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# Effects of diacylglycerol on the structure and phase behaviour of non-bilayer forming phospholipid

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**Abstract**

The phase behaviour of mixed aqueous dispersions of the monomethyl derivative of dioleoylphosphatidylethanolamine and dipalmitoylglycerol has been characterised by X-ray diffraction, differential scanning calorimetry and freeze-fracture electron microscopy for mixtures containing dipalmitoylglycerol in the concentration range 0–20 mol%. Dispersions prepared at temperatures where the phospholipid exhibits a liquid-crystalline lamellar phase show that dipalmitoylglycerol is completely phase separated into aggregates of stable crystal phase ($\beta'$-phase). Heating mixed dispersions results in transformation of lamellar into hexagonal-II structure commencing at approximately 45°C. This temperature coincides with a disappearance of $\beta'$-phase of DPG which becomes incorporated into hexagonal-II phase. The pure phospholipid is transformed upon cooling from hexagonal-II into characteristic cubic phases; the formation of cubic phase is prevented by the presence of dipalmitoylglycerol and mixed dispersions initially form a lamellar liquid-crystalline phase in which the lipids are phase separated. The X-ray and thermal data suggest that relatively small domains of metastable crystal phase ($\alpha$-phase) of DPG form initially on cooling and these subsequently coalesce and transform to $\beta'$-phase. 

*Keywords:* Diacylglycerol; Phosphatidylethanolamine; Hexagonal-II phase; Freeze-fracture electron microscopy; Differential scanning calorimetry; X-ray diffraction

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## 1. Introduction

Diacylglycerol is a product of membrane phospholipid hydrolysis associated with cell activation. Its role as an important intracellular messenger is well established and its primary functions appear to be to activate protein kinase C [1] and phospholipases [2,3]. Diacylglycerol is also believed to mediate certain membrane fusion events [4]. It is likely that the functions of diacylglycerol in cell membranes described above are mediated by the modulation of the physical properties of cell membranes due to the presence of diacylglycerols.

In this regard, diacylglycerols are known to induce structural perturbations in phospholipid bilayers [4–13]. When phosphatidylcholines are mixed with diacylglycerol of the same fatty acyl composition stoichiometric complexes are formed. Two types of the complex have been reported [9,10,13]. One is a complex with a 1:1 stoichiometry and the other is a 1:2 complex. The 1:1 complex is immiscible with domains of fluid phase phospholipid [11] presumably because of strong van der Waals attractive forces between the diglyceride and phospholipid in the complex. This implies that in membranes, diacylglycerol will not distribute randomly within the lipid bilayer matrix but will tend to be present in the form of 1:1 complexes with the bilayer-forming phospholipid. There is evidence to suggest that phase separation of 1:1 complex with disordered hydrocarbon chains is preserved in fluid bilayers of phospholipids, at least in the temperature region close to the fluidus phase boundary [11], however, at higher temperatures non-bilayer phases are formed [4–7,9,10,12,13]. This tendency to form non-bilayer phases increases with increasing proportions of diglyceride in the phospholipid.

Taking into account the fact that some biological membranes contain considerable amount of non-bilayer forming phospholipid (phosphatidylethanolamine, etc.), it is important to understand the effects of diacylglycerol on the phase behaviour of non-bilayer forming phospholipids. Some preliminary studies of the phase behaviour of the monomethyl derivative of dioleoylphosphatidylethanolamine (DOPE-Me) codispersed with 2 mol% diacylglycerol have been reported [4,8]. These have shown that the diacylglycerol causes a marked decrease in the temperature of transition between the bilayer and non-bilayer phase of the phospholipid. The present work was undertaken to investigate the detailed molecular mechanism underlying the effect of diacylglycerol on the phase behaviour of non-bilayer forming phospholipid.

## 2. Materials and methods

### 2.1. Materials and sample preparation

The monomethyl derivative of L-$\alpha$-dioleoylphosphatidylethanolamine (DOPE-Me) was obtained as a solution in chloroform from Avanti Polar Lipids Inc. (Alabaster, AL, USA) and 1,2-dipalmitoyl-*sn*-glycerol (DPG) was purchased as a dry powder from Sigma Chemical, Co. (St Louis, MO, USA). The lipids gave a single spot on a silica gel thin-layer plate developed with a solvent system consisting of chloroform: methanol: 30% aqueous ammonia = 10:10:3 (by volume) and they were used without further purification. The water used in this study was prepared by using a Milli-Q water purification system (Millipore Corp., MA, USA).

Chloroform solutions containing DOPE-Me and appropriate amounts of DPG in glass tubes were evaporated under a stream of oxygen-free dry nitrogen, and the remaining traces of solvent were removed by storage of the sample for 16 h under reduced pressure. The lyophilized samples were dispersed in buffer (10 mM TES, 100 mM NaCl, 0.1 mM EDTA, pH 7.4) with shaking for 20 min at a temperature $< 30$°C, i.e. below the lamellar liquid-crystalline to hexagonal-II phase transition temperature of DOPE-Me (approx. 66°C). The lipid concentrations were 30 wt.% and 2.5 wt.% for X-ray diffraction and DSC measurements, respectively.

### 2.2. X-ray diffraction

X-ray diffraction experiments using syn-

chrotron X-radiation were performed at beamline 15A [14] of the Photon Factory at Tsukuba, Japan. On this beamline, the X-ray beam is focused and monochromatized (0.1506 nm) with a bending fused silica mirror and a Ge crystal monochromator, respectively. The incident X-rays were focused to a spot size of $2 \times 1$ mm at the sample position. The patterns of scattered X-rays were recorded using a one-dimensional position-sensitive proportional counter with 512 channels or a two-dimensional area detector (Imaging plate, Fuji Photo Film Co. Ltd., Tokyo, Japan). Time-resolved data obtained with the electronic detector were stored in a SUN S-4/LX workstation. Data obtained by imaging plates were digitised using a BAS2000 data reading system (Fuji Photo Film Co. Ltd., Tokyo). Typical sample-to-detector distances were 1200–1400 mm. The diffraction spacings were calibrated using the lamellar spacings of anhydrous cholesterol [15]. Sample temperature was controlled with a modified differential scanning calorimeter (FP84, Mettler Instrument Corp., Hightstown, NJ). The modifications have been reported elsewhere [16,17]. Temperature scan experiments were performed with a scan at a rate of 2.5°C/min and 90 consecutive scattering patterns were recorded, each of 20 s. Samples were sealed into an aluminium pan in which windows of polyimide films were inserted to provide access of the beam though the sample. The position of the sample cell in the calorimeter was changed after each scan so as to minimise any possible radiation damage.

Some static X-ray diffraction measurements were carried out using a Ni-filtered $CuK_{\alpha}$ radiation source (RU200BEH, Rigaku, Tokyo, Japan) and a two-dimensional area detector (Imaging plate, Fuji Photo Film Co. Ltd., Tokyo, Japan). The X-ray beam was focused with a double-mirror optical system. The sample was sealed in a thin wall quartz capillary of 1.5 mm diameter (Hilgenberg, Malsfeld, Germany). The capillary was cradled into a hollow brass holder. Temperature of the sample was controlled within $\pm 0.1$°C by circulating water from a temperature-controlled water bath (B. Braun, Melsungen, Germany) through the sample mount.

### 2.3. Calorimetry

Calorimetric scans were performed with a Privalov-type differential adiabatic mirocalorimeter, DASM-4 [18] at a heating scan rate of 0.5°C/min with 0.5-ml volume cells under a pressure of 2.5 atm to prevent bubble formation. The data were processed on a personal computer NEC PC-9801Vm (Tokyo, Japan). The heat absorption was calibrated by joule's of heat from the internal circuit.

### 2.4. Freeze-fracture electron microscopy

Mixed aqueous dispersions for freeze-fracture were prepared as described above but equilibrated at 20°C for approximately 1 h prior to examination. A lipid concentration of 30 wt.% was used. The temperature of the specimen was regulated by placing the sample contained in a glass capillary on a block heater fitted with a metal cover for thermal insulation. The temperature was monitored by a thermocouple attached directly to the sample capillary. The average heating and cooling rates were 3°C/min and 2.3°C/min, respectively. After experiencing the desired course of temperature change, the specimen was transferred quickly onto a small copper cell and incubated at the final temperature for approximately 2 min before thermal quenching into nitrogen slush which was prepared by placing liquid nitrogen in a vacuum chamber [19]. The speed of thermal quenching appeared to be comparable to thermal quenching into liquid freon, judging from the state of the fractured surface of water. The frozen samples were fractured and the exposed surfaces were coated with platinum and carbon in a JOEL freeze-fracture apparatus (JFD-9010, Tokyo, Japan). The replicas were cleaned with solvent and examined under an electron microscope (JEOL JEM-1010, Tokyo, Japan) at an accelerating voltage of 80 kV.

## 3. Results

To determine the effect of the presence of DPG on the structure of DOPE-Me aqueous dispersions of the phospholipid containing 5, 10,

15 and 20 mol% DPG were examined by static X-ray diffraction methods. Small-angle scattering patterns recorded from these mixed aqueous dispersions are shown in Fig. 1a. In the observed small-angle region, pure DOPE-Me gave a pattern with two orders of reflection, typical of a lamellar phase (data not shown). The lamellar spacing is 6.2 nm. Two following features can be seen from Fig. 1: (1) a peak located at a position corresponding to spacing of 4.35 nm appears when DPG is present and the intensity of this peak increases with increasing proportion of DPG in the mixtures; and (2) the position of the lamellar diffraction peak of DOPE-Me at 6.2 nm is unaffected by the presence of up to 20 mol% DPG. The X-ray diffraction peaks at 4.35 nm and the first order lamellar diffraction peak centred at 6.2 nm were fitted using a Lorentznian shape function to estimate integrated diffraction intensities. We calculated a ratio of diffraction intensity from the two peaks (6.2 and 4.35 nm) by normalising the intensity of the first order lamellar diffraction of DOPE-Me to be unit. This data is plotted as a function of the mol% of DPG in Fig. 1b. There is a direct relation between the two parameters which passes through a point close to the origin. This is consistent with an assignment of the peak at 4.35 nm to a repeat structure of pure DPG in a stable crystal phase ($\beta'$-phase) [20] which is almost completely phase separated from the phospholipid. When the samples were heated to temperatures above the lamellar to hexagonal-II ($H_{II}$) phase transition temperature of the phospholipid and the melting temperature of the $\beta'$-phase of DPG, the two components may be mixed because the spacing of the $H_{II}$ repeat structure progressively decreases with increasing mol% of DPG in the mixture as shown in Fig. 1c.

The effect of DPG on the phase behaviour of DOPE-Me was examined by temperature scan X-ray diffraction methods using a synchrotron radiation source. Mixed aqueous dispersions of DOPE-Me containing up to 20 mol% DPG were heated at 2.5°C/min from 5 to 80°C. The *d*-spacings of the lamellar liquid-crystalline ($L_\alpha$), $H_{II}$ and $\beta'$-phase of DPG are plotted as a function of temperature in Fig. 2. For the pure phospholipid, there is a two-state transition between $L_\alpha$ and

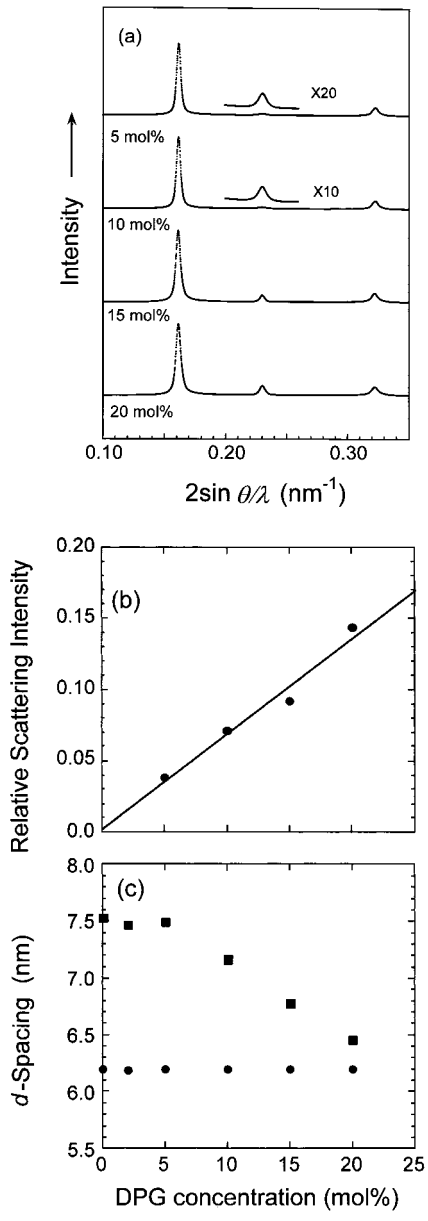


Fig. 1. (a) Static X-ray scattering patterns recorded at 20°C from mixed aqueous dispersions of DOPE-Me containing 5, 10, 15 and 20 mol% DPG. Diffraction intensity in the scattering pattern corresponding to spacing of approximately 4.35 nm is also scaled ×20 and ×10 in dispersions containing 5 and 10 mol% DPG, respectively; (b) relative scattering intensity (see text) of the first-order lamellar repeat spacing of DOPE-Me (6.2 nm) and the intensity of the peak centred at 4.35 nm as a function of the proportion of DPG in the mixture; (c) spacing of first-order lamellar repeat at 20°C (●) and first-order *d*-spacing of hexagonal-II structure recorded after heating to 65°C (■).

$H_{II}$ phase in the temperature range 62–70°C with coexistence of the initial and final phases throughout the transition region. The onset temperature of the transition, as judged by the first appearance of the $H_{II}$ phase, decreases to approximately 45°C when DPG is present even in a proportion as low as 2 mol%. The temperature of completion of the transition where the $L_\alpha$ phase can no longer be detected, decreases from 75 to 45°C with proportions of DPG up to 10 mol% and reaches a limiting value which does not change with higher proportions of DPG up to 20 mol%. Another feature seen in this figure is the effect of DPG on the temperature-dependent change in *d*-spacing of the $H_{II}$ phase. Temperature dependence of *d*-spacing is approximately $-0.02$ nm/°C in the temperature ranges where there is no coexistence of $\beta'$-phase of DPG crystals and the $H_{II}$ phase. At higher proportions of DPG biphasic behaviour is clearly seen (Fig. 2d–f); the *d*-spacing decreases with a decrement of approximately $-0.08$ nm/°C in the temperature ranges where the DPG $\beta'$-phase and the $H_{II}$ phase coexist.

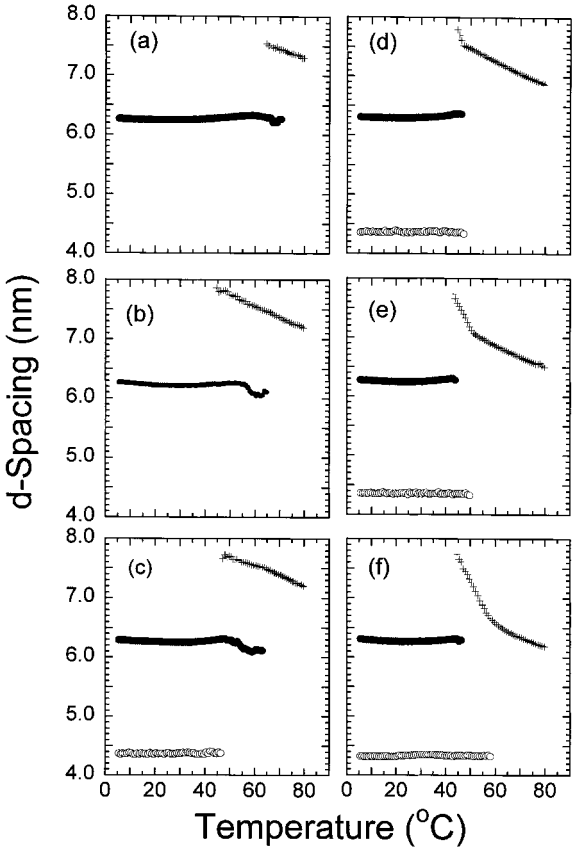


Fig. 2. Small-angle spacings of DOPE-Me containing (a) 0; (b) 2; (c) 5; (d) 10; (e) 15; and (f) 20 mol% DPG during heating at 2.5°C/min from 5° to 80°C. (●) *d*-spacings of the $L_\alpha$ phase of DOPE-Me, (+) the $H_{II}$ phase, and (○) the $\beta'$ phase of DPG.

Fig. 3 shows a partial phase diagram of DOPE-Me/DPG in mole ratios of DPG up to 20 mol% over the temperature range 0–80°C has been constructed from the temperature scan X-ray diffraction data shown in Fig. 2. In the low temperature region of the phase diagram ($T < 45$°C) the $L_\alpha$ phase of DOPE-Me was assigned from the *d*-spacing of the lamellar repeat and a broad scattering band in the wide-angle region which is consistent with disordered hydrocarbon chains (data not shown). $\beta'$-structure of stable DPG crystals was distinguished from $\alpha$-structure of metastable DPG crystals by characteristic repeat spacings of 4.35 and 4.83 nm, respectively [20]. In the high temperature region of the phase diagram ($T > 45$°C) the structure is $H_{II}$ but the temperature of the transition decreases from approximately 66 to 45°C with increasing DPG up to 10 mol% whereupon at higher mole ratios of DPG the transition temperature is increased. The

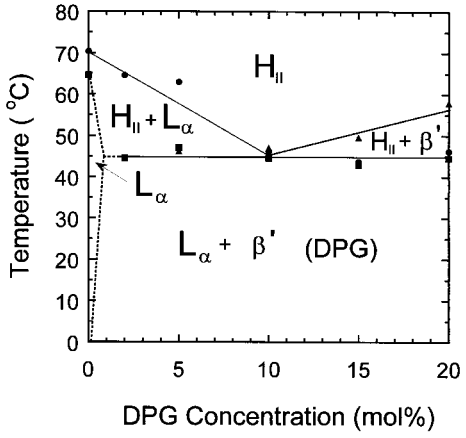


Fig. 3. Partial phase diagram of DOPE-Me/DPG over the concentration range 0–20 mol% DPG derived from the data presented in Fig. 2. $L_\alpha$, lamellar liquid crystalline; $H_{II}$, hexagonal-II structure; $\beta'$, $\beta'$-crystal phase of DPG.

aqueous mixture of DOPE-Me and DPG exhibits behaviour analogous to an eutectic, which is observed for two component systems and strongly suggests that the DOPE-Me/DPG system has an eutectic point.

To further characterise the structure of the mixture in the region of phase coexistence and to examine the possibility of a phase separation process on cooling, temperature scan X-ray experiments were performed during heating and subsequent cooling of aqueous dispersions of pure DOPE-Me and DOPE-Me/20 mol% DPG (Fig. 4). Two-dimensional projections of X-ray scattering intensity are presented in Fig. 4. The heating profile of an aqueous dispersion of pure DOPE-Me (Fig. 4a) shows a typical two-state transition between $L_\alpha$ and $H_{II}$ phase, i.e. in the temperature range of 62–70°C, the two phases coexist without the appearance of other phases. The cooling profile of the dispersion of phospholipid shows that the transition is not reversible under the experimental conditions employed. Below approximately 74°C, in addition to the diffraction peaks from the $H_{II}$ phase, three diffraction peaks appear at smaller angles than the (10) reflection of the $H_{II}$ phase. At 73.8°C, the spacings of these new peaks are 12.12, 9.01 and 7.70 nm, respectively. Judging from these long spacings, the formation of a cubic phase or phases is implied, however, from only three diffraction peaks it is not possible to determine the space group. The $H_{II}$ structure transforms to a cubic phase on cooling below 62°C. Six spacings of a cubic phase could be identified and the spacings are in the ratio of $1/\sqrt{2} : 1/\sqrt{3} : 1/\sqrt{4} : 1/\sqrt{6} : 1/\sqrt{8} : 1/\sqrt{9}$ (Fig. 5). This series is consistent with the indexes of Pn3m and Pn3 space groups. The lattice constant is $17.68 \pm 0.15$ nm at 24.6°C. Gruner et al. [21] have reported that two cubic phases (Pn3m/Pn3 and Im3m) form in pure DOPE-Me after a 1.5-year incubation at room temperature. In the present study, the existence of Im3m cubic phase could not be detected. The lattice constant of the Pn3m/Pn3 cubic phase (17.68 nm at 24.6°C) observed in our experiment is slightly longer than that reported by Gruner et al. (13.6 nm at 25°C) [21]. This disagreement may be due to the differences in the buffer conditions and thermal history between the study of Gruner et al. [21] and the present one. Recently, Tenchov et al. [22] have been shown that sorts of sodium salts, their concentrations and the number of temperature cycles

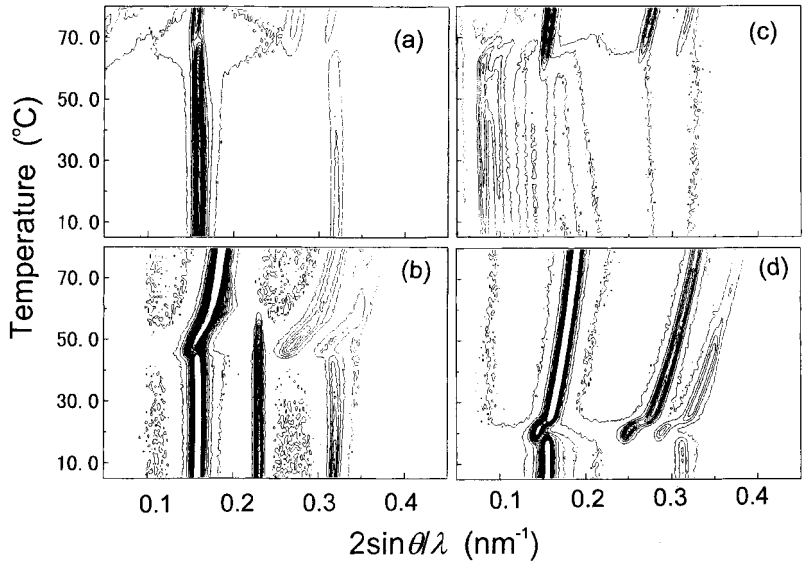


Fig. 4. Two-dimensional projections of X-ray scattering intensity recorded from aqueous dispersions of DOPE-Me (a,c) and DOPE-Me/20 mol% DPG (b,d) during heating (a,b) and immediate cooling (c,d) at 2.5°C/min.

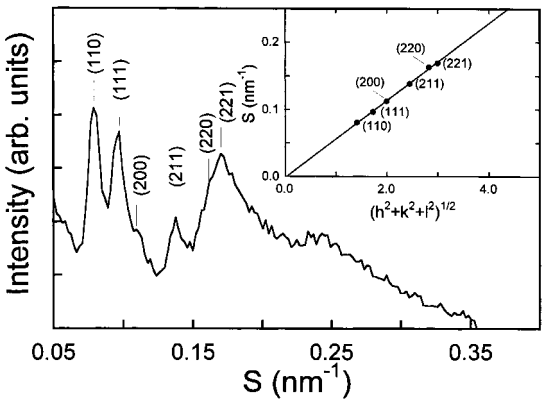


Fig. 5. X-ray scattering patterns of pure DOPE-Me recorded at 24.6°C after cooling from the $H_{II}$ phase with a rate of 2.5°C/min. The bar marks are expected positions of the diffraction peak of Pn3m cubic lattice that are indexed (*hkl*). Inset shows reciprocal spacings ($S$) vs. $(h^2+k^2+l^2)$ for the *hkl* indices. All points fall on a straight line. The lattice constant obtained as the reciprocal slope is $a = 17.68 \pm 0.15$ nm.

affect on the lattice constants of cubic phases in phosphatidylethanolamine dispersions and that the repeating of temperature cycles to the cubic phases results in the relaxation of the lattice constants from a larger value to smaller value. We observed the cubic phase formed after the first cooling scan for pure DOPE-Me dispersed into a TES buffer. On the other hand, Gruner et al. [21] observed the sample dispersed into pure water after a 1.5-year incubation at a room temperature.

The presence of 20 mol% DPG in the DOPE-Me dispersion reduced the onset temperature of the $H_{II}$ phase, which can be seen to coexist with the peak assigned as the first-order repeat of the $\beta'$-phase of DPG (Fig. 4c). The intensity of diffraction from the pure DPG phase decreases progressively through the transition and coincides with the rapid decrease in spacing of the $H_{II}$ phase. The phase transition is clearly irreversible under the conditions of the experiment. Although there is a progressive increase in *d*-spacing of the $H_{II}$ phase with decreasing temperature down to the transition region (22°C, Fig. 4d), there is no indication of phase separation of pure DPG. Furthermore, the $H_{II}$ phase transforms into a lamellar liquid-crystal phase rather than into a cubic phase.

Phase separation of DPG from the phospholipid in DOPE-Me/20 mol% DPG mixtures at temperatures below the phase transition were examined by conventional X-ray diffraction methods and the results are presented in Fig. 6. X-ray diffraction peaks at 6.16 and 4.35 nm correspond to *d*-spacings of pure DOPE-Me and $\beta'$-phase of DPG, respectively. The wide-angle scattering pattern is typical of $\beta'$-phase of DPG superimposed on a broad scattering band of disordered chains of phospholipids. When the dispersion is heated to 80°C and recooled to 4°C the peak at 4.35 nm corresponding to $\beta'$-phase of DPG is absent. Close examination of the wide-angle scattering region indicates weak reflections at spacings of 0.417 and 0.377 nm consistent with the presence of both $\alpha$- and $\beta'$-phase of DPG [20]. A scattering pattern in both a small- and wide-angle of a typical $\beta'$-structure of DPG gradually emerges during incubation of the dispersion at 4°C.

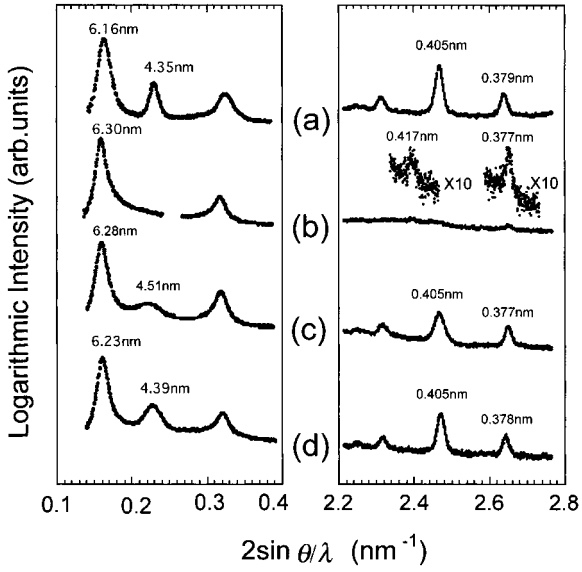


Fig. 6. Small- and wide-angle X-ray scattering intensity patterns recorded from dispersions of DOPE-Me/20 mol% DPG. (a) initial sample at 20°C, (b) sample after immediately heating to 80°C and cooling to 4°C, (c) sample recorded after 6 h equilibration at 4°C, (d) sample recorded after 140 h equilibrium at 4°C.

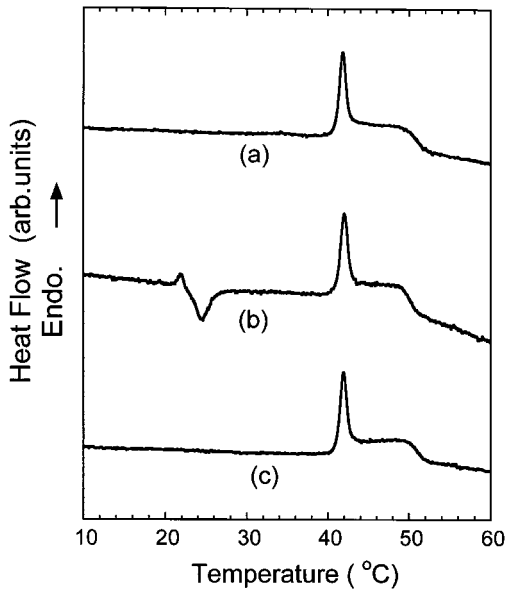


Fig. 7. Differential scanning calorimetric heating curves recorded from aqueous dispersions of DOPE-Me/15 mol% DPG between 10 and 60°C with a heating rate of 0.5°C/min. (a) initial heating scan; (b) heating scan recorded immediately after cooling from 80 to 4°C; (c) heating scan recorded from sample incubated for 24 h at 4°C after cooling from 80°C.

Previous DSC studies [20,23] have revealed that thermal quenching of pure DPG from the melt results in formation of the metastable $\alpha$-phase which subsequently transforms into the $\beta'$-phase with endothermic and exothermic heat flows upon heating. The existence of $\alpha$-phase of DPG in the recooled dispersion of DOPE-ME/DPG was examined using calorimetry. The thermograms of the mixed dispersion of DOPE-Me/15 mol% DPG are shown in Fig. 7. This shows that in addition to an endotherm at 42°C, the thermal anomalies appear at approximately 25°C in samples reheated immediately following the initial cooling scan, indicating the existence of $\alpha$-phase of DPG in the mixture. With 24 h incubation at 4°C the thermal transitions at 25°C are absent and only the endotherm at 42°C remains. The present DSC results suggest that conversion of the $\alpha$-phase to the $\beta'$-phase of DPG takes place during incubation of the mixed dispersion at 4°C.

Phase separation of pure DPG in mixtures containing 20 mol% DPG in DOPE-Me prior to heating is further confirmed by the freeze-fracture electron microscopy. Among the rounded vesicular surfaces there appear stacks of flat planes, which may correspond to a pure DPG crystal domain (Fig. 8A). The continuity between the flat and rounded surfaces suggests the lateral phase separation, i.e. the DPG crystals are not isolated from the DOPE-Me bilayer membranes. An angular structure seem in some areas of the replica is characteristic of DPG in the crystal phase. After heating to 80°C and subsequent cooling to 20°C there were no flat regions discernible as a DPG crystal; all the fracture planes

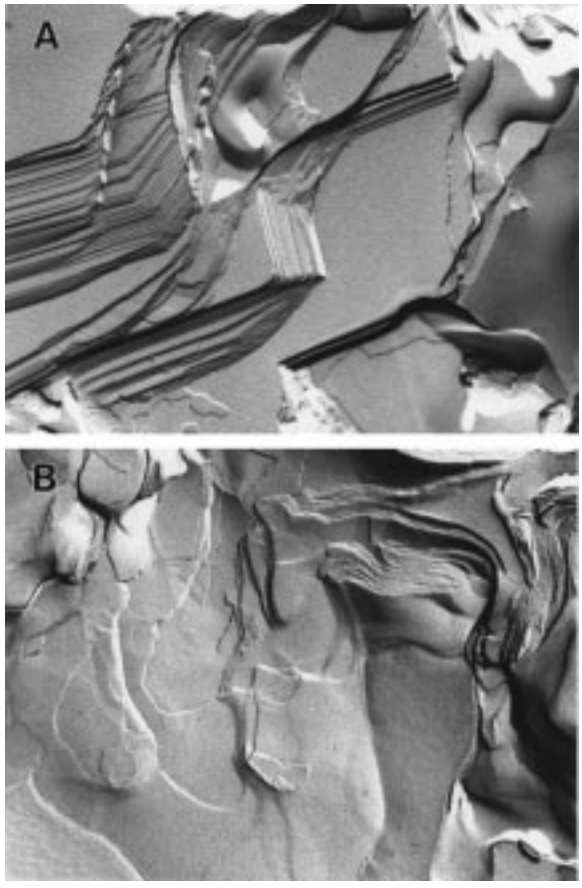


Fig. 8. Electron micrographs of freeze-fracture replicas prepared from a dispersion of DOPE-Me containing 20 mol% DPG thermally quenched from approximately 20°C. (A) initial sample at 20°C; (B) sample after heating to 80°C and cooling to approximately 20°C.

are found to have a rounded appearance (Fig. 8B).

## 4. Discussion

### 4.1. Phase diagram

A partial phase diagram of the mixture of DOPE-Me and DPG in mole ratios of DPG up to 20 mol% has been constructed over the temperature range 0–80°C. The diagram is based mainly on structural (and thermal) data obtained from the temperature scan X-ray diffraction experiments described in Fig. 2. The phase diagram exhibits apparent eutectic behaviour with a composition at the eutectic point of 9:1, DOPE-Me/DPG. The phase boundaries in the coexistence region of $<2$ mol% DPG could not be defined with the data presently available and are indicated by dashed lines.

Changes in structure in mixed aqueous dispersions of DOPE-ME/DPG containing more than 10 mol% DPG during heating may be inferred from the information provided in this phase diagram. A molecular rearrangement model for the heating process is illustrated schematically in Fig. 9. Below approximately 45°C, the $\beta'$-phase of DPG and the $L_\alpha$ phase coexist in separate domains. Upon heating, the first appearance of the $H_{II}$ structure begins at approximately 45°C and this coincides with the onset of melting of the $\beta'$-phase. The melted DPG mixes with DOPE-Me, i.e. the DPG is incorporated into the $H_{II}$ structure. During this process, the *d*-spacing of the $H_{II}$ phase decreases progressively with increasing temperature. At the point when all the DPG has been completely incorporated into the $H_{II}$ phase, there is a change in the inverse relation between *d*-spacing of the $H_{II}$ phase and temperature which can be seen in Figs. 2 and 4.

Phase diagrams of several types of binary mixtures of phospholipid and diacylglycerol have already been reported [9,10,13,24,25]. This is the first report of an almost complete phase separation between the phospholipid in the $L_\alpha$ phase and diacylglycerol. In mixtures of phosphatidylcholine and diacylglycerol, the formation of lamellar complexes has been reported [9,10,13].

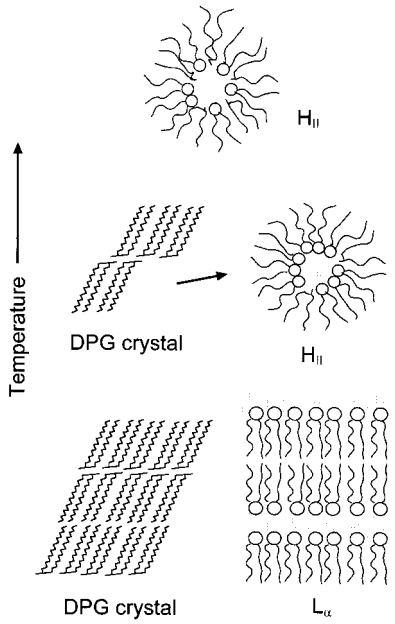


Fig. 9. Molecular model showing phase separation and mixing during heating of mixed aqueous dispersions of DOPE-Me and DPG.

Clearly in the case of phosphatidylethanolamine there is phase separation in the temperature region where the phospholipid is in a $L_\alpha$ phase. These facts may be interpreted in terms of the difference of the interaction between the headgroups. Unlike phosphatidylcholines, there is a strong hydrogen bond network between phosphatidylethanolamine headgroups [26]. It may be expected that disruption of this hydrogen bond network would be required for formation of a lamellar phosphatidylethanolamine/diacylglycerol complex. As a consequence DPG is phase separated from phosphatidylethanolamine bilayers, where it exhibits phase behaviour analogous to the pure diacylglycerol.

### 4.2. Effect of DPG on the structure of the $H_{II}$ phase

Siegel et al. [4,8] reported that the presence of 2 mol% diacylglycerol reduces the lattice constant of DOPE-Me in the $H_{II}$ phase. We have confirmed that this tendency is also true for the mixtures containing higher proportions of DPG. As they pointed out [4,8], the effect of diacylglyc-

erol on the dimensions of the $H_{II}$ phase may be due to the preponderance of hydrophobic character and the molecular shape of DPG. Both factors can serve to decrease in spontaneous radius of curvature of the lipid/water interface. Similar reduction of *d*-spacing of the $H_{II}$ phase has been reported for the case of addition of cholesterol [27,28]. In contrast, an expansion of the *d*-spacing of the $H_{II}$ phase has been observed upon addition of alkane [29] or squalene [30]. These results have been explained by suggesting that alkane and squalene molecules pack into the perimeter or the corner of the hexagon, but that diacylglycerol and cholesterol molecules are located near the hydrocarbon chains and glycerol moiety of phospholipids [4,8,27,28]. On the whole, the *d*-spacing of the $H_{II}$ phase of DOPE-Me tends to decrease with increasing DPG concentrations. The present data (Fig. 1c), however, may be possible to be interpreted as showing that the *d*-spacings are almost constant up to 5 mol% DPG concentrations. Thus, a following situation would be expected. For the case of addition of a small amount of DPG to DOPE-Me, some of the DPG molecules might be able to penetrate into the interstitial space of the corner of the hexagon and as a result, these molecules contribute to the increasing of *d*-spacing, but this effect may be compensated by the effect of the DPG molecules which are located near the interface between water and lipids. Above 5 mol% DPG concentrations, only the effect of the decreasing of *d*-spacing may be observed, because the interstitial space is filled. Nevertheless, more detailed studies must be required to conclude and also to determine the phase boundaries at the low DPG concentration regions precisely.

### *4.3. Phase behaviour on cooling*

It has been noted previously that pure DOPE-Me exhibits a complex pattern of phase behaviour [21,31,32]. In a heating scan, the $L_{\alpha}$ phase transforms directly into the $H_{II}$ phase. In a cooling scan, however, the $H_{II}$ phase forms a cubic phase rather than an $L_{\alpha}$ phase. Our results (Fig. 4c) are consistent with these earlier findings [21,32], however, the diffraction pattern of the cubic phase in the present study is slightly broader, which suggests the coexistence with some other structure (Fig. 4c). This is supported by freeze-fracture electron microscopic studies that showed that the cubic structure coexists with an $H_{II}$-like structure and multilamellar vesicle structures at approximately 25°C (data not shown). Gruner et al. [21] have reported that temperature cycling or prolonged incubation is required to create a uniform cubic phase.

The present study shows that on cooling from the $H_{II}$ phase the presence of DPG prevented the formation of some cubic phases (Fig. 4d). Furthermore, X-ray scattering profiles recorded from mixed dispersions cooled to below approximately 15°C showed a single lamellar repeat spacing rather than two lamellar repeats observed immediately after sample preparation (Fig. 4d). One way to interpret this data is by reference to the polymorphic phase behaviour of DPG. DPG exists in two crystal forms; one is a stable crystal ($\beta'$-phase) and the other is a metastable crystal ($\alpha$-phase) [20]. For pure DPG, endothermic and exothermic thermal events occur associated with the $\alpha$- to $\beta'$-phase transformation on heating [20,23]. The DSC data obtained from a mixed dispersion containing 15 mol% DPG during reheating after cooling from 80°C (Fig. 7b) is consistent with the formation of $\alpha$-phase of DPG in the mixture, though the temperatures of the endothermic and exothermic peaks (approx. 25°C) are somewhat lower than those of pure DPG [20,23]. This strongly suggests that the presence of the $L_{\alpha}$ phase of DOPE-Me affects the phase transition behaviour of DPG. It is noteworthy that the diffraction pattern characteristic of the $\alpha$-phase of DPG is not clearly distinguished in the scattering patterns recorded immediately after cooling (Fig. 4d and Fig. 6b). Taking this X-ray result into consideration, we can interpret the process on cooling as follows: first, DPG mixed with DOPE-Me partitions out of the $H_{II}$ structure, which coincides with a transformation of the mixture into the $L_{\alpha}$ phase. The DPG crystallises into relatively small domains of the $\alpha$-phase which do not give rise to distinct Bragg reflections in the X-ray scattering profile. The presence of these small crystals of DPG in the phospholipid bilayers

may be the reason why DOPE-Me does not form characteristic cubic structures of the pure phospholipid. With time, the DPG gradually coalesces into large aggregates which are converted into the stable $\beta'$-phase and ultimately an almost complete phase separation between the $L_\alpha$ phase of DOPE-Me and the $\beta'$-phase of DPG occurs. The present DSC results suggest that 24 h incubation at 4°C induces nearly complete transformation of DPG in DOPE-Me mixtures to the $\beta'$-phase (Fig. 7c), whereas in the case of pure DPG, even after 24:00 h incubation at 4°C after cooling from the melt, approximately 20% of DPG remains as the $\alpha$-phase (Takahashi et al., unpublished results). The relaxation time of the conversion from the $\alpha$- to $\beta'$-phase under isothermal conditions in the mixture may be faster than that observed in pure DPG.

### *4.4. Biological relevance*

The formation of lamellar complexes of diacylglycerols with bilayer forming phospholipids and its tendency to lower the temperature of non-lamellar phase transitions in mixtures with non-bilayer forming phospholipids may represent the molecular mechanism responsible for mediating enzyme activation and membrane fusion. DPG is known to have effects on phospholipase activation that distinguishes it from other molecular species of diacylglycerol [2]. One explanation could be that DPG has a greater tendency to phase separate from the lamellar phase of phosphatidylethanolamines compared to other molecular species of diacylglycerol.

The origin of the diglyceride is important not only from the viewpoint of the particular effects that different molecular species may have on the membrane lipid matrix but also that the properties of the lipid matrix itself will be altered according to the class and molecular species of the phospholipid that is removed by hydrolysis to produce the diglyceride. Since most of the diglyceride formed in membranes appears to originate from bilayer-forming phospholipids (phosphatidylinositols and phosphatidylcholines), the balance that exists between bilayer and non-bilayer forming phospholipids must shift in favour of the latter. This shift is further exacerbated by the effect of diacylglycerol on the remaining phospholipids. Whether such a shift in the phase behaviour of lipids comprising the bilayer matrix of biological membranes is responsible for activation of protein kinase C or in mediation of membrane fusion process has yet to be determined.

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