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THE INFLUENCE OF CHARGE ON BILAYER MEMBRANES

CALORIMETRIC INVESTIGATIONS OF PHOSPHATIDIC ACID BILAYERS

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Summary

The pH-dependence of the phase transition of dimyristoyl phosphatidic acid and dihexadecyl phosphatidic acid has been investigated using differential scanning calorimetry. Varying the pH induces different degrees of ionization of the polar head group. The changes in transition temperature with pH as observed by calorimetry are in good agreement with those obtained by measuring the changes in light scattering, whereas the transition temperatures reported by the fluorescent probe *N*-phenylnaphthylamine do not always coincide with those determined from calorimetry [1]. The observed maximum of the transition temperature at pH 3.5 corresponds to a minimum in the transition enthalpy vs. pH diagram. At this pH a particular stable bilayer phase is formed. Full protonation of phosphatidic acids leads to suspensions of mycrocrystals. The transition enthalpy approaches the value of the melting enthalpy of crystalline anhydrous phosphatidic acid. The decrease in the transition enthalpy at high pH values is due to a change in the hydrocarbon chain interactions induced by the doubly charged head groups. The cooperativity of the transition varies with the degree of ionization of the head group, being lower for doubly charged phosphatidic acids.

Introduction

Calorimetric investigations of the phase transition of charged phospholipids are rather limited. Jacobson and Papahadjopoulos reported phase transition

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

data for dipalmitoyl-phosphatidylglycerol and dipalmitoyl-phosphatidic acid [2], McDonald et al. [3] investigated the phase behaviour of dipalmitoyl-phosphatidylserine, Verkleij et al. and van Dijck et al. [4,5] reported transition enthalpies for dilauroyl- and dimyristoyl-phosphatidyl-glycerol, and very recently van Dijck et al. [6] measured the pH-dependence of the transition temperature of several charged phospholipids using calorimetry. However, up to now a complete determination of the pH-dependence of the transition enthalpy of a charged phospholipid has not been made.

On the other hand, there are considerable discrepencies in the reported phase characteristics of phosphatidic acid and phosphatidylserine bilayers at different pH values as measured by calorimetry and spectroscopy with fluorescent probes. Some of these differences may be attributed to the use of fluorescent probes, which disturb the bilayer and for this reason do not report the true phase transition temperatures, others to the deterioration of the phospholipids at extreme pH values.

To determine whether the phase transition data obtained with the fluorescent probe *N*-phenyl-naphthylamine are correct [1], we investigated the same phosphatidic acid systems using calorimetry, this being a non-perturbing intrinsic method of measuring phase transitions. As well as the ester compound DMPA we used the ether analogue DHPA, which is stable to hydrolysis at low and high pH values [1]. The data obtained with the phospholipid can be directly compared to those obtained with ester phospholipids, since their ether analogues are shown to have similar phase transition characteristics.

Materials and Methods

Lipid dispersions for calorimetry were prepared by sonicating the desired amount of lipid (20—30 mg) for 2—3 min in approx. 20 ml of bidistilled water at a temperature above the respective phase transition temperature of the lipid. This sonification procedure yields multilamellar liposomes comparable to those obtained by vigorous stirring or shaking. The pH was adjusted to the desired value with dilute HCl or NaOH with a Radiometer PHM 26 pH-meter with microglasselectrode (Schott and Gen., Mainz). After adjustment of the pH value, the dispersion was again sonicated for 30 s at low power. The pH was controlled before and after each calorimetric run. The lipid concentration was 2 mM. The calorimetric measurements were made using an adiabatic differential scanning calorimeter as described before [7,8]. The heating rate was 13.5 degrees/h. At least three runs were made with each lipid dispersion to test the reversability of the phase transition. The transition enthalpies Δh were determined by measuring the area under the excess heat vs. temperature curve by paper weighing. The molar ΔH values were calculated for the known amount of dispersed lipid. In some cases the phospholipid content of the dispersion was checked by the determination of the inorganic phosphate after heating with perchloric acid. The concentrations of lipid determined by these two different methods varied by only 1.5%. For the determination of the enthalpy of fusion of crystalline phosphatidic acid approx. 15 mg of the lipid was sealed in a small glass tube, which was immersed in the calorimetric vessel filled with 25 g of water.

Results

Figs. 1A and 1B show calorimetric scans of DMPA dispersions in water and 0.1 M NaCl solution at different pH values. The pH-dependence of the transition temperature T_t determined from our calorimetric scans is in accordance with the results obtained before by fluorescence spectroscopy or calorimetry [1,6,9]. The transition enthalpies of DMPA as a function of pH are shown in Fig. 2. The ΔH values for dispersions containing 0.1 M NaCl are appreciably larger when states of equivalent degree of ionization are compared. The decrease of the ΔH value in the alkaline pH-region reflects the dissociation of the second proton of DMPA. This decrease in ΔH is shifted to lower pH values in dispersions containing 0.1 M NaCl in accordance with the shift in pK [9]. The transition enthalpy for the doubly charged form of DMPA is approx. 3.5 kcal/mol lower than for the singly charged form. The decreae in the transition enthalpy below pH 4.0 was reproducible and not due to the deterioration of the lipid. This decrease is in the same pH range where the transition temperature is still rising. With the ester lipid DMPA measurements below pH 3 and above pH 11 were not possible due to lipid hydrolysis.

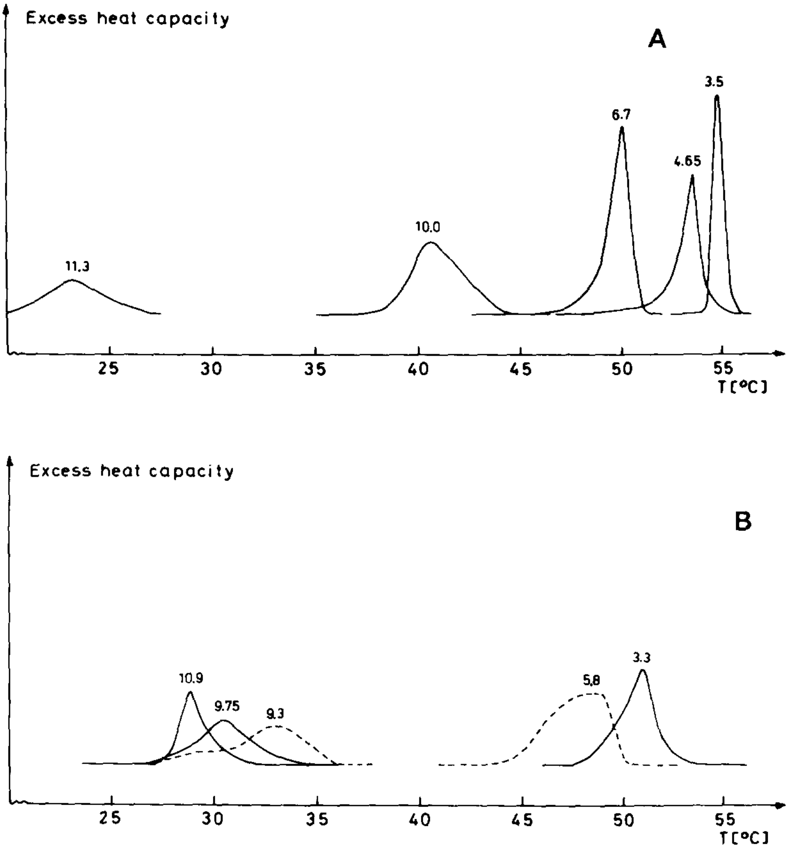


Fig. 1. Differential scanning calorimetry curves of 2 mM DMPA dispersions in (A) pure water and (B) 0.1 M NaCl-solution.

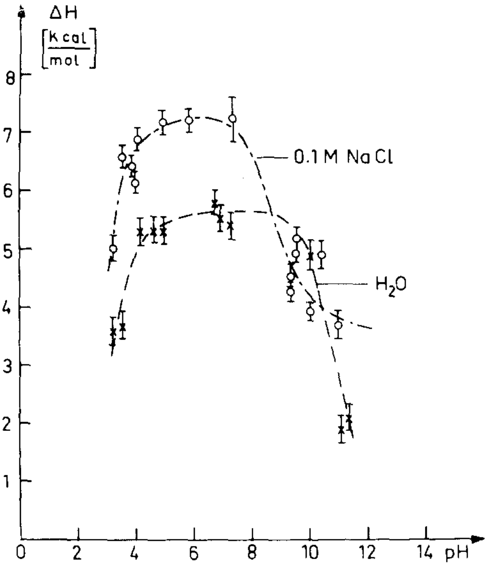


Fig. 2. Dependence of the transition enthalpy ΔH on the pH value for DMPA dispersions in water and 0.1 M NaCl solution. Bars represent standard deviations of at least three different measurements.

The knowledge of the calorimetric ΔH_{cal} values and the $\Delta H_{\mathrm{van'tHoff}}$ values taken from the normalized transition curves at $\theta = 0.5$, using the expression

$$\left(\frac{\mathrm{d}\theta}{\mathrm{d}T}\right)_{\theta=0.5} = \frac{\Delta H_{\mathrm{van't\ Hoff}}}{4RT_t^2}$$

makes it possible to evaluate the ratio $\Delta H_{\mathrm{van'tHoff}}/\Delta H_{\mathrm{cal}} = n$, i.e., the size of the cooperative unit involved in the phase transition [10]. Table I summarizes the values for n for DMPA dispersions in water and in 0.1 M NaCl solution. The cooperativity of the transition for doubly charged DMPA is somewhat lower than for the singly charged form. In the pH region of the second pK the transitions are especially broad since the pK changes during the transition [3,11]. The addition of 0.1 M NaCl leads to a decrease in cooperativity.

To confirm our results found for DMPA we investigated the phase transitions of the ether analogue DHPA which is extremely stable to hydrolysis.

TABLE I

SIZE OF THE COOPERATIVE UNIT n FOR DMPA DISPERSIONS

In water		in 0.1 M NaCl solution	
pH	n	pH	n
3.5	288	3.3	53
4.6	101	5.8	30
6.7	100	9.3	35
10.0	43	9.75	48
11.3	83	10.9	93

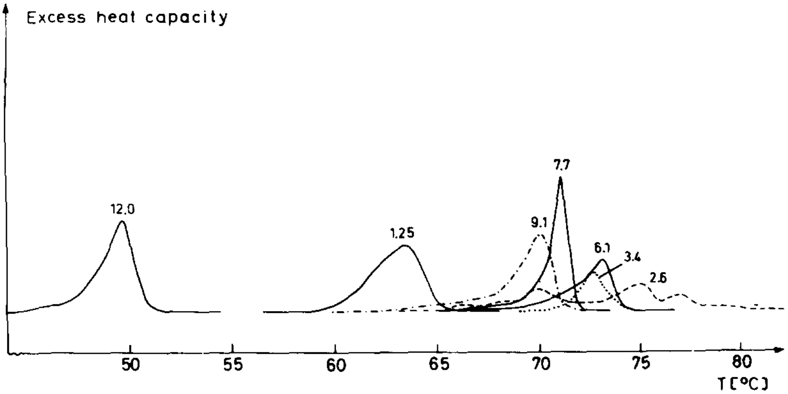
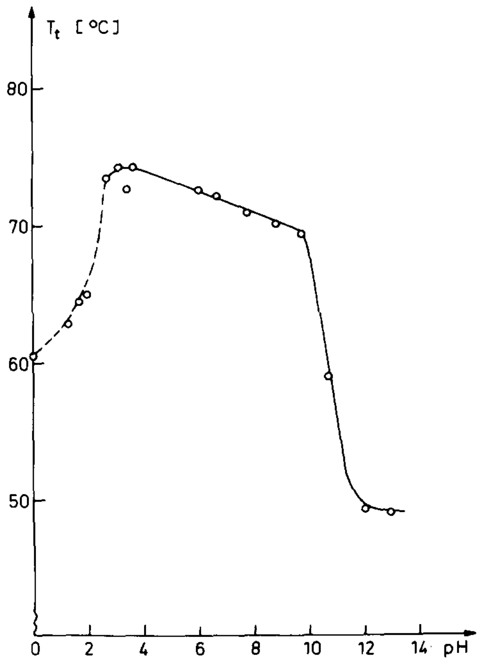


Fig. 3. Differential scanning calorimetry curves of aqueous 2 mM DHPA dispersions at different pH values.

Calorimetric scans of DHPA dispersions in water at various pH values are shown in Fig. 3. The pH-dependence of T_t (Fig. 4) for DHPA corresponds closely to the results obtained by 90° light scattering [1]. The T_t value at pH 0.0 shown in Fig. 4 represents the melting point of pure crystalline DHPA. It was not possible to measure the phase transition of DHPA below pH 1.5 because at



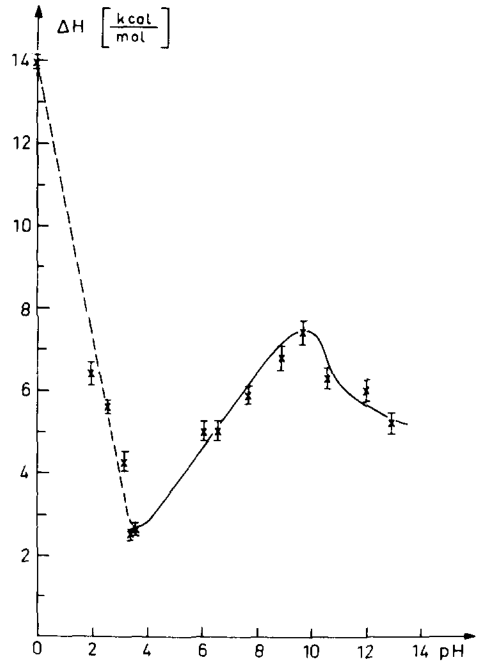


Fig. 4. Dependence of the transition temperature T_t of DHPA on the pH value. In the pH range indicated by the dashed line DHPA does not form stable dispersions (see text).

Fig. 5. Dependence of the transition enthalpy ΔH on the pH value for DHPA dispersions in water. Bars represent standard deviations of at least three different measurements. For explanation of the dashed line see Fig. 4.

TABLE II

SIZE OF THE COOPERATIVE UNIT n FOR DHPA DISPERSIONS IN WATER

pH	n
3.4	360
6.1	158
7.7	183
9.1	90
12.0	80

the higher concentrations used for calorimetry compared to light scattering or fluorescence, the lipid could not be dispersed sufficiently well to enable transfer to the calorimetric vessel. An additional complication in these cases was, that the lipid concentration could not be determined accurately since the lipid stuck to the walls of the glass tube used for sonification as well as to the walls of the calorimetric vessel.

The transition enthalpies of DHPA as a function of pH are shown in Fig. 5. Again, a decrease of the ΔH value at high pH is observed, as the head group becomes doubly charged. This decrease is not as pronounced, however, as that observed for DMPA. The transition enthalpy decreases considerably between pH 9.0 and 5.0 in contrast to the observations for DMPA-dispersions, where this decrease starts at lower pH-values. The minimal value for ΔH is found at pH 3.5 where the transition temperature T_t has a maximum. At yet lower pH values the transition enthalpy rises again. Because of the reasons mentioned above molar ΔH values could not be determined exactly for dispersions with a pH below 1.5. The ΔH value of 14.0 kcal/mol at pH 0.0 in Fig. 5 represents the enthalpy of fusion of fully protonated crystalline DHPA. The evaluation of the size of the cooperative unit for DHPA (Table II) gives results which are similar to those obtained for DMPA, i.e., the cooperativity is smaller at pH 12.0 but increases drastically on lowering the pH to 3.5.

Discussion

The results obtained by two independent methods, 90° light scattering measurements and differential scanning calorimetry are very similar regarding the pH-dependence of the T_t values of the two phospholipids DMPA and DHPA [1]. The transition temperatures for DHPA in the pH range between 3.5 and 9.5 as determined by fluorescence intensity measurements with N-phenylnaphthylamine, however, do not coincide with the correct transition temperatures determined by calorimetry. These differences were discussed before as being due to the tight packing of the hydrocarbon chains of the ether analogue DHPA in this particular pH range [1]. Complete protonation of DHPA apparently leads to the formation of mycrocrystals of DHPA in water so that the T_t value approaches the melting point of crystalline DHPA [1]. This was verified by calorimetry. The lipid phase in the pH range below 3.5 does not necessarily have to be lamellar. The structures predominantly formed by saturated phospholipids are of the lamellar type, however. Preliminary X-ray experiments with DMPA at pH 3.5 and 20°C showed the typical wide angle reflection at 4.18 Å arising from the tightly packed hydrocarbon chains (unpublished observations). Thus, it seems to be quite certain, that at least in the pH range between 3.5 and 12.0 the lamellar phase is maintained [12]. The experimental values for the total decrease in T_t between pH 3.5 and 12 are the sum of two terms, the electrostatic shift ΔT_t^{el} and the shift due to the change in tilt angle ΔT_t^{tilt} [1,12]. ΔT_t^{el} was found for DHPA to be 6–7°C in the pH range mentioned above [1]. A comparison of this value with the theoretical prediction for ΔT_t^{el} enables us to calculate the transition entropy for uncharged DHPA bilayers. ΔT_t^{el} was given by Träuble et al. [11] to:

$$\Delta T_t^{el} = 2kT \cdot \frac{L}{\Delta S^*} \cdot \frac{\Delta f}{f}$$

with k = Boltzmann constant; T = absolute temperature, L = Avogradro's number, ΔS^* = transition entropy of the uncharged species and $\Delta f/f$ = relative change in area at the transition.

Inserting the values for k, L and $\Delta f/f = 0.136$ (pH 7) [12], $T = 343$ K and $\Delta T_t^{el} = 4$ K for $\Delta\alpha = 1$, we obtain a value of 46 cal · degrees^{-1} · mol^{-1} for ΔS^*. This value is quite reasonable, when we compare it with the melting entropy of crystalline protonated DHPA, which was determined from the melting enthalpy to 42 cal · degrees^{-1} · mol^{-1}.

The change in the electrostatic internal energy accompanying the change in area per charged lipid molecule at the phase transition can be calculated by using a modified Gouy-Chapman theory for the electrical double-layer developed by Träuble et al. [11] and Jähnig [13]. The contribution of the changes in electrostatic internal energy at the transition to the observed transition enthalpy is small (—0.1 kcal/mol) [13,16] and cannot account for the observed differences in ΔH for the singly and doubly charged form, which are 3.5 kcal/mol for DMPA and 2.2 kcal/mol for DHPA. This decrease in the transition enthalpy with increasing surface charge is thus mainly due to the change of the internal energy and the van der Waals interactions of the system induced by the tilting of the hydrocarbon chains at high pH [12]. Up to now there is no satisfactory explanation for the observation that the addition of 0.1 M NaCl leads to higher transition enthalpies. We assume that the screening of the charges of the head groups by Na^+, which decreases the surface potential, leads to a tighter packing of the hydrocarbon chains in the gel phase and to a correspondingly larger change in molecular area at the transition.

The transition enthalpy for DHPA decreases continuously between pH 9.0 and 3.5 in contrast to the results found DMPA. These differences may reflect a difference in chain packing for these two lipids due to the absence of the hydrated ester carbonyl groups in DHPA. To explain the very low transition enthalpy of 2.6 kcal/mol at pH 3.5 we assume that at this particular pH, where DHPA is half protonated [1,9], a specifically stable bilayer phase is formed. For this phase only restricted lateral expansion is possible at the transition, leading to only small increases in internal energy and corresponding small changes in van der Waals energy. These conclusions are supported by the observation, that the absolute value as well as the increase of the fluorescent intensity of the probe N-phenyl-naphthylamine are very small for DHPA-dispersions at this pH [1]. The lipid molecules in this phase may be arranged alternatively, one single-charged next to a protonated lipid molecule, with one hydrogen bond between every phosphate group (similar to the arrangement of the phosphatidylethanolamine head groups [17]) and an additional hydrogen bond between every second phosphate group, connecting a protonated with a single-charged lipid molecule. This additional hydrogen bond may lead to the observed stabilization of the bilayer in the gel phase and to a smaller change in molecular area at the transition, thus increasing the transition temperature and lowering the transition enthalpy. Further protonation of DHPA will break up this stable arrangement of alternating protonated and single-charged molecules. A segregation of domains of fully protonated DHPA molecules eventually leads to the crystallization of the phosphatidic acid. This phase separation can be observed between pH 2.0 and 3.0 (see calorimetric scan at pH 2.6 in Fig. 3). Below pH 2.0 T_t and ΔH finally approach the melting point and melting enthalpy of pure crystalline DHPA. This phenomenon that a lipid crystallizes when its polar group can no longer be sufficiently hydrated was observed with phosphatidylserine, too. In this case the addition of Ca^{2+} leads to the crystallization of the lipid [18]. The experimentally observed dependence of the cooperativity of the transition on the charge of the head group is qualitatively in accordance with the theoretical predictions made by Forsyth et al. [19]. At high pH the cooperativity is slightly smaller for the doubly charged form of phosphatidic acids. The high cooperativity for dispersions at pH 3.5 again indicates that a tightly packed structure is formed, which increases the size of the cooperative unit (see Table I and II).

Finally, we want to compare our data for DMPA and DHPA with those reported by other workers. Jacobson and Papahadjopoulos [2] measured the phase transition of DPPA by calorimetry and found T_t values of 67°C at pH 6.5 and 58°C at pH 9.1. The transition temperature at pH 6.5 agrees with our data for DPPA (71°C) and DHPA (72°C) (see Fig. 4 and Ref. 1). The value for ΔT of 9°C between pH 6.5 and 9.1 for DPPA, however, is considerably lower than the values we found (27°C between pH 4.0 and 12.5). Apparently DPPA was not fully dissociated at pH 9.1, as is indeed indicated by the titration curves reported by these authors [2]. Their ΔH values for DPPA (5.2 kcal/mol at pH 6.5 and 2.9 kcal/mol at pH 9.1) are considerably lower than the transition enthalpies we found for DHPA (see Fig. 5). Normally ΔH values for ether- and ester-phospholipids are very similar [15,16]. A control experiment with DPPA at pH 8.0 indeed gave a ΔH value of 7.8 kcal/mol. It has to be noted that the DPPA transition reported by Jacobson and Papahadjopoulos is relatively broad (10°C). This is normally an indication of a perturbation of the bilayer by impurities, thus leading to lower ΔH values.

Recently, van Dijck et al. [6] measured the pH-dependence of DMPA by differential scanning calorimetry. Their T_t values are qualitatively in agreement with ours for dispersions with 0.1 M NaCl and with those reported earlier by Träuble and Eibl [9]. Unfortunately, these authors show no values for the pH-dependence of the transition enthalpy. The ΔH value at pH 7.0 taken from one of their figures (approx. 7 kcal/mol at 0.1 M NaCl), however, is in accordance with the ones we found (see Fig. 2).

The results, obtained by calorimetry for the pH-dependence of the phase transition of phosphatidic acids, show that care has to be taken when phase transition values can only be measured with spectroscopic probes. Only an intrinsic non-perturbing method can definitely report the true phase transition temperature at all states of ionization of the respective phospholipids. With phosphatidic acids fluorescence measurements with *N*-phenyl-naphthylamine report correct T_t values for the ester compounds DMPA and DPPA, but not for the ether lipid DHPA. This shows that the phase transition data obtained with spectroscopic probes should be checked using an intrinsic method before a quantitative interpretation of these data is attempted.

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