Formation of a new stable phase of phosphatidylglycerols

Richard M. Epand,* Brent Gabel,* Raquel F. Epand,* Arindam Sen,‡ Sek Wen Hui,‡
Arturo Muga,§ and Witold K. Surewicz§

*Department of Biochemistry, McMaster University Health Sciences Centre, Hamilton, Ontario, Canada, L8N 3Z5;
‡Department of Biophysics, Roswell Park Memorial Institute, Buffalo, New York 14263 USA; and §Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, K1A 0R6

ABSTRACT Dilauroyl and dimyristoylphosphatidylglycerol (DMPG) form a more stable gel state when aqueous suspensions are incubated several days at low temperature (0–2°C), pH 7.4 with 0.15 M NaCl. This gel state is characterized by a higher transition temperature and a higher transition enthalpy. The geometry of this gel state is distinguishable from the metastable gel state that forms rapidly upon hydration on the basis of its x-ray diffraction pattern. Infrared spectra in the CH_2 scissoring region indicate that the stable gel phase of DMPG is also characterized by reduced reorientational fluctuations of acyl chains and increased interchain interactions. Analysis of vibrational bands due to ester carbonyl groups of DMPG suggests that the transition to a new gel phase is initiated by changes in the interfacial and/or headgroup region of the bilayer, most likely via formation of interlipid hydrogen bonds. The melting of the stable gel phase of DMPG is accompanied by a gross morphological change resulting in vesiculation.

INTRODUCTION

Phosphatidylglycerols containing saturated fatty acids have been extensively used in studies of model membranes. Interest in their use in model systems comes, in part, because they are anionic lipids that, in freshly prepared suspensions at pH 7 and with 0.15 M NaCl, have almost identical phase transition properties as zwitterionic phosphatidylcholines, with the same length fatty acid chains. Thus, one can assess the role of membrane charge on the interactions of substances with membranes by comparing model membranes composed of phosphatidylcholine versus phosphatidylglycerol. Phosphatidylcholines, however, are also known to form a more stable phase after prolonged incubation at low temperature (Chen et al., 1980). No comparable phenomenon has been shown for anionic lipids. In this paper, we demonstrate that phosphatidylglycerols form a different type of stable phase and that the melting of this phase is accompanied by an unusual morphological change.

MATERIALS AND METHODS

Materials. Dilauroyl (12:0), dimyristoyl (14:0), dipalmitoyl (16:0), distearoyl (18:0), and dioleoyl (18:1) phosphatidylglycerols were purchased from Avanti Polar Lipids (Pelham, AL). They will be referred to as DLPG,[1] DMPG, DPPG, DSPG, and DOPG, respectively. All these lipids showed a single spot on TLC.

Sample preparation. The phospholipid was dissolved in chloroform/methanol, 2/1 (vol/vol). Lipid films were formed on the walls of a glass test tube by evaporating the solvent with a stream of nitrogen. Final traces of solvent were removed in a vacuum desiccator kept at 40° for 90 min and using a liquid nitrogen trap. The dry lipid film was then suspended in 20 mM Pipes, 1 mM EDTA, 150 mM NaCl with 0.002% NaN_3 at pH 7.4 by vigorous vortexing at 45°C. For some experiments the buffer was made up in ^2H_2O (D_2O) instead of H_2O. The pH of this buffer was also adjusted to a pH meter reading of 7.40. The lipid suspensions at a concentration of 0.3 mg/ml were then incubated in an ice bath. For complete conversion to the stable phase, it was found important to keep the lipid suspension in an ice bath, rather than simply storing them in a refrigerator at ~4°C.

Samples for x-ray diffraction were prepared as described above, except that 2 mM histidine and 2 mM TES were used instead of 20 mM Pipes in the buffer solution. The lipid suspensions were concentrated by centrifugation in an Eppendorf centrifuge to loose pellets, which were then loaded into 1.5-mm diameter thin wall glass tubes (Charles Supper, Cambridge, MA). Data collection started either immediately after loading, or after the samples were placed in an ice bath at 2°C for a given period.

Samples for infrared spectroscopy were prepared essentially as described above, except that the buffer was 50 mM Hepes, 100 mM NaCl, pH 7.4, and the lipid concentration was 10 mg/ml. The buffer was made up in H_2O (for studies of the methylene scissoring mode) or in D_2O (for studies of the carbonyl stretching mode). These samples were immediately placed between two calcium fluoride windows (separated by a 25 or 50 μm thick Teflon spacer) and mounted in a thermostated compartment of the infrared spectrometer that was preequilibrated at 2°C.

Differential scanning calorimetry (DSC). Lipid suspensions and buffer were degassed on ice under vacuum and then loaded into the sample and reference cell, respectively, of a MicroCal MC-2 high sensitivity scanning calorimeter (Amherst, MA) that had been precooled to 0°C. Heating scans were run at 39K/h. The data were acquired on an IBM-PC and analyzed using software provided by MicroCal.

X-ray diffraction. X-ray diffraction data were obtained by Frank-type cameras mounted on a Rigaku H300 rotating anode generator (Danvers, MA), using a copper target. The patterns were recorded on Kodak DEF x-ray film. Due to the poor packing and lamellar ordering of the PG samples, the reflections are weak and broad. Data collection usually requires over 10 h exposure for reasonable signal-to-noise ratio. Samples were transferred and placed in a thermostatic holder throughout data collection.

Freeze fraction electron microscopy. Hydrated lipid samples (0.1 μl) were sandwiched between thin copper sheets, and frozen by rapidly plunging into liquid propane, maintained at liquid N_2 temperature (Boni and Hui, 1983). Freeze fracture replicas were prepared using a modified Polaron freeze fracture instrument at a temperature of −120°C and to 10^{-7} Torr vacuum. Replicas were floated off on dilute nitric acid and after washing with water were placed on grids. The

Arturo Muga's permanent address is Department of Biochemistry, University of Basque Country, Bilbao, Spain.

Address correspondence to Dr. R. Epand.

[1] Abbreviations used: DL, dilauroyl; DM, dimyristoyl; DO, dioleoyl; DP, dipalmitoyl; DS, distearoyl; PG, phosphatidylglycerol; D_2O, ^2H_2O; DSC, differential scanning calorimetry.

0006-3495/92/08/327/06 $2.00

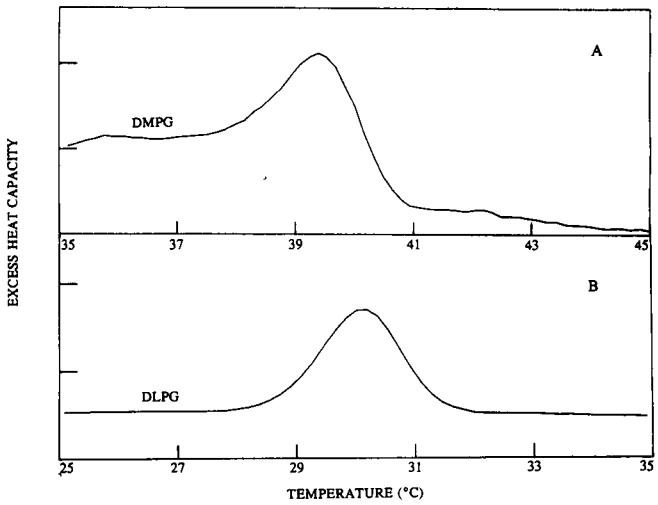

FIGURE 1 DSC heating scans of a suspension of DMPG (*A*) or DLPG (*B*) that had been incubated 3 d at 0° in Pipes buffer. No transitions are observed above 10° or 25° for freshly prepared suspensions of DLPG or DMPG, respectively. Each tick mark on the verticle axis represents 5 kcal/K/mol.

replicas were then washed with chloroform:methanol 2:1, and were examined in a Hitachi H600 electron microscope (Tokyo) operating at 100 kV.

Fourier-transform infrared spectroscopy. Infrared spectra were recorded at ~2°C with a Digilab FTS-60 or Nicolet 520 instrument. The temperature was controlled by the computer and was stable within 0.5°C. For each spectrum, 100 interferograms were co-added and Fourier transformed to give a resolution of 2 cm^{-1}. To eliminate spectral contributions of atmospheric water vapor, the instrument was continuously purged with dry nitrogen. The frequencies of infrared bands were determined by use of a center of gravity algorithm (Cameron et al., 1982). Overlapping bands were resolved by using Fourier self-deconvolution procedures (Kauppinen et al., 1981).

RESULTS

Differential scanning calorimetry. Incubation of a suspension of DLPG or DMPG at 0° for a period of 20–30 h results in the formation of a phase that has a higher transition temperature and enthalpy than freshly prepared samples (Fig. 1 and Table 1). The formation of this new phase occurs with a half-time of ~10–15 h. The rate of formation does not follow simple first order kinetics but exhibits an initial lag phase, the duration of which is somewhat variable among different samples. The formation of the higher melting phase in DMPG is accompanied by a loss of the 23° transition. After scanning an aged sample of DMPG to 50°, cooling to 0° and rescanning the same sample, the 39.6° transition is lost and the 23° transition reappears as for a fresh sample. In the case of DLPG the 29.9° transition is lost on rescanning. This behavior represents the slow formation of a more stable phase that occurs upon incubation of a lipid suspension in the gel state. This phenomenon is not observed with DPPG or DSPG, and with DOPG no transition is observed above 0° even after 4 wk of incubation at 0°.

X-ray diffraction. DMPG samples show only a single, broad reflection at the small angle region. The spacing corresponds to 47.5 Å. The broadness of the reflection indicates that the lamellar ordering is poor. Freshly prepared samples show a moderately sharp wide angle reflection at 4.2 Å, typical of a metastable gel phase such as that of phosphatidylcholine. During a 16-h incubation at 2°C, a new set of wide angle reflections begin to appear. The spacings of these reflections are 4.5, 4.15, 4.0, and 3.85 Å, with the 4.15 Å reflection being the strongest (Fig. 2). It is not possible to determine if the 4.2 Å reflection has disappeared or has merged with the 4.15 Å reflection after incubation. The set of reflections resembles that of the Lc phase of phosphatidylcholine. However, the number of reflections recorded is not sufficient to unambiguously determine the structure of the hydrocarbon packing subcell. This pattern remains after the samples were placed at 2°C for more than 60 h. Samples kept at 5°C do not show this series of wide angle reflections after 20 h. The x-ray pattern corresponding to the stable gel state, formed after low temperature incubation, is maintained to higher temperatures. Subsequent to melting of the gel state at 30° or 40° for DLPG or DMPG, respectively, the sample no longer shows a wide angle x-ray diffraction pattern.

Freeze fracture electron microscopy. Freeze fracture electron microscopy shows flat sheets for aged DMPG at low temperature (Fig. 3 *A*). At 50°, the lipid spontaneously vesiculates (Fig. 3 *B*) and on cooling down to 22°, ripple formation, characteristic of the P'_β phase is observed (Fig. 3 *C*). The size of the vesicles is not uniform but they are generally larger than some of the vesicles and fragments of DMPG that form at low ionic strength (Epand and Hui, 1986). All specimens in this study contain 0.15 M NaCl and do not show loss of multilayer periodicity unless the sample is aged at low temperature and then heated above the gel to liquid crystalline transition temperature.

Fourier-transform infrared spectroscopy. Fig. 4 shows the time dependence of the methylene scissoring region

TABLE 1 Shift in the thermotropic transitions of phosphatidylglycerol as a result of incubation at 0°

	Fresh		Aged	
Lipid	T_m	ΔH	T_m	ΔH
DLPG	*	*	29.9 ± 0.1	12 ± 1
DMPG	23.0 ± 0.1	4.8 ± 0.7	39.6 ± 0.2	14 ± 1
DPPG	42.0 ± 0.3	14 ± 1	42.0 ± 0.3	15 ± 1

T_m, the temperature of the single van't Hoff component and is given in units of °C. ΔH, the transition enthalpy in units of kcal/mol. *Not determined because T_m too close to 0°.

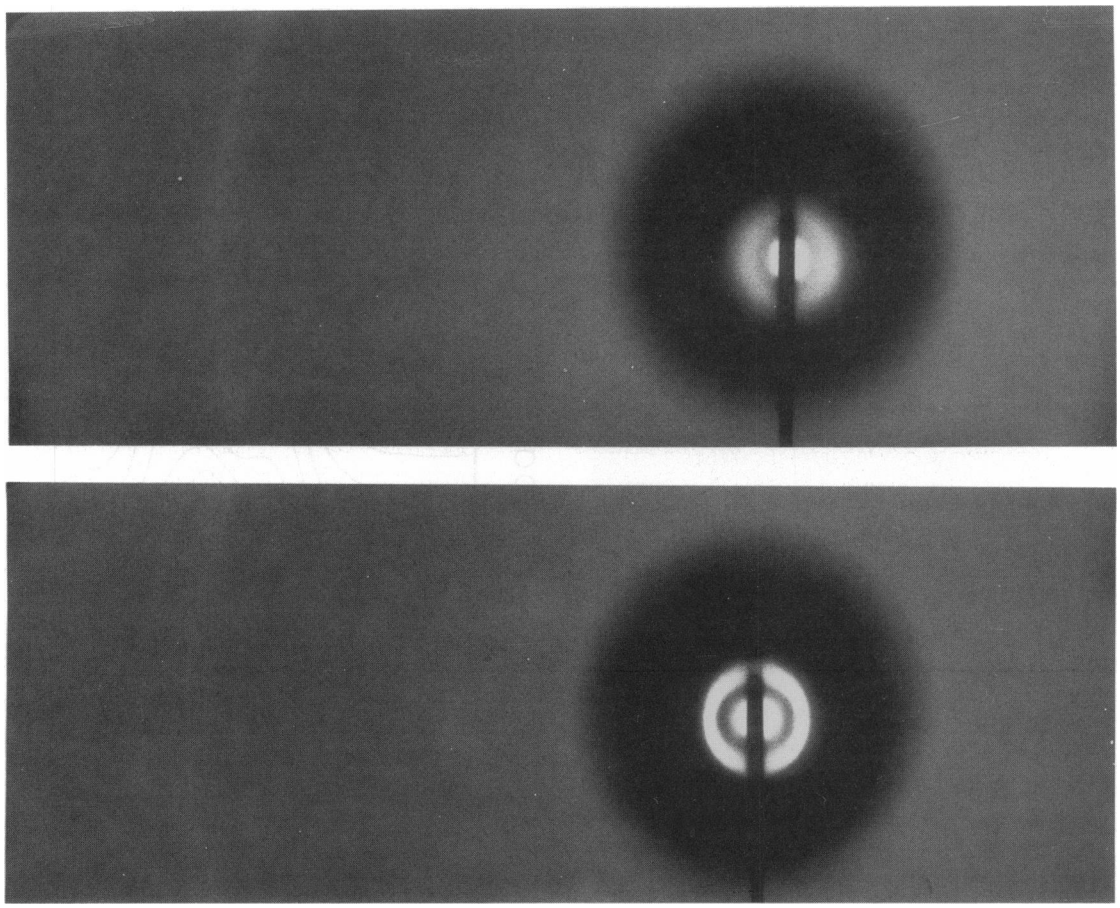

FIGURE 2 X-ray diffraction patterns of (*A*) (*top*) freshly prepared DMPG sample kept at 5° and exposed to x-rays from 0 to 16 h after sample preparation. (*B*) (*bottom*) Sample kept at 2° for 33 h and exposed to x-rays for another 15 h. Only one side of the wide angle pattern is shown. Spacings are given in the text.

of the infrared spectrum of DMPG at 2°C. The methylene scissoring mode (CH_2) is known to be sensitive to the packing and interchain interactions between the lipid hydrocarbon chains (Snyder, 1961). The spectrum measured within the first hours of incubation at 2°C shows a single CH_2 scissoring band at 1468 cm^{-1}. This band is indicative of a hexagonal packing which permits significant orientational fluctuations of the acyl chains (Snyder, 1961; Casal and Mantsch, 1984). Longer low temperature incubation of DMPG results in a correlation field splitting of the CH_2 mode. The first manifestation of this splitting is the appearance, after an initial lag period of 15–20 h, of a shoulder on the high wavenumber side of CH_2. This shoulder gradually gains intensity and shifts towards higher wavenumbers until a well defined correlation field component is fully developed after ~45 h. Longer incubation of the sample at 2°C produces no further changes in the methylene scissoring mode (Figs. 4 and 5).

The presence of a correlation field splitting in the CH_2 mode of low temperature incubated DMPG is indicative of a transition to a new stable gel phase in which the reorientational fluctuations of acyl chains are significantly reduced and there is a considerable increase in chain–chain interactions (Wong et al., 1988). A similar type of chain packing was observed for a number of phospholipids under high pressure (Wong et al., 1988) or for the complexes of phosphatidylserine with calcium or lithium ions (Casal et al., 1987).

Incubation of DMPG at low temperature results also in significant changes in the vibrational mode of the phospholipid carbonyl group. The ester C=O stretching mode of freshly prepared DMPG in the gel phase consists of a broad band with a maximum at 1740 cm^{-1} and a shoulder on the low wavenumber side (Fig. 6). Band narrowing by Fourier self-deconvolution (not shown) allows identification of two component bands: at 1741 and 1724 cm^{-1}. The presence of more than one band in the C=O stretching mode of phospholipids may reflect the conformational nonequivalence of the two carbonyl groups and/or the presence of hydrogen bonded and nonbonded populations of C=O groups in both *sn*-1 and *sn*-2 chains (Myshayakarara and Levin, 1982; Blume et al., 1988). Upon incubation at 2°C there is a narrowing and low wavenumber shift of the overall band contour (Fig. 6). These spectral changes start to

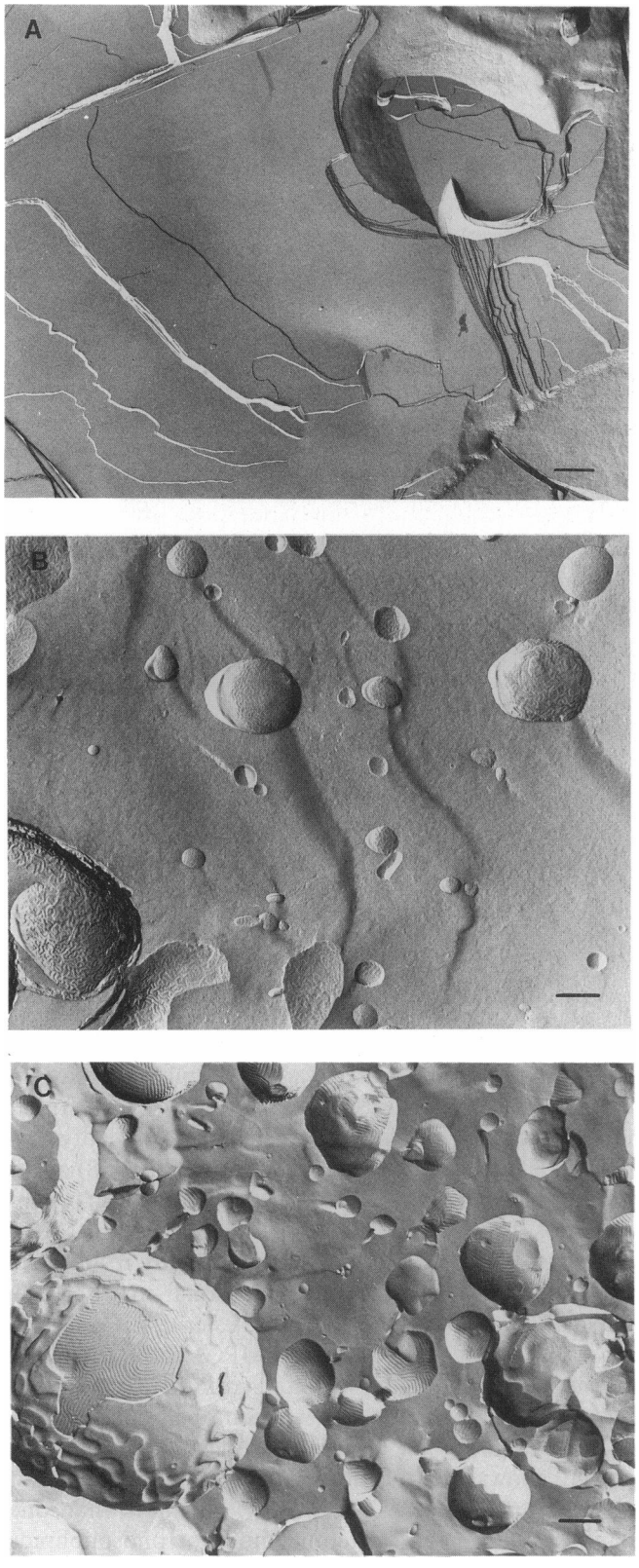

FIGURE 3 Freeze fracture electron micrographs of DMPG. Bar represents a distance of 250 nm. Sample of DMPG aged in Pipes buffer for 7 d at 0°C and then: (*A*) equilibrated to 4° and quench frozen from this temperature; (*B*) equilibrated to 50° and quench frozen at this temperature; (*C*) equilibrated to 50° then cooled to 22° and quench frozen at this temperature.

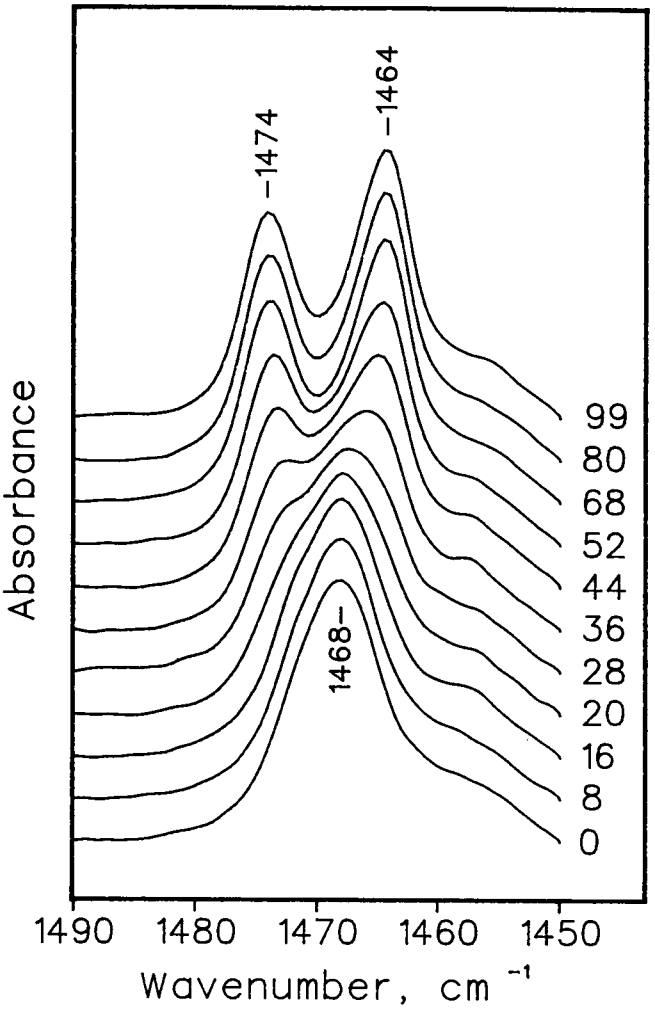

FIGURE 4 Time dependence of infrared spectrum in the CH_2 scissoring region of DMPG at 2°C. Numbers on the right side of each spectrum indicate hours of incubation at 2°C.

appear after an initial lag period of ~15 h and are completed after ~45 h of incubation (Fig. 7). The new spectrum has a maximum at 1732 cm^{-1} and a pronounced shoulder at ~1712 cm^{-1}. Fourier self-deconvolution of this spectrum (not shown) reveals a major band at 1731 cm^{-1} and a smaller band at 1714 cm^{-1}.

DISCUSSION

The well documented transitions of DLPG between P'_β and L_α phases and of DMPG between L_β, P'_β, and L_α phases represent transitions from metastable gel phases. Therefore, caution must be exercised in interpreting the effects of membrane additives on these transitions. In particular, substances that raise the phase transition temperature and enthalpy of these transitions may simply be accelerating the formation of a more stable, higher melting gel phase.

The newly discovered stable gel phase of DLPG and DMPG probably forms as a result of increased interlipid

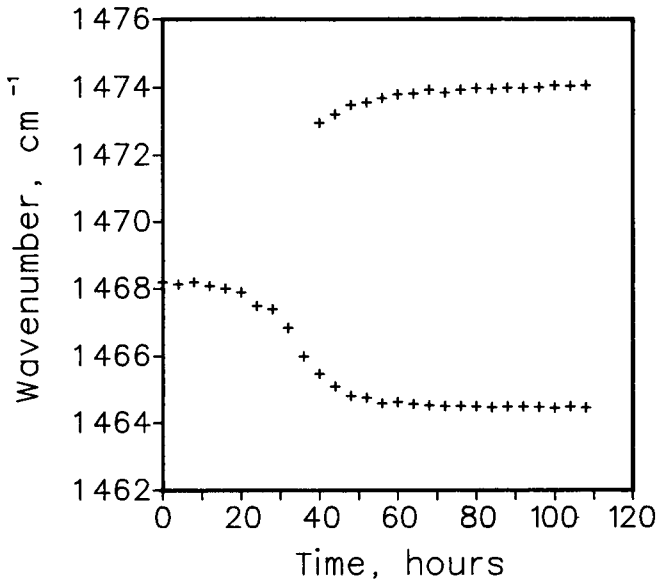

FIGURE 5 Time dependence of the frequency of the CH_2 scissoring mode of DMPG incubated at 2°C.

H-bonding. The headgroup of phosphatidylglycerol has considerable H-bonding capability. However, the lamellar repeat of DMPG is not regular enough to give high resolution reflections necessary for the determination of hydration and bilayer thicknesses by x-ray diffraction. The hydrocarbon packing nevertheless shows increasing order in the stable gel state which may be a consequence of an increase in interlipid headgroup H-bonding. In agreement with possible headgroup H-bonding, Fourier-transform infrared spectra clearly indicate time-dependent alterations in the interfacial zone of DMPG. The low frequency shift of the bands due to ester carbonyl groups could indicate a direct involvement of the C=O groups of aged DMPG in hydrogen bonding with hydroxyl groups (Mushayakarara et al., 1986). An alternative possibility is that alterations in carbonyl bands reflect change in the conformation and/or orientation of one of the C=O groups, without direct participation of these groups in hydrogen bonding. Such conformational change in the interfacial region of the bilayer could arise from interlipid hydrogen bonding that involves other potential H-bond donors and acceptors, i.e., the hydroxyl and phosphate groups of the DMPG headgroup. The discrimination between these two possibilities is at present not possible. Nevertheless, the changes in the interfacial region of the low temperature aged DMPG and the shorter lag period preceding these changes, compared with the corresponding changes in the hydrocarbon region (cf. Fig. 5 and Fig. 7), strongly suggest that the transition from metastable to stable gel phase in DMPG is initiated by changes within the headgroup region, most likely via formation of intermolecular H-bond.

In accord with the suggestion that aging of DMPG or DLPG increases the hydrogen bonding among phospholipid headgroups is also the observation that substitution of D_2O for H_2O results in an increase of approximately one degree in the phase transition of aged samples of these lipids, whereas it has no effect on the Tm of freshly prepared DMPG (data not shown). The deuterium bond is stronger than the hydrogen bond (Eisenberg and Kauzmann, 1969). If the stable gel state had more H-bonding than the rapidly formed metastable gel state and, in addition, the liquid crystallizing states formed from the two kinds of gel states were similar, then it would explain the observed increase in T_m of aged samples of DLPG and DMPG in D_2O compared with H_2O. Phosphatidylethanolamine, which also forms H-bonds in the gel state, exhibits a shift to higher melting temperatures for the gel to liquid crystalline transition in D_2O (Epand, 1990).

The formation of a new gel phase occurs only with phosphatidylglycerols containing short acyl chains. Neither DPPG nor DSPG exhibit any change in thermotropic properties on aging. It is possible that the kinetic

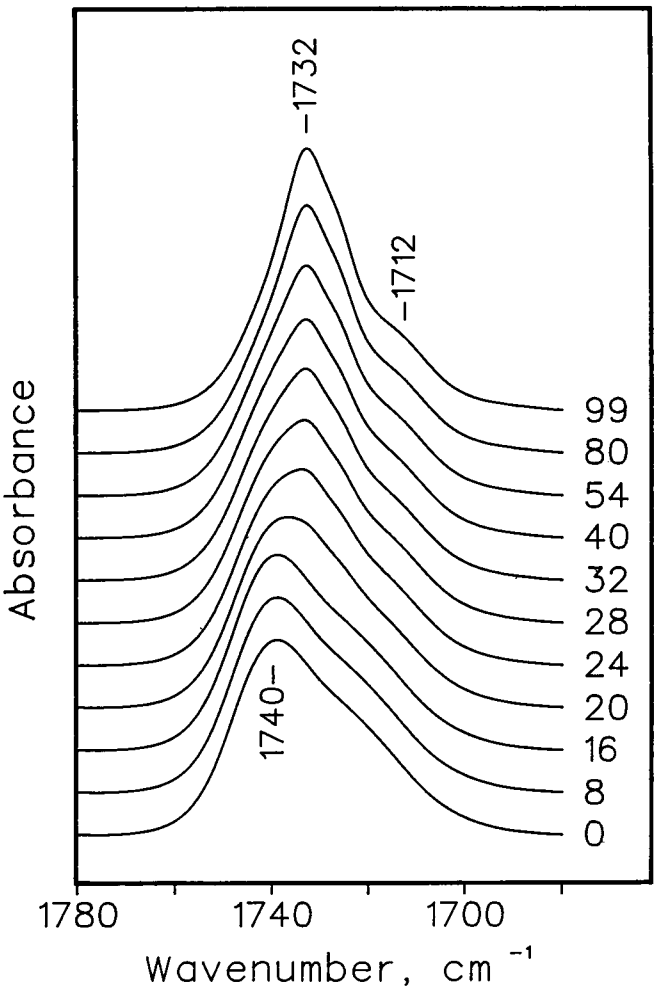

FIGURE 6 Time dependence of infrared spectrum in the ester C=O stretching region of DMPG incubated at 2°C. Numbers on the right side of each spectrum indicate hours of incubation at 2°C.

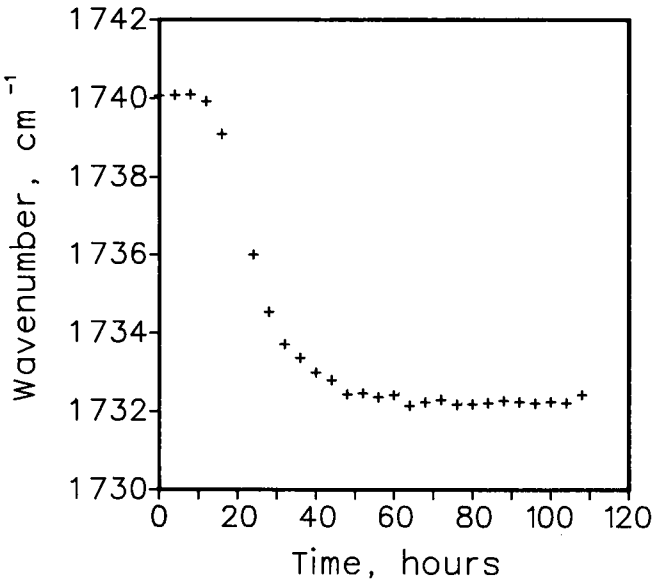

FIGURE 7 Time dependence of the frequency of the maximum of C=O stretching band contour of DMPG incubated at 2°C.

barrier for forming a more stable gel state with the longer homologues is too great for the process to occur in a short time. Although we cannot rule out this possibility completely, we believe it is unlikely. The DPPG sample was incubated at 0° for ~10 times the length of time required for complete conversion of DLPG or DMPG to the stable phase, yet not even partial conversion to a higher melting form of DPPG was observed. We also incubated DPPG at room temperature, rather than 0°, for one week without observing any change in thermotropic behavior. It thus appears that with longer chain phosphatidylglycerols, the gel phase of freshly prepared samples is thermodynamically stable, likely as a result of increased hydrophobic interactions among the acyl chains.

One of the unusual features of the melting of the stable gel state of DLPG and DMPG is the morphological change that accompanies the transition. In the stable gel state, the lipid is arranged as flat sheets. Upon undergoing the transition to the liquid crystalline state, the interlipid hydrogen bonds are broken, resulting in an expansion of the headgroup cross-sectional area. This would increase the tendency toward micellization and would help to break up extended bilayer sheets. A similar phenomenon may also occur with sphingomyelin (Hui et al., 1980). This does not occur with DPPG perhaps because the increased hydrophobic interactions among the acyl chains stabilize an extended bilayer structure.

This work was supported by a grant from the Medical Research Council of Canada.

Received for publication and in final form 9 March 1992.

REFERENCES

Blume, A., W. Hubner, and G. Messner. 1988. Fourier transform, infrared spectroscopy of $^{13}C=O$-labelled phospholipids hydrogen bonding to carbonyl groups. *Biochemistry.* 27:8239–8249.

Boni, L. T., and S. W. Hui. 1983. Polymorphic phase behaviour of dilinoleoylphosphatidylethanolamine and palmitoyloleoylphosphatidylcholine mixtures. Structural changes between hexagonal, cubic and bilayer phases. *Biochim. Biophys. Acta.* 731:177–185.

Cameron, D. G., J. K. Kauppinen, D. J. Moffatt, and H. H. Mantsch. 1982. Precision in condensed phase vibrational spectroscopy. *Appl. Spectrosc.* 36:245–250.

Casal, H. L., and H. Mantsch. 1984. Polymorphic phase behaviour of phospholipid membranes studied by infrared spectroscopy. *Biochim. Biophys. Acta.* 779:381–401.

Casal, H. L., H. H. Mantsch, and H. Hauser. 1987. Infrared studies of fully hydrated saturated phosphatidylserine bilayers effect of Li^{++} and Ca^{++}. *Biochemistry.* 26:4408–4416.

Chen, S. C., J. M. Sturtevant, and B. J. Gaffney. 1980. Scanning calorimetric evidence for a third phase transition in phosphatidylcholine bilayers. *Proc. Natl. Acad. Sci. USA.* 77:5060–5063.

Eisenberg, D., and W. Kauzmann. 1969. The Structure and Properties of Water. Oxford University Press, London. 171–182.

Epand, R. M. 1990. Hydrogen bonding and the thermotropic properties of phosphatidylethanolamines. *Chem. Phys. Lipids.* 52:227–230.

Epand, R. M., and S. W. Hui. 1986. Effect of electrostatic repulsion on the morphology and thermotropic transitions of anionic phospholipids. *FEBS (Fed. Eur. Biol. Organ.) Lett.* 209:257–260.

Hui, S. W., T. P. Stewart, and P. L. Yeagle. 1980. Temperature-dependent morphological and phase behaviour of sphingomyelin. *Biochim. Biophys. Acta.* 601:271–281.

Kauppinen, J. K., D. J. Moffatt, H. H. Mantsch, and D. G. Cameron. 1981. Fourier self-deconvolution: a method for resolving intrinsically overlapped bands. *Appl. Spectrosc.* 35:271–276.

Mushayakarara, E., and I. W. Levin. 1982. Determination of acyl chain conformation at the lipid interface region: raman spectroscopy study of the carbonyl stretching mode region of dipalmitoyl phosphatidylcholine and structurally related molecules. *J. Phys. Chem.* 86:2324–2327.

Mushayakarara, E., P. T. T. Wong, and H. H. Mantsch. 1986. The effect of pressure on the hydrogen bonding between carbonyl and hydroxyl moieties in 1,2-dipalmitoylglycerol: a Fourier transform infrared spectroscopy study. *Biochim. Biophys. Acta.* 857:259–264.

Snyder, R. G. 1961. Vibration spectra of crystalline n-paraffins. *J. Mol. Spectrosc.* 7:116–141.

Wong, P. T. T., D. J. Siminovitch, and H. H. Mantsch. 1988. Structure and properties of model membranes: new knowledge from high pressure vibrational spectroscopy. *Biochim. Biophys. Acta* 947:139–141.