Schmidt, 1938; Johansson et al., 1978). Given the knowledge of the orientation, the directions of the transition moments of the different electronic transitions giving rise to various absorption bands can be determined; conversely, if the directions of the transition moments are already known, information regarding the orientation of the chromophore can be extracted. For highly symmetric molecules, the directions of the transition moments are relatively easy to ascertain; unfortunately, flavins, like most other biological chromophores, do not fall in this convenient category, which makes the determination of the directions of the transition moments a demanding task, but a task that must nonetheless be undertaken if the orientation of FMN is to be studied.

In the absorption spectrum of an oxidized flavin, the first three bands, to be denoted here as I, II, and III, occur at 450, 375–330 (depending on the solvent polarity), and 270 nm, respectively; all three have been assigned to $\pi^* \leftarrow \pi$ transitions (Weber, 1966; Palmer & Massey, 1968; Sun et al., 1972; Yu et al., 1976). Though a great deal of attention has been paid to the spectroscopic aspects of the flavins, no previous work has grappled with the problem which is considered here, viz., the location, in the molecular framework, of all three transition moments. To reach this goal, we have studied the fluorescence polarization of lumiflavin, riboflavin, and FMN and the electric linear dichroism (ELD) of lumiflavin. The results of this study have allowed us to grasp, and describe, the orientation of FMN in model membranes.

Materials and Methods

The absorption spectra were recorded on a Cary 118C spectrometer. Measurements of LD and ELD (Davidsson &

† From the Division of Physical Chemistry 2, Chemical Centre, University of Lund, S-220 07 Lund 7, Sweden (L.B.-Å.J. and G.L.), Inorganic Chemistry 1, Chemical Centre, University of Lund, S-220 07 Lund 7, Sweden (Å.D.), and the Department of Physics, NLHT, University of Trondheim, N-7000 Trondheim, Norway (K.R.N.). *Received April 6, 1979.* This work was supported by the Swedish Natural Science Research Council and the Norwegian Research Council for Science and Humanities.

0006-2960/79/0418-4249$01.00/0