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# Stabilization of non-bilayer structures by the etherlipid ethanolamine plasmalogen

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**The thermotropic phase behavior of mixtures between diradylphosphatidylethanolamines and diacylphosphatidylcholine was studied using polarized light microscopy, $^{31}$P-NMR spectroscopy and synchrotron X-ray diffraction. Multilamellar liposomes composed of alkenylacylphosphatidylethanolamine (ethanolamine plasmalogen) undergo a phase transition from a lamellar to an inverse hexagonal lipid structure at 30 °C, which is about 20 C° and 30 C° lower as compared to its alkylacyl- and diacyl-analog, respectively. These results indicate a higher affinity to non-bilayer structures for the ether lipids. In the presence of the bilayer stabilizing phospholipid, palmitoyloleoylphosphatidylcholine, the transition is shifted to higher temperature without any significant changes in the overall structural parameters as revealed by X-ray diffraction experiments. Again, ethanolamine plasmalogen stabilizes the inverted hexagonal phase to the highest extent, i.e. even in the presence of 40 mol% palmitoyloleoylphosphatidylcholine a pure inverse hexagonal phase is formed at 60 °C. Such a result was not reported so far for a diacylphosphatidylethanolamine. This property of ethanolamine plasmalogen might be predominantly explained by an optimized packing of the hydrocarbon chains in the corners and interface region of the hexagonal tubes, owing to a different conformation of the *sn*-2 chain, which was deduced from $^{2}$H-NMR experiments (Malthaner, M., Hermetter, A., Paltauf, F. and Seelig, J. (1987) Biochim. Biophys. Acta 900, 191–197). Data obtained by time resolved X-ray diffraction show a coexistence of lamellar and inverse hexagonal structures in the phase transition region, but do not indicate the existence of non-lamellar intermediates or disorder within the sensitivity limits of the method.**

## Introduction

Etherlipids are widely abundant in mammalian cell membranes and in microorganisms [1,2]. Usually they represent two types, namely 1-*O*-alkyl and 1-*O*-(1′-*Z*-alkenyl) analogs of glycerophospholipids. 1-*O*-(1′-*Z*-alkenyl)-2-acyl-phospholipids (plasmalogens) are important components of most animal and human cell membranes, e.g., ethanolamine plasmalogen represents a high percentage of the total phospholipids in nerve tissue, bone marrow and red blood cells. Although information on the impact of plasmalogens on membrane properties is increasing, there is still little known about their physiological significance [3].

From earlier studies [4,5] it is known that ethanolamine plasmalogen exhibits a very high affinity for the inverse hexagonal ($H_{II}$) phase. Other examples of lipids occurring in non-lamellar arrangements under physiological conditions are unsaturated diacylphosphatidylethanolamines [6,7] and mono-glycosyldiacylglycerols [8]. On the other hand, phosphatidylcholines and sphingomyelins are lipids clearly preferring the lamellar phase [9,10]. Also the charged lipids, phosphatidylglycerol, -inositol and -serine, tend to form this lipid structure, which is, however, dependent on pH conditions and on the presence of divalent cations [10,11]. Many biological membranes contain a substantial amount of lipids preferring non-lamellar structures [12]

Abbreviations: $L_\beta$, lamellar gel-phase; $L_\alpha$, lamellar liquid-crystalline phase; $H_{II}$, inverse hexagonal phase; $T_{bh}$, $L_\alpha$–$H_{II}$ phase transition temperature; PE, phosphatidylethanolamine; DOPE, 1,2-dioleoyl-PE; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoyl-PC; DOPC, 1,2-dioleoyl-PC.

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and it is widely speculated that this may have biological implications. Yet, there is considerable debate if natural membranes actually do maintain a balance of these different types of lipids. First studies on the phase behavior of mixtures consisting of phosphatidylethanolamine (PE) and -choline (PC) of natural and synthetic origin [7,13–16] were reported already a decade ago, showing that the $H_{II}$ phase was abolished in the presence of about 25 mol% PC. This observation led to the conclusion that a major functional role of PC in biological membranes is to stabilize the bilayer structure. We were now interested to know to what extent ethanolamine plasmalogen is able to maintain the $H_{II}$ phase in the presence of such a bilayer stabilizing phospholipid.

Therefore, we performed a comparative study of the thermotropic lipid polymorphism of ethanolamine plasmalogen and its alkylacyl and diacyl analog in the presence of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC). In addition to synchrotron X-ray diffraction and $^{31}$P-NMR spectroscopy, we studied the phase behavior of these lipid mixtures using polarized light microscopy, the latter method allowing easy and fast detection of the lamellar to inverse hexagonal phase transition. Continuous structural information of these phases over the entire temperature range was obtained by X-ray diffraction with highly intense synchrotron-radiation. This technique provides also information on transition kinetics and possible intermediate structures, leading to a better understanding of the mechanisms of lipid phase transitions [17,18].

## Materials and Methods

### *Lipids*

1-Acyl-2-oleoylphosphatidylethanolamine (acyl-oleoyl-PE) [19], 1-*O*-alkyl-2-oleoyl-PE [5] and 1-*O*-(1'-*Z*-alkenyl)-2-oleoyl-PE (alkenyloleoyl-PE and ethanolamine plasmalogen are used synonymously) [20] were synthesized as described. These ethanolaminephospholipids contain identical, mainly saturated, hydrocarbon side chains in position 1, as already reported in an earlier paper [5]. Fatty acids were purchased from NuCheck Prep., MN, U.S.A. and used without further purification (99% pure). Phospholipase $A_2$ from bee venom was obtained from Boehringer Mannheim, F.R.G., and synthetic POPC (99% pure) from Sigma, F.R.G.. The purity of phospholipids was tested by thin-layer chromatography before and after prolonged experiments. Only one single spot was detected using $CHCl_3/CH_3OH/H_2O$ (65 : 25 : 4, v/v) or $CHCl_3/CH_3OH/NH_3$ (50 : 25 : 6, v/v) as a solvent.

### *Liposome preparation*

Phospholipids dissolved in $CHCl_3/CH_3OH$ 2 : 1 (v/v) were dried under a stream of $N_2$ and finally in vacuo over $P_2O_5$ for at least 4 h. Subsequently, the lipids were dispersed in excess water. The lipid to water ratio of samples for polarized light microscopy as well as for $^{31}$P-NMR spectroscopy was 1 : 2 (w/w) and for X-ray diffraction between 1 : 5 and 1 : 10 (w/w). The exact amount of phospholipid was determined according to the method of Bartlett [21]. To obtain homogeneous hydrated lipid pastes, the suspensions were extensively mixed above their gel-liquid crystalline phase transition temperature for about 3 h. Samples containing ethanolamine plasmalogen were centrifuged additionally through narrow constrictions in the tubes to achieve full equilibration.

### *Polarized light microscopy*

Samples, placed in a glass capillary of 1 mm inner diameter, were heated to about 70 °C and cooled to room temperature a couple of times before measurement with a Zeiss microscope with crossed polarizers. It was equipped with a cooling-heating stage consisting of Peltier elements. A thermal filter avoided heating of the sample by the microscope lamp. Heating and cooling scans were performed at 1 C°/min. Phase transition temperatures were determined from changes in the intensity of transmitted light, which correspond to changes in the birefringence of the samples [22]. The integral intensity of transmitted light was monitored by a photodiode on the microscope tube.

### *$^{31}$P-NMR*

A Bruker WH 90 FT-NMR spectrometer operating at 36.4 MHz was used to record $^{31}$P-NMR spectra, which were proton noise decoupled with an input power of 15 W. Accumulated free induction decays were obtained from up to 10 000 transitions employing a 45° radiofrequency pulse and a spectral width of 10 kHz. $H_3PO_4$ was used as an external reference.

### *X-ray diffraction*

Time-resolved small-angle X-ray diffraction experiments were performed on the double-focussing monochromator-mirror camera X-33 installed at the European Molecular Biology Laboratory (Hamburg Outstation at DESY, F.R.G.). The X-ray flux ($\lambda = 1{,}5$ Å, $\Delta\lambda/\lambda \approx 10^{-4}$), originating from the bending magnet section of the storage ring DORIS II (3.7 GeV, 40–80 mA, multibunch mode), through the sample was in the order of $10^{11}$ photons/s. The sample cell consisted of a brass block containing bores for perfusion with thermostat fluid. The sample was placed in an elliptical chamber (1 × 0.5 cm, optical thickness 0.1 cm) with 40 μm mica windows. Temperatures within the sample cell were measured by a Pt-100 resistance microprobe. Heating and cooling experiments were performed as described elsewhere [23]. The diffractograms were recorded by a four quadrant detector [24] at a sample-to-detector distance of approx. 1 m.

Wide-angle X-ray diffraction experiments were carried out in a pin-hole camera (A. Paar, Graz, Austria) with Ni-filtered $CuK_\alpha$-radiation ($\lambda = 1.54$ Å) from a conventional X-ray generator (50 kV, 40 mA). The camera was equipped with a Peltier controlled variable temperature cuvette. Diffractograms were recorded with a linear one-dimensional position sensitive detector OED 50-M (MBraun, Garching, F.R.G.) at a sample to detector distance of 15 cm. Exposure times of 1000 s were chosen for each temperature.

## Results

Typical intensity traces, as obtained by temperature scans of aqueous dispersions of ethanolaminephospholipids placed between crossed polarizers in the light microscope, are shown in Fig. 1. Two transitions are observed, the first one is characterized by a sharp increase of intensity of the transmitted light and the second one by a decrease of light intensity. In order to assign the corresponding phospholipid phases we performed small- and wide-angle X-ray diffraction experiments. Time-resolved small-angle X-ray powder diffractograms of alkyloleoyl-PE during temperature scans are shown in Fig. 2. Increasing the temperature leads at first to a decrease of the lamellar repeat period from 63 Å to about 53 Å (Table I). Concomitantly, the wide-angle pattern (not shown) changes from a sharp reflection at 4.2 Å to a diffuse reflection around 4.5 Å, which is typical for the chain melting from the $\beta$- to the $\alpha$-conformation [25]. Hence, we can conclude that the low-temperature transition seen in the polarized light microscopy experiments is related to the gel to liquid-crystalline ($L_\beta$-$L_\alpha$) phase transition. A further increase of temperature gives rise to three small-angle reflections indexing in the ratio of $1 : 1/\sqrt{3} : 1/2$, characteristic for hexagonally packed tubes of the $H_{II}$ phase [26], indicating a transition from the liquid-crystalline to the inverse-hexagonal ($L_\alpha$-$H_{II}$) phase. Again, the wide-angle region shows a diffuse reflection at about 4.5 Å as expected from melted hydrocarbon chains. The $d_{10}$-reflection, describing the separation of the planes of the hexagonally packed lipid cylinders, is strongly temperature dependent. This phenomenon can be clearly seen in the contour-line plots of the intensities as shown for alkyloleoyl-PE (Fig. 2b) or for a mixture of 90 mol% ethanolamine plasmalogen and 10 mol% POPC (Fig. 5b). Such behavior was also found by Tate and Gruner [27] for aqueous dispersions of dioleoyl-PE (DOPE) with and without dioleoyl-PC (DOPC). Additionally, one can notice that the lamellar repeat of the $L_\alpha$ phase is reduced by 3–4 Å prior to the transition and during the regime of coexisting lamellar and inverse hexagonal structures, which is in accordance with early observations by Hui et al. [15]. It should be mentioned that in general higher diffraction intensities were observed for

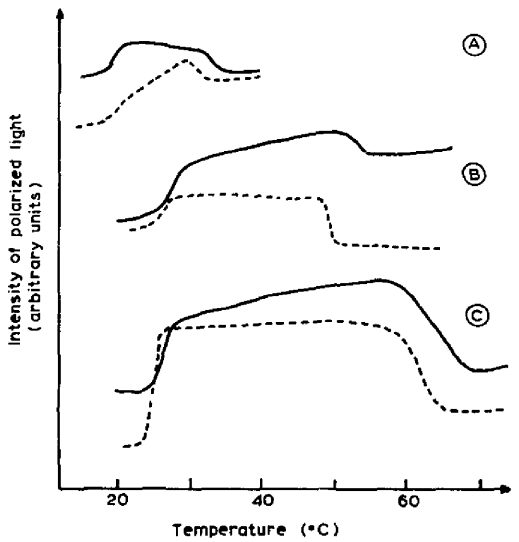


Fig. 1. Traces from polarized light microscopy experiments obtained from heating (solid line) and cooling (dashed line). For details see Materials and Methods. A, alkenyloleoyl-PE; B, alkyloleoyl-PE; C, acyloleoyl-PE.

the acyl- and alkyloleoyl-PE as compared to the alkenyloleoyl-PE, probably due to the difficulties encountered with the preparation of homogeneous samples for the latter species and connected therefore with a lower concentration of these samples.

Comparing the traces obtained from polarized light microscopy experiments (Fig. 1) it is obvious that ethanolamine plasmalogen has by far the lowest $L_\alpha$–$H_{II}$ phase transition temperature (Table II). The $L_\beta$–$L_\alpha$

TABLE I

*Characteristic small-angle X-ray diffraction spacings (d) of the first-order peaks from liposomes of ethanolamine-phospholipids with and without admixture of pOPC*

| Lipid | mol% POPC | $d$(Å) [a] | | |
|---|---|---|---|---|
| | | $L_\beta$-phase | $L_\alpha$-phase | $H_{II}$-phase [b] |
| Alkenyloleoyl-PE | 0 | 63±0.3 [c] | 54±0.3 | 62±1.0 |
| | 10 | 62±0.3 | 54±0.3 | 60±0.5 |
| | 19 | 63±0.5 | 55±0.8 | 62±0.8 |
| Alkyloleoyl-PE | 0 | 63±0.3 | 53±0.3 | 60±0.4 |
| | 14 | 64±0.7 | 53±0.5 | 61±0.6 |
| Acyloleoyl-PE | 0 | 63±0.5 | 53±0.5 | 60±0.6 |

[a] $d = 1/s = \lambda/2 \sin \Theta$, where $\lambda$ is the wavelength (1.54 Å) and $\Theta$ is half of the scattering angle.

[b] Due to the temperature dependance spacings were calculated at $T = T_{bh} + 15$ C°.

[c] Error limits were estimated from the experimental variations in peak maximum position in subsequent time frames of the same structure.

phase transition, however, is less affected by the different molecular structures of the investigated phospholipids (21°C for ethanolamine plasmalogen vs. 27°C and 26°C for alkyl- and acyioleoyl-PE, respectively). These results agree well with our previous data obtained by $^{31}$P-NMR spectroscopy and differential scanning calorimetry [5]. The cooling scans obtained by polarized light microscopy show the same features as compared to the heating scans and indicate a substantial hysteresis ($\approx 5$ C°) for the $L_\alpha$–$H_{II}$ phase transition under the described experimental conditions (Fig. 1). A similar hysteretical effect was described by Epand and Epand [28] upon cooling 1-palmitoyl-2-oleoyl-PE as measured by high sensitivity differential scanning calorimetry at a

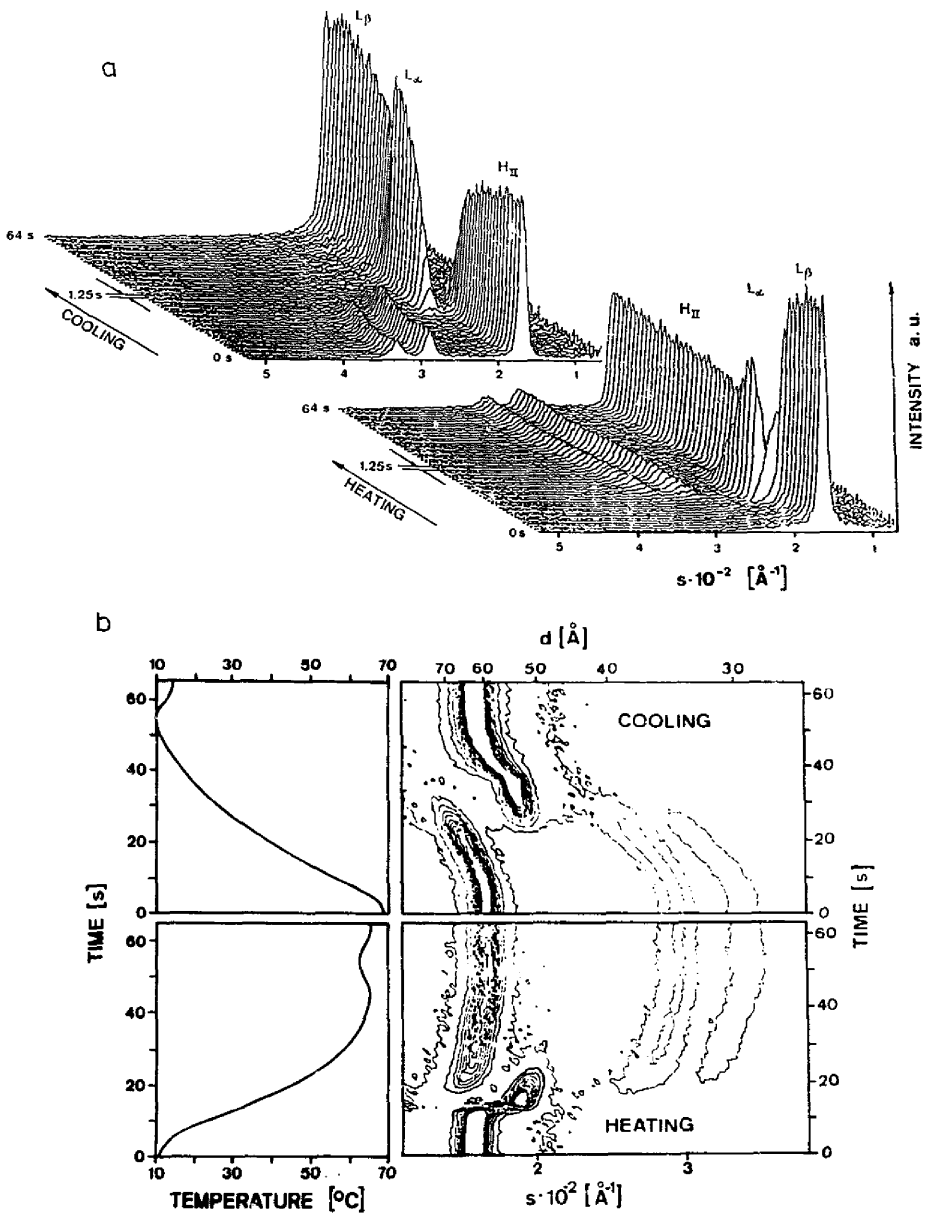


Fig. 2. (a) Time-resolved small-angle X-ray diffractograms from aqueous dispersions of alkyloleoyl-PE in the presence of excess water ($c_{Lip} \approx 0.2$), during a heating and cooling experiment. (b) Contour-line plots of the intensities and temperature course of the experiment.

TABLE II

*$L_\alpha \rightarrow H_{II}$ phase transition temperatures ($T_{bh}$) of aqueous dispersions of diradyl-PE/POPC mixtures obtained from polarized light microscopy*

| Diradyl-PE | mol% POPC | $T_{bh}$(°C) |
|---|---|---|
| Aikenyloleoyl-PE | 0 | 33 |
| | 5 | 31 |
| | 6[a] | 34 |
| | 18 | 45 |
| | 22[a] | 43 |
| | 34 | 52 |
| | 38[a] | 56 |
| Alkyloleoyl-PE | 0 | 54 |
| | 9 | 51 |
| | 20 | 70 |
| | 29 | ≈ 90 |
| Acyloleoyl-PE | 0 | 64 |
| | 14 | 72 |

[a] Results obtained by $^{31}$P-NMR spectroscopy.

scan rate of 0.5 C°/min. A structural hysteresis is also observed from X-ray diffraction measurements, indicating that the first cylindrical tubes formed on heating are smaller in diameter than the last ones disappearing (e.g., 68 Å vs. 73 Å for alkyloleoyl-PE, see Fig. 2).

Fig. 3 shows traces obtained by polarized light microscopy for the ethanolamine phospholipids in the presence of various amounts of POPC. Increasing the admixture of POPC generally raised the $L_\alpha$–$H_{II}$ phase transition temperature ($T_{bh}$) and broadened the transition range. Epand and Bottega [29] observed the same effects (increase of $T_{bh}$ for ≈ 10°C and of the transition range by a factor of ≈ 2) for a mixture of palmitoyloleoyl-PE 91 mol% POPC 9 mol%, which closely resembles the diacyl-PE/PC mixture in this study. Again, a hysteresis of about 5 C° is observed upon cooling the samples (not shown for clarity only). This phenomenon can be used to relate the decrease of light intensity, as seen in the heating for a mixture of 71 mol% alkyloleoyl-PE/29 mol% POPC, to the onset of the $L_\alpha$–$H_{II}$ phase transition. We are able to determine a midpoint transition temperature, $T_{bh}$, of about 85°C for this mixture in the cooling experiment. Assuming a hysteresis of 5 C° we can estimate then a $T_{bh}$ of about 90°C for the mixture in the heating scan. On the same basis we can conclude that liposomes of acyloleoyl-PE with 27 mol% POPC do not undergo a $L_\alpha$–$H_{II}$ phase transition in the temperature range investigated, as no change of light intensity was observed in the cooling scan. This observation is in accordance with results described for POPE/POPC mixtures [30].

$^{31}$P-NMR and X-ray diffraction experiments of mixtures between POPC and ethanolamine plasmalogen confirmed that we are dealing with a $L_\alpha$–$H_{II}$ phase transition. Fig. 4 shows $^{31}$P-NMR spectra of ethanolamine plasmalogen in the presence of 6 and 38 mol%

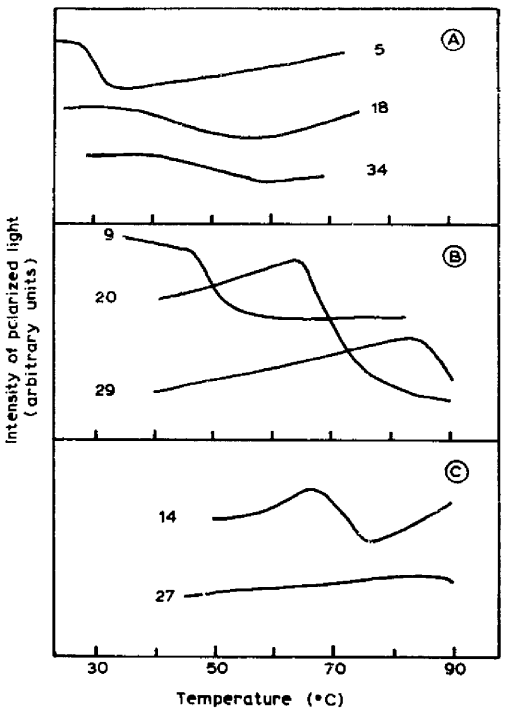


Fig. 3. Traces from polarized light microscopy experiments obtained from aqueous dispersions of mixtures between ethanolamine phospholipids and POPC. The molar admixture of POPC is indicated by the numbers in the panel. A, alkenyloleoyl-PE; B, alkyloleoyl-PE; C, acyloleoyl-PE.

POPC, respectively, as a function of temperature. At lower temperatures both mixtures exhibit $^{31}$P-NMR spectra with a low-field shoulder and a high-field peak, which is typical for lipids arranged in multilamellar bilayers [9,31]. Upon heating the high-field peak disap-

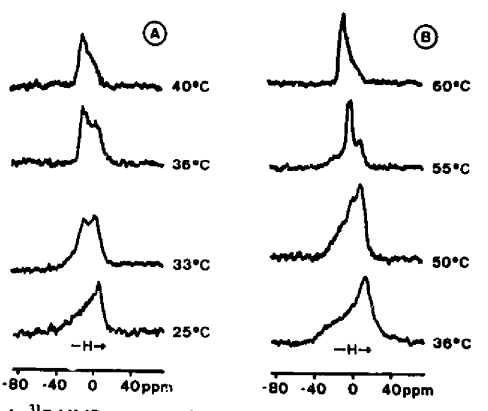


Fig. 4. $^{31}$P-NMR spectra of aqueous dispersions of aikenyloleoyl-PE/POPC mixtures. 25 Hz line broadening was applied. A, 6 mol% POPC; B, 38 mol% POPC.

pears with a concomitant rise of a low-field peak. Finally, $^{31}$P-NMR spectra are observed like those seen for phospholipids in the $H_{II}$-phase showing a reversed asymmetry and a narrower width by a factor of about 2 as compared to the bilayer spectra. This fact is due to additional motional averaging caused by lateral diffusion of the lipid molecules around the small aqueous channel of the hexagonal tubes [9,31]. X-ray diffractograms recorded for alkenyloleoyl-PE containing 10 and 19 mol% POPC, respectively, as well as for alkyloleoyl-PE with 14 mol% POPC confirm this phase assignment. Their characteristic small-angle X-ray diffraction spacings are listed in Table I and do not differ significantly from the corresponding aqueous dispersions of the pure

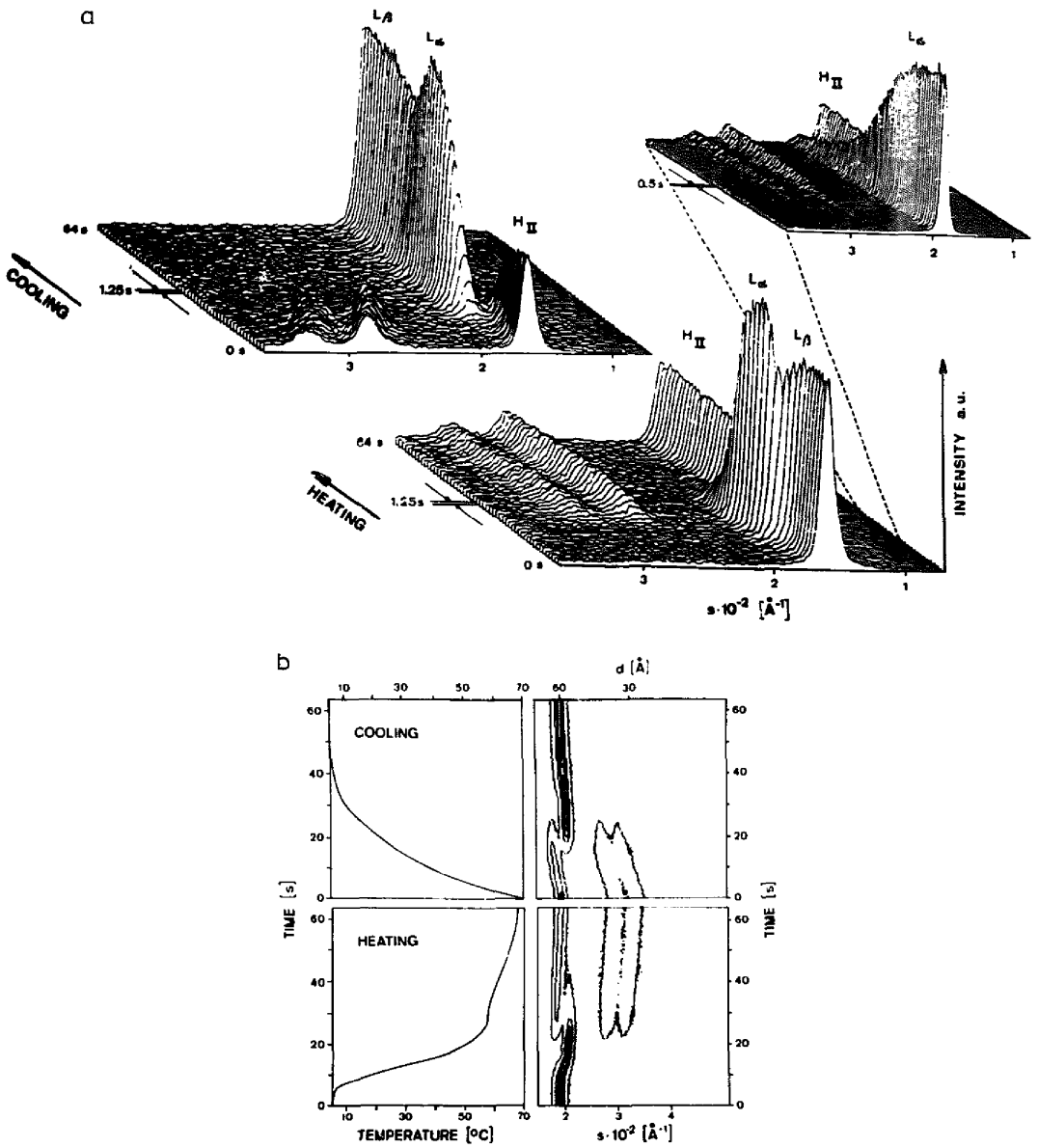


Fig. 5. (a) Time-resolved small-angle X-ray diffractograms from aqueous dispersions of mixtures of alkenyloleoyl-PE 90 mol%/POPC 10 mol% in the presence of excess water ($c_{L,p} = 0.15$), during a heating and cooling experiment. The overlapping of the $L_\alpha$- and $H_{II}$-phase is shown in the inset. (b) Contour-line plots of the intensities and temperature course of the experiment.

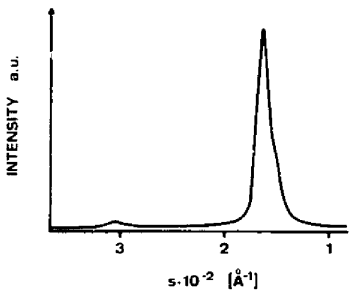


Fig. 6. X-ray diffractogram from aqueous dispersions of mixtures of alkenyloleoyl-PE 81 mol%/POPC 19 mol% in the presence of excess water ($c_{Lip} = 0.15$) at 8°C.

ethanolamine phospholipids. Again, a coexistence of lamellar and inverse hexagonal lipid structures (inset Fig. 5a) is observed in the transition range as already seen for the individual phospholipids. Examination of the X-ray diffractograms show complete miscibility for the alkyloleoyl-PE 86 mol%/POPC 14 mol% and alkenyloleoyl-PE 90 mol%/POPC 10 mol% system in the temperature range investigated. For the mixture alkenyloleoyl-PE 81 mol%/POPC 19 mol%, however, a phase separation seems to occur in the $L_\beta$-gel phase, indicated by a shoulder of the first order reflection and by the presence of their second order reflection (Fig. 6), which are related to the diffraction pattern of POPC. It has to be mentioned that these reflections disappeared on additional heating and cooling, which might indicate that we are dealing with kinetic effects and not with an equilibrium phenomenon. Our observations concerning the miscibility behavior agree with results from calorimetric studies on lipid mixtures of PE and PC [32]. Also X-ray diffraction studies on DOPE/DOPC mixtures up to a molar ratio of 3 : 1 did not indicate immiscibility in the liquid-crystalline phase [27].

## Discussion

In a recent paper [22], it was shown that lamellar phase transitions in lipid-water systems can be detected by measuring the intensity of the transmitted light through a sample placed between crossed polarizers as a function of temperature. We were able to demonstrate now that polarized light microscopy can also be used for the determination of the lamellar to inverse hexagonal phase transition, which is characterized by a decrease of intensity of the polarized light. As is the case with differential scanning calorimetry, one can gain rapid information about the phase behavior of liposomes. But, in contrast to calorimetric measurements, the recorded parameter (light intensity) is insensitive to scan rate, which might be especially important for the detection of the low enthalpic $L_\alpha$-$H_{II}$ phase transition. A feature common to both methods is the fact that no direct phase assignment can be made, which has to be achieved by X-ray diffraction experiments.

From X-ray diffraction experiments we can conclude that the type of linkage between the hydrocarbon side chain in position 1 and the glycerol backbone has no influence on the overall structure of the lamellar phases as evidenced by the same lamellar repeat period for the $L_\beta$ and $L_\alpha$ phase (63 Å and $\approx$ 53 Å, respectively). Identical repeat spacings for the gel or liquid-crystalline phase were also reported by Seddon et al. [11] for diacyl- and dialkyl-PE containing saturated hydrocarbon side chains of the same length. The same authors also studied the lipids' dimension of the $H_{II}$ phase in dependence of the hydrocarbon chain length. They observed a substantial difference between the acyl- and alkyl-PE of 16 carbon atoms, whereas they found only a negligible difference between the acyl- and alkyl-PE of 18 carbon atoms. We also found that the inverse hexagonal phase is characterized by very similar reflections for all three lipids investigated. From monolayer studies [34], it is known that the investigated ethanolamine phospholipids do not differ with regard to packing density and it was concluded that polar interactions of the headgroups are predominant between these lipid molecules. In plasmalogens, however, the carboxylester in position 1 is replaced by an enolether group, leading to a decrease of the polarity of the membrane interface, and therefore they are less hydrophilic, which should increase the preference for $H_{II}$ phases [35,11]. The same holds for the alkylacyl-PE, for which $T_{bh}$ is about 10 C° lower as compared to the diacyl analog. The further decrease of $T_{bh}$ for the ethanolamine plasmalogen ($\approx$ 30 C° lower than the analogous diacyl-PE) might be explained by a different hydrocarbon chain ordering near the lipid/water interface. Recently, Malthaner et al. [36,37] concluded from $^2$H-NMR experiments that the *sn*-2 chain of ethanolamine plasmalogen is perpendicular to the bilayer surface at all chain segments, resulting in a greater effective chain length of the *sn*-2 chain as compared to the analogous diacyl lipid. Based upon results obtained for mixtures of DOPE and PC of various chain length [38], Malthaner et al. [37] concluded further that the extended conformation of the *sn*-2 chain may be in part responsible for the increased tendency of ethanolamine plasmalogen to form the inverse hexagonal phase. Since this transition can be considered as a symmetry-heterologous phase transition involving a change of the type of lattice symmetry, the question of intermediate structures naturally arises. In fact, from $^{31}$P-NMR and electron microscopy experiments [39–41] micellar intermediates were proposed, and also postulated by a theoretical approach [42]. However, our results as well as results published by Caffrey [43] from time-resolved X-ray diffraction studies on 1,2-dihexadecyl-PE show no detectable nonlamellar intermediates or disorder. Still, it has to be noted

that small amounts of the sample may lack long-range order and may be undetected due to the difficulties in detecting diffuse scattering with this experimental approach. The coexistence of $L_\alpha$ and $H_{II}$ structures over a relatively long time and temperature range (Figs. 2 and 5) might indicate, that the two structures belong to one coherent phase in equilibrium. However, owing to the heat conductivity of the various materials (metal, glass, sample) involved in the present temperature scanning experiment, the time constant for equilibration is only in the order of 1 s, which is too slow to obtain information about the intrinsic kinetics of the process. Laggner et al. [44] overcame this problem using a powerful infrared Er-laser-pulse, thereby achieving a time-resolution in the millisecond range. A mechanistic model of the $L_\alpha$–$H_{II}$ transition based on this experimental approach will be described elsewhere [45].

Already a decade ago, Cullis and De Kruijff [13] as well as Hui et al. [15] performed systematical studies on the thermotropic phase behavior of lipid mixtures using naturally occurring lipids, namely soya-PE and egg-PC. Their results showed that no inverted hexagonal phase was formed above an admixture of $\approx 25$ mol% PC, indicating the bilayer-stabilizing effect of this lipid species. In the meantime, this effect was confirmed by many groups using synthetic phospholipids [14,16,29, 30,32]. Our data agree with the above observation insofar as we also could not detect a hexagonal phase for the diacyl lipid mixture at this molar ratio within the temperature limit of our experiments (about 30 C° above $T_{bh}$ of acyloleoyl-PE). However, both the alkyl- and alkenylacyl analog exhibit a $L_\alpha$–$H_{II}$ phase transition in the presence of 30 mol% POPC. The transition temperatures increased for about 35 C° and 20 C°, respectively. Again, ethanolamine plasmalogen stabilizes the inverted hexagonal phase to the highest extent. Furthermore, even at a molar ratio of 62 mol% ethanolamine plasmalogen/38 mol% POPC, this ether-lipid can adopt the hexagonal phase at reasonable temperature (60 °C) as evidenced by $^{31}$P-NMR measurements (Fig. 4).

To adopt favorably the inverted hexagonal phase the interaction of several forces, minimization of the hydrophilic surface area of the lipids [46], hydrocarbon packing constraints [47] and increase of disorder in the hydrocarbon chains [48], is necessary. Gruner and coworkers established a model based on experiments with addition of alkanes [49,50] and of minor fraction of long-chain monounsaturated PC's [51,38] to DOPE, both compounds lowering drastically $T_{bh}$. It was concluded that the constraints of hydrocarbon chain packing are reduced by filling the corner regions of the hexagon with the hydrophobic additives, thereby lowering the costs in free energy. In respect of our results, one might assume that in the presence of POPC ethanolamine plasmalogen favorably arranges in the corners of the hexagon owing to the postulated extended conformation of the *sn*-2 chain, i.e., it costs less energy to fit the cylindrical shaped PC into the hexagonal tubes of ethanolamine plasmalogen then into tubes formed by diacyl-PE. However, Scherer [52] doubts the importance of the presumed stress in controlling the $L_\alpha$-$H_{II}$ phase transition. Relying on a new definition of the hydrophilic/hydrophobic boundary in lipid bilayers [53], he suggests that stress in the hydrocarbon chain region from low density packing is avoided by chain interdigitation, which might be facilitated for ethanolamine plasmalogen, again due to the postulated extended conformation of the acyl-chain. Since the arguments in favor of one or the other model rely heavily on the details of the chain conformations, which are, however, still not clear enough, we are not able to decide between the two models. Further studies in our laboratories employing mixed-chain acyl lipids and computer modeling should lead to a sound basis for decision.

Biological membranes contain a great variety of phospholipids including PC, in addition to phospholipids that may favor the formation of non-bilayer phases. In this context, it seems to be very important that ethanolamine plasmalogen forms non-bilayer phases in the presence of high amounts of lipids preferring the bilayer arrangement. Some naturally occurring ethanolamine plasmalogens display even a higher degree of unsaturation as compared to our semisynthetically prepared one, e.g., a major portion of ethanolamine plasmalogen of the myelin membrane is 18 : 1 in both positions [54]. Therefore, they might be able to maintain the $H_{II}$ phase even to a higher extent and may induce local formation of non-bilayer phases which can exist temporarily as, e.g., during membrane fusion or due to interaction of the membrane with external factors such as ions or proteins.

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