ELSEVIER

Biochimica et Biophysica Acta 1237 (1995) 59–69

BBA Biochimica et Biophysica Acta

Effects of short-chain alcohols on the phase behavior and interdigitation of phosphatidylcholine bilayer membranes

Ludwig Löbbecke, Gregor Cevc *

Medizinische Biophysik, Technische Universität München, Klinikum r.d. Isar, Ismaningerstr. 22, 81675 Munich, Germany

Received 10 October 1994; revised 16 February 1995; accepted 9 March 1995

Abstract

Solute effects on the polymorphism and phase transitions in the suspensions of dipalmitoylphosphatidylcholine (DPPC) were studied by means of carboxyfluorescein (CF) and phosphatidylethanolamine rhodamine (PERho) fluorescence, differential scanning calorimetry, and X-ray diffraction. Specifically, the shifts of the lipid chain-melting phase transition, pretransition and subtransition temperature as a function of the bulk alcohol concentration were determined calorimetrically. The chain-melting phase transition temperature, T_m, was found to depend on the chain-length of the added alcohol: for short-chain alcohols (up to n-propanol), T_m first decreases and then increases with increasing alcohol concentration. For longer-chain alcohols, however, T_m decreases over the whole investigated alcohol concentration range. The pretransition and the subtransition temperature of DPPC both decrease monotonously (but non-linearly) with increasing alcohol concentration, but the former transition disappears at some characteristic, chain-length dependent alcohol concentration, $c_{L_{\beta i}}$. This point in the solute-dependent phase diagram of DPPC is diagnostic of the complete hydrocarbon interdigitation. It was determined for a series of short-chain alcohols ranging from methanol through to 1-hexanol. A quantitative formula for the calculation of such limiting alcohol concentration is introduced. This formula relates the $c_{L_{\beta i}}$ values to the free energy of transfer of alcohols from the aqueous sub-phase into the DPPC sub-phase. By using the concept of an apparent chain-length this formalism can also be used for the alcohols with polar OH-groups at the second or third position on the hydrocarbon chain. Alcohol-induced hydrocarbon interdigitation in the phospholipid bilayers is thus shown to result chiefly from the solute-induced perturbation (lateral expansion) in the lipid headgroup region. Longer-chain alcohols, which balance this effect by disordering the phospholipid chains, therefore do not induce chain interdigitation.

Keywords: Lipid polymorphism; X-ray diffraction; DSC; Phospholipid structure; Phase transition; Partition coefficient

1. Introduction

The interaction of foreign molecules with phospholipid bilayers has been the topic of a host of investigations. Perhaps the most interesting and new consequence of such interaction is the solute-dependent shift of phase transition temperature and the induction of new lamellar phases. The complete hydrocarbon chain interdigitation caused by the addition of certain agents to phospholipid/water suspensions has attracted most interest in this respect. Indeed, many molecules, ranging from ions, such as thiocyanate [1], to peptides, such as polymyxin B [2,3] have been reported to induce hydrocarbon interdigitation, this is, the formation of a $L_{\beta i}$-phase (see Slater and Huang for a review [4]). The only common feature of these agents, recognized until now, is their ability to be taken up by the phospholipid membranes in the interfacial region.

We desired to get a deeper understanding of the phenomena underlying hydrocarbon interdigitation at the molecular level. We have therefore started a systematic study of the interdependence between the $L_{\beta i}$-phase formation and the inducer characteristics. Owing to their relative structural simplicity and the ease with which their molecular properties can be varied we have chosen short-chain alcohols as 'model agents' for such studies of the solute-induced interdigitation.

Alcohols ranging from methanol to 1-heptanol have been previously reported to induce hydrocarbon chain interdigitation in phosphatidylcholine bilayers [5–12]. To date, however, clarity has not been achieved with regard to

* Corresponding author. Fax: +49 89 41805119.

SSDI 0005-2736(95)00076-3

the relative contribution of various molecular parts to the process of bilayer structure modification.

The thermodynamics of chain interdigitation has also been the topic of several publications. Detailed phase diagrams in particular of the phospholipid/ethanol mixtures in water were thus elaborated [8,9,13,14]. We wanted to find out if other short-chain alcohols will similarly affect the phase diagram of DPPC [10,11].

For the very short alcohols, ethanol and 1,3-propanediol, several structural investigations have also been done by means of X-ray diffractometry [6,14,15]. These studies have shed some light on the packing characteristics of the corresponding systems. Moreover, partition coefficient measurements [10,16], densitometry [13], Fourier-transform infrared spectroscopy [17], electron spin resonance spectroscopy [18], and carbon-13 nuclear magnetic resonance spectroscopy [19] were used to reveal the details of the interaction between the short-chain alcohols and phospholipids in a suspension.

However, none of these studies has as yet succeeded in explaining the relationship between the inducer properties and the limiting concentration needed for causing complete lipid chain interdigitation, $c_{L_{\beta i}}$. We have thus determined $c_{L_{\beta i}}$ for the systems containing DPPC and n-alcohols ranging from methanol through to 1-hexanol. We have also looked at the effects of changing the polar OH-group position along the alcohol hydrocarbon chain. In addition to this, one diol (1,2-hexanediol) has also been studied. Fluorescence spectroscopy, calorimetry and X-ray diffraction were used for this purpose.

Our data reveal a very simple relation between the short-chain alcohol characteristics and the $L_{\beta i}$-phase inducing concentration. They, moreover, suggest that this concentration can be calculated with a high degree of accuracy from the known partition coefficient of the interdigitation-inducing alcohol in the $L_{\beta'}$-phase. Based on this we argue that the main reason for the hydrocarbon chain interdigitation is the perturbation induced by the presence of alcohols in the headgroup region. Other effects, however, which predominantly operate in the lipid layer interior, are likely to dominate for the alcohols with long hydrocarbon chains, starting with n-octanol.

2. Materials and methods

1,2-Dipalmitoyl-*sn*-glycero-3-phosphorylcholine (DPPC) was purchased from Sygena (Liesthal, Switzerland), Boehringer-Mannheim (Mannheim, Germany), or Fluka (Neu-Ulm, Germany). Lipid purity was checked by high pressure liquid-chromatography (HPLC) using a Pharmacia-LKB system (Pharmacia, Freiburg, Germany) and found to be greater than 99%, independent of the source of lipid. Alcohols were from Merck (Darmstadt, Germany), except for 1-pentanol, 1-hexanol and 1,2-hexanediol which were from Fluka (Neu-Ulm, Germany). These alcohols were always of the highest commercially available quality and were used without purification. 5(6)-Carboxyfluorescein (CF) was bought from Kodak (Rochester, USA) and rhodamine PE (R18) was purchased from Molecular Probes (Eugene, USA). Water (18 MΩ cm) was doubly distilled in an all glass apparatus and reprocessed by a water purification unit (Elgastat HQ, Elga, UK).

2.1. Preparation of unilamellar vesicles

A stock suspension of DPPC was prepared either in water or in a 30 mM CF solution with a pH value of 7.0. DPPC concentration in each stock suspension was 5 mg/ml. To obtain a homogeneous suspension of multilamellar vesicles such preparation was heated to 50° C and vortexed.

To prepare small unilamellar vesicles (SUV), 1.5 ml of this stock suspension were sonicated at approx. 41° C for 1 h by a Branson Sonifier Model B12 (Branson, Danbury, CT) using a standard titanium micro tip. After overnight equilibration, the vesicle diameter was measured by the quasi-elastic light scattering (QELS) to be 60 ± 10 nm; most of the resulting vesicles were thus unilamellar.

Prior to each experiment the suspension was further equilibrated at 41° C for 1 day and then for at least two weeks at room temperature. This ensured the investigated bilayers to be tension-free. During the equilibration period the vesicle diameter gradually increased to $100 \cdots 120$ nm. Even longer storage times did not further increase the vesicle size indicating that, under the circumstances given, the vesicles have reached an energetically favorable, quasi-stable state.

Vesicles with a diameter of 100 nm were also prepared by manually extruding part of the stock suspension with an extruder (Avestin, Ottawa, Canada) using polycarbonate filters with 100 nm pore size.

2.2. Membrane permeability measurements

Membrane permeability measurements were based on the CF-fluorescence increase upon the marker leakage from the vesicle interior. Experiments were done with a Perkin-Elmer LS-5 spectrofluorimeter using a fixed wavelength of 490 nm for the excitation and 524 nm for the emission. Data acquisition was done by an IBM-XT computer using a laboratory-written software. To suppress the signal from the trivial light scattering, a 500 nm cut-off filter (Schott, Mainz, Germany) was placed in the emission path. The temperature of the cuvette in the fluorimeter was kept at 22° C by a Lauda cooling bath (± 0.1 K) during each measurement.

Prior to doing the fluorimetric measurements, the non-encapsuled CF was separated from the fluorescent vesicles by chromatography on a Sephacryl S-400 column (Pharmacia, Uppsala, Sweden). Each sample cuvette then contained 2 ml of bidestilled water with triethanolamine/HCl

buffer (pH ~ 7), 10 μl of the filtered DPPC/CF suspension, and the required amount of the investigated alcohol.

Samples were allowed to equilibrate for 24 h before the fluorescence intensity was determined. Since the total amount of CF in each sample differed, due to the preparation variability, the 100% reference point was measured for each sample separately. This was done by solubilizing the vesicles by the addition of 10 μl of Triton X-100. After 5 min of equilibration, the fluorescence intensity was then measured again and identified with 100% contents-release (longer equilibration periods did not further increase the fluorescence intensity).

2.3. PE rhodamine fluorescence measurements

PERho and DPPC (1:50 mol/mol) were dissolved in chloroform. Chloroform was then removed by a stream of N_2 gas and then under vacuum ($\leq$ 80 Pa, 12 h). SUVs in a 10 mM lipid suspension in water were obtained as described in previous sections. The sample cuvette contained 10 μl of such lipid suspension, 2 ml of bidestilled water with triethanolamine/HCl buffer, and the specified amount of alcohol.

Experiments with PERho were done at a fixed excitation wavelength of 300 nm. The emission wavelength was scanned between 450 nm and 620 nm to determine the maximum fluorescence intensity. Final results were obtained by normalizing the measured fluorescence intensities with regard to the value determined for an alcohol-free sample.

2.4. Differential scanning calorimetry (DSC)

Multilamellar vesicles for the DSC measurements were prepared by mixing DPPC with bidestilled water. The resulting 5 mM DPPC suspension was then heated to 50° C for at least 1 h.

The appropriate amount of the investigated alcohol was added to the resulting suspension 1 week before each measurement in order to insure a homogeneous solute partitioning into the bilayer. During this period, the samples were kept at 4° C in a refrigerator. Shorter equilibration periods were insufficient for reaching the sample equilibrium.

The samples containing DPPC and different concentrations of 3-hexanol were kept at 4° C for 7 months in order to assess the long-term equilibration subtransition temperature of DPPC in this system. Lipid purity after these measurements was checked by HPLC and found to be greater than 95%.

DSC measurements were done in a MC-2 scanning calorimeter (MicroCal, Amherst, MA) with the original data acquisition and analysis software (ORIGIN). For the cooling-scan measurements the calorimeter was connected to a computer-controlled refrigerated bath (F3C, Haake, Germany). Samples were heated and cooled in the temperature range 4° C · · · 50° C at a scanning rate of 30 K/h.

2.5. X-ray measurements

Multilamellar vesicles were prepared by adding lipid (~ 20 mg) to approx. 1.5 ml of doubly distilled water with the required amount of the investigated alcohol. The resulting suspension was allowed to equilibrate for at least 24 h above the chain melting temperature in order to reach full hydration. Before each experiment the samples were allowed to equilibrate at room temperature for one week, at least.

Prior to each experiment the suspension was centrifuged in order to collect the lipid. The resulting pellet, together with some extra water, was mounted into a thermostated (±0.1° C) sample holder between two mica windows (sample width ~ 1 mm). After measurements, the presence of excess water was confirmed.

The X-ray source was a conventional Seifert SF 60 copper anode (Seifert, Ahrensburg, Germany) with line focus. It was typically run at 1.3 kW. For the unoriented samples a focusing Ge-(111) monochromator was used to select the Cu K_{α_1} line ($\lambda = 1.5405$ Å). The beam was focused onto the position sensitive detector (OED 50, Braun, Munich, Germany) with an effective resolution of 0.2 mm. The distance between the sample and the detector was 20 cm.

Sample temperature was measured with a Pt100 and controlled with an accuracy of ±0.1° C with an Eurotherm unit (Eurotherm Regler, Limburg, Germany). Each sample was measured at three temperatures in the $L_{\beta'}$ or $L_{\beta i}$-phase (for example at 20° C, 24° C and 28° C) and at two temperatures in the fluid-lamellar phase (typically 45° C and 50° C). At each measuring temperature the sample was first allowed to equilibrate for 30 min and then inspected for 3 h to 24 h, depending on the intensity of the Bragg peaks [1]. In the $L_{\beta'}$-phase 3–5 Bragg peaks were usually observed whereas the $L_{\beta i}$-phase only gave two or three Bragg peaks. The number of the observed Bragg peaks strongly depended on the alcohol used. As a rule, the number of detectable Bragg peaks got lower with increasing number of carbon atoms of the investigated alcohol. In the L_{α}-phase, two well defined Bragg peaks were nearly always measurable.

The experimental background scattering from the sample holder and mica windows was measured separately. It was subtracted from the sample signal prior to the definitive data analysis.

[1] It was found out that the intermediate-chain alcohols like 1-pentanol or 2-hexanol cause much disorder in DPPC membranes even in the lipid gel phase. Therefore, longer measuring times were necessary to get a good signal-to-noise ratio.

3. Results

3.1. CF measurements

Fluorescence measurements were first done with DPPC/ethanol mixtures in order to assess the reliability of our test method. The corresponding results are given in Fig. 1. The inset to this picture shows cartoons of different bilayer states expected to exist in the various ethanol-concentration regions. The molecular rationale for these states is given in the recent article by Vierl et al. [14].

Fig. 1 reveals that the relative fluorescence from the vesicle 'encapsulated' CF increases linearly with the bulk ethanol concentration in the range from 0 to 0.5 M. This change is from zero to 0.25 relative units. In the same concentration interval the tilt angle of the hydrocarbon chains in the DPPC bilayers has been reported to increase from approx. 30° (in water) to 50° (in 0.5 M ethanol) [14,20]. Correspondingly, in a 0.45 M ethanol solution the relative fluorescence intensity jumps nonlinearly to 1.0, this is, to the maximum possible value. This is indicative of the onset of partial hydrocarbon interdigitation and of the generation of domain boundaries. Fluorescence increase at this concentration is thus due to the facilitated CF flux through the leaky domain boundaries.

Alcohol concentrations higher than 0.45 M decrease the relative fluorescence intensity to 0.5. If ethanol concentration in the bulk exceeds 1.0 M, however, the measured fluorescence intensity is statistically distributed around one. This suggests that carboxyfluorescein molecules are now homogenously distributed throughout the whole sample even before the injection of a 'lytic' ethanol concentration. In previous publications the alcohol concentration leading to the induction of complete hydrocarbon interdigitation ($c_{L_{\beta'} \rightarrow L_{\beta i}}$) for the multilamellar DPPC vesicles was reported to be $1.0 \cdots 1.2$ M [7,13,14] at room temperature.

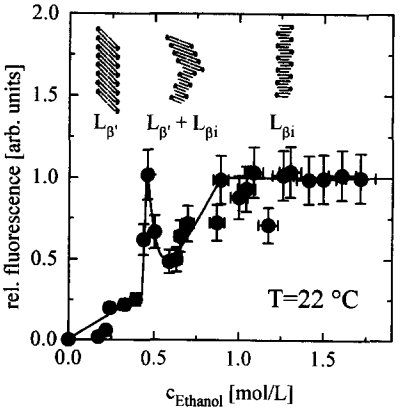

Fig. 1. Relative fluorescence of carboxyfluorescein, CF, that was originally enclosed into the interior of small unilamellar DPPC vesicles, 5 h after the addition of specified amounts of ethanol to the lipid suspension. Cartoons illustrate the state of DPPC bilayers for three representative alcohol concentrations, according to the previously published phase diagram.

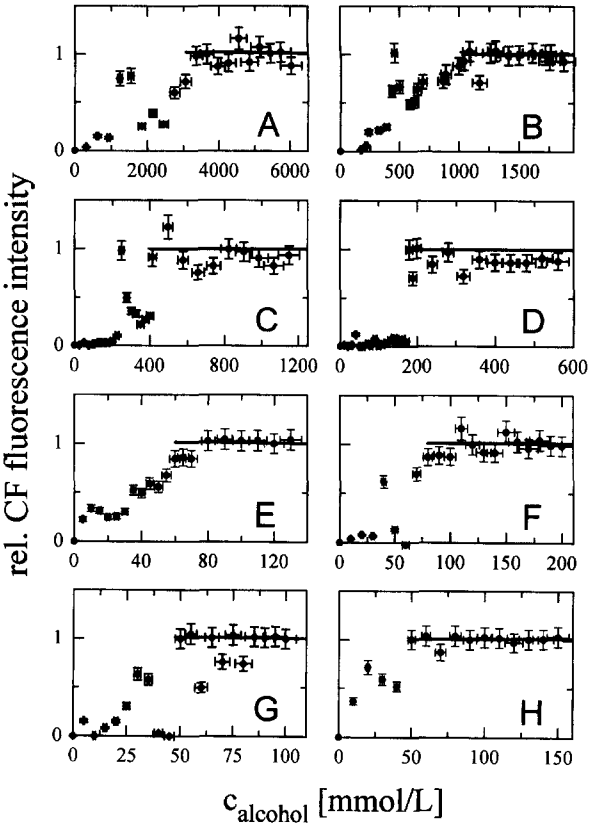

Fig. 2. Relative intensity of the CF-fluorescence 24 h after the addition of various alcohols to a 0.01 mM DPPC suspension in water. CF was originally enclosed into the DPPC vesicles under self-quenching conditions. All values are normalized with regard to the fluorescence intensity measured after the addition of Triton X-100, which completely solubilizes lipid vesicles. A, methanol; B, ethanol; C, 1-propanol; D, 1-butanol; E, 1-pentanol; F, 2-pentanol; G, 1-hexanol; H, 2-hexanol.

Similar measurements were also done for other alcohols ranging from methanol ($n_c = 1$) through to hexanol ($n_c = 6$). Fig. 2 illustrates the corresponding behavior of the relative fluorescence intensity with respect to the bulk alcohol concentration. In each case, a linear increase of the relative fluorescence intensity in the low concentration region is observed. The more or less pronounced first maximum is always found at the alcohol concentrations corresponding, within experimental error, to $c_{L_{\beta i}}/2$ (see further discussion). After the subsequent decrease in the relative fluorescence intensity its value then again reaches 100% for concentrations exceeding $c_{L_{\beta i}}$.

We have found the $c_{L_{\beta i}}$ values to be 2500 ± 500 mM for methanol, 1000 ± 100 mM for ethanol, 400 ± 50 mM for 1-propanol, 180 ± 30 mM for 1-butanol, 60 ± 10 mM for 1-pentanol, 50 ± 10 mM for 1-hexanol, 75 ± 10 mM for 2-pentanol and 50 ± 10 mM for 2-hexanol, respectively. Other alcohols such as 3-pentanol and 1,2-pentanediol were also tested but gave ambiguous results. The

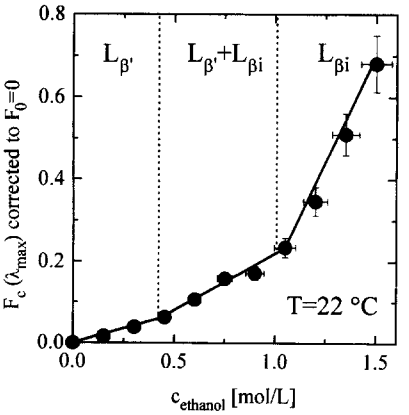

Fig. 3. Relative fluorescence at the intensity maximum of PERho in the DPPC vesicles as a function of bulk ethanol concentration. All fluorescence values are corrected so as to give $F(c_{EtOH} = 0) = 0$.

DPPC concentration in these measurements was approx. 10^{-5} M.

It should be noted that error margins in Fig. 2 only take into account the experimental and statistical errors related to the performance and further interpretation of the fluorimetric data. The 'true' $c_{L_{\beta'} \rightarrow L_{\beta i}}$ value may still differ significantly from the $c_{L_{\beta i}}$ value evaluated by the CF fluorescence method owing to the neglect of other shortcomings of this experimental method in the described evaluation (see Discussion).

3.2. PERho measurements

In contrast to CF, PERho is lipophilic and thus always partitions preferentially into the lipid bilayer. This fluorescent dye is hence sensitive to the local polarity of its binding surrounding, higher fluorescence intensities always signifying a higher local polarity.

Again, we have first measured the PERho fluorescence intensity as a function of the bulk ethanol concentration (see Fig. 3). The corresponding data can roughly be divided into three sections. In each of these the maximum fluorescence intensity increases linearly: for the alcohol concentrations between 0 and 0.45 M, a smooth increase of the maximum fluorescence intensity is detected. Between 0.45 and 1.05 M ethanol the fluorescence intensity has a slightly more positive trend with respect to the bulk alcohol concentration and at concentrations exceeding 1.05 M the steepest increase is measured.

Relating to these data, the results obtained with other alcohols were interpreted by identifying the onset of the steep increase in the PERho fluorescence intensity with $c_{L_{\beta i}}$. This gave values of 130 ± 20 mM for 2-butanol, 75 ± 10 mM for 2-pentanol, 70 ± 10 mM for 3-pentanol, 50 ± 10 mM for 2-hexanol, 60 ± 20 mM for 3-hexanol, and 200 ± 100 mM for 1,2-pentanediol.

Fig. 4 shows these data for different alcohols. The point which was tentatively assigned to the induction of the complete DPPC interdigitation is marked with a vertical line. Relatively large error pertaining to the 1,2-pentanediol data indicate that the interpretation of these results is far from being unambiguous. It is also not possible to get a good value for $c_{L_{\beta i}}$ in the DPPC/1,2-hexanediol system by means of PERho fluorescence spectroscopy.

3.3. Differential scanning calorimetry

Differential scanning calorimetry was used to investigate the interaction between DPPC and 1-pentanol as well as 1-, 2-, 3-hexanol and 1,2-hexanediol. Fig. 5 shows the corresponding pretransition temperature and the chain-melting phase transition temperature as a function of the bulk alcohol concentration.

All investigated alcohols suppress the pretransition of DPPC at some characteristic concentration. It is well established that the complete interdigitation of hydrocarbon chains in the phospholipid bilayers is thermodynamically equivalent to the direct transition from an interdigitated gel phase into the liquid crystalline state. The former concentration was thus denoted as $c_{L_{\beta i}}$. For 1-pentanol this gave 35 mM, for 1-hexanol $c_{L_{\beta i}} = 10 \pm 2$ mM, for 2-hexanol

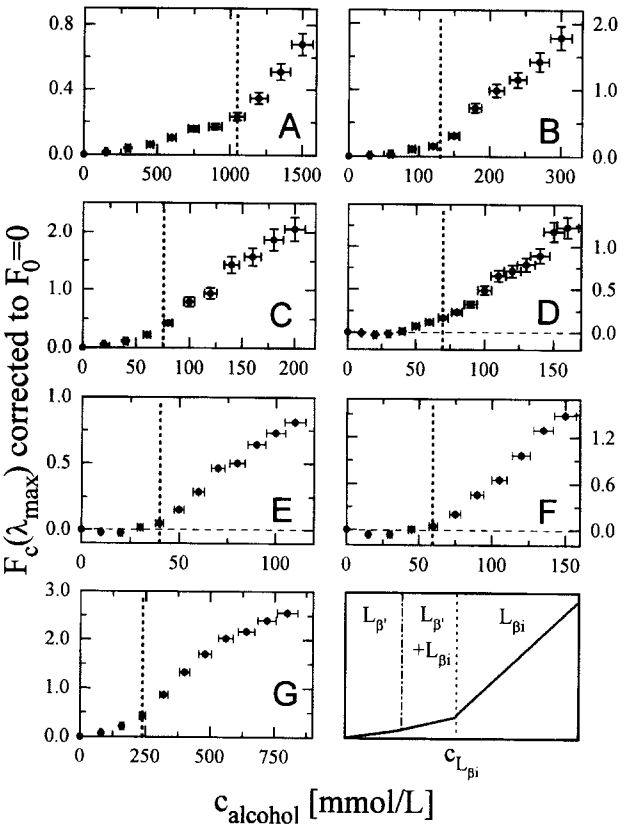

Fig. 4. Effect of the bulk alcohol concentration on the maximum fluorescence intensity of PERho in DPPC vesicles. A, ethanol; B, 2-butanol; C, 2-pentanol; D, 3-pentanol; E, 2-hexanol; F, 3-hexanol; G, 1,2-pentanediol. Dotted lines tentatively identify complete bilayer interdigitation and thus determine the value of $c_{L_{\beta i}}$. The last panel illustrates the positions of different phase boundaries.

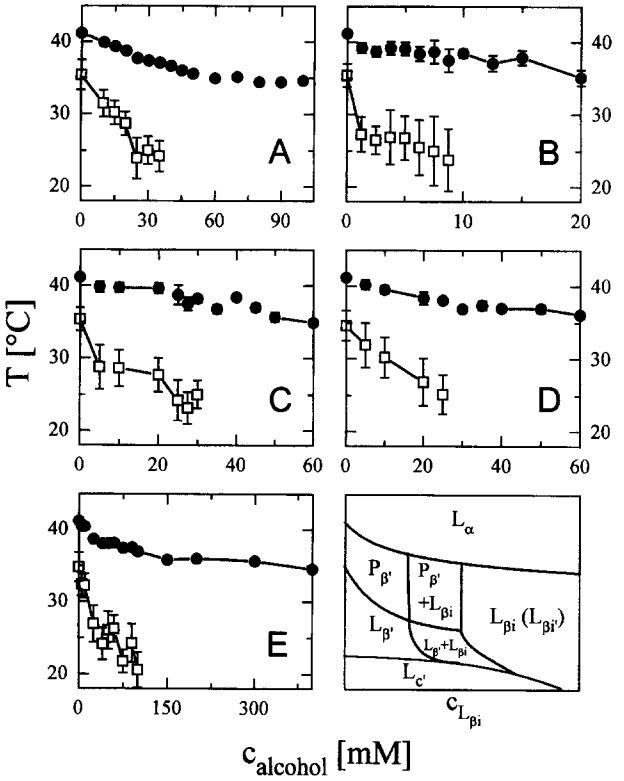

Fig. 5. Pretransition (□) and chain-melting phase transition temperature (●) of DPPC multibilayers in the presence of various alcohols as a function of the bulk solute concentration. A, 1-pentanol; B, 1-hexanol; C, 2-hexanol; D, 3-hexanol and E, 1,2-hexanediol. The lowest right panel gives the appropriate phase assignments (see also Ref. [14]).

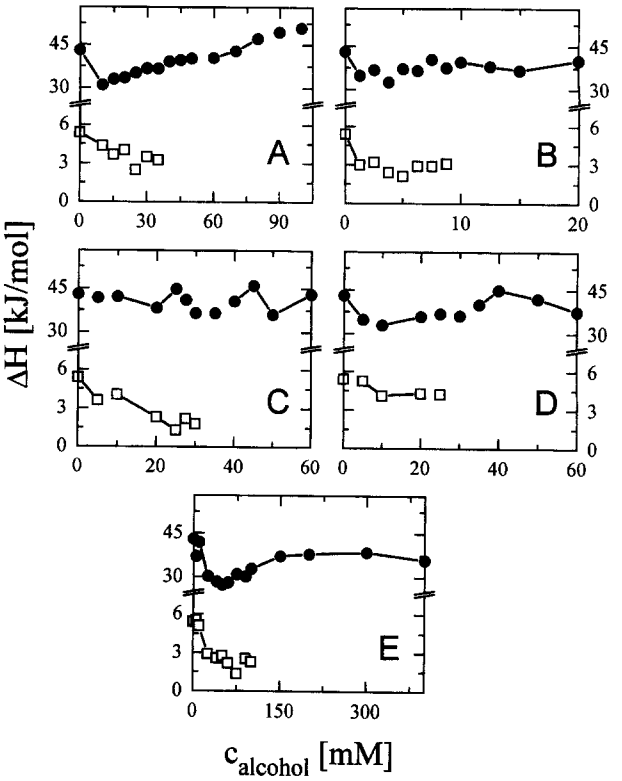

Fig. 6. The enthalpy of dipalmitoylphosphatidylcholine pretransition (□) and chain-melting phase transition (●) as a function of the bulk alcohol concentration. A, 1-pentanol; B, 1-hexanol; C, 2-hexanol; D, 3-hexanol and E, 1,2-hexanediol.

$c_{L_{\beta i}} = 35 \pm 5$ mM, for 3-hexanol $c_{L_{\beta i}} = 25 \pm 5$ mM and for 1,2-hexanol $c_{L_{\beta i}} = 100 \pm 20$ mM.

Within experimental error, the effect of intermediate chain alcohols on the pretransition temperature of DPPC is thus quite similar to that observed in ethanol-DPPC mixtures. Maximum lowering of the pretransition temperature depends strongly on the alcohol used, however: in the presence of 1-pentanol the lowest measured pretransition temperature is 24.2° C, for 1-hexanol one gets 23.8° C, for 2-hexanol 23.2° C, for 3-hexanol 25.1° C and for 1,2-hexanediol 20.6° C.

In Fig. 5 the chain-melting phase transition temperature is also seen to decrease linearly with the bulk alcohol concentration over the whole concentration range studied, even for concentrations exceeding $c_{L_{\beta i}}$. The lowest measured chain-melting phase transition temperature of DPPC, 35° C, therefore, always corresponds to the highest alcohol concentration used.

Fig. 6 shows the measured DPPC pretransition and chain-melting phase transition enthalpies in the presence of the investigated alcohols. It is seen, that all investigated alcohols affect the enthalpy of the pretransition in very much the same way as ethanol. At low alcohol concentrations this enthalpy decreases slightly with the bulk alcohol concentration. If alcohol concentration exceeds approximately $c_{L_{\beta i}}/2$, however, the enthalpy gets smaller until the disappearance of pretransition at $c_{L_{\beta i}}$. The halfwidth of the DPPC pretransition is approx. 2.5° C, independent of the alcohol concentration. Only in 4–8 mM 1-hexanol solutions this value increases to 4° C.

The enthalpy of the chain-melting phase transition of DPPC is 43 kJ/mol in water. In the presence of low

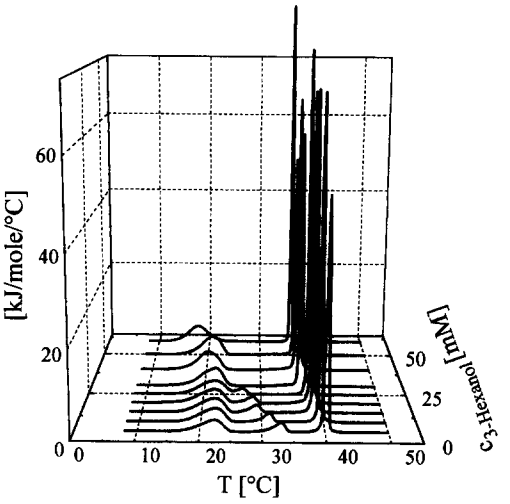

Fig. 7. Original calorimetric scans with DPPC in various 3-hexanol solutions in water. The samples were kept at 4° C for 7 months before the experiments.

Table 1
Alcohol concentration, $c_{L_{\beta i}}$, that induces complete DPPC interdigitation as measured by different methods

Alcohol	Chain length		$c_{L_{\beta i}}$ (FS) (mM)	Fluorescent dye	$c_{L_{\beta i}}$ (other methods) (mM)	Method and reference
	n_c	$n_{c,app}$				
Methanol	1	1	3000 ± 500	CF	2500	[7]
Ethanol	2	2	1100 ± 100	CF, PERho	1100 ± 100	X-ray, DSC
1-Propanol	3	3	400 ± 50	CF	330 ± 20	[7,11]
1-Butanol	4	4	180 ± 20	CF	120 ± 10	DSC [10]
2-Butanol	4	3.6	130 ± 20	PERho		
1-Pentanol	5	5	60 ± 5	CF	35 ± 5	DSC
2-Pentanol	5	4.5	75 ± 10	CF, PERho		
3-Pentanol	5	4.3	70 ± 10	PERho		
1-Hexanol	6	6	50 ± 10	CF	10 ± 2	X-ray, DSC
2-Hexanol	6	5.5	50 ± 10	CF, PERho	35 ± 5	X-ray, DSC
3-Hexanol	6	5.3	60 ± 20	PERho	25 ± 5	X-ray, DSC
Diol						
1,2-Pentanediol	5		240 ± 100	PERho		
1,2-Hexanediol	6				100	DSC

The column labeled 'FS' gives the values of $c_{L_{\beta i}}$ as determined by the membrane permeability or PERho-fluorescence studies with the given fluorescent dye; DSC denotes the results of differential scanning calorimetry data. All $c_{L_{\beta i}}$ values were evaluated at room temperature. They are related to 5 mM DPPC concentration as used for the DSC measurements. $n_{c,app}$ is the apparent alcohol chain-length (see Discussion).

amounts of alcohol this value decreases to approx. 32 kJ/mol. At higher alcohol concentrations, however, the enthalpy of the chain-melting transition of DPPC again increases to approx. 45 kJ/mole or even to 53 kJ/mol in a 100 mM 1-pentanol solution.

In accord with previously published ΔT_m values for DPPC and ethanol [14] or 1-butanol [10] the halfwidth of the chain-melting phase transition of DPPC increases to approx. 0.6° C in all investigated aqueous alcohol solutions.

The subtransition of DPPC was investigated in more detail for the 3-hexanol solutions of different bulk concentrations. For this purpose, lipid samples were allowed to equilibrate for 7 months at 4° C. Fig. 7 shows the results of the observed first DSC heating scans. After such an equilibration period, the subtransition temperature of DPPC in water is 22.8° C [21]. This value is unaffected by a 3-hexanol concentration of 30 mM, the corresponding enthalpy then being approx. 16 kJ/mol. Higher concentrations of 3-hexanol decrease the subtransition temperature of DPPC linearly to 17.8° C (in 60 mM 3-hexanol), the concomitant subtransition enthalpy being 21 kJ/M. After 20 h of equilibration at 4° C the subtransition temperature of DPPC for the samples with 40 mM 3-hexanol is 18.5° C and the corresponding enthalpy is 12 kJ/mol.

3.4. X-ray diffraction

X-ray diffraction was used to determine the repeat distance, d_r, of DPPC bilayers in the presence of an aqueous alcohol solution. In excess water, one has d_r = 6.37 ± 0.03 nm [22,23]. Complete hydrocarbon interdigitation diminishes this value to approx. 4.9 nm [15]. X-ray diffraction thus unambiguously confirms the hydrocarbon interdigitation.

It would, however, be a formidable task to determine various $c_{L_{\beta i}}$ values via X-ray diffraction measurements. In this study, X-ray diffraction was thus used solely to control the values obtained by other described methods. Selected positions in the phase diagram only and just for some alcohols were checked in order to ensure that DPPC was indeed in the $L_{\beta i}$-phase for all alcohol concentrations exceeding $c_{L_{\beta i}}$ and in the $L_{\beta'}$-phase for alcohol concentration below $c_{L_{\beta i}}$.

Table 1 lists the corresponding values. Except for 1-hexanol, where a considerable deviation was uncovered, the spectroscopic results agree well with those obtained from the X-ray diffraction data.

4. Discussion

4.1. Membrane permeability

CF fluorescence intensity is concentration-dependent. For the dilute solutions with CF concentrations below 0.1 mM, the CF fluorescence intensity increases linearly with the CF concentration. In the more concentrated CF solutions ($c \geq 1$ mM), the CF fluorescence is self-quenched. Our experiments thus always started with a highly concentrated –and thus non-fluorescent –CF solution confined into the vesicle interior. An increase of CF fluorescence was therefore always diagnostic of the CF passage from the inside of DPPC vesicles into the bulk subphase. The temporal evolution of the resulting fluorescence was thus

taken to be proportional to the efflux of CF molecules through the bilayer membrane.

Boni et al. [24] have pointed out that the unilamellar vesicles with a diameter below $100 \cdots 200$ nm rapidly form a stack of extended interdigitated sheets when brought into contact with ethanol at concentrations sufficient to induce chain interdigitation. Komatsu et al. [25] have also reported that the sonicated SUV with a diameter of 54 ± 26 nm (according to the QELS measurements) aggregate and/or fuse in the presence of sufficiently concentrated ethanol. According to the latter authors, however, the extruded vesicles remain stable when suspended in different ethanolic solutions in spite of the fact that their lipid chains may interdigitate. All these authors agree in the belief that the minimal ethanol concentrations necessary for the complete induction of hydrocarbon interdigitation in DPPC decreases with increasing vesicle size.

Fusion or even destruction of lipid vesicles would lead to an abrupt release of all 'encapsulated' CF from the vesicles within minutes, as observed after adding Triton X-100 to the lipid suspension. To test this, we have measured the CF fluorescence at half hour intervals for twelve hours starting after the alcohol injection. No extraordinary increase in the CF fluorescence intensity was detected. We therefore conclude that the sonication-induced defects on SUVs, which were responsible for the limited stability of such vesicles in the presence of alcohol as reported in previous studies, have annealed during our long equilibration. This made our sonicated vesicles similar to the extruded vesicles. Due to our experimental method we cannot rule out, however, that some vesicle aggregation might have occurred upon the addition of ethanol as such aggregation would not have altered our results.

It is to be expected that complete hydrocarbon interdigitation will increase the permeability of DPPC membranes. Our results confirm this assumption. By measuring the temporal development of CF fluorescence it is even possible to calculate the permeability change due to the DPPC-chain interdigitation. CF molecules are thus estimated to leak through the interdigitated bilayers approx. 100-times more rapidly than through the standard DPPC bilayers (to be published). This is in qualitative agreement with the results of the published proton permeability studies for the interdigitated PC bilayers [26].

In a recent article [14] we have detected the coexistence of $L_{\beta'}$-and $L_{\beta i}$-phases in DPPC vesicles. The lowest ethanol concentration at which an interdigitated phase was identified was approx. equal to $c_{L_{\beta i}}/2$. In our CF fluorescence measurements we have fairly regularly measured a fluorescence maximum exactly at this concentration, independent of the alcohol used. We thus conclude that all alcohols induce a phase coexistence region ($L_{\beta'} + L_{\beta i}$) in the DPPC bilayers. CF fluorescence maximum is hence indicative of the membrane destabilization during the course of bilayer interdigitation.

4.2. PERho fluorescence

PERho fluorescence is polarity-sensitive. The intensity of this marker fluorescence is thus expected to increase when alcohol is present in the headgroup region. Zhang et al. [10] have shown that 1-butanol partitions between the bulk and the lipid phase with approx. 3–4-times higher affinity for the interdigitated gel phase compared to the non-interdigitated phase. According to the latest phase diagram [14] of ethanol/DPPC/water mixtures we thus expect the partitioning of alcohols in the headgroup region to involve three regimes:

(1) In the non-interdigitated gel phase the local alcohol concentration in the headgroup region increases slowly with increasing bulk alcohol concentration.
(2) In membranes with partially interdigitated hydrocarbon chains the mean partition coefficient is a function of the bulk alcohol concentration. The local alcohol concentration in the headgroup region, however, now increases faster than in the normal gel-, L_{β}-phase.
(3) For alcohol concentrations exceeding $c_{L_{\beta i}}$, the solute molecules have the highest membrane affinity. Alcohol concentration in the headgroup region still increases linearly with the bulk alcohol concentration, but the gradient with respect to the this concentration is approx. 3-to 4-times higher than in the cases 1 and 2.

These assumptions are mirrored in our results. With some fantasy one can divide each of the given data sets in three regions depending on the bulk alcohol concentration.

Fig. 4, however, also shows the limits of such rationale. For the alcohols with the polar group at the second or third position in the carbon chain, in the low concentration interval (corresponding to item 1), the alcohol accumulation in the headgroup region does not increase but rather decreases the local polarity. Bearing in mind that the alcohol OH-groups are located near the phosphate groups of the phospholipid molecules [17], whereas hydrocarbon chains of the alcohol molecules point towards the membrane interior, this decrease can be explained easily by the presence of the shorter unpolar part of each alcohol in the region that affects the PERho fluorescence. Following this idea, our results suggest this unpolar disturbance to be screened in the interdigitated phase. At the molecular level, this means that in the interdigitated phases enough room exists between the neighboring heads for both nonpolar parts of the 2-or 3-alcohols to stretch themselves into the hydrocarbon surrounding (membrane interior).

4.3. Differential scanning calorimetry

Most of previous information on the phase diagrams of phosphatidylcholine-alcohol mixtures was based, at least partially, on the results of DSC measurements. The following phase behavior of DPPC in the presence of interdigitation-inducers was thus established: relatively dilute alcohol

solutions (concentration values depending on the alcohol used) decrease the chain-melting phase transition temperature as well as the pretransition temperature of DPPC. Upon complete chain interdigitation, which is reached at sufficiently high alcohol concentrations, the pretransition disappears, however. For methanol, ethanol and n-propanol the chain-melting phase transition temperature, T_m, then increases again with increasing alcohol concentration [7]. A further decrease of T_m is measured in the presence of 1-butanol, however [10].

Our results show that 1-pentanol, 1-, 2-, and 3-hexanol, as well as 1,2-hexanediol all decrease the chain-melting temperature approx. linearly with increasing alcohol concentration. This is even true when the gel phase of DPPC bilayers is completely interdigitated, as suggested by the X-ray diffraction measurements. This lack of biphasic effect implies that these alcohols partition more effectively into the L_α-phase than into the $L_{\beta i}$-phase. Such behavior of the chain-melting phase transition temperature has also been reported for the aqueous 1-octanol and 1-decanol solutions [27,28]. Despite this similarity the situation is different in both cases, however: In contrast to 1-hexanol, 1-octanol does not induce chain interdigitation in DPPC.

The effects of alcohols on lipid bilayers can thus be divided into two classes:

First, the presence of polar OH-groups in the headgroup region increases the effective lipid headgroup volume and induces a lateral expansion in the interfacial region. This lowers the pretransition temperature and, finally, provokes lipid chain interdigitation. The quantitative strength of this effect is nearly independent of alcohol chain-length.

Second, the hydrocarbon chains of alcohol molecules disorder lipid chains. This is reflected in the decreased chain-melting phase transition temperature. Sufficiently high alcohol concentrations may also induce a non-lamellar phase (data not shown).

Headgroup effects dominate for short-chain alcohols. Such alcohols, consequently, can induce complete hydrocarbon interdigitation in DPPC bilayers. Alcohols with more than seven or eight carbon atoms per chain are inefficient in this respect since the interfacial expansion caused by the polar alcohol segments is fully absorbed by the chain-induced repulsion in the hydrocarbon region of the lipid bilayer.

Following this argument we have used the disappearance of lipid pretransition as an unambiguous indication for the induction of the complete hydrocarbon interdigitation.

4.4. Connection between $c_{L_{\beta i}}$ and alcohol chain length

The chief motivation for this work was to determine the relation between the interdigitation-inducing alcohol concentrations and the alcohol chain-length and -type. Since the phase behavior of DPPC in the suspensions containing small organic molecules is rather well understood we also wanted to predict experimental results with reasonable accuracy.

In the first approximation, we have assumed that the decisive factor for the induction of chain interdigitation is the number of alcohol molecules in the headgroup region. Our assumption was thus the comparison of the partition coefficient for an alcohol with n_c carbon atoms per chain and the bulk concentration of this alcohol that induces the complete chain interdigitation in the DPPC membranes.

Such partition coefficient for the liquid lamellar phase, K_p, can be calculated from the free energy of inducer transfer from the bulk water into the lipid subphase [29]. According to Cevc and Marsh [30] this (molar) free energy of transfer for the straight-chain alcohols and DPPC is given by:

$$\mu_{trans}/RT = -0.9 - 1.11 n_c = \log(K_p) \quad (1)$$

The partition coefficient is then $K_p = \exp(\mu_{trans}/RT)$. R is the universal gas constant and T is the temperature. The free energy of transfer between the bulk water and the DPPC bilayer subphase thus depends linearly on the number of carbon atoms in each alcohol molecule, n_c. The actual alcohol concentration in the interfacial region is hence given by the Boltzmann factor containing the above expression. Starting with the best available data are used [2], (and making corrections for the values obtained by the fluorescence measurements whenever justified by the DSC or X-ray diffraction results) one gets by the least-squares fit:

$$c_{L_{\beta i}}(n_c) = 2.31 \cdot 10^4 \, e^{-0.9 - 1.11 n_c} \ (\text{mmol/l}) \quad (2)$$

Fig. 8 illustrates the quality of this fit. The measured and the calculated data are nearly identical for $1 < n_c < 5$.

Due to its high affinity for the organic phase, 1-hexanol takes an exceptional position in the short-chain alcohol series. This alcohol probably partitions immediately into the boundary region of the bilayer. It is, therefore, important to know and control the total lipid concentration to get reliable $c_{L_{\beta i}}$ values in harmony with Eq. (2). In other words, $c_{L_{\beta i}}$ must be related to the phospholipid concentration whenever alcohols with a strong preference for the lipid phase are used. (An indirect support to this assumption comes from the observation that $c_{L_{\beta i}}$ for the short-chain alcohols decreases with increasing phospholipid chain-length whereas for 1-hexanol this value remains essentially constant, independent of lipid chain length (unpublished data).)

[2] We are aware of the problems arising from the combination of the data pertaining to unilamellar and multilamellar vesicles. In spite of this we believe that such combination is justified by the good correlation between the results obtained with different methods. The restriction to one type of the experimental system (SUV or LMV), moreover, would merely change the constants in our fit but leave the basic conclusion unaltered.

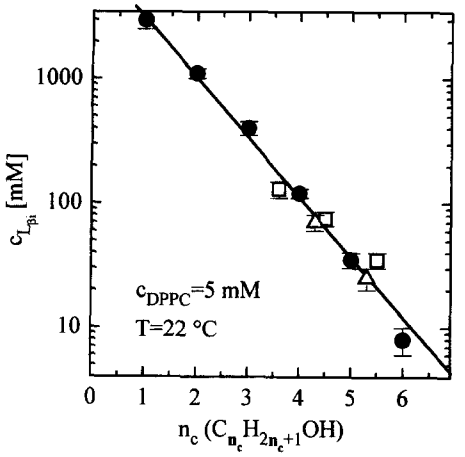

Fig. 8. The effect of apparent alcohol chain length, $n_{c,app}$, on the limiting interdigitation-inducing alcohol concentration, $c_{L_{\beta i}}$. The straight line is described by $K = 2.31 \cdot 10^4 \exp(-0.9 - 1.11n)$ mM, as given in the text. ●, 1-alcohols; □, 2-alcohols and △, 3-alcohols. For the latter two types of alcohols, the concept of apparent chain-length was used to get the appropriate $n_{c,app}$-values (see the text).

We have also investigated the effect of varying the position of the polar OH-group on the value of $c_{L_{\beta i}}$. In order to explain the resulting $c_{L_{\beta i}}$ changes quantitatively we have used the concept of the apparent chain-length which has proven useful, for example, for explaining the wetting power of various alcohols. In this approach, which has first been used in the combination with alcohols by Traber [31] one assigns an apparent chain-length of $n_{c,app} = 3.55$ to 2-butanol, $n_{c,app} = 4.3$ to 3-pentanol, $n_{c,app} = 4.5$ to 2-pentanol, $n_{c,app} = 5.3$ to 3-hexanol, and $n_{c,app} = 5.5$ to 2-hexanol.

The results of such a procedure, together with the original data, are given in Fig. 8. Good agreement is achieved for the 2-and 3-pentanol as well as for the 3-hexanol solution. The $c_{L_{\beta i}}$ values of 2-butanol and 2-hexanol are less satisfactory, however. This may be due to the steric constraints in the latter molecule which are likely to be particularly eminent after the partitioning of alcohol into the interfacial region.

It is also noteworthy that the rather long equilibration period is crucial for the success of the phase-transition studies with the mixtures of phospholipids and intermediate-or long-chain alcohols. It is notable that for the alcohols with the longest equilibration period, the agreement between the results of Eq. (2) and the measured $c_{L_{\beta i}}$ value is the best.

To date, no corresponding data of the apparent chain length have been proposed for diols. By comparing our $c_{L_{\beta i}}$ values for such bivalent alcohols with the predictions of Eq. (2), the values of $n_{c,app} = 3.3$ and $n_{c,app} = 4.1$ can be assigned to 1,2-pentanediol and 1,2-hexanediol, respectively. Partition coefficients for these two diols can thus also be estimated.

5. Summary

The effects of amphiphilic solutes from the class of alcohols on the lipid chain-melting phase transition temperature as well as on the pretransition and subtransition of dipalmitoylphosphatidylcholine suspensions were shown experimentally to increase monotonously, but non-linearly, with the bulk solute concentration. The resulting phase transition temperature shift was concluded to be proportional to the concentration of alcohol molecules in the interfacial region. This concentration, moreover, was related to the (apparent) alcohol chain-length and its effects on the alcohol partitioning into the lipid bilayer subphase. The latter was described quantitatively by a simple exponential law. Interdigitation was found to depend crucially on the lipid bilayer disturbance by the alcohol molecules partitioned into the headgroup region, provided that this perturbation is stronger than the disorder caused by the alcohol chain in the hydrocarbon region.

6. Addendum

After this work has been submitted for publication we became aware of the paper by Elizabeth S. Rowe and Janet M. Champion [32], which presents similar, but less detailed results.

Acknowledgements

We thank S. Kirchner and U. Vierl for their support with X-ray diffraction experiments and analysis. This work is supported by the Deutsche Forschungsgemeinschaft (grants Ce 2/2-2) and SFB 266 project C8.

References

[1] Cunningham, B.A., Tamura-Lis, W., Lis, L.J. and Collins, J.M. (1989) Biochim. Biophys. Acta 984, 109–112.
[2] Ranck, J.L. and Tocanne, J.F. (1982) FEBS Lett. 143, 175–178.
[3] Boggs, J.M. and Tmmler, B. (1993) Biochim. Biophys. Acta 1145, 42–50.
[4] Slater, J.L. and Huang, C.-H. (1988) Prog. Lipid Res. 27, 325–359.
[5] Rowe, E.S. (1983) Biochemistry 22, 3299–3304.
[6] McIntosh, T.J., McDaniel, R.V. and Simon, S.A. (1983) Biochim. Biophys. Acta 731, 109–114.
[7] Rowe, E.S. (1985) Biochim. Biophys. Acta 813, 321–330.
[8] Nambi, P., Rowe, E.S., and Macintosh, T.J. (1988) Biochemistry 27, 9175–9182.
[9] Rowe, E.S. and Cutrera, T.A. (1990) Biochemistry 29, 10398–10404.
[10] Zhang, F. and Rowe, E.S. (1992) Biochemistry 31, 2005–2011.
[11] Veiro, J.A., Nambi, P., Herold, L.L. and Rowe, E.S. (1987) Biochim. Biophys. Acta 900, 230–238.
[12] Rowe, E.S. (1987) Biochemistry 26, 46–51.
[13] Ohki, K., Tamur, K. and Hatta, I. (1990) Biochim. Biophys. Acta 1028, 215–222.
[14] Vierl, U., Löbbecke, L., Nagel, N. and Cevc, G. (1994) Biophys. J. 67, 1067–1072.

[15] Simon, S.A. and McIntosh, T.J. (1984) Biochim. Biophys. Acta 773, 169–172.
[16] Janes, N., Hsu, J.W., Rubin, E. and Taraschi, T.F. (1992) Biochemistry 31, 9467–9472.
[17] Chiou, J.-S., Krishna, P.R., Kamaya, H. and Ueda, I. (1992) Biochim. Biophys. Acta 1110, 225–233.
[18] Bartucci, R., Páli, T. and Marsh, D. (1993) Biochemistry 32, 274–281.
[19] Rowe, E.S., Fernandes, A. and Khalifah, R.G. (1987) Biochim. Biophys. Acta 905, 151–161.
[20] Nagel, N.E., Cevc, G. and Kirchner, S. (1992) Biochim. Biophys. Acta 1111, 263–269.
[21] Ruocco, M.J. and Shipley, G.G. (1982) Biochim. Biophys. Acta 691, 309–320.
[22] Wiener, M.C., Suter, R.M. and Nagle, J.F. (1989) Biophys. J. 55, 315–325.
[23] Kirchner, S. and Cevc, G. (1994) Europhys. Lett. 28, 31–36.
[24] Boni, L.T., Minchey, S.R., Perkins, W.R., Ahl, P.L., Slater, J.L., Tate, M.W., Gruner, S.M. and Janoff, A.S. (1993) Biochim. Biophys. Acta 1146, 247–257.
[25] Kumatsu, H., Guy, P.T. and Rowe, E.S. (1993) Chem. Phys. Lipids 65, 11–21.
[26] Zeng, J., Kirk, E.S. and Chong, P.L.-G. (1993) Biophys. J. 65, 1404–1414.
[27] Eliasz, A.W., Chapman, D. and Ewing, D.F. (1976) Biochim. Biophys. Acta 448, 220–230.
[28] Kaminoh, Y., Nishimura, S., Kamaya, H. and Ueda, I. (1992) Biochim. Biophys. Acta 1106, 335–343.
[29] Diamond, J.M. and Katz, Y. (1974) J. Membr. Biol. 17, 121–154.
[30] Cevc, G. and Marsh, D. (1987) Phospholipid Bilayers, pp. 30–34, Wiley, New York.
[31] Traber, R.H. (1992) Textilveredelung 27, 312–316.
[32] Rowe, E.S. and Champion, J.M. (1994) Biophys. J. 67, 1888–1895.