Lateral Phase Separation in Phospholipid Membranes†

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ABSTRACT: The phase diagrams of aqueous dispersions of binary mixtures of dimyristoyl-, dipalmitoyl-, and distearoylphosphatidylcholine and dipalmitoylphosphatidylethanolamine have been determined. The method used is based on the partition of the spin label, 2,2,6,6-tetramethylpiperidine-1-oxyl (Tempo) between the fluid hydrophobic regions of the lipids and the aqueous regions. As the hydrophobic regions of the phospholipid mixtures "freeze," Tempo is excluded and its solubility in the lipids decreases. This solubility, measured by means of a Tempo spectral parameter for different lipid mixtures, exhibits abrupt changes in slope at characteristic temperatures corresponding to the onset and completion of lateral phase separations. These temperatures are used to define points on the equilibrium phase diagrams for five binary phospholipid mixtures. A model is presented in which one can calculate the Tempo spectral parameter as a function of temperature from the experimentally determined phase diagrams. Phospholipid phase separations in biological membranes are discussed.

Recent studies have provided evidence that some properties of biological membranes can be understood in terms of a two-dimensional fluid. This evidence is of two types. First, diffusive-like lateral motions of fluorescent-labeled surface antigens (probably glycoproteins) have been observed directly in cells and cell hybrids (Frye and Edidin, 1970; Edidin, 1972). Electron microscope studies of specifically labeled cell surface antigens also show different degrees of clustering in normal cells, as contrasted to trypsin-treated cells and virus transformed tumor cells, again indicating the likelihood of lateral mobility of these surface components (Nicolson, 1972; Singer and Nicolson, 1972). These studies tie in nicely with determinations of rapid lateral diffusion of spin-label phospholipids in model lipid bilayer membranes (Devaux and McConnell, 1972; Träuble and Sackmann, 1972), as well as in biological membranes (McConnell *et al.*, 1972a; Scandella *et al.*, 1972). Thus, a current picture of biological membranes suggests that if tightly bound intrinsic proteins (or glycoproteins) can move laterally in biological membranes, then so must the phospholipids. This is analogous to the diffusion of a solute in an aqueous solvent, which requires mobility of the water molecules. The lateral mobility of the phospholipids is in turn related to the high flexibility of their hydrophobic fatty acid chains, a flexibility that has been extensively studied and documented in both biological membranes and in model membranes (phospholipid bilayers) (Hubbell and McConnell, 1968, 1971; Phillips *et al.*, 1969; Levine and Wilkins, 1971; Luzzati, 1968; Jost *et al.*, 1971). Lateral mobility of phospholipids also indicates that some membrane proteins may have rotational freedom about an axis perpendicular to the membrane surface; optical methods have been used to measure a rotational correlation time of about 10^{-6} sec for rhodopsin in photoreceptor membranes (Brown, 1972; Cone, 1972).

Other evidence for the lateral motion of membrane components is derived from freeze-fracture studies of protein "particles" in various membranes under conditions where they sometimes show a clustering and where under other conditions they show a diffuse distribution (Branton, 1971; Tourtellotte *et al.*, 1970; Pinto da Silva *et al.*, 1971). The possibility of lateral motion serving specific biological functions is raised by the observations of patch and cap formation in lymphocyte and fibroblast membranes under specific stimulation of the cell surface, for example, stimulation by concanavalin A antigens and/or antibodies (Taylor *et al.*, 1971; Yahara and Edelman, 1972; Edidin and Weiss, 1972; see also Betel and van den Berg, 1972).

Taken individually, some of these studies might be criticized for one reason or another; taken collectively, they leave no doubt that the lateral motion of certain components takes place in some regions of some biological membranes.

The second line of evidence for lateral mobility (of phospholipids) is less direct but is almost equally compelling. The isolation of a number of unsaturated fatty acid auxotrophs of *Escherichia coli* has permitted the study of the temperature dependence of the rates of active uptake of specific sugars into cells having a relatively simple fatty acid composition (Overath *et al.*, 1970, 1971; Wilson and Fox, 1971; Schairer and Overath, 1969; Esfahani *et al.*, 1970; Wilson *et al.*, 1970; Fox, 1971, 1972). The temperature dependence of each of these uptake curves shows abrupt changes in slope at certain characteristic temperatures, T^*.[1] Under specified growth schedules involving various temperatures, exogenous unsaturated fatty acids, and time of induction of the sugar permease, the characteristic temperature(s) T^* can depend on the average fatty acid composition of the bacterial membrane, rather than on one or the other of the unsaturated fatty acids present at the time of induction of the sugar permease. This result strongly suggests, but does not prove, that this dependence of T^* on the average fatty acid composition results from a mixing of the various phospholipids present in the membrane by lateral diffusion. Further evidence for this interpretation of these data comes from other studies, where diffusion and intermixing of the different phospholipids can only occur at sufficiently high temperatures (Fox and Tsukagoshi, 1972).

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[1] Such changes in slope are also observed in the activation of ($Na^+ + K^+$)-ATPase by dipalmitoylphosphatidylglycerol (Kimelberg and Papahadjopoulos, 1972) and in the ($Na^+ + K^+$)- and Mg^{2+}-ATPase activity of rat brain microsomes (Gruener and Avi-Dor, 1966).

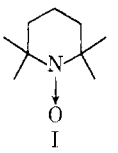
All of these studies obviously rest on the inference that the characteristic temperatures T^* reflect some physical property of the phospholipids, which in turn affects the activity of the sugar permeases. Overath *et al.* (1970) have extracted the phospholipids from such fatty acid auxotrophs and have measured the temperatures at which Langmuir films of these phospholipids change from the "liquid expanded state" to the "condensed state(s)." These "physical" temperatures are closely correlated with the characteristic "physiological" sugar transport temperatures T^*, leaving little doubt that the physical properties of the lipids do play a central role in determining the characteristic temperature T^*. (Studies by Melchior *et al.* (1970) of the lipid "phase transition" in intact *Acholeplasma laidlawii* membranes, and in the extracted lipids, also show that the temperature of the bulk lipid phase transition is not greatly perturbed by the presence of membrane proteins.) However, the studies by Overath *et al.* (1970) still leave open the question of the precise physical interpretation of the characteristic temperatures T^*. In fact, it has recently been shown that in the unsaturated fatty acid auxotrophs of *E. coli* supplied with a single exogenous unsaturated fatty acid, there are always at least *two* characteristic temperatures T^* (Linden *et al.*, 1973). (Also see a later section titled Biological Significance in the present paper.) On the basis of spin-label studies of bacterial membranes given elsewhere (Linden *et al.*, 1973), as well as on the basis of the present paper, it seems highly probable that these characteristic temperatures can be understood in terms of the phase diagrams of two-dimensional lipid (and lipid–protein) systems. The present paper is concerned with showing how the paramagnetic resonance spectra of spin labels can be used to determine the phase diagrams of pure binary, essentially two-dimensional lipid membranes.

In contrast to "phase transitions" in pure substances, the existence of phase diagrams of the type considered in the present paper *requires* lateral diffusion of the membrane components and the separation (in two dimensions) of a relatively *solid* phase at lower temperatures.

In earlier studies, Phillips *et al.* (1970) have reported two binary phase diagrams of the type studied in the present paper, using the technique of differential scanning calorimetry. Comparisons between their results and ours are discussed later.

Lipid phase diagrams thus established can be correlated not only with other spin-label studies of intact biological membranes (Linden *et al.*, 1973; Mehlhorn and Keith, 1972), but also can be used to facilitate quantitative comparisons of recent studies of pure lipid mixtures and biological membranes using freeze-fracture, electron microscopic techniques. Very recent freeze-fracture studies of lipid mixtures and biological membranes appear to provide direct and dramatic evidence for the lateral phase separations considered in the present paper (Verkleij *et al.*, 1972; Ververgaert *et al.*, 1972).

Our approach to this problem follows closely from our earlier studies of the distribution of the spin label, 2,2,6,6-tetramethylpiperidine-1-oxyl or Tempo (I),[2] between the aqueous and the fluid hydrophobic phases which are simultaneously present when biological membranes (or phospholipids) are dispersed in water (Hubbell and McConnell, 1968). In these earlier studies it was shown that this fluid hydrophobic phase is provided by the flexible fatty acid chains of phospholipids at physiological temperatures, but that at lower temperatures where the chains are less flexible the Tempo solubility can be greatly reduced. In fact, this Tempo solubility in the fluid hydrophobic phase has been used as an assay for the fraction of the lipids in a biological membrane that have chains in a highly flexible or fluid state (McConnell *et al.*, 1972b; Metcalfe *et al.*, 1972). Since the problem under consideration in the present paper involves the lateral separation and growth of phases having chains with differing flexibilities, we have used this Tempo solubility to study these phase separations.



I

Materials and Methods

O-(1,2-Dipalmitoyl-*X*-glycero-3-phosphoryl)choline (DPPC) was purchased from Schwarz-Mann and *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)ethanolamine (DPPE) was purchased from Calbiochem. Traces of impurities were detected by thin-layer chromatography in the DPPC and small amounts (on the order of 1%) were found in the DPPE; each was used without further purification. *O*-(1,2-Dimyristoyl-*sn*-glycero-3-phosphoryl)choline (DMPC) and *O*-(1,2-distearoyl-*sn*-glycero-3-phosphoryl)choline (DSPC) were synthesized by the method of Cubero Robles and van den Berg (1969) from *O*-(*sn*-glycero-2-phosphoryl)choline (available as the $CdCl_2$ adduct from Sigma) and the anhydride and sodium salt of myristic and stearic acids. The products were purified on a silica gel column (Bio-Sil A, 100–200 mesh) eluted with chloroform–methanol. Stock solutions of the phosphatidylcholines in ethanol were prepared, and their concentrations were determined by the method of McClare (1971). In order to determine their configuration, the phospholipids were hydrolyzed with phospholipase A by the method of van Deenen and de Haas (1963) and the products examined by thin-layer chromatography. Only the DPPC appears to be a mixture of the two configurations, with an estimated 80% of the 3-*sn*-phosphatidylcholine present.

The interior of a 10-ml round-bottom or pear-shaped flask was coated with a thin film of phospholipids by the evaporation to dryness under vacuum of an ethanol or chloroform solution containing approximately 35–40 μmol of the desired mixture of lipids; 200–300 μl of 0.01 M sodium phosphate buffer at pH 7.0 and a glass bead were then added to the flask which was then shaken vigorously on a Vortex mixer for several minutes. The temperature during agitation was kept above the higher transition temperature of the two lipids. A 5×10^{-3} M aqueous Tempo solution (30 μl) was added to the phospholipid dispersion which was then transferred to a 50-μl capillary pipet used as a sample cell. The final lipid concentration was approximately 8–11 wt %.

The sample cell was sealed and centered in a hollow Kel-F cylindrical sample holder, 23 cm in length, 0.8-cm i.d., and 1.1-cm o.d., which was then inserted in the microwave cavity. Silicone oil (Dow Corning 200 electronic fluid) was pumped through the Kel-F tube by a Haake constant-temperature circulator.

The temperature could be controlled to within $\pm 0.25°$ over a range of 20–70° and was measured with a Smith–Florence potentiometric microvoltmeter and a copper–con-

[2] Abbreviations used are: Tempo, 2,2,6,6-tetramethylpiperidine-1-oxyl (structure I); DPPC, *O*-(1,2-dipalmitoyl-*X*-glycero-3-phosphoryl)-choline; DPPE, *O*-(1,2-dipalmitoyl-*sn*-glycero-3-phosphoryl)ethanolamine; DMPC, *O*-(1,2-dimyristoyl-*sn*-glycero-3-phosphoryl)choline; DSPC, *O*-(1,2-distearoyl-*sn*-glycero-3-phosphoryl)choline.

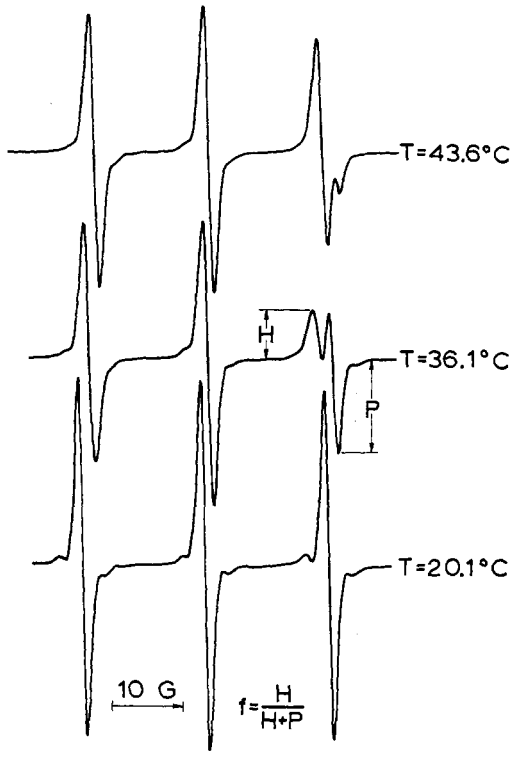


FIGURE 1: Paramagnetic resonance spectra of Tempo dissolved in an aqueous dispersion of DPPC (201 mg/ml) above the primary transition temperature, between the pretransition and primary transition temperatures, and below the pretransition temperature.

stantan thermocouple immersed in the silicone oil near the sample cell.

All spectra were obtained on a Varian E-12 spectrometer at *X* band with the microwave cavity oriented such that the long axis of the sample cell was horizontal. This was done in order to minimize the settling of the phospholipid liposomes in the sample tube. In most of the experiments, spectra were obtained as a function of temperature with a cooling rate no greater than 5–10°/hr.

Results

First derivative paramagnetic resonance spectra of Tempo in aqueous dispersions of DPPC at three temperatures are illustrated in Figure 1. Each spectrum is a superposition of two spectra: one is due to Tempo dissolved in the fluid, hydrophobic region of the lipid; the other is due to Tempo in the aqueous phase. Small differences in the isotropic hyperfine coupling constants and *g* factors for the spin label in each environment result in a partial resolution of the high-field hyperfine line, whereas the low and middle field lines are not resolved.

Experimentally we measure two amplitudes, H and P, of the high-field nitroxide hyperfine signals which are labeled in Figure 1. To a first approximation, H is proportional to the amount of spin label dissolved in the membrane bilayer and P is proportional to the amount dissolved in the aqueous region. The change in the relative values of H and P as a function of temperature for DPPC reflects the fact that, in the presence of excess water, this phospholipid undergoes a transition from a "gel phase" to a lamellar smectic liquid crystalline phase (Chapman *et al.*, 1967). According to Luzzati *et al.* (1972), this transition is between a lamellar phase having

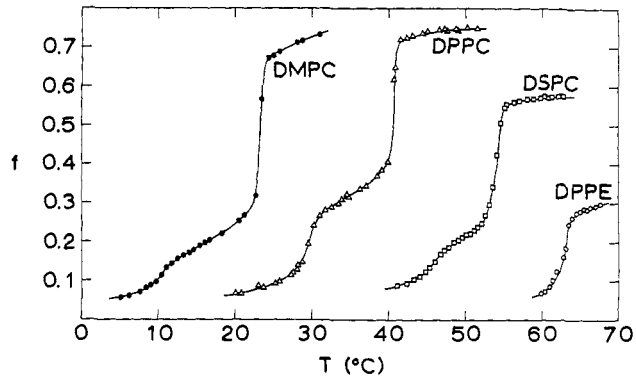


FIGURE 2: The Tempo spectral parameter, *f*, *vs.* temperature for aqueous dispersions of: (●) DMPC (108 mg/ml), (△) DPPC (201 mg/ml), (□) DSPC (124 mg/ml), and (○) DPPE (80 mg/ml).

flexible fatty acid chains (designated L_α) and a lamellar phase having stiff extended chains (designated L_β). In the case of the phosphatidylcholines, the phase having relatively stiff extended chains is sometimes referred to as the "gel" phase, where the hydrocarbon chains are packed into an hexagonal subcell. In the present work we prefer to use a less specific terminology, S and F, where S refers to a region of a two-dimensional bilayer membrane in which the fatty acid chains are stiffer or less fluid than in the contiguous F phase, where the fatty acid chains are more flexible or fluid.

This phase transition has been detected and studied by diverse physical techniques (Chapman and Dodd, 1971; Phillips, 1972; Oldfield and Chapman, 1972) such as differential scanning calorimetry (Chapman *et al.*, 1967; Ladbrooke and Chapman, 1967; Hinz and Sturtevant, 1972), X-ray diffraction (Chapman *et al.*, 1967; Dupont *et al.*, 1972), electron paramagnetic resonance (Hubbell and McConnell, 1971; Barrat *et al.*, 1969; Jost *et al.*, 1971; Metcalfe *et al.*, 1972; Sackmann and Träuble, 1972; Mehlhorn and Keith, 1972), nuclear magnetic resonance (Levine *et al.*, 1972; Sheetz and Chan, 1972; Oldfield and Chapman, 1971), fluorescence spectroscopy (Träuble, 1971; Vanderkooi and Chance, 1972), and light scattering (Träuble, 1971).

The transition temperature depends strongly on both the length and degree of unsaturation of the fatty acid chains and also on the nature of the phospholipid head group (Chapman and Wallach, 1968; Ladbrooke *et al.*, 1968; Phillips *et al.*, 1969). For the saturated chain lipids, the transition temperature increases as the length of the hydrocarbon chain increases (Chapman *et al.*, 1967).

In Figure 2, plots of a Tempo spectral parameter f, equal to $H/(H + P)$, which is approximately the fraction of spin label dissolved in the membrane bilayer, as a function of temperature for four phospholipids dispersed in excess buffer, exhibit abrupt decreases in the magnitude of the spectral parameter at temperatures corresponding to the calorimetrically measured S-phase to F-phase transition temperatures. These calorimetry temperatures for the phosphatidylcholines (Hinz and Sturtevant, 1972) and for the phosphatidylethanolamine (Abramson, 1970) are given below in parentheses. DMPC with an acyl chain length of 14 carbons has a transition temperature, defined as the midpoint of the Tempo transition curve, of 23.2° (23.70°); that of DPPC with a chain length of 16 is 40.5° (41.75°); and the transition temperature of DSPC with 18 carbons is 54.0° (54.24°). DPPE, on the other hand, has a transition temperature of 63.0° (62–65°).

This reduction of the spectral parameter for Tempo in

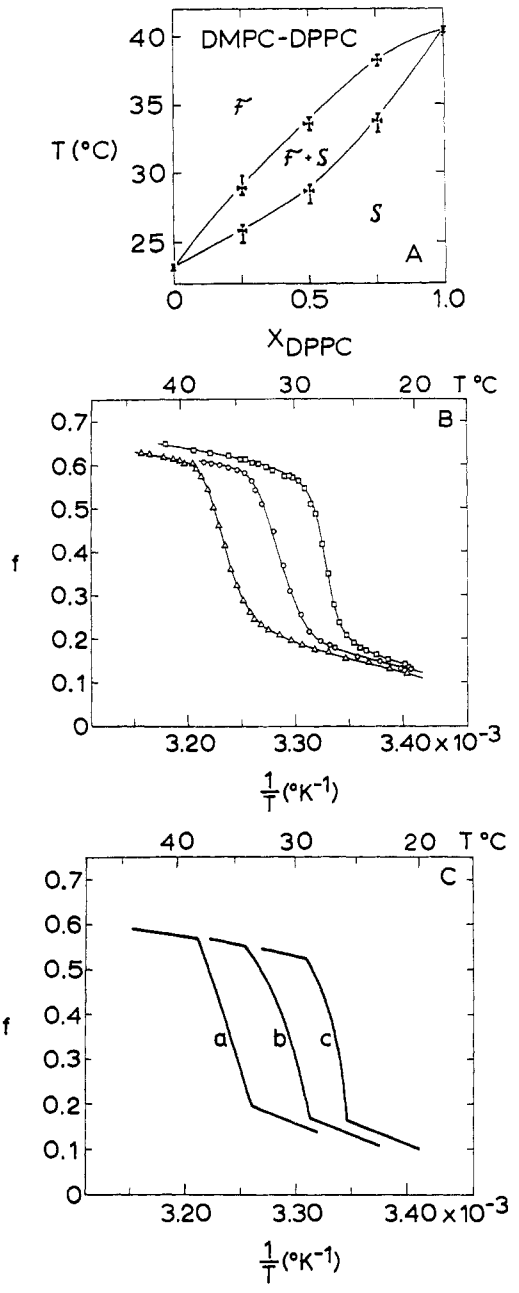


FIGURE 3: (A) The fluid–solid equilibrium phase diagram for aqueous dispersions of the DMPC–DPPC binary system: temperature *vs.* mole fraction of DPPC. The regions of the phase diagram containing F phase only, an equilibrium mixture of F and S phases, and S phase only are indicated. (B) Experimental Tempo spectral parameter, f, as a function of $1/T$ for: (△) 76 mol % DPPC, (○) 51 mol %, and (□) 26 mol %. (C) Calculated Tempo spectral parameter, f, as a function of $1/T$ for (a) 75 mol % DPPC, (b) 50 mol %, and (c) 25 mol %.

dispersions of phospholipids which undergo these phase transitions can be interpreted as an exclusion of the spin label from the fluid, hydrophobic regions of the lipids upon "solidification."

Immediately below this sharp transition, there is still an appreciable Tempo solubility in the hydrophobic regions of each of the three phosphatidylcholine dispersions. This solubility is almost totally eliminated in a secondary transition which occurs at a lower temperature, has a greater width than the primary transition, and gives rise to a smaller decrease in the spectral parameter. This secondary transition corresponds to the "pretransition" observed with differential scanning calorimetry (Ladbrooke and Chapman, 1967; Hinz and Sturtevant, 1972). We observed "pretransition" temperatures of 46.1° (49.1°) for DSPC, 29.5° (34.0°) for DPPC, and 10.1° (13.5°) for DMPC. This pretransition is not observed in DPPE dispersions.

Ladbrooke and Chapman (1967) and Chapman *et al.* (1967) have associated this pretransition with the motion of the polar head groups of the lipids. Whatever this transition is due to, our data show that this pretransition produces effects which are not limited solely to the bilayer surface, but are also extended throughout the bilayer. Because of the enhanced Tempo solubility in the hydrophobic region above the pretransition it is possible that the phospholipid lattice is in a more expanded array above this transition than it is below.

The concentrations of spin label used in these experiments are sufficiently small such that no appreciable reduction of the primary phase transition temperature occurs. The transition temperature of DPPC was unchanged, to within experimental error, for DPPC to Tempo mole ratios of 400 to 800. Since only about half of the Tempo is dissolved in the lipid bilayer, the DPPC to spin label ratio *in* the membrane bilayer is actually double the above.

Figures 3B and 5B to 7B show plots of the Tempo spectral parameter as a function of $1/T$ for binary mixtures of phospholipids. We interpret each of these curves in terms of a phase diagram which describes a two-dimensional equilibrium between an F phase and an S phase, each having a different phospholipid composition. This phase separation is a two-dimensional phase separation in which the two phases are contiguous in the plane of the phospholipid bilayer. This process cannot be a three-dimensional physical separation of the lipids into different (noncontinuous) bilayers of F and S phase, since the rate of fusion of purified phospholipid bilayer vesicles and liposomes (and thus the fission rate also) is very slow under our experimental conditions (Phillips *et al.*, 1970; Taupin and McConnell, 1973; Kornberg and McConnell, 1971), whereas the lateral diffusion rate of phospholipids is very fast. When the temperature of a dispersion of a mixture of phospholipids of a specific composition, which is initially in the F phase, is reduced slowly, a small amount of S phase (in equilibrium with the F phase) forms at a characteristic temperature, T_f (see Figure 8A). This phase separation, characterized by the formation of regions of S phase surrounded by regions of F phase continues to increase as the temperature is reduced until, finally, all of the lipids are in the S phase, at temperatures equal to and below T_s. The onset of the phase separation results in a decrease in the number of spin labels dissolved in the membrane and a decrease in the Tempo parameter. This decrease is not as pronounced as the sharp transition that exists for a pure component but is, however, readily observable as an abrupt change in slope of the spectral parameter–temperature curve. We also observe a similar break in the slope at a second, lower temperature. These abrupt changes in slope occur at the onset and completion of phase separation at definite temperatures, T_f and T_s, for a specific phospholipid composition, on the *fluidus* and *solidus* curves, respectively, of an equilibrium phse diagram. In a number of cases there appear to be more than two abrupt changes in slope in the spectral parameter–temperature curves. However, the analysis of the shapes of the Tempo parameter curve given below indicates that only the high- and low-temperature breaks define the points, T_f and T_s. The shape of the curve between these two points, including the existence of any other inflection points, depends on the specific details of

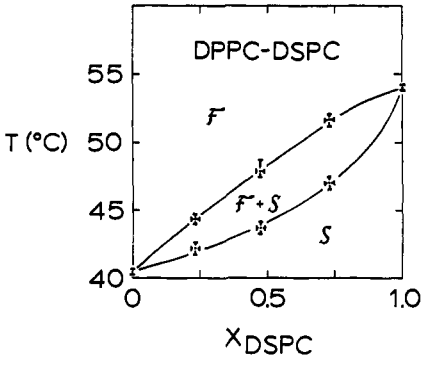


FIGURE 4: Phase diagram for aqueous dispersions of the DPPC–DSPC binary system.

phase diagrams, that is, on the shapes of the *fluidus* and *solidus* curves between the two extreme temperatures.

An assumption made in the present work is that, throughout the course of each experiment, the S phase is uniform in composition and is in equilibrium with the coexisting F phase (except when more than a single S phase is present, see below). This equilibrium between the composition of the F phase and the composition of the *entire* S phase can be achieved by phospholipid lateral diffusion in the F phase (which is known) and sufficiently rapid phospholipid lateral diffusion in the S phase. (Equilibrium within the S phase would also be achieved if the boundaries between the F and S phases were to undergo sufficiently rapid lateral motion.) Our evidence for composition equilibrium within the S phase is discussed at the end of this section of the present paper. The assumption of uniform *solidus* composition is often made in discussions of phase diagrams, but is not always valid (Findlay, 1951; Zernike, 1955).

The Tempo spectral parameters for all of the binary lipid mixtures were plotted as a function of the reciprocal of the absolute temperature. This was done primarily because we wished to compare results from model systems of this type with the temperature-dependent behavior of transport processes in the membranes of bacterial fatty acid auxotrophs, where such plots have been found to be convenient (Overath *et al.*, 1970; Wilson and Fox, 1971).

Binary mixtures of DMPC and DPPC exhibit spectral parameter–temperature curves in Figure 3B, with very distinct high- and low-temperature changes in slope. Their shapes can be qualitatively described as broadened transition curves. T_f and T_s are determined by the intersections of straight lines which are drawn through the three distinct regions in each curve. The phase diagram derived from these straight line intersections is illustrated in Figure 3A. This phase diagram is one in which the lipids form a complete range of *solid* or S phase solutions.[3]

The phase diagram for the DPPC–DSPC system is illustrated in Figure 4. These mixtures exhibit Tempo solubility curves (not shown) similar to those in Figure 3B; and, in fact, such spectral parameter *vs.* $1/T$ curves with only two breaks in slope appear to be characteristic of those lipid systems which

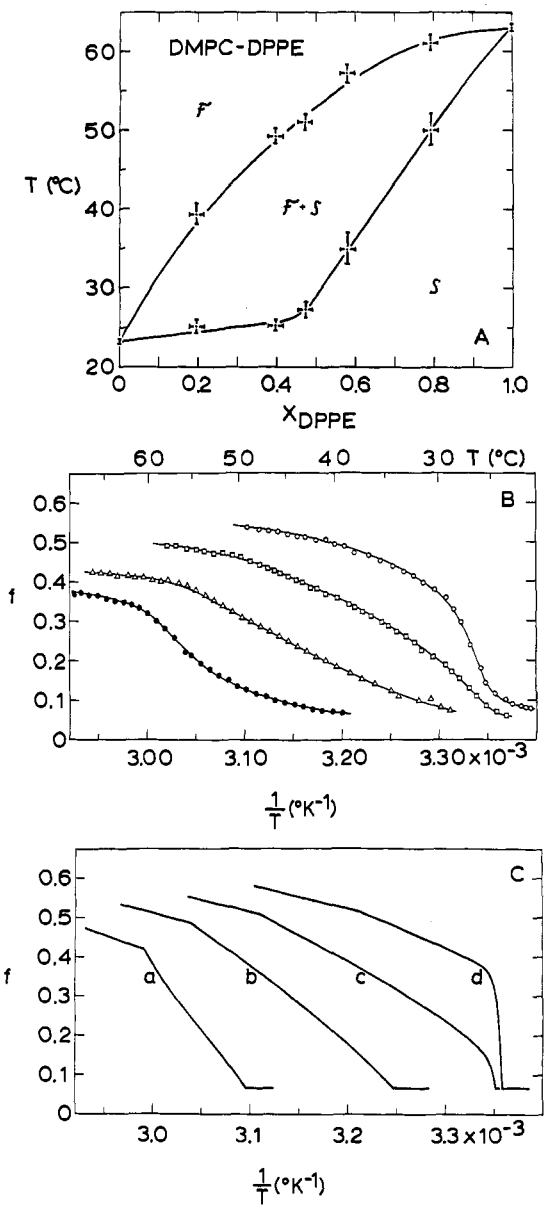


FIGURE 5: (A) Phase diagram for aqueous dispersions of the DMPC–DPPE binary system. (B) Experimental Tempo spectral parameter, f, as a function of $1/T$ for: (●) 79 mol % DPPE, (△) 58 mol %, (□) 40 mol %, and (○) 20 mol %. (C) Calculated Tempo spectral parameter, f, as a function of $1/T$ for (a) 79 mol % DPPE, (b) 58 mol %, (c) 40 mol %, and (d) 20 mol %.

have phase diagrams that indicate complete S-phase miscibility, as in Figures 3A and 4.

Binary mixtures of DMPC–DPPE and DPPC–DPPE have Tempo spectral parameter curves and phase diagrams given in Figures 5A,B and 6A,B. These phase diagrams indicate that phosphatidylcholine and phosphatidylethanolamine mixtures do not form a complete range of S-phase solutions but instead have a limited S-phase miscibility.[3] The spectral parameter curves are moderately complex and their shapes depend on the relative amounts of each lipid in the mixture. At high DPPE concentrations, they resemble curves obtained for the systems which have complete S-phase miscibility; at lower concentrations, their shapes are radically different. The high- and low-temperature abrupt change in slope were obtained by the straight-line extrapolation procedure described above.

[3] Immiscibility within the S phase is suggested by two features of these phase diagrams. A discontinuity in slope of the *solidus* curve of a phase diagram is usually the point of intersection of a third line representing immiscibility of the S-phase components. In the present work, we have no means of determining the position of this third line. Also, a horizontal *solidus* line indicates immisicbility within the S phase and this condition is approached in some of these phase diagrams.

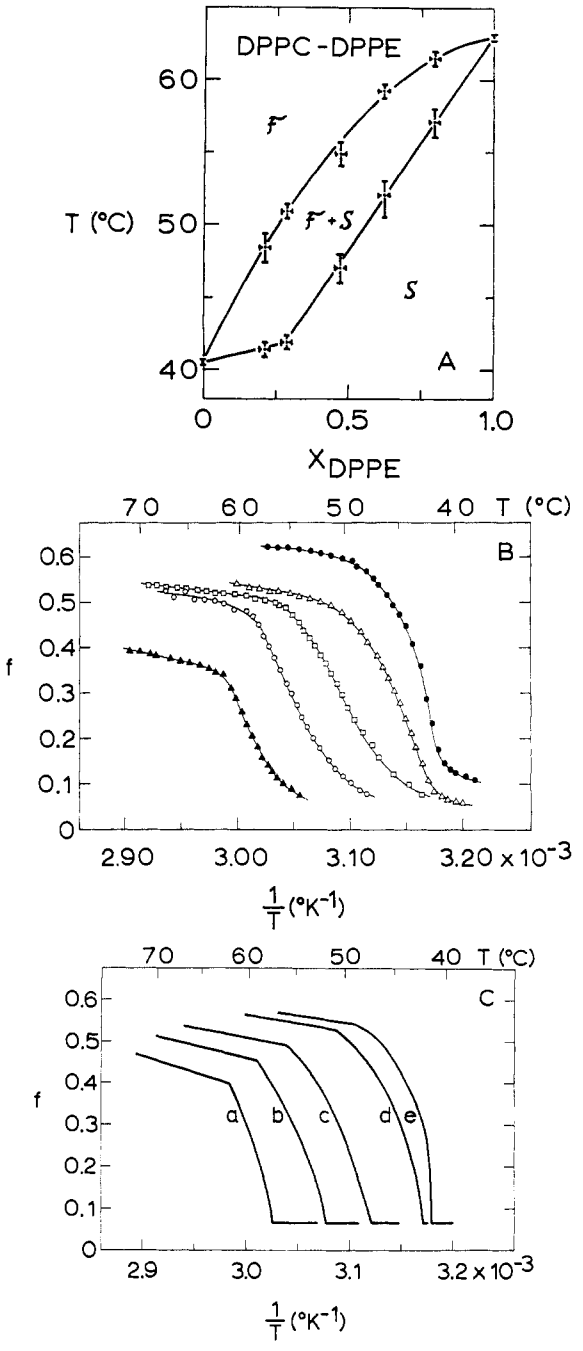


FIGURE 6: (A) Phase diagram for aqueous dispersions of the DPPC–DPPE binary system. (B) Experimental Tempo spectral parameter, f, as a function of $1/T$ for: (▲) 80 mol % DPPE, (○) 62 mol %, (□) 47 mol %, (△) 28 mol %, and (●) 21 mol %. (C) Calculated Tempo spectral parameter, f, as a function of $1/T$ for: (a) 80 mol % DPPE, (b) 62 mol %, (c) 47 mol %, (d) 28 mol %, and (e) 21 mol %.

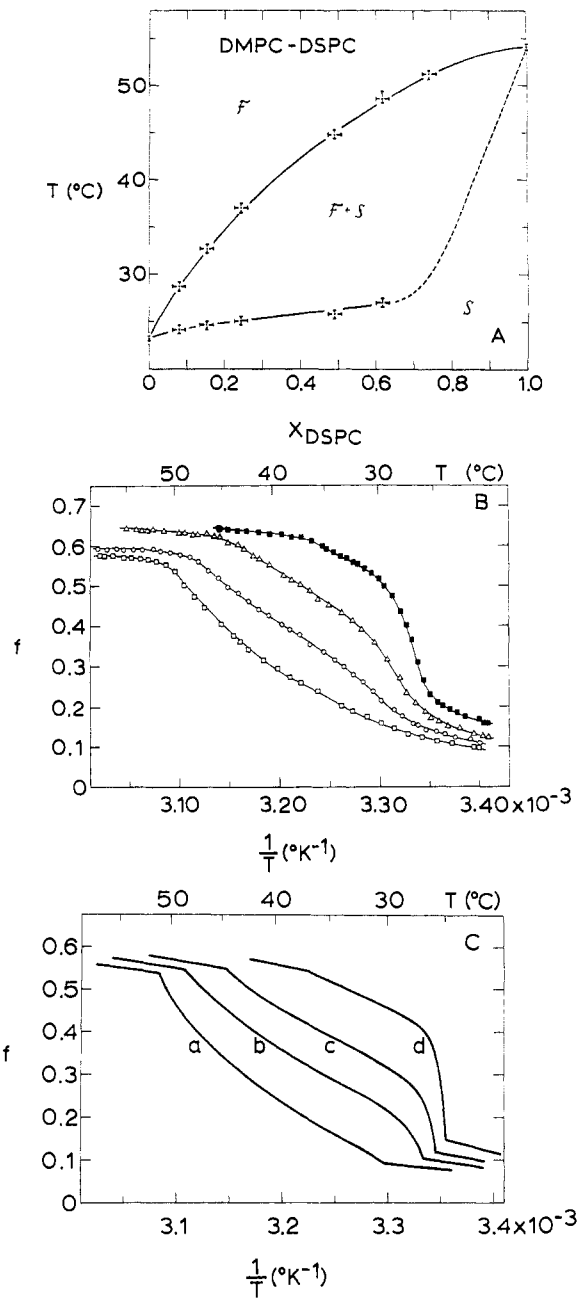


FIGURE 7: (A) Phase diagram for aqueous dispersions of the DMPC–DSPC binary system. (B) Experimental Tempo spectral parameter, f, as a function of $1/T$ for: (□) 74 mol % DSPC, (○) 62 mol %, (△) 49 mol %, and (■) 24 mol %. (C) Calculated Tempo spectral parameter, f, as a function of $1/T$ for: (a) 75 mol % DSPC, (b) 63 mol %, (c) 50 mol %, and (d) 25 mol %.

Binary mixtures of DMPC–DSPC show the phase diagram given in Figure 7A which indicates an S-phase immiscibility.[8] The Tempo spectral parameter curves for these mixtures in Figure 7B have at least three relatively abrupt changes in slope. At high DSPC concentrations, the low-temperature break is not observed. As a result, the *solidus* curve is indeterminate in the region where the DSPC mole fraction is greater than about 0.62. This effect may be due to a negligible difference in spin-label solubility in the S phase and in the F phase at these lower temperatures so that the discontinuity in the spectral parameter curve is not discernible. This same region of the phase diagram is also undefined in scanning calorimetry measurements (Phillips *et al.*, 1970).

As mentioned above, in our experiments, we do not have separate bilayers composed of only the pure lipids since transitions for each of the pure components are not observed in the lipid mixtures used to determine the phase diagrams. These same conclusions were reached by Phillips *et al.* (1970). However, if DMPC and DPPC are mixed in a dry state and then dispersed in buffer, distinct bilayers are formed. The spectral parameter curve then shows two transitions associated with each of the pure components.

The spectral parameter–temperature curves in general are reversible, although occasionally we observed a slight hys-

teresis of about 1°. This, however, may have been due to an excessive rate of heating. These results are consistent with the assumption that our measurements do refer to the thermal properties of the lipid mixtures at equilibrium.

We have also performed some preliminary temperature-jump experiments on the kinetics of the melting process in these mixed lipid systems. (Dupont *et al.* (1972) have studied by X-ray diffraction the kinetics of the thermal transitions in lipoprotein and bacterial membranes.) Our experiments have involved, for example, temperature jumps from a point below the *solidus* curve of an equimolar DMPC–DPPC mixture to a higher temperature, above the *liquidus* curve, or between the *liquidus* and the *solidus* curves. In both cases, the system appears to come to equilibrium within 10–20 sec, or less, as judged by the time dependence of the Tempo spectral parameter. One mechanism for this equilibration could involve lateral diffusion within the S phase (together with the known rapid lateral diffusion within the F phase) and/or lateral motion of the S phase–F phase boundaries. We emphasize that even though we have referred to the S phase as "solid" in the sense of having stiffer and more extended fatty acid chains for the phospholipids, the rate of lateral diffusion in this phase may nevertheless be high.

Quantitative Analysis

In order to check our phase diagrams (deduced from the temperatures of abrupt changes in slope of the Tempo spectral parameter) with all the spectral data, we can work backward from the phase diagrams and make approximate calculations of the Tempo spectral parameters as a function of temperature.

From the definition of f, we make the approximation

$$f = n_h/(n_h + n_p) \tag{1}$$

where n_h is the number of moles of Tempo dissolved in the hydrophobic membrane bilayer and n_p is the number of moles of Tempo dissolved in the polar aqueous phase. This expression would be exact if f were determined, for example, from the integrated absorptions of the two components using a spectrometer that operated at 35 GHz, where the high-field hyperfine lines are almost completely resolved. The principal errors in eq 1 arise from signal overlap, line-width differences, and neglect of the underlying ^{13}C hyperfine satellites. The solubilities of the spin label in the lipid and aqueous phases can be related by a partition coefficient, K

$$K = n_h V_p/n_p V_h \tag{2}$$

where V_h and V_p are the volumes of the lipid and aqueous regions, respectively. A combination of the above definitions leads to an equation for the spectral parameter

$$= \frac{KV_h/V_p}{1 + KV_h/V_p} \tag{3}$$

There is an appreciable Tempo solubility in the S phase. In order to account for this, we consider partition coefficients, K_S, for the S phase, and K_F, for the F phase, and assume that these can be combined linearly to give the observed partition coefficient. Thus, we assume

$$KV_h = K_F V_{Fh} + K_S V_{Sh} \tag{4}$$

where V_{Sh} and V_{Fh} are the volumes of the lipids in the S and F phases, respectively.

Let F_F be the "fraction of the lipids in the F phase"

$$F_F = N_{Fh}/N_h \tag{5a}$$

$$\approx V_{Fh}/V_h \tag{5b}$$

where N_{Fh} is the number of moles of lipid in the F phase, and N_h is the total number of moles of lipid. The approximation (5b) neglects differences in molecular weights of the lipids and the densities of the different phases. Therefore

$$K = K_F F_F + K_S(1 - F_F) \tag{6}$$

and

$$f = \frac{[K_F F_F + K_S(1 - F_F)]V_h/V_p}{1 + [K_F F_F + K_S(1 - F_F)]V_h/V_p} \tag{7}$$

Since K_F is usually very much greater than K_S, the spectral parameter decreases as the fraction of "frozen" lipids increases.

For a binary mixture of phospholipids, F_F can be calculated by material balance directly from the phase diagram.

$$F_F = (X^0 - X_S)/(X_F - X_S) \tag{8}$$

At a temperature at which the F and S phases are in equilibrium, X_F is the mole fraction of the higher melting component in the F phase, and X_S is the mole fraction of that component in the S phase. X^0 is the (total) mole fraction of the higher melting component. In Figure 8B, F_F is calculated as a function of temperature for several values of X^0 for a phase diagram which represents complete *solid* miscibility. In Figure 9, F_F is also calculated for a phase diagram representing complete immiscibility in the *solid* phase. The shape of the F_F curve clearly determines the shape of the spectral parameter curve and the number of discontinuities in slope. For the systems we have examined, the shape of fraction fluid curve ranges between the two extreme cases given in Figures 8B and 9B.

The partition coefficients for the S and F phases can be assumed to be linear combinations of the partition coefficients for each of the pure components in these phases. Thus, for two components, A and B, of which B is the higher melting, the partition coefficients for Tempo in the pure components in the F phase, K_{FA} and K_{FB}, and those in the S phase, K_{SA} and K_{SB}, are combined to obtain K_F and K_S

$$K_F = (1 - X_F)K_{FA} + X_F K_{FB}$$

and (9)

$$K_S = (1 - X_S)K_{SA} + X_S K_{SB}$$

Partition coefficients can be derived from the spectral parameter–temperature plots for the pure phospholipids above and below their transition temperatures. These coefficients can be represented by the empirical equation $K = c - (d/T)$, where c and d are constants. The partition coefficients for the pure components in the F phase are: DMPC, 107–29,000/T; DPPC, 63–15,500/T; DSPC, 52–13,800/T; and

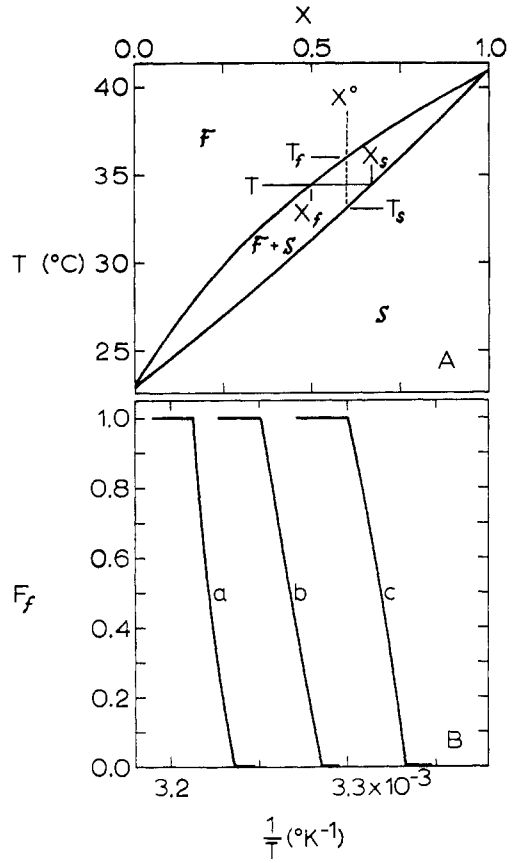


FIGURE 8: (A) A model phase diagram for two components which are completely miscible in the S phase. The regions in which the F and S phases are stable are labeled. For a constant composition, X^0, the temperatures corresponding to the onset and completion of phase separation, T_F and T_S, are shown. For a constant temperature, the compositions of the F and S phases in equilibrium, X_F and X_S, are indicated. (B) The mole fraction of the binary mixture in the F phase, F_F, as a function of $1/T$ for mole fractions of the higher melting component, X^0, equal to: (a) 0.75, (b) 0.50, and (c) 0.25.

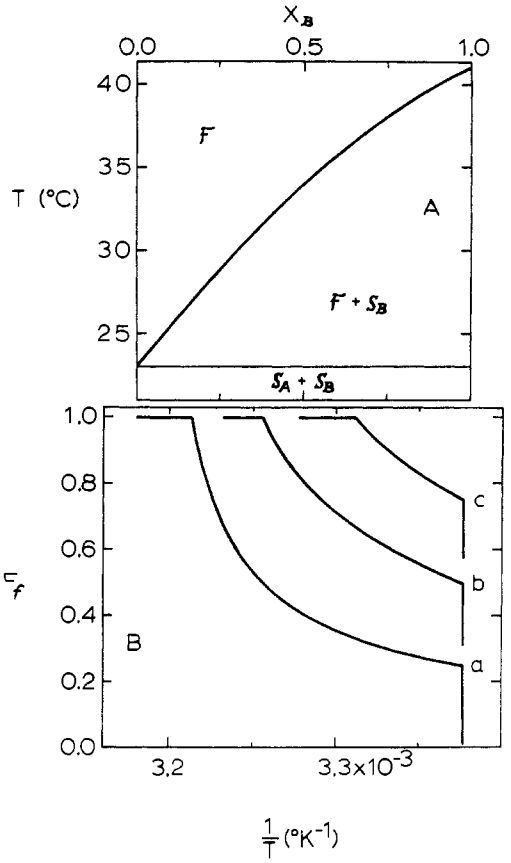


FIGURE 9: (A) A model phase diagram for two components, A and B, which are completely immiscible in the S phase. The regions consisting of F phase, an equilibrium mixture of F and S_B phases, and a heterogeneous mixture of S_A and S_B phases are indicated. (B) The mole fraction of the mixture in the F phase, F_F, as a function of $1/T$ for mole fractions of the higher melting component, X^0, equal to: (a) 0.75, (b) 0.50, and (c) 0.25. Immediately above the transition temperature of the lower melting component, F_F is equal to $1 - X^0$.

DPPE, 80–25,400/T. The choice of the partition coefficients for each of the pure components in the S phase depends on the nature of both of the phospholipids in the binary lipid system. For mixtures of phosphatidylcholines, the effective partition coefficients in the S phase are assumed to be: DMPC, 42–11,900/T; DPPC, 49–14,400/T; and DSPC, 0.4. For mixtures of phosphatidylcholine and phosphatidylethanolamine, on the other hand, we observed that the Tempo solubility in the S phase is effectively eliminated by the addition of small amounts of the phosphatidylethanolamine. (Oldfield and Chapman (1972) also observed that the pretransition in DMPC is suppressed by the addition of dimyristoylphosphatidylethanolamine.) As a result, we assume that the S-phase partition coefficient for each of the pure components in such mixtures is a small number, chosen as 0.7, in order to calculate values for the S-phase spectral parameter close to 0.05, which is approximately the minimum value observed experimentally.

We have calculated spectral parameter *vs.* $1/T$ curves from our experimentally derived phase diagrams and have compared them with the experimental spectral parameter curves in order to check the validity of our phase diagrams. The compositions of the F and S phases were determined at 1° intervals from our measured phase diagrams. These values were used to calculate K_F, K_S, and F_F at each temperature for several mixtures of lipids. The spectral parameter, f, was then calculated at these temperatures for a ratio V_h/V_p equal to 0.1. Plots of f as a function of reciprocal temperature are given in Figures 3C, 5C, 6C, and 7C for four binary lipid systems. Since the *solidus* curve for the DMPC–DSPC system is indeterminate at high DSPC concentrations, we have used, for purposes of calculation only, the dashed portion of the curve indicated in Figure 7A. This region is critical for the determination of the exact shape of the spectral parameter curve. When a calculation was done for the *solidus* curve extrapolated linearly for DSPC mole fractions greater than 0.62, the theoretical and experimental curves were in striking disagreement. Thus, it is highly probable that the *solidus* curve for this sytem at high DSPC concentrations is similar to, but not necessarily identical with, the dashed portion shown in Figure 7A. This behavior would be analogous to that of the *solidus* curves for DMPC–DPPE and DPPC–DPPE. The agreement between the calculated and experimental spectral parameter curves for all four systems is not exact but is quite good considering the approximations involved. Since the shape of the spectral parameter curve depends on the shapes of the *fluidus* and *solidus* curve between T_F and T_S, we are justified in concluding that we have measured the equilibrium phase diagrams of these lipid systems as determined by the temperatures at which abrupt changes in the spectral parameter slopes occur.

Discussion

The *solidus* curve in the phase diagram of binary mixtures of phosphatidylcholines that have acyl chain lengths differing by only two carbons has a continuously varying slope, and thus the S phase is one in which the lipids form a continuous range of solutions.[3] The *solidus* curves for mixtures of phosphatidylcholine and phosphatidylethanolamine that have the same chain lengths or which differ by two carbons have an apparent discontinuity in slope. This discontinuity occurs at approximately 30% DPPE in DPPE–DPPC mixtures and 50% DPPE in DPPE–DMPC. Such a discontinuity indicates a limited S-phase miscibility in which the area below the *solidus* curve is composed of regions containing only *solid* solutions or only *solid* heterogeneous mixtures. The region under the *solidus* curve above and to the right of the discontinuity is a *solid* solution of phosphatidylcholine and phosphatidylethanolamine. The nature of the region below and to the left of that point, however, is undetermined. At the present time, we have not determined with sufficient precision the shape of the *solidus* curve at low phosphatidylethanolamine concentrations and, consequently, cannot determine the phase equilibria in the S phase at these concentrations. If the precise shape of the *solidus* curve were known, we could decide among such possibilities as *solid* phase compound formation, equilibria between two *solid* solutions, or equilibria between a pure *solid* component and a *solid* solution.

Phillips *et al.* (1970) have published phase diagrams for the DMPC–DSPC and DPPC–DSPC systems. These mixtures contain 50 wt % lipid as opposed to the 10 wt % used in our experiments. Phillips' *et al. fluidus* curve for DMPC–DSPC is 3–5° higher than ours over the entire composition range. The *solidus* curves agree, to within experimental error, up to approximately 60 mol % DSPC. The calorimetrically measured curve is indeterminate beyond about 80 mol % DSPC whereas our curve is indeterminate beyond about 62 mol %.

Phillips *et al.* report a DPPC–DSPC phase diagram in which the *fluidus* and *solidus* curves are shifted to higher temperatures relative to our curves at high DSPC concentrations. However, they report a transition temperature of 58° for DSPC instead of the 54° we observe.[4] The temperature difference between the *fluidus* and *solidus* curves is 2–3° greater at high DSPC concentration in our experiments than in theirs, whereas it is about 2° smaller at low DSPC concentrations.

In more recent work Phillips *et al.* (1972) have pointed out that binary mixtures of phosphatidylcholines whose saturated chains differ by only two carbon atoms show broad endothermic gel to liquid-crystal phase transitions, and that such mixtures give ideal mixing and a single homogeneous "gel" phase. The present work is in agreement with this conclusion providing the experimental conditions are such that equilibrium within the S phase (or "gel" phase) is maintained through the heating and cooling cycles. Phillips *et al.* (1972) also state that with pairs of phosphatidylcholine molecules having saturated chains with lengths differing by more than two carbon atoms, there are two thermal transitions and a heterogeneous "gel" phase; as crystallization begins, the phosphatidylcholine molecules migrate within a bilayer to form clusters of the two components. With respect to the conclusions by Phillips *et al.* (1970, 1972) we emphasize that our interpretation of the spin-label data, as well as their thermal data requires that *under equilibrium conditions* lateral diffusion (or equivalent motion) is necessary for the phase separations in *all* the binary mixtures considered, and that in no case is there convincing evidence for the lateral separation of a pure lipid component.

[4] The most recent and precise transition temperature for DSPC is 54.24° (Hinz and Sturtevant, 1972).

Biological Significance

As mentioned earlier, there is now strong evidence for lateral diffusion and phase separations in intact, functional biological membranes. It is an interesting question as to whether lateral phase separations of phospholipids play any functional role in cell membranes. Of the various functions that lateral phase separations might serve, those that involve a coupling between lateral motion and motion perpendicular to the membrane are particularly interesting. For thermodynamic reasons, a biological membrane whose lipids are partially in the F state and partially in the S state should have a high lateral compressibility and extensibility. A lateral compressibility could facilitate the insertion of newly synthesized protein, lipid, or new membrane into old membrane without a corresponding expansion in the area of the membrane. A high lateral compressibility and extensibility may also facilitate the kinetics of the translocation of protein carriers from one side of a membrane to the other (or motion of proteins within the membrane), again without an overall increase or decrease in the membrane area during the translocation step. If this mechanism for achieving enhanced extensibility and compressibility is utilized in biological cells, one would expect both F phase and S phase to be simultaneously present under normal growth conditions. A number of authors have already mentioned the possibility that cells may grow under conditions "within the phase transition" (Melchior *et al.*, 1972; Linden *et al.*, 1973; Ashe and Steim, 1971).

Strong experimental evidence that the coexistence of two lipid phases does facilitate transport through membranes has been obtained in the case of sugar uptake by *E. coli* (Linden *et al.*, 1973) and also in the case of valinomycin mediated K^+ transport through phospholipid bilayers (Wu and McConnell, 1973).

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