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Hexagonal Phases in Phospholipids with Saturated Chains: Phosphatidylethanolamines and Phosphatidic Acids†

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ABSTRACT: The structure of phospholipids with saturated chains was investigated by differential scanning calorimetry and by X-ray diffraction. It is shown that both phosphatidylethanolamines and phosphatidic acids can exhibit a hexagonal phase at high temperature. The temperature of the transition to the hexagonal phase is dependent on chain length and sodium salt concentration. Increasing the chain length or the sodium salt concentration results in a decrease in the transition temperature. In addition to this transition at high temperature, a calorimetric transition at low temperature is detected in some phospholipids.

Phospholipid/water systems can be regarded as simple models for biological membranes. It has been demonstrated that phospholipids can form lamellar structures as well as adopt so-called hexagonal phases. As the lamellar phase is dominant in phospholipids, most previous studies have dealt with lamellar structures. Hexagonal phases have been reported mainly for phospholipids with unsaturated chains, for example, for dioleoylphosphatidylethanolamine (Cullis & de Kruijff, 1976; Van Dijck et al., 1976), for phosphatidylethanolamines from natural sources (Reiss-Husson, 1967; Rand et al., 1971; Cullis & de Kruijff, 1978), and for (egg) phosphatidylserine (Hope & Cullis, 1980). For negatively charged phospholipids, it has been shown that divalent cations can induce hexagonal phases (Deamer et al., 1970; Rand & Sengupta, 1972; Papahadjopoulos et al., 1976; Cullis et al., 1978; Harlos & Eibl, 1980a,b). This study deals with the appearance of hexagonal phases in phospholipids with saturated chains in the presence of monovalent cations.

In the present paper, the term "hexagonal phase" is used solely to indicate the appearance of small-angle diffraction lines in the ratio of $1:1/3^{1/2}:1/2$ and is not restricted to a particular

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0006-2960/81/0420-2888$01.25/0

model. However, with regard to the specific models which have been suggested for hexagonal phases (Marsden & McBain, 1948; Luzzati & Husson, 1962; Luzzati, 1968), the H_{II} model would seem to be applicable.

In this study, the structure of synthetic phosphatidylethanolamines and phosphatidic acids is investigated by differential scanning calorimetry and by X-ray diffraction. Phospholipids with ester and phospholipids with ether bonds were examined. Ether lipids are known to possess slightly higher transition temperatures than the corresponding ester lipids (Vaughan & Keough, 1974; Blume, 1976). The advantage of ether phospholipids lies in their high chemical stability, and a number of studies have therefore been performed by using ether lipids (Blume & Eibl, 1979; Eibl & Blume, 1979; Harlos et al., 1979; Jähnig et al., 1979; Stümpel et al., 1980; Harlos & Eibl, 1980a,b).

Materials and Methods

The phosphatidylethanolamines DMPE, DPPE, DSPE, and DHPE (all puriss. grade) were obtained from Fluka, Neu-Ulm, F.R.G. The phosphatidylethanolamine DTPE (Eibl, 1978) and the disodium salts of phosphatidic acid (Eibl, 1980a,b) were synthesized in our laboratory. Thin-layer chromatograms of the starting lipids with a variety of solvent systems did not reveal any impurities. The stability of the lipids during the calorimetric and X-ray measurements was checked separately by thin-layer chromatography. When the samples were heated to 5 °C above T_H[1] (heating rate 2.5 °C/min) and then cooled, decomposition could not be detected. When DTPE (pH 7) and DTPA (pH 4.6) were stored at 98 °C for 4 h, there was no decomposition for DTPE, and for DTPA the decomposition was less than 2%.

Calorimetry. A differential scanning calorimeter (Perkin-Elmer "DSC 2" with "Intracooler I") was employed for the calorimetric measurements. The transition temperatures and enthalpies were calibrated with indium. A total of 6–7 mg of each lipid was weighed into stainless steel pans ("large volume capsules"), and 50 μL of adjusted buffer solution was added before the pans were sealed. The samples were equilibrated in the calorimeter at $T > T_t$ for more than 10 min before the first scan. The reference pan contained 50 μL of the respective buffer solution. The heating rate was 2.5 °C/min, and the sensitivity range was 1 mcal/s (full scale). The transition temperature was defined as the point of intersection of the tangent of the rising slope with the base line. The enthalpies were calculated from the peak areas, which were determined by weight.

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 Cu $K\alpha_1$ line (λ = 1.5405 Å). A camera with a movable film was used in order to record a number of exposures on the same film so as to allow a direct comparison of the X-ray diffraction lines. Further details of the experimental setup have been reported elsewhere (Harlos, 1978).

The preparation of the lipid samples for the X-ray diffraction experiments was the same as that for the calorimetric measurements. After equilibration at $T > T_t$ for more than 10 min, the lipid samples were sealed with Teflon between two mica plates in such a way that no water could evaporate even at temperatures above 100 °C. The samples were thermostated, and the temperature was kept constant (± 0.5 °C) during the measurement. For temperatures up to 70 °C, a circulating water/ethylene glycol mixture was used. For higher temperatures, an electric heating instrument was employed. Before the actual X-ray exposure was started, the sample was kept at the desired temperature for more than 5 min. The exposure times of the photographic films (Kodak, "Kodirex, une face") varied between 15 min and 2 h. The density of the reflections was scanned with a Joyce-Loebl microdensitometer type 3CS.

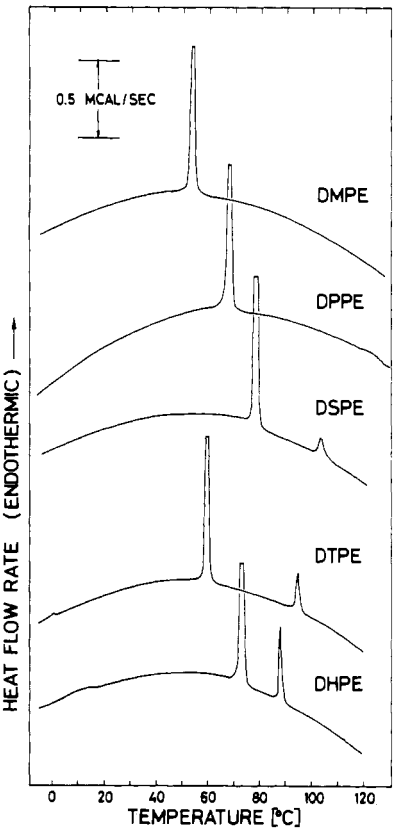

FIGURE 1: Differential scanning calorimeter traces of phosphatidylethanolamines with saturated chains at pH 7 (1 M NaCl, 0.25 M phosphate). The heating rate was 2.5 °C/min.

Results and Discussion

Phosphatidylethanolamines. The calorimetric scans of several phosphatidylethanolamines with saturated chains are shown in Figure 1. The buffer (1 M NaCl, 0.25 M phosphate) used in all samples was adjusted to pH 7 with sodium hydroxide. In the sensitivity range shown in the figures, the peak of the main transition was beyond the recorder scale. Above the main transition temperature, DSPE, DTPE, and DHPE show a small and sharp transition. The scan of DPPE exhibits a small and broad transition at around 118 °C. In the case of DMPE (pH 7, 1 M NaCl), a transition at high temperature could not be detected.

The high-temperature endotherm does not exhibit the same chain-length dependence as the main transition temperature. Although T_t increases from 58 (DTPA) to 71.5 °C (DHPE), the high-temperature endotherm decreases from 93.5 (DTPE) to 87 °C (DHPE). This behavior was also found for phosphatidic acids.

In addition to the transition at high temperature, DTPE and DHPE also show a very small transition at low temperature.

[1] Abbreviations used: T_t, gel to liquid-crystalline phase transition temperature; T_l, low-temperature transition temperature; T_H, high-temperature transition temperature; ΔH, transition enthalpy; DMPE, DPPE, DSPE, DTPE, and DHPE, 1,2-dimyristoyl-*sn*-, 1,2-dipalmitoyl-*sn*-, 1,2-distearoyl-*sn*-, 1,2-ditetradecyl-*rac*-, and 1,2-dihexadecyl-*sn*-glycero-3-phosphoethanolamine; DPPA, DTPA, and DHPA, 1,2-dipalmitoyl-*sn*-, 1,2-ditetradecyl-*rac*-, and 1,2-dihexadecyl-*rac*-glycero-3-phosphoric acid.

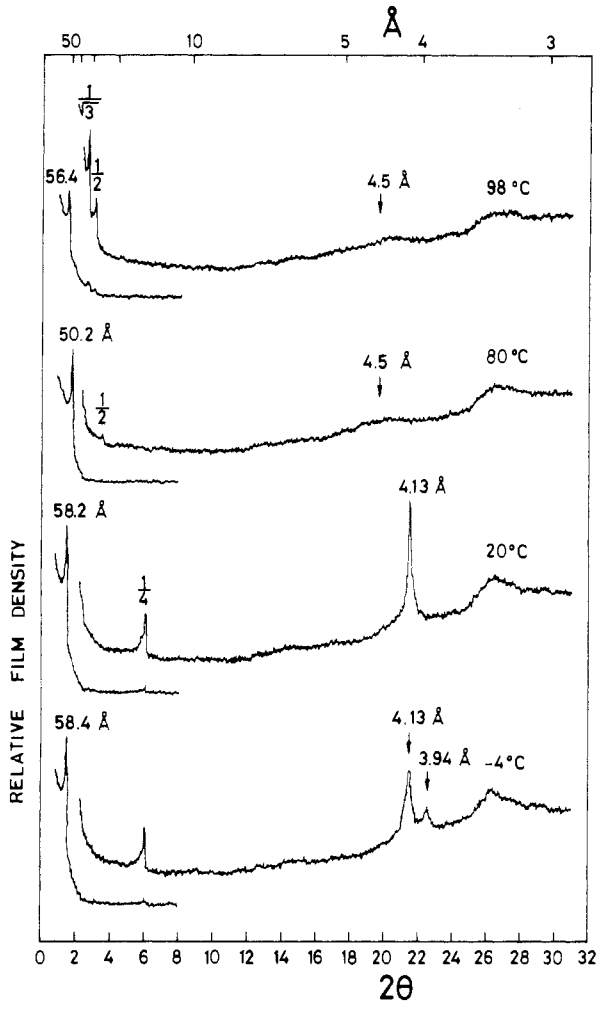

FIGURE 2: X-ray diffraction lines of DTPE at pH 7 (1 M NaCl, 0.25 M phosphate) as a function of temperature.

In the case of DTPE, this transition was found at −2 °C, and for DHPE, the transition was very broad and started at approximately +2 °C. For DMPE, DPPE, and DSPE, this low-temperature transition could not be detected calorimetrically.

In order to understand more about the nature of these calorimetric transitions, X-ray experiments were carried out. Typical X-ray diffraction patterns of DTPE in 1 M NaCl (0.25 M phosphate buffer adjusted to pH 7) are shown in Figure 2. The density of the film is plotted as a function of the diffraction angle 2θ. At the top of the figure, the corresponding Bragg spacings are marked. The same sample was used for all the patterns shown in Figure 2. For each temperature, the exposure time was 15 min for the innermost reflection ($2\theta <$ 2) and 2 h to record the rest of the pattern. The broad diffuse line in the range of 27° (2θ) is due to water.

In Figure 2, a single small-angle reflection and its fourth order can be seen at low temperature. This spacing hardly changes when the temperature is raised from −4 to 20 °C. Because of the similarities to the reflections reported for DSPE (Harlos, 1978), it can be assumed that DTPE is in the lamellar phase at −4 and at 20 °C. In the wide-angle region, a single sharp reflection at 4.13 Å is present at 20 °C. This line splits up into two lines at low temperature. Thus the small transition detected in DTPE at −2 °C in Figure 1 can be attributed to this change in the wide-angle diffraction lines.

This type of transition observed at low temperatures was originally reported in the case of rubidium soaps of stearic acid (Vincent & Skoulios, 1966) but was subsequently also detected in DPPE and DSPE (Harlos, 1978), in DHPE after the addition of 1 M RbOH (Harlos, 1980), and in DHPA at pH 7 (Jähnig et al., 1979). The original interpretation by Vincent & Skoulios (1966), which was also applied for DSPE (Harlos, 1978), can be used to describe the structure of DTPE as well. Thus, at −4 °C, the lateral chain packing is characterized by a rectangular I lattice with the lattice planes $d_{110} = d_{\bar{1}10} = 4.13$ Å and $d_{200} = 3.94$ Å. At 20 °C, an exact hexagonal lattice is present with $d_{110} = d_{\bar{1}10} = d_{200} = 4.13$ Å. In both cases, the two-dimensional lattice applies to the plane perpendicular to the chain axes.

The low-temperature transition observed calorimetrically in DTPE and DHPE at pH 7 is not the so-called "pretransition" which was originally described for phosphatidylcholines. Neither is this low-temperature transition associated with the appearance of the so-called "ripple" structure. To avoid confusion, we recommend the use of the designation "pretransition" strictly for the ripple transition [phosphatidylcholines (Janiak et al., 1976; Luna & McConnell, 1977) and phosphatidylglycerols (Watts et al., 1978) at pH 7; phosphatidylethanolamines (Stümpel et al., 1980) and phosphatidic acids (Harlos et al., 1979) at pH 12]. The lattice transformation discussed here for phosphatidylethanolamine at pH 7 is defined as the low-temperature transition T_1.

At 80 °C, the hydrocarbon chains of DTPE are in the disordered state, because the sharp wide-angle line disappears and only a very broad diffuse line could be detected in the 4.5-Å region (see Figure 2). At the same time, the small-angle reflection decreases to 50.2 Å. A weak second order of this reflection was also present, and it can therefore be assumed that the structure is lamellar. There is no change in the wide-angle region with increasing temperature. In the small-angle region, however, a new diffraction pattern is observed. The reflections at 98 °C are in the ratio $1:1/3^{1/2}:1/2$ and therefore indicate the presence of a hexagonal phase. Thus the calorimetric endotherm of DTPE at high temperature represents a transition from the (lamellar) liquid-crystalline state to the hexagonal phase. (*Hexagonal phase* should not be confused with *hexagonal packing*, which is deduced from the wide-angle diffraction lines as discussed above.)

The X-ray measurements on other phosphatidylethanolamines show that the high-temperature transition of DSPE and DHPE also represents a transition from the lamellar to the hexagonal phase. However, there is no indication from X-ray analysis that the broad calorimetric transition at 118 °C in DPPE (1 M NaCl) is a transition to the hexagonal phase. For DMPE (1 M NaCl), a hexagonal phase was also not detected by X-ray diffraction.

The high-temperature transition was found to depend strongly on the NaCl concentration. This is shown in Figure 3. It can be seen that the high-temperature transition is also present in the absence of NaCl. Whereas the temperature (T_H) of this transition decreases with increasing salt concentration, the main transition temperature rises slightly.

The calorimetric data of all the phospholipids studied are given in Table I. The T_t and ΔH values of the main transition are only given for comparison. These values of the main transition agree basically with the values other authors have obtained (Vaughan & Keough, 1974; Blume, 1976). It was confirmed by X-ray diffraction that the high-temperature transition of DSPE both with 4 M NaCl as well as in the absence of NaCl is, in fact, a transition to the hexagonal phase.

As this salt dependence can also be expected with the other phosphatidylethanolamines, the structure of DMPE and DPPE was subsequently investigated in the presence of 4 M NaCl.

Table I: Calorimetric Data of All the Phospholipids Which Were Used in the Present Study

phospholipid	T_t (°C)	$\Delta H(T_t)$ (kcal/mol)	T_t (°C)	$\Delta H(T_t)$ (kcal/mol)	T_H (°C)	$\Delta H(T_H)$ (kcal/mol)
DMPE (1 M NaCl, pH 7)	*a*		52	5.81	*b*	
DMPE (4 M NaCl, pH 7)	*a*		56.5	5.55	109.5[c]	0.26[c]
DPPE (1 M NaCl, pH 7)	*a*		64.5	8.38	118[c]	0.39[c]
DPPE (4 M NaCl, pH 7)	*a*		70.5	7.93	92	1.09
DSPE (without NaCl, pH 7)	*a*		75.5	10.43	109.5	0.76
DSPE (1 M NaCl, pH 7)	*a*		77	10.24	101.5	0.91
DSPE (2 M NaCl, pH 7)	*a*		78	9.68	95.5	1.10
DSPE (4 M NaCl, pH 7)	*a*		80.5	9.30	88.5	1.50
DTPE (1 M NaCl, pH 7)	−2	0.07	58	5.94	93.5	0.95
DHPE (1 M NaCl, pH 7)	≃2	≃0.46	71.5	7.76	87	1.56
DPPA (1 M NaCl, pH 4,6)	*a*		69	7.87	104.5	0.13
DTPA (1 M NaCl, pH 4,6)	1.5	0.08	67	5.74	85.5	1.20
DHPA (1 M NaCl, pH 4,6)	12	0.10	79.5	*d*	≃81	*d*

[a] Not detected by calorimetry. [b] Not detected by calorimetry and X-ray diffraction. [c] The X-ray diffraction results indicate that this transition is probably not a transition to the hexagonal phase. [d] $\Delta H(T_t) + \Delta H(T_H) = 8.57$ kcal/mol.

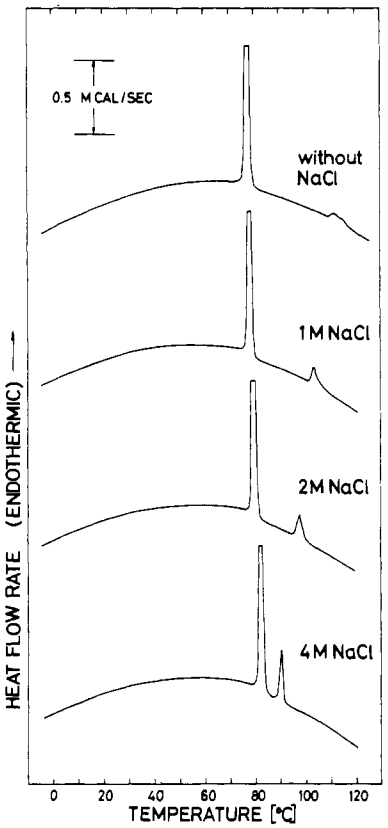

FIGURE 3: Differential scanning calorimeter traces of DSPE (pH 7, 0.25 M phosphate) as a function of the NaCl concentration. The heating rate was 2.5 °C/min.

In both cases, a high-temperature transition could be detected (the exact T_H and ΔH values are given in Table I). The X-ray experiments revealed that the hexagonal phase is present in DPPE (4 M NaCl) but probably not in DMPE (4 M NaCl) at high temperature.

The reflections of the hexagonal phases studied exhibited the usual temperature dependence: with increasing temperature, the lines moved toward smaller spacings. It should be mentioned that in some cases a mixture of the (lamellar) liquid-crystalline phase and the hexagonal phase was already detected at temperatures just below T_H. The T_H values given in Table I were determined from the calorimetric scans by using a heating rate of 2.5 °C/min. It is possible that slightly lower T_H values will be obtained if an infinitely slow heating rate is employed.

Phosphatidic Acids. The results obtained with several phosphatidic acids at pH 4.6 are basically very similar to the above results for phosphatidylethanolamines at pH 7. Typical calorimetric scans of the phosphatidic acids used in the present study are shown in Figure 4. The low-temperature transition could be detected calorimetrically only for DTPA and DHPA. The transition T_H was found for all three phosphatidic acids, and the X-ray measurements confirmed the presence of a hexagonal phase in all cases. In DPPA, the T_H transition is rather small, and in DHPA, the transition is so close to the main transition temperature that it forms a high-temperature shoulder of the main transition peak. In DTPA, T_t and T_H are clearly separated.

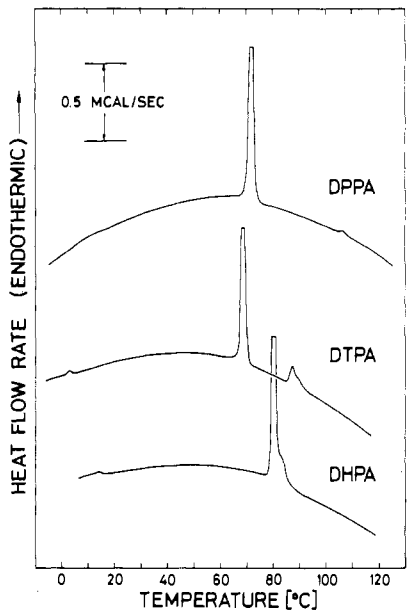

FIGURE 4: Differential scanning calorimeter traces of phosphatidic acids with saturated chains at pH 4.6 (1 M NaCl, 0.25 M sodium acetate). The heating rate was 2.5 °C/min.

The X-ray results obtained with DTPA are shown in Figure 5. At low temperature, a splitting of the wide-angle reflection is observed clearly whereas the lamellar reflections remain constant. A similar line splitting has been described above for phosphatidylethanolamines and has already been reported for DHPA at pH 7 (Jähnig et al., 1979). At 80 °C, DTPA is present in the (lamellar) liquid-crystalline structure, and at 98 °C, the small-angle reflections are again in the ratio of $1:1/3^{1/2}:1/2$ and therefore demonstrate the existence of a hexagonal phase.

In the course of the present study, the structure of phosphatidic acid was also investigated at pH 7. However, a hexagonal phase could then not be detected. The presence of

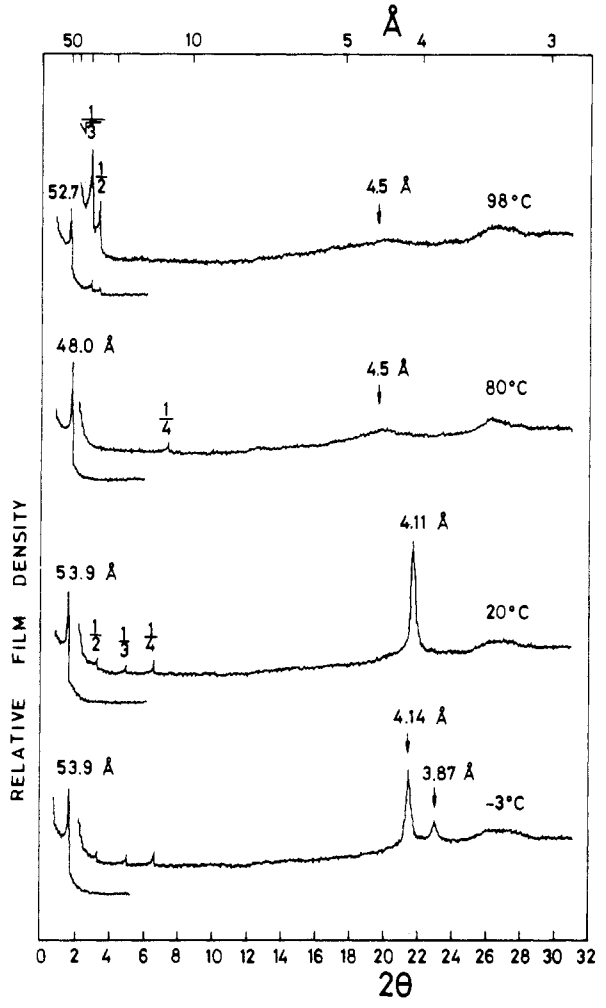

FIGURE 5: X-ray diffraction lines of DTPA at pH 4.6 (1 M NaCl, 0.25 M sodium acetate) as a function of temperature.

a hexagonal phase at pH 4.6 is probably connected with the first p*K* of the phosphate group. The titration characteristics of phosphatidic acids have been dealt with in detail by Träuble & Eibl (1974), Eibl & Blume (1979), and Eibl & Woolley (1979).

Previous studies have already described a similar dependence on pH for the hexagonal phase in phosphatidylserine and phosphatidylethanolamine. Hope & Cullis (1980) showed that a hexagonal phase can be induced in (egg) phosphatidylserine by lowering the pH from 7.4 to 3. They attributed this behavior to the p*K* of the carboxyl group. In the case of DHPE, it has been suggested that the hexagonal phase is present at pH 8 but not at pH $\geq$ 12 (Harlos & Eibl, 1980b). This suggestion has been confirmed by X-ray diffraction in the course of the present study. Thus, the absence of the hexagonal phase at pH $\geq$ 12 in phosphatidylethanolamine is due to the deprotonation of the amino group.

It can be concluded that the physical behavior of phosphatidylethanolamine and phosphatidic acid at pH 12 differs largely from that at pH 7 (PE) and 4.6 (PA). The removal of protons from the membrane surface results in systems which closely resemble the structures described by Janiak et al. (1976) and by Luna & McConnell (1977) for phosphatidylcholines at pH 7. This is indicated by the appearance of a pretransition with a characteristic ripple structure in PA (Harlos et al., 1979) and PE (Stümpel et al., 1980). An induction of hexagonal phases at high pH was not possible with monovalent cations in the case of DHPE. Therefore the structure of both phosphatidylethanolamine and phosphatidic acid differs greatly according to the degree of protonation.

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