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INVESTIGATIONS ON THE ORDER-DISORDER BEHAVIOUR OF VARIOUS PHOSPHOLIPIDS OF NATURAL AND SYNTHETIC ORIGIN BY OPTICAL AND CALORIMETRIC TECHNIQUES

K. Brandenburg, U. Seydel

Research Institute Borstel, Institute of Experimental Biology and Medicine, Division of Biophysics, D-2061 Borstel, Federal Republic of Germany

Abstract

The regulation of metabolic processes in cells (e.g. active and passive transport, enzymatic activity) is highly governed by the ccmposition of the cell membrane usually composed as a bilayer of phospholipids. Under physiological conditions the membrane is - at least partially - in the liquid crystalline state. This is achieved by biosynthesis of appropriate phospholipids varying in the kind of polar head-groups and in length and degree of saturation of the hydrocarbon chains in dependence on the ambient temperature and milieu.

Starting with pure phospholipids (PL) of synthetic and natural origin the thermodynamic states of physiological relevant PL's and PL-mixtures and the thermotropic gel to liquid crystalline phase transition were investigated. From a combination of DTA and different optical techniques statements are deduced about structure and orientation of lipid molecules, microviscosity of the lipid bilayer, cooperativity of the phase transition, and permeability of the membrane.

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Introduction

Metabolic processes of the cell are influenced by structural and functional properties of the cell membrane. Thus, the physical composition and the spatial arrangement of the membrane components and their behaviour in respect to diffusion and transport processes to interactions with ions, proteins, and drugs are of high interest

The order-disorder or gel to liquid-crystalline phase transition is one of the most important parameters for the physical investigation of lipids. The temperature T_c of this transition - characterized by melting of the lipid hydrocarbon chains - and the enthalpy change ΔH_c associated with it are special features of phospholipids, but also lipopolysaccharides. It depends on several parameters - as for instance the pH-value, the concentration of certain divalent ions and for bacteria the growth temperature, which has be observed to influence the composition especially of the outer membrane (consisting of phospholipids, proteins, and lipopolysacharide rather than the cytoplasmic membrane (consisting mainly of phospholipids) of E. coli in a strong manner /1-3/.

The present paper is concerned with the order-disorder transition of physiological relevant phospholipids (PL). For this purpose pure PL's and PL-mixtures of synthetic and natural origin, namely phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylglycerol (PG), being the main components in many biological membranes, were investigated. The zwitterionic character of PC's and PE's are jointly responsible for the enzymatic activity, as for example for α-1,2- mannosidase /4/. Additionally, however, the special characters of the polar head groups give rise to differen-

ces in their ability to catalyse enzymatic processes. PE's exhibit great activity regarding the synthesis of bacterial lipopolysaccharides (LPS) whereas PC's are completely inactive /5/. Moreover, the magnitude of activity depends on the kind of hydrocarbon chains, i.e. length and saturation degree.

In addition to these zwitterionic PL's the negatively charged PG was employed, playing a prominent role in bacterial membranes /6/.

The investigations were performed with different optical (fluorescence and fluorescence polarisation) and calorimetric techniques with the aim of getting information about structure and orientation of the PL molecules in the different phases and of deriving parameters like state of order, microviscosity, cooperativity of the phase transition and permeability for dyes.

Materials and methods

As lipid material dimyristoyl-L-α-phosphatidylcholine (DMPC), dipalmitoyl-L-α-phosphatidylcholine (DPPC), distearoyl-L-α-phosphatidylcholine (DSPC), dilauroyl-L-α-phosphatidylethanolamine (DLPE), dipalmitoyl-L-α-phosphatidylethanolamine (DPPE), dipalmitoyl-L-α-phosphatidylglycerol (DPPG), and the lipid L-α-cephalin (natural phosphatidylethanolamine) from E. coli purchased from Sigma (München F.R.G.) in high purity (> 98%) were used without further purification. The preparation of the lipid dispersions for optical measurements was done as described before /7/ with following exceptions: In some cases the sonication time was varied, in other cases the lipids were vortexed for 2 to 3 minutes rather than sonicated.

The calorimetric measurements were performed in a heat-flow DTA device based on a microcalorimeter described earlier /8/. The instrument was calibrated with decanoic acid (T_c = 31.3 °C, ΔH_c = 27.7 kJ), dodecanoic acid (T_c = 43.7 °C, ΔH_c = 36.6 kJ), tetradecanoic acid (T_c = 53.8 °C, ΔH_c = 44.9 kJ), and hexadecanoic acid (T_c = 62.6 °C, ΔH_c = 55.2 kJ) as reference samples. The phase transition temperature and its enthalpy are determined by the midpoint of the endotherm peak and the area of the endotherm, respectively. From this calibration a sensitivity of 0.1 mJ/s was deduced. Heating-rates were adjusted to 1 - 2 K min^{-1}, and each DTA run was repeated at least twice. The sample pans were filled with 1 to 4 mg of lipid, being dissolved in chloroform and evaporated in a nitrogen stream. The resulting films were vortexed in 20 to 100 µl of water or of 10 mM Tris/100 mM NaCl buffer, in some cases in phosphate buffer at different pH, and vortexing was usually perfomed at $T > T_c$. The reference pan was filled with the same amount of the above fatty acids as well as with suitable amounts of water for balancing the thermal capacity.

For the measurement of fluorescence polarisation the dye diphenylhexatriene (DPH) purchased from Fluka, Buchs,Switzerland, was used. From the stock solution of $2 \cdot 10^{-3}$ M DPH in tetrahydrofurane 100 µl were mixed with 10 ml water or with phosphate buffer at different pH and vigorously stirred for 0.5 hour. For labelling of lipids, 2 ml DPH solution was incubated with 100 µl lipid dispersion at $T > T_c$ for 45 min. The dye not bound to the lipid was removed in some cases by gel filtration on a sephadex column G-75. Steady-state fluorescence intensities $I_{\parallel}$ and $I_{\perp}$ were recorded on an Aminco-Bowman spectrophotofluorometer at an exciting wavelength of 360 nm and emission wavelength of 425 nm in dependence on temperature at heating-rates of $3 \cdot$ K min^{-1}.

As fluorophores for fluorescence measurements with non-polarized light N-phenylnaphtylamine (NPN) and anilonaphtalensulfonat (ANS) obtained from Fluka were used. NPN (excitation wavelength λ_{ex} = 360 nm, emission wavelength λ_{em} = 425 nm) was dissolved in a methanolic solution (3 %) to give a final concentration of $1 \cdot 10^{-5}$ M. ANS (λ_{ex}=360 nm, λ_{em}= 470 nm) was dissolved in water to a final concentration of $1 \cdot 10^{-5}$ M. The labelling of the lipid dispersions was performed as described for DPH with the only difference, that no incubation was necessary or only for a short period of 5 to 10 min.

In some cases 90° light-scattering measurements 400 nm/400 nm without addition of fluorophores were carried out.

Permeability measurements were performed with the dyes 6-carboxyfluorescin (6-CF, λ_{ex} = 490 nm, λ_{em} = 520 nm) obtained from Sigma and with hydroxyterephtalicacid (HTA , λ_{ex} = 314 nm, λ_{em} = 425 nm) synthesized in our laboratory /9/. In contrast to the other dyes used, these fluorophores show a very high selfquenching and do not bind to lipids. Therefore they are extremely suitable for studying their release from liposomes or cells /10/. For the entrapment of dye molecules in lipid vesicles a $1 \cdot 10^{-4}$ dye solution in phosphate buffer (10 mM) was sonicated with the dried lipid film (~1mg ml^{-1}) at $T > T_c$. The dye molecules not entrapped in vesicles were removed by gel-filtration on a sephadex G-75 column.

Preparation of specimens for electron microscopy was as follows: The lipid suspensions were negatively stained with 2 % aqueous solution of potassium phosphotungstate and placed on formvar-coated copper grids. Specimens were examined in a Philips EM 301 operating at 80 kV

and micrographs were obtained by standard procedures. Gas-chromatographic analysis of the fatty acids of natural cephalin was performed on the Hewlett-Packard GC/MS device HP 5985. The cleavage of the hydrocarbon chains was done by acidic alkaline hydrolysis and conversion to fatty acid methylester by addition of diazomethane.

Mathematical treatment

Fluorometric and 90° light-scattering measurements were evaluated by either direct plotting or by taking the slope dI/dT of fluorescence intensity I versus temperature T. Moreover, the van't Hoff enthalpy of the phase transition,

$\Delta H_{vH} = 4 \cdot R \cdot T_c^2 \left(\frac{d\theta}{dT}\right)_{\theta = 1/2}$ (R gas constant, T_c temperature main transition, θ molar ratio melted to non-melted lipid) was calculated from NPN curves as well as the true enthalpy ΔH_{cal} from calorimetric measurements. The expression $\xi = \Delta H_{vH} / \Delta H_{cal}$ giving the size of the cooperative unit (molecules) - was used as a measure of the cooperativity of the phase transition /11/. The value for ξ approaches unity for vanishing and infinity for high cooperativity.

In DTA scans of mixed samples of lipids A and B with masses m_A, m_B respectively, the enthalpy change was calculated according to

$\Delta H_{mix} = \frac{m_A \Delta H_A + m_B \Delta H_B}{m_A + m_B}$ and compared with the measured value ΔH_{cal}.

Measurements of fluorescence polarisation were evaluated according tc newest available approximations essentially presented in /12-14/. From the steady-state Perrin equation the fluorescence anisotropy

(1) $r = r_\infty + \frac{(r_0 - r)\,\eta_a}{C(r) \cdot T \cdot \tau}$ can be calculated from steady-state measurements via $r = \frac{I_\parallel - I_\perp}{I_\parallel + 2 \cdot I_\perp}$;

r_0 limiting value of r at infinite viscosity (r_0 = 0.362 for DPH), r_∞ infinitely slow decaying component of fluorescence anisotropy or residual fluorescence anisotropy for $t \longrightarrow \infty$, i.e. r_∞ = 0 for an isotropic medium; C (r) parameter depending on molecular shape and location of transition dipoles of rotating fluorophores; τ lifetime of excited state; T absolute temperature; η_a microviscosity of the lipid sensed by the probe. Several investigations indicate that the factor $C(r) \cdot T \cdot \tau$ can be approximated by a value of 2.4 P (poise) /15/. r_∞ is related to r by the empirical relation: $r_\infty = 4/3 \cdot r - 0.1$ for $0.13 \leq r \leq 0.28$, for values beyond this range r_∞ can be taken from the plot in the paper of Blitterswijk et al /13/.

An estimated value for the microviscosity of the bulk lipid is given by

(2) $\bar{\eta}' = \eta_a/(1 - r_\infty / r)$, and the "true" microviscosity by

(3) $\bar{\eta} = \bar{\eta}' \frac{(1 - r_\infty / r)}{(1+2\,R\,(r_\infty / r_0)}$ with R = - 0.5 in the case of non-isotropic viscosity of the lipid ($\eta_\perp \gg \eta_\parallel$).

The values for $\bar{\eta}'$ and $\bar{\eta}$ were calculated and compared with approximations presented in literature, especially with that of Shinitzky and Barenholz /15/

(4) $\bar{\eta}_{app} = \frac{2\,P}{0.46 - P}$ (P = polarisation $= \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$),

which results from equation (1) neglecting r_{∞}.

Furthermore, the "order parameter" $S^2(DPH) = r_{\infty}/r_0$ and an estimation of its relative error $\Delta S/S(\%) \sim 50 \cdot (1-2.5\ r_0)^2$ were determined. ln ($\bar{\eta}$) versus 1/T and $d(\ln \bar{\eta})/d(1/T)$ were plotted, the last giving a peak which should be proportional to the heat of transition according to Lentz /15/.

Results

In fig. 1. the NPN fluorescence intensity I_{425} of pure and mixed vesicles prepared from DPPC, DPPG, and DPPE is plotted versus temperature T. DPPG exhibits a pretransition at T = 40 °C which can be related to a structural transformation $L_{\beta}' \rightarrow P_{\beta}'$. A corresponding pretransition appears for DPPC, but seems to be strongly dependent on the details of preparation.

The graph for mixed DPPC/DPPG solutions shows that despite common sonication above T_c (DPPG), i.e. 60 °C, the fusion is incomplete. During the experiments, DPPG behaved somehow confusing with respect to fluctuations of T_c. A more systematic investigation of the DPPG phase transition yielded a strong dependence on pH as illustrated in fig. 2.

In the protonated state a maximum T_c of 54.5 °C can be observed with a sharp rise of I_{425} over a range of 6 °C, whereas in the negatively-charged state the phase transition becomes broader

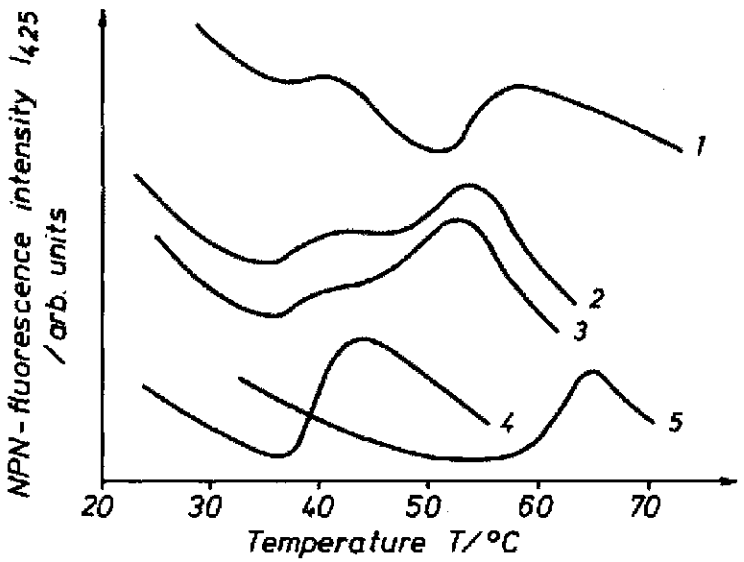

Fig. 1: Fluorometric scans for pure and mixed phospholipid-water dispersions at pH = 6.5
1. DPPC:DPPE 1:1, 2. DPPC:DPPG 1:1, 3. DPPG,
4. DPPC, 5. DPPE

with a pretransition located at approx. 38 °C and a main transition at 44.5 °C. A further lowering of pH caused a flaking-out of particle aggregates making further observations impossible by optical techniques.

A phase separation even stronger than with DPPG-despite common sonication at T = 65 °C - can be diagnosed for the system DPPC: DPPE (fig. 1). This behaviour is in accordance with our observation that it was impossible to get closed vesicles from DPPE. After sonication of DPPE with 6-CF or HTA the attempt of segregating free dye molecules from the lipid by gel filtration failed,

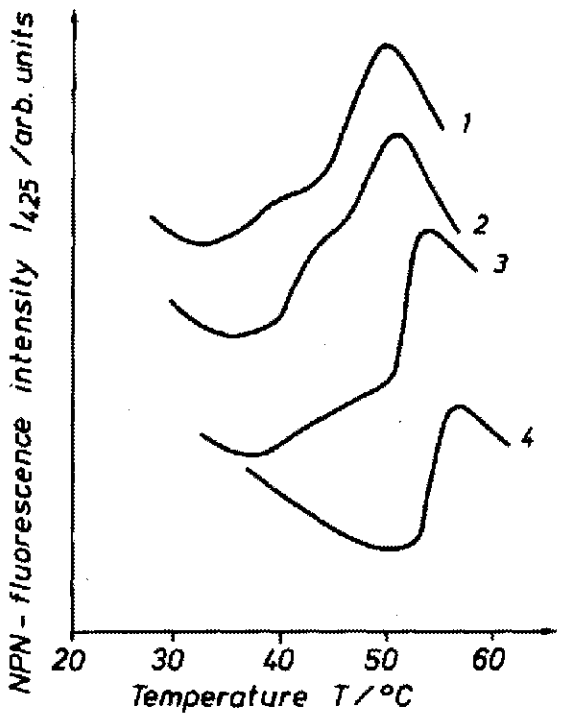

Fig. 2: Fluorometric scans for the DPPG-water system
1. pH = 11, 2. pH = 7, 3. pH = 6, 4. pH = 4

at neutral or acidic pH only one photometer peak appeared which could be assigned to pure dye. In some experiments at pH > 7 two peaks - one assigned to lipid and the other to dye - were observed, but in no case the fluorometric measurement resulted in an increasing release of dye molecules above the phase transition temperature T_c. Electron micrographs of DPPE suspensions (fig. 3a, b) show - in the case of the appearance of only one photometer peak after gel filtration-amorphous and structureless configurations. However, in cases where a lipid peak was observed after gel filtration spherical structures can be stated, indicating a somehow ordered - lammelar or crystalline - but not vesi-

cular structure and being in accordance with the measurement of the DPPE phase transition using other techniques as presented in fig. 4.

Measurements of the release of 6-CF and HTA from vesicles were also perfomed with DPPC, DMPC, DPPG and DLPE. Only for the lecithins a steep increase of dye fluorescence intensity was observed when passing T_c (without figure). From this it can be concluded that also the nature of the polar head groups of phospholipid molecules plays an important role for the geometrical arrangement in membranes.

Furtner experiments with NPN-labelled mixed lipids from DLPE and DPPC or DPPE showed that the system DLPE:DPPE exhibits a very broad transition over a range of approx. 30 °C (fig. 5) even after common sonication at 65 °C indicating the appearance of phase separation. In contrast, the system DLPE:DPPC seems to be more miscible over a wide concentration range as illustrated in fig. 5, demonstrating that beside the dependence on the polar head group there is also a dependence on the hydrocarbon chain length.

The investigations of mixed suspensions of DPPC and natural L-α-phosphatidylethanolamine (cephalin) yielded a nearly complete miscibility after incubation at T = 45 °C. As demonstrated in fig. 6, at increasing concentrations of cephalin the transition becomes broader and less pronounced apparently due to the fact that the hydrocarbon moiety of natural cephalin is a mixture of chains of different length and degree of saturation. This assumption was confirmed by gas-chromatographic analysis, which resulted in the follo

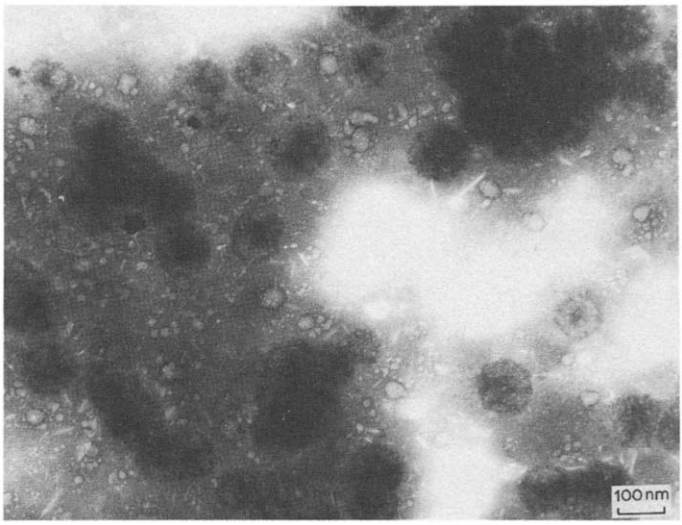

Fig. 3 a

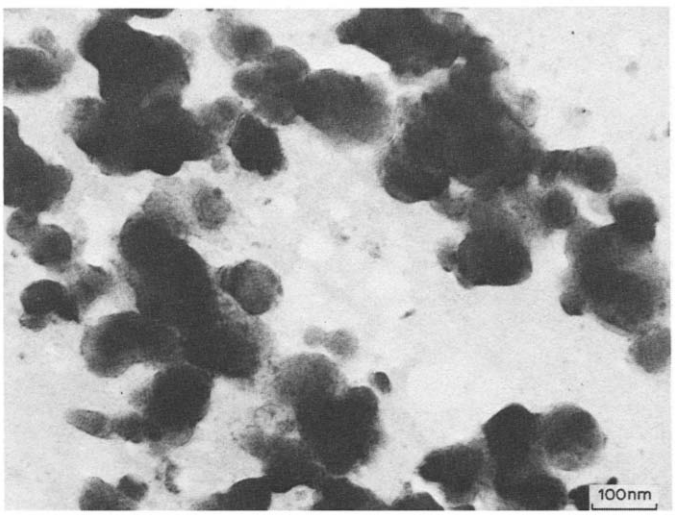

Fig. 3 b

Electron micrographs for DPPE preparations stained wi
2 % potassium phosphotungstate

a) after standard preparation
b) as in a) but additionally gel-filtrated

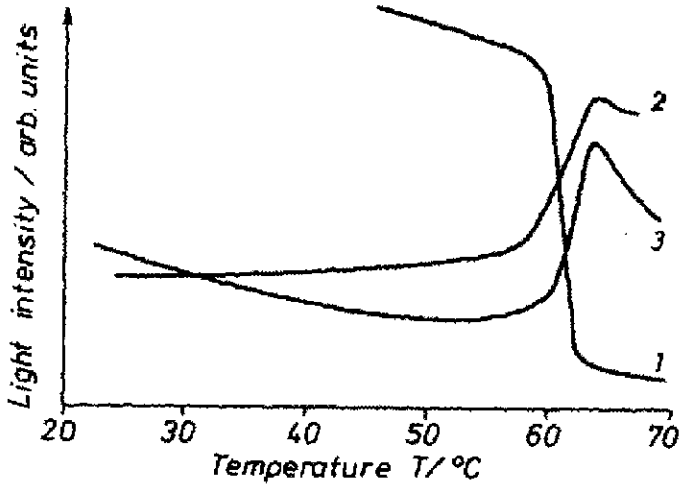

Fig. 4: Light scattering and fluorometric scans of the DPPE-water system
1. 90° - light scattering 400 nm/ 400 nm
2. Fluorometric scan with ANS
3. Fluorometric scan with NPN

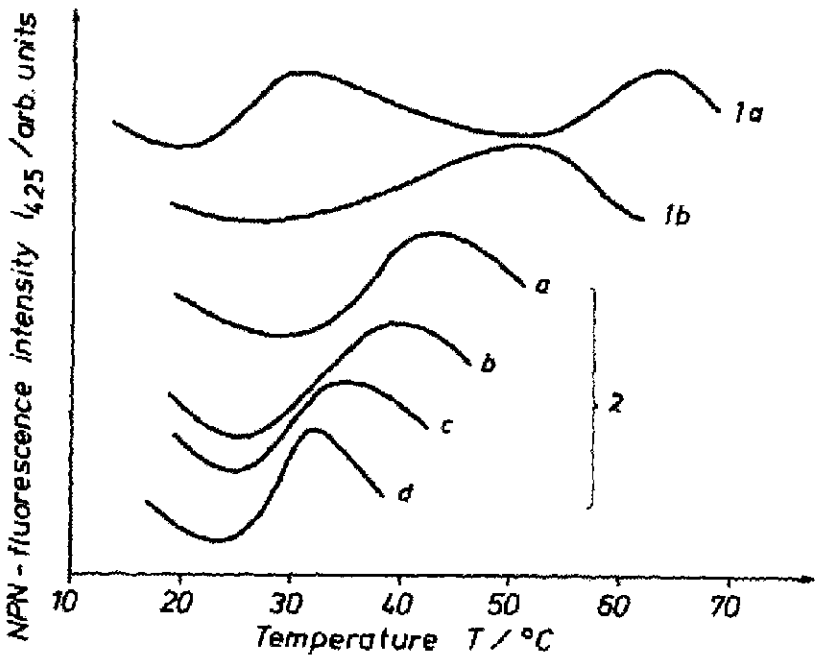

Fig. 5: Fluorometric scans of mixed phospholipid-water dispersions
1. DLPE:DPPE 1:1, 2. DLPE:DPPC
a. separate sonication a. 1:5 c. 5:1
b common b. 1:1 d. 1:0

wing distribution of fatty acids: C 16:0 (relative intensity 1 cis-9,10-methylen-C 16 (0.63) and cis-10,11-methylen-C 18 (0.16 and C 14:0 (0.013).

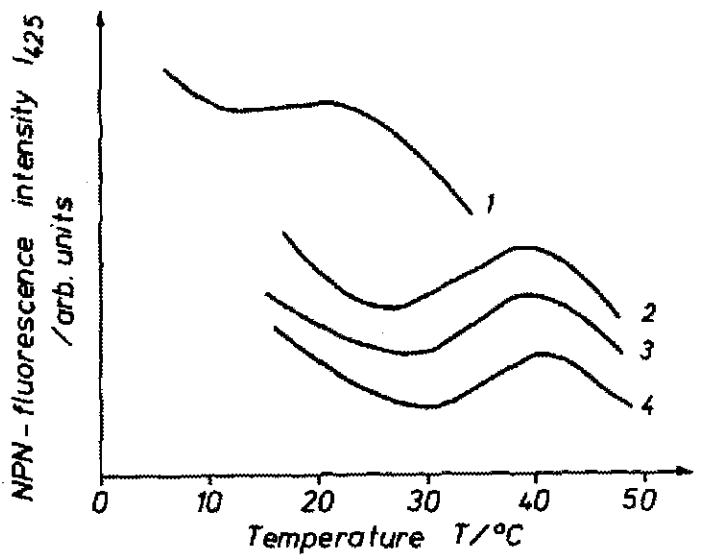

Fig. 6: Fluorometric scans of the DPPC:cephalin-water syster 1. 0:1, 2. 1:1, 3. 2:1, 4. 3:1

In fig. 7 dI/dT for various samples is plotted versus T leading a more appropriate representation of the peak locus.

The results from the DTA measurements for pure and mixed PL's a illustrated in figures 8 and 9 a. For DPPG (fig. 8) at pH - 7, lorimetric scans show a pretransition similar to that observed optical measurements (fig.2). At lower pH a broadening or a spl ting of endotherms is observed. At pH = 1 the heat of transitio decreases dramatically indicating a destruction of molecular or

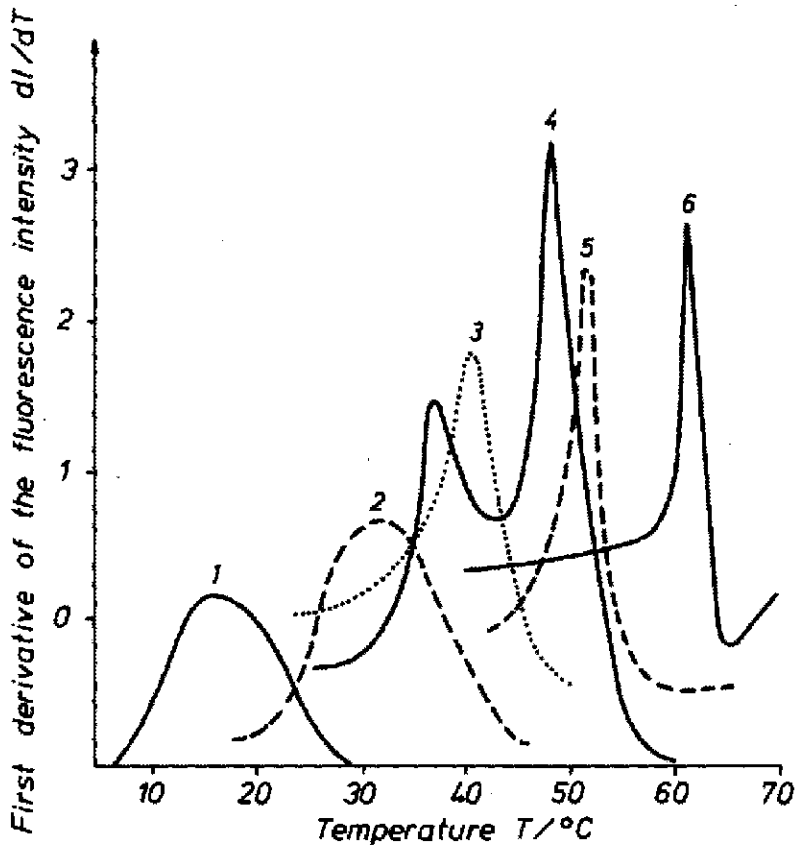

Fig. 7: Slopes dI/dT of the fluorescence intensity versus T for various phospholipids

1. Cephalin, T_c = 16.3 °C
2. DPPC:cephalin 2:1, T_c = 32.8 °C
3. DPPC, T_c = 42.2 °C
4. DPPG, T_p = 38.3 °C, T_c = 50.1 °C
5. DSPC, T_c = 52.5 °C
6. DPPE, T_c = 62.8 °C

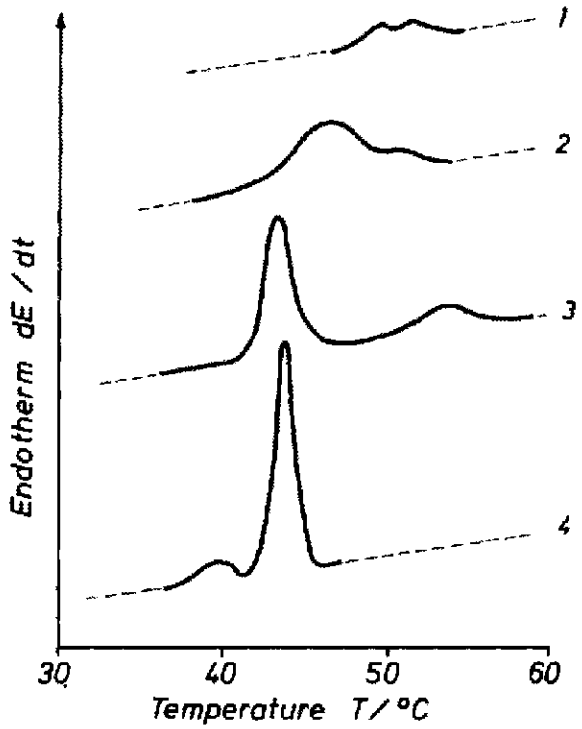

Fig. 8: DTA scans for the DPPG-water system
1. pH≈1 2. pH≈3 3. pH≈4 4. pH≈6.5

DTA scans of DPPC:DPPG mixtures (without figure) show restricted miscibility even after repeated heating similar to the results fro NPN-fluorescence measurements.

For the synthetic cephalin DLPE the DTA scans exhibit in the cases in which the aqueous suspensions were vortexed at room temperature (fig. 9 a) during the first run a sharp endotherm at T_{c2} = 45 °C. A lowering of T_{c2} and a splitting of the endotherm as well as a decrease in the heat of transition occurs during the 2nd run. A corresponding phenomenon was observed when the lipid suspension wa vortexed at T> 30 °C. During the 3rd and further scans a phase tra sition at T_{c1}≈ 30.5 °C occurs with a maximum value for the enthalp of 14.3 kJ $Mole^{-1}$.

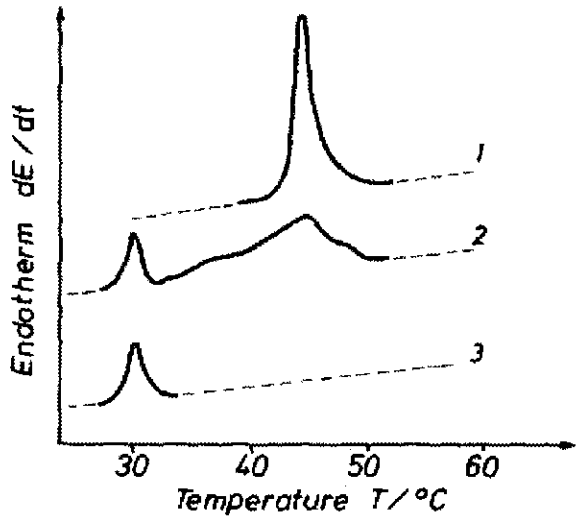

Fig. 9 a: DTA scans for the DLPE-water system prepared at room temperature
1. 1st scan, H_1=54.5 kJ, 2. 2nd scan, H_2(right peak)= 40.8 kJ, H_2(left peak)= 12.2 kJ, 3. 3rd scan, H_2=14.3 kJ

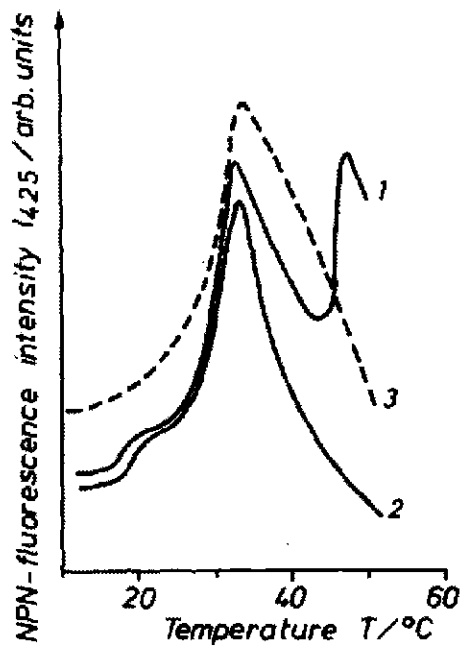

Fig. 9 b: Fluorometric scans of the DLPE-water system
1. water content 94%, 1st scan, 2. water content 94%, 2nd scan
3. water content 99.9%

Fluorometric scans of DLPE with NPN in no case revealed the calori metrically observed transition at 45 °C when the lipid was prepare as described in Methods, i.e. with a water content of 99.9 %. Sinc in typical DTA measurements the water content is as low as 80 % th preparation technique was modified by raising the lipid concentration as far as possible. The result is demonstrated in fig. 9 b for a lipid content of 6 %, showing a pronounced phase transition at 46 °C with extremely high cooperativity. The well-known phase transition can be observed as well as a pretransition at approx. T_p = 20 °C. Consecutive fluorometric scans led to the disappearanc of the peak at 46 °C as already observed with the DTA measurements

For DPPE, at concentrations 10 to 40 mg/ml at pH> 10 a broad endotherm could be measured reproducibly, but with an enthalpy of as low as 14.7 kJ $Mole^{-1}$. Only when the concentration was reduced dra stically to values lower than 0.5 mg/ml, an enthalpy change of ΔH_c 33.5 kJ $Mole^{-1}$ was measured being comparable to literature data (Mantsch et al /16/ and Wilkinson and Nagle /17/: 8.8 kcal $Mole^{-1}$= 36.8 kJ $Mole^{-1}$, Blume /11/: 8.2 kcal $Mole^{-1}$ = 34.3 kJ $Mole^{-1}$).

In table 1 the transition temperatures T_p and T_c and the respective heats of transition ΔH_p, ΔH_c for some PL's and PL-mixtures are listed and compared with data from literature. Moreover, the van't Hoff enthalpy ΔH_{vH} obtained from NPN-measurements and the size of the cooperative unit ξ are listed. The values ξ are comparable for DPPC and DPPG, only the ξ-value for DPPG at low pH indicates significantly higher cooperativity for this compound in the protonated state. Higher ξ-values also are observed for PE's, the compoun

with the shorter hydrocarbon chain length (DLPE) exhibiting a still higher cooperativity of the phase transition at 30 °C as well as at 45 °C than DPPE.

Compound	T_p/°C	ΔH_p/kJ	T_c/°C	ΔH_c/kJ	ΔH_{vH}/kJ	ξ	Remark/reference
DPPC	37.2	7.9	42.3	37.1	293.6	8.1	Pretransition dependent on preparation technique
DPPC	37	5.02	42.4	32.2	-	-	/2/ 1975
DPPC			41.2	36.4	-	-	/18/ 1982
DPPC			41.2	35.2	-	-	/19/ 1976
DPPC				37.7	-	-	/20/ 1981
DPPG	39.5	4.1	43.7	37.6	268.3	7.1	pH = 6.5 to 11
DPPG	35	2.1	41.0	33.1	-	-	/2/ 1975
DPPG			43.5	40.4	575.9	14.3	pH = 4
DLPE			44.5	54.5	1210.1	28.4	Crystalline phase, 1st scan
DLPE			30.3	14.3	342.8	24.0	Phase P_β'
DLPE			30.5	14.7	-	-	/17/ 1981
DPPE			64.2	(14.7)	622.0	(42.3)	Incomplete P_β', 20 to 30 mg/ml
DPPE			63.8	33.5	622.0	18.6	<0.5 mg/ml
DPPE			63.1	36.8	-	-	/17/ 1981
DLPE:DPPC 1.5 : 1			36.2	17.1	-	-	ΔH_{mix}= 25.1 kJ
DLPE:DPPC 0.6 : 1			37.5	34.7	-	-	ΔH_{mix}= 30.6 kJ

Tab. 1: Pre- and main transition temperatures T_p and T_c and the respective enthalpy changes ΔH_p and ΔH_c, van't Hoff enthalpy ΔH_{vH} and the resulting cooperativity ξ of the phase transition as compared to literature data

In fig. 10 the courses of fluorescence polarisation P versus T are shown for DMPC, DPPC, DPPG, DLPE, DPPE and a mixture DPPC: cephalin 2:1. Attempts of measuring the phase transition of pure cephalin by labelling with DPH failed possibly due to the hetero genity of the acyl chains.

Fig. 11 gives a plot of $\ln\bar{\eta}$ versus 1/T and figures 12 a and 12 the slopes $d(\ln\bar{\eta}_{app})/d(1/T)$ and of $d(\ln\bar{\eta})/d(1/T)$, respectively versus T. The comparison of these graphs (fig. 12 a and b) shows that for DPPC the pretransition at $T_p \sim 33.5$ °C is very distinct the $\bar{\eta}$, but only weak in the $\bar{\eta}_{app}$-presentation. Similarly, the re tive plot of $\bar{\eta}_{app}$ for a mixture of DPPC:cephalin 2:1 exhibits a broad transition at $T_c = 30.2$ °C, whereas that of $\bar{\eta}$ indicates two different transitions, one initiated by a more cephalin-containi domain with a value of $T_{c1} = 27.9$ °C and another by a mainly DPP containing domain with a value of $T_{c2} = 39.5$ °C. It is noteworth that this domain formation could neither be seen from the depend of P and r on T nor from those of $d(\ln\bar{\eta})/d(1/T)$ and $d(\ln\bar{\eta}_{app}$ d(1/T) on T when evaluating the same measurements.

The assumption of Lentz (cited in /15/) that the plot of $d(\ln\bar{\eta})$ d(1/T) versus T should give peaks with areas proportional to the enthalpies of the phase transitions, could not be confirmed. Usi the uncorrected value $\bar{\eta}_{app}$, the evaluation shows too high values for DLPE when compared with calorimetric data. This holds true f the corrected microviscosities $\bar{\eta}$, too. Also, in this case the va for DPPE is much lower than for DPPC and DPPG and corresponds to own DTA measurements of the DPPE transition from the incomplete P_{β}'-phase to L_{α} (see tab. 1).

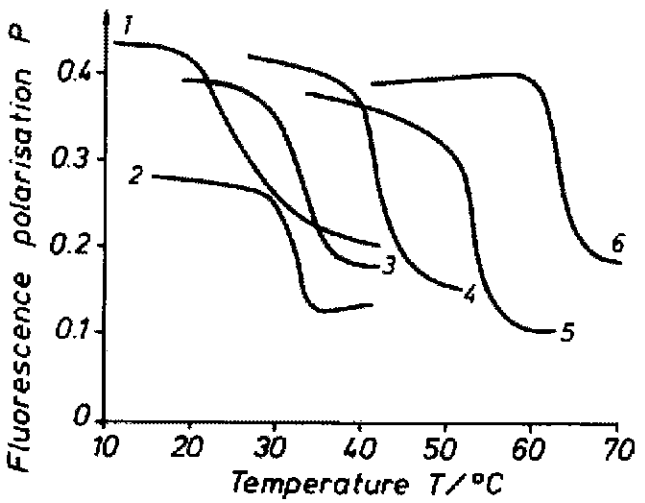

Fig. 10: Scans of the fluorescence polarisation P for various phospholipid-water systems
1. DMPC, 2. DPPC:cephalin 2:1, 3. DLPE, 4. DPPC, 5. DPPG, 6. DPPE

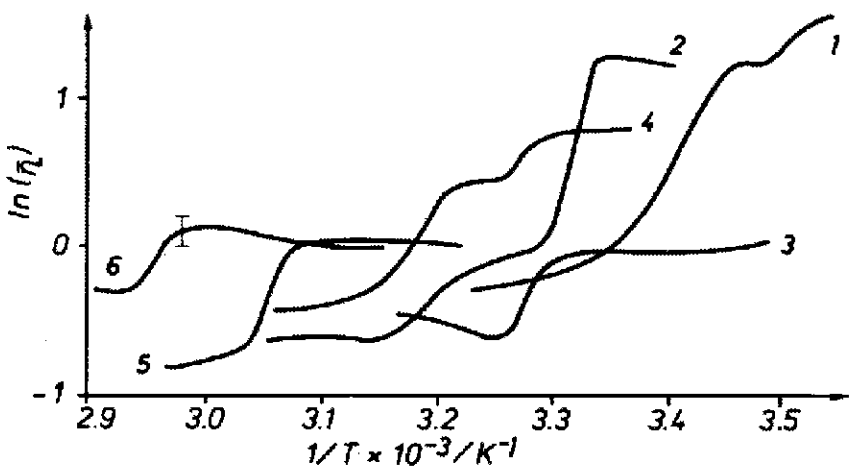

Fig. 11: Plots of $\ln\bar{\eta}$ versus 1/T for various phospholipid-water systems. The standard deviation of the measurement is indicated by the error bar in graph 6.
1. DMPC, 2. DPPC:cephalin 2:1, 3. DLPE, 4. DPPC, 5. DPPG, 6. DPPE

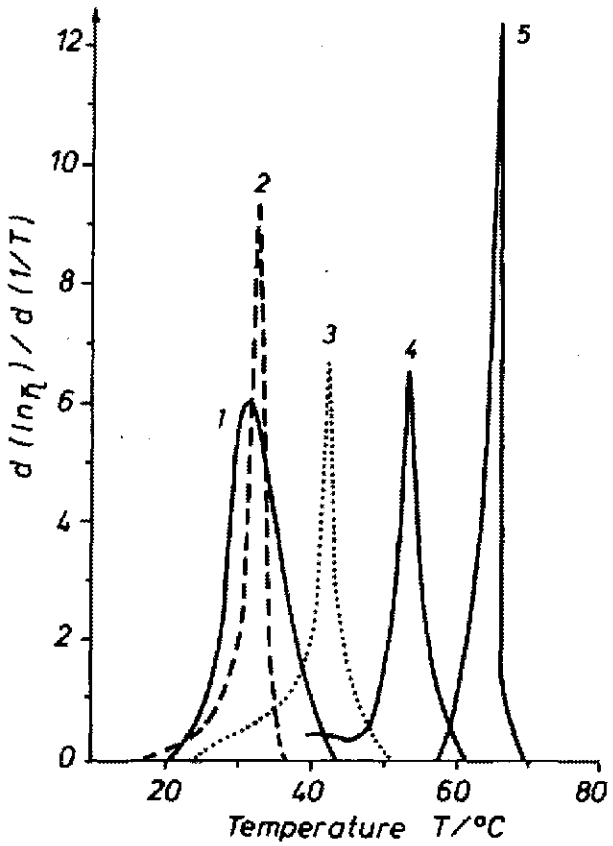

Fig. 12 a: Plots of $d(\ln \bar{\eta}_{app})/d(1/T)$ versus T for variou phospholipid-water systems

1. DPPC:cephalin 2:1, 2. DLPE, 3. DPPC, 4. DPPG, 5. DPPE

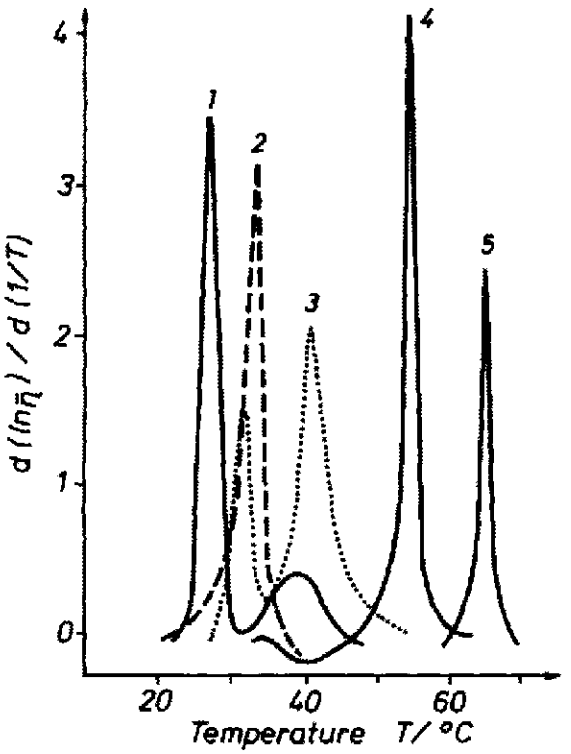

Fig. 12 b: Plots of $d(\ln \bar{\eta})/d(1/T)$ versus T for various phospholipid-water systems

1. DPPC:cephalin 2:1
2. DLPE
3. DPPC
4. DPPG
5. DPPE

In tab. 2 the parameters r, S ($t_{\Delta S}$), $\bar{\eta}_{app}$, $\bar{\eta}'$ and $\bar{\eta}$ are listed for some values of T below and above the respective phase transition temperature T_c as calculated from equations (2) to (4). Values for anisotropies r are similar for DPPC and DPPE but reduced for DPPG and DLPE, the order parameter S as well as the

approximations $\bar{\eta}_{app}$ and $\bar{\eta}'$ show a decrease in the sequence DPPC→ DPPE→ DPPG→ DLPE. In contrast to this the values for $\bar{\eta}$ lie much closer together, i.e. the ratio of $\bar{\eta}$-values below and above T_c is only 1.5 to 3, whereas the respective ratios for $\bar{\eta}_{app}$ and $\bar{\eta}'$ are in the range 4 to 16. Striking is the similarity for DLPE and DPPE with respect to $\bar{\eta}$ whereas the respective ratios for $\bar{\eta}_{app}$ and $\bar{\eta}'$ show great differences for these compounds.

Compound	T/°C	r	S	ΔS	Microviscosities/ poise $\bar{\eta}_{app}$	$\bar{\eta}'$	$\bar{\eta}$	T_p/°C	T_c/°C
DMPC	16	0.33	0.95	0.01	25.9	27.7	3.55	14.5	24.5
	22	0.30	0.90	0.03	11.8	12.5	1.43		
	28	0.21	0.70	0.08	3.2	3.1	0.90		
	34	0.17	0.58	0.10	2.0	1.9	0.75		
DPPC	32	0.32	0.93	0.08	18.0	18.4	2.30	33.4	40.7
	36	0.31	0.91	0.04	12.8	13.1	1.45		
	42	0.23	0.75	0.05	4.2	4.3	1.02		
	48	0.12	0.40	0.04	1.1	1.2	0.70		
DPPG	42	0.26	0.83	0.04	6.4	6.5	1.03		55.1
	48	0.25	0.81	0.04	5.5	5.6	1.05		
	54	0.14	0.48	0.05	1.4	1.5	0.76		
	60	0.08	0.26	0.02	0.6	0.7	0.48		
DLPE	18	0.20	0.69	0.08	3.1	3.1	0.94		33.4
	24	0.21	0.71	0.08	3.3	3.3	0.96		
	30	0.19	0.64	0.09	2.5	2.5	0.89		
	36	0.09	0.31	0.09	0.8	0.6	0.82		
DPPE	52	0.30	0.91	0.03	11.5	11.7	0.99		64.5
	58	0.31	0.92	0.02	13.9	14.1	1.06		
	64	0.25	0.79	0.05	5.0	5.1	1.05		
	70	0.13	0.47	0.05	1.4	1.4	0.75		
DPPC : cephalin 2:1	28	0.29	0.88	0.03	9.8	10.0	1.79	and	27.9 and 39.5
	32	0.26	0.83	0.05	6.2	6.3	1.04		
	36	0.20	0.66	0.08	2.8	2.8	0.92		
	40	0.10	0.32	0.08	0.9	0.9	0.65		

Tab. 2: Anisotropy r, order parameter S, approximations $\bar{\eta}_{app}$ $\bar{\eta}'$, and $\bar{\eta}$ for microviscosities at different temperatures T and phase transition temperatures T_p and T_c from measurements of polarized light intensities $I_\parallel$, $I_\perp$ with DPH

In table 3 some values from literature for microviscosities at different temperatures for the same or similar phospholipids are compared with our results.

Compound	Microviscosities / poise $\bar{\eta}_{25}$	$\bar{\eta}_{37}$	$\bar{\eta}_{50}$	$\bar{\eta}_{65}$	Remark/reference
Mixture of phospholipids	1.8	0.7			Not including r_{∞}, /21/ 1976
PC from egg yolk	0.95				/21/ 1976
Egg yolk PC: cholesterol					Including r_{∞},
1 : 0.2	1.59				/12/ 1979
1 : 0.5	2.23				
Lupine phospholipids	1.23				Not including r_{∞} /24/ 1982
DPPC	9.4	3.9	0.94 (45 °C)		Not including r_{∞}, perylen as probe /23/ 1973
DPPC	12	7.5	1.0 (42 °C)		Not including r_{∞}, apparent microviscosity /22/ 1976
DPPC	1.42	1.50	0.66	-	Own data
DPPG	-	0.93	1.05	0.48	
DLPE	0.94	0.59	-	-	
DPPE	-	-	0.98	0.75	

Tab. 3: Microviscosities $\bar{\eta}$ of various phospholipids with DPH as probe (Cogan et al /23/: Perylen) for 25, 37, 50 and 65 °C as compared to literature data

Discussion

For DPPG a discrepancy between DTA- and NPN-fluorescence measurements (fig. 2 and 8) is observed: whereas the fluorescence measurements clearly show an increase of T_c already at pH < 7, the calorimetric data indicate the beginning of the shift for pH < 4. The increase of the phase transition temperature can be explained as an induction of a closer bound between molecules via decreasing repulsive forces by the protonation of the negatively-charged PG head group. In DTA measurements the water or buffer content of the lipid dispersions is approximately 80 %, in typical optical measurements, however, as high as 99.9 %.The ratio of lipid molecules to protonated water molecules can be estimated to be unity at pH = 2 for calorimetric and at pH = 4 for optical samples, thus the difference can be understood readily.

The results for DPPG agree with those of Watts et al /25/ with respect to the appearance of the pretransition only in the negatively-charged state, but slightly deviate from them as for the location of T_c. Watts et al observed by means of the ESR spin-probe TEMPO a transition in fully protonated state (pH = 2) at 54 °C, in the negatively-charged state (pH > 4) at approximately 40 °C. As an explanation a disturbance of the phospholipid structure by the spin-probe - as already measured for other phospholipid labels - can be assumed to cause a shift in T_c, which can be excluded for calorimetric scans.

The surprising but strongly reproducible behaviour of DLPE in the DTA experiments can be supposed to arise from a crystalline phase with a transition to the known liquid crystalline state at approx.

45 °C. The existence of this phase, which disappears when repeating calorimetric or optical scans, has been described in recent publications during preparation of this paper by Chang and Epand /26/ and Mantsch et al /16/. Mantsch et al obtained a value of 13.3 kcal $Mole^{-1}$ (55.7 kJ $Mole^{-1}$) at pH = 7 for the enthalpy change being in much better agreement with our value of 54.5 kJ $Mole^{-1}$ at pH = 6.5 than with those of Chang and Epand (10.0 kcal $Mole^{-1}$ at pH = 5.5 and 12.0 kcal $Mole^{-1}$ at pH 9.5). In the cited papers the crystalline phase of DLPE is assigned to a single crystal-structure, which may be stable even in the presence of excess water. The phase transition at nearly 45 °C then corresponds to both a hydration of the polar head-group and a melting of the acyl chains.

These results are in accordance with our observations regarding the strong dependence of the phase transition on the preparation technique. Preparing the lipid suspensions at higher temperatures ($T > 30$ °C) causes at least a partial hydration of the DLPE head-groups and an appearance of both transitions. Moreover, our measurements at higher water content (99.9 %) indicate that the crystalline phase - resulting from the close packing of the DLPE molecules - cannot develop in the presence of high excess water even when preparing the lipid sample very carefully at room temperature or lower. Regarding the phase transition $P_{\beta}' \longrightarrow L_{\alpha}$ at approx. 30 °C, our result ΔH_c = 14.3 kJ $Mole^{-1}$ agrees well with those of Wilkinson and Nagle /17/ (14.6 kJ $Mole^{-1}$) and Mantsch et al (3.5 kcal $Mole^{-1}$ = 14.65 kJ $Mole^{-1}$).

For PE's until now a pretransition has been observed only for distearoyl-PE, but far below the main transition (T_c (DSPE)=80 °C,

$T_{p.}$ between 5 and 20 °C according to /27/), and for dihexadecyl-PE in the protonated state at pH > 13, accompanied by the appearance of a "ripple" structure /28/.

The fact that the DLPE transition at 20 °C was only observed at a water content of 94 % verifies the statement of Sackmann /29/ that upon addition of water to PL rather longlived metastable structures - depending on the water concentration - may be formed, which are not thermodynamically stable. However, whether the transition observed at 20 °C corresponds to the pretransition $L_{\beta}' \longrightarrow P_{\beta}'$ seen for example in PC's cannot be answered now.

The strange behaviour of DPPE in nearly all kinds of measurements is backed by observations of Singer /30/ who also was not successful in preparing closed vesicles from DPPE. As Fringeli /31/ stated, DPPE tends to form microcrystals when spread from a chloroform solution over a substrate. Similarly, Seddon et al /32/ investigated the transition of diacyl-PE's from the bilayer to the inverted hexagonal-phase ($L_{\alpha} \rightarrow H_{II}$), which takes place at temperatures decreasing with increasing chain length. Our results indicate that - similar to DLPE - the lipid:water ratio is of uppermost importance regarding the phase behaviour of DPPE. Moreover, the differences between the results for DLPE and DPPE show that the composition of the hydrocarbon chains in biomembranes cannot be a free variable but must rather meet the geometrical requirements of the membrane of the living cell.

The investigations of mixed phospholipids demonstrate that the tendency of phase separation is quite distinct for mixtures with the

same hydrocarbon moiety, i.e. dipalmitoyl, but different head-groups (PC, PE, PG). In contrast to this, domain building seems to be much lower for mixtures of DPPC with DLPE and cephalin. From these and similar results - taking into account also those of Singer /33/ who investigated mixed preparations of saturated PE's and PC's by light absorbance at 450 nm and permeability determination - it can be concluded that the geometrical arrangement in biomembranes is strongly dependent on the nature of the head-groups as well as the length and saturation degree of the hydrocarbon chains. This agrees also with results in previous papers, in which this strong influence of chain length (PC's/34/) and of the nature of the head-groups by slight modifications of PE's /35/ has been discussed. Regarding the fact that biomembranes are responsible for the permeability of the cell for many substances, the intimate study of the behaviour of single membrane components with the aim of reconstituting biomembranes still is of great importance.

The evaluation of the results of microviscosity measurements led to large differences in the values for $\bar{\eta}_{app}$ and $\bar{\eta}'$ on the one hand between DLPE and DPPE and on the other hand between DPPC and DPPG, indicating that the introduction of r_∞ as well as the application of the empirical relation between r_∞ and r (see Methods) presented in this paper to obtain the "true" viscosity seem to be necessary for getting reasonable values for $\bar{\eta}$. This statement is confirmed by the comparison with the few existing literature data and by the fact that the pretransitions of DMPC and DPPC as well as a domain building in a DPPC:cephalin mixture (see tab. 2) could only be observed when applying the corrected value of $\bar{\eta}$.

Remarkable are the low differences of the order parameter S and especially the "true" microviscosity $\bar{\eta}$ above and below T_c for the PE's in contrast to the PC's and to DPPG. Although the phase transition enthalpies ΔH_c are comparable and - in a first approximation - independent of the kind of the polar head-group (compare Tab. 1), the strong interaction of the tightly packed PE head-groups apparently prevents the lipid from building vesicles leading to an enhancement of the mean interchain distances and to lower microviscosities as compared to PC's and PG's. These statements are supported by the fact that the hydrocarbon cross section of DLPE (0.193 nm^2) and DPPE (0.195 nm^2) are slightly larger than that of DMPC (0.190 nm^2)/36-37/ despite the smaller cross-section of the PE-head-groups.

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