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THE INFLUENCE OF CHARGE ON PHOSPHATIDIC ACID BILAYER MEMBRANES

HANSJÖRG EIBL [a] and ALFRED BLUME [b]

[a] *Max-Planck-Institut für Biophysikalische Chemie, Am Fassberg, D-3400 Göttingen and* [b] *Institut für Physikalische Chemie II, Albertstr. 23a, D-7800 Freiburg (F.R.G.)*

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Summary

A complete titration of phosphatidic acid bilayer membranes was possible for the first time by the introduction of a new anaologue, 1,2-dihexadecyl-*sn*-glycerol-3-phosphoric acid, which has the advantage of a high chemical stability at extreme pH values. The synthesis of this phosphatidic acid is described and the phase transition behaviour in aqueous dispersions is compared with that of three ester phosphatidic acids; 1,2-dimyristoyl-*sn*-glycerol-3-phosphoric acid, 1,3-dimyristoylglycerol-2-phosphoric acid and 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoric acid.

The phase transition temperatures (T_t) of aqueous phosphatidic acid dispersions at different degrees of dissociation were measured using fluorescence spectroscopy and 90° light scattering. The T_t values are comparable to the melting points of the solid phosphatidic acids in the fully protonated states, but large differences exist for the charged states.

The T_t vs. pH diagrams of the four phosphatidic acids are quite similar and of a characteristic shape. Increasing ionisation results in a maximum value for the transition temperatures at pH 3.5 (pK_1). The regions between the first and the second pK of the phosphatidic acids are characterised by only small variations in the transition temperatures (extended plateau) in spite of the large changes occurring in the surface charge of the membranes. The slope of the plateau is very shallow with increasing ionisation. A further decrease in the H^+ concentration results in an abrupt change of the transition temperature. The slope of the T_t vs. pH diagram beyond pK_2 becomes very steep. This is the

Abbreviations: 1,2-DHPA, 1,2-dihexadecyl-*sn*-glycerol-3-phosphoric acid; 1,3-DMPA, 1,3-dimyristoyl-glycerol-2-phosphoric acid; 1,2-DMPA, 1,2-dimyristoyl-*sn*-glycerol-3-phosphoric acid; 1,2-DPPA, 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoric acid.

result of reduced hydrocarbon interaction energy, which was demonstrated by differential scanning calorimetry (Blume, A. and Eibl, H., unpublished data).

Introduction

Discussion of the structure and function of biomembranes has gained much information from studies on model membrane systems [1] which may be formed by sonication of phospholipids in water. A characteristic property of such membranes is the phase transition, the transition from the ordered to the fluid state of the lipid chains or vice versa. It describes abrupt changes in the fluidity and packing density of the fatty acid chains at a distinct temperature, the phase transition temperature (T_t).

Since lipid phase transitions have been observed not only in simple model systems but also in natural membranes [2—4], interest has been focussed on mechanisms which can lead to changes in lipid chain fluidity and thus control regulatory functions in biomembranes [5,6]. Such changes in the fluidity of membranes at the phase transition temperature are detected by optical methods such as fluorescence spectroscopy and 90° light scattering and also by differential scanning calorimetry. It was shown in earlier work that the phase transition temperature of a given bilayer membrane can be influence by altering the surface charge [7—10], or by a redistribution of phospholipid molecules within the lipid matrix, which includes phase separation [11—13], or by the incorporation of guest molecules [14—17].

In the present paper, the discussion is restricted to charge alteration in bilayer membranes of synthetic phosphatidic acids. This system has been already studied by different groups, for example Träuble and Eibl [7], Jacobson and Papahadjopoulos [18] and Galla and Sackmann [19]. There has been general agreement, that an increase in surface charge results in a decrease of the phase transition temperature, at least in the region of the second pK. However, considerable discrepancies exist with regard to the extent of the effects when the various reports on this subject are compared. Therefore, a re-examination of the phosphatidic acid system was undertaken by introducing new analogues of increased chemical stability against pH variations. Besides the use of an ether analogue, 1,2-dihexadecyl-*sn*-glycerol-3-phosphoric acid (1,2-DHPA), an increased stability was also expected for 1,3-dimyristoyl-glycerol-2-phosphoric acid (1,3-DMPA), which lacks the labile fatty acid ester of the secondary hydroxyl of glycerol [20]. The pH dependences of the transition temperature for the more stable analogues were compared with those for 1,2-dimyristoyl-*sn*-glycerol-3-phosphoric acid (1,2-DMPA) and 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoric acid (1,2-DPPA). Earlier observations for lecithins and cephalins had revealed similar phase transition temperatures for the ester and ether anlogues [21]. It was assumed that this is also true for the ester and ether phosphatidic acids in this study.

Experiments were carried out to acertain whether the more stable analogues would allow a complete titration of the phosphate group in phosphatidic acid bilayer membranes and if the titration of the first pK with increasing charge would result in a similar decrease of the phase transition temperature as

observed for the second pK. Titration of the first pK was not possible in the earlier work due to precipitation and chemical decomposition of the bilayer membranes [7].

Materials and Methods

All solvents were of p.a. grade. 1,3-Dimyristoylglycerol was synthesized as described earlier [22]. 1,2-Dihexadecyl-*sn*-glycerol, 1,2-DMPA and 1,2-DPPA were purchased from Medmark (München-Grünwald, F.R.G.). 1,3-Dimyristoylglycerol and 1,2-dihexadecyl-*sn*-glycerol were shown to be pure by TLC on boric acid plates [23] with the solvent system chloroform/hexane/diisopropylether (5 : 4 : 1, v/v). The 1,3-dimyristoylglycerol ($R_F = 0.3$) did not contain 1,2-isomer ($R_F = 0.2$) and the 1,2-dihexadecyl-*sn*-glycerol ($R_F = 0.4$) was free of 1,3-isomer ($R_F = 0.5$). *N*-Phenylnaphthylamine was purchased from Eastman. Phosphate was analyzed with the reagent Kit from Serva (Heidelberg, F.R.G.).

Synthesis of phosphatidic acids. Freshly distilled phosphorus oxychloride (boiling range 105—107°C), 1.8 g (0.012 mol), was dissolved in 20 ml of tetrahydrofuran, stirred and cooled in an ice bath. Triethylamine, 2 g (0.02 mol), in 20 ml of tetrahydrofuran, was added followed by the dropwise addition of 1,2-dihexadecyl-*sn*-glycerol, 5.4 g (0.01 mol), or 1,3-dimyristoylglycerol, 5.0 g (0.01 mol), in 60 ml of tetrahydrofuran. Stirring was continued and the ice bath was replaced by a water bath of 20°C for the phosphorylation of 1,2-dihexadecyl-*sn*-glycerol and of 40°C for the phosphorylation of 1,3-dimyristoylglycerol. The phosphorylation was completed after 30 min, as shown by TLC. The starting material, with an R_F value of about 0.3 in 1 : 1 diisopropylether/hexane (v/v), was completely converted to a new substance, R_F value of about 0.6, giving a deep-blue colour with molybdate spray [24]. The reaction mixture was filtered to remove the precipitated triethylamine hydrochloride. The filtrate was cooled in an ice bath under continuous stirring and hydrolysis of the phosphorus chlorides was achieved by the addition of 0.5 M sodium hydrogen carbonate, 100 ml, and 0.5 M EDTA, 20 ml. The hydrolysis was completed after 6 h. The tetrahydrofuran layer (upper) was filtered and 100 ml acetone added to the filtrate. The precipitate was collected and repartitioned in a mixture of $CHCl_3/CH_3OH/H_2O$ (1 : 1 : 1), 450 ml, to remove sodium acetate from the lipid. The chloroform layer was evaporated and the residue dissolved in 40 ml chloroform. 400 ml acetone was added to precipitate the phospholipids. The purity of the phosphatic acids was checked by TLC and elemental analysis: (a) 1,2-dihexadecyl-*sn*-glycerol-3-phosphoric acid, disodium salt, yield 90%, mol. wt. 682.9 (calculated form $C_{35}H_{71}Na_2O_6P \cdot 1H_2O$; expected: C, 61.56%; H, 10.78%; P, 4.54%; found: C, 61.87%; H, 10.69%; P, 4.51%); (b) 1,3-dimyristoylglycerol-2-phosphoric acid, disodium salt, yield 78%, mol. wt. 654.8 (calculated for $C_{31}H_{59}Na_2O_8P \cdot 1H_2O$; expected: C, 56.87%; H, 9.39%; P, 4.73%%; found: C, 56.41%; H, 9.45%; P, 4.62%).

The melting points of the phosphatidic acids were determined for free acid, mono- and disodium salts of solid. The different salt forms were obtained by dissolving 200 mg of disodium salt in chloroform. The addition of 1 M HCl, or 1 M sodium acetate (pH 4.8), resulted in the formation of the free acid and

monosodium salt, respectively. Methanol was added to facilitate phase separation. The chloroform layer was evaporated and the residue crystallised from hexane (free acid) or chloroform/acetone (monosodium salt).

Sample preparation and spectrophotometric measurements. Dispersions of the phosphatidic acids, $5 \cdot 10^{-3}$ M, were prepared by shaking the respective amount of phosphatidic acid (disodium salt) with double-distilled water and incubating the mixture for 30 min at 50° C for 1,2-DMPA and 1,3-DMPA and at 70° C for 1,2-DPPA and 1,2-DHPA. The resulting lipid dispersions of pH 8.5—9.5 were freshly prepared each day. No decomposition products were observed by TLC in the stock solution up to a period of at least 12 h at 20° C.

The concentration of the lipid dispersions for the determination of the phase transition temperatures was $2.5 \cdot 10^{-4}$ M and $2 \cdot 10^{-6}$ M in *N*-phenylnaphthylamine, which did not influence the 90° C light-scattering signal in these experiments. For 1,2-DMPA and 1,2-DPPA dispersions, the measurements were performed with and without the addition of 5% ethylene glycol with no observed differences in the transition behaviour. The pH of the phosphatidic acid dispersions was adjusted to the desired values by the addition of HCl or NaOH in water. The pH of the dispersions at 25° C was controlled immediately before and after one cooling-heating cycle. To equilibrate the system it was most important to temper the lipid dispersion in the fluid state, about 5° C above T_t for 10 min, before starting with the cooling run. In the case of the ester phosphatidic acids, the dispersions at pH values larger than 10 or smaller than 4 were only used for one temperature cycle, because of their instability.

The ordered fluid phase transition in phosphatidic acid dispersions was monitored following the change in the fluorescence [25] and in 90° light scattering at 400 nm [26] in the same experiments. The phase transitions is indicated by a large increase in the fluorescence intensity as shown in Fig. 1 for 1,2-DHPA or in a decrease of 90° ligth-scattering intesity as shown in Fig. 2 for 1,2-DMPA. The fluorescence intensity of *N*-phenylnaphthylamine was monitored at 430 nm (emission) and 350 nm (excitation). The increase (ordered to fluid transition) or decrease (fluid to ordered transition) in the fluorescence intesity at the phase transition temperature T_t is the result of the more favourable partitioning of the dye into the hydrocarbon phase of the fluid bilayers. The dye does not influence the phase transition of phosphatidic acid dispersions at a molar ratio of dye/lipid smaller than 10^{-2} as demonstrated by almost identical T_t values obtained by fluorescence, 90° C light scattering or calorimetry (Blume, A. and Eibl, H., unpublished data). The degree of dissociation (α) was determined by acid-base titrations of the different phosphatidic acids as described earlier [7]; pK_1 of 3.5 and pK_2 of 9.0 were found for 1,2-DMPA. Almost identical titration curves and p*K* values were obtained for the structural analogues. The higher p*K* of the phosphate groups in phosphatidic acids in comparison to glycerol phosphate is due to electrostatic forces attracting the protons to the negatively charged bilayer surface.

Results and Discussion

The described procedure for the synthesis of 1,2-DHPA and of 1,3-DMPA allows the complete conversion of the respective alcohols to phosphatidic acids

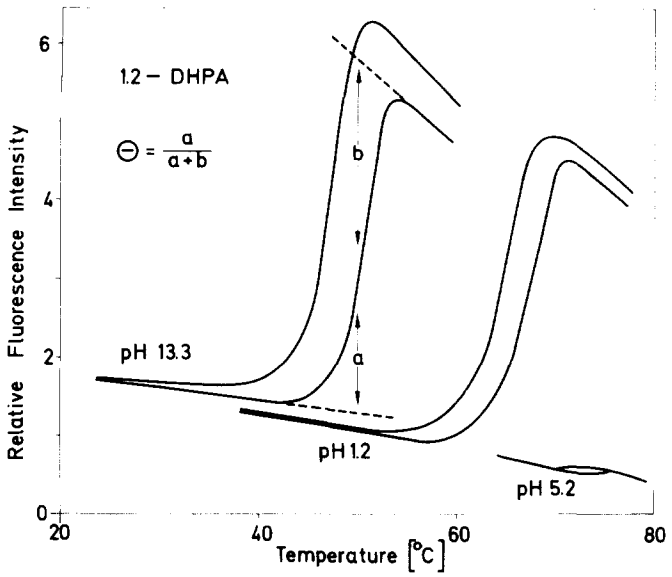

Fig. 1. Fluorescence indication of lipid phase transition for 1,2-DHPA at different degrees of ionisation (original tracings). Excitation, 350 nm; emission, 420 nm. Dispersion: $2.5 \cdot 10^{-4}$ M lipid, $2 \cdot 10^{-6}$ M *N*-phenylnaphthylamine, pH 1.2, 5.2 and 13.3. The value of Tt is defined as the temperature where the degree of transition $\theta = 0.5$.

by phosphorylation with phosphorus oxychloride. Byproducts such as bisphosphatidic acids [27], which have been reported when phosphorus oxychloride is used in such synthetic procedures, were not observed in our experiments. We conclude that the formation of such byproducts is avoided by the use of a large excess of phosphorus oxychloride in the phosphorylation step.

The phase transition behaviour of phosphatidic acid dispersions was followed by observing changes in the *N*-phenylnaphthylamine fluorescence or 90° light-scattering intensity. Typical fluorescence and 90° light-scattering scans are

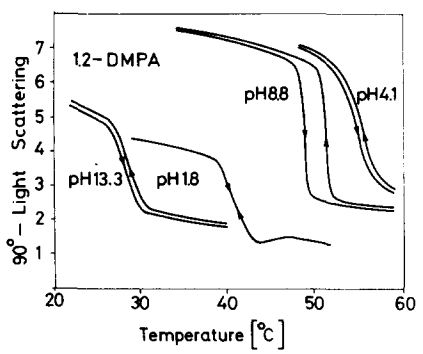

Fig. 2. 90° light-scattering indication at 400 nm of the lipid phase transition at different degrees of ionisation (original tracings). Dispersion: $5 \cdot 10^{-4}$ M lipid, pH 1.8, 4.1, 8.8 and 13.3. In some experiments *N*-phenylnaphthylamine, $2 \cdot 10^{-6}$ M, was also included.

shown in Figs. 1 and 2, respectively. They allow the evaluation of T_t values which are derived from the original curves as indicated in Fig. 1. The T_t values determined by fluorescence or light scattering correspond to each other closely. In addition there was good agreement with T_t values obtained by differential scanning calorimetry (Blume, A. and Eibl, H., unpublished data).

T_t values at the corresponding pH values or degrees of dissociation (α) allow the construction of T_t vs. pH diagrams as shown in Figs. 3 and 4, which are the main subject of the following discussion. For simplification, hysteresis effects are neglected. Hysteresis, the difference in T_t values taken from one cooling and one heating scan, may amount to about 4°C at high pH; but these differences are not shown in the figures. The T_t values given represent averaged values from one cooling and one heating cycle.

A variation of the pH from 2 to 12 corresponds to bilayers with no residual charge up to minus two negative charges/phosphate moiety. Such titrations were possible for the four different phosphatidic acids: 1,2-DMPA, 1,3-DMPA, 1,2-DPPA and 1,2-DHPA. The following discussion concentrates on specific states of the bilayers which are determined by the charge/phosphate group of

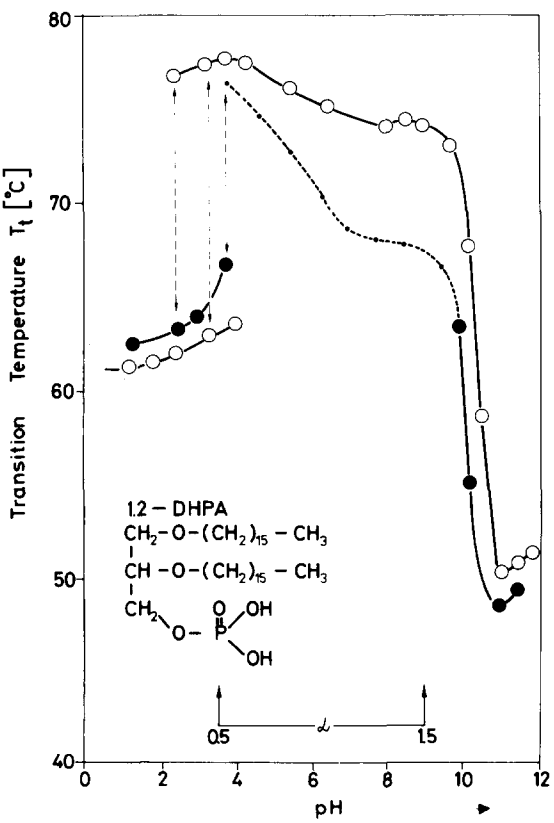

Fig. 3. The effect of increasing ionisation on the phase transition temperature of 1,2-DHPA. Two distinct points of the degree of dissociation α are shown; 0.5 and 1.5 (pK_1 and pK_2). ○, indication by 90° light-scattering; ●, indication by *N*-phenylnaphthylamine fluorescence; - - - - - -, see text.

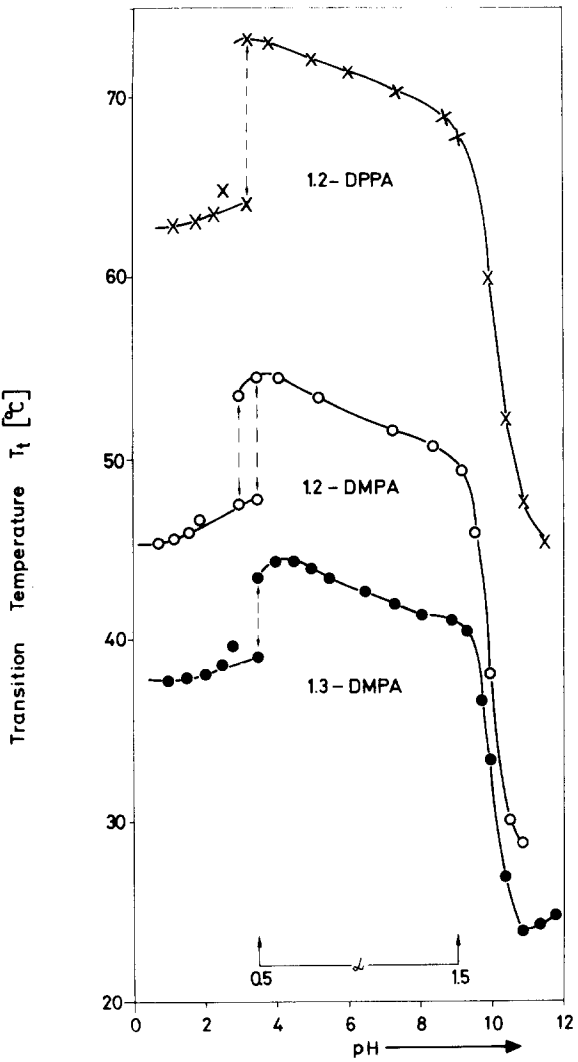

Fig. 4. The effect of increasing ionisation on the phase transition temperature of 1,2-DPPA (X), 1,2-DMPA (○) and 1,3-DMPA (●). Two distinct points of the degree of dissociation α are shown: 0.5 and 1.5 (pK_1 and pK_2). Indication by *N*-phenylnaphthylamine fluorescence and 90° light-scattering (see text).

the lipid molecule, this being the degree of dissociation, α. The behaviour of the chemically stable 1,2-DHPA at increasing α, the pH range from 2 to 12, is described in detail. In principal, the ester phosphatidic acids exhibit a similar charge behaviour to 1,2-DHPA.

In Table I the melting points of the different solid, water-free phosphatidic acids in the various ionization states, i.e. free acid, monosodium and disodium salts, are compared with their aqueous bilayer phase transition temperature T_t, at different degrees of dissociation, α = 0, 1 and 2. In the case of 1,2-DHPA for complete protonation, that is α = 0, the melting point of the water-free system (62°C) and T_t of the lipid dispersion (62°C, $5 \cdot 10^{-4}$ M) are the same as each

TABLE I

MELTING POINTS AND PHASE TRANSITION TEMPERATURE OF PHOSPHATIDIC ACIDS AT DIFFERENT DEGREES OF DISSOCIATION α

		α		
		0.0	1.0	2.0
1,2-DMPA	melting point	59	165	260
	transition temperature	45	52	28
1,3-DMPA	melting point	47	158	254
	transition temperature	38	42	23
1,2-DPPA	melting point	69	160	253
	transition temperature	62	71	46
1,2-DHPA	melting point	62	166	257
	transition temperature	62	75	50

other, indicating that the presence of water does not at all influence the state of the fully protonated lipid. The dispersion thus seems to represent a distribution of solid lipid in water with negligible interaction between 1,2-DHPA and water molecules. This is substantiated by a comparison of the transition enthalpy values which were found to be identical for the solid free acid and the aqueous dispersion at $\alpha = 0$ (Blume, A. and Eibl. unpublished data).

The presence of excess water with phosphatidic acids containing ester bonds, that is for 1,2-DMPA, 1,3-DMPA and 1,2-DPPA, lowers T_t in comparison to the melting points of the fully protonated lipids. The effect is more pronounced for the shorter chain analogues with differences between melting point and T_t of 14°C for 1,2-DMPA, 9°C for 1,3-DMPA and 7°C for 1,2-DPPA. The ester bonds therefore facilitate the penetration of water into the phosphatidic acid molecules, which was already indicated from earlier monolayer experiments [28].

Inducing a charge on 1,2-DHPA bilayers results in the formation of a second phase with $T_t = 77°C$ as shown in Fig. 3, that is 15°C above the first transition. In an intermediate region from pH 2.5 to 3.5 both transitions are detected by 90° light-scattering. At pH 4, $\alpha \approx 0.5$, the transition of the fully protonated state disappears completely and the maximum in the T_t vs. pH diagram is reached. This increase in T_t with increasing charge was not expected and is reported here for the first time. Destabilisation of the bilayers by electrostatic repulsion of the negatively charged lipid molecules within the bilayer is predicted to result in a decrease of the phase transition temperature. The observed increase in T_t, however, is explained by the presence of protons and negatively charged phosphate groups within the membrane surface, and the destabilizing effect could therefore be overcompensated by strong intermolecular hydrogen bonding. It was shown in other systems that the presence of protons and phosphate groups in membrane surfaces may lead to strong interactions which stabilize the lipid matrix (Eibl, H. and Woolley, P., unpublished data).

The behaviour of the ester phosphatidic acids is quite similar. But the

TABLE II

The maximum difference in the phase transition temperature from $\alpha = 0.5—2.0$ (ΔT_t); and the slope ($dT/d\alpha$) from the T_t vs. pH diagram for the plateau region $\alpha = 0.5—1.5$ for different phosphatidic acids. The values for ΔT_t^{el} from $\alpha = 0.5—2.0$ are obtained by extrapolation of $dT/d\alpha$ to $\alpha = 2.0$. The values for ΔT_t^{tilt} are then obtained by subtracting ΔT_t^{el} from ΔT_t.

	ΔT_t	$dT/d\alpha$	ΔT_t^{el}	ΔT_t^{tilt}
1,2-DPMA	26	4.0	6.0	20.0
1,3-DMPA	21	3.3	5.0	16.0
1,2-DPPA	27	4.7	7.0	20.0
1,2-DHPA	27	4.3	6.5	20.5

increase in T_t for $\alpha = 0—0.5$ is less pronounced with 10°C for 1,2-DMPA, 7°C for 1,3-DMPA and 11°C for 1,2-DPPA.

For $\alpha = 0.5$ and 1.5 which corresponds to the dissociation of one proton/phosphate residue, a large but slightly descending plateau is observed in the T_t vs. pH diagram for 1,2-DHPA and also for the ester phosphatidic acids as shown in Figs. 3 and 4. At neutral pH the phosphatidic acid bilayers are in the plateau region and scarcely influenced by charge variations or changes in ionic concentrations. The slope of the plateau, $dT_t/d\alpha \approx -4.1$°C, is almost the same for all the different phosphatidic acids. The values, $dT_t/d\alpha$, for the plateau region are summarized in Table II. The values $dT_t/d\alpha$ obtained experimentally may be compared with the theoretical deviation for the electrostatically induced shift in T_t for the effect of one charge/lipid molecule:

$$\Delta T_t^{el} = -70.7\ \Delta f/f\ (°C)\ [7,29]$$

Inserting $\Delta f/f = 0.07$ for pH 12 as determined for 1,2-DHPA [29] results in $\Delta T_t^{el} = -5$°C. The experimental values $dT/d\alpha = -4$°C for 1,2-DMPA, −4.7°C for 1,2-DPPA and −4.3°C for 1,2-DHPA for the plateau are close to the theory. The slope of the plateau region represents the electrostatic destabilisation effect with increasing charge. The small decrease in T_t for an increase in charge from $\alpha = 0.5$ to 1.5 can be explained by an increased electrostatic repulsion within the membrane surface. Because of the very similar results for the different ester and ether phosphatidic acids it appears that relatively large differences in the chemical structure of these molecules do not change the phase transition behaviour of these lipids in the region between the first and second pK. It also demonstrates that the lipid structure is particularly stable when negatively charged phosphate groups and protons are located simultaneously within the membrane surface (pK regions).

A dramatic change in the T_t vs. pH diagram of the phosphatidic acids is observed in the region of the second pK that is $\alpha = 1.5$. Small changes in the degree of dissociation, α, lead to large changes of T_t (Figs. 3 and 4). The slopes in $dT/d\alpha \approx -100$°C indicate a change in the bilayer properties, which cannot be explained by electrostatic considerations.

From X-ray diffraction studies and Raman spectroscopy of the chemically stable 1,2-DHPA at pH 7 and pH 12 ($T < T_t$; $\alpha = 1.0$ and 2.0) it has been concluded that the alkyl chains in the ordered phase increase their angle of tilt with respect to the bilayer plane at the higher pH [29]. This results in a loss of

van der Waals interaction energy which leads to the large and sudden decrease in T_t. The electrostatically induced tilt of the alkyl chains is most probably due to the penetration of water into the destabilized surface region of 1,2-DHPA. This was discussed also for phosphatidylglycerol by Watts et al. [10] and for lecithin dispersions by Tardieu et al. [30] and Janiak et al. [31].

The stepwise titration of 1,2-DHPA allowed the exact determination of the degree of dissociation α, where the tilting of the chains occurs. It is clearly indicated by the sudden and deep drop in the T_t vs. pH diagram and corresponds to $\alpha = 1.5$.

It should be mentioned here, that X-ray analysis is only available in the case of 1,2-DHPA. This analysis was possible because of the high chemical stability of this lipid at extreme pH values. However, the similarity of the T_t vs. pH diagrams of 1,2-DHPA to that for the ester phosphatidic acids indicates that similar mechanisms are involved in the case of the ester phosphatidic acids when α becomes larger than 1.5. As shown in Fig. 4, the ester phosphatidic acids do contain the smoothly descending plateau from pH 4 to 9 in the T_t vs. pH diagram which at $\alpha = 1.5$ is terminated by a sudden decrease in T_t for 1,2-DMPA, 1,3-DMPA and 1,2-DPPA.

Whereas for the fully protonated lipids ($\alpha = 0$) the solid lipid melting point temperatures and aqueous dispersions phase transition temperatures are very similar (see Table I), at higher values of α the differences become much larger and depend upon the alkyl chain length. At $\alpha = 1.0$, the difference between melting point and T_t is 115°C for 1,2- and 1,3-DMPA and about 90°C for 1,2-DHPA and 1,2-DPPA. At $\alpha = 2.0$ the temperature difference is even greater: about 231°C for 1,2- and 1,3-DMPA and 207°C for 1,2-DHPA and 1,2-DPPA (see Table I).

For $\alpha = 2.0$ the stabilization of membranes by interfacial hydrogen bonds is very much reduced. Water molecules can penetrate and lead to the described tilting of the fatty acid chains [29]. As shown by Fig. 3 the titration of the chemically stable 1,2-DHPA not only resulted in a reliable end value (minimum) in the T_t vs. pH diagram but also showed the gradual increase in T_t with increasing Na^+ concentration owing to the screening effect of counterions [32]. A stable end value in the T_t vs. pH diagram means that T_t does not decrease any further with increasing pH. Similar chemical stability was indicated for 1,3-DMPA, a structural analogue of 1,2-diacylphosphatidic acids (see Fig. 4). The lack of reliable end values in the T_t vs. pH diagram of 1,2-DMPA and 1,2-DPPA is due to chemical decomposition at high pH. It was found that one heating-cooling cycle at pH 12 results in 5—10% decomposition products for the 1,2-diacylphosphatidic acids. However, 1,2-DHPA at pH 12 and 80°C (above T_t) was stable for at least 12 h.

In our earlier work on 1,2-DMPA [7], the observed decomposition of 1,2-DMPA at high pH did not lead to incorrect values for the decrease in T_t of about 24°C altering α from 1.0 to 2.0. In comparison to other studies [18,19], the smaller decrease in T_t of about 9°C for this ionization step could be explained either by incomplete deprotonation or by the inability of the reporter molecules to indicate the correct phase transition. Such an effect was recognized in this study for the system 1,2-DHPA and *N*-phenylnaphthylamine.

As shown in Fig. 1, *N*-phenylnaphthylamine is of limited use for the deter-

mination of T_t in the system 1,2-DHPA in the region between pK_1 and pK_2. The change in the fluorescence intensity is small and hardly detectable (see original tracing for pH 5.2). This might be caused by a very tight packing of the alkyl chains in this pH region. The lower T_t values for 1,2-DHPA obtained from fluorescence indication are hence less accurate and marked with a dotted line (see Fig. 3). The difference in the T_t values amount to about 8°C when compared with 90° light scattering or calorimetry (Blume, A. and Eibl, H., unpublished data).

It should be mentioned here that earlier experiments with 1,2-DMPA [7] did not allow full protonation owing to precipitation of the dispersion at pH values below pH 4. This problem has now been overcome by using 1,2-DMPA and 1,2-DPPA dispersions in the presence of 5% ethylene glycol. This small amount of ethylene glycol successfully inhibited precipitation of the dispersions at low pH and did not influence the phase transition behaviour at pH values from 4 to 12 (measurements with and without ethylene glycol resulted in identical T_t values). For 1,2-DHPA and 1,3-DMPA the addition of ethylene glycol was not necessary and was therefore omitted.

The results with phosphatidic acids are of general importance for phospholipids carrying two dissociable protons. The ionization effect of phosphatidic acids, which leads to the dissociation of two protons/molecule in the membrane surface, is also observed for phosphatidylethanolamines. In this case one proton is dissociated from the phosphate group, the other one is liberated from the nitrogen. The plateau region for this phospholipid is even more extended owing to the greater difference between the first and the second pK [1]. The similarity between singly charged phosphatidic acids and phosphatidylethanolamines is further reflected in the phase transition temperature, since T_t for 1,2-DMPA at pH 7 is 50.5°C and T_t for 1,2-dimyristoyl-*sn*-glycerol-3-phosphorylethanolamine is 49.6°C. In the case of phosphatidylethanolamines full deprotonation also leads to a large drop in the phase transition temperature [1] as described for the phosphatidic acids and most probably also includes tilting of the acyl or alkyl chains. The cephalin system is now being analyzed by our group intensively to demonstrate the more general aspects of the work with phosphatidic acids.

The behaviour of phospholipids with only one dissociable proton is quite different. Only one dissociation step is involved and in consequence of the lack of the second pK the characteristic plateau region of the phosphatidic acids is replaced by a maximum in the phase transition temperature at $\alpha = 0.5$. For phosphatidic acid methylesters this maximum in T_t was believed to represent the phase transition of the bilayer membranes at full protonation, $\alpha = 0.0$ [5,32]. However, due to the presence of salts and the respective shift of the pK to lower values, the fully protonated state was not reached in these experiments. This was the reason that the described drop in T_t for phosphatidic acids by altering α from 0.5 to 0.0 was not observed in phosphatidic acid alkylesters.

To summarize one can say that increasing the charge in bilayer membranes of phosphatidic acids will result in three different states: Firstly the neutral state, where the phase transition temperature of the phosphatidic acids corresponds to the melting point of the fully protonated crystalls. The system represents a microcrystalline dispersion of phosphatidic acid in water.

Secondly, the extended plateau region between pH 4 and 9 which corresponds to a stable lipid bilayer arrangement. An increase in charge by one unit (α = 0.5—1.5), which is equivalent to the dissociation of one proton/phosphatidic acid molecule, lowers T_t only by a value of about 4—5°C. This characteristic plateau region in the T_t vs. pH diagram for the four phosphatidic acids in general has T_t values above those of the fully protonated state. In addition, it is interesting to note that the phase transition temperature of the phosphatidic acids at α = 1.5, where half of the phosphatidic acid molecules carry one negative charge and half of the phosphatidic acid molecules carry two negative charges, is still above T_t of the fully protonated state. This demonstrates the strong stabilisation effect of protons in the surface of phosphatidic acid bilayers. Thirdly the fully deprotonated state, where the phosphatidic acid bilayers are characterized by a minimum in the phase transition temperature. The chains in the ordered state are more tilted thus reducing the van der Waal's interaction energy between the lipid chains. The stronger tilt is induced if the degree of dissociation α becomes larger than 1.5.

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