Intermolecular Interactions in Dry and Rehydrated Pure and Mixed Bilayers of Phosphatidylcholine and Digalactosyldiacylglycerol: A Fourier Transform Infrared Spectroscopy Study

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ABSTRACT Glycolipids are an important part of almost all biological membranes. Their effects on membrane structure and their interactions with phospholipids, however, have not been extensively studied so far. We have investigated the phase behavior and intermolecular interactions in dry and rehydrated bilayers made from the phospholipid egg phosphatidylcholine (EPC) and the plant chloroplast glycolipid digalactosyldiacylglycerol (DGDG), or from a mixture (1:1) of these lipids, using Fourier transform infrared spectroscopy. We show that there are extensive interactions between EPC and DGDG in mixed membranes, and also between DGDG molecules in pure DGDG membranes, involving sugar OH groups and C=O, P=O, and choline moieties in dry membranes. These interactions persist to a certain degree even after rehydration. We present evidence that these interactions influence the mixing behavior in phosphatidylcholine/DGDG membranes and also the phase behavior of both EPC/DGDG and pure DGDG membranes in the dry state.

INTRODUCTION

Glycolipids are present in all biological membranes. They are structurally divers, comprising glycerolipids, sterols, ceramides, and sphingolipids. Our knowledge of their exact physiological roles and their effects on the physical behavior of their host membranes is still incomplete. The sugar headgroups can give rise to intermolecular and interbilayer interactions that may be unique to glycolipids. For example, interactions between the sugar headgroups of glycosphingolipids may stabilize lipid rafts in membranes (Simons and Ikonen, 1997), although no simple correlation exists between headgroup structure and partitioning into rafts (Wang and Silvius, 2003). Also, in the myelin sheath around nerve cells, membrane stacking may at least in part be mediated by Ca^{2+}-induced interactions between galactosylceramide or glucosylceramide, and cerebroside sulfate (Stewart and Boggs, 1993; Koshy and Boggs, 1996; Koshy et al., 1999; Boggs et al., 2000). For plant chloroplast thylakoids, model membrane studies implicate digalactosyldiacylglycerol (DGDG) in such interactions (Webb et al., 1988; Menikh and Fragata, 1993; Hincha, 2003). DGDG is, together with monogalactosyldiacylglycerol, one of the major components of thylakoid membranes (Webb and Green, 1991; Lee, 2000). It is a bilayer-forming lipid that comprises ~30% of the total thylakoid lipid content.

The physiological role of DGDG in plant membranes has been investigated with the help of knockout mutants in the biosynthetic pathway leading to galactolipid formation (Dörmann and Benning, 2002). A mutant in *Arabidopsis thaliana* with a 90% reduction in DGDG content showed dramatic changes in the composition of the photosynthetic apparatus, suggesting an important role for this lipid in the structural organization of membrane protein complexes (Härtel et al., 1997). In addition, the import of nuclear encoded genes from the cytoplasm into the chloroplasts is severely inhibited in this mutant, indicating a crucial structural role of DGDG also for the chloroplast envelope membranes and their protein import machinery (Chen and Li, 1998).

In addition to their important physiological roles, glycolipids may also have interesting technical applications. It has, for instance, been shown that synthetic glycolipids can stabilize phospholipid liposomes during freeze-thawing (Goodrich and Baldeschwieler, 1991; Park and Huang, 1992a) or freeze-drying (Bendas et al., 1996), making glycolipids interesting potential stabilizers for liposome-encapsulated drugs. It has been suggested that these lipids exert their stabilizing activity in the frozen or dry state by interactions of their headgroups with the phospholipids, similar to the interactions of soluble sugars with phospholipids under such conditions (Goodrich et al., 1988, 1991; Park and Huang, 1992b; Testoff and Rudolph, 1992). However, detailed investigations into the interactions between phospholipids and glycolipids in the dry and hydrated states have not been published to date.

Here we present a comparative study of three types of liposomes, composed either of pure egg phosphatidylcholine (EPC), or pure DGDG, or a 1:1 mixture of EPC and DGDG, both in the dry and rehydrated state. We have used Fourier transform infrared spectroscopy (FTIR) to investigate the thermotropic phase behavior of the membranes and possible interactions between the lipid molecules. FTIR is uniquely suited for this type of investigation, as it does not rely on externally added probes that may influence membrane

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properties; it can be used on both dry and hydrated systems, and it gives information on different parts of the lipid molecules simultaneously (see Lewis and McElhaney, 1996; Brandenburg and Seydel, 1998; Lewis and McElhaney, 1998, for reviews). We have therefore been able to investigate lipid-lipid interactions at the levels of the carbonyl, the phosphate, and the choline groups, in addition to the fatty acyl chains.

MATERIALS AND METHODS

Materials

EPC, 1,2-dimyristoyl-*sn*-glycero-3-phosphatidylcholine (DMPC), 1,2-dimyristoyl(D54)-*sn*-glycero-3-phosphatidylcholine (D54DMPC), and 1-palmitoyl(D31)-2-oleoyl-*sn*-glycero-3-phosphatidylcholine (D31POPC) were obtained from Avanti Polar Lipids (Alabaster, AL). The chloroplast glycolipid DGDG was purchased from Lipid Products (Redhill, Surrey, UK). It is isolated from spinach leaves and contains mainly 18:3 fatty acids.

Liposome preparation

Liposomes were composed of either EPC, DGDG, or 50% EPC and 50% DGDG by weight. The lipids (10 mg) were dried from chloroform under a stream of N_2 and stored overnight under vacuum to remove traces of solvent. The lipids were hydrated in 200 μl of distilled water and extruded using a hand-held extruder (MacDonald et al., 1991) (LiposoFast, Avestin, Ottawa, Canada) with two layers of 100 nm pore filters. Fifty μl of the liposome suspension were spread on a CaF_2 window and dried at 0% relative humidity in a desiccator at 28°C for 48 h in the dark (Hincha et al., 2002). The absence of a water absorption band at 1650 cm^{-1} in the FTIR spectra indicates that samples were nearly anhydrous. For rehydration, the dry samples were stored in a closed container over distilled water and were allowed to absorb water from the vapor phase for 24 h at 28°C in the dark.

FTIR spectroscopy

Dry or rehydrated lipid samples were sandwiched between two CaF_2 windows and fixed in a vacuum chamber with windows, situated in the infrared beam (Hincha et al., 2002). Sample temperature was controlled by a coolant reservoir and an electrical heater (Specac Eurotherm, Worthington, UK) and monitored by a fine thermocouple attached to the surface of the window. The dry samples were cooled to −30°C by filling the coolant reservoir with liquid N_2. The rehydrated samples were cooled to 0°C by a mixture of NaCl and ice. In both cases, samples were equilibrated at the respective temperature for 10 min. Then the temperature was increased with a constant rate of 1°C/min. Infrared spectra were recorded from 4000 to 940 cm^{-1} with a PerkinElmer GX 2000 Fourier transform infrared spectrometer connected to a computer, equipped with the Spectrum 2000 software. After normalization of absorbance and baseline correction of the spectra by the interactive abex and flat routines (Tsvetkova et al., 1998), the peak frequencies of the symmetric CH_2 or CD_2 stretching, the asymmetric P=O stretching and the asymmetric N-$(CH_3)_3$ stretching peaks were determined by the software. The lipid melting temperature (T_m) was determined from the temperature-dependent changes in the peak frequencies of the symmetric CH_2 or CD_2 vibrations as the midpoint of the lipid melting curves (Crowe et al., 1997). Hydrogen bonding interactions in the carbonyl region of the lipid molecules were determined by analyzing the contours of the C=O stretching peak (1760–1700 cm^{-1}) before and after deconvolution. The deconvolution routine in the Spectrum 2000 software was used for enhanced peak resolution.

RESULTS

We used FTIR to investigate the structure of model lipid membranes, both in the dry and rehydrated state. Liposome solutions were dried for 48 h. After this time, under the conditions described under Materials and Methods, the samples were dry, as indicated by the absence of a water peak at 1650 cm^{-1} in the FTIR spectra (data not shown). In addition, further treatment of such samples for 24 h under vacuum did not result in any measurable changes in the FTIR spectra (data not shown). However, it has been shown that such samples can still contain two molecules of water per molecule of lipid, which would also be consistent with the observed position of the P=O vibration at 1250 cm^{-1} (Wong and Mantsch, 1988). For rehydration, dry samples were allowed to take up water from the vapor phase for 24 h. We did not determine the water content of the rehydrated samples. However, after rehydration for more than 24 h (up to 72 h), the FTIR spectra did not change anymore and the position of the P=O peak was the same as in freshly prepared liposome suspensions (data not shown). We therefore conclude that a saturating water content had been reached after 24 h under our experimental conditions.

Gel to liquid crystalline phase transitions and lipid mixing

The physical state of the hydrophobic interior of lipid bilayers was determined from the vibrational frequency of the symmetric CH_2 stretching mode at around 2850 cm^{-1}. As the lipid acyl chains melt and go from the gel to the liquid crystalline state, the position of this peak is shifted to higher wavenumbers (Mantsch and McElhaney, 1991).

Fig. 1 *A* shows the temperature dependence of the symmetric CH_2 stretching vibration between −20°C and 60°C of liposomes composed of pure EPC, of pure DGDG, or of a mixture of 50% EPC and 50% DGDG. The vibrational mode of the CH_2 groups of dry EPC liposomes was shifted from 2851.6 cm^{-1} in the gel state to 2853.7 cm^{-1} in the liquid crystalline state. A phase transition temperature (T_m) of 40°C was determined from these experiments, in good agreement with data published previously by us (Hincha et al., 2000, 2002) and others (Crowe et al., 1997) for this lipid.

In liposomes containing only DGDG, the symmetric CH_2 vibration was shifted to higher wavenumbers compared to EPC, from 2856 cm^{-1} to 2857 cm^{-1} between −20°C and 60°C. These high wavenumbers, the gradual increase in the vibrational mode of the symmetric CH_2 stretch with temperature, and the lack of a phase transition indicate that for the whole temperature interval, the lipid system was in the liquid crystalline state. The curve of the third type of liposomes, composed of 50% EPC and 50% DGDG, had an intermediate position between those of the pure lipids, and no phase transition was apparent. This indicates again that the lipids were in the liquid crystalline state.

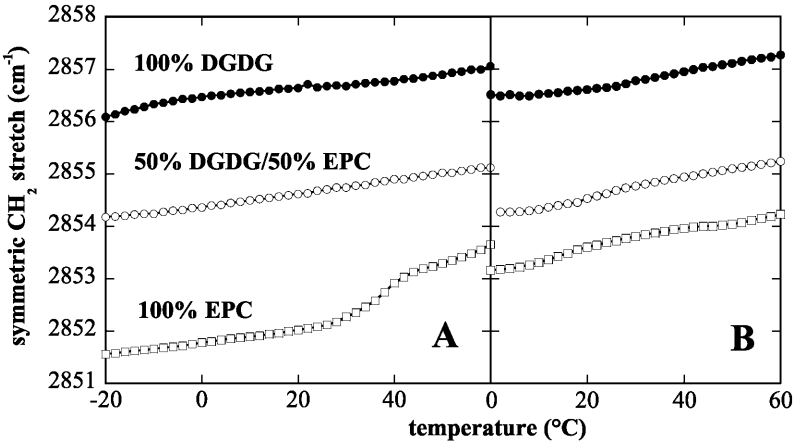

FIGURE 1 Temperature dependence of the symmetric CH_2 stretching band of fatty acyl chains of the three types of liposomes, studied in a dry (*A*) and in a rehydrated state (*B*). Only in the case of dry membranes made from pure EPC could a chain melting phase transition be determined, with a T_m of 40°C. For all other liposomes, the lipids were in the liquid crystalline state at all investigated temperatures.

The temperature dependence of the wavenumbers of the symmetric CH_2 stretching peaks for rehydrated samples are shown in Fig. 1 *B*. In this case, spectra were only taken between 0°C and 60°C, as we found that cooling the rehydrated samples to lower temperatures induced ice crystallization, which led to artifacts in the FTIR spectra (data not shown). With increasing temperature, the wavenumbers of the symmetric CH_2 stretching vibration increased gradually for all three types of liposomes, without an apparent phase transition. This, together with the high wavenumbers, indicates that after rehydration these lipid systems were all in the liquid crystalline state at all temperatures. As in the dry state, the acyl chains in pure EPC bilayers were in the most ordered state, and with increasing content of DGDG the disorder of the fatty acyl chains increased.

The lack of phase transitions in dry and rehydrated mixed membranes made from EPC and DGDG (Fig. 1) suggests that both lipids were well mixed in the bilayers. To determine the degree of mixing between phosphatidylcholines and DGDG, we used synthetic lipids with deuterated fatty acyl chains—D54DMPC and D31POPC. The higher mass of the CD_2 groups in the phospholipids in comparison to the CH_2 groups in DGDG shifts the infrared vibration to lower wavenumbers. Therefore, the phase behavior of both lipids in the mixture can be analyzed simultaneously (Mendelsohn and Moore, 1998). DMPC is a short chain (C14), fully saturated lipid, whereas POPC contains one saturated (*sn*-1 C16) and one monounsaturated (*sn*-2 C18) fatty acid. EPC contains ~60% POPC (Avanti Polar Lipids product information). In D54DMPC, both fatty acyl chains are perdeuterated, whereas in D31POPC only the *sn*-1 palmitoyl chain is perdeuterated. DGDG contains mainly C18 fatty acids with three double bonds (Quinn and Williams, 1983; Klaus et al., 2002). Since it had already been reported that DMPC and DGDG are well mixed under fully hydrated conditions (Tomczak et al., 2002), we only investigated lipid mixing in dry membranes. The T_m of pure DMPC in the dry state was 89°C (data not shown), whereas the T_m of dry POPC is 61°C (Koster et al., 1994).

Three different binary lipid mixtures, EPC/D54DMPC (Fig. 2 *A*), DGDG/D54DMPC (Fig. 2 *B*), and DGDG/D31POPC (Fig. 2 *C*) were investigated. We included mixed bilayers of EPC and DMPC in this study, to facilitate a comparison between the miscibility of two phospholipids, and of a phospholipid and a glycolipid. Complete demixing would result in two phase transitions at the temperatures of the pure lipids (40°C and 89°C for EPC and DMPC, respectively), whereas complete mixing would result in a single phase transition, situated between those of the two lipids. It can be clearly seen that the saturated short-chain DMPC and the monounsaturated longer-chain EPC were partially demixed during their phase transitions, with phase transition temperatures of 70°C and 53°C, respectively.

The liposomes composed of 50% DGDG and 50% D54DMPC showed a similar tendency (Fig. 2 *B*). However, in this case the T_m of DMPC is decreased to 47°C. DGDG, which by itself is in the liquid crystalline state over the whole temperature range (Fig. 1 *A*), showed a T_m of 44°C in the mixed bilayer. This indicates that the degree of mixing between DGDG and D54DMPC in the dry state is nearly complete and certainly more pronounced than between EPC and DMPC.

The third binary lipid mixture contained 50% DGDG and 50% D31POPC. Fig. 2 *C* shows that with increasing temperature, the wavenumbers of both the CH_2 and CD_2 vibrations increased gradually without an apparent phase transition. Both lipids were in the liquid crystalline state, and the curves are very similar to those from membranes composed of 50% EPC and 50% DGDG (Fig. 1 *A*). From these data we conclude that galactolipids and phospholipids in dry mixed bilayers made from DGDG and POPC, and from DGDG and EPC, are well mixed under our experimental conditions.

Effects of lipid composition and hydration on the lipid C=O moiety

The difference in lipid mixing between samples containing DMPC and EPC, or DMPC and DGDG (Fig. 2, *A* and *B*),

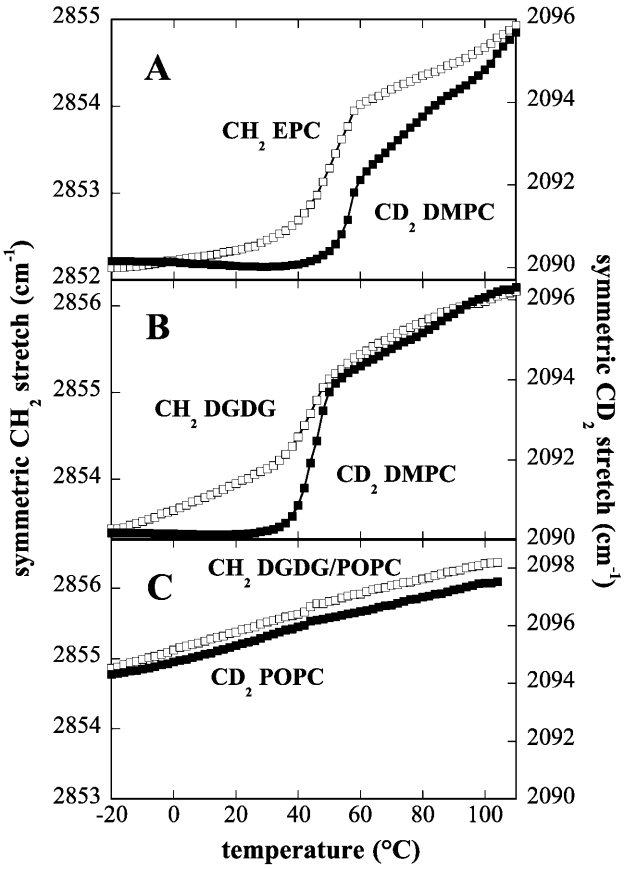

FIGURE 2 Melting curves of dry liposomes prepared from a 1:1 mixture of lipids containing either CH_2 or CD_2 in their fatty acyl chains. (*A*) 50% EPC/50% D54DMPC; (*B*) 50% DGDG/50% D54DMPC; and (*C*) 50% DGDG/50% D31POPC. The D31POPC contains only one deuterated fatty acyl chain. Therefore, the CH_2 frequencies reported in *C* result from the two acyl chains of DGDG and the *sn*-2 oleoyl chains of POPC, whereas the CD_2 frequencies result exclusively from the perdeuterated *sn*-1 palmitoyl chains of POPC. Phase transition temperatures (T_m) were determined as the midpoint of each melting curve. (*A*) EPC, $T_m = 53°C$; DMPC, $T_m = 70°C$. (*B*) DGDG, $T_m = 44°C$; DMPC, $T_m = 47°C$. (*C*) Curves indicate that the lipids are in the liquid crystalline state at all investigated temperatures.

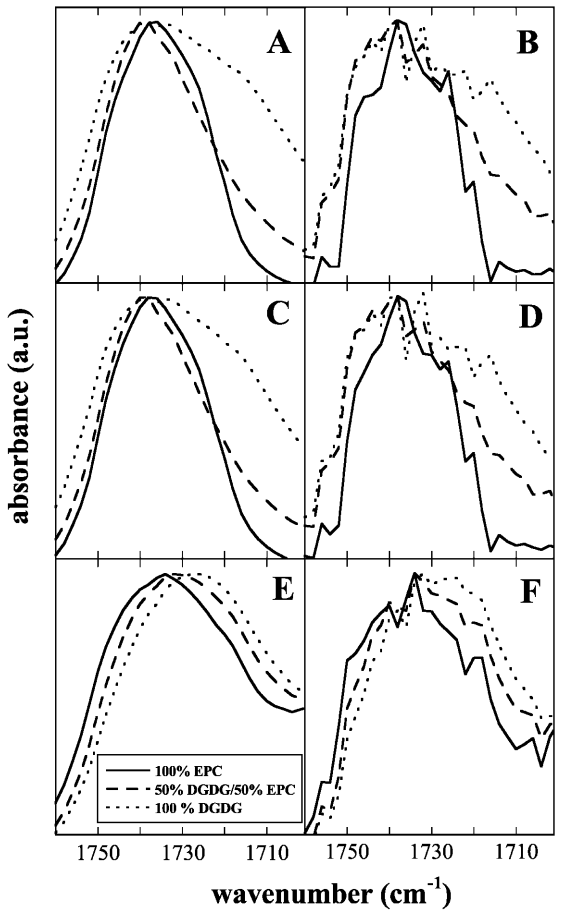

FIGURE 3 Infrared spectra in the carbonyl stretching region. Normalized C=O stretching band contours (*A*, *C*, and *E*) and contours after resolution enhancement through deconvolution (*B*, *D*, and *F*) of FTIR spectra of liposomes composed of 100% EPC, 50% DGDG/50% EPC, or 100% DGDG. (*A* and *B*) Dry liposomes at −20°C. (*C* and *D*) Dry liposomes at 60°C. (*E* and *F*) Rehydrated liposomes at 60°C.

suggests the involvement of interactions between the headgroups of glycolipids and phospholipids that may stabilize mixed bilayers. Such interactions were first investigated at the level of the carbonyl groups, which are situated at the interface between the hydrophobic hydrocarbon chains and the more hydrophilic headgroup region. The C=O band in FTIR spectra of diacyl lipids is split into at least two bands, with the upfield peak due to nonhydrogen bonded C=O groups and the downfield peaks due to hydrogen-bonded C=O groups (Blume et al., 1988; Lewis and McElhaney, 1996).

At −20°C, where dry EPC liposomes are in the gel phase (Fig. 1 *A*), the absorbance contour of the C=O vibration shows a well-defined peak with a maximum at 1738 cm^{-1} and a slight shoulder at lower wavenumbers (Fig. 3 *A*), which becomes clearly visible as a peak after deconvolution (Fig. 3 *B*). In the liquid crystalline state (60°C, Fig. 3 *C*), the peak position was not changed, but the peak became slightly more narrow, indicating a more homogeneous population of C=O groups after chain melting. The low-field peak was no longer resolved by deconvolution (Fig. 3 *D*). This may indicate hydrogen bonding between lipids and residual H_2O molecules in the more condensed bilayers at low temperatures, which is reduced at higher temperatures due to increased lipid spacing (see also Discussion). In liposomes containing 50% or 100% DGDG, no such difference between spectra at −20°C and 60°C was visible, in accordance with the fact that these bilayers did not show a phase transition (Fig. 1 *A*).

In liposomes containing only DGDG, the C=O peak was much broader, with a clear shoulder at ~1720 cm^{-1}, indicating extensive hydrogen bonding between the carbonyl esters and the OH groups of the galactose moieties. It is interesting to note that there was no indication of H-bonding between sugar OH groups and C=O groups in membranes

containing 50% EPC and 50% DGDG. We attribute this to enhanced H-bonding of the sugars to P=O groups in EPC, as discussed below.

On rehydration of the liposomes, the C=O vibrations are represented by a single broad absorbance peak (Fig. 3 *E*), which has been shown to encompass at least two peaks (Fig. 3 *F*), corresponding to hydrogen-bonded and free carbonyl groups (Blume et al., 1988). The maximum for EPC liposomes is centered at 1734 cm^{-1}, a downfield shift by four wavenumbers compared to the dry membranes, which is consistent with the increase of hydration in the interfacial region. Addition of 50% DGDG shifts the position of the maximum downfield by another 2 cm^{-1}. For rehydrated liposomes containing only DGDG, the C=O peak is located at 1726 cm^{-1}. This shift in peak position is due to a relative increase in the absorbance of C=O groups at lower wavenumbers (Fig. 3 *F*) These changes in the C=O band are consistent with an increase in hydrogen bonding both due to the presence of water and in addition to the sugar headgroups and indicate H-bonding interactions between C=O and sugar OH groups in the dry and hydrated state.

The phosphatidylcholine headgroup region: interactions of the P=O and choline groups

A vibrational mode that can be used to obtain structural information about the interfacial region of the bilayer is the asymmetrical stretching mode of P=O (Fig. 4). The peak position is sensitive to the formation of H-bonds, shifting to lower frequencies with increased H-bonding (Wong and Mantsch, 1988; Lewis and McElhaney, 1998). The temperature dependence of the asymmetric P=O stretching vibration in EPC and in EPC/DGDG membranes in the dry and rehydrated state is shown in Fig. 5. With increasing temperature, a gradual shift to higher frequencies was observed in all cases. However, the phase transition in dry EPC at 40°C (Fig. 1) was not reflected in the position of the P=O band, in accordance with published observations (Lewis and McElhaney, 1998).

As expected, rehydration had a dramatic effect on the position of the P=O peak in EPC membranes (Crowe et al., 1988; Hübner and Blume, 1998). The value in the rehydrated state was nearly 20 cm^{-1} lower than in the dry state (Fig. 4). For the liposomes containing 50% DGDG, the decrease in wavenumber on hydration was only 10 cm^{-1}. A contribution of the DGDG molecules to H-bonding to the P=O groups of EPC is strongly indicated by the fact that in the dry state the P=O peak is shifted by 14 wavenumbers downfield. This shift is still evident in the hydrated samples, although it is reduced to ~4 wavenumbers.

The terminal part of the PC molecule is the choline group. It has a characteristic asymmetric stretching vibration around 970 cm^{-1} that is sensitive to dipolar interactions, e.g., with water (Akutsu, 1981) (Fig. 6). The temperature dependence of the frequency of the $^{+}N(CH_3)_3$ asymmetric stretching vibration is shown in Fig. 7. In the dry state (Fig. 7 *A*), there is a difference of ~6 cm^{-1} in the position of the peak between EPC liposomes and those containing 50% EPC/50% DGDG, indicating an interaction between EPC and DGDG also at the level of the choline moiety. With increasing temperature, the choline maximum is shifted downfield in both types of membrane. After rehydration (Fig. 7 *B*), the position of the choline peak is shifted upfield for both types of membrane, indicating an interaction of water molecules with the surface-exposed choline group (Akutsu, 1981). However, the shift in the position of the choline band due to rehydration was bigger in membranes made from pure EPC than in membranes containing DGDG, even in the presence of water, analogous to the situation found for the P=O group (Fig. 5).

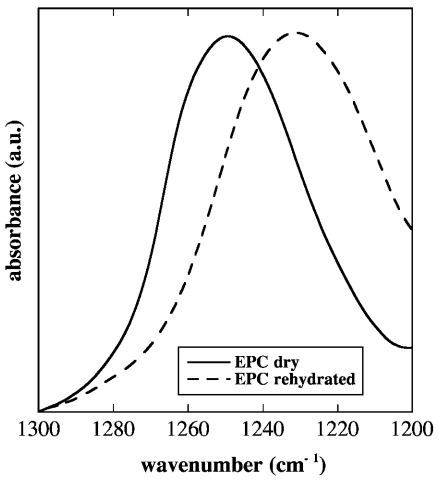

FIGURE 4 Infrared spectra in the asymmetric P=O stretching region. Normalized P=O stretching band contours of FTIR spectra of dry and rehydrated liposomes composed of 100% EPC at 60°C. The solid line represents a dry sample and the dashed line a rehydrated sample.

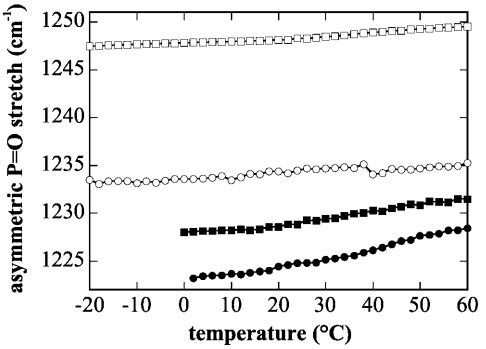

FIGURE 5 Temperature dependence of the P=O asymmetric stretching frequency of dry (*open symbols*) and rehydrated (*solid symbols*) liposomes. Liposomes were composed either of pure EPC (*squares*) or 50% EPC/50% DGDG (*circles*).

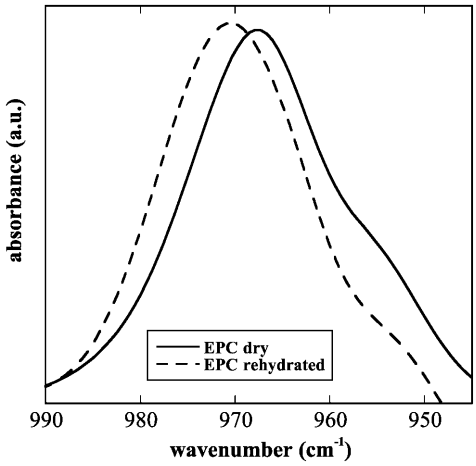

FIGURE 6 Infrared spectra in the asymmetric N-$(CH_3)_3$ stretching region. Normalized N-$(CH_3)_3$ stretching band contours of FTIR spectra of liposomes composed of 100% EPC at 60°C. The solid line represents a dry sample and the dashed line a rehydrated sample.

DISCUSSION

Phase transitions and lipid mixing

The most often-used spectral band for determining lipid hydrocarbon chain order is the symmetric CH_2 stretching mode at 2850 cm^{-1}. The frequency of this band is sensitive to acyl chain conformation. It responds to changes in the ratio of *trans*/*gauche* rotamers of the fatty acyl chains of model and natural membrane systems (Casal and Mantsch, 1984; Mantsch and McElhaney, 1991). In the gel phase, the fatty acyl chains are tightly packed in all-*trans* conformation. With increasing temperature through T_m, the hydrocarbon chains increase their conformational disorder, and *gauche* rotamer formation takes place. This transition from the gel to the liquid crystalline state is accompanied by an increase in the frequency of the CH_2 symmetric stretching band by ~1.5–2.5 cm^{-1}. The magnitude of this increase is dependent not only on the type, length, and degree of unsaturation of the hydrocarbon chains, but also on the nature of the lipid polar headgroup (Casal and Mantsch, 1984).

Pure EPC liposomes in the dry state undergo a transition from the gel to the liquid-crystalline phase at 40°C (Fig. 1 *A*), but at 60°C, the peak wavenumber was still below 2854 cm^{-1}. Dry liposomes containing only DGDG had much more disordered fatty acyl chains than EPC, as indicated by the higher wavenumbers of the CH_2 band (2856–2857 cm^{-1}). The DGDG bilayers were in the liquid crystalline state over the whole temperature region investigated. A characteristic feature of DGDG is the high degree of unsaturation of its fatty acyl chains. The predominantly found fatty acid residue is 18:3 (Shipley et al., 1973; Foley et al., 1988; Klaus et al., 2002). By differential scanning calorimetry, it was estimated that for the hydrated lipid the transition temperature is ~−50°C (Shipley et al., 1973). Our data indicate that in the dry state, T_m of DGDG must still be well below −20°C. Liposomes composed of 50% EPC and 50% DGDG showed lipid order intermediate between the pure bilayers. There was no phase transition apparent, which indicates that the liposomes were again in the liquid crystalline state at all investigated temperatures (Fig. 1 *A*). We will discuss evidence below, indicating that the high fluidity and low T_m of membranes containing DGDG is not only the result of the high degree of unsaturation of the fatty acids in DGDG, but is also a consequence of strong intermolecular interactions.

An assumption underlying our discussion of the phase behavior of EPC/DGDG bilayers is that both lipids are well mixed and do not phase separate even during desiccation. We performed experiments with perdeuterated PCs to test this assumption (Fig. 2). We found that EPC and D54DMPC were not completely mixed, whereas DGDG formed almost uniform bilayers with D54DMPC and D31POPC. Since POPC is the major constituent of EPC, we conclude from these and the results in Fig. 1 *A* that EPC/DGDG bilayers are indeed well mixed in the dry state. In addition, these results indicate that mixtures of PC and DGDG are less prone to phase separation than mixtures of different PCs with widely

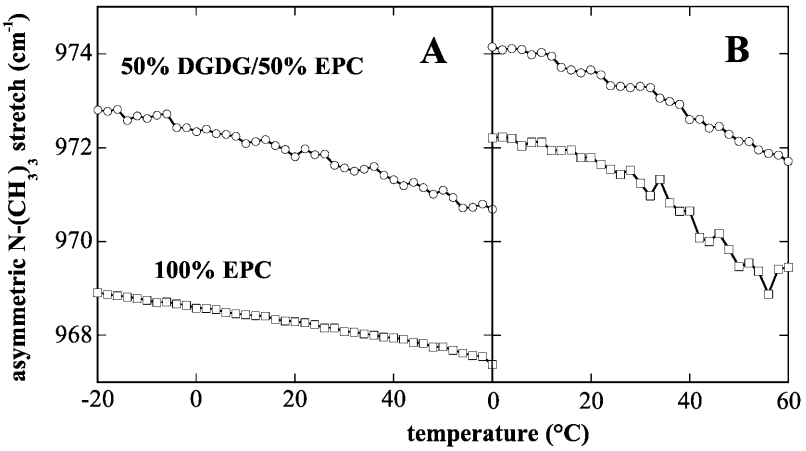

FIGURE 7 Temperature dependence of the infrared frequency of the N-$(CH_3)_3$ asymmetric stretching peak of (*A*) dry and (*B*) rehydrated liposomes.

different T_m (e.g., EPC and DMPC). Although DGDG is much more unsaturated than EPC, it showed substantially less phase separation from DMPC. This indicates that headgroup interactions play a dominant role in the physical behavior of DGDG-containing membranes and probably also in membranes containing other glycolipids.

When the three types of liposomes were allowed to absorb water, the temperature dependence of the symmetric CH_2 stretching band indicated that both lipids were in the liquid crystalline state (Fig. 1 *B*), as expected from their published phase behavior (Foley et al., 1988; Crowe et al., 1994). Lipid mixing was not investigated in this case. It has been shown that DGDG and DMPC are well mixed in hydrated membranes (Tomczak et al., 2002) and the same can be assumed for DGDG and EPC.

Interactions between EPC headgroups in the dry state

The C=O vibration band in the FTIR spectra of diacyl lipids is composed of at least two bands. The position and intensity of these bands depend on the degree of hydrogen bonding to the C=O groups (Blume et al., 1988). In pure EPC in the dry state, we observed a maximum at 1738 cm^{-1} and a shoulder at lower wavenumbers (Fig. 3, *A* and *B*) in the gel state lipid. In the liquid crystalline phase, the upfield peak remained unchanged, but the shoulder was reduced (Fig. 3, *C* and *D*). The position of the C=O peak in anhydrous lipid in CCl_4 has been shown to be at 1742 cm^{-1} (Blume et al., 1988). Such a peak could be identified after deconvolution in all spectra, even in the presence of water (Fig. 3 *F*). However, our data indicate some H-bonding between EPC molecules and residual H_2O molecules in the dry state that is enhanced when the lipids are tightly packed and reduced when they move apart after chain melting (Fig. 3, *B* and *D*). In addition, there could be Coulombic interactions between the choline and phosphate groups of different EPC molecules. There are several lines of evidence in the literature that argue in favor of such interactions. It has been shown that the PC headgroup is oriented almost parallel to the membrane surface (Büldt et al., 1979) in the hydrated state. Molecular dynamics simulations indicate that this bending toward the surface increases during dehydration (Mashl et al., 2001), which would make such interactions more likely. Interactions between the choline and the phosphate group in hydrated membranes have been shown by NMR (Yeagle et al., 1975, 1976, 1977) and similar interactions have been suggested between the choline and both the P=O and C=O groups by molecular dynamics simulations (Pasenkiewicz-Gierula et al., 1999).

The antisymmetric P=O stretching band monitors the polarity and hydration state of the lipid polar headgroup region. On formation of hydrogen bonds, the position of the absorbance band is shifted to lower frequencies (Fig. 4). In a nonbonded (anhydrous) state, the frequency in DMPC is found at ~1260 cm^{-1}, whereas in a fully hydrated state it is situated at ~1220 cm^{-1} (Wong and Mantsch, 1988). The frequency is ~1230 cm^{-1} for EPC (Fig. 5) and this band position is not changed when the liposomes are suspended in the original volume (50 μl) of water (data not shown). The temperature dependence of the P=O peak position in dry liposomes composed of pure EPC showed no effect of the gel to liquid crystalline phase transition. However, there was a small increase in the wavenumber by 2 cm^{-1} (Fig. 5), indicating disturbance of H-bonds or Coulombic interactions (see above). The same is also indicated by the gradual downfield shift of the choline band (Fig. 7) with temperature.

Interactions between EPC and DGDG in the dry state

In the case of liposomes containing 50% EPC and 50% DGDG, there was evidence for interactions between the two lipids at the levels of the P=O and choline groups. The influence on the C=O vibrations, however, was marginal (Fig. 3, *A–D*). The P=O peak (Fig. 5) was shifted by 14 cm^{-1}, indicating strong hydrogen bonding interactions between EPC and DGDG (DGDG does not contain phosphate). This shift is almost as big as that induced by the addition of water to the system. Similarly, the choline peak is shifted by 4 cm^{-1} in the presence of DGDG, the same amount as the shift after rehydration (Figs. 6 and 7).

This strong interaction between the headgroups is probably facilitated by the fact that the DGDG headgroup, similar to the EPC headgroup, is oriented parallel to the membrane surface (McDaniel, 1988). Although there is no information in the literature about interactions between phospholipids and DGDG, or about the effects of such interactions on the orientation of the headgroups, there are reports about such interactions between phospholipids and synthetic glycolipids (Goodrich et al., 1988, 1991; Park and Huang, 1992b; Testoff and Rudolph, 1992). All these studies show clear evidence for H-bonding interactions, similar to those described above. Interestingly, the same shift in the P=O vibration is also seen, when PC membranes are dried in the presence of soluble sugars, such as sucrose or trehalose (Tsvetkova et al., 1998). Both soluble (see Oliver et al., 2002, for a recent review) and membrane-bound carbohydrates (Goodrich et al., 1988, 1991; Park and Huang, 1992b; Testoff and Rudolph, 1992) reduce the T_m of dry PC membranes. It is therefore suggested that the low T_m of dry EPC/DGDG membranes (below −20°C) is not only due to the high degree of unsaturation of the fatty acid chains in DGDG, but also to headgroup interactions between the galactose units in DGDG and the P=O and choline groups in EPC. These interactions could lead to an increased spacing of the lipids in the dry state, as suggested in the ''water replacement hypothesis'' for soluble sugars and dry membranes (Oliver et al., 2002).

Interactions between DGDG headgroups in the dry state

The IR vibrations attributable to the galactose in the DGDG headgroups are only poorly characterized and have therefore not been included in our analysis. The formation of H-bonds between DGDG molecules is clearly evident at the level of the C=O band (Fig. 3, *B* and *D*), where two low-field peaks can be distinguished in addition to the high-field peaks present in all lipids. In analogy to our argument above for the EPC/DGDG membranes, we would suggest that this intermolecular hydrogen bonding contributes to the low T_m of dry DGDG membranes (below −20°C). H-bonding to the C=O groups in pure DGDG membranes may be stronger than in mixed EPC/DGDG membranes, because DGDG does not contain P=O groups, which are situated above the C=O groups in EPC and may therefore limit the accessibility of the C=O for sugar OH groups.

Effects of rehydration on lipid headgroups

As expected, the C=O peaks in all three types of liposomes were shifted to lower wavenumbers (Fig. 3), indicating H-bonding of water at the solution-membrane interface. The same was also true for the P=O peak, which was shifted downfield by almost 20 wavenumbers in pure EPC membranes and by ~10 cm^{-1} in EPC/DGDG membranes (Fig. 5) and for the choline peak, which was shifted upfield in both types of membranes (Fig. 7). In all cases, the spectral shifts induced by rehydration were in the same direction and of the same magnitude as those induced in EPC membranes by the addition of DGDG. This fact again emphasizes the strong interactions between these two lipids in the dry state.

The data in Figs. 3–7 also provide evidence that interactions with water and DGDG sugar headgroups are additive and not competitive. In the case of the C=O group, the maximum is situated at 1734 cm^{-1} in the case of pure EPC. In the case of rehydrated liposomes made of 50% EPC and 50% DGDG, rehydration results in shifting the peak downfield by an additional 2 cm^{-1}, whereas for rehydrated liposomes composed only of DGDG, the peak is centered at 1726 cm^{-1}. Similarly, the P=O peak in rehydrated EPC/DGDG liposomes is situated ~5 wavenumbers downfield from that of rehydrated EPC vesicles, whereas the choline peak is situated 2 cm^{-1} more upfield. This indicates that interactions with DGDG may occupy sites that are not available for water. This is different from the interactions of soluble sugars with lipid headgroups, as soluble sugars are replaced by water already at very low levels of hydration (Nagase et al., 1997).

The magnitude of the shift in the P=O peak position in EPC membranes upon rehydration is obviously much bigger than that in EPC/DGDG membranes (Fig. 5). This indicates that the extent of hydration in EPC liposomes is higher than in EPC/DGDG membranes. This is in good agreement with reports about the low degree of hydration of glycolipid headgroups in general (Wieslander et al., 1978; Baba et al., 2001), which has been interpreted as evidence for strong interlipid H-bonding, leading to a partial exclusion of water from the solute-membrane interface.

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