The Phase Behavior of Mixed Aqueous Dispersions of Dipalmitoyl Derivatives of Phosphatidylcholine and Diacylglycerol

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ABSTRACT The phases and transition sequences for aqueous dispersions of mixtures of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycerol (1,2-DPG) have been studied by differential scanning calorimetry, dynamic x-ray diffraction, freeze-fracture electron microscopy, ^{31}P-nuclear magnetic resonance spectroscopy, and Fourier-transform infrared spectroscopy. The results have been used to construct a dynamic phase diagram of the binary mixture as a function of temperature over the range 20°–90°C. It is concluded that DPPC and 1,2-DPG form two complexes in the gel phase, the first one with a DPPC/1,2-DPG molar ratio of 55:45 and the second one at a molar ratio of approximately 1:2, defining three different regions in the phase diagram. Two eutectic points are postulated to occur: one at a very low 1,2-DPG concentration and the other at a 1,2-DPG concentration slightly higher than 66 mol%. At temperatures higher than the transition temperature, lamellar phases were predominant at low 1,2-DPG concentrations, but nonlamellar phases were found to be predominant at high proportions of 1,2-DPG. A very important aspect of these DPPC/1,2-DPG mixtures was that, in the gel phase, they showed a ripple structure, as seen by freeze-fracture electron microscopy and consistent with the high lamellar repeat spacings seen by x-ray diffraction. Ripple phase characteristics were also found in the fluid lamellar phases occurring at concentrations up to 35.6 mol% of 1,2-DPG. Evidence was obtained by Fourier transform infrared spectroscopy of the dehydration of the lipid-water interface induced by the presence of 1,2-DPG. The biological significance of the presence of diacylglycerol in membrane lipid domains is discussed.

INTRODUCTION

The formation of 1,2-diacylglycerols from phospholipids is known to be stimulated by hormones and neutrotransmitters, and its accumulation within the membrane acts to modulate the activity of protein kinase C (Berridge, 1987). The examination of the molecular species of 1,2-diacylglycerols produced has shown that the substrates for phospholipase C action are phosphatidylinositol 4,5-bisphosphate, generally hydrolyzed immediately after agonist stimulation (Nishizuka, 1984) and phosphatidylcholine, which results in elevated membrane levels of 1,2-diacylglycerols over a longer time period (Pelech and Vance, 1989).

It is widely assumed that because the molecular species of diacylglycerols produced in biological membranes are relatively hydrophobic, they will remain associated with the membrane. The resulting interaction of the diacylglycerol with other membrane constituents, particularly the phospholipids, is then directly relevant to their role as membrane second messengers.

A number of studies have shown that diacylglycerols may induce structural changes in membranes (Dawson et al., 1984; Das and Rand, 1986; Cheng and Hui, 1986) and destabilize lipid bilayers (Siegel, et al., 1989a; Siegel et al., 1989b). Such effects have been described in some cases in systems where the proportion of diacylglycerol is relatively low (<2 mol%), a concentration that is similar to that found in some physiological situations (Preiss et al., 1986). However, a study looking at molar ratios of diacylglycerol to phosphatidylcholine of 1.5:20 mol% has been carried out (Hamilton et al., 1991) showing that even at 20 mol% there is no hexagonal inverted phase (H_{II}) formation and that the transbilayer exchange rate calculated using ^{13}C-nuclear magnetic resonance was about 62/s at 38°C in the L_α phase. Furthermore, the conformation around the glycerol region of 1,2-diacylglycerol was found to be similar to that in phosphatidylcholines and unlike that in crystalline diacylglycerols (Hamilton et al., 1991). Evidence for the modification of the membrane structure by diacylglycerols are obtained from ^{31}P-nuclear magnetic resonance (^{31}P-NMR) studies of codispersions of phospholipids with diacylglycerols, which reveal an appearance of the phospholipid component in nonlamellar structures, which may include inverted lipid micelles (Siegel et al., 1989a). Such structures have been shown to be associated with fusion between lipid vesicles (Siegel et al., 1989a; Ortiz et al., 1992; Nieva et al., 1989; Burger et al., 1991; Van Gorkom et al., 1992) leading to the formulation of molecular models of the process of fusion

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Abbreviations used: ^{31}P-NMR, ^{31}P-Nuclear magnetic resonance; H_{II}, Hexagonal inverted phase; DPPC, 1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine; DPPC-d_{62}, 1,2-Dipalmitoyl-d_{62}-*sn*-glycero-3-phosphocholine; [*sn*-1,2-1-^{13}C]-DPPC, 1,2-Dipalmitoyl-*sn*-1-^{13}C-glycero-3-phosphocholine; 1,2-DPG, 1,2-Dipalmitoyl-*sn*-glycerol; 1,3-DPG 1,3-Dipalmitoyl-*sn*-glycerol; DSC, Differential scanning calorimetry; FTIR, Fourier transform infrared spectroscopy; ΔH, Enthalpy change of the gel to liquid-crystalline phase transition; T_c, Onset temperature of the gel to liquid-crystalline phase transition; T_m, Midpoint temperature of the gel to liquid-crystalline phase transition; $\Delta\sigma$, Chemical shift anisotropy.

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between biological membranes. Mixed aqueous dispersions of diacylglycerols and phosphatidylcholine have also been shown to form cubic phases, which have been examined in some detail by x-ray diffraction and other techniques (Seddon, 1990; Luzzati et al., 1992). There is also some evidence that diacylglycerols do not form ideal mixtures with phosphatidylcholines and that lateral phase separations result in domains that are enriched in diacylglycerols, whereas others are relatively depleted of diacylglycerol (Ortiz et al., 1988; Cunningham et al., 1989; De Boeck and Zidovetzchi, 1989; Heimburg et al., 1992).

These effects of diacylglycerols on phospholipid phase behavior and bilayer stability seem to manifest in the susceptibility of the phospholipid to hydrolysis by specific phospholipases. Thus phosphatidylcholine and phosphatidylinositol bilayers were found to be poor substrates for endogenous phospholipase A_2 or phospholipase C, respectively, but the action of these enzymes was greatly stimulated by inclusion of diacylglycerols in the substrate dispersions (Pelech and Vance, 1989; Cunningham et al., 1989; Dawson et al., 1983). This suggests that presentation of the substrate may be an important factor in regulation of phospholipid turnover in membranes and represent the biochemical mechanism for membrane lipid homeostasis.

One way to determine the consequences of the effect of diacylglycerols on membrane lipid structure and stability is to examine different binary mixtures of the two constituents and to construct a phase diagram. In this paper we describe the characterization of the structure of mixtures of 1,2-dipalmitoylphosphatidylcholine and 1,2-dipalmitoylglycerol dispersed in excess water using differential scanning calorimetry (DSC), time-resolved x-ray diffraction, freeze-fracture electron microscopy, ^{31}P-NMR, and Fourier transform infrared spectroscopy (FTIR).

MATERIALS AND METHODS

1,2-Dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) and 1,2-dipalmitoyl-*sn*-glycerol (1,2-DPG) were purchased from Avanti Polar Lipids (Birmingham, Alabama) and Sigma Chemical Co. (Poole, Dorset, UK), respectively. Their purity was determined as described previously (Ortiz et al., 1988). 1,2-Dipalmitoyl-d_{62}-*sn*-glycero-3-phosphocholine (DPPC-d_{62}) was also from Avanti Polar Lipids and 1,2-dipalmitoyl-*sn*-1-^{13}C-glycero-3-phosphocholine ([*sn*-1,2–1-^{13}C]-DPPC) was purchased from Serdary Research Laboratories (London, Ontario, Canada). Water was twice distilled in an all-glass apparatus and deionized in a Milli-Q apparatus from Millipore Ibérica (Madrid, Spain).

DSC

Samples containing 4 μmol DPPC and the appropriate amount of 1,2-DPG in chloroform were dried under a stream of oxygen-free dry N_2, and the last traces of solvent were removed by keeping the samples under high vacuum for more than 3 h. Water (0.5 ml) was added to the dry lipid, and the sample was heated to 70°C and vortex mixed during 30 min of incubation. The hydrated samples were equilibrated at 40°C for 4 days, centrifuged at 25,000 × g for 30 min, and the pellets were carefully transferred to small aluminum pans. Thermograms were recorded using a Perkin-Elmer (Norwalk, CT) DSC-4 calorimeter, using an empty sample pan as reference. The DSC instrument was calibrated using indium as standard. The samples were scanned over a temperature range of 20–90°C at a heating rate of 4°C/min and a sensitivity of 2 mcal/s. The first and the fifth consecutive heating scans were always used for transition enthalpy calculations and display. Peak areas were measured by weighing paper cutouts of the peaks. After the thermal measurements, the phospholipid content of the pans was determined by phosphorus assay of perchloric acid digests (Bartlett, 1959). The incorporation of 1,2-DPG in the vesicles has been determined as previously (Ortiz et al., 1988). To determine the acyl migration of 1,2-DPG to 1,3-Dipalmitoyl-*sn*-glycerol (1,3-DPG) after the experiments were carried out, and because it is known that some acyl migration may occur in 1,2-DPG (Serdarevich, 1967; Kodali et al., 1990b), the different samples, as well as pure 1,2-DPG as standard for transmigration during the separation, were dissolved in chloroform/methanol 2:1 (v/v), spotted on silica gel thin layer chromatography plates, and immediately developed in chloroform/acetone 95:5 (v/v) at room temperature (development time approximately 60 min). The plates were stained with iodine, and the spots corresponding to 1,2-DPG and 1,3-DPG were scraped off and quantitatively analyzed for fatty acid, by standard gas liquid chromatography, after transmethylation, in a Shimadzu (Tokyo, Japan) GC-Mini3 chromatograph using heptadecanoic acid as an internal standard. In our experimental conditions, and after completion of the experiments, acyl migration in 1,2-DPG led to the formation of 6–9% of 1,3-DPG over total diacylglycerol.

X-ray diffraction

Samples for x-ray diffraction were prepared using 34 μmol of pure DPPC and the appropriate amount of 1,2-DPG as described above and by heating and cooling the samples through the phase transition temperature several times, but without incubation. The samples contained more than 75% (w/w) of water. Time-resolved x-ray diffraction experiments were performed using a monochromatic (0.15 nm), focused x-ray beam at station 8.2 of the Daresbury Synchrotron Radiation Laboratory (U.K.). A purpose-built camera (Lis and Quinn, 1991) allowed clear resolution of reflections between 0.35 and 10 nm. The samples were sandwiched between thin mica sheets, 1 mm apart, and were mounted on a modified cryostage (Linkam Scientific Instruments Ltd., Tadworth, U.K). Temperature scans in heating and cooling modes were performed at 4°C/min over a temperature range of 20°–90°C. X-ray scattering intensities were recorded on a multiwire quadrant detector constructed at the Daresbury Laboratory. X-ray scattering data were acquired in 255 consecutive time frames of 3 s separated by a dead time between frames of 50 μs. Data were stored in a VAX 11/785 computer (Maynard, MA), and the experimental data was analyzed using OTOKO software (EMBL, Hamburg, Germany) program (Boulin et al., 1986). Scattering intensities were corrected for detector response recorded from an ^{59}Fe source, and spatial calibrations were obtained using Teflon (0.48 nm) as a calibration standard (Bunn and Howell, 1954). Under the conditions used to record the dynamic x-ray data no apparent radiation damage was evident either from the x-ray behavior of the samples or by detection of lipid breakdown products in thin-layer chromatograms of the lipid, performed after completion of the diffraction experiments. The radiation dose was well below the level where radiation damage has been previously observed (Caffrey, 1985).

Freeze-fracture electron microscopy

Samples for freeze-fracture examination were prepared as described above for x-ray studies but using 13.6 μmol of DPPC and an appropriate amount of 1,2-DPG. Samples were sandwiched in Balzers BUO 12 055-t sample holders (Balzers, Liechtenstein) and were thermally quenched at >10^4°C/min using a BioRad (Cambridge, MA) Liquid Nitrogen jet freezer. The frozen specimens were transferred to a Polaron E7500 freeze-fracture apparatus (Polaron Equipment, Watford, UK) and were fractured at −150°C. Replicas were prepared by platinum/carbon shadowing with carbon overlay by using resistive evaporation sources. Replicas and sample-holders were immersed in chloroform/methanol 2:1 to dissolve the lipids. The replicas were subsequently floated off onto distilled water and were mounted on 400-mesh uncoated grids. The replicas were examined using a Philips EM301G transmission electron microscope (Eindhoven, The Netherlands).

FTIR

Samples for FTIR containing 3.7 μmol DPPC-d_{62} or [*sn*-1,2–1-^{13}C]-DPPC and the appropriate amount of 1,2-DPG in chloroform were dried under a stream of oxygen-free dry N_2, and the last traces of solvent were removed by desiccation under high vacuum for more than 3 h. After the addition of 50 μl of either H_2O or ^2H_2O, multilamellar liposomes were formed by careful mixing using a bench vibrator and keeping the samples at 70°C. Mixing was continued until a homogeneous and uniform suspension was obtained. The samples were finally maintained at 70°C for 30 min with occasional vortex mixing.

Infrared spectra were recorded using a Philips PU9800 Fourier transform infrared spectrometer equipped with a deuterated triglycine sulfate detector. Samples were examined in a thermostated Specac 20710 cell (Specac, Kent, UK) equipped with CaF_2 windows and using 25-μm Teflon spacers. The samples were equilibrated at 27°C for 20 min in the infrared cell before recording spectra. Each spectrum was obtained by collecting 100 interferograms with a nominal resolution of 2 cm^{-1} and triangular apodization using a sample shuttle accessory to obtain average background spectra recorded between each consecutive sample spectra. The sample chamber of the spectrometer was continuously purged with dry air to prevent atmospheric water vapor obscuring the bands of interest. Samples were scanned between 27°C and 75°C at 2°C intervals with a 5-min delay between each consecutive scan using a circulation water bath interfaced to the spectrometer computer. Spectral subtraction was performed interactively using the Spectra-Calc program (Galactic Industries Corp., Salem, NH). The spectra were subjected to Fourier selfdeconvolution and derivatization using the same software. The spectra were subjected to Fourier selfdeconvolution using Spectra-Calc FD function, which is analogous to the method of Kauppinen et al. (1981). A Gaussian band of 15 cm^{-1} half-bandwidth and a narrowing factor, k, of 1.3 have been found to be optimal.

^{31}P-NMR

DPPC (20–65 μmol) and the appropriate amount of 1,2-DPG were mixed in a final volume of 0.3 ml of chloroform in a small glass tube (5-cm length, 8-mm outer diameter) and were evaporated to dryness under a stream of oxygen-free dry N_2. The remaining traces of solvent were removed by storage for 3 h under high vacuum. Water (0.4 ml) was added to the dry lipid mixtures and the samples were heated at 70°C for 30 min to hydrate the phospholipid. The tube was then inserted into a conventional 10 mm NMR tube with external ^2H_2O. ^{31}P-NMR spectra were recorded in the Fourier transform mode using a Bruker AC-200 spectrometer (81 MHz) interfaced with an Aspect 3000 computer (Bruker, Rheinstetten, Germany). Temperature was controlled to ±0.5°C with a standard Bruker B-VT-1000 variable temperature control unit. The $\Delta\sigma$ values were calculated as 3 times the chemical shift difference between the high-field peak and the position of the peak recorded for lipid molecules in isotropic motion (Seelig, 1978). All chemical shift values are quoted in parts per million (ppm) from micelles of pure lysophosphatidylcholine (0 ppm); positive values referring to low-field shifts. All spectra were obtained in the presence of a gated-broad band decoupling pulse (5 watts input power during acquisition time), and accumulated free induction decays were obtained from up to 2000 transients. A spectral width of 50 kHz, a memory of 8000 data points, a 0.2-s interpulse time, and a 80° radio frequency pulse were the parameters used to record the spectra. Before Fourier transformation an exponential multiplication was applied resulting in a 100-Hz line broadening.

RESULTS

Thermal studies

DSC of aqueous dispersions of DPPC/1,2-DPG have been performed, and the range of proportions of the two components has been extended beyond that examined in previous studies (Ortiz et al., 1988). The results obtained agree precisely with the earlier, more limited thermal study of mix-

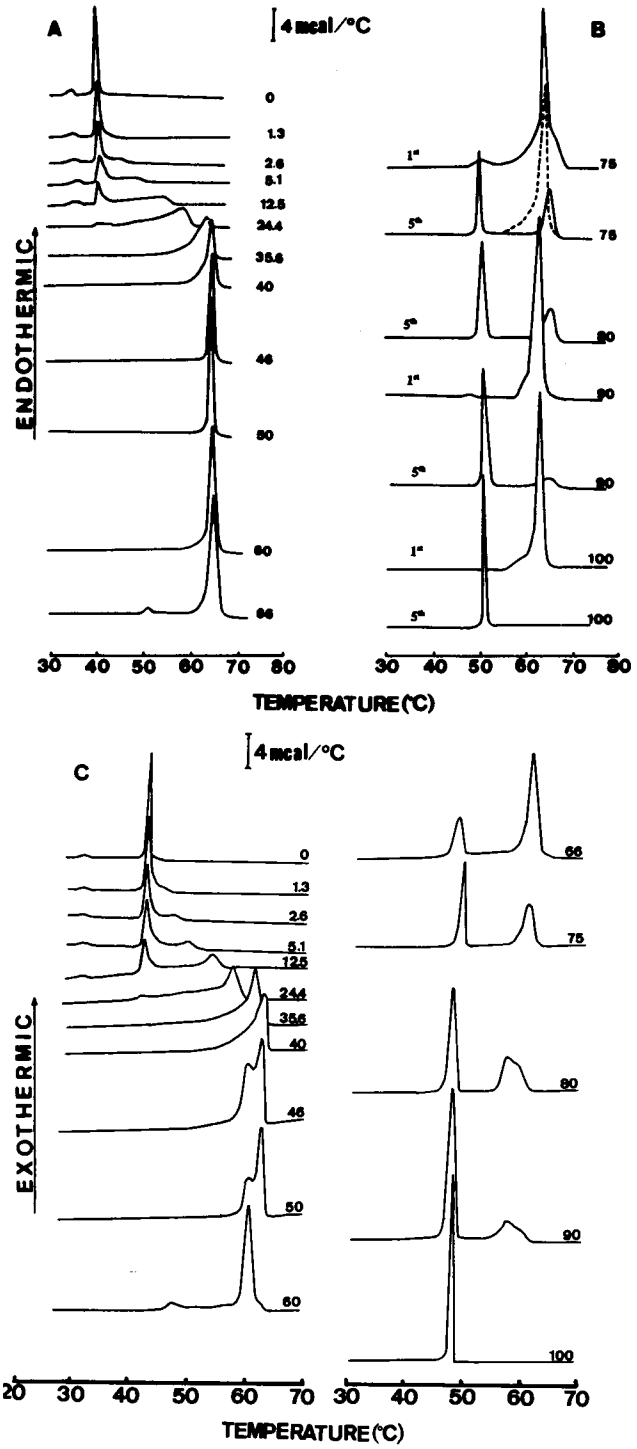


FIGURE 1 DSC thermograms of aqueous dispersions of mixtures of DPPC and 1,2-DPG. The mol% of 1,2-DPG in the mixture is indicated on each of the thermograms. (*A*) First heating thermograms obtained from pure DPPC to 66 mol% of 1,2-DPG. (*B*) Heating thermograms obtained for mixtures containing DPPC and 1,2-DPG ranging from 75 mol% to pure 1,2-DPG. Both the first and the fifth scans are shown as indicated. The dashed line on the fifth scan of the 75 mol% of 1,2-DPG mixture is the result of subtracting the first and the fifth thermograms of the same mixture. (*C*) First cooling thermograms obtained from pure DPPC to pure 1,2-DPG as indicated.

tures up to 30 mol% of 1,2-DPG. Thermograms recorded in heating scans of all the mixtures examined in the present study are illustrated in Fig. 1, *A* and *B*. It can be seen that for mixtures containing 1.3 mol% 1,2-DPG the only noticeable effect is a slight broadening of the phase transitions of the lamellar gel to the ripple gel phase ($L_{\beta'} \rightarrow P_{\beta'}$) and the subsequent transition to the lamellar liquid-crystalline phase ($P_{\beta'} \rightarrow L_{\alpha}$) (Fig. 1 *A*). Over the range of 2.6–24.4 mol% of 1,2-DPG there is a clear evidence of two endothermic transitions, one corresponding to pure DPPC and the other one appearing at progressively higher temperatures, indicating a phase separation within the mixture (gel phase immiscibility, as deduced from the phase diagram below). It is noteworthy that the pretransition of DPPC is clearly visible in heating scans of mixtures containing up to 12.5 mol% of 1,2-DPG, suggesting that 1,2-DPG is phase separated from a relatively pure phospholipid phase. Also at 24.4 mol% of 1,2-DPG a transition, although small, is still seen at a temperature corresponding to that of pure DPPC. However, this transition is not detected at 35.6 mol%. At 40 mol% and up to 60 mol% of 1,2-DPG the transition peak is already detected at 63°C (Fig. 1 *A*).

It is known that 1,2-DPG presents metastable behavior, the β' structure being the most stable form (Kodali et al., 1984; Shannon et al., 1992). Upon rapid cooling the melted 1,2-DPG is transformed to the α form. As described by Kodali et al. (1984), only one endotherm is observed corresponding to the melting of the α form if the melted form is rapidly cooled to temperatures below T_c and then rapidly reheated. It was necessary to equilibrate for some time to detect the transition to the β' form and its subsequent melting. A complex transition from α to β' was observed at about 50°C, in agreement with previous observations (Kodali et al., 1984; Shannon et al., 1992). As it has been previously shown, the hexane-crystallized β' polymorph of dry 1,2-DPG shows a single, sharp endothermic peak at 67.5°C (Shannon et al., 1992) or 64°C as described by other authors (Howe and Malkin, 1951). These different values could be due to either different scanning rates or the way of measuring the transition (T_c or T_m). However, we have observed that, in the presence of water, T_c decreases about 3°C without changing ΔH as compared with dry 1,2-DPG. The presence of small quantities of 1,3-DPG (<10%) also may induce a small decrease in T_c of about 12°C. We have observed the transition of the β' form of hydrated 1,2-DPG to be 62.5°C. The addition of both effects, hydration and the presence of small quantities of 1,3-DPG, could be the origin of the small differences found in this work and other previously reported values (Shannon et al., 1992; Howe and Malkin, 1951).

Samples containing 60 mol% or less of 1,2-DPG showed the same thermal pattern after incubation for 4 days at 40°C as when they were immediately scanned after being rapidly cooled from the melted form. The same pattern was also detected for the first scan compared with up to five successive scans, when the samples were left at low temperatures (approximately 30°C) for <2 min before rescanning. However, the behavior of the samples containing more than 60 mol% of 1,2-DPG was different, inasmuch as they exhibited transitions attributable to pure 1,2-DPG (Fig. 1 *B*). The new transitions were confirmed to be the result of pure 1,2-DPG by their metastable behavior. When these samples were incubated for 4 days at 40°C, the first scan showed a broad peak transition starting at about 60°C (Fig. 1 *B*). This pattern was changed in successive scans, when heating immediately after rapid cooling to 30°C. Samples appeared to be stabilized with respect to their thermal behavior from the fifth scan on, so that further recycling did not alter their thermal pattern anymore. Fig. 1 *B* shows also the fifth scan of all the samples. It can be observed that the transition of pure 1,2-DPG in the β' form overlaps with the transition of the DPPC/1,2-DPG complexes, hence broad peaks appear. It can be observed also that the size of the peak ascribed to pure 1,2-DPG increased at the expense of the peak arising from the DPPC/1,2-DPG mixture as the concentration of 1,2-DPG was increased. The difference from the fifth and first heating scans of the 75 mol% mixture obtained by computer subtraction (Fig. 1 *B*, *dashed line*) is nearly identical to the pure β' form of 1,2-DPG, indicating that the complexity of the transition peak is because of the superposition of two different endotherms. We did not observe any thermal event in the DSC thermograms because of the $L_{\alpha} \rightarrow H_{II}$ transition, which, given the limited sensitivity of our calorimeter, may be explained if this transition has a very small cooperativity.

The DSC first cooling scans of the binary mixtures of DPPC with 1,2-DPG are shown in Fig. 1 *C*. It can be observed that both heating and cooling scans are very similar, i.e., the phase boundaries of the gel and the fluid region of the mixtures are in a good agreement, except for the 46 and 50 mol% of 1,2-DPG containing samples, indicating a possible heterogeneity in this range of 1,2-DPG concentration. A similar behavior has been described before for similar systems as that for systems composed of dimyristoylphosphatidylcholine and 1,2-dimyristoylglycerol (Heimburg et al., 1992).

The molar enthalpies of the transitions in the different mixtures obtained from heating endotherms are plotted in Fig. 2. ΔH values were calculated by adding together all the different peaks appearing in each thermogram. It can be observed that the enthalpy, calculated in this way, remains relatively constant (about 8.7 kcal/mol) in mixtures containing up to 40 mol% of 1,2-DPG. At higher concentrations of 1,2-DPG the enthalpy increases to about 14.2 kcal/mol at 60–66 mol%. Up to these concentrations similar values of total ΔH were found independently of the preparation method of the sample, i.e., with or without preincubation, and irrespective of whether the first or other successive scans were taken into account. However, for mixtures containing proportions of 1,2-DPG higher than 66 mol%, different values of ΔH were obtained depending on the thermal history of the sample. As it can be seen in Fig. 2, samples preincubated at 40°C for 4 days yielded a progressive increase in ΔH as the proportion of 1,2-DPG was increased approaching 28.2 kcal/mol, which is the value found for pure 1,2-DPG in the β' form. On the other hand, when these samples were scanned for the fifth

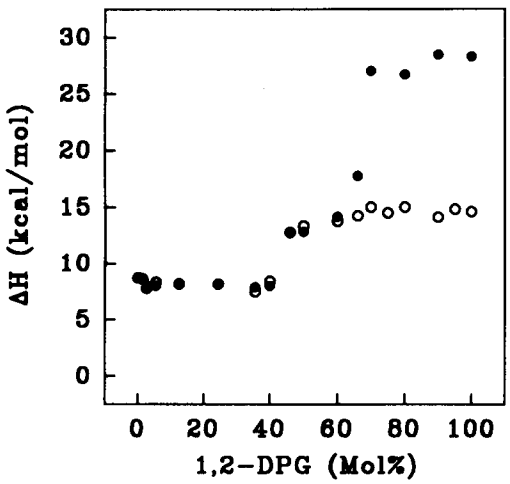


FIGURE 2 Dependence of the enthalpy change (ΔH) obtained from heating thermograms in Fig. 1 as a function of 1,2-DPG in codispersion with DPPC. ●, Samples preincubated for 4 days at 40°C; ○, directly scanned samples upon mixing. See text for details.

time or without preincubation, they yielded ΔH values very similar to those samples containing <66 mol% of 1,2-DPG, approaching 14.6 kcal/mol, which is the ΔH value corresponding to pure 1,2-DPG in the α form.

Dynamic x-ray diffraction studies

The intensity of synchrotron x-rays has been exploited to relate the phase structure to the enthalpic phase transitions recorded by DSC. Fig. 3 shows the sequence of diffraction patterns in the low-angle scattering region recorded during heating of a sample of pure DPPC and a sample containing 66 mol% of 1,2-DPG, whereas Fig. 4 shows the relative intensity and wide-angle spacings for all samples studied in this work. The transition from a gel to a disordered liquid-crystalline phase can be clearly observed in the wide-angle scattering region, because the former is characterized by a single, sharp peak centered at a spacing of 0.41 nm but the latter is typified by a broad scattering band centered at a spacing of 0.47 nm (Fig. 4 *B*). All these samples were prepared without preincubation at 40°C.

The sample of pure DPPC gave a lamellar spacing of 6.17 nm up to 35°C (Table 1). The pretransition, leading to a phase change from $L_{\beta'}$ to $P_{\beta'}$, induces the appearance of spacings of 8.3 nm, and after the main transition $P_{\beta'} \rightarrow L_{\alpha}$ has occurred the repeat spacing decreases to 6.17 nm at 46°C (Fig. 3 *A*). A 20% decrease in the wide-angle relative scattering intensity of the peak centered at 0.41 nm is observed at 35°C corresponding to a phase change from $L_{\beta'}$ to $P_{\beta'}$. This peak disappears at 41°C in good agreement with the DSC data (Fig. 4, *A* and *B*). The sample containing 2.6 mol% of 1,2-DPG shows a similar behavior to that of pure DPPC (Fig. 4, *A* and *B* and Table 1).

The sample containing 12.5 mol% of 1,2-DPG showed a lamellar gel phase at temperatures less than 35°C (spacing of 7.22 nm, Table 1). A slight decrease in intensity of the wide-angle diffraction maxima centered at a spacing of 0.41 nm is observed above 35°C ($L_{\beta'}$ to $P_{\beta'}$ phase transition) (Fig. 4 *A*). The lamellar repeat spacing increases to 7.9 nm at 41°C. The change in mesophase structure results in a decrease in spacing from 7.9 to 7.42 nm (Table 1). At higher temperatures (>45°C) there is a further decrease in intensity in the wide-angle diffraction maxima corresponding to the progressive disappearance of the gel phase packing of the hydrocarbon chains which finally disappears at 55°C (Fig. 4). At about 56°C there is a sharp transition, evident from a decrease in spacing from 7.18 to 6.77 nm (Table 1). Because of the unusually large spacing of many of these lamellar phases together with data obtained from freeze-fracture electron microscopy (see below), these lamellar structures are designated as ripple phases, or mixed ripple and lamellar liquid-crystal L_{α} phases. There is evidence from the wide-angle scattering profiles (Fig. 4 *A*) of the coexistence of lamellar gel and liquid-crystalline phases over the temperature range of about 45–55°C, above which only a liquid-crystalline phase is observed in the wide-angle scattering profile. The temperatures of the transitions recorded as structural changes in the x-ray experiments agree closely with the endothermic peaks observed in the heating thermograms.

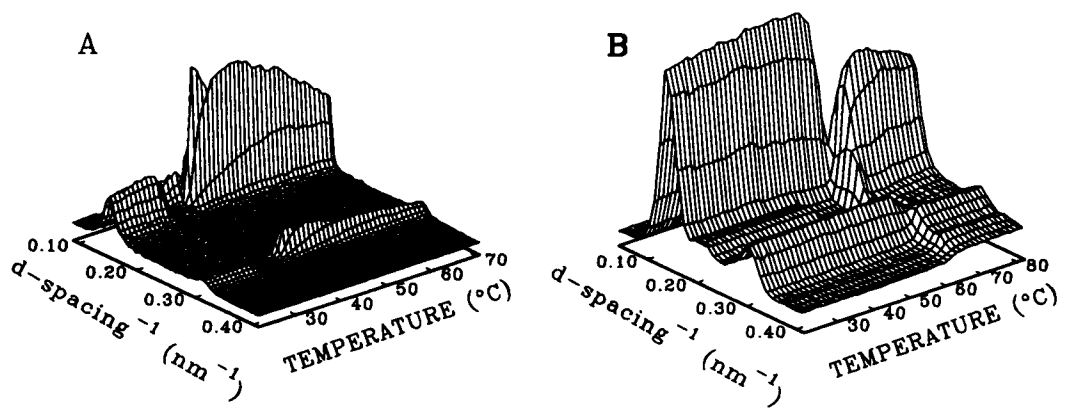


FIGURE 3 Three -dimensional plots of low-angle x-ray scattering intensity versus reciprocal dispersions of pure DPPC (*A*) and with 66 mol% of 1,2-DPG (*B*) during a heating scan at 4°C/min. Each diffraction pattern represents the counts recorded over 15 s.

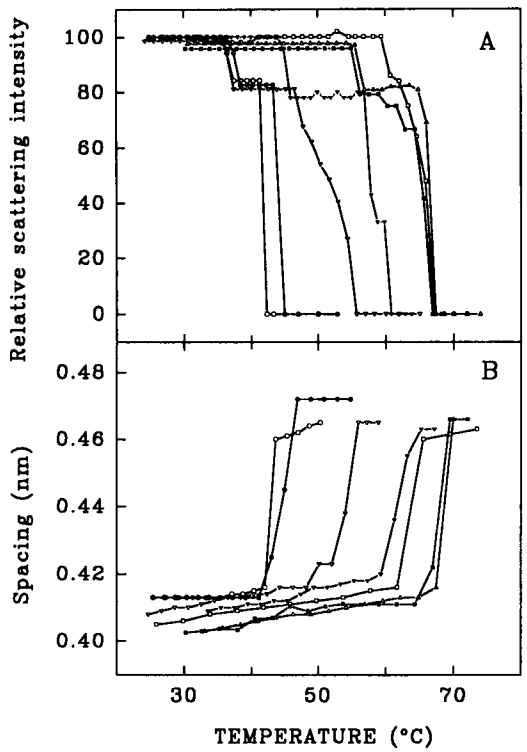


FIGURE 4 X-ray scattering intensity (as percentage of the maximum intensity) (*A*) and spacings (*B*) in the wide-angle region obtained from mixed aqueous dispersions of DPPC and 1,2-DPG as a function of temperature. DPPC (○), 2.6 mol% of 1,2-DPG (●), 12.5 mol% of 1,2-DPG (▽), 24.4 mol% of 1,2-DPG (▼), 35.6 mol% of 1,2-DPG (□), 46 mol% of 1,2-DPG (■), and 66 mol% of 1,2-DPG (△).

TABLE 1 Repeat spacings, d (nm), in the low-angle region of mixtures of DPPC/1,2-DPG

Mixture	Temperature (°C)	h, k	d (nm)*
DPPC	35	1, 0	6.17
		2, 0	3.00
	37	1, 0	8.30
	46	1, 0	6.17
		2, 0	3.00
12.5% 1,2-DPG	35	1, 0	7.90
		2, 0	3.56
	41	1, 0	7.90
	43	1, 0	7.42
	55	1, 0	7.18
	57	1, 0	6.77
		2, 0	3.22
24.4% 1,2-DPG	35	1, 0	7.74
		2, 0	3.71
	45	1, 0	7.85
		2, 0	3.71
	57	1, 0	7.63
	60	1, 1.09	6.89
35.6% 1,2-DPG	65	1, 0	7.19
		2, 0	3.48
		3, 0	2.32
	66	1, 0	6.90
		2, 0	3.41
	73	1, 0	6.90, 6.03
		1, 1	3,35
		2, 0	3.41, 2.95
	85	1, 0	6.03
		1, 1	3.35
		2, 0	2.95
46% 1,2-DPG	64	1, 0	7.29
		2, 0	3.40
	66	1, 0	6.04
		1, 1	3.36
		2, 0	3.00
66% 1,2-DPG	50	1, 0	6.90, 4.85
		2, 0	3.37
	65	1, 0	7.03
		2, 0	3.40
	72	1, 0	5.52
		1, 1	3.23
		2, 0	2.84

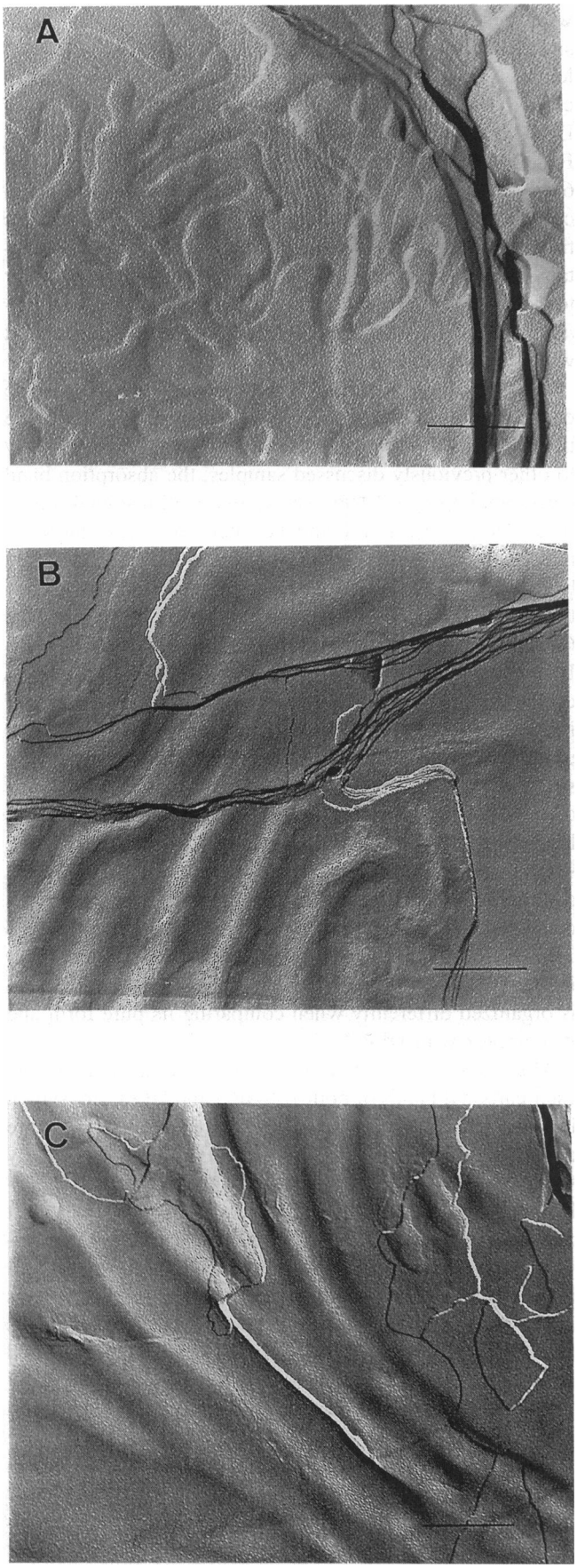
* Doubles entries correspond to two independent patterns.

The sample containing 24.4 mol% of 1,2-DPG showed a spacing of 7.74 nm at 35°C and 7.85 nm at 45°C, corresponding to a lamellar phase (Table 1). A decrease in spacing was observed between 45 and 57°C from 7.85 to 7.63 nm (Table 1) and in the intensity of the peak in the wide-angle region (Fig. 4 *A*), probably related to the small transition peak observed by DSC (Fig. 1). A bigger decrease was found in spacing, starting at 58°C (spacing of 7.41 nm) and finishing at 60°C (spacing of 6.89 nm), that corresponds to a gel to liquid-crystalline phase transition (Table 1). The disappearance of the gel phase was detected through the wide-angle diffraction at approximately 58°C (Fig. 4 *B*), confirming the observation made in the corresponding low-angle diffraction region.

Mixed dispersions of DPPC containing 35.6 mol% 1,2-DPG were examined to study the structure of the phases and transition sequence of the endotherm observed at about 60°C in the heating thermogram of this mixture observed by DSC (Fig. 1). A structural change in the low-angle scattering region from a lamellar phase with a repeat spacing of 7.19 nm (65°C) to another lamellar phase with a repeat spacing of 6.9 nm (66°C) could be observed (Table 1). The corresponding wide-angle scattering intensities and spacings, shown in Fig. 4, *A* and *B*, indicate that this is a gel to liquid-crystalline phase transition and it coincides with the large endotherm seen in the thermogram in Fig. 1. At temperatures greater than about 73°C a nonlamellar phase begins to appear in the low-angle scattering region characterized by 3 reflections at spacings of 6.03, 3.35, and 2.95 nm that follow the sequence $1:1/\sqrt{3}:1/\sqrt{4}$ characteristic of hexagonal phases (H_{II}). The lamellar liquid-crystalline phase coexists with the hexagonal phase up to a temperature of about 84°C above which only the nonlamellar phase can be distinguished in the scattering pattern.

The sample containing 46 mol% of 1,2-DPG showed a transition at about 65°C from a lamellar phase (characterized by a spacing of 7.29 nm) to a nonlamellar phase (characterized by a spacing of 6.04 nm) (see Table 1). This transition is also detected in the wide-angle region as shown in Fig. 4, *A* and *B*. The pattern recorded in the wide-angle region shows that the peak centered at a spacing of 0.41 nm disappears at about 65°C, a temperature that agrees with the transition detected by DSC.

The dynamic x-ray scattering patterns of an aqueous dispersion of DPPC containing 66 mol% 1,2-DPG are shown in



Figs. 3 *B* and 4. The diffraction patterns at temperatures up to about 50°C indicate the presence of a lamellar structure with a spacing of 6.9 nm (Table 1) with a gel phase hydrocarbon chain packing evidenced by a reflection at 0.409 nm (Fig. 4 *B*). Another somewhat weaker low-angle reflection at about 4.85 nm is superimposed on the lamellar repeat structure and disappears from the scattering profile at about 50°C. The scattering band at 4.85 nm is tentatively assigned to phase-separated 1,2-DPG in the gel phase, which melts, according to the thermal data in Fig. 1, at about 50°C. A spacing of 4.9 nm was described before for pure 1,2-DPG in α form (Kodali, et al., 1990a). A 20% decrease in the intensity of the gel peak in the wide-angle region could be assigned to the disappearance of the peak at 4.85 nm (Fig. 4 *A*). Only a nonlamellar phase is observed at temperatures higher than 65°C, probably hexagonal phases consistent with three reflections at spacings that follow the sequence $1:1/\sqrt{3}:1/\sqrt{4}$ (5.52 nm, 3.23 nm, and 2.84 nm).

Freeze-fracture electron microscopy

The origin of the large lamellar repeat-spacing characteristic of ripple phases in DPPC was examined by freeze-fracture electron microscopy and the results are illustrated in Fig. 5. An electron micrograph of a replica obtained from an aqueous dispersion of DPPC containing 12.5 mol% 1,2-DPG thermally quenched from 40°C (Fig. 5 *A*) shows the presence of a smooth fracture face together with regions of characteristic ripple structure with a periodicity of about 12.1 nm. This suggests phase separation of components within the mixture. Replicas of samples thermally quenched from temperatures above 58°C showed only a smooth lamellar structure.

The electron micrographs shown in Fig. 5, *B* and *C* were obtained from replicas prepared from an aqueous dispersion of DPPC containing 35.6 mol% of 1,2-DPG thermally quenched from 50°C and 70°C, respectively. Lamellar ripple phases are clearly observed in both fracture faces with periodicities of about 112 and 92 nm, respectively. In the fluid lamellar phase, bending of the surfaces is a dynamic phenomenon, and distortion of these surfaces could also be caused by the freezing process. But, together with data provided by x-ray scattering experiments, these phases may be assigned to a gel lamellar ripple phase and a fluid lamellar ripple phase, respectively.

Vibrational spectroscopy

FTIR has been employed to confirm the origin of the transitions observed in the thermal studies. Mixed dispersions of the deuterated derivative of DPPC (DPPC-d_{62}) and 1,2-DPG

FIGURE 5 Electron micrographs of freeze-fracture replicas prepared from aqueous dispersions of (*A*) DPPC/12.5 mol% of 1,2-DPG thermally quenched from 40°C, (*B*) DPPC/35.6 mol% of 1,2-DPG thermally quenched from 50°C, and (*C*) from 70°C. The bar represents 200 nm for all the micrographs.

were prepared as described in the Methods section and examined at different temperatures. The wavelength maxima of the symmetric CH_2 and C^2H_2 stretching modes of the lipid acyl chains are plotted as a function of temperature in Fig. 6. It is well established that a shift in frequency of this vibration is a reliable index of the gel to liquid-crystalline phase transitions in phospholipid dispersions (Cameron et al., 1980; Casal and Mantsch, 1984). Moreover, in this mixture of isotopically labeled lipids it is possible to observe the phase transitions of DPPC-d_{62} through the C^2H_2 symmetric stretching vibrational modes independently from the phase transitions of 1,2-DPG through the changes in the CH_2 symmetric stretching vibrational modes.

The dependence of the frequency of the C^2H_2 symmetric stretching band on temperature (Fig. 6 *A*) indicates a broad phase transition of DPPC-d_{62} in a mixed dispersion containing 12.5 mol% of 1,2-DPG, which spans the temperature range from the transition temperature of DPPC-d_{62} at 35–54°C. The gel to liquid-crystalline phase transition of 1,2-DPG in this mixture, on the other hand, begins at 45°C and is completed at about 54°C. The broad transition of the phospholipid suggests that an excess of pure phospholipid is present at the same time that a stoichiometric complex with the diacylglycerol is formed. These results agree quite well with those reported from DSC (Fig. 1), although the transitions due to pure DPPC and DPPC/1,2-DPG mixtures are less well resolved, probably the result of the lower resolution capacity of FTIR. Similar data recorded for mixtures of DPPC containing 35.6 mol% of 1,2-DPG are also shown in Fig. 6. In this mixture it is apparent that both DPPC-d_{62} (Fig. 6 *A*) and 1,2-DPG (Fig. 6 *B*) undergo only one transition centered at a temperature of 61°C similar to the enthalpic transition recorded by calorimetry (Fig. 1). Samples containing 50 mol% of 1,2-DPG showed a behavior very similar to that of 35.6 mol%, with a single transition for both DPPC-d_{62} and 1,2-DPG at approximately 61°C in close agreement with DSC results.

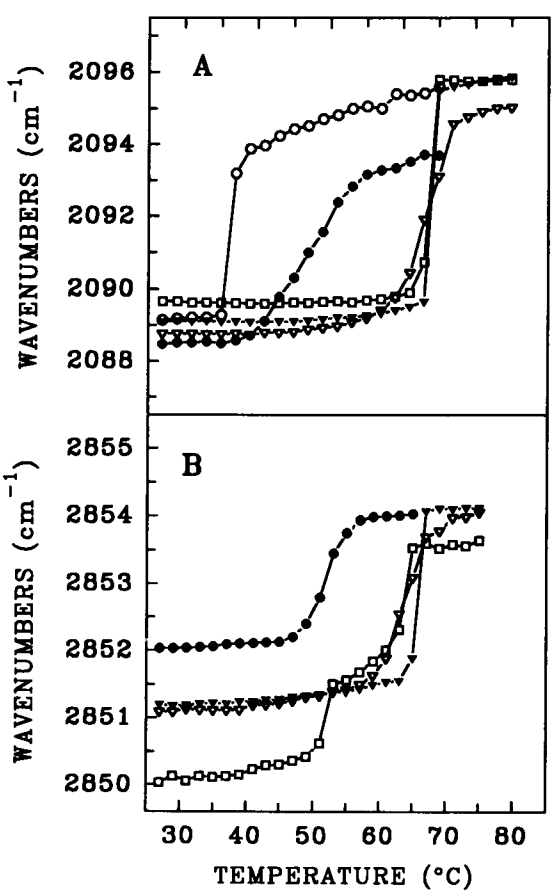


FIGURE 6 Temperature dependence of the frequency of the symmetric C^2H_2 (DPPC-d_{62}) stretching (*A*) and symmetric CH_2 (1,2-DPG) stretching (*B*) of pure DPPC-d_{62} (○), and mixtures of DPPC-d_{62} containing 12.5 mol% (●), 35.6 mol% (▽), 50 mol% (▼), and 80 mol% (□) of 1,2-DPG.

A totally different result can be observed for a sample containing 80 mol% of 1,2-DPG. In this case, whereas the transition of DPPC-d_{62} is observed to occur at 61°C, similarly to other previously discussed samples, the absorption band corresponding to 1,2-DPG shows two well resolved transitions, one of them at 51°C and the other at 61°C, i.e., the same temperatures at which endothermic transitions were detected by DSC (Fig. 1). These spectroscopic observations confirm that the 51°C transition corresponds to pure 1,2-DPG, and the 61°C transition corresponds to the DPPC/1,2-DPG complex. It should be said that this sample was subjected to several cycles of heating and cooling through the phase transition, i.e., between 20 and 80°C, so that the formation of the α phase of 1,2-DPG was induced. Hence, the expected transition of pure 1,2-DPG is 51°C (Kodali, et al., 1984). The other interesting point with respect to this sample is that at low temperatures the maximum of the CH_2 symmetric stretching band of 1,2-DPG was observed at 2850 cm^{-1}, i.e., a shift of approximately 1 cm^{-1} to lower frequencies. This could indicate that the proportion of *trans* isomers of pure 1,2-DPG in the sample is higher than the proportion of *trans* isomers of 1,2-DPG in the complex, indicating that 1,2-DPG is organized differently when comparing its pure form and its complex with DPPC.

The results obtained from the observation of the C=O ester carbonyl bands of both 1,2-DPG and DPPC are shown in Fig. 7. At 35°C, i.e. below the T_c transition temperature of the pure lipids and their mixtures (Fig. 7 *A*), it is clearly seen that the progressive increase in the proportion of 1,2-DPG leads to the widening of the C=O band and to a shift of the maximum toward higher wavenumbers. After deconvolution the component appearing at the highest wavelength is increasingly predominant as the proportion of 1,2-DPG is increased (Fig. 7 *C*). It is also shown in Fig. 7 that, whereas the α form of pure 1,2-DPG exhibits two maxima centered at 1735 cm^{-1} and 1712 cm^{-1}, two peaks are very clearly separated in the β' form with maxima at 1731 cm^{-1} and 1707 cm^{-1}.

Above the main phase transition of pure DPPC, it is known that the maximum of the ester C=O band is shifted to a lower wavelength (Casal and Mantsch, 1984) because of the increase of the peak height of the component found at lower wavelength as resolved by deconvolution (Cameron et al.,

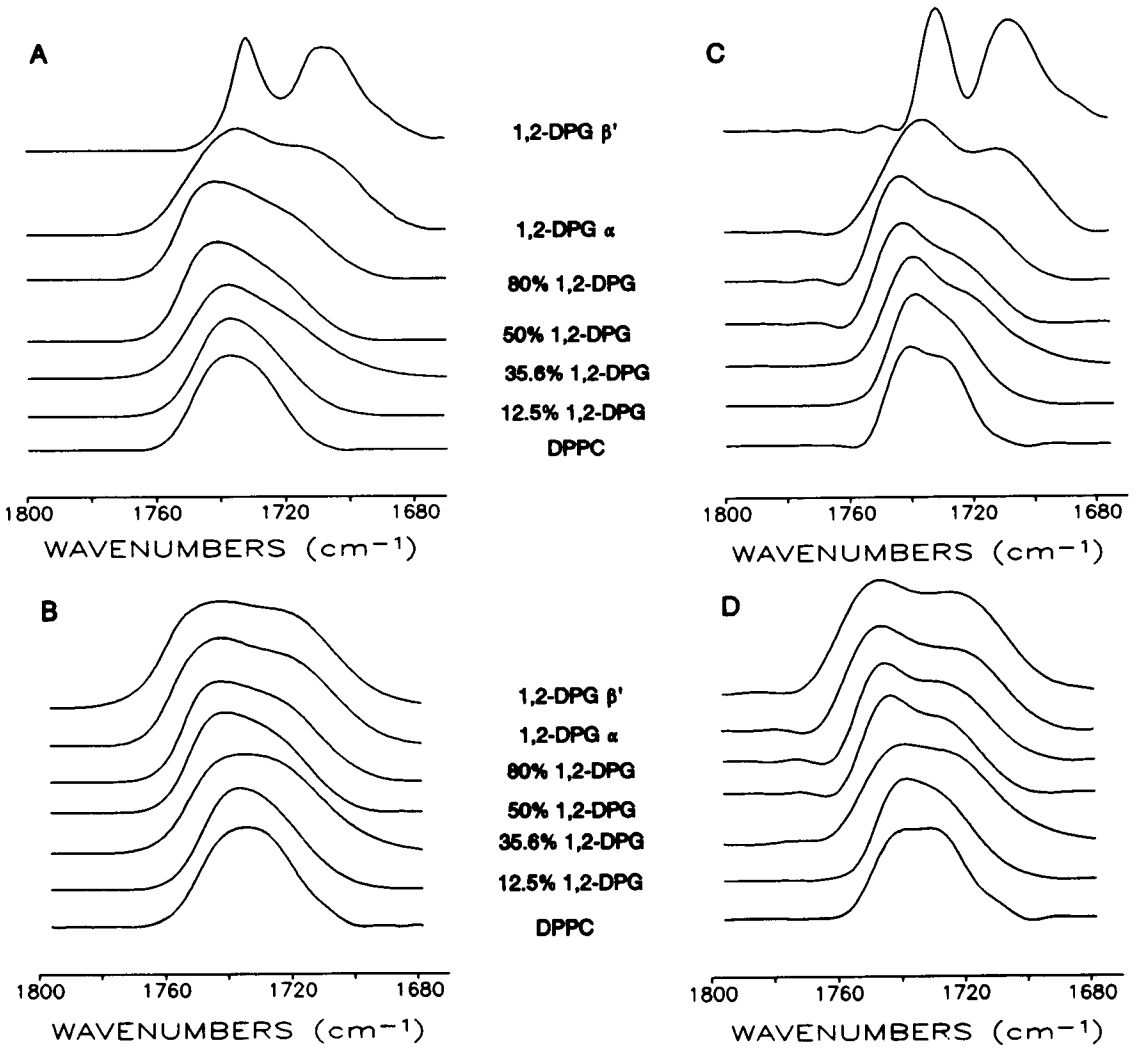


FIGURE 7 Infrared spectra in the region 1800–1670 cm^{-1} of pure DPPC, pure 1,2-DPG in both α and β forms and different mixtures at 35°C (*A*, *C*) and 70°C (*B*, *D*), as indicated. The deconvolved spectra for the 35 and 70°C original spectra are shown in *C* and *D*, respectively.

1980). Fig. 7 *B* shows the C=O ester carbonyl band of DPPC and its mixtures with 1,2-DPG at 70°C, i.e., above the phase transition temperature of the pure lipids and their mixtures. It can be seen that increasing concentrations of 1,2-DPG lead to a situation in which the component appearing at the lowest wavelength predominates. This can be clearly observed in the deconvoluted spectra (Fig. 7 *D*). We will discuss later that this observation can be taken as an indication of the dehydration of these C=O groups.

To distinguish between the C=O groups coming from 1,2-DPG and those from DPPC and identify the possible effects of hydrogen bonding, a further set of experiments was carried out using DPPC labeled with ^{13}C in both carbonyl ester groups ([*sn*-1,2–1-^{13}C]-DPPC). The spectrum of the C=O carbonyl region and its deconvolution for an aqueous dispersion of pure [*sn*-1,2–1-^{13}C]-DPPC is shown in Fig. 8 *A*. A characteristically broad ^{13}C=O vibration band of [*sn*-1,2–1-^{13}C]-DPPC is observed at 1694 cm^{-1}; a weaker ^{12}C=O vibration band is also observed at 1737.5 cm^{-1} because of unlabeled carbonyl groups (<10% as confirmed by a comparison of the relative intensities of the two bands). The deconvolved spectrum of the ^{13}C=O carbonyl region shows that the broad band can be resolved into two components, one at higher frequency (approximately 1699 cm^{-1}), which corresponds to the dehydrated ^{13}C=O groups, and the other at lower frequency (approx. 1689 cm^{-1}), which corresponds to the hydrated (hydrogen bonded) ^{13}C=O groups (Blume et al., 1988). The intensity of the dehydrated component is clearly greater than that of the hydrated component.

The presence of 35.6 mol% of 1,2-DPG in codispersion with [*sn*-1,2–1-^{13}C]-DPPC results in a change in the overall bandshape and frequency of the maximum of the ^{13}C=O vibration band of [*sn*-1,2–1-^{13}C]-DPPC as seen in Fig. 8 *B*. The maximum of the ^{13}C=O vibration band of [*sn*-1,2–1-^{13}C]-DPPC is shifted from a frequency of 1694 cm^{-1} in pure [*sn*-1,2–1-^{13}C]-DPPC to 1697.8 cm^{-1} in the presence of 35.6 mol% of 1,2-DPG. Deconvolution of the spectrum shows that the presence of 1,2-DPG results in a significant reduction in intensity of the hydrated component relative to the de-

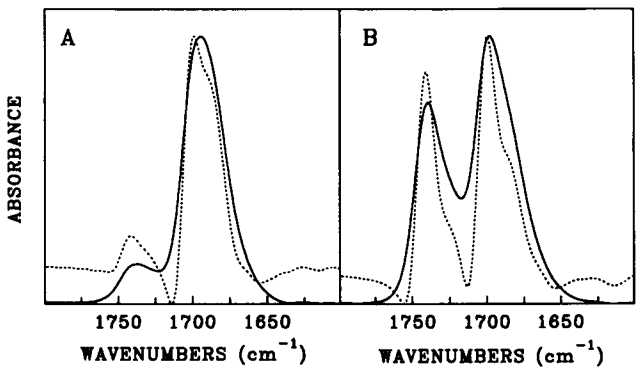


FIGURE 8 Infrared spectra recorded of an aqueous dispersion of (*A*) pure [*sn*-1,2–1-^{13}C]-DPPC and (*B*) a mixture of [*sn*-1,2–1-^{13}C]-DPPC plus 35.6 mol% of 1,2-DPG recorded at 35°C in the region 1800–1600 cm^{-1}. Broken lines show spectral deconvolutions.

hydrated component (Fig. 8, *A* and *B*). Therefore, the difference in frequency maximum of the ^{13}C=O band results from the difference in intensity of the two components of the C=O carbonyl group of [*sn*-1,2–1-^{13}C]-DPPC in the presence of 1,2-DPG. These data suggest that the presence of 1,2-DPG results in a significant reduction in the interaction of the carbonyl groups of the phospholipid with water.

^{31}P-NMR spectroscopy

The effect of 1,2-DPG on the phase polymorphism of DPPC was further investigated by ^{31}P-NMR spectroscopy. The ^{31}P-NMR spectra of aqueous dispersions of DPPC codispersed with different amounts of 1,2-DPG recorded at various temperatures are presented in Fig. 9. Codispersion of DPPC with either 12.5 or 25 mol% of 1,2-DPG (Fig. 9, *A* and *B*) gives rise to an asymmetric lineshape with a high-field peak and a low-field shoulder at all temperatures between 40 and 70°C, characteristic of an axially symmetrical shift tensor and consistent with the arrangement of the phospholipids in a bilayer configuration. In the gel state, the lineshape is broadened ($\Delta\sigma$ = 73 ppm) compared with the liquid-crystalline state (50 and 70°C, respectively) where the lineshape is considerably narrower ($\Delta\sigma$ = 49 ppm).

^{31}P-NMR spectra of DPPC in the presence of 35.6 mol% of 1,2-DPG (Fig. 9 *C*) shows that at a temperature of 50°C an asymmetric lineshape with a high-field peak and a low-field shoulder is produced, characteristic of bilayer structures in the gel state. Similar spectra were recorded at temperatures down to 20°C. As the temperature is increased to 70°C, the asymmetric line shape narrowed indicating that a gel to liquid-crystalline phase transition has taken place. A noteworthy feature of this spectrum is the appearance of a smaller component at frequencies between 8 and 0 ppm, indicating the coexistence of the lamellar phase with another phase, either hexagonal or isotropic. At 80°C, three different lineshapes are clearly visible, namely, hexagonal, isotropic, and lamellar phases. The isotropic component indicates that in the presence of 1,2-DPG some of the DPPC molecules undergo rapid motion leading to nearly complete averaging of the chemical shift anisotropy. In some ^{31}P-NMR spectra it was possible to detect three components (lamellar, hexagonal, and isotropic) at the same time. This does not seem to be a result of the existence of a triple point but possibly to the formation of small particles or regions of the bilayer surface with a relatively high curvature. Something similar has been observed previously by other authors examining analog mixtures such as dimyristoylphosphatidylcholine and 1,2-dimyristoylglycerol (Heimburg et al., 1992).

The ^{31}P-NMR spectra of DPPC codispersed with 66 mol% of 1,2-DPG are shown in Fig. 9 *D*. At temperatures lower than 55°C an asymmetric lineshape with a high-field peak and a low-field shoulder is observed, characteristic of bilayer

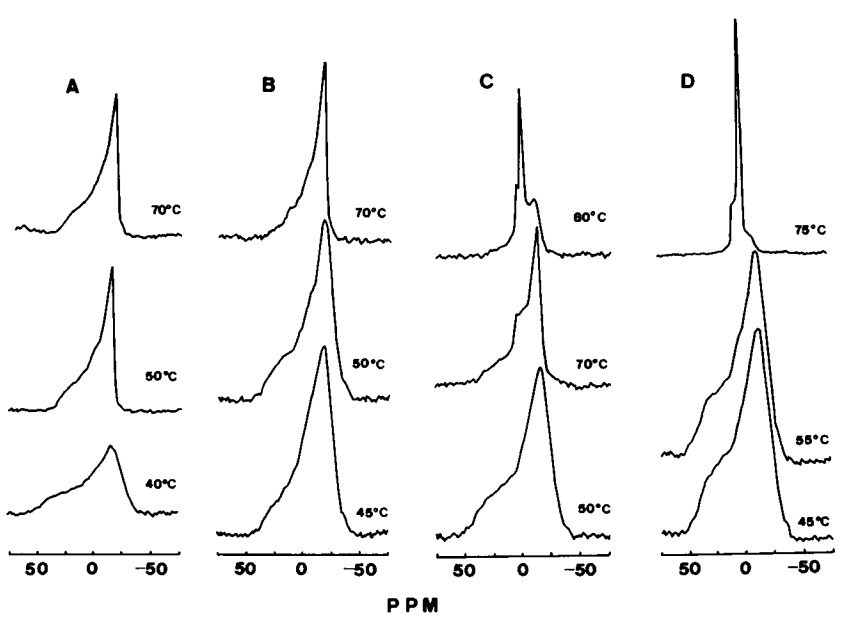


FIGURE 9 ^{31}P-NMR of aqueous dispersions of mixtures of DPPC/1,2-DPG as a function of temperature; (*A*) 12.5 mol% of 1,2-DPG; (*B*) 25 of mol% 1,2-DPG; (*C*) 35.6 mol% of 1,2-DPG; and (*D*) 66 mol% of 1,2-DPG.

structures in the gel state ($\Delta\sigma$ = 68 ppm). At higher temperatures (75°C) two different types of lineshapes can be distinguished in the spectrum, a slightly broadened isotropic peak (frequency at 0 ppm and half-width of 3 ppm), which is superimposed on a lineshape with reverse asymmetry, i.e., a high-field shoulder and a low-field peak. The $\Delta\sigma$ value of this component is approximately 22 ppm, which is characteristic of a hexagonal phase arrangement of phospholipid, and, presumably, this is a H_{II} phase. This indicates a coexistence of an isotropic and a hexagonal phase phospholipid at this temperature. Nevertheless, it should be said that ^{31}P-NMR lineshape measurements do not unambiguously sense phospholipid phase structure, because changes in headgroup conformation can theoretically affect spectral shape in a manner consistent with a phase change (Thayer and Kohler, 1980). Therefore, ^{31}P-NMR results should be considered in the context of data obtained by other techniques like x-ray diffraction and freeze-fracture electron microscopy. Good agreement between our interpretation of ^{31}P-NMR results and the other techniques was achieved in this study.

DISCUSSION

Studies of the behavior of mixed phospholipid-diacylglycerol systems are necessary to understand the processes of lipid-mediated signal transduction across biological membranes. Many combinations of aqueous phospholipid and diacylglycerol systems have been examined using a range of biophysical methods, but so far there are few data that enable construction of complete phase diagrams of these mixtures. The present study, in constructing a phase diagram of DPPC/1,2-DPG in water, has exploited some novel techniques that provide complementary data on both static and dynamic phase behavior of the mixture. Dynamic x-ray diffraction measurements, for example, were performed under identical conditions to the DSC studies, so that both thermal and structural information could be derived. It should be commented here that the 001 spacing of 8.3 nm at 37°C for pure DPPC may seem very large in comparison with previous measurements such as those of Janiak et al. (1976) who reported a value of 66 Å for the $P_{\beta'}$ phase. However, the conditions used in our experiments were totally different. The spacing of 8.3 nm was consistent under the conditions used in our dynamic x-ray measurements. The spacing of the $P_{\beta'}$ lamellar repeat is known to be highly dependent on the rate of temperature scan and the ionic composition of the medium in which DPPC is dispersed as pointed out by Caffrey et al. (1990). Values of up to 7.9 nm have been reported from Hatta's group in dynamic x-ray studies of the $P_{\beta'}$ phase (Tenchov et al., 1989). On the other hand, the primary lattice spacings corresponding to the ripple repeat could not be detected in the camera configuration employed in this study, and higher order reflections are relatively weak in dynamic measurements. The broad lamellar repeat spacing observed suggests that the structure is not well correlated, and this may also be expected of the ripple structure. It may not be surprising, therefore, that lattice orders are not detected. It should be said also here that some lack of precise agreement of some of the second orders, with respect to the first orders in the lamellar repeats, can be observed in Table 1. This could be due to the dynamic method used to obtain these diffraction data, because the continuous changes in temperature may introduce some noise in the diffractograms.

Isotopic substitution of the lipid constituents, moreover, provided information on vibrational modes related to the extent of hydration of the two components. The use of palmitoyl derivatives of both phospholipid and diacylglycerol greatly simplifies the interpretation of the data and establishes the principles upon which more complex mixtures that more closely resemble biological membranes can be based.

A consensus phase diagram of DPPC/1,2-DPG in excess water based on data obtained in this multifaceted approach is presented in Fig. 10. The phase boundaries of the solidus and fluidus lines have been established from the respective heating and cooling scans of appropriate mixtures of phospholipid and diacylglycerol. The thermal and structural data were in close agreement in delineation of these boundaries. The phase assignments have been determined from dynamic x-ray diffraction measurements. The method was able to distinguish clearly where coexistence between phases occurs. Structural assignments also were confirmed in static measurements under carefully controlled conditions using ^{31}P-NMR and freeze-fracture electron microscopy. For the sake of simplicity, the pretransition of DPPC has been omitted in pure DPPC and also in those DPPC/1,2-DPG mixtures that display it (Fig. 1).

The phase diagram presents at least three well differentiated regions, which are very similar to those previously described for dimyristoylphosphatidylcholine/1,2-dimyristoylglycerol (Heimburg et al., 1992). The first one corresponds to concentrations of 1,2-DPG lower than 45 mol%. This region can be approached by a eutectic model, so that

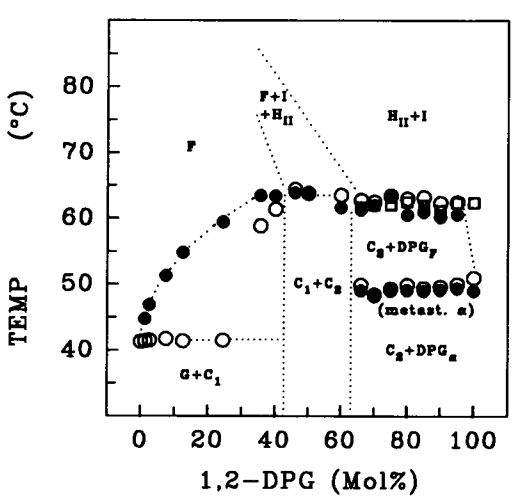


FIGURE 10 Phase diagram of aqueous dispersions of DPPC/1,2-DPG constructed from data derived from calorimetry and dynamic x-ray diffraction. Phase assignments were confirmed by freeze-fracture electron microscopy, FT-IR, and ^{31}P-NMR. The open and closed circles were obtained from T_c of the heating and cooling scans, respectively.

the eutectic point will be located at a very low concentration of 1,2-DPG, e.g., close to pure DPPC. The immiscibility in the gel phase is presumed to occur between pure DPPC and a compound, called C_1, with a DPPC/1,2-DPG molar ratio of about 55:45.

On the other hand, at the highest mole fractions of 1,2-DPG within this region, such as 35.6 mol% of 1,2-DPG, there are clear deviations from total immiscibility in the gel phase, indicating a limited solubility of DPPC in the higher melting compound, C_1. This is a situation very similar to that previously observed for dilauroylphosphatidylcholine/DPPC mixtures (Van Dijck et al., 1977). The gel phase immiscibility within the lamellar structure is clearly observed through freeze-fracture electron microscopy (Fig. 5). Thus smooth lamellar surfaces can be distinguished from a ripple phase with a periodicity of about 12.1 nm, corresponding to pure DPPC in $P_{\beta'}$ phase. The high values obtained for the low-angle lamellar repeat (approximately 7 nm), approaching that of $P_{\beta'}$ for DPPC in the gel phase, can be explained by the adoption of a ripple phase by the compound C_1. The large spacing observed in both gel and liquid-crystal lamellar phases of the mixtures containing 35.6 mol% of 1,2-DPG were found to be associated with ripple structures as shown by the freeze-fracture electron microscopy.

Theoretical predictions (Cevc, 1991) suggest that interlamellar headgroup-water-headgroup interactions are the primary factor that induces a lamellar phase to adopt a ripple structure. The balance of forces resulting from interchain (*ch*) and headgroup (*hg*) interactions is said to conform to the relationship:

$$\psi = \psi_{ch} + \psi_{hg} = A_{ch}\cos(m\alpha) + B_{ch}P_2\cos(\gamma) + C_{hg}\cos(n\alpha),$$
$$m, n = 1, 2, \cdots,$$

where γ is the angle between chain and headgroup axes and α is the sum of the relative angles of rotation of the two neighboring chains. P_2 is a second-order Legendre polynomial and A_{ch}, B_{ch}, and C_{hg} are coupling constants. The greater the interaction energy between molecules in the bilayer phase, the more likely that the $P_{\beta'}$ phase will occur at lower temperatures. This will also hold for situations where the bilayer phase is stabilized at higher temperatures by reduced repulsion between headgroups.

On the other hand, the phase diagram indicates that above of the phase transition a good miscibility occurs between 1,2-DPG and DPPC. Nevertheless, the low-angle lamellar repeats, as observed by x-ray diffraction (Table 1), again indicate high values compared with pure DPPC. This suggests the formation of lamellar ripple phases when the system is in fluid state as well. This is confirmed by the observation of freeze-fracture electron microscopy replicas of samples containing 35.6 mol% of 1,2-DPG (Fig. 5), where a 92-nm periodicity ripple can still be observed at temperatures as high as 70°C.

At very high temperatures and at concentrations higher than 35.6 mol% of 1,2-DPG, nonlamellar phases are detected by ^{31}P-NMR and x-ray diffraction. Whereas at 35.6 mol% of 1,2-DPG the transition to nonlamellar phases is from a fluid (ripple) lamellar phase, this transition is directly from gel phases at higher concentration of 1,2-DPG. This is a similar case to others described previously, e.g., mixtures DPPC/free palmitic acid (Marsh and Seddon, 1982). It should be remarked that the FTIR data presented in Fig. 6 confirms the phase diagram as it has been designated in Fig. 10, so that the assignment of the different calorimetric peaks and transition temperatures is supported by the infrared results.

The other important feature that defines the phase diagram presented in Fig. 10 is the appearance of free 1,2-DPG at concentrations $\geq$66 mol%, indicating the presence of a new compound with a DPPC/1,2-DPG molar ratio of about 1:2, referred to as C_2. An additional eutectic mixture is probably present at a composition very close to that of the second compound. In the central region, i.e., that corresponding to samples containing 1,2-DPG in a proportion higher than about 46 mol% and lower that about 66 mol% a mixture between both C_1 and C_2 should be present. Nevertheless, the evidence for the presence of these two compounds is not too strong, inasmuch as we have no evidence supporting it from x-ray diffraction. The only experimental evidence comes from the DSC cooling scans in the range comprising this region. Another possibility that could exist is the presence of a single solid solution with limited composition.

It is noteworthy that the low-angle lamellar repeats, observed by x-ray diffraction, present very high values for all these samples containing high proportions of 1,2-DPG. Obviously, this is the case for gel phases only, because, as discussed above, these samples do not maintain lamellar structures at temperatures higher than that of their gel-to-fluid transition. Therefore, ripple phases are to be expected also at low temperatures for samples with higher proportions of 1,2-DPG.

The ΔH plot shown in Fig. 2 provides additional evidence of complex formation, inasmuch as it suggests the formation of the complexes C_1 and C_2 with the stoichiometries suggested above. It seems that the C_1 complex has a ΔH similar to DPPC. At proportions of 1,2-DPG higher than about 40 mol% ΔH increases, indicating the appearance of a new species, i.e., the C_2 complex. This second complex has, on the other hand, a ΔH similar to that of pure 1,2-DPG in the α form, but it is a quite different one from that of the β' form of 1,2-DPG.

These results can be summarized by supposing that, in the gel phase, DPPC forms two types of complexes with 1,2-DPG. The first one, having a 55:45 DPPC/1,2-DPG molar ratio, will adopt a ripple structure as characterized by high lamellar repeats by x-ray diffraction and seen through freeze-fracture electron microscopy. This complex will have a transition to a fluid phase also with high lamellar repeat spacings and a ripple structure as seen by electron microscopy. This ripple phase possibly may be of the $P_{\alpha\beta}$ type proposed in other lipid systems (Ranck, 1983). Nonlamellar phases can be detected only at very high temperatures and only for relatively high concentrations of

1,2-DPG. The second complex will have a 1:2 DPPC/1,2-DPG molar ratio, and it will also exhibit large lamellar repeat spacings in the gel state; however, it undergoes a direct lamellar gel to a H_{II} phase transition.

The FTIR results are also worthy of comment, inasmuch as they suggest that, in the presence of 1,2-DPG, water is not able to penetrate as extensively into the interfacial region of the bilayer compared with pure DPPC. This seems to be inconsistent with previous models of phospholipid bilayers containing intercalated diacylglycerols, which have envisaged that spreading the polar head groups of the phospholipids results in greater exposure of the hydrocarbon interface to water (Das and Rand, 1986).

On the other hand, the closer packing of the hydrocarbon chains and increased stability of the resulting bilayer could be explained by reduced repulsion between the phosphocholine head groups of the phospholipid and the consequent increase in van der Waal's cohesive forces between the hydrocarbon chains.

The biological relevance of this study may be focused in principle on the region of the phase diagram where the proportion of diacylglycerol in phospholipid is low. There is compelling evidence that gel phase immiscibility occurs even in mixtures containing less that 2.6 mol% of 1,2-DPG in DPPC. It should be emphasized that diacylglycerols have been found to be present in biological membranes at concentrations of 2 mol% at most, with respect to total lipid (Preiss et al., 1986). Models of the type $P_{\alpha\beta}$ imply domains concentrated with respect to diacylglycerol, and such domains may be generated in regions of phospholipid bilayer surrounding the molecules of phopholipase producing them. Local high concentrations of diacylglycerols could also depend on their diffusion capability and on the existence of membrane domains where they could be particularly concentrated. As shown in this work, the existence of local accumulation of diacylglycerols will facilitate the existence of effects such as ripple phase formation or dehydration of certain membrane groups located near the interface. Further studies will be required to characterize these complexes to explain the physiological consequences of diacylglycerols in membranes.

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