# Ceramides in Phospholipid Membranes: Effects on Bilayer Stability and Transition to Nonlamellar Phases

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ABSTRACT The effects of ceramides of natural origin on the gel-fluid and lamellar-inverted hexagonal phase transitions of phospholipids (mainly dielaidoylphosphatidylethanolamine) have been studied by differential scanning calorimetry, with additional support from infrared and $^{31}$P nuclear magnetic resonance (NMR) spectroscopy. In the lamellar phase, ceramides do not mix ideally with phospholipids, giving rise to the coexistence of domains that undergo the gel-fluid transition at different temperatures. The combination of differential scanning calorimetry and infrared spectroscopy, together with the use of deuterated lipids, allows the demonstration of independent melting temperatures for phospholipid and ceramide in the mixtures. In the lamellar-hexagonal phase transitions, ceramides (up to 15 mol %) decrease the transition temperature, without significantly modifying the transition enthalpy, thus facilitating the inverted hexagonal phase formation. $^{31}$P-NMR indicates the coexistence, within a certain range of temperatures, of lamellar and hexagonal phases, or hexagonal phase precursors. Ceramides from egg or from bovine brain are very similar in their effects on the lamellar-hexagonal transition. They are also comparable to diacylglycerides in this respect, although ceramides are less potent. These results are relevant in the interpretation of certain forms of interfacial enzyme activation and in the regulation and dynamics of the bilayer structure of cell membranes.

## INTRODUCTION

The role of phospholipids in metabolism was considerably enlarged by the identification of the products of glycerophospholipid cleavage as intracellular signals, or second messengers (Berridge, 1987). No less important has been the more recent discovery of the role of the sphingomyelin derivatives ceramides in cell signaling (Michell and Wakelam, 1994; Hannun and Obeid, 1995). These novel metabolic signals originate in the cell membranes, as a result of the operation of specific lipases. In the case of sphingomyelin, or ceramidephosphorylcholine, the action of sphingomyelinase gives rise to ceramide and water-soluble phosphorylcholine. Ceramides are amphiphiles, but they are virtually insoluble in water. Their hydrophobicity explains the other important biological role of ceramides, as components of the stratum corneum that constitutes the permeability barrier of the skin (Elias et al., 1977). Skin ceramides can be divided into two main groups, those that contain $\alpha$-hydroxy fatty acids and those that do not (Gray and White, 1978). The physical properties of anhydrous and hydrated ceramides as well as the effect of fatty acid hydroxylation have been explored by x-ray diffraction and differential scanning calorimetry (Han et al., 1995; Shah et al., 1995a,b) and by infrared spectroscopy (Moore and Rerek, 1997; Moore et al., 1997).

Because of their nonpolar character, ceramides are likely to exert their role as metabolic signals at least in part from within the cell membrane bilayers. Thus in this work, we have examined the bilayer-perturbing effects of ceramides. Most of our studies have been carried out with ceramide derived from egg-yolk lipids, containing mainly palmitic acid.

Synthetic membranes consisting of aqueous dispersions of dielaidoylphosphatidylethanolamine (DEPE) have been used in most cases. Ceramides have been tested with respect to their ability to modify the gel-fluid ($L_\beta$-$L_\alpha$) and lamellar-hexagonal ($L_\alpha$-$H_{II}$) phase transitions of DEPE, which occur respectively at $T_m \approx 37.5$ and $T_h \approx 65$°C. These phase transitions can be accurately examined, and the associated enthalpy change measured, using high-sensitivity differential scanning calorimetry (DSC). In addition, some peculiarities of the phase changes have been studied by infrared or $^{31}$P nuclear magnetic resonance (NMR) spectroscopy. Ceramides have been found to mix poorly with phospholipids in bilayers and to facilitate the formation of inverted hexagonal phases.

## MATERIALS AND METHODS

DEPE, *N*-monomethyldioleoylphosphatidylethanolamine (DOPE-Me), dipalmitoylphosphatidylcholine with fully deuterated fatty acyl chains ($d_{54}$-DPPC), and brain and egg ceramide were supplied by Avanti Polar Lipids (Alabaster, AL). Typical fatty acid distributions are, for brain ceramide, 2% C16:0, 58% C18:0, 6% C20:0, 9% C22:0, 7% C24:0, 15% C24:1, and 3% others; for egg ceramide, 78% C16:0, 8% C18:0, 4% C22:0, 3% C24:1, 2% C20:0, 3% C24:0, and 2% others. Egg diacylglycerol obtained by phospholipase C cleavage of egg phosphatidylcholine was grade I from Lipid Products (South Nutfield, UK). Its fatty acid composition was 34% C16:0, 11% C18:0, 31% C18:1, 18% C18:2, 3% C20:4, and 3% others. All of these lipids were >99% pure according to the suppliers and were used without further purification.

Phospholipid and ceramide were co-dissolved in chloroform, and the mixture was evaporated to dryness under a stream of nitrogen. Traces of solvent were removed by evacuating the samples under high vacuum for at

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least 2 h. The samples were dispersed at 45°C with shaking in 20 mM PIPES, 150 mM NaCl, 1 mM EDTA, pH 7.4. The hydrated samples were put in glass tubes ~5 × 60 mm, containing a constriction ~0.5 mm in diameter at half-height. The tubes were sealed and the hydrated mixtures forced through the constriction backwards and forwards 10 times at 45°C by centrifuging the tubes in a bench centrifuge, with the aim of improving the mixing. The amount of phospholipid was kept constant while the amount of additive and, correspondingly, that of total lipid, varied.

Both lipid suspensions and buffer were degassed before being loaded into the sample or reference cell of an MC-2 high-sensitivity scanning calorimeter (MicroCal, Northampton, MA). The final concentration of phospholipid was 0.4 mM for samples where gel-to-fluid transitions were measured and 7 mM for those in which fluid-to-inverted-hexagonal transitions were studied. Three heating scans, and occasionally a cooling one, at 45°C/h were recorded for each sample. After the first one, successive heating scans on the same sample gave always superimposable thermograms. Thermogram decomposition, transition temperatures, enthalpies, and widths at half-height were determined using the software ORIGIN (MicroCal) provided with the calorimeter. This software uses the Levenberg/Marquardt nonlinear least-squares method for curve fitting. A model assuming independent non-two-state transitions provided the best fit to the experimental data.

$^{31}$P-NMR spectra were recorded in a VXR 300 Varian spectrometer operating at 300 MHz for protons (121.4 MHz for $^{31}$P). The final phospholipid concentration was 130 mM. Spectral parameters were 45° pulses (10 $\mu$s), pulse interval of 3 s, sweep width of 16 kHz, and full proton decoupling. One thousand free induction decays were routinely accumulated from each sample; the spectra were plotted with a line broadening of 80 Hz. Samples were equilibrated for 10 min at each temperature before data acquisition.

Infrared spectra were recorded in a Nicolet Magna II 550 spectrometer, equipped with a mercury cadmium telluride detector. Lipid mixtures were resuspended in buffer at a 25 mM final phospholipid concentration. Samples were placed in a temperature-regulated cell with $CaF_2$ windows and heated at 60°C/h in the 20–60°C temperature range; 12-$\mu$m spacers were used, and 304 scans/°C were taken using a rapid-scan software. Band maxima were determined from derivative spectra, Fourier derivation being performed with a power of 3 and a breakpoint of 0.3.

## RESULTS

### The gel-fluid transition

Representative DSC thermograms (heating scans) of the gel-fluid transition of pure DEPE and of mixtures of DEPE/ceramide are shown in Fig. 1 *A*. Ceramide has the effect of spreading the phase transition over a wide range of temperatures, ~15°C at 25 mol % and above, while increasing the midpoint transition temperature by ~8°C. The latter figure is difficult to establish with accuracy, because the endotherms are clearly asymmetric and reveal the existence of several components. These complex endotherms preserve their overall shape when the PIPES buffer is substituted by 10 mM Tris/HCl, 150 mM NaCl, pH 7.4, and although there may be differences between the first and second scan they do not change afterwards; thus, the thermograms are probably indicating some degree of immiscibility, with the coexistence of mixtures of somewhat different compositions.

The complex nature of the DEPE/ceramide DSC signals was further explored by decomposing the endotherms with the ORIGIN software as indicated under Methods (Fig. 1 *B*). The simplest fitting requires at least two components for mixtures containing 5–10 mol % ceramide and three com-

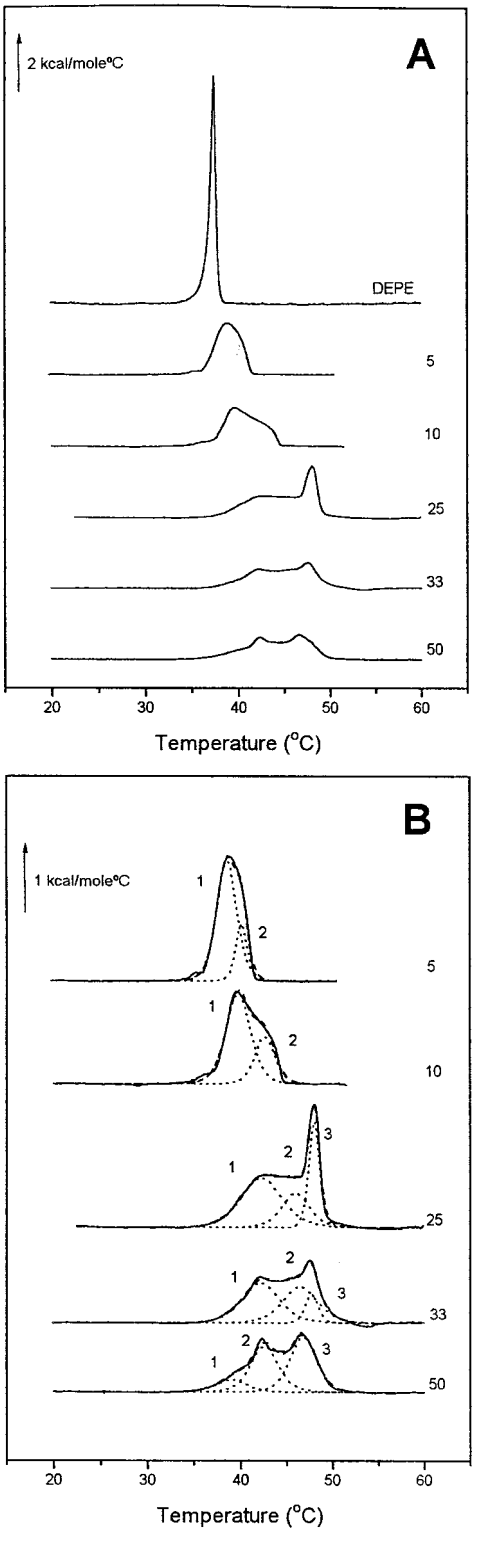


FIGURE 1 The gel-fluid transitions of pure DEPE and DEPE/ceramide mixtures as detected by differential scanning calorimetry. (*A*) Representative thermograms (second heating scan). (*B*) The same thermograms as in *A*, except the one corresponding to pure DEPE, drawn at a larger scale to show the component peaks (in broken lines). The component peaks are numbered 1, 2, 3, in increasing order of melting temperatures. The figures at the right of each thermogram represent the ceramide contents, in mole percentages.

ponents for the remaining samples. We have designated component 1 the one that appears to be derived from the main transition endotherm of pure DEPE. The various novel components all follow the same pattern as ceramide concentration is increased (Figs. 1 *B* and 2); they appear at the high-T side of the endotherm as narrow bands, and increasing ceramide proportions make them wider, at least up to 30% ceramide, and shift them toward higher temperatures, thus contributing to the overall increase in the midpoint transition temperature that was mentioned above. Such an increase in *Tm* is easy to understand considering that the pure hydrated ceramide is expected to have an order-disorder transition well above the *Tm* of DEPE (Shah et al., 1995a; Moore et al., 1997).

The nature of the various components that are detected in the DSC thermograms was further explored by spectroscopic methods. Infrared (IR) spectroscopy is useful in this respect because it can provide independent information on the melting of the ceramide and phospholipid chains, provided one of the molecules has their chains fully deuterated (Echabe et al., 1995). With this purpose in mind a series of samples was prepared with ceramide and $d_{54}$-DPPC, a dipalmitoylphosphatidylcholine with both fatty acyl chains fully deuterated. ($d_{54}$-DPPC is much more readily available than the corresponding DEPE deuterated derivative.) DSC thermograms of ceramide/DPPC mixtures show the same kind of complex endotherms that were seen with DEPE (data not shown).

The gel-fluid transition in ceramide/$d_{54}$-DPPC mixtures can be detected by IR spectroscopy through changes in the C—H (C—D) stretching frequencies. Fig. 3 shows the plot of the asymmetric C—H (ceramide) and C—D (phospholipid) stretching frequencies as a function of temperature. The corresponding symmetric frequencies gave rise to very similar plots (not shown). The IR data show clearly that pure $d_{54}$-DPPC undergoes a sharp gel-fluid transition with a mid-point transition temperature $Tm \approx 38°C$ (fatty acyl deuteration downshifts *Tm* by 2–3°C). However,in mixtures containing ceramide the transition is considerably broadened and shifted to higher temperatures, in agreement with the calorimetric data (Fig. 3 *A*). Meanwhile, the ceramide, when included in the DPPC bilayer, undergoes a transition

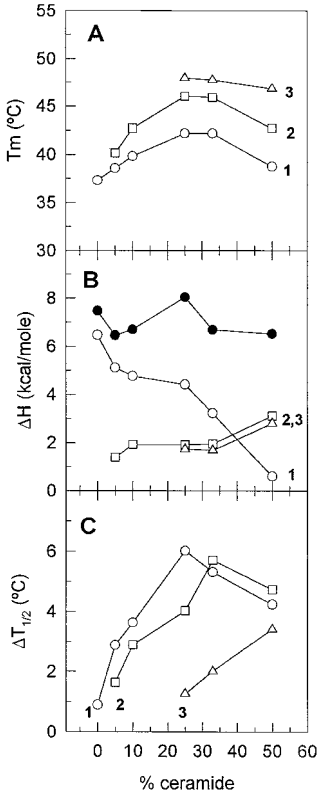


FIGURE 2 Thermodynamic parameters of the gel-fluid transitions of pure DEPE and DEPE/ceramide mixtures. Data are derived from thermograms as shown in Fig. 1. (*A*) Midpoint transition temperatures of the component peaks. ○, peak 1; □, peak 2; △, peak 3. (*B*) Transition enthalpies of the overall transition and of its components. ●, overall transition $\Delta H$; other symbols as in *A*. (*C*) Width at mid-height of the component transition peaks. Symbols are as in *A*.

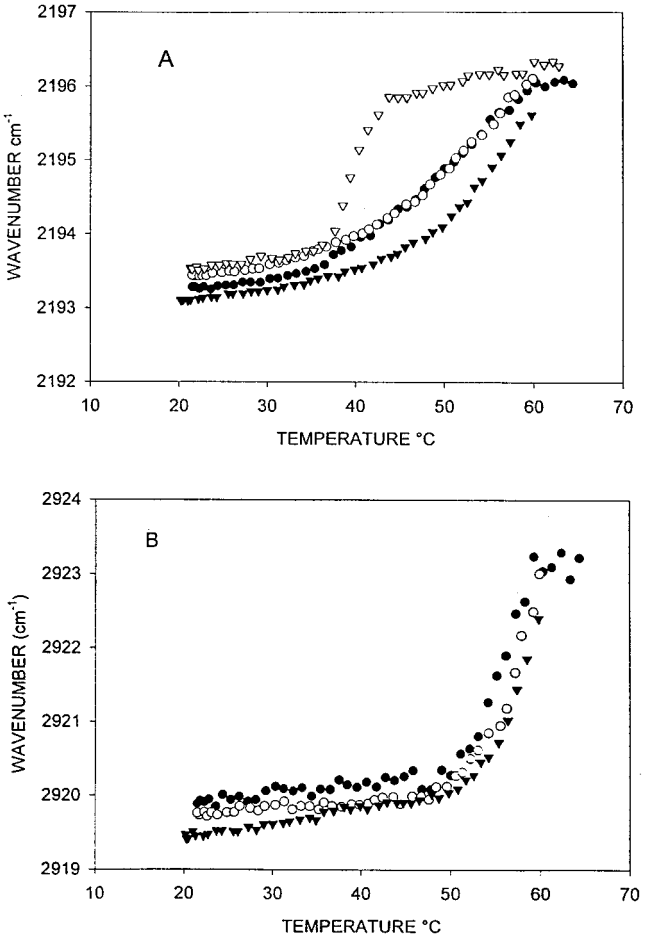


FIGURE 3 The gel-fluid transitions of pure $d_{54}$-DPPC and $d_{54}$-DPPC/ceramide mixtures as detected by IR spectroscopy. The maximal frequencies of the asymmetric C—H or C—D stretching bands are plotted as a function of temperature (data from the second heating scan). (*A*) C—D vibrations of $d_{54}$-DPPC. ▽, pure phospholipid; ●, ○, ▼, phospholipid/ceramide mixtures containing, respectively, 15, 20, and 25 mol % ceramide. (*B*) C—H vibrations of ceramide chains. Symbols are as in *A*.

at a *Tm* ≈ 55°C that is hardly modified by increasing the ceramide proportion, apart from a small increase in *Tm* (Fig. 3 *B*). The fact that in ceramide/$d_{54}$-DPPC mixtures ceramide melts at temperatures clearly above the phospholipid is an obvious indication of poor mixing. This, together with the high order-disorder transition temperature of the pure ceramide (Shah et al., 1995b; Moore and Rerek, 1997) explains the broadening and shift to higher temperatures of the calorimetric transitions (Figs. 1 and 2). In addition, the differences in the transition patterns of ceramide and phospholipid (Fig. 3) support the idea that the low-*Tm* components seen in Fig. 1 *B* correspond to domains rich in phospholipid, whereas the high-*Tm* components would correspond to the transition of ceramide-rich domains.

## The lamellar-hexagonal transition

Ceramide modifies the lamellar ($L_\alpha$) to hexagonal ($H_{II}$) transition of DEPE even at small proportions (Fig. 4). The $L_\alpha$-$H_{II}$ transition endotherm of DEPE is widened and shifted to lower temperatures, whereas a shoulder is seen at the high-temperature side of the endotherm, which becomes more prominent as ceramide proportions increase. This asymmetric DSC signal can be analyzed in terms of two components (Figs. 4 and 5), with the high-temperature one presumably related to ceramide-rich membrane domains. The overall shape of the endotherms was not modified by repeated scanning of the samples (after the second scan).

The enthalpy change $\Delta H$ associated to the $L_\alpha$-$H_{II}$ transitions is much lower, in absolute figures, than that of the $L_\beta$-$L_\alpha$ transition. Ceramide has a very small effect on the overall $\Delta H$ of the transition (Fig. 5).

As ceramides have an obvious structural analogy with diacylglycerols, although both lipid classes exhibit physiological actions very different from each other (Hannun and Obeid, 1995; Ruiz-Argüello et al., 1998), the effects of egg diacylglycerol on the lamellar-hexagonal phase transition of DEPE may be relevant in comparison with the effects of ceramide. These results are shown in Figs. 6 and 7. The effect of diacylclycerol on the $L_\alpha$-$H_{II}$ transition is similar to that of ceramide in that 1) the transition is shifted to lower temperatures, i.e., hexagonal phase formation is facilitated, 2) the overall enthalpy change is modified but slightly, 3) a multicomponent transition is observed, and 4) the overall transition endotherm, as well as each of the component peaks, becomes wider with increasing diacylglycerol pro-

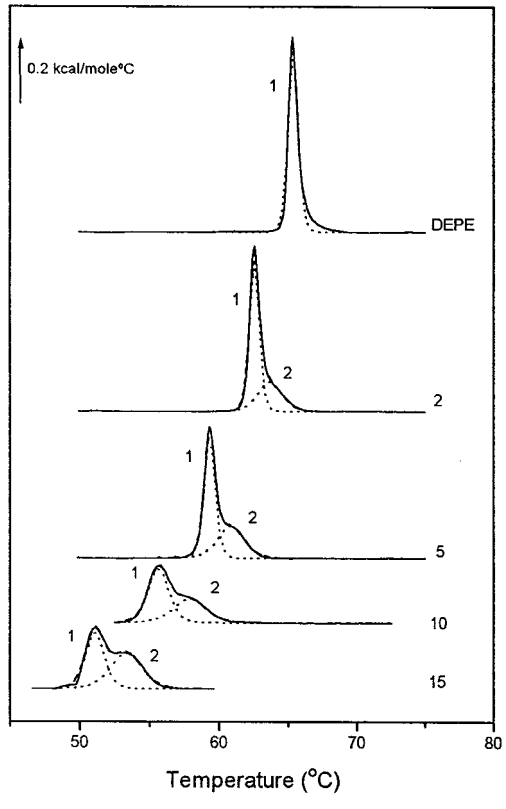


FIGURE 4 The lamellar-to-inverted hexagonal ($L_\alpha$-$H_{II}$) transitions of pure DEPE and DEPE/ceramide mixtures as detected by differential scanning calorimetry. In broken lines, the transition components are as obtained by decomposition and fitting of the experimental signal. The component peaks are numbered 1 and 2. The figures at the right of each thermogram correspond to the ceramide contents, in mole percentages.

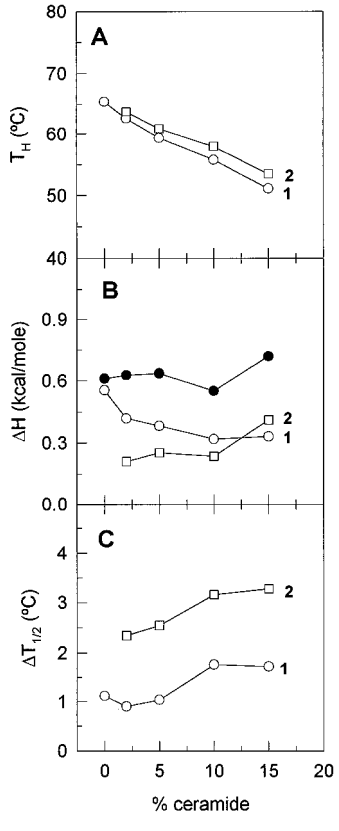


FIGURE 5 Thermodynamic parameters of the lamellar-hexagonal transitions of pure DEPE and DEPE/ceramide mixtures. Data are derived from thermograms as shown in Fig. 4. (*A*) Midpoint transition temperatures of the component peaks. (*B*) Transition enthalpies of the overall transition and of its components. (*C*) Width at mid-height of the component transition peaks. ●, overall transition; ○, peak 1; □, peak 2. Peaks 1 and 2 are shown in Fig. 4.

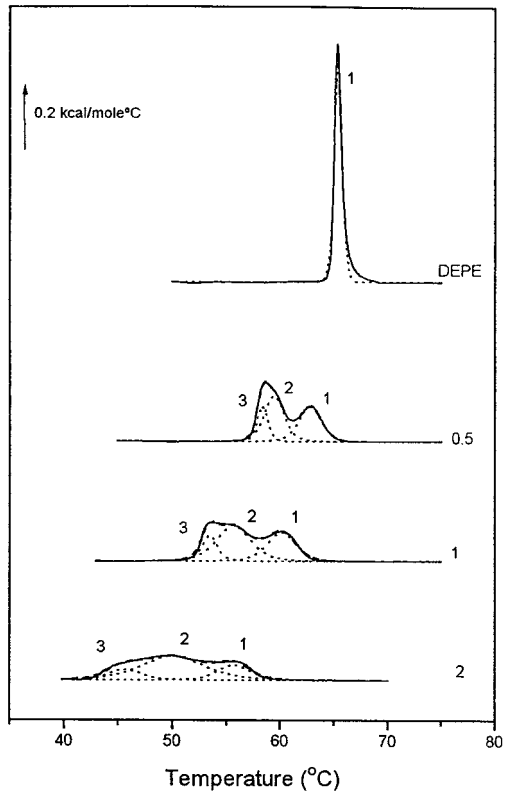


FIGURE 6 The lamellar-to-inverted hexagonal ($L_\alpha$-$H_{II}$) transitions of pure DEPE and DEPE/egg diacylglycerol mixtures as detected by differential scanning calorimetry. In broken lines, the transition components are as obtained by decomposition and fitting of the experimental signal. The component peaks are numbered 1, 2, and 3. The figures at the right of each thermogram correspond to the diacylglycerol contents, in mole percentages.

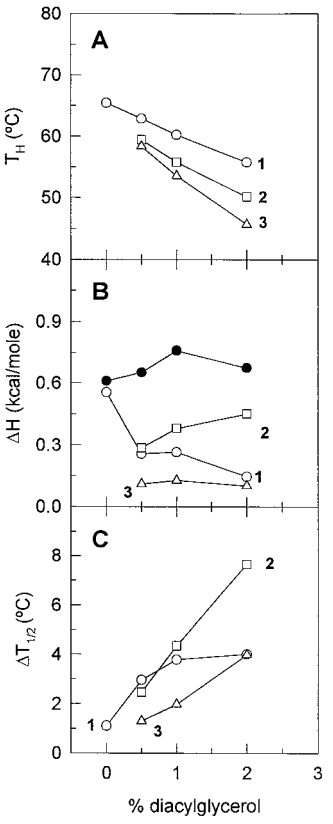


FIGURE 7 Thermodynamic parameters of the lamellar-hexagonal transitions of pure DEPE and DEPE/egg diacylglycerol mixtures. Data are derived from thermograms as shown in Fig. 6. (*A*) Midpoint transition temperatures of the component peaks. (*B*) Transition enthalpies of the overall transition and of its components. (*C*) Width at mid-height of the component transition peaks. ●, overall transition; ○, peak 1; □, peak 2; △, peak 3. Peaks 1, 2, and 3 are shown in Fig. 6.

portions. There is, however, an important difference in the effects of these lipids, which lies in their respective potencies, diacylglycerol being considerably more potent (see the quantitative data in Table 1). It is doubtful that this difference can be attributed to the different acid composition of diacylglycerol and ceramide, because egg and brain ceramide differ significantly in fatty acid composition, yet they have very similar effects on the $L_\alpha$-$H_{II}$ transition (see below).

Some ceramide samples were prepared by mixing this lipid with *N*-methyl dioleoylphosphatidylethanolamine (DOPE-Me). The latter phospholipid has a $L_\alpha$-$H_{II}$ transition at $T_h \approx 63$°C. The effect on the lamellar-hexagonal transition is the same as described for DEPE, although the absolute figures may differ (data not shown). As an example, 2 mol % ceramide in DOPE-Me decreases $T_h$ from 63.3 to 57.5°C, increases the transition enthalpy $\Delta H$ from 220 to 319 cal/mol, and increases the endotherm width at half-height from 1.45°C to 2.07°C. (See Table 1 for comparative data). $T_h$ of DOPE-Me appears to be more sensitive to the presence of foreign lipids than that of DEPE. In fact, DOPE-Me has been found to form nonlamellar (cubic) phases more readily than DEPE (Siegel and Banschbach, 1990). The table shows as well data derived from unpublished studies by D. P. Siegel and J. Banschbach using bovine brain ceramide. The effects on DOPE-Me are very similar, even quantitatively, to the ones found by us with egg ceramide.

The origin of the high-temperature shoulder observed in most lamellar-hexagonal endotherms was further explored by $^{31}$P-NMR. Mixtures containing DEPE and 5 mol % ceramide were analyzed by this technique. It should be noted that, although the NMR samples contained excess water, so that no lyotropic effects can be expected, still lipid concentration was much higher in NMR than in DSC experiments. Also, the thermal histories of samples were not identical in both techniques, due to their inherent limitations (e.g., continuous heating is virtually impossible with $^{31}$P-NMR if a large number of transients are to be accumulated). With these caveats in mind, $^{31}$P-NMR results can be a useful complement of the DSC observations. It was found (Fig. 8) that the $L_\alpha$-$H_{II}$ transition occurred gradually over a temperature range of ~5–7°C. In this interval, the lamellar and hexagonal spectral line shapes coexisted. The temperature interval is about the same as the full width of the DSC endotherms, i.e., completion minus onset temperatures. The absolute temperatures at which the transitions are detected

**TABLE 1 Effects of ceramide and diacylglycerol on the lamellar-hexagonal transition of phospholipids, as detected by differential scanning calorimetry**

| Lipid | Additive | Data source | $\Delta T_h$ (°C/mol %) | $\Delta\Delta H$ (cal/mol %) | $\Delta$width (°C/mol %) |
|---|---|---|---|---|---|
| DOPE-Me | Ceramide (egg) | TW | −2.9 | +50 | +0.31 |
| DOPE-Me | Ceramide (brain) | SB | −4.3 | +41 | ND |
| DEPE | Ceramide (egg) | TW | −1.3 | +75 | ~0 |
| DEPE | Diacylglycerol (egg) | TW | −10.8 | +88 | +5.74 |

Average values per 1 mol % ceramide (or diacylglycerol) were obtained from measurements with mixtures containing between 1 and 5 mol % ceramide or between 0.5 and 2 mol % diacylglycerol. Widths were measured at half-height of the endothermal signal. TW, this work; SB, Siegel and Banschbach, personal communication.

by DSC and by $^{31}$P-NMR do not coincide, presumably due to the different heating rates and lipid concentrations, or to reasons intrinsic to the resonance technique (Epand and Lemay, 1993).

## DISCUSSION

The main experimental results in this paper show that ceramides of natural origin do not mix ideally with phospholipids, favor the stability of the gel over the fluid lamellar phase, and facilitate the lamellar-hexagonal transition of the phospholipids. These properties are important by themselves, but in addition they may be related to certain functional effects of ceramides. Moreover, a comparison of the effects of ceramides with those of their structural analogues diacylglycerols may provide some interesting information.

### Physical data

The influence of ceramides on phospholipid phase transitions has been the object of recent attention by this and other laboratories. Huang et al. (1996) have shown, in mixtures of bovine brain ceramide and DPPC, using $^{2}$H-NMR, the same phenomenon of gel immiscibility seen in our DSC thermograms (Figs. 1 and 2). Also, Holopainen et al. (1997), measuring excimer formation with a pyrene-labeled phospholipid probe, describe the formation of microdomains concomitant with the formation of a distinct ceramide-enriched phase at ceramide molar fractions $X_{cer} > 0.10$ in dimyristoylphosphatidylcholine, in agreement with our calorimetric and IR data. IR spectroscopy, when combined with the use of selectively deuterated lipids (as in Fig. 3), is particularly useful in revealing unambiguously poor lipid miscibility along a phase transition process. The other significant effect of ceramide is the stabilization of the gel versus the fluid lamellar phase, as detected through the dose-dependent increase in *Tm* (Figs. 1–3). This is to be expected from the high temperature of the order-disorder chain transition of the pure, hydrated ceramide (Han et al., 1995; Shah et al., 1995a,b; Moore et al., 1997) and was also observed by Holopainen et al. (1997) in their ceramide-dimyristoylphosphatidylcholine system. The data in Figs. 1 and 2 are particularly meaningful as they are obtained with the same lipid (DEPE) in which the lamellar-hexagonal transition has been explored, thus allowing a more direct comparison of the effects of ceramides on both phase transitions.

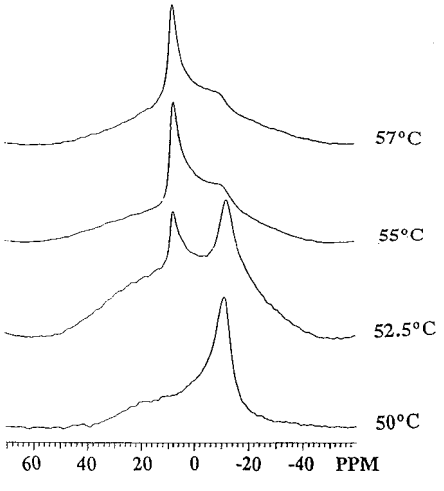


FIGURE 8 $^{31}$P-NMR spectra of aqueous dispersions of DEPE/ceramide. Ceramide content was 5 mol %. The spectra were recorded at increasing temperatures, as indicated (in °C) by each curve; 0 ppm corresponds to *o*-phosphoric acid. Line broadening, 80 Hz.

The effect of ceramides on the lamellar-hexagonal transition of phospholipids had not been studied up to now, to the authors' knowledge. Ceramides clearly facilitate the $L_\alpha$-$H_{II}$ transition in phospholipids. Our results (Figs. 4 and 5 and Table 1) have been carried out with egg ceramides, the fatty acid of which is most frequently relatively short and saturated, e.g., palmitic acid. However, the unpublished data kindly provided by D. P. Siegel and J. Banschbach, some of which are also included in Table 1, were obtained with bovine brain ceramides, containing usually much longer fatty acids, yet the ceramide effect on the $L_\alpha$-$H_{II}$ transition is qualitatively and quantitatively very similar in both cases. Thus, the observations described in Figs. 4 and 5 can be considered as representative of the effects of naturally occurring ceramides in phospholipid systems.

The calorimetric data in Fig. 4 suggest the presence of two populations of lipids undergoing the $L_\alpha$-$H_{II}$ transition at different temperatures. The $^{31}$P-NMR data in Fig. 8 corroborate this interpretation, showing that within a certain range of temperatures, signals attributable to both lamellar and hexagonal structures coexist. The poor miscibility of DEPE

and ceramide, already discussed, could explain this situation of coexisting domains of different composition, each of them undergoing the $L_\alpha$-$H_{II}$ transition at its own temperature. However, the NMR hexagonal signal (and perhaps the DSC shoulder, or peak 2 in Fig. 4) may also be indicating the actual formation not of hexagonal phase structures but of their topologically related precursors, such as the aggregates of *trans*-monolayer contacts suggested by Siegel and Epand (1997). See in particular Figs. 6–8 of the latter paper.

As the ceramide content in the DEPE membranes is increased, the gel-fluid and lamellar-hexagonal transition temperatures approach each other. Although the data in the present paper are insufficient for constructing a detailed phase diagram of the PE-ceramide-water system, the calorimetric data in Fig. 1 indicate that at least some of the lipid remains in the lamellar gel phase below ~35°C even at $X_{cer} = 0.50$. IR observations confirm the same point for $X_{cer}$ up to 0.25. At these high ceramide concentrations the calorimetric signal becomes too broad to be detected; ceramide immiscibility may also occur under these conditions. However, the available experimental data may be compatible with a direct gel lamellar to fluid hexagonal phase transition at high ceramide concentrations, as observed in PE-diacylglycerol mixtures (Castresana et al., 1992; Basáñez et al., 1996).

Leikin et al. (1996) have measured, using x-ray diffraction and osmotic stress, the effects of diacylglycerol on the structural and elastic properties of dioleoylphosphatidylethanolamine monolayers in the $H_{II}$ phase of this phospholipid. These authors conclude that the diacylglycerol favors or induces hexagonal phase formation by reducing the intrinsic radius of monolayer curvature of the phospholipid. Because of the structural similarities, ceramides could also facilitate the $L_\alpha$-$H_{II}$ transition through a similar mechanism. Also by analogy with diacylglycerols, ceramides may favor the $L_\alpha$-$H_{II}$ transition by decreasing the hydration of the bilayer surface. Diacylglycerols are known to have this effect (López-García et al., 1993), and headgroup dehydration occurs during the lamellar-to-inverted hexagonal transition in PE (Castresana et al., 1992).

## Physiological implications

Ceramides are well known as inductors of apoptosis, or programmed cellular death, and as second messengers for various aspects of cell regulation (Kolesnick, 1989; Hannun and Obeid, 1995). The hydrophobic character of ceramides suggests that they may exert at least part of their actions at the membrane level. In situ production of these biomolecules by their synthetic enzymes may give rise to high local concentrations and perhaps to an asymmetric distribution of the enzyme products. This may in turn lead to local conditions, very different from the average membrane properties, in which the ceramide concentrations tested in our studies could certainly occur. Our data are relevant to at least two important biological phenomena, namely, membrane enzyme activation and membrane destabilization and fusion.

In several instances membrane enzyme activation has been related to the formation of lipid packing defects, such as those arising from lateral phase separations. This would be the case of phospholipase C (Basáñez et al., 1996a) or of protein kinase C (Dibble et al., 1996). The poor miscibility of ceramide and phospholipid, and subsequent coexistence of different domains (Fig. 2), will give rise to such packing defects; thus, ceramide could be modulating membrane-bound enzyme activities in this way. In fact, Huang et al. (1996) described by $^2$H-NMR the gel immiscibility of ceramide and phospholipid and proposed a relationship with the activation of phospholipase $A_2$.

Ceramides have also been found to induce certain forms of membrane destabilization, namely, leakage of aqueous solutes from vesicles (Ruiz-Argüello et al., 1996). In the in vivo situation, the ensuing ion fluxes might be responsible for important metabolic changes. Under certain conditions, ceramides have also been seen to induce fusion of liposomes (Basáñez et al., 1997). Cell membrane fusion is believed to occur through structural intermediates similar to those involved in the lamellar-to-nonlamellar (inverted hexagonal or inverted cubic) phase transitions (Siegel, 1993; Nieva et al., 1995; Siegel and Epand, 1997; Luzzati, 1997; Basáñez et al., 1998, and references therein). Thus, our observations in this paper that ceramides favor the $L_\alpha$-$H_{II}$ transition of phospholipids may be related to their role in facilitating bilayer fusion.

## Ceramides and diacylglycerols

The structural similarity between these two groups of compounds make almost compelling a comparative study of their membrane effects. The effects of diacylglycerols on the gel-fluid transitions of phospholipid bilayers have been extensively studied (Ortiz et al., 1988; Heimburg et al., 1992; López-García et al., 1994; Boeck and Zidovetski, 1989). Diacylglycerols give rise, as well as ceramides, to phenomena of immiscibility and phase separation in phospholipid bilayers. Both groups of compounds appear to be about equally immiscible with phospholipids in bilayers.

The influence of diacylglycerols on the $L_\alpha$-$H_{II}$ transition of phospholipids has also been the object of several studies. Particularly relevant are the DSC data by Epand and coworkers (Epand, 1985; Epand et al., 1988). They showed that diacylglycerols at small mole fractions lower the $T_h$ transition temperature of DEPE, the unsaturated ones being more potent. Similar findings were reported by Siegel et al. (1989). Our results with diacylglycerol (Figs. 6 and 7 and Table 1) are qualitatively similar to the thermogram of 2% 1-oleoyl-2-arachidonoyl-*sn*-glycerol in DOPE-Me shown by Siegel et al. (1989), and the quantitative effect on $T_h$ is also similar to the value given by Epand et al. (1988) for diolein. Das and Rand (1984, 1986), using x-ray diffraction,

showed that diacylglycerol induces a $L_\alpha$-$H_{II}$ transition in egg PE and that $T_h$ decreases with diacylglycerol concentration. These authors also show a phase diagram in which the lamellar and hexagonal phases are separated by a region of coexistence of $L_\alpha$ and $H_{II}$, just as observed in our system (Figs. 4, 5, and 8). More recently, Leikin et al. (1996) have explained the tendency of diacylglycerols to stabilize the $H_{II}$ phase by showing that the intrinsic radius of monolayer curvature is significantly reduced by those amphiphiles and that the bending modulus of hexagonal phase monolayers increases with increasing diacylglycerol content. Hexagonal phases have also been observed in the fully saturated phosphatidylcholine/diacylglycerol systems described by Heimburg et al. (1992) and López-García et al. (1994) but only at high temperatures and diglyceride concentrations. In general, the effects of ceramides on the $L_\alpha$-$H_{II}$ transitions are similar to those of diacylglycerols, although ceramides are clearly less potent. This is illustrated, e.g., by the data in Table 1.

In this context, a study was carried out in our laboratory (Ruiz-Argüello et al., 1996) to compare the abilities of diacylglycerols and ceramides in modifying the lamellar-to-nonlamellar (inverted cubic) transition of the lipid mixture egg PC:egg PE:cholesterol (2:1:1, mole ratio). Diacylglycerol had been found to decrease the transition temperature of the system (Nieva et al., 1995; Basáñez et al., 1996a,b). Ceramides also facilitate the transition, although they are less potent than diglycerides in this respect (Ruiz-Argüello et al., 1996). Thus, ceramides appear to be in general less able than diacylglycerols in the promotion of nonlamellar inverted lipidic phases. Ceramides and diacylglycerols have similar physical properties, but their chemical structure is rather different. The fact that their membrane effects differ from each other quantitatively rather than qualitatively suggests that the physical rather than the chemical properties of these compounds are responsible for the observed differences. If it is assumed that ceramides, diacylglycerols, and other nonpolar lipids may have a physiological role in the (transient) destabilization of the lamellar structures, the different potencies of the various lipid groups add a new possibility in the modulation of membrane structure and dynamics.

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