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CHARGE-INDUCED PRETRANSITION IN PHOSPHATIDYLETHANOLAMINE MULTILAYERS

THE OCCURRENCE OF RIPPLE STRUCTURES

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Summary

The influence of pH and ionic strength on the phase transition behaviour of 1,2-dihexadecylphosphatidylethanolamine was studied calorimetrically. In the range of ionic strength from 0.75 to 1.5 M NaCl at pH $\geqslant$ 13, where the amino group of the phosphatidylethanolamine is in the deprotonated state, resulting in one negative charge per lipid molecule, the calorimetric scan shows a pretransition before the main transition. Accompanying freeze-fracture electron microscopic studies on these preparations in the temperature range between the pre- and main transitions show a regular surface, the so-called ripple structure. These are comparable with the structures seen in phosphatidylcholine-water systems at temperatures between the pre- and main transition.

Introduction

Phosphatidylcholine-water systems have been intensively investigated in recent years as models for biological membranes [1,2]. In these systems a phase transition may be thermally induced which results in a transition of the chains (acyl chains) from a crystalline to a liquid-crystalline state and as a consequence leads to a sudden change in membrane fluidity.

This work tries to explain the known differences in the phase transition behaviour of phosphatidylethanolamines and phosphatidylcholines. As an

Abbreviations: 1,2-HH-G-PE, 1,2-dihexadecyl-*sn*-glycerol-3-phosphoethanolamine; 1,2-HH-G-PC, 1,2-dihexadecyl-*sn*-glycerol-3-phosphocholine; T_t, phase transition temperature; ΔH, phase transition enthalpy.

example, the phase transition temperature of 1,2-dipalmitoylphosphatidylethanolamine T_t = 64°C, is different from that of the corresponding phosphatidylcholine, T_t = 42°C. Besides differences in the phase transition temperature, phosphatidylethanolamines in contrast to phosphatidylcholines do not show any pretransition or ripple structures [3,4].

It was shown in earlier reports that deprotonation of phosphatidylethanolamines results in a sudden decrease of the phase transition temperature [5,6]. However, a quantitative study of this effect was impossible with diacylphosphatidylethanolamines due to ester hydrolysis at high pH values. The chemically more stable 1,2-dihexadecylphosphatidylethanolamine, the ether analogue of 1,2-dipalmitoylphosphatidylethanolamine, was taken to circumvent this problem. This approach has been successfully taken in studies on the thermotropic behaviour of phosphatidic acids at extreme pH values [7—10].

Materials and Methods

1,2-Dihexadecyl-*sn*-glycerol-3-phosphoethanolamine was a product of Fluka (Buchs, Switzerland). Thin-layer chromatography in different solvent systems did not show impurities.

The calorimetric measurements were performed using a Perkin Elmer 'DSC 2' (with Intracooler I). Weighted amounts of lipid (3—4 mg) and 50 μl of water or the respective salt solution were sealed in stainless steel pans and equilibrated for 60 min at a temperature of 5°C above T_t. The reference pan contained 50 μl of the corresponding water or salt solution. For each sample at least three scans were performed with heating rates of 2.5°C/min in the sensitivity range of 2 mcal/s. Repetitive scans did not shown significant differences in T_t or in the ΔH values. The T_t values given are taken as the temperature of the peak inversion and enthalpies are calculated from the peak areas.

The stability of the 1,2-HH-G-PE at pH 7 and at pH $\geqslant$ 13 was confirmed by thin-layer chromatography. None of the possible decomposition products, phosphatidic acid or 1,2-dihexadecyl-*sn*-glycerol, were observed after heating to 80°C for 5 h applying the experimental conditions.

The samples for freeze-fracture electron microscopy were prepared in the same way as the calorimetric ones. Because of the sometimes observed hysteresis of the pretransition the lipid dispersions were kept at $T < 5$°C for at least 1 h after equilibration at $T > T_t$. The dispersion was then stored at the desired temperature for 15 min before droplets (2—4 μl) of the dispersion were pipetted onto gold planchets (Balzers) thermostated at the same temperature. The samples were quenched by plunging the planchets into liquid Freon 22 and then stored under liquid nitrogen.

Fracturing was carried out on a Balzer freeze-etch device (type BA 360) at —105°C with no etching. The replicas were floated off, cleaned in a mixture of $CHCl_3/CH_3OH$/1 N HCl (4 : 4 : 1, by vol.) and examined on a Siemens 101 electron microscope (at 80 kV; instrumental magnification 20 000).

Results

As described earlier, deprotonation of phosphatidylethanolamine will result in a drop in T_t [5,6]. The pH dependence of T_t for the stable 1,2-HH-G-PE can

be taken from Fig. 1. The calorimetric scans of 1,2-HH-G-PE in the pH region from 7 to 11.5 show a large plateau for T_t with T_t values of about 74°C. At pH 12 the transition region is broadened and spans 8°C in comparison to approx. 3°C for the lower pH values. In addition, at pH 12.5 T_t is lowered and at pH $\geqslant$ 13 a lower transition temperature appears, at T_t = 46°C. The transition is sharp and in the presence of 1 M NaCl also accompanied by a well-separated pretransition. T_t also matches the transition temperature of the respective 1,2-dihexadecyl-*sn*-glycerol-3-phosphocholine, T_t = 45°C.

The appearance of a pretransition in phosphatidylethanolamines is described here for the first time. The close similarity with phosphatidylcholines is striking. It includes T_t, the pretransition and also the transition enthalpies.

As shown in Fig. 2, high pH alone will not suffice to induce a pretransition. In addition to pH $\geqslant$ 13 it requires a 1 M Na^+ concentration. At lower concentrations (0.2—0.6 M Na^+) a pretransition is not observed. At 0.75 M Na^+ a pretransition separates from the main transition. With increasing ionic strength the pretransition is shifted towards lower temperature and the distance between main and pretransition is increased largely. At 0.75 M Na^+ the difference is approx. 5°C and at 1.5 M Na^+ it amounts to approx. 15°C in comparison to approx. 10°C for the system 1,2-HH-G-PC at pH 7 in water.

At higher Na^+ concentrations of 2 M Na^+ and above, the pretransition is no longer observed, but the increasing salt concentration affects the main transi-

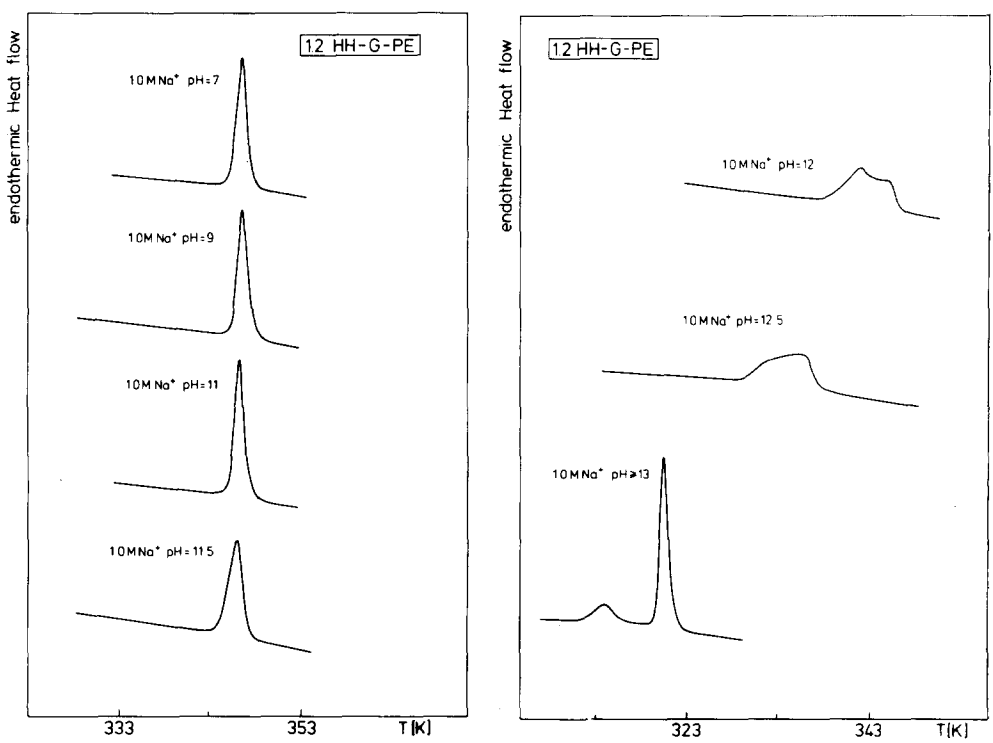

Fig. 1. Calorimetric heating curves of 1,2-dihexadecyl-*sn*-glycerol-3-phosphoethanolamine. Variation of pH at constant Na^+ concentration. Heating rate: 2.5° C/min; sensitivity range: 2 mcal/s.

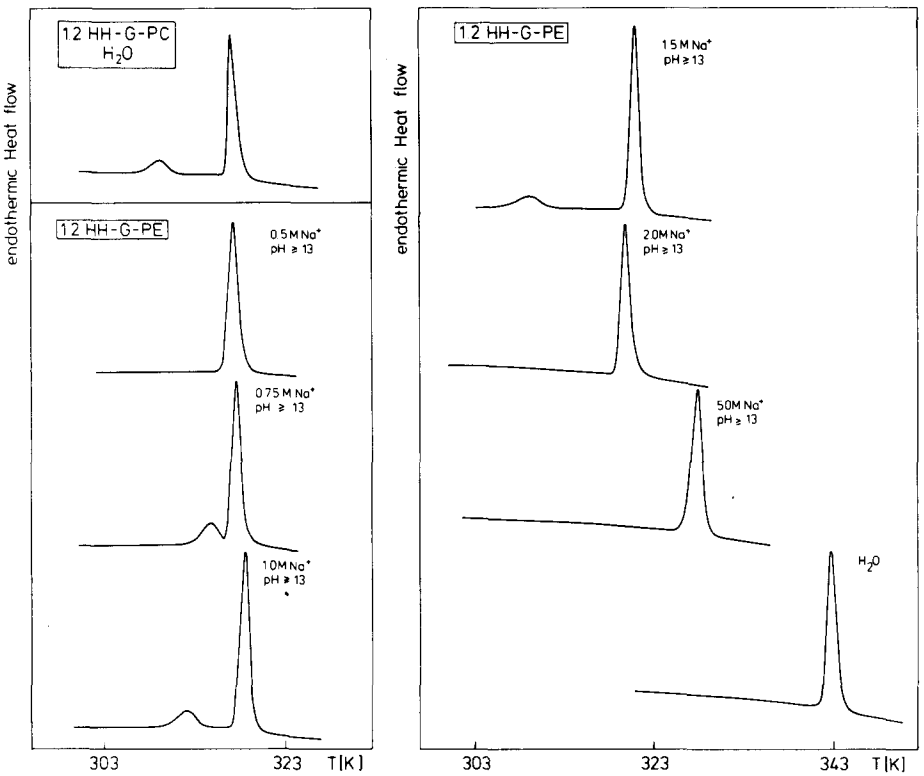

Fig. 2. Calorimetric heating curves of 1,2-dihexadecyl-*sn*-glycerol-3-phosphoethanolamine. Variation of Na^+ concentration at constant pH (pH ⩾ 13). Heating rate: 2.5°C/min; sensitivity range: 2 mcal/s.

tion and results in a shift to higher temperatures with a T_t value of approx. 55°C for 5 M Na^+ and a T_t value of approx. 64°C for 10 M Na^+. Note the close coincidence between the T_t values of 1,2-HH-G-PE at pH 7, no salt and at pH ⩾ 13 in the presence of 10 M Na^+.

TABLE I

TRANSITION ENTHALPIES OF 1,2-DIHEXADECYL-*sn*-GLYCEROL-3-PHOSPHOCHOLINE AND 1,2-DIHEXADECYL-*sn*-GLYCEROL-3-PHOSPHOETHANOLAMINE

Data are in kcal/mol.

1,2-HH-G-PE (1 M Na^+ concentration)								1,2-HH-G-PC in H_2O
(a) Variation of pH value								
pH	7	9	11	11.5	12	12.5	⩾13	7
ΔH_p	—	—	—	—	—	—	1.4	1.2
ΔH_M	6.6	6.4	6.7	6.6	6.8	6.7	7.1	6.7
(b) Variation of Na^+ concentration								
1,2-HH-G-PE at pH ⩾13								
Na^+	0.5	0.75	1.0	1.5	2.0	5.0		
ΔH_p		≈1.4	1.5	1.4				
ΔH_M	8.1	9.6	7.2	7.1	6.7	6.9		

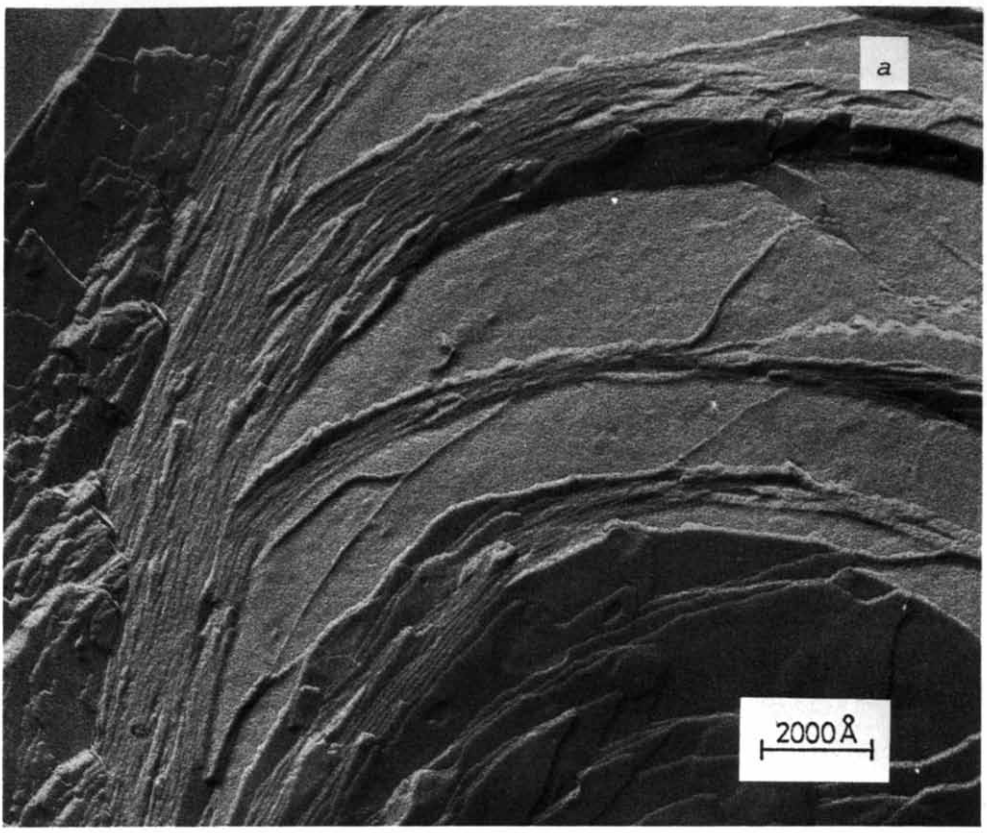

Fig. 3. Electron micrographs (freeze-etch technique) of 1,2-dihexadecyl-*sn*-glycerol-3-phosphoethanolamine at pH ⩾ 13 and 1 M Na^+ concentration for (a) $T = 20^\circ C$, and (b) $T = 43^\circ C$.

It has been shown in earlier studies on phosphatidylcholines that the observation of a pretransition in phospholipid-water systems is always accompanied by the appearance of a ripple structure detectable by electron microscopy [17].

It was interesting to see whether this is also true for phosphatidylethanolamine, a structural quite different system. Therefore freeze-etch samples of phosphatidylethanolamine were analyzed by electron microscopy in the temperature range below pretransition and between pre- and main transition. As can be seen in Fig. 3, only between pre- and main transition there appears the rippled structure with a periodic length of approx. 200 Å under the conditions of 1 M Na^+ and pH ⩾ 13.

This demonstrates with the methods used, that there is no difference in the physical behaviour of phosphatidylcholine in the neutral state (pH 4—11) and phosphatidylethanolamine in the charged state (pH ⩾ 13, 1 M Na^+). The high salt concentration in the case of phosphatidylethanolamine is most probably necessary to minimize the differences in the structural behaviour between phosphatidylcholine and phosphatidylethanolamine.

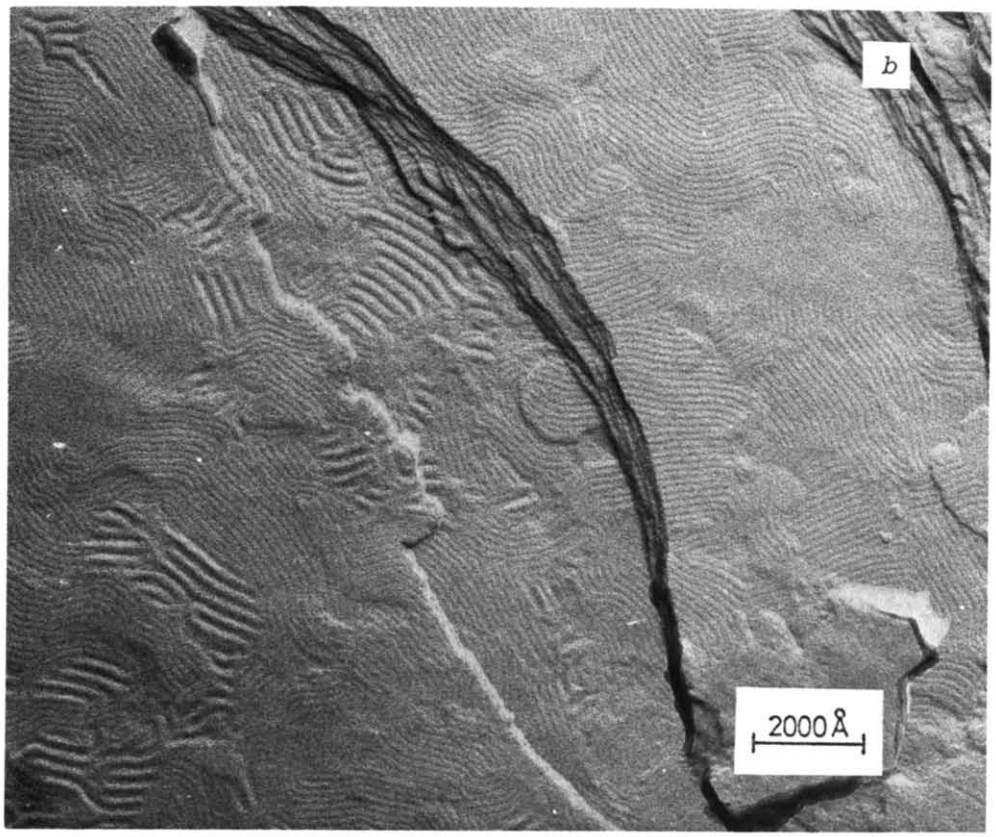

Fig. 3b.

If we consider the transition enthalpy and compare the experiment at high and low pH and also the variation in the salt concentration, it is obvious that ΔH is quite constant despite the fact that the milieu is varied largely. This most probably reflects the situation that the main contribution for ΔH comes from chain-chain interactions. As shown in Table I, ΔH for the main transition of 1,2-HH-G-PC at pH 7.0 in water and of the respective 1,2-HH-G-PE in 1 M NaCl from pH 7.0 to 12.5 is almost constant, however, with no pretransition in the case of phosphoethanolamine. At pH $\geqslant$ 13 a pretransition is observed in the phosphatidylethanolamine system. ΔH of the main and of the pretransition of 1,2-HH-G-PE is slightly increased (approx. 10—20%, respectively) in comparison to 1,2-HH-G-PC. A variation of the salt concentration at pH $\geqslant$ 13 will result in a disappearance of the pretransitions, for instance there is no pretransition detected at pH $\geqslant$ 13 and 0.5 M Na^+, but note the similarity with the ΔH value of the phosphocholine. (ΔH of 8.1 kcal/mol for the phosphoethanolamine and 7.9 kcal/mol for the phosphocholine.) An increase in the salt concentration up to 2 M Na^+ will also remove the pretransition.

Discussion

Differences in the T_t values for phospholipid systems at neutral pH are small

at constant apolar region and large structural variations in the polar part of the molecule [5]. For instance, the phase transition temperature for 1,2-dipalmitoyl-*sn*-glycerol-3-phosphocholine and of the respective analogues with increased phosphate-trimethylammonium distance differ only slightly in T_t [11,12]. The respective phosphatidyl-*sn*-glycerol [13] shows the same T_t which is also true for the respective alkylesters such as phosphatidylpropylester or 2,2-dimethylbutylester which differs from the respective phosphatidylcholine only by substitution of nitrogene by carbon [5]. This demonstrates that the positive charge of phosphocholine is of a minor influence to the physical properties of such membranes.

A striking difference is observed for such molecules where positive charged ions are still left in the membrane surface as in the case of phosphatidylethanolamines or phosphatidic acids at pH values below the second pK [7,10]. Therefore dramatic changes of membrane properties can be expected if these ions in the membrane surface are removed. Earlier experiments in this respect were not conclusive due to marked decomposition of 1,2-dimyristoyl-*sn*-glycerol-3-phosphoethanolamine at high pH [14]. A reinvestigation of the phosphatidylethanolamine system was now undertaken using the more stable 1,2-HH-G-PE.

An important result of this study is the observation that the large differences in the phase transition temperatures and the physical behaviour between 1,2-HH-G-PE and 1,2-HH-G-PC is mainly a result of structural variations triggered by external parameters. The differences introduced by the structural diversity in the polar parts can be minimized if the external conditions such as pH and salt concentration are chosen properly. For instance the T_t values of 1,2-HH-G-PC and of 1,2-HH-G-PE at pH $\geqslant$ 13 are almost identical. In addition, an increase in the salt concentration to about 1 M Na^+ will introduce a pretransition in the phosphatidylethanolamine system.

The pretransition in 1,2-HH-G-PE is only observed at complete deprotonation of the amino function and in a certain range of salt concentration. In this range the distance between pretransition and main transition depends on the salt concentration. With increasing salt concentration the distance increases, an effect, which was also found for the phosphocholines (Stümpel, J. and Eibl, H., unpublished results).

If we now turn from a more macroscopic observation of the system to a molecular level we may ask, how differences in phase transition behaviour were created in phospholipid systems. Pretransitions were first shown for phosphocholine-water systems and it was reported to be a characteristic property of phosphatidylcholine [1]. Later on it was also shown for the phosphatidic acid system [8] and phosphatidylglycerol [13].

Considering the following charge relationships (Table II), which cannot be exactly established with the methods used, the ionic strength and the pH dependence of T_t can be understood qualitatively.

In the region below pH 3 (column A, Table II), the negative charge of the phosphate groups is neutralized in the phosphatidylcholines and cephalins as a result of protonation. Electrostatic repulsion then begins due to the positively charged amino groups. The consequence of this repulsion should be a reduction of T_t, which is not observed experimentally.

TABLE II

CHARGE DISTRIBUTION IN THE POLAR HEAD GROUPS OF PHOSPHATIDYLCHOLINE AND CEPHALIN AT DIFFERENT pH

A	B	C
$PO_4H\text{-}(CH_2)_2\text{-}N^+(CH_3)_3$	$PO_4^-\text{-}(CH_2)_2\text{-}N^+(CH_3)_3$	$PO_4^-\text{-}(CH_2)_2\text{-}N^+(CH_3)_3$ Y^+
$PO_4H\text{-}(CH_2)_2\text{-}N^+H_3$	$PO_4^-\text{-}(CH_2)_2\text{-}N^+H_3$	$PO_4^-\text{-}(CH_2)_2\text{-}NH_2$ Y^+
pH < 3	pH 3—12.5	pH > 12.5

For instance 1,2-HH-G-PE in 1 M HCl has a phase transition temperature of T_t = 74°C. A similar result, namely an increase in T_t with decreasing pH, is observed for 1,2-HH-G-PC in 1 M HCl with a phase transition temperature of T_t = 53°C. The reason for this opposite behaviour in the low pH region is most probably the presence of protons in the membrane surface as discussed in a separate paper [6].

In the pH range from 3 to 12.5 the charge relationships are shown in column B (Table II). The repelling PO_4^--PO_4^- and NX_3^+-NX_3^+ interactions are superimposed by the PO_4^--NX_3^+ attractions which, however, are somewhat more pronounced in the cephalins than in the phosphatidylcholines.

From the different strengths of the charge relationships result the known differences in the phase transition behaviour. At neutral pH the cephalins have about a 20°C higher phase transition temperature than the analogue phosphatidylcholines.

A further increase in pH (pH > 12.5) leads to the charge relationships shown in column C (Table II). In the cephalins, the amino group is deprotonated and the phosphate group attracts a positively charged counterion from the bulk phase. Since in this case the repulsion NX_3^+-NX_3^+ interaction is absent, there is only a repulsive PO_4^--PO_4^- interaction. This leads to a reduction of T_t. Due to the positively charged counterions from the bulk phase, which at high ionic strengths (greater than 1 M Na^+) are associated with the phosphate groups, the PO_4^--PO_4^- repulsive interaction is weakened so that increasing ionic strength leads to an increase in T_t [15,16].

Therefore it is possible to explain the differences in the phase transition behaviour of phosphatidylcholines and cephalins qualitatively taking into account the preceding discussion of the electrostatic interactions in the pH regions given in Table II.

These experiments were made possible due to the fact that ether-phosphatidylcholines and phosphatidylethanolamines are stable in the entire pH range. Differences between diacyl compounds and the investigated diether compounds are of minor significance for the above arguments. Therefore the described principles are applicable to all types of phosphatidylcholines and cephalins.

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