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INFLUENCE OF pH ON PHOSPHATIDIC ACID MULTILAYERS A RIPPLED STRUCTURE AT HIGH pH VALUES

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Summary

The influence of pH on the structure of 1,2-(ditetradecyl)-phosphatidic acid was investigated by differential scanning calorimetry and freeze-fracture electron microscopy. At pH 13.5—14 (2.6 M K^+), where phosphatidic acid has two negative charges, calorimetric scans show a small transition (pretransition) below the main phase transition temperature. Freeze-fracture studies of the same dispersions reveal regular band patterns (so-called ripples) in the plane of the bilayers, when the lipid is quenched from below the main phase transition temperature. This rippled structure is similar to the well-known rippled structure of phosphatidylcholines.

Introduction

It is well-known that phospholipids in aqueous dispersions exhibit a phase transition from an ordered structure to a more liquid-like disordered state. Besides this main phase transition, calorimetric scans have shown a further transition (termed pretransition) for phosphatidylcholine [1—3] and phosphatidylglycerol [4], which takes place a few degrees below the main phase transition temperature.

The pretransition of phosphatidylcholines has been associated with the appearance of regular band patterns in the plane of the bilayer (so-called ripples), both by X-ray diffraction [5] and by freeze-fracture studies [6]. The rippled structure was found only when the dispersion was quenched from temperatures between the pre- and the main phase transition temperatures [6]. These findings did not agree with earlier studies, where a rippled structure was also

Abbreviations: T_t, main phase transition temperature; T_p, pretransition temperature.

reported below the pretransition temperature [7—9]. A recent freeze-fracture study of phosphatidylglycerol [10] reported similar results to the ones found by Luna and McConnell [6] for phosphatidylcholines.

Several models have been suggested to explain the rippled structure [5,11—14]. However, it is still unclear whether the hydrocarbon chains remain tilted or become perpendicular at the pretransition. In the present paper the term 'rippled structure' is used solely to indicate the appearance of the regular bands and is not restricted to a particular molecular model. It has been pointed out that the choline headgroup is not associated with the pretransition of phosphatidylcholine and that the pretransition should therefore be attributed to the hydrocarbon chains [15]. The present study confirms this suggestion by showing that the calorimetric pretransition and the appearance of a rippled structure can also be found in the case of phosphatidic acid.

Materials and Methods

1,2-Ditetradecyl-*sn*-glycerol-3-phosphoric acid was synthesized from 1,2-ditetradecyl-*sn*-glycerol [16,17] by phosphorylation with phosphorus oxychloride as described elsewhere [18]. The purity of the phosphatidic acid was checked by thin-layer chromatography and elemental analysis: 1,2-ditetradecyl-*sn*-glycerol-3-phosphoric acid, disodium salt, molecular weight 626.8 ($C_{31}H_{63}Na_2O_6P \cdot 1H_2O$: Expected: C 59.40%, H 10.45%, P 4.94%; Found: C 59.16%, H 10.22%, P 4.83%). Phosphate was analysed according to Eibl and Lands [19] with the reagent kit from Serva (Heidelberg, F.R.G.). Throughout the present study the disodium salt was used.

For the calorimetric measurements a differential scanning calorimeter (Perkin-Elmer 'DSC 2' with 'Intracooler I') was employed. The transition temperatures and enthalpies were calibrated with indium. Desired amounts of lipid (usually 4 mg) were weighed into stainless steel pans ('large volume capsules') and 50 μl of H_2O were added before the pans were sealed. The samples were equilibrated at $T > T_t$ for at least 10 min in the calorimeter before the first scan. The reference pan contained 50 μl of the corresponding H_2O solution. For each sample at least three scans were carried out with heating rates of 2.5°C/min in the sensitivity range of 2 mcal/s (full scale). Repetitive scans showed no change in the transition temperatures or in the enthalpies. The enthalpies were calculated from the peak areas, which were determined by weight.

The freeze-fracture samples were prepared in the same way as the calorimetric ones. Because of the well-known hysteresis of the pretransition [6] and the possibility that the kinetics of the pretransition takes place in the order of minutes [20], the lipid dispersion was kept for at least 1 h at $T < 5$°C after the 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), which were kept at the same temperature. The samples were quenched by plunging the planchets into partially liquid Freon 22 and then the samples were stored under liquid nitrogen until use.

Fracturing was carried out on a Balzers freeze-etch device (type BA 360) at —105°C with no etching. The replicas, which were floated off and cleaned in

$CHCl_3/CH_3OH$/1 N HCl (4 : 4 : 1, v/v/v), were examined on a Siemens 101 electron microscope operating at 80 kV with instrumental magnification of 20 000.

Results

The stability of 1,2-ditetradecyl-*sn*-glycerol-3-phosphoric acid was checked after the calorimetric measurements had been carried out. The two possible decomposition products, 1,2-ditetradecyl-*sn*-glycerol and free phosphate, were not detected by thin-layer chromatography or phosphate analysis.

The calorimetric scans of ditetradecyl phosphatidic acid dispersions in 2.6 M K^+ as a function of pH are shown in Fig. 1. At pH 7 (0.05 M Tris), where phosphatidic acid has one negative charge, the transition temperature lies at approx. 61.5°C ($\Delta H \approx 4.7$ kcal/mol). A calorimetric pretransition could not be detected in the whole range below pH 13. At pH 13.5—14, when the lipid has two negative charges, the transition temperature drops down to approx. 31.5°C ($\Delta H \approx 5.4$ kcal/mol) and at the same time a pretransition is detected at approx. 23.5°C ($\Delta H \approx 0.24$ kcal/mol).

Taking into consideration that the transition temperatures for the ether lipids lie a few degrees higher than for the ester compounds (2.4—5.3°C according to Ref. 21), then the transition temperature at pH 14 is very similar to the transition temperature reported for dimyristoyl phosphatidic acid at pH 11

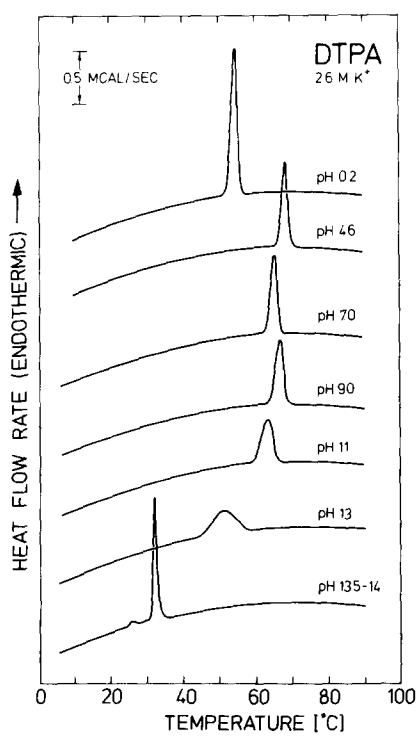


Fig. 1. Differential scanning calorimeter traces of ditetradecyl phosphatidic acid (DTPA) in 2.6 M K^+ as a function of pH. The scan rate was 2.5°C/min.

(27°C according to Ref. 22). Furthermore the transition temperature is in the range of T_t of dimyristoyl phosphatidylcholine. The relative enthalpy of the pretransition compared with the main transition is approx. 5% and therefore smaller than the ratio reported for dimyristoyl phosphatidylcholine (approx. 17% according to the values given in Ref. 5). It should be mentioned, however, that the enthalpy of the pretransition showed some dependence on the ionic strength, as higher values for ΔH (T_p) were measured for 2 M K^+ (Fig. 3a).

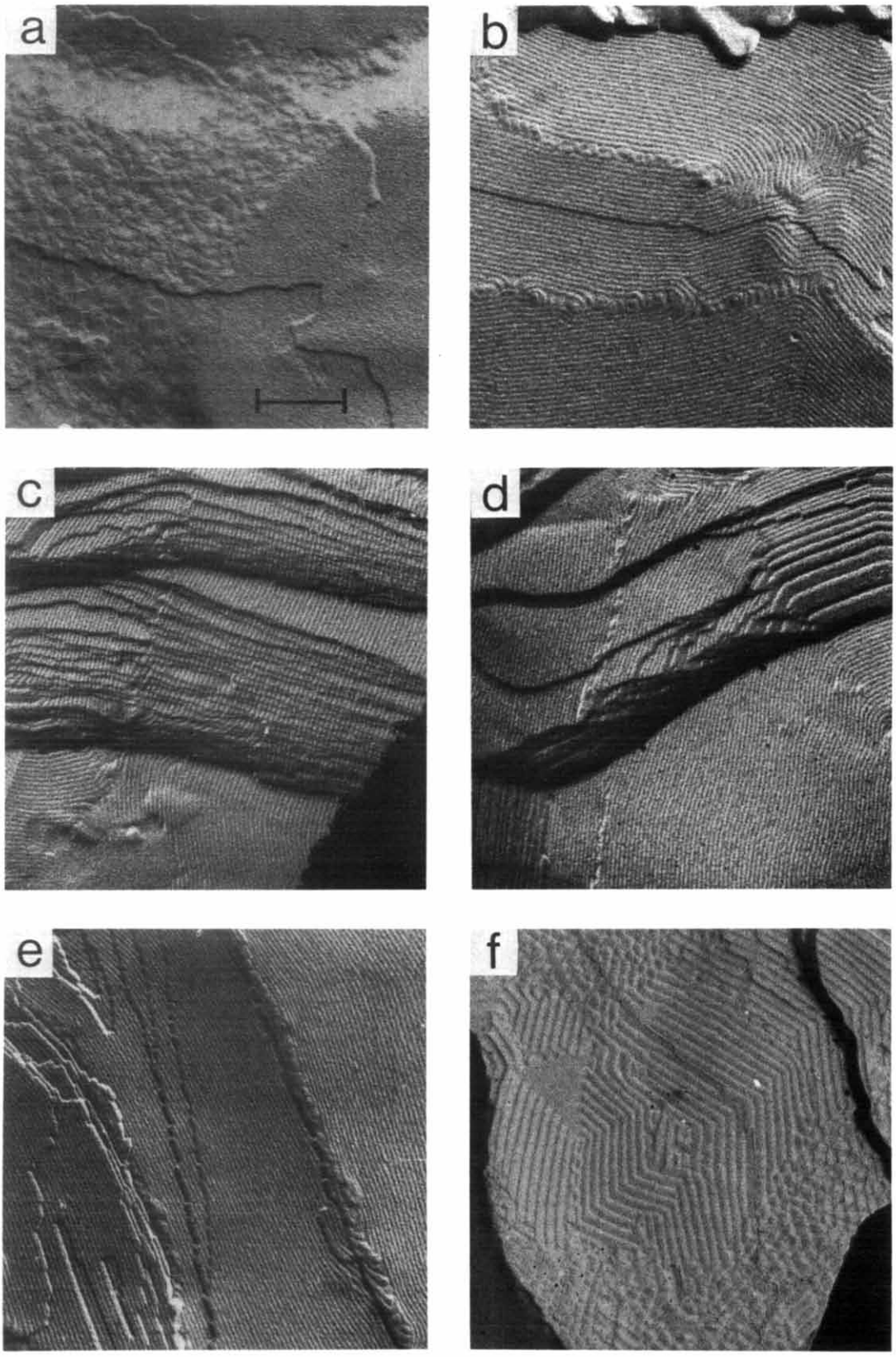
Freeze-fracture studies carried out at pH 7 (2.6 M K^+/0.05 M Tris) only showed smooth fracture planes, whereas studies carried out at pH 13.5—14 revealed similarities to the structures of phosphatidylcholines. Typical freeze-fracture electron micrographs of the lipid dispersion (2.6 M K^+) at pH 13.5 - 14 are shown in Fig. 2. When the lipid is quenched from a temperature above T_t, the lipid bilayers mostly show a smooth or jumbled structure (Fig. 2a). When the lipid is quenched from temperatures between the pre- and the main transition, the electron micrographs reveal a very regular banded structure in the plane of the bilayer (Fig. 2b—2e). The periodicity of the ripples is approx. 100 Å and is therefore smaller than the reported periodicities for dimyristoyl phosphatidylcholine (130 ± 10 Å according to Ref. 6) and dimyristoyl phosphatidylglycerol (215 ± 20 Å according to Ref. 10).

When the lipid dispersion is quenched from 5°C, a jumbled structure with occasional regular bands was found (Fig. 2f). Occasionally areas with smooth bilayer planes were also detected. Below the pretransition (at 5°C) the structure sometimes resembled the well-known spiral growth patterns of crystals [23] and the spiral growth pattern reported for dimyristoyl phosphatidylcholine [14]. It is also interesting to note that the periodicity of the bands at 5°C is about double the periodicity found at 25.5°C.

Some features of the rippled structure, which can be seen in Fig. 2b—2e, possibly give an insight into the molecular organisation. In all the electron micrographs studied, a packing disorder was visible in areas where the ripples turned by 180°C and joined together again (Fig. 2b). Packing disorders of this sort have not been detected in the case of phosphatidylcholine or phosphatidylglycerol. Assuming that the hydrocarbon chains are tilted in the rippled structure, then the tilt of the chains changes its direction when the ripples turn by 180°. In areas where the ripples join together again this should then cause a high degree of packing disorder. It has been concluded from similar symmetry considerations that for phosphatidylcholines the chains could not be tilted at temperatures between T_p and T_t [14]. This suggestion did not agree with the original model put forward by Tardieu et al. [11]. In the case of ditetradecyl phosphatidic acid the observed strong packing irregularities in areas where the ripples turn by 180° and join together again do not exclude the possibility that the hydrocarbon chains are tilted between T_p and T_t.

In Fig. 2c and d it can be seen that the ripples of a large stack of bilayers have the same direction and that the ripples are in register. This observation differs from the one reported for dimyristoyl phosphatidylglycerol [10]. There

Fig. 2. Freeze-fracture electron micrographs of ditetradecyl phosphatidic acid (DTPA) multilayers at pH 13.5—14 in 2.6 M K^+ quenched from (a) 36°C; (b—e) 25.5°C, and (f) 5°C. The magnification is approx. 63 000. (The length of the inserted bar in (a) indicates 2000 Å.)


a
b
c
d
e
f

the freeze-fracture electron micrographs showed that the direction of the ripples could change in a stack of bilayers. The reason for this could lie in a different average distance between the bilayers. In the case of dimyristoyl phosphatidylglycerol 0.1 M KCl was used, and it is known that under these conditions the water uptake between adjacent bilayers is not limited (Harlos, K., unpublished results). Thus the distance between two bilayers might easily become very large. In the present case 2.6 M K^+ was used and thus it can be expected that the water uptake is limited by the high ionic strength and, therefore, the distance between adjacent bilayers will be very small. In Fig. 2d it can be seen that the ripples are not only in register, but, furthermore, that areas of packing disorders extend right through a stack of bilayers.

Some details of the actual fracturing are shown in Fig. 2e. The bilayer preferentially fractures in the form of bands parallel to the direction of the ripples. It thus seems that the stability parallel to the ripples differs from that between adjacent ripples. At present, however, the meaning of the rippled structure on a molecular level still remains to be solved.

Differences in the influence of K^+ and Na^+ on phosphatidic acid multilayers were found at high pH (Fig. 3a and b). When using Na^+ the main transition temperature decreases at high pH in a way similar to K^+. T_t at pH 13.5–14,

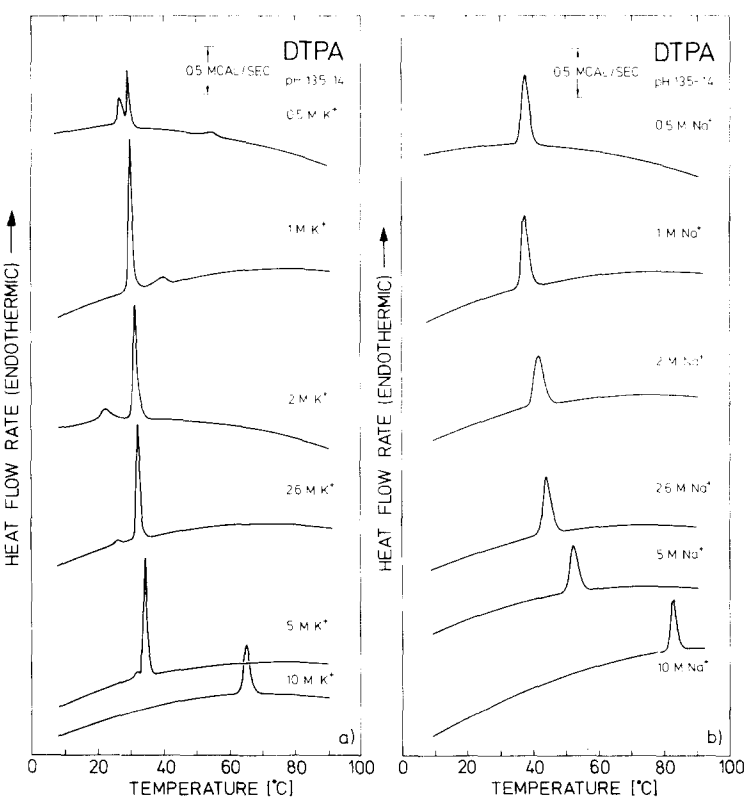


Fig. 3. Differential scanning calorimeter traces of ditetradecyl phosphatidic acid (DTPA) at high pH values (pH 13.5—14). The salt dependence of K^+ and Na^+ is shown in (a) and (b), respectively.

however, was found to be slightly higher than for the corresponding dispersion with K^+. A small transition above the main phase transition temperature could be detected at 1 M K^+ (Fig. 3a), but not with Na^+ (Fig. 3b). Furthermore a calorimetric pretransition and the appearance of a rippled structure could not be detected in the case of Na^+.

Discussion

It is well-known that the ordered-disordered phase transition temperature depends not only on the particular chemical structure of each phospholipid, but also on parameters of the aqueous phase, such as the H^+, Na^+, K^+ or Ca^{2+} concentration [22,24—27]. The change in T_t has been related to the dissociation characteristics of the phospholipid headgroup by Träuble and Eibl [22]. The study of phosphatidic acid is particularly interesting, as the number of negative charges/lipid molecule can be varied from zero to two by a change in the pH of the aqueous phase. The effect of pH on the transition temperature of dimyristoyl phosphatidic acid therefore shows a decrease in T_t from 52°C at pH 7 (where the lipid bears one negative charge) to about 27°C at pH 11 (where the lipid has two negative charges) [22]. A somewhat smaller decrease in T_t has been reported by Galla and Sackmann [27]. A pretransition, the existence of a rippled phase and a tilt of the hydrocarbon chains have already been suggested for phosphatidic acid at pH 9 [14].

Recently diether analogues of phosphatidic acid have been used to investigate the structure of phospholipids at extreme pH values [18,28]. The ether lipids are known to possess slightly higher transition temperatures [21], but otherwise show the same titration characteristics as the diester lipids [18,29]. A study of dihexadecyl phosphatidic acid showed that the hydrocarbon chains tilt when the lipid bears two negative charges [28]. However, in this study Na^+ was used, as opposed to K^+ in the present case. The exact tilt angle has not yet been defined in the present study.

In a recent paper a change in the hydrocarbon chain packing has been reported for distearoyl phosphatidylethanolamine at a temperature of approx. 17.5°C [30], which is about 53.5°C below the main phase transition temperature. It was pointed out that this lattice transformation (termed pretransition), which is characterized by a change to a hexagonal chain packing, should also be found with other phospholipids. For phosphatidylcholine this change takes place at the same temperature as the calorimetrically observed pretransition [5, 11]. It would therefore be interesting to see whether the calorimetrically detected pretransition in the case of ditetradecyl phosphatidic acid at high pH is also associated with this change in the hydrocarbon chain packing.

As it is now known that the calorimetric pretransition and the appearance of a rippled structure can be induced by a change in pH in the case of phosphatidylglycerol [10] and phosphatidic acid, it seems likely that the rippled structure can also be induced with other phospholipids by a change in pH.

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