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# Phase transitions and fatty acid spin label behavior in interdigitated lipid phases induced by glycerol and polymyxin

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**Glycerol and polymyxin have been shown by X-ray diffraction to induce interdigitated bilayers in phosphatidylcholine (PC) and phosphatidylglycerol (PG), respectively (McDaniel, R.V., et al. (1983) Biochim. Biophys. Acta 731, 97–108; Ranck, J.-L. and Tocanne, J.-F. (1982) FEBS Lett. 143, 175–178). In the present study we have investigated the phase behavior of PC and PG in the presence of glycerol and polymyxin by differential scanning calorimetry and the use of fatty acid spin labels. Interdigitation causes a large increase in the order parameter of a fatty acid spin labeled near the terminal methyl, 16-doxylstearate, so that it was similar to that of a fatty acid labeled much closer to the polar head group region, 5-doxylstearate. Thus interdigitation abolishes the fluidity gradient found in a non-interdigitated bilayer. 16-Doxylstearate may be useful in detecting interdigitation of lipid bilayers caused by other substances. The different samples all went through two transitions on heating or cooling, or both. However, use of the fatty acid spin label showed that the molecular events during these transitions varies for different samples. The results suggested that PC-glycerol freezes from the liquid-crystalline phase into a non-interdigitated gel phase. This subsequently becomes interdigitated upon lowering the temperature a few degrees, in a low enthalpy transition. PG-polymyxin shows a similar behavior except that the enthalpy of the non-interdigitated gel to interdigitated phase transition is greater and the transition is reversible on heating. Thus on heating PG-polymyxin first goes through a transition from the interdigitated phase to a non-interdigitated gel phase and then, in a separate transition, to the liquid-crystalline phase. This occurs because the fatty acid chains in the presence of polymyxin become too disordered with increase in temperature to maintain the interdigitated state. PG-glycerol goes into the interdigitated state less readily than the other mixtures. If cooled rapidly, PG-glycerol freezes into a metastable phase which is more disordered than the interdigitated phase. It goes into the interdigitated phase in an exothermic transition on heating. An increase in fatty acid chain length causes greater steric hindrance to interdigitation but also increases the stabilizing energy gained by interdigitation.**

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Abbreviations: DSC, differential scanning calorimetry; ESR, electron spin resonance; PG, phosphatidylglycerol; PC, phosphatidylcholine; DMPX, dimyristoyl form of phospholipid (X = choline or glycerol); DPPX, dipalmitoyl form; DSPX, distearoyl form; DAPX, diarachidoyl form; DBPX, dibehenoyl form; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

## Introduction

Myelin basic protein causes saturated fatty acid forms of phosphatidylglycerol to undergo interesting reversible metastable phase behavior which was detected by differential scanning calorimetry [1–4]. A metastable phase formed on cooling from the liquid-crystalline phase. On heating it underwent an exothermic transition to a stable phase which subsequently melted at a similar temperature and with a similar enthalpy as the pure lipid. The maximum hyperfine splitting, $T_{max}$, of the spectrum of a fatty acid, spin labeled near the terminal methyl, 16-doxylstearate, was increased in the stable phase to a similar value as that of a fatty acid spin labeled closer to the aqueous interface, 5-doxylstearate. This could be a result of a decrease in motion of 16-doxylstearate or an increase in its order parameter. The increase in $T_{max}$ occurred at the temperature of the exothermic transition to the stable phase. The order parameter of 16-doxylstearate is normally much less than that of 5-doxylstearate even in the gel phase of phospholipids. This effect on 16-doxylstearate led us to suggest that the stable phase of the BP-DPPG complex was interdigitated, causing the spin label moiety of 16-doxylstearate to be located in the same region of the interdigitated bilayer as 5-doxylstearate. Basic protein has a number of hydrophobic residues which are believed to penetrate partway into the bilayer causing lateral expansion and separation of the lipid fatty acid chains (see Ref. 5 for a recent review). We have argued that interdigitation would be a stabilizing consequence of penetration since it would increase the van der Waals interactions between the lipid acyl chains and compensate for this potentially disordering effect of the protein [1].

Several amphipathic substances, such as glycerol, polymyxin, acetylcholine, chlorpromazine and alcohols were shown recently by X-ray diffraction to cause interdigitation in dipalmitoylphosphatidylcholine (DPPC) and DPPG [6–11]. These substances have in common with basic protein their amphiphilicity causing them to penetrate partway into the bilayer and laterally separate the lipids. Like the hydrophobic residues of BP, they would also shield the ends of the lipid acyl chains in the interdigitated state from water. This demonstration of interdigitation caused by such a wide variety of substances suggests that interdigitated gel states of saturated lipids may be much more common than previously thought.

In the present study we investigated the calorimetric and fatty acid spin label behavior in polymyxin- and glycerol-induced interdigitated phases of saturated forms of phosphatidylcholine and phosphatidylglycerol and show that they are very similar to the BP-DPPG complex. This provides further evidence that the BP-DPPG complex may be interdigitated and also indicates that 16-doxylstearate may be useful for detecting interdigitation in many cases.

## Materials and Methods

Dimyristoylphosphatidylcholine (DMPC) and dipalmitoylphosphatidylcholine (DPPC) were from Sigma Chemical Co., St. Louis, MO. Distearoylphosphatidylcholine (DSPC), diarachidoylphosphatidylcholine (DAPC), dibehenoylphosphatidylcholine (DBPC) and distearoylphosphatidylglycerol (DSPG) were from Avanti Polar Lipids, Birmingham, AL. Dimyristoylphosphatidylglycerol (DMPG) was from Supelco, Bellefonte, PA and dipalmitoylphosphatidylglycerol (DPPG) was from Calbiochem, La Jolla, CA. The L-α-forms were used in all cases. All lipids were chromatographically pure. $Ca^{2+}$ was removed from the DSPG as described in Ref. 4. Polymyxin B sulfate was from Burroughs Wellcome, Inc., Kirkland, Quebec, Canada. Glycerol was from Matheson, Coleman and Bell. The fatty acid spin labels, 5-doxylstearate and 16-doxylstearate and the methyl ester of 16-doxylstearate were from Syva, Palo Alto, CA.

*Preparation of vesicles.* Vesicles of PC in water at pH 6 were prepared by dispersing the dry PC in distilled water at a concentration of 2 mg/0.5 ml. Complexes of PG with polymyxin B sulfate at a 5 : 1 mole ratio of lipid to polymyxin were prepared by dissolving the lipid and polymyxin together in chloroform/ methanol (1 : 1, v/v), evaporating the solvent, and dispersing the complex in 2 mM Hepes buffer containing 0.1 M NaCl (pH 7.4), at a concentration of 2 mg lipid per 0.5 ml buffer. The lipids were dispersed by vortex mixing at a temperature above the lipid phase

transition temperature. The samples were centrifuged in an Eppendorf bench centrifuge for 5 min and the wet pellets were loaded into aluminum DSC pans. PC was mixed with glycerol by adding 2 drops of glycerol (about 20 $\mu$l) to 1 mg of dry, powdered PC at room temperature. About half of the sample was loaded into the DSC pan. DPPG was mixed with glycerol by evaporating a chloroform/methanol (1 : 1, v/v) solution of the DPPG (1 mg) and mixing with 2 drops glycerol by vortexing at 45°C. The sample was loaded into the DSC pan. The results were independent of the temperature used except in the case of DPPG-glycerol. Vortexing of DPPG in glycerol at other temperatures above or below the phase transition temperature gave different calorimetric results, and affected the rate of the transition from the lower melting state to the higher melting state. Samples labeled with spin label were prepared similarly except that the lipid and spin label were first dissolved together at a 150 : 1 mole ratio in chloroform/methanol (1 : 1, v/v), the solvent was evaporated and the lipid was dispersed as described above. The samples were loaded into 100 $\mu$l capillary tubes for ESR measurements, sealed with a flame and centrifuged at 2000 rpm. The pellets for the aqueous samples were positioned in the ESR cavity; however, the lipid floated in glycerol and therefore the top of the glycerol column in the capillary tube was positioned in the cavity.

*Differential scanning calorimetry.* Samples were run on a Perkin-Elmer DSC-2 equipped with a Perkin-Elmer data station, at heating and cooling rates of 0.62 deg. C/min to 20 deg. C/min. The temperature of maximum heat absorption was defined as the phase transition temperature $T_m$. The areas of the peaks were obtained using the data station and the amount of lipid in the pan was determined by phosphorus analysis [12] after opening the pan, dropping it into 1 ml chloroform/methanol (1 : 1, v/v), and sonicating briefly with a bath sonicator to dissolve the lipid. Three or four pans of each sample were used for enthalpy detrminations.

*Electron spin resonance measurements.* ESR spectra were measured on a Varian E-104B spectrometer equipped with a Varian temperature controller and a DEC LSI-11 based microcomputer system. The maximum hyperfine splitting, $T_{max}$, of the ESR spectra or motional parameter, $\tau_o$, were measured as described earlier [1]. $T_{max}$ was used as a measure of the order parameter, the amplitude of motion of the fatty acid chains about their average orientation with respect to the normal to the bilayer surface. The microwave power used was 10 mW.

## Results

*Glycerol-phosphatidylcholine*

McDaniel et al. [8] showed that glycerol substitutes for water in multilamellar vesicles of phosphatidylcholine and allows a gel to liquid-crystalline phase transition with similar thermodynamic parameters to that of the lipid in water. They found further, using X-ray diffraction, that the lipid gel state in the presence of glycerol is interdigitated, with a bilayer thickness of 30 Å for DPPC, rather than 43 Å for the $L_\beta$ phase of DPPC in water. In glycerol the lipid also does not undergo the premelt transition to the $P_\beta$ phase. We have investigated the calorimetric behavior of PC in glycerol in more detail, including the effect of fatty acid chain length. DSC heating and cooling scans of DPPC in the presence of water and glycerol are shown in Fig. 1. As reported by McDaniel et al. [8] the heating scans are very similar in the presence of glycerol or water except that the premelt is abolished in glycerol. The phase transition temperature, $T_m$, was 1 deg. C higher and the enthalpy, $\Delta H$, was 2 kcal/mol greater in the presence of glycerol than in water (Table I). On cooling, however, two peaks were present in the thermogram of the glycerol sample (Fig. 1d). The enthalpy of the lower temperature one was about 30% of that of the higher temperature one. The sum of the enthalpies of both peaks observed on cooling, $\Delta H_T$, was similar to that observed on heating.

The effect of fatty acid chain length on the thermodynamic parameters of the phase transition is given in Table I. The difference between the $T_m$ values observed in glycerol and water increased with fatty acid chain length except for DBPC, while the difference in enthalpies was relatively independent of chain length, ranging between 1.6 and 2.7 kcal/mol. The entropy of the phase tran-

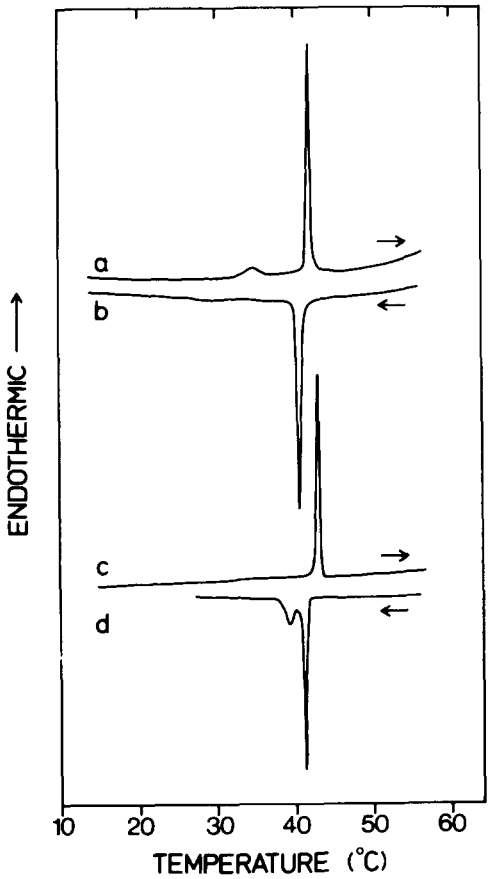


Fig. 1. DSC heating (a,c) and cooling scans (b,d) at a rate of 1.25 deg. C/min of DPPC in (a,b) distilled water; (c,d) glycerol. Arrows indicate the direction of the change in temperature. Sensitivity settings in mcal/s were (a,b) 0.5; (c,d) 0.8. See Table I for enthalpies.

sition was greater in the presence of glycerol suggesting a greater degree of order in the interdigitated gel state than in the $L_\beta$ phase. On cooling, the enthalpy of the lower temperature transition was lowest for DMPC and greatest for DBPC, but relatively constant for intermediate chain lengths. The temperature difference between the two transitions increased with chain length.

ESR spectra of 16-doxylstearate and 5-doxylstearate in the $L_\beta$ phase of DPPC-water and the interdigitated phase of DPPC-glycerol at 9°C are compared in Fig. 2. Both 16-doxylstearate and 5-doxylstearate show anisotropic motion in DPPC-water (Fig. 2 A,C). However, $T_{max}$ is 30.6 G for 5-doxylstearate and 23 G for 16-doxylstearate indicating that 5-doxylstearate has a much greater order parameter. In DPPC-glycerol, 16-doxylstearate and 5-doxylstearate give spectra with $T$max of 31.4 G and 31.9 G, respectively (Fig. 2 B,D). The line shape of the spectrum of 16-doxylstearate indicates that it still has anisotropic motion, like 5-doxylstearate, and is not immobilized. Thus the order parameter of the spin label located near the ends of the fatty acid chains has been increased to the degree experienced further up on the chain, and the fluidity gradient, normally found even in the gel state of lipids, both by spin labels and by NMR [13,14], is abolished in the interdigitated phase. Similar behavior was found in the BP-DPPG complex, although the spectrum in that case could arise from either im-

TABLE I

COMPARISON OF TRANSITION TEMPERATURES, ENTHALPIES AND ENTROPIES OF SATURATED FORMS OF PHOSPHATIDYLCHOLINE IN WATER OR GLYCEROL

At heating and cooling rates of 1.25 deg. C/min.

| | Water | | | | Glycerol | | |
|---|---|---|---|---|---|---|---|
| | heat | | | cool | heat | | |
| | $T_m$ (°) | $\Delta H$ (kcal/mol) | $\Delta S$ (cal/mol per deg.) | $T_m$ (°C) | $T_m$ (°C) | $\Delta H$ (kcal/mol) | $\Delta S$ (cal/mol per deg.) |
| DMPC | 24.0 | 5.9±0.3 | 19.9 | 23.0 | 24.0 | 8.6±0.4 | 28.9 |
| DPPC | 42.0 | 8.3±0.5 | 26.3 | 40.7 | 43.0 | 10.2±0.7 | 32.3 |
| DSPC | 55.3 | 11.6±0.5 | 35.3 | 53.9 | 56.8 | 13.9±0.4 | 40.0 |
| DAPC | 65.2 | 14.6±0.5 | 43.2 | 63.9 | 67.1 | 17.3±0.5 | 50.9 |
| DBPC | 73.1 | 17.7±0.6 | 51.1 | 71.7 | 73.9 | 20.0±0.3 | 57.6 |

[a] $\Delta T = T_2 - T_1$.

[b] Total enthalpy of both transitions ($\Delta H_1 + \Delta H_2$).

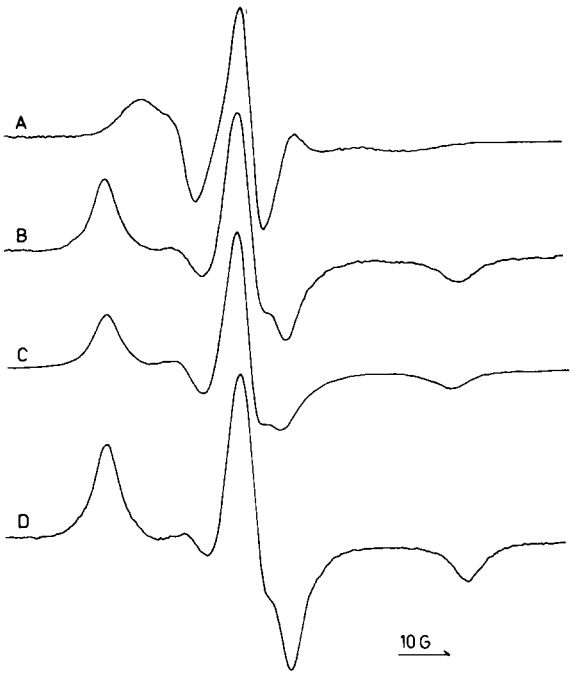


Fig. 2. ESR spectra of (A, B) 16-doxylstearate and (C, D) 5-doxylstearate at 9°C in DPPC in (A, C) distilled water; (B, D) glycerol.

mobilization or an increase in order parameter [1–4].

The p$K$ of fatty acids is 7.2–7.4 in PC and increases with increase in the negative surface charge [18]. Therefore, the fatty acid spin label should be predominantly in the protonated neutral form under the conditions used here. However, in order to rule out the possibility that ionization of the fatty acid spin label, which might cause a change in location of the probe in the bilayer, was responsible for the increase in order observed in the interdigitated phase, the effect of pH on the spectrum of 16-S-SL in DPPC was determined. The $T_{max}$ value at 9°C at pH 9 was 22.9 G, a little less than at pH 4 (23.4 G). The spectra resembled that in Fig. 2A except that at pH 9 there was a small amount of isotropic signal from spin label free in solution as a result of its increased aqueous solubility in the ionized state. The spectra of the methyl ester of 16-doxylstearate in DPPC at 9°C in glycerol or distilled water were also compared. The spectrum in the presence of distilled water had two components with $T_{max}$ values of 22.3 and 27.6 G, a little less and a little greater than that of 16-S-SL. In glycerol, the spectrum of the methyl ester was very similar to that of 16-doxylstearate in Fig. 2B, with $T_{max}$ = 31.5 G. Therefore, a change in ionization of the spin label cannot account for the dramatic ordering of 16-S-SL or its methyl ester in the interdigitated bilayer.

The change in $T_{max}$ and $\tau_o$ of 16-doxylstearate with temperature for the water and glycerol samples is compared in Fig. 3. The large increase in $T_{max}$ in glycerol is maintained up to the phase transition temperature, where the highly oriented anisotropic motion is abruptly transformed over a temperature interval of 2 deg. C into an isotropic

| Glycerol | | | | | |
|---|---|---|---|---|---|
| cool | | | | | |
| $T_1$ (°C) | $T_2$ (°C) | $\Delta T$ [a] (°C) | $\Delta H_1$ (kcal/mol) | $\Delta H_2$ (kcal/mol) | $\Delta H_T$ [b] (kcal/mol) |
| 20.2 | 21.5 | 1.3 | 0.8 | 7.2 | 8.0 |
| 39.0 | 41.2 | 2.2 | 3.2±0.3 | 9 ±1 | 11.6±1.5 |
| 50.5 | 55.3 | 4.8 | 3.5±0.7 | 11.4±0.9 | 14.9±0.8 |
| 59.5 | 65.4 | 5.9 | 3.4±0.5 | 13.7±1.2 | 17.8±0.6 |
| 65.2 | 72.5 | 7.3 | 6.5±0.1 | 14.4±1.0 | 20.9±1.0 |

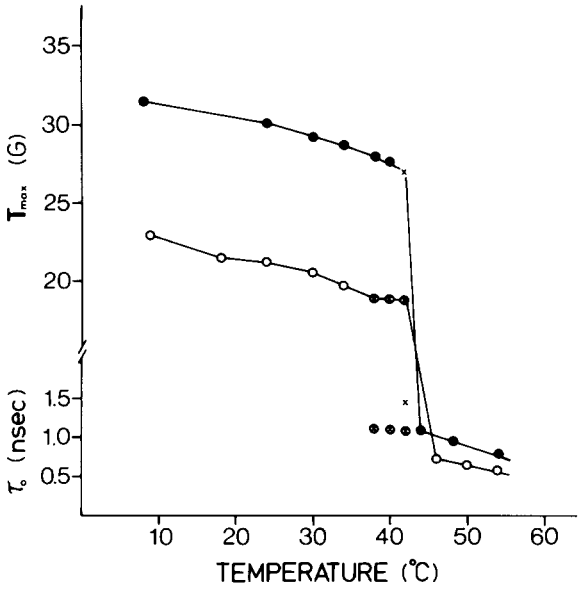


Fig. 3. Effect of temperature on spectral parameters, $T_{max}$ and $\tau_0$, of 16-doxylstearate in DPPC in distilled water (○) and glycerol (●). ⊗ indicates presence of two components in spectrum for DPPC-water and × for DPPC-glycerol.

type of motion characterisitc of 16-doxylstearate in the liquid-crystalline phase of lipids. In contrast, the transition sensed by the spin label is broader in DPPC-water. The spectrum changes from an anisotropic type spectrum, with $T_{max}$ of 20–23 G, characteristic of the $L_\beta$ phase, to an isotropic one over a temperature range of 8 deg. C; in this range, indicated by the symbol ⊗ in Fig. 3, two component spectra are obtained. The motional parameter of 16-doxylstearate above the phase transition temperature is greater in the presence of glycerol than water indicating less motion in the liquid-crystalline phase of the glycerol sample.

*Glycerol-phosphatidylglycerol*

The effect of glycerol on DPPG, determined by X-ray diffraction, has not yet been reported; hence it is not known if glycerol induces interdigitation in this lipid. However, we investigated the effect of glycerol on the phase transition and spectrum of 16-doxylstearate in DPPG in order to compare its behavior to that of the BP-DPPG complex. On the first heating scan a high enthalpy (15 kcal/mol) transition at 68°C was observed (Fig. 4). For

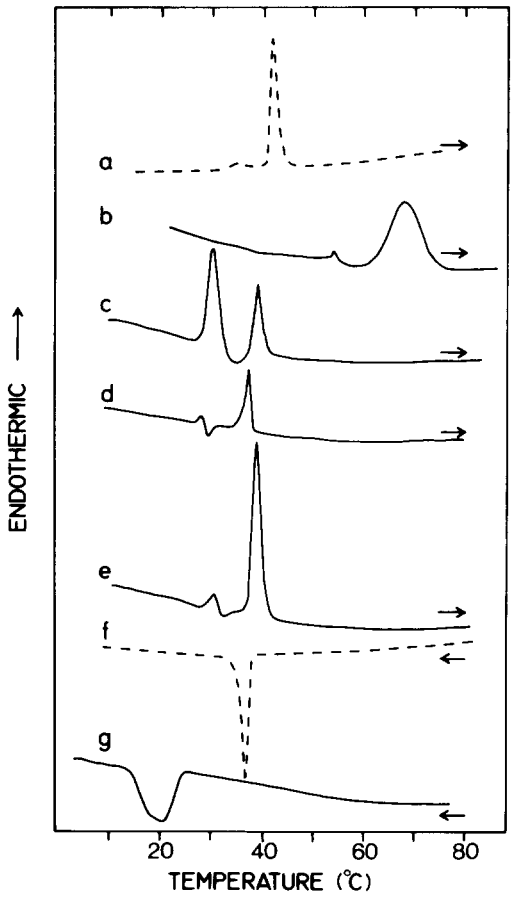


Fig. 4. DSC heating and cooling scans of DPPG in distilled water and glycerol. (a) Heating scan at 10 deg. C/min of DPPG in distilled water; (b) first heating scan at 10 deg. C/min of DPPG in glycerol; (c) reheating scan of DPPG-glycerol from −3°C at 10 deg. C/min after fast (20–40 deg. C/min) cooling scan; (d) similar reheating scan of DPPG-glycerol at 1.25 deg. C/min; (e) reheating scan of DPPG-glycerol at 10 deg. C/min after slow (1.25 deg. C/min) cooling scan; (f) cooling scan at 10 deg. C/min of DPPG in distilled water; (g) cooling scan at 10 deg. C/min of DPPG in glycerol. Sensitivity settings in mcal/s are (a,f) 1.5; (b–d,g) 0.5.

samples prepared near the phase transition temperature, metastable phase behavior was observed on the second and subsequent heating scans, obtained after cooling at a rapid rate, 20–40 deg.C/min. An endothermic transition at 30°C was followed by an exothermic transition and a second endothermic transition at 39°C (Fig. 4c). The sample could be completely converted to the stable, higher melting phase by heating at a slow rate as shown for a 1.25 deg.C/min scan in Fig. 4d, by

incubating at the temperature of the first endothermic transition, or by cooling at a rate of 10 deg. C/min or less, as shown for a heating scan obtained after cooling at 1.25 deg. C/min in Fig. 4e. The transition temperature of the stable phase, 39°C, was a few degrees less than that of DPPG in water, 41.7°C, and the enthalpy, 11 kcal/mol, was greater than that of DPPG-water, 8.3 kcal/mol. On cooling at 10 deg. C/min a broad transition occurred (Fig. 4g) which was centered at 23.5°C (after correction for instrumental hysteresis), considerably lower than that of the first transition at 30°C observed on heating. However, the enthalpy was similar to that of the transition of the stable phase indicating that the sample went into the stable phase at this cooling rate.

ESR spectra of 16-doxylstearate in the metastable and stable phases at 9°C and 30°C are shown in Fig. 5. The metastable phase was obtained by heating to 75°C and cooling rapidly to 9°C. The stable phase was obtained by heating to 30°C, the temperature of the first endothermic transition, and incubating for 50 min. The spectrum of 16-doxylstearate in the stable phase at 9°C (Fig. 5B) with $T_{max}$ of 31.2 G indicates a high order parameter, while that in the metastable phase with $T_{max}$ of 29.2 G (Fig. 5A), indicates a lower order parameter. The order in the $L_{\beta}$ phase of DPPG is even lower (see Fig. 8A) with a $T_{max}$ of 24 G. The spectrum in the metastable phase immediately after heating to 30°C and in the stable phase after incubation at 30°C for 50 min are compared in Fig. 5 C and D. Conversion to the stable phase causes an increase in the amount of a component with greater $T_{max}$ and decrease in the amount of the less ordered component. This ordering of the spin label at the temperature of the first endothermic transition suggests that the stable phase of DPPG in glycerol is interdigitated like that of PC-glycerol, while the metastable phase may be less completely interdigitated. The results indicate further that interdigitation of PG in the presence of glycerol does not occur instantaneously with freezing; if the sample was cooled at a fast rate, it does not occur completely until reheating.

Changes in the spectral parameters of 16-doxylstearate in the stable phase of DPPG-glycerol and DPPG-water with temperature are compared in Fig. 6. As was the case with PC-glycerol, $T_{max}$ is maintained at a high value up until the phase

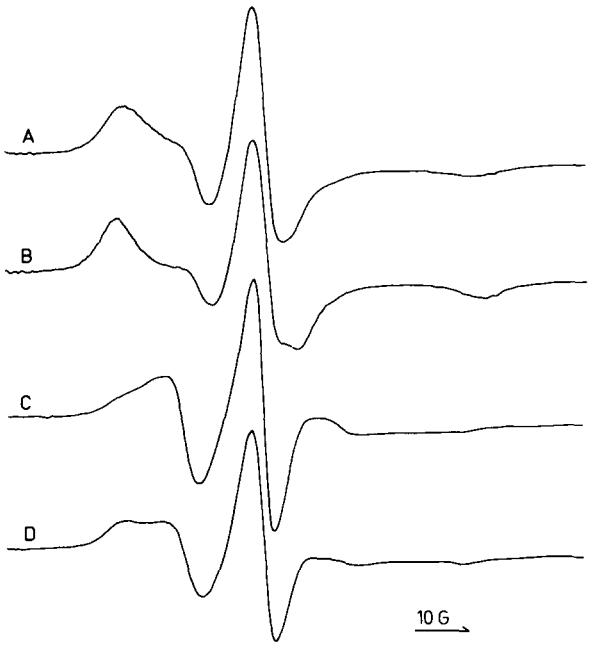


Fig. 5. ESR spectra of 16-doxylstearate in DPPG-glycerol. (A) in metastable state at 9°C, (B) in stable state at 9°C after incubation at 30°C for 50 min; (C) in metastable state at 30°C; (D) in stable state at 30°C after 50 min incubation at 30°C.

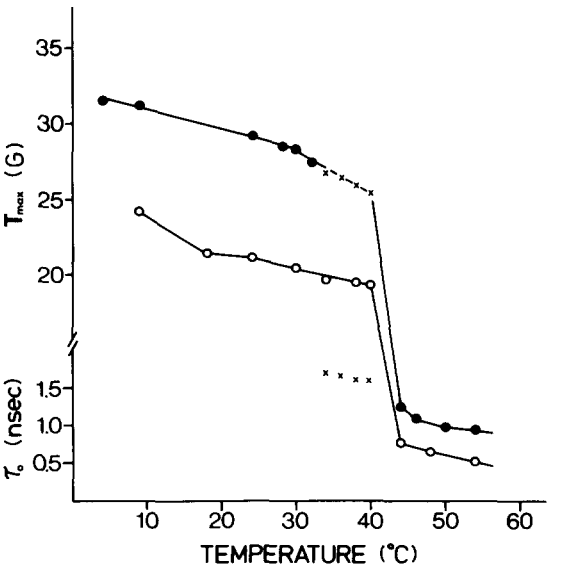


Fig. 6. Effect of temperature on spectral parameters, $T_{max}$ and $\tau_o$, of 16-doxylstearate in DPPG in distilled water (○) and glycerol (⊙). × indicates presence of two components in spectrum for DPPG-glycerol.

transition. However, unlike PC-glycerol, but like PC and PG in water, the phase transition is broad with an isotropic component characteristic of the liquid-crystalline phase starting to appear at 34°C.

The slow rate of interdigitation and metastable phase behavior were found only for PG-glycerol samples prepared near the phase transition temperature. If the sample was prepared at a much lower or higher temperature the sample went through a single transition on heating and cooling, at a somewhat higher temperature and with a greater enthalpy than the pure lipid. 16-Doxylstearate was highly ordered in these samples suggesting that they were interdigitated; the order was maintained up to the phase transition temperature. The difference in behavior may be a result of greater penetration of glycerol into the bilayer when the sample is prepared at the phase transition temperature, where the lateral compressibility of the lipid is at a maximum, than when prepared at lower or higher temperatures.

*Polymyxin-phosphatidylglycerol*

Ranck and Tocanne [7] using X-ray diffraction, showed that polymyxin B caused interdigitation of DPPG when present at a 1 : 5 mole ratio of polymixin to lipid. Polymixin caused a decrease in the repeat distance of the gel phase from 58 Å to 45 Å. They showed further by DSC that the polymyxin-DPPG complex underwent two endothermic phase transitions on heating similar to those shown in Fig. 7 with the first at a temperature about 2 deg. C below that of the pure lipid and the second at a similar temperature as the pure lipid [10]. We have investigated the calorimetric behavior in more detail, the effect of fatty acid chain length, and the effect on the order of 16-doxylstearate.

The temperatures and enthalpies of the two transitions for DMPG, DPPG, and DSPG in the presence and absence of polymyxin are given in Table II. The temperature difference between the two transitions decreased with increase in chain length. The total enthalpy of the two transitions was about 2 kcal/mol greater than that of the pure lipid. The ratio between the enthalpies of the two transitions depended somewhat on heating rate as shown in Fig. 7a–d and Table III. However, an exothermic transition between the two endothermic transitions was never observed. Furthermore the sample went through the same two transitions at similar temperatures and with similar enthalpies on cooling as shown for DPPG in

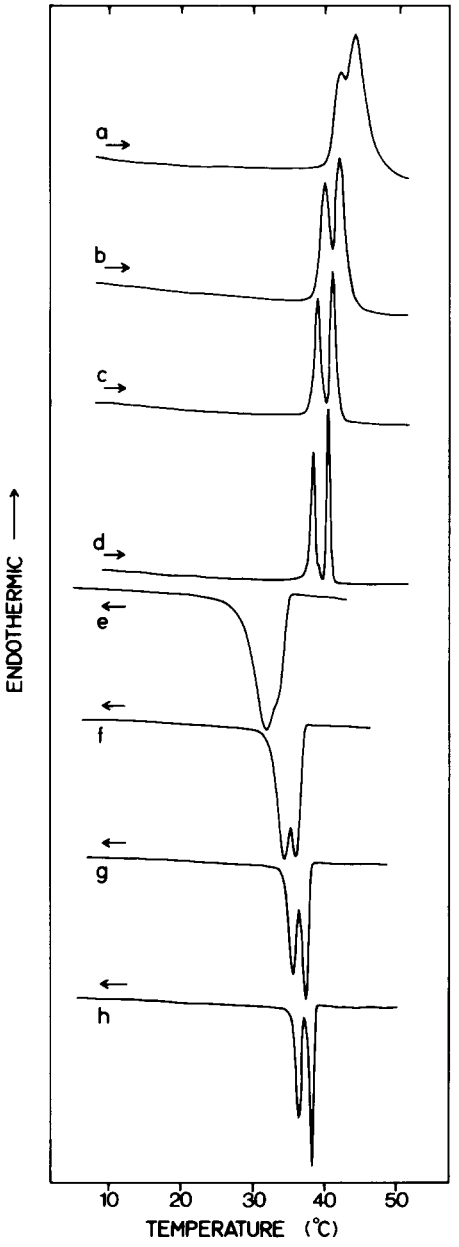


Fig. 7. DSC heating (a–d) and cooling (e–h) scans of DPPG/polymyxin (5 : 1 molar ratio) and heating and cooling rates of (a,e) 20 deg. C/min; (b,f) 10 deg. C/min; (c,g) 5 deg. C/min; (d,h) 2.5 deg. C/min. Sensitivity setting in mcal/s are (a,e) 3; (b,f) 2; (c,g) 1.5; (d,h) 1. Arrows indicate direction of temperature change.

TABLE II

EFFECT OF POLYMYXIN ON TRANSITION TEMPERATURES AND ENTHALPIES OF SATURATED FORMS OF PHOSPHATIDYLGLYCEROL

Temperatures determined at a heating rate of 10 deg. C/min, enthalpies at 1.25 deg. C/min.

| | $T_1$ (°C) | $\Delta H_1$ (kcal/mol) | $T_2$ (°C) | $\Delta H_2$ (kcal/mol) | $\Delta H_T$ [a] (kcal/mol) |
|---|---|---|---|---|---|
| DMPG | | | 24.0 | 6.4±0.2 | |
| DMPG-polymyxin | 19.4 | 3.7 | 22.4 | 4.6 | 8.5 |
| DPPG | | | 41.7 | 8.3±0.7 | |
| DPPG-polymyxin | 40.2 | 5.1±0.4 | 42.3 | 5.3±0.2 | 10.5±0.6 |
| DSPG | | | 56.3 | 12.5 | |
| DSPG-polymyxin | 54.1 | 7.0 | 55.7 | 7.6 | 14.6 |

[a] Total enthalpy of both transitions ($\Delta H_1 = \Delta H_2$).

Fig. 7e–h. The ratio of the enthalpies of peaks 1 and 2 from heating scans increased as the heating rate decreased down to a heating rate of 5 deg. C/min. The ratio of the same transitions observed on cooling decreased as the cooling rate decreased down to a cooling rate of 2.5 deg. C/min. Further decreases in the heating or cooling rate had no effect. This was not indicative of metastable phase behavior. The presence of the two transitions could indicate that there were two different and independent populations of lipid. However, the small but definite dependence of the ratio of the two peaks on heating and cooling rate suggested that there was only one population which went through the interdigitated gel state to liquid crystalline phase transition in a two stage process. Monitoring of the spectra of 16-doxylstearate through the two phase transitions provided a clue about the mechanism of these two stages.

The order of 16-doxylstearate was increased in the interdigitated phase of DPPG-polymixin at 9 deg. C as shown in Fig. 8B. The $T_{max}$ value was 30.5 G compared with a value of 24 G for pure DPPG (Fig. 8A). Similar increases in $T_{max}$ were obtained in the complexes of polymyxin with DMPG and DSPG. However, this increase persisted only until 30–35°C (for DPPG) as shown in Fig. 9A, below the temperature of the first peak observed by DSC. At the temperature of the first DSC transition the $T_{max}$ value was 23 G, only a little greater than the value in the pure lipid and not characteristic of interdigitation. At the temperature of the second peak observed by DSC the spin label acquired isotropic motion as in the pure

TABLE III

EFFECT OF HEATING RATE ON RATIOS OF ENTHALPIES OF PEAKS 1 AND 2 OF POLYMYXIN-PHOSPHATIDYLGLYCEROL

Molar ratio of polymyxin to PG, 1:5.

| Heating or cooling rate (deg. C/min) | Ratio $\Delta H_1/\Delta H_2$ | | | | | |
|---|---|---|---|---|---|---|
| | DMPG | | DPPG | | DSPG | |
| | heat | cool | heat | cool | heat | cool |
| 20 | 0.5 | [a] | 0.4 | >1.5 [a] | 0.4 | [a] |
| 10 | 0.8 | 1.6 | 0.8 | 1.5 | 0.6 | 1.8 |
| 5 | 0.9 | 0.9 | 1.0 | 1.1 | 0.8 | 1.2 |
| 2.5 | 0.9 | 0.8 | 1.0 | 1.0 | 0.7 | 1.0 |
| 1.25 | 0.8 | 0.9 | 1.0 | 1.0 | 0.9 | 1.1 |

[a] Two peaks were not resolved.

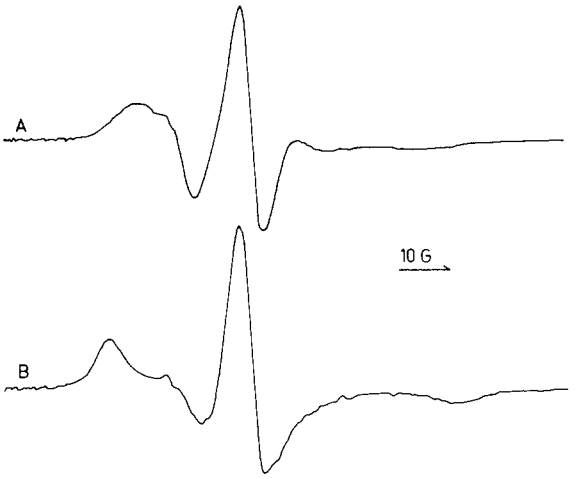


Fig. 8. ESR spectra of 16-doxylstearate at 9°C in (A) DPPG in buffer and (B) DPPG/polymyxin (5 : 1, molar ratio).

lipid. This large increase in motion causes an abrupt increase in the height of the center line of the spectrum as shown in Fig. 9B. The decrease in $T_{max}$ value before the first transition did not produce a relatively significant increase in the height because a much smaller increase in motion is involved, and thus the first transition cannot be detected in Fig. 9B.

These results suggest that during the first transition detected by DSC the lipid becomes non-interdigitated but does not yet melt. Its structure may not be identical to that of the $L_\beta$ or $P_\beta$ phase of the pure lipid, however, because of the perturbing effect of the polymyxin. The phase transition to the liquid crystalline phase does not occur until the temperature of the second transition, close to the transition temperature of the pure lipid. The enthalpy of this transition, obtained at a sufficiently slow heating rate that the enthalpy is independent of heating rate, is 20–30% less than that of the pure lipid (Table II) which can be ascribed to the perturbing effect of polymyxin. If the heating rate is too fast for de-interdigitation to occur completely at the lower temperature it occurs simultaneously with melting and the enthalpy of peak 2 increases. Similarly if the cooling rate is too fast, the freezing transition from the liquid-crystalline phase occurs simultaneously with interdigitation at the lower temperature and the enthalpy of peak 1 increases. This dependence of the ratio of

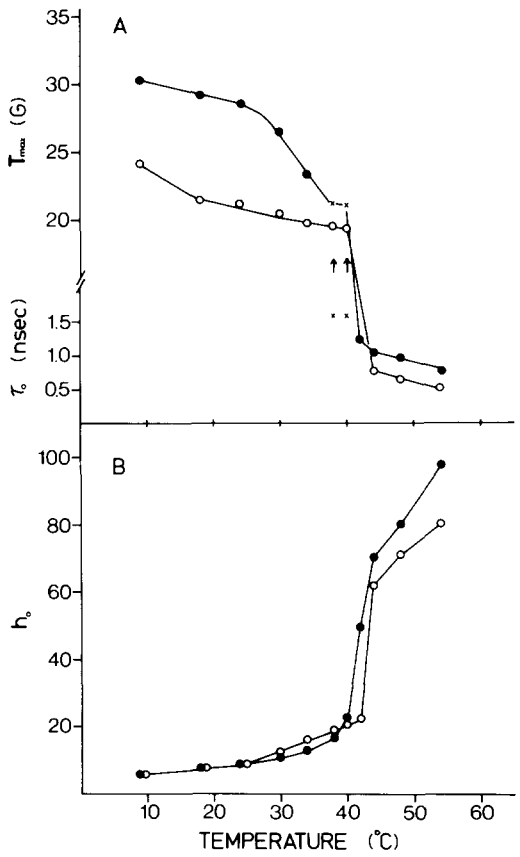


Fig. 9. Effect of temperature on ESR spectral parameters of 16-doxylstearate in DPPG only (○) and DPPG/polymyxin (5 : 1, molar ratio) (●). (A) $T_{max}$ and $\tau_o$ and (B) $h_o$, the height of the center line in arbitrary units. Arrows in (A) indicate the temperatures of the transitions detected by DSC. (×) indicates presence of two components in spectrum for DPPG-polymyxin.

the enthalpies of the two peaks on heating and cooling rate is a little more pronounced as the chain length increases (Table III) suggesting that the rates of refreezing and interdigitation decrease as the chain length increases.

## Discussion

We have shown that the order of 16-doxylstearate is increased in interdigitated states of lipids induced by glycerol and polymyxin, so that it is comparable to that of 5-doxylstearate in the non-interdigitated $L_\beta$ phase. 5-Doxylstearate is much less affected by interdigitation. Thus the fluidity gradient found in the $L_\beta$ phase as well as

the liquid-crystalline phase is abolished in the interdigitated phase. These results are to be expected since the terminal ends of the lipid fatty acid chains of lipids in one monolayer would be located in the region of methylene segments much closer to the ester linkage of lipid in the opposite monolayer of the interdigitated bilayer. Glycerol has also been shown to cause changes in the Raman spectra of DPPC which are interpreted as being a result of increased lateral interactions between the chains in the interdigitated state [15].

X-ray diffraction is probably the best technique for proving that interdigitation occurs. However, the increased order of 16-doxylstearate may make this spin label useful for detecting interdigitation in other systems. The spectrum obtained cannot by itself be completely diagnostic of interdigitation since other phenomena might also cause a similar type of spectrum, particularly motional restriction. However, motional restriction of 16-doxylstearate is very unusual except in the presence of intrinsic proteins, and then it occurs in the liquid-crystalline state as well as the gel state [16]. Thus a spectrum of 16-doxylstearate with large $T_{max}$ value which occurs only in the gel state, accompanied by information about the system which may allow ruling out of other mechanisms, may be useful in predicting whether or not interdigitation is likely. Use of a phospholipid spin label containing 16-doxylstearic acid acylated to the glycerol may be even more useful in detecting interdigitation.

Observation of motional restriction of 16-doxylstearate in DPPG-glycerol, for which X-ray diffraction results have not yet been reported, allows us to predict that glycerol induces interdigitation in DPPG as in DPPC. We also predict that DPPG-polymyxin, shown to be interdigitated in the gel state by X-ray diffraction [7], becomes deinterdigitated during the first transition observed by calorimetry on heating, based on the decrease in order of 16-doxylstearate which occurs before this transition, and that the transition to the liquid-crystalline phase occurs during the second calorimetric transition.

We predicted earlier that myelin basic protein (BP) induces interdigitation in DPPG [1,2]. The similar increase in $T_{max}$ of 16-doxylstearate in known interdigitated systems and the similar calorimetric behavior of DPPG-glycerol to that of BP-DPPG helps to support this prediction although X-ray diffraction studies will be necessary for further evidence.

The rate of interdigitation and the cause of the transitions detected by DSC depended on the particular system. Thus in the case of DPPG-glycerol, the results indicate that if the sample is cooled quickly, complete interdigitation does not occur until the sample is reheated to the temperature of the first transition observed by DSC, when it then occurred with a release of heat. On cooling from the liquid-crystalline phase the sample refroze at a much lower temperature than the $T_m$ of the interdigitated state. It refroze into a metastable state, which may have been partially interdigitated, based on the spectrum of 16-doxylstearate. If the sample was cooled slowly, complete interdigitation, resulting in further ordering of 16-doxylstearate, could occur on cooling. Very similar behavior was observed for DPPG with basic protein [1,2]. Thus for these two systems the first transition observed in DSC heating scans was caused by melting of the metastable state, followed by an exothermic transition to the interdigitated state, and the second transition was that of the interdigitated state to the liquid-crystalline state.

In contrast, for DPPG-polymyxin, the sample froze into a non-interdigitated gel state which then became interdigitated at a few degrees lower temperature with further release of heat even at fast cooling rates. These steps were reversible on heating. Changes in the spectrum of 16-doxylstearate indicated that the lipid went into a noninterdigitated gel state during the first transition observed in DSC heating scans and then went into the liquid-crystalline phase during the second transition. The chain disorder may have become too great with increase in temperature, as indicated by the decrease in $T_{max}$ values in Fig. 9A, for interdigitation to be maintained.

PC-glycerol also went into the interdigitated state on cooling, even at rapid rates. Like DPPG-polymyxin two transitions were observed on cooling, but the first was of higher enthalpy while the second was of much lower enthalpy than observed for DPPG-polymyxin. Only one transition was observed on heating whose enthalpy was similar to the sum of the two transitions on cooling. The second, lower temperature transition could be due

to interdigitation and the first higher temperature transition to freezing into a non-interdigitated gel state as for DPPG-polymyxin. The two transitions were too close together to be able to detect two separate states with different degrees of ordering of 16-doxylstearate. Alternatively, the second low enthalpy transition might be due to repartitioning of glycerol into the gel phase. The fact that glycerol increases the transition temperature relative to water, could indicate that the partition coefficient is higher in the gel phase than in the liquid-crystalline phase; however, in fact, the reverse has been found [17]. Furthermore, the fact that the enthalpy of the lower temperature transition increases with chain length, and in fact is quite significant for DBPC, supports the first interpretation.

The smaller enthalpy of the lower temperature transition for PC-glycerol compared to PG-polymyxin indicates that less stabilization energy is gained by interdigitation for PC-glycerol than PG-polymyxin, i.e. that the enthalpy of the non-interdigitated and interdigitated phases of PC-glycerol are very similar while they are quite different for PG-polymyxin. This is an expected consequence of the greater penetration and greater perturbing effect of polymyxin on the bilayer.

The increase in enthalpy of the lower temperature transition on cooling with fatty acid chain length for PC-glycerol and PG-polymyxin is consistent with the conclusion that this transition is due to interdigitation since the stabilizing energy to be gained by interdigitation would be expected to increase with chain length. However, the increase in temperature interval between the two transitions, i.e. decrease in $T_1$ relative to $T_2$, for PC-glycerol with increase in chain length indicates an increasing steric hindrance to interdigitation. Thus the longer chain length forms had to be cooled to a lower temperature, relative to the first transition temperature, where the fatty acid chains would have greater order, before interdigitation could occur.

Glycerol-induced interdigitation did not occur as readily in PG as in PC. Furthermore, glycerol lowered the transition temperature of the interdigitated phase of PG but increased it for PC. The effect of glycerol on the rate of interdigitation was found to depend on the temperature used for preparation, however; the slowest rate was found for samples prepared near the phase transition temperature. This may be due to greater penetration of glycerol into PG when prepared at this temperature, so that it causes greater lateral expansion. Based on the calorimetric behavior reported here and previously for BP-PG, the results suggest that the rate of interdigitation decreases in the order PC-glycerol > PG-polymyxin > PG-glycerol > PG-BP. Of these mixtures, only PG-polymyxin becomes disordered and deinterdigitated on heating. This is probably due to a greater disordering effect of the hydrophobic tail of polymyxin in the bilayer. The slower rate of interdigitation of PG-BP compared to PG-glycerol suggests that the hydrophobic residues of myelin basic protein (BP) may penetrate a little further into the bilayer than glycerol.

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