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FLUORESCENCE STUDIES OF CHLOROPHYLL *a* INCORPORATED INTO LIPID MIXTURES, AND THE INTERPRETATION OF "PHASE" DIAGRAMS

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SUMMARY

The fluorescence of chlorophyll *a* incorporated into liposomes of mixtures of phosphatidylcholines and phosphatidylethanolamines is reported. Plots of fluorescence intensities against temperature show breaks at characteristic temperatures which can be attributed to the onset and completion of solid phase lipid formation. These temperatures can be plotted to give diagrams analogous to the phase diagrams obtained for macroscopic systems. Complications due to "small-system effects" are discussed, and the experimental diagrams are compared with theoretical phase diagrams calculated for ideal mixing. Introduction of cholesterol leads to a reduction in fluorescence intensity, most readily explained by a 1 : 1 lipid : cholesterol interaction with exclusion of monomeric, fluorescent, chlorophyll *a*. Interaction of divalent ions with mixtures of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylserine leads to exclusion of chlorophyll *a* from the phosphatidylserine.

INTRODUCTION

The lipid composition of most biological membranes is complex, both with respect to the lipid head groups and with respect to the fatty acid chains. Further, model membrane studies have shown that the physical properties of lipids are very sensitive to environment: all lipids seem to undergo a gel to liquid crystalline phase transition at some characteristic temperature, and those lipids with a net negative charge interact with divalent ions with a decrease in motional fluidity (reviewed in ref. 1).

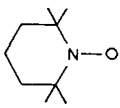
In simple systems containing a single class of lipid, it has been shown that the state of the lipid, gel or liquid crystalline, has a marked effect on the activity of a transport protein incorporated into the lipid membrane [2, 3]. Similarly, it would be expected that a phase change in the surrounding lipid would affect the environment of any lipophilic molecule present in the membrane, and indeed it has been shown that the aggregation of chlorophyll incorporated into a lipid bilayer is greater when the lipid is in the gel state than when it is in the liquid crystalline state [4]. Since,

Abbreviation: TEMPO, 2,2,6,6-tetramethylpiperidine-*N*-oxyl.

however, biological membranes contain complex mixtures of lipids, it becomes important to extend these model studies from systems containing single classes of lipids to systems containing mixtures of lipids.

In recent years the properties of lipid mixtures have been probed using differential scanning calorimetry [5–9] freeze-fracture [10], electron spin resonance [11–15] and X-ray diffraction [33]. For binary mixtures of lipids, the general conclusion is that most phospholipids are completely miscible in the liquid crystalline phase, but that in the gel phase there is limited solid solution formation if the lipids differ in their headgroups, or if the lipid fatty acid chains differ in unsaturation. These conclusions are of importance since it appears that at physiological temperatures, most cell membranes contain mixtures of gel and liquid crystalline phase lipids [1, 16]. The presence of both gel and liquid crystalline phases in the same membrane confers special thermodynamic properties on the membrane, of which the most significant is probably a high compressibility [1, 17, 18]

This paper reports on the effects of temperature on the degree of aggregation of chlorophyll *a* incorporated into lipid mixtures. Equilibrium between monomeric and oligomeric forms of chlorophyll can readily be followed by fluorescence intensity measurements, since only the monomeric form is fluorescent. It has been found that at chlorophyll · lipid molar ratios of 1 : 400 or greater, the fluorescence intensity measurements closely parallel the TEMPO (I) partition studies of Shimshick and McConnell [11], so that "phase diagrams" can be readily established using this technique The nature of these phase diagrams will be discussed later



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EXPERIMENTAL

Dipalmitoyl phosphatidylcholine and dimyristoyl phosphatidylcholine were obtained from Koch-Light, dimyristoyl phosphatidylethanolamine and dipalmitoyl phosphatidylethanolamine from Fluka and dipalmitoyl phosphatidylserine from Serdary. Cholesterol (from B.D.H.) was twice recrystallized from ethanol. Chlorophyll *a* was purified by column chromatography on powdered sugar columns by the method of Strain and Svec [19]. Chlorophyll *a* concentrations were estimated from absorption spectra, using the extinction coefficients given by Strain and Svec [19].

Lipids plus chlorophyll *a* ($1.6 \cdot 10^{-9}$ mol) dissolved in chloroform or chloroform-methanol were mixed in 10-ml stoppered flasks and evaporated to dryness under a stream of nitrogen and in a vacuum desiccator. Buffer (4 ml; 0.01 M Tris · HCl pH 7.2, NaCl 0.1 M) was added and the mixture shaken on a Vortex mixer. For all systems reported here the lipid : chlorophyll molar ratios were equal at 400 : 1.

Fluorescence measurements were made on an Aminco Bowman SPF Fluorimeter. Samples were continuously stirred during the fluorescence experiments, and temperatures were measured by a thermocouple. Fluorescence intensities were standardized between measurements, so that direct comparison is possible between samples Fluorescence of chlorophyll *a* was excited at 420 nm and recorded at 670 nm. Effects of scatter were very small at the lipid concentrations used (approx. 3 % of the

fluorescence intensity in liquid crystalline dipalmitoyl phosphatidylcholine) and were corrected for when necessary by subtraction.

Electron spin resonance (ESR) spectra were obtained using a Varian E3 spectrometer with a variable temperature controller, at TEMPO concentrations of $1 \cdot 10^{-4}$ M. Lipid concentrations of 100 mg/ml were used.

For studies of sonicated lipid vesicles, lipid and chlorophyll dispersions in buffer were degassed with nitrogen and sonicated in glass vials under nitrogen until the sample was translucent and the residual light scattering was minimised.

RESULTS

Chlorophyll a and single lipids

Previous studies [4] have shown that chlorophyll *a* incorporated into liposomes at a chlorophyll : lipid molar ratio of 1 : 400 does not affect the observed temperature of the gel to liquid crystalline phase transition. In Fig. 1, plots of the fluorescence intensity of chlorophyll *a* in liposomes as a function of temperature show abrupt decreases in magnitude at temperatures corresponding to the calorimetrically determined phase transition. This can be attributed to the formation of non-fluorescent, aggregated chlorophyll species, as discussed later. If the transition temperature is defined as the midpoint of the fluorescence transition curve, then the following temperatures are obtained, which can be compared to the calorimetric transition temperatures [8, 9, 20] given in parenthesis: dimyristoyl phosphatidylcholine 23 °C (23.7 °C); dipalmitoyl phosphatidylcholine 40.5 °C (41.75 °C); dimyristoyl phosphatidylethanolamine 46 °C (48 °C); dipalmitoyl phosphatidylethanolamine, 62 °C (63 °C). As described elsewhere [4], the fluorescence intensity changes in dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine also indicate the presence of a pre-transition, as found in TEMPO partition studies.

Although the definition of the phase transition temperature as the mid-point of the transition is generally useful, the actual range of the transition is also significant. This range is readily determined by the intersections of the straight lines which can be

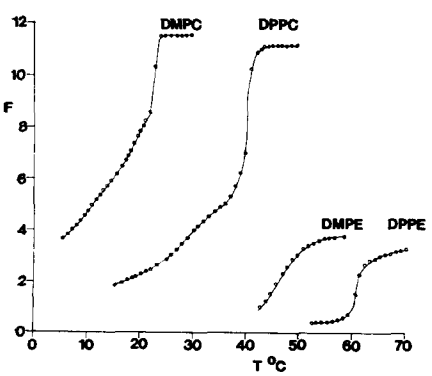


Fig 1. Fluorescence intensity (arbitrary units) at 670 nm of chlorophyll *a* in liposomes vs temperature, at a chlorophyll : lipid ratio of 1 : 400. DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; DMPE, dimyristoyl phosphatidylethanolamine; DPPE, dipalmitoyl phosphatidylethanolamine.

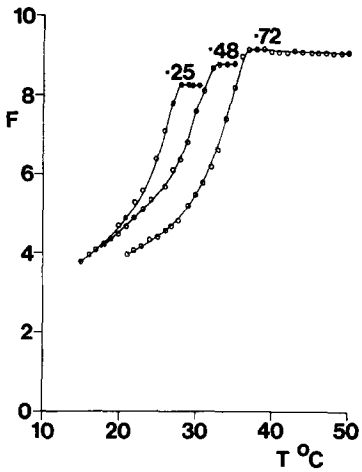
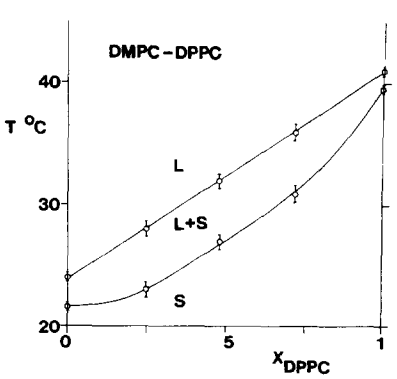


Fig 2 Fluorescence intensity vs temperature for chlorophyll *a* incorporated into liposomes of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylcholine, at the given molar ratios of dipalmitoyl phosphatidylcholine

drawn through the three district portions of each curve, and is about 2 °C for the lipids studied here.

Chlorophyll a and lipid mixtures

Fig. 2 shows plots of the fluorescence intensity of chlorophyll *a* as a function of temperature, when incorporated into mixtures of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine. The shape of the plots are remarkably similar to those obtained by Shimshick and McConnell [11] for TEMPO partitioning into the same lipid mixtures. The fluorescence plots can be interpreted in a manner analogous to that of Shimshick and McConnell [11] to give "phase" diagrams that for the dimyristoyl phosphatidylcholine : dipalmitoyl phosphatidylcholine mixture is given



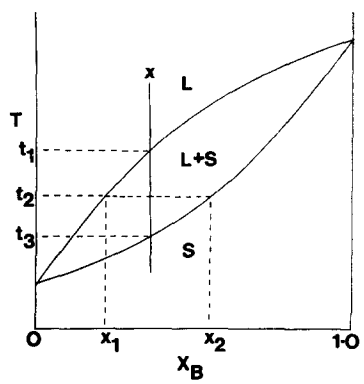


Fig. 3 Temperatures of onset and completion of solid lipid separation in dimyristoyl phospatidylcholine/dipalmitoyl phosphatidylcholine mixtures

Fig. 4. Theoretical phase diagram for two component system, miscible in both solid and liquid phases

in Fig. 3. When the temperature of a lipid mixture of some specific composition, initially in the fluid phase, is reduced, at some characteristic temperature t_1 (see Fig. 4) lipid in the gel, or solid, phase will appear. As the temperature is lowered further, the regions of solid lipid will increase and those of fluid lipid will decrease, until at the temperature t_3 and below only solid phase lipid will be present. The onset of the formation of solid phase lipid is signalled by a decrease in fluorescence, since chlorophyll *a* will be excluded from the solid lipid, leading to increased aggregation and consequent fluorescence quenching. Two breaks are observed in the slope of the fluorescence plot, corresponding to the temperatures of onset and completion of solid phase separation. It is these onset and completion temperatures that are plotted in Fig. 3 For consistency the transition ranges for the pure lipids are plotted in the figure rather than the transition temperature defined as the midpoint of the transition. Although such diagrams can loosely be termed "phase diagrams" such a term has certain implications which may not be true for diagrams such as Fig. 3. this point will be discussed later.

The fluorescence-temperature curves are generally completely reversible. For technical reasons, it was found to be more convenient to study cooling curves rather than heating curves, but for systems where both were measured there were no significant differences.

Fig. 5 shows the fluorescence intensity plots for mixtures of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine. Again, the shapes of the curves are very similar to those for TEMPO partitioning into the same mixtures [11]. Although the shapes of these curves are rather complex, the upper and lower transition temperatures can be determined by the intersections of straight lines drawn through the distinct portions of each curve. The resulting temperatures are plotted in Fig. 6. The corresponding data for mixtures of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine are shown in Figs 7 and 8.

Fig. 9 shows data for mixtures of dimyristoyl phosphatidylcholine and cholesterol. For these mixtures, the results are very different from those described

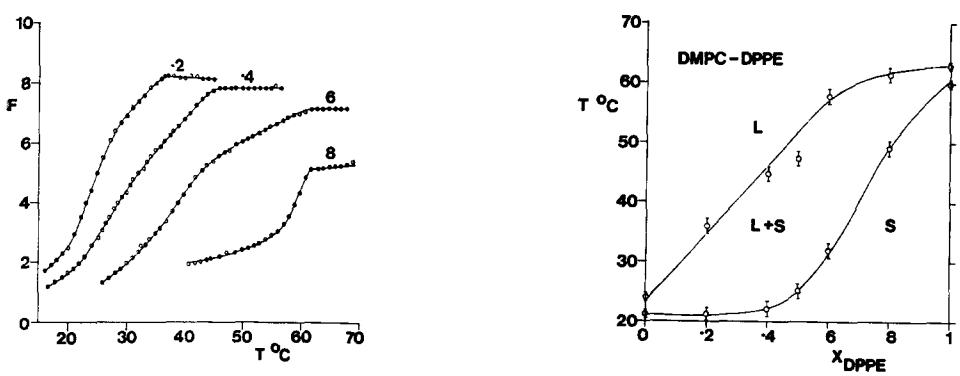
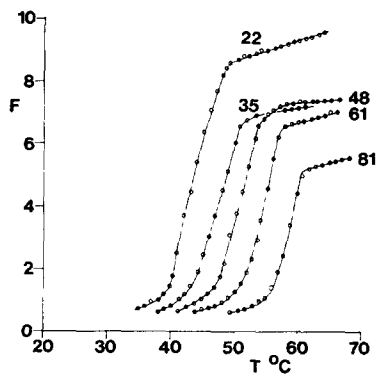


Fig 5 Fluorescence intensity vs temperature for chlorophyll *a* incorporated into liposomes of dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylethanolamine, at the given molar ratios of dipalmitoyl phosphatidylethanolamine.

Fig. 6. Temperatures of onset and completion of solid lipid separation in dimyristoyl phosphatidylcholine/dipalmitoyl phosphatidylethanolamine



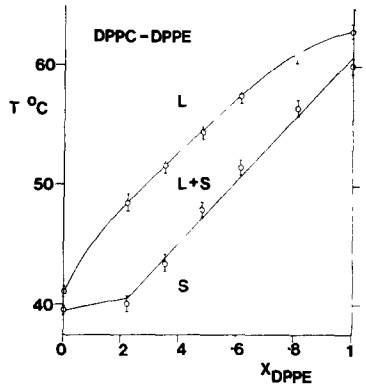


Fig 7 Fluorescence intensity vs temperature for chlorophyll *a* incorporated into liposomes of dipalmitoyl phosphatidylcholine/dipalmitoyl phosphatidylethanolamine, at the given molar ratios of dipalmitoyl phosphatidylethanolamine

Fig 8. Temperatures of onset and completion of solid lipid separation in dipalmitoyl phosphatidylcholine/dipalmitoyl phosphatidylethanolamine mixtures

above. It is clear that with an increasing mole fraction of cholesterol, the lipid phase transition becomes less distinct as the fluorescence intensity at higher temperatures decreases. It has previously been observed [4] that there is a marked concentration quenching of fluorescence for chlorophyll *a* incorporated into lipid bilayers, both when the lipid is in the gel phase and when it is in the liquid crystalline phase. One possibility is therefore that the decrease in chlorophyll *a* fluorescence intensity corresponds to the decrease in the amount of phospholipid present. This however is not the case. Thus when the mol fraction of cholesterol present is 0.33, the amount of dimyristoyl phosphatidylcholine present is $4 \cdot 10^{-7}$ mol, and the phospholipid : chlorophyll molar ratio is 260 : 1, with a fluorescence intensity of 34 (arbitrary units) at 55 °C : chlorophyll *a* incorporated into liposomes of dimyristoyl phosphatidylcholine at the same molar ratio, has a fluorescence intensity of 76 at 55 °C The observed data can, however, be fitted to a model in which a 1 : 1 dimyristoyl phosphatidylcholine-cholesterol interaction occurs, and monomeric chlorophyll *a* is displaced when

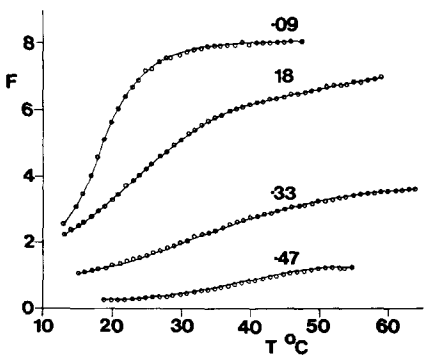


Fig 9. Fluorescence intensity vs temperature for chlorophyll *a* incorporated into liposomes of dimyristoyl phosphatidylcholine/cholesterol, at the given ratios of cholesterol

dimyristoyl phosphatidylcholine and cholesterol interact. The fluorescence intensity will then reflect the instantaneous proportion of dimyristoyl phosphatidylcholine not involved in interaction with cholesterol. The curve of fluorescence intensity for the mixture with cholesterol against the calculated molar ratio of "free" lipid to chlorophyll is identical to that found for mixtures of dimyristoyl phosphatidylcholine and chlorophyll, in which the lipid : chlorophyll molar ratio was varied by varying the amount of dimyristoyl phosphatidylcholine present.

Some studies have also been performed with the charged lipid dipalmitoyl phosphatidylserine. Incorporation of chlorophyll *a* into liposomes of dipalmitoyl phosphatidylserine leads to irreversible decomposition, presumably because of the large negative charge on such liposomes. It has been reported [21] that incorporation of chlorophyll *a* into monolayers of fatty acids leads to pheophytinization. Decomposition does not occur, however, when the dipalmitoyl phosphatidylserine is diluted with uncharged lipid. Table I shows some of the results obtained with a 1 : 2 dipalmitoyl phosphatidylserine : dipalmitoyl phosphatidylcholine mixture with a chlorophyll : lipid molar ratio of 1 : 400. In the absence of any divalent metal ions, there were no significant differences in fluorescence intensity between the mixture and dipalmitoyl phosphatidylcholine alone. On addition of 0.01 M Ca^{2+} or 0.04 M Mg^{2+}, however, there was a marked drop in fluorescence intensity for the mixture, to a level characteristic of a chlorophyll : lipid molar ratio of 1 : 260. Interaction of dipalmitoyl phosphatidylserine with divalent ions therefore appears to lead to an exclusion of chlorophyll *a*.

TABLE I

FLUORESCENCE INTENSITIES (ARBITRARY UNITS) FOR CHLOROPHYLL *a* IN LIPOSOMES

Conditions	Chlorophyll · lipid (molar ratio 1 400)		Chlorophyll . lipid (molar ratio 1 · 260)
	Dipalmitoyl phosphatidylserine dipalmitoyl phosphatidylcholine (1:2)	Dipalmitoyl phosphatidyl-choline (alone)	Dipalmitoyl phosphatidyl-choline (alone)
55 °C, no divalent ions	103	111	76
35 °C, no divalent ions	44	49	25
15 °C, no divalent ions	19	19	12
55 °C, 0.01 M Ca^{2+}	68	111	–
55 °C, 0.04 M Mg^{2+}	55	111	–
35 °C, 0.01 M Ca^{2+}	18	46	–
35 °C, 0 04 M Mg^{2+}	18	49	–

The effect of sonication

After sonication of liposomes containing chlorophyll *a*, there is a decrease in fluorescence intensity, typically by about 30 %. For brief sonication periods, the fluorescence intensity is fairly reproducible, but prolonged sonication leads to further loss of fluorescence, presumably due to decomposition of chlorophyll. Although it is not therefore possible to attach any significance to the decrease of intensity observed after sonication, a broadening of the transition is also observed, which is reproduced in all samples. The transition width increases from 2 °C in the unsonicated samples to 8 °C in the sonicated samples. To confirm that this observation is not artifactual, measurements have also been made on the partitioning of TEMPO into sonicated liposomes, since in this case it is possible to add the TEMPO after sonication, so that decomposition of the label is no longer a problem. It has previously been shown that sonication under the conditions employed here does not produce any degradation of the lipid [22].

The electron spin resonance spectrum of TEMPO in an aqueous lipid dispersion is the superposition of the spectrum of TEMPO in an aqueous environment and in a lipid environment The amplitudes B and F of the high-field signal sare approximately proportional to the amount of TEMPO dissolved in the lipid and in the aqueous phase respectively [11, 14]. Plots of the parameter f, given by

$$f = B/(F+B)$$

as a function of temperature, then show discontinuities at temperatures corresponding to the onset and completion of the phase transition. For unsonicated aqueous dispersions of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine, the observed width of the transition is 2 °C, whereas in the sonicated lipid dispersions it is approx. 8 °C

DISCUSSION

The concentration of chlorophyll within photosynthetic membranes is high, and in chloroplasts has been estimated to be 0.1 M [23] The majority of this chlorophyll is present as "antenna" chlorophyll, organised for efficient energy transfer to a reactive centre. In a previous paper [4] it has been argued that both monomeric, fluorescent, and oligomeric, non-fluorescent, forms of chlorophyll are present in liposomes, both when the lipid is in the gel phase and when it is in the liquid crystalline phase. However, the proportion of oligomeric chlorophyll is much higher in the gel phase lipid than in the liquid crystalline. This is readily understandable in view of the structure of chlorophyll *a* .The lipophilic part of the molecule is phytol, a fatty alcohol whose hydrocarbon chain is linked through an ester bond to the propionic acid side chain of the chlorophyllide residue. Along the sixteen carbon chain of the phytol there are four methyl groups at positions 3, 7, 11 and 15. Although these bulky groups could be relatively well accommodated when the lipid is highly fluid, when it crystallized in the gel phase, the chlorophyll *a* will be excluded and so form separate aggregates

In this paper, the studies of chlorophyll *a* in liposomes are extended to mixtures of phosphatidylcholines and phosphatidylethanolamines, two of the lipids found, for example, in the membranes of photosynthetic bacteria [24]. In such mixtures monomeric chlorophyll *a* effectively "partitions" between aggregated chlorophyll and the

lipid, so that the proportion of monomeric chlorophyll reflects the proportion of fluid lipid. The plots shown in Figs 2, 5 and 7 show the relative proportions of monomeric chlorophyll in lipid mixtures at various temperatures. At the same time, they can be used to detect phase transitions in lipid mixtures, in much the same way as ESR measurements of TEMPO partitioning. The fluorescence measurements, however, have the advantage of being faster and of requiring less material.

The low fluorescence yield in the phosphatidylethanolamines in the liquid crystalline phase is of interest since this is a major lipid of photosynthetic bacteria [24]. thus in *Rhodospirillum rubrum* the lipid composition is 57 % phosphatidylethanolamine, 6 % phosphatidylcholine and 29 % phosphatidylglycerol [25]. The small proportion of monomeric chlorophyll in dimyristoyl phosphatidylethanolamine and dipalmitoyl phosphatidylethanolamine can be attributed to a more tightly packed fatty acid chain region in the bilayer, caused by strong interaction between the phosphatidylethanolamine headgroups, with the positively charged amine group of one molecule interacting electrostatically with the negatively charged phosphate group of an adjacent molecule.

For both dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine, a pretransition is observed which can be associated with a change in headgroup conformation [4]. No pretransition is apparent in dimyristoyl phosphatidylethanolamine or dipalmitoyl phosphatidylethanolamine in agreement with other studies [9, 11]. Interestingly, in mixtures of phosphatidylcholines and phosphatidylethanolamines containing greater than 20 % phosphatidylethanolamine, the pretransition is abolished. This suggests both that the pre-transition in the phosphatidylcholines is highly cooperative, and that the long range order in the lipid headgroup region is easily perturbed.

From these fluorescence-temperature plots for mixtures of phospholipids it is also possible to measure temperatures corresponding to the onset and completion of the formation of solid phase lipid. These are presented in Figs 3, 6 and 8 for the mixtures studied here. The shape of the plots are very similar to those reported by Shimshick and McConnell [11] and their significance will be discussed later. The results obtained by fluorescence measurements for dimyristoyl phosphatidylcholine/cholesterol mixtures are, however, significantly different from those observed by Shimshick and McConnell [12] using TEMPO partitioning. A very tentative explanation of the different results obtained using TEMPO and chlorophyll *a*, is that cholesterol significantly reduces the fluidity of the lipid fatty acid chains [34] with relatively little effect on the glycerol backbone region, and that TEMPO is dissolving in that part of the membrane close to the glycerol backbone region, whereas chlorophyll *a* is sensitive to changes in the whole of the fatty acid chain region. But whatever the reason for the different effects of cholesterol, the important observation in our case is the marked decrease in fluorescence on addition of cholesterol, so that at a dimyristoyl phosphatidylcholine : cholesterol molar ratio of 1 : 1 there is virtually no fluorescence. The results can be explained if it is assumed that a 1 : 1 interaction occurs between cholesterol and dimyristoyl phosphatidylcholine with the effect of excluding chlorophyll *a*. It should perhaps be emphasized that this stoichiometry does not necessarily imply complex formation between lipid and cholesterol, but could represent some optimum geometrical packing arrangement Calorimetric experiments appear to indicate a 2 : 1 stoichiometry for the lipid-cholesterol interaction, but this has been

challenged by Phillips and Finer [27] Largely on the basis of NMR data, Phillips and Finer [27] propose a 1 1 stoichiometry for lipid-cholesterol interaction, with the formation of a long lived complex, but this, in its turn, has also been challenged [28].

The results obtained with mixtures of dipalmitoyl phosphatidylcholine and dipalmitoyl phosphatidylserine are as would have been expected from previous studies with phosphatidylserines Interaction with divalent metal ions results in a decrease in fluidity for the dipalmitoyl phosphatidylserine with exclusion of chlorophyll *a* and a decrease in fluorescence.

On phase diagrams

The plots of onset and completion of solid phase formation in lipid mixtures (Figs 3, 6, 8) closely resemble traditional phase diagrams. Before their identity can be accepted, however, it is necessary to consider the consequences of the fact that lipid systems are small systems in a thermodynamic sense [29]. One important consideration concerns the nature of the phase-transitions that can be expected. The sharpness of a phase transition depends on the number of molecules forced to cooperate in the transition: this number is called the co-operative unit A first-order phase transition is defined as a transition for which there is a discontinuous change in the first derivatives of the Gibbs free energy Such a "sharp" transition will only occur for an infinite co-operative unit and would correspond to the melting of a perfect infinite crystal. Since real crystals contain many imperfections, the co-operative unit will be much smaller than the total solid, and a phase transition will be more or less gradual. No exact thermodynamic treatment is possible for such transitions [29], but the phase transition temperature can usefully be defined as the mid-point of the transition

In lipid bilayers, the co-operative unit has been shown to be quite small. The width of the gel to liquid crystalline phase transition for dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine depends on sample pre-history, and is found calorimetrically to be between 0 2 °C and 2 °C, corresponding to cooperative units of 200 and approx 100 respectively [20]. The significant width of the transition is not due simply to problems in establishing thermal equilibrium within the samples [9] since X-ray diffraction studies have also shown that the transition takes place over a finite temperature range [30]. The phase rule cannot then be applied directly to such transitions. According to the phase rule, a two component system (i.e. dimyristoyl phosphatidylcholine and water or dipalmitoyl phosphatidylcholine and water) with three phases (i e. gel, liquid crystalline and pure water phases) should have no degrees of freedom at constant pressure, that is, the transition between gel and liquid crystalline phase should be truly isothermal, which it is not for lipid systems. The problem arises because the Gibbs phase rule was introduced to explain the behaviour of macroscopic systems [31] where phase transitions can be expected to be very sharp because the co-operative units are very large. With small co-operative units and broad transitions, the phase rule cannot be strictly applied, and consequently phase diagrams cannot be rigorously constructed.

Fig. 4 shows a typical phase diagram for a macroscopic system of two very similar compounds, miscible both in the liquid and solid phases, and will serve to illustrate the problems outlined above. If we consider a mixture of compositon X, then at any temperature down to t_1, there will be a single liquid phase of composition X. Similarly, at temperatures below t_3, there will be a single solid phase of composi-

tion X. At some intermediate temperature t_2, however, both liquid and solid phases will be present, the liquid phase having composition X_1 and the solid phase composition X_2. As required by the phase rule, there is just one single temperature t_2 at which liquid of composition X_1 and solid of composition X_2 are in equilibrium.

For lipid mixtures, we determine experimentally a diagram showing the onset and completion temperatures for the formation of lipid in the solid phase. If the transition between liquid and solid phases in such systems were very sharp, then these diagrams would be conventional phase diagrams. If, however, the co-operativity of the transition is low, then the width of the transition for a mixture of composition X (i.e. from temperature t_1 to t_3) could be primarily due to the low co-operativity, and not imply much difference in the composition of the solid and liquid phases. Although the co-operativity of the transition in lipid mixtures is not known, at least it is known that for single lipids, the co-operativity can be highly variable. Hinz and Sturtevant [20] report a co-operative unit of between approx. 200 and 100 depending on sample prehistory, and experiments reported here show that in sonicated lipid dispersions the co-operativity can drop to approx 10, with a corresponding increase in the width of the transition to 8 °C.

Considerations such as these mean that it is probably not possible to interpret the finer details of these diagrams, although the grosser aspects are probably meaningful, in that large deviations from the expected phase diagrams for ideal mixing can be attributed to immiscibility. The phase diagrams for ideal mixing can be calculated from the latent heats of fusion and the melting points of the two components. Consider two components A and B, with melting points T_A and T_B, respectively, and molal heats of fusions ΔH_A and ΔH_B respectively, with $T_B > T_A$. The mol fraction of B in the liquid phase at temperature T, N_B, is given by [32]

$$N_B = \frac{1 - k_A e^{\Delta H_A/RT}}{k_B e^{\Delta H_B/RT} - k_A e^{\Delta H_A/RT}}$$

where

$$k_A = 1/e^{\Delta H_A/RT_A}$$

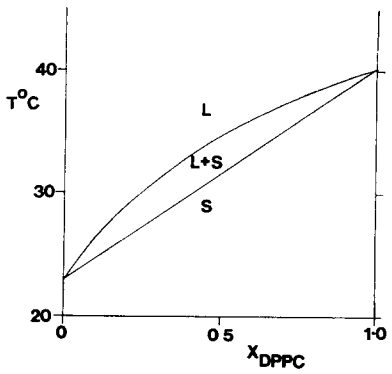
and

$$k_B = 1/e^{\Delta H_B/RT_B}$$

The mol fraction of B in the solid phase, N'_B, at the same temperature is

$$N'_B = N_B k_B e^{\Delta H_B/RT}$$

For ideal mixing in mixtures of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine, the phase diagram in Fig. 10 is obtained, assuming a highly co-operative phase transition: in the calculations, transition enthalpies of 6.2 and 9.7 kcal · mol^{-1} and transition temperatures of 23.7 °C and 41.7 °C were assumed for dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylcholine, respectively [20]. The theoretical phase diagram is similar to the experimental Fig. 3, in that both the solidus and fluidus curves are continuous. The major difference is in the separation between solidus and fluidus curves: at a 1 : 1 dimyristoyl phosphatidycholine : dipalmitoyl phosphatidylcholine molar ratio, the width of the transition in the theoretical curve is 3 °C, whereas in the experimental curve it is 5 °C. The experimental



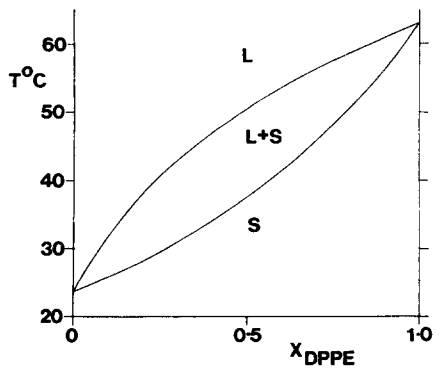


Fig. 10 Theoretical phase diagram calculated for ideal mixing of dimyristoyl phosphatidylcholine/ dipalmitoyl phosphatidylcholine.

Fig. 11 Theoretical phase diagram calculated for ideal mixing of dimyristoyl phosphatidylcholine/ dipalmitoyl phosphatidylethanolamine

onset and completion temperatures for solid state separation reported by Chapman et al. [9] using differential scanning calorimetry are all approx. 2.5 °C higher than those that we observe, but correcting for this our curves are superimposable. Similarly, the temperatures reported by Shimshick and McConell [11] are approx. 1 °C higher than those reported here, but again the transition widths are the same The extra width of the experimental transition appears to be largely attributable to a lowering of the solidus curve, but the deviations from ideality appear to be relatively small.

The shape of the "phase" diagram for mixtures of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine suggests a much greater deviation from ideal mixing The phase diagram for ideal mixing is shown in Fig 11, using an enthalpy of transition of 8.0 kcal · mol^{-1} and a transition temperature of 63 °C for dipalmitoyl phosphatidylethanolamine [8]. At a 1 1 dimyristoyl phosphatidylcholine : dipalmitoyl phosphatidylethanolamine molar ratio the width of the transition is 13 °C, very considerably greater than that found for a 1 : 1 dimyristoyl phosphatidylcholine : dipalmitoyl phosphatidycholine mixture The most noticeable difference between the experimental and theoretical curves for the dimyristoyl phosphatidylcholine : dipalmitoyl phosphatidylethanolamine mixtures is the horizontal portion of the experimental solidus curve, an indication of solid state immiscibility. An important consequence of this immiscibility for the functioning of the cell membrane could be the corresponding increase in the proportion of lipid in the fluid state at lower temperatures. The proportion of lipid in the solid and liquid phases can be calculated from a phase diagram as follows (see Fig. 4). If n_l is the sum of the number of molecules of components A and B in the liquid phase, and n_s is the corresponding sum for the solid phase, then for a mixture with mol fraction X of component B at temperature t_2,

$$X(n_l+n_s) = X_l n_l + X_2 n_s$$

$$\frac{n_l}{n_s} = \frac{(X_2-X)}{(X-X_l)}$$

For ideal mixing of dimyristoyl phosphatidylcholine and dipalmitoyl phosphatidylethanolamine, a mixture containing 40 mol % dipalmitoyl phosphatidylethanolamine would be all in the solid phase below 35 °C, and at 40 °C would contain 47 % lipid in the liquid phase. From the experimental diagram (Fig. 6) we can see however that because of deviations from ideality already at 30 °C for the same mixture, there is 37 % lipid in the liquid phase, and by 40 °C this has risen to 75 %.

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