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INFLUENCE OF CALCIUM ON PHOSPHATIDYLETHANOLAMINE

AN INVESTIGATION OF THE STRUCTURE AT HIGH pH

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Summary

The influence of calcium on the structure of 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine was investigated at pH 8 and 12 by differential scanning calorimetry and by X-ray diffraction. At pH 12, where the amino group of phosphatidylethanolamine is partly deprotonated, two separate lamellar phases are observed at 60°C in the presence of $CaCl_2$; one phase is metastable and the other is present in a crystalline structure. At high temperature (90°C), the small-angle diffraction lines indicate the existence of a hexagonal phase at pH 8. At pH 12 a hexagonal phase is detected only in the presence of calcium.

Introduction

The properties of phospholipid/water systems are of general interest for an understanding of biological membranes. Early studies of lipid membranes revealed that phospholipids exhibit a phase transition from an ordered structure to a more disordered state. It was subsequently found that the phase transition temperature does not only depend on the particular chemical structure of each phospholipid, but can be influenced by parameters of the water phase. Thus, several authors have shown that the H^+, Na^+, K^+, Ca^{2+} and Mg^{2+} concentrations can influence the phase transition temperature [1—8]. The influence of calcium on the structure of phospholipids is of particular interest because calcium plays a central role in many biological processes.

Abbreviation: DHPE, 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine.

In the present study the influence of Ca^{2+} on the structure of 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine is investigated by differential scanning calorimetry and by X-ray diffraction. The ether analogue of dipalmitoyl phosphatidylethanolamine was chosen because of the high chemical stability of the ether lipids. The ether analogues are known to possess slightly higher transition temperatures than the ester lipids [9], but otherwise show the same titration characteristics [10,11].

At high pH values (pH > 11) the deprotonation of the amino group results in negatively charged phosphatidylethanolamine. As Ca^{2+} has a pronounced effect on negatively charged lipids [3,4,6,12–15], one would expect Ca^{2+} also to influence the conformation of phosphatidylethanolamine at high pH. It is therefore the purpose of the present study to investigate the influence of Ca^{2+} on phosphatidylethanolamine as a function of pH.

It has been shown that phosphatidylethanolamines can be present not only in certain lamellar phases, but also in hexagonal phases. The existence of hexagonal phases has been reported for dioleoyl phosphatidylethanolamine [16,17] and for phosphatidylethanolamines from natural sources [18–20], but not as yet for synthetic phosphatidylethanolamines with saturated chains. Specific models for hexagonal phases have been presented by Marsden and McBain [21] and by Luzzati and Husson [22,23]. In the present paper the term 'hexagonal phase' is used solely to indicate the appearance of small-angle diffraction lines in the ratio $1 : 1/\sqrt{3} : 1/2$ and does not imply a particular molecular arrangement of the phospholipid molecules.

Materials and Methods

1,2-Dihexadecyl-*sn*-glycero-3-phosphoethanolamine (DHPE) (puriss.) was obtained from Fluka, Neu-Ulm, F.R.G. Thin-layer chromatograms using a variety of solvent systems did not reveal any impurities. After the experiments the stability of the DHPE/water (pH 12) system at 95° C was checked. None of the possible decomposition products, 1,2-dihexadecyl-*sn*-glycerol or 1,2-dihexadecyl-*sn*-glycero-3-phosphoric acid, were detected after an incubation period of 36 h. It can therefore be concluded that DHPE was completely stable under the conditions of these experiments.

Sample preparation. Approx. 10 mg of lipid were weighed into glass tubes and 20 ml of the desired $CaCl_2$ solution was added (all the solutions contained 10 mM Na^+ and a 10 mM Tris buffer was used at pH 8). The samples were then equilibrated at $T \geqslant 95°C$ for at least 15 min. After equilibration the pH was checked and the lipid suspension was centrifuged briefly. Samples from the wet pellet were used for the calorimetric and the X-ray diffraction experiments. All the samples still contained excess of the desired $CaCl_2$ solution. The present study was carried out at pH 12 in order to avoid complex formation and precipitation of $Ca(OH)_2$ which occurs at higher pH values.

Calorimetry. For the calorimetric measurements a differential scanning calorimeter (Perkin-Elmer DSC 2 with Intracooler I) was employed. Approx. 50 µl of the lipid dispersion (wet pellet) were pipetted into stainless-steel pans ('large volume capsules') which were then sealed. The reference pan contained 50 µl of H_2O. For each sample several scans were carried out with

a heating/cooling rate of 1.25°C/min in the sensitivity range of 1 mcal/s (full scale).

X-ray diffraction. For the X-ray diffraction experiments a Guinier camera (operating under vacuum) with a bent quartz crystal monochromator was used (R. Huber, 8211 Rimsting, F.R.G.). The monochromator was set to isolate the $CuK\alpha_1$ line ($\lambda = 1.5405$ Å). A camera with a movable film was used in order to record a number of exposures on the same film. The X-ray diffraction lines could then be compared more easily. Further details of the experimental set-up have been reported elsewhere [24].

The lipid samples were sealed with Teflon between mica plates. Before starting the actual X-ray exposure, the sample was kept at the desired temperature for 5 to 10 min. The exposure times of the photographic films (Kodak, Kodirex, une face) varied between 15 min and 4 h. The density of the reflections was scanned with a Joyce-Loebl microdensitometer type 3CS.

Results

Calorimetric scans of DHPE in the absence and in the presence of 10 mM $CaCl_2$ are shown in Fig. 1. At pH 8, when the phosphatidylethanolamine head group has a positive and a negative charge, the presence of 10 mM $CaCl_2$ causes practically no change in the phase transition temperatures (Fig. 1a and b). In both cases the main transition lies at 69°C and is followed by a small transition at about 85°C.

At pH 12 (without $CaCl_2$) the main transition temperature decreases from 69 to 60°C (Fig. 1c), thus indicating that the amino group of DHPE is partially deprotonated at this pH [1,25]. The transition at pH 12 is less sharp than that at pH 8. Similar measurements have already been performed for DHPE in the presence of 1 M Na^+ [25]. The small transition at high temperature could not be detected at pH 12. The calorimetric scan at pH 12 in the presence of $CaCl_2$ (Fig. 1d) shows a number of transitions which will be discussed in more detail later.

The X-ray diffraction patterns of DHPE at pH 8 (10 mM $CaCl_2$, 10 mM Na^+, 10 mM Tris) are shown in Fig. 2. At 60°C the lamellar repeat is 60.3 Å. The fourth and the sixth order of this repeat could also be detected. In the wide-angle region the pattern shows a single sharp line at 4.22 Å. A single sharp wide-angle diffraction line is characteristic for a hexagonal lateral packing of the hydrocarbon chains in a plane perpendicular to the chain axes (see, for example, Ref. 24). At 80°C the diffuse line at approx. 4.55 Å indicates that the chains are in the disordered state. At the same time the small-angle reflection decreases from 60.3 to 50.4 Å. The 50.4 Å reflection is a lamellar repeat because the fourth order could also be observed. However, the intensity of the fourth order at 80°C is much smaller than the intensity of the fourth-order reflection at 60°C.

In Fig. 2 and in the following figures a short exposure time of 15 min was used to record the innermost reflection ($2\theta < 2°$). The broad diffuse line at $27°(2\theta) \approx 3.3$ Å originates from the water. For all the patterns shown in Fig. 2 the same sample was used (the reflections of all the different phases are listed in Table I).

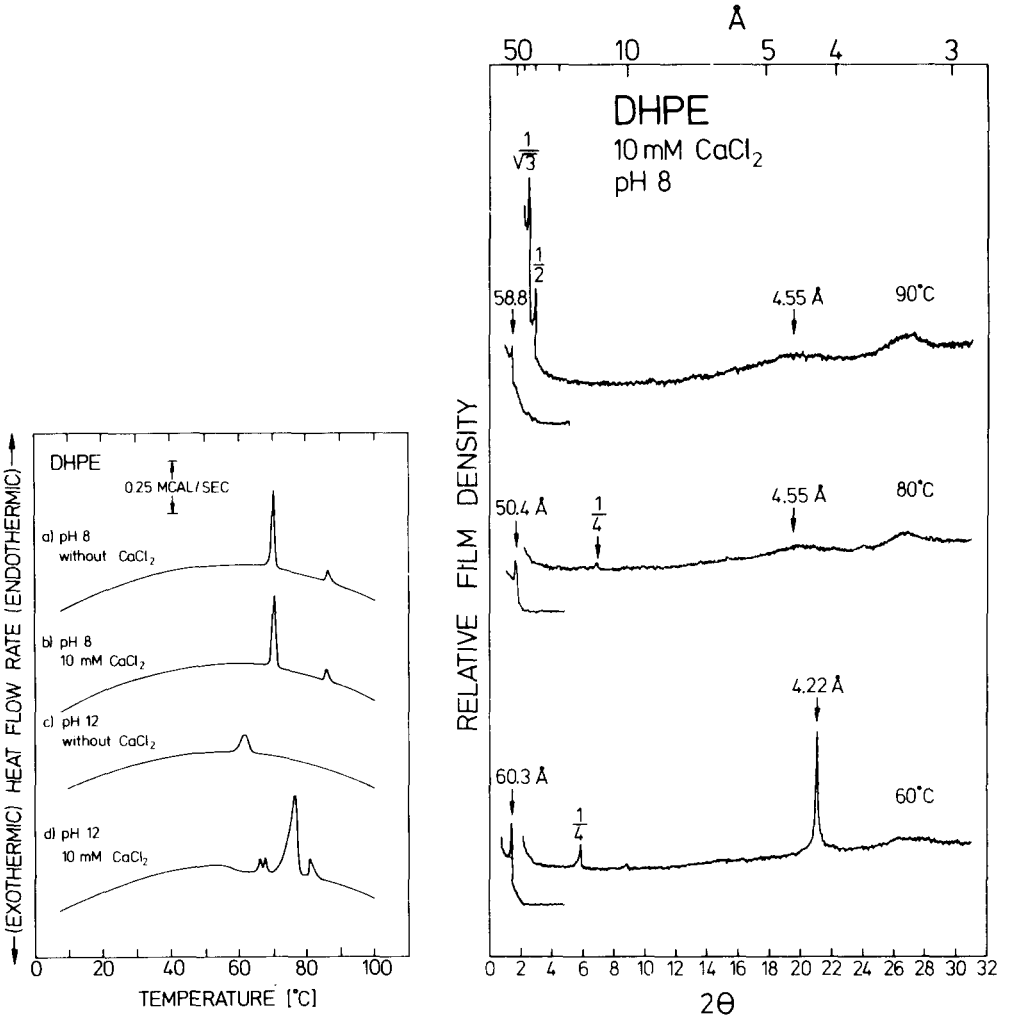


Fig. 1. Differential scanning calorimetric traces of DHPE at pH 8 (scans a and b) and at pH 12 (scans c and d) in the presence and in the absence of 10 mM $CaCl_2$. The heating rate was 1.25°C/min.

Fig. 2. X-ray diffraction lines of DHPE in 10 mM $CaCl_2$ (pH 8) at 60, 80 and 90°C. The broad diffuse line at 27°(2θ) is not due to the lipid, but to water which is present in the sample.

Heating the sample from 80 to 90°C causes a change in the small-angle diffraction lines, whereas the wide-angle pattern remains unchanged. The small-angle reflections at 90°C are in the ratio $1 : 1/\sqrt{3} : 1/2$ and thus indicate the presence of a so-called hexagonal phase. It is clear from the diffraction patterns shown in Fig. 2 that the calorimetric transition in Fig. 1b at 69°C is the well known ordered-disordered (gel-liquid-crystalline) lipid phase transition. The peak at 85°C in Fig. 1b represents a transition from the (lamellar) liquid-crystalline to the hexagonal phase. It can be assumed that the high-

TABLE I

OBSERVED X-RAY DIFFRACTION SPACINGS OF DHPE (10 mM $CaCl_2$)

Weak lines (w) are specially marked.

	T (°C)	d (Å)	
pH 8	60	60.3, 15.07, 10.07 (w)	4.22
	80	50.4, 12.6	broad line at 4.55 Å
	90	58.8, 33.9, 29.4	broad line at 4.55 Å
pH 12	60, phase A	59.6, 14.9, 9.9 (w)	4.22
	60, phase B	53.8, 26.9, 17.9, 10.76	7.93, 7.75, 4.27, 4.18, 3.97, 3.93, plus several lines between 7.75 and 4.27 Å
	70	53.8, 26.9, 17.9, 10.76	7.94, 7.77, 4.28, 4.20, 3.98, 3.94, plus several lines between 7.77 and 4.28 Å
	79	48.6, 24.3 (w), 12.15 (w)	broad line at 4.55 Å
	90	52.2, 30.1, 26.1	broad line at 4.55 Å

temperature transition found at pH 8 in the absence of $CaCl_2$ (Fig. 1a) is also due to a transition to the hexagonal phase.

In order to obtain some insight into the nature of the different transitions detected at pH 12 (10 mM $CaCl_2$), a number of heating and cooling scans were necessary. The curves A—C in Fig. 3 show heating scans using the same sample but with different pretreatments. In curve A the sample was kept at $T \geqslant 95°C$ for $t \geqslant 10$ min, cooled to 60°C, and then scanned in the calorimeter. In curve B the sample was equilibrated at $T \geqslant 95°C$ for $t \geqslant 10$ min and also cooled to 60°C, but was then stored at 70°C for $t \geqslant 15$ min. The scan in Fig. 3C is the same as in Fig. 1d.

It can be seen that all the scans show a small high-temperature transition at approx. 80°C. With respect to the main transition, however, curves A and B differ completely. Curve A shows two sharp transitions at 65.5 and 66.5°C, whereas curve B exhibits only two very small transitions at these temperatures. The main transition of curve B lies at 74°C and the transition enthalpy is considerably higher than the enthapy of the transitions in curve A. In curve A a transition at 74°C cannot be detected. As the calorimetric scans A and B in Fig. 3 were found to be characteristic for two different lipid structures, the phases (at 60°C) corresponding to scan A and scan B are defined as phase A and phase B, respectively, in the present paper. In curve C, where the sample was heated from 1°C, it can be seen that all the transitions are present and that the transition enthalpies lie in between the corresponding enthalpies of curve A and B.

A number of cooling scans were performed to find out more about these different transitions. The cooling curves from 100 and 79°C did not show a dependence on the pretreatment of the sample. Thus, the curves D and E in Fig. 3 were always obtained, irrespective of whether the sample was originally in phase A or phase B at 60°C. In both cooling curves the 74°C transition

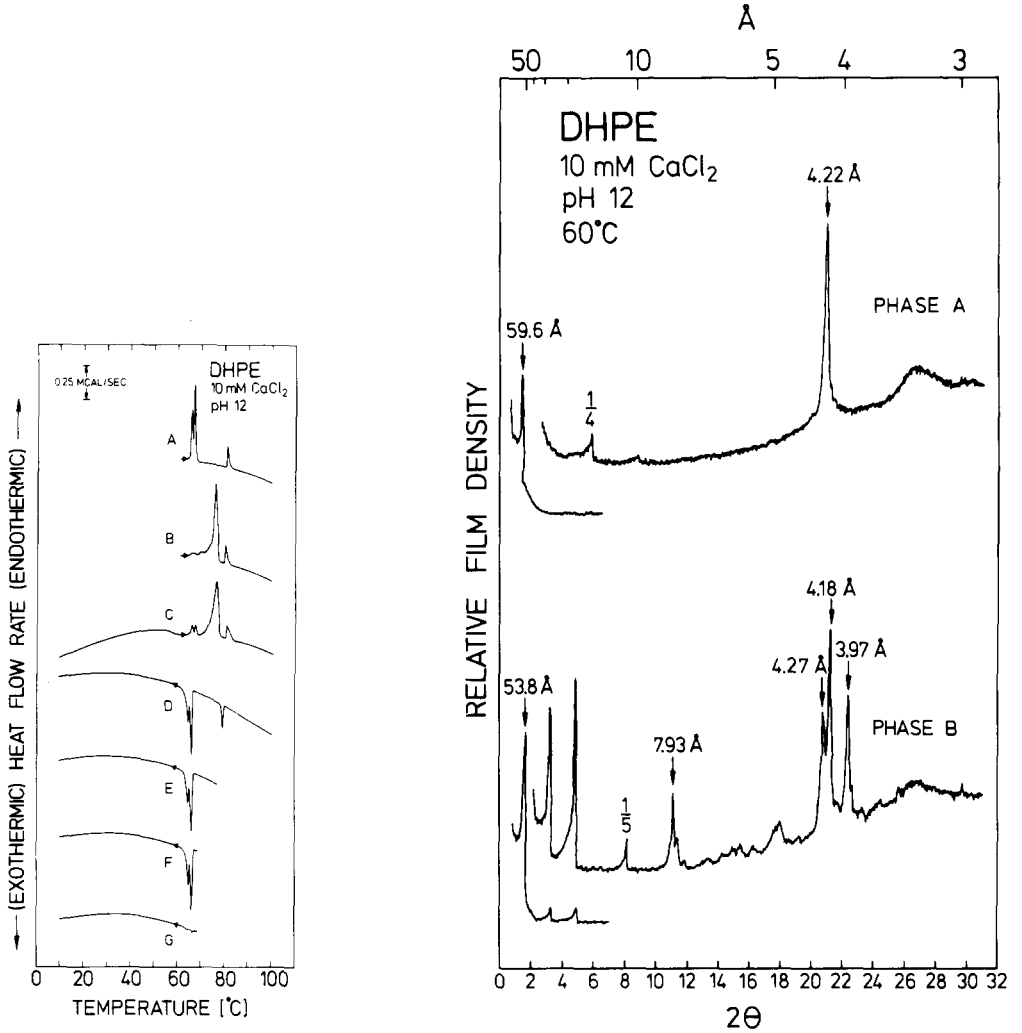


Fig. 3. Differential scanning calorimetric traces of DHPE in 10 mM $CaCl_2$ at pH 12 as a function of the pretreatment of the sample: (A) heating from 60°C, after equilibration at $T \geqslant 95$°C; (B) heating from 60°C, after equilibration at $T \geqslant 95$°C, cooling to 60°C and storage at $T = 70$°C for $t \geqslant 15$ min; (C) heating from 1°C, after equilibration at $T \geqslant 95$°C; (D) cooling scan after heating to 100°C; (E) cooling scan after heating to 79°C; (F) cooling scan from 70°C immediately after equilibration at $T \geqslant 95$°C, cooling to 60°C and heating to 70°C; (G) cooling scan from 70°C with the same treatment as in F, except that the sample was stored at $T = 70$°C for $t \geqslant 15$ min. For all the scans in this figure the same sample was used. The scan rate was 1.25°C/min.

Fig. 4. X-ray diffraction lines of DHPE in 10 mM $CaCl_2$ (pH 12) at 60°C. The pretreatment to obtain phase A and phase B was the same as in Fig. 3A and B. All the diffraction patterns of this figure and of Fig. 5 were obtained with the same sample. The broad diffuse line at 27° (2θ) in both this figure and in Fig. 5 is not due to the lipid but to water which is present in the sample.

of phase B is absent, whereas the other transitions are all present (even without any remarkable hysteresis).

When the sample was cooled from 70°C, however, the scans depended on the particular pretreatment of the sample. Both curves F and G show cooling scans from 70°C of the sample which was originally in phase A at

60°C. When the sample was cooled immediately after being heated to 70°C, curve F was obtained. However, when the sample was stored at $T = 70°C$ for $t \geqslant 15$ min, curve G was obtained. It can be seen that the transition of phase A is present in curve F and absent in curve G. As heating the G sample resulted in a curve similar to Fig. 3B, it can be concluded that storing the sample at 70°C caused the sample to change from phase A to phase B.

The X-ray diffraction patterns of DHPE in 10 mM $CaCl_2$ (10 mM Na^+, pH 12) at 60°C are shown in Fig. 4. In order to obtain phase A and phase B, the sample was prepared in the same way as for curve A and curve B in Fig. 3. It can be seen that the diffraction lines of phase A and B differ completely. Therefore, the sample is present in two separate phases, as has already been deduced from the calorimetric results. Phase A and B both show a lamellar structure with a repeat distance of 59.6 and 53.8 Å, respectively. In A and B higher orders of the lamellar repeat are present.

The wide-angle reflections of phase A and B also show remarkable differences. In phase A a single sharp line at 4.22 Å is detected. As has already been pointed out, this is characteristic for a hexagonal packing of the chains. It is interesting to note the similarities between the diffraction lines of phase A (60°C) in Fig. 4 and those at pH 8 (60°C) shown in Fig. 2. Phase B gives rise to a large number of wide-angle diffraction lines, indicating a high degree of lateral order. The most intense lines are at 7.93, 4.27, 4.18 and 3.97 Å (see Table I). Because of the large number of wide-angle diffraction lines, it is reasonable to assume that the packing of the chains in phase B is crystalline. All the diffraction patterns of Figs. 4 and 5 were obtained with the same sample and with identical exposure times.

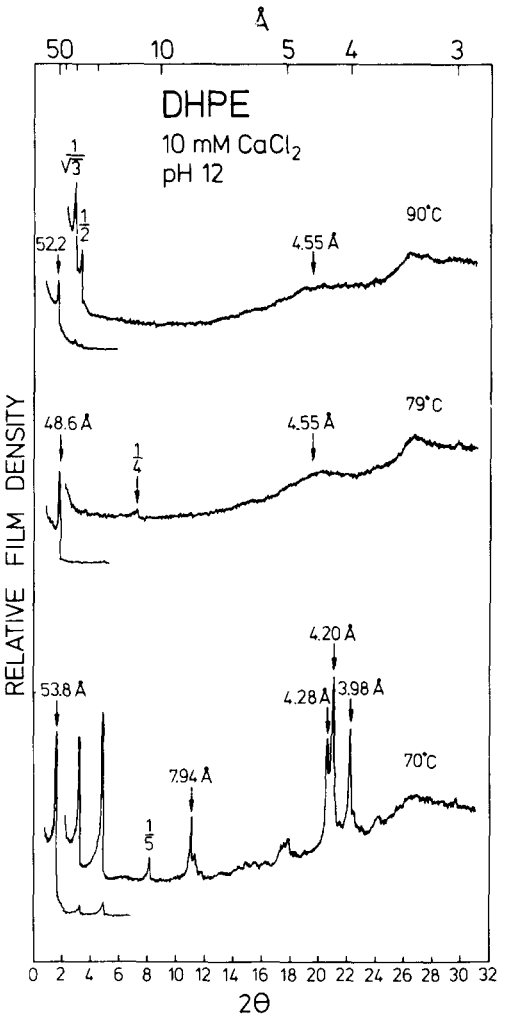
The X-ray diffraction lines of DHPE in 10 mM $CaCl_2$ (10 mM Na^+, pH 12) at 70, 79 and 90°C are shown in Fig. 5. Irrespective of whether the sample was originally in phase A or B at 60°C, heating the sample to 70°C resulted in these diffraction lines. The same was also true when the sample was heated to 79 and 90°C. The sharp wide-angle reflections at 70°C indicate that the lipid is in the ordered state. The position of the reflections is almost the same as that of the reflections observed for phase B at 60°C. Therefore, at 70°C the lipid is in the B phase. As heating phase A to 70°C also resulted in the diffraction lines shown in Fig. 5, a transformation from phase A to phase B must have taken place. The same conclusion has already been drawn from the calorimetric measurements. Phase A can be considered as a metastable phase, which is obtained after cooling the sample from $T \geqslant 79°C$ and which can be transformed to phase B by storing the sample at a low temperature (see Fig. 3, curve C) or annealing the sample at 70°C.

At 79°C the broad diffuse line at approx. 4.55 Å indicates that the hydrocarbon chains are now in the disordered state. In the small-angle region a reflection at 48.6 Å with very weak higher lamellar orders could be detected. The sample is therefore in a liquid-crystalline phase at 79°C. The main endotherm in Fig. 3A can be regarded as the well known ordered-disordered (gel-liquid crystalline) lipid phase transition. In Fig. 6 the structure present at 79°C is therefore designated as phase A′. Phase A′ should then also be present at 70°C. However, it could not be detected by X-ray diffraction because the transformation to phase B is relatively fast at this temperature. Why the

66° C endotherm is split up into two peaks is not clear at present. The main transition at 74° C in Fig. 3B represents a transformation from a crystalline structure to the (lamellar) liquid-crystalline phase A'.

At 90° C the diffuse wide-angle halo at 4.55 Å again indicates the disordered state of the hydrocarbon chains. In the small-angle region the reflections are in the ratio $1 : 1/\sqrt{3} : 1/2$. The high-temperature peak at 80° C in Fig. 3 thus represents a transition to a hexagonal phase. A schematic diagram showing all the different phases of DHPE (10 mM $CaCl_2$, pH 12) is given in Fig. 6.

In the course of the present study calorimetric measurements were also



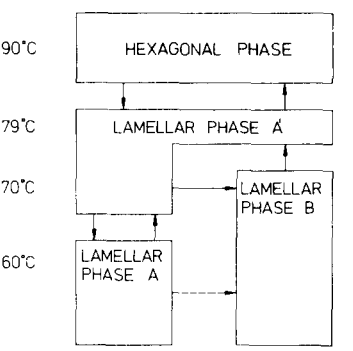


Fig. 5. X-ray diffraction lines of DHPE in 10 mM $CaCl_2$ (pH 12) at 70, 79 and 90° C.

Fig. 6. Schematic diagram showing the different phases of DHPE (10 mM $CaCl_2$, pH 12). Phase A is obtained by cooling the sample from a high temperature ($T \geqslant 79°$ C). Phase B is reached by storing the sample at $T = 70°$ C. The transformation of phase A to phase B was also observed at $T \leqslant 60°$ C, although at a slower rate than at 70° C. At pH 8 (10 mM $CaCl_2$) the lamellar phases A and A' as well as the hexagonal phase are present, but phase B could not be detected.

carried out with 1,2-ditetradecyl-*rac*-glycero-3-phosphoethanolamine, which was synthesized in our laboratory. The calorimetric transitions found in the absence and presence of Ca^{2+} are very similar to the ones described here in the case of DHPE.

Discussion

These results (with DHPE) are similar to the results obtained with 1,2-ditetradecyl-*rac*-glycero-3-phosphoglycerol after the addition of 1 M $CaCl_2$ [26]. In the present study the transition to the hexagonal phase was clearly separated from the main phase transition temperature of phase B and it was therefore possible to record the spacings of the intermediate phase A'. In the case of phosphatidylglycerol the transition from phase A to phase B can be exothermic. It has been suggested [26] that this transition might be responsible for a number of exothermic transitions, which can be observed in some calorimetric scans reported in the literature. The same is probably true of the exothermic transition which has been detected recently with stearoylsphingomyelin [27].

A comparison of phosphatidylglycerol and phosphatidylethanolamine indicates that both phospholipids are influenced by Ca^{2+}. However, interaction of calcium is only possible in the deprotonated states of the bilayer membranes. For example, phosphatidylethanolamine at pH 8 is not affected by Ca^{2+}, owing to the presence of the aminoproton. The removal of the proton makes the membrane sensitive to calcium and results in the appearance of similar structures to those already described for phosphatidylglycerol. The specific properties of protonated as compared to deprotonated membranes have already been described in an earlier paper [28].

The present results, obtained by adding $CaCl_2$, resemble results which other authors have obtained in the absence of calcium. For example, calorimetric transitions which depend on the sample history have already been reported for some synthetic phosphatidylethanolamines at low water content [29,30] and for dry dimyristoyl phosphatidylethanolamine, which was crystallized from chloroform/methanol [29].

The experiments reported recently with dimyristoyl phosphatidylcholine at 10% water content are particularly interesting [31]. There seem to be several similarities between the phosphatidylcholine system and the system described in the present paper. Firstly, the heating scans depended on the pretreatment of the sample (see Fig. 1A of Ref. 31). Furthermore, the authors report a crystalline phase (designated as phase C) at temperatures between T_3 and T_4. In the phosphatidylcholine system (10% water) one would therefore expect to find the crystalline phase C at 20°C, after storing the sample at a temperature between T_4 and T_3. Care would have to be taken not to heat the sample above T_3. It would be interesting to study the structures of DHPE using freeze-fracture electron microscopy to determine whether this hexagonal phase reveals similar fracture faces to other hexagonal phases [17,32–34] and also to find out whether there are any differences between the fracture faces of phase A and phase B.

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