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Influence of Calcium on Phosphatidylglycerol. Two Separate Lamellar Structures†

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ABSTRACT: The influence of calcium on the structure of *rac*-1,2-ditetradecylglycerol-3-phosphoglycerol is investigated by differential scanning calorimetry and by X-ray diffraction. It is shown that 1 M $CaCl_2$ (pH 4.6) induces two separate lamellar phases in the same sample at 20 °C. These two phases can be clearly distinguished by their X-ray diffraction patterns. The type of phase observed depends on the pretreatment of the sample. At high temperature (90 °C), when the hydrocarbon chains are in the disordered state, the small angle reflections are in the ratio $1:1/\sqrt{3}:1/2$ and thus indicate the presence of a hexagonal phase.

It is well-known that phospholipids in aqueous systems exhibit a phase transition from an ordered structure to a more disordered state. The phase transition temperature T_t[1] does not only depend on the particular chemical structure of each phospholipid but can also be influenced by parameters of the aqueous phase. Several authors have shown that the H^+, Na^+, K^+, Ca^{2+}, and Mg^{2+} concentrations can influence T_t (Träuble & Eibl, 1974; Tocanne et al., 1974; Verkleij et al., 1974; Ververgaert et al., 1975; Jacobson & Papahadjopoulos, 1975; Galla & Sackmann, 1975; MacDonald et al., 1976). The influence of calcium on the structure of phospholipids is of particular interest, because calcium plays a central role in many biological processes.

The influence of calcium on the phase transition temperature of negatively charged phospholipids, such as phosphatidic acid, phosphatidylglycerol, phosphatidylserine, or cardiolipin, is particularly pronounced. A number of studies have reported a large increase in T_t ($\Delta T_t > 20$ °C) when using a high calcium concentration (Tocanne et al., 1974; Verkleij et al., 1974; Van Dijck et al., 1978; Portis et al., 1979). In the case of phosphatidylserine and phosphatidic acid, a phase transition could not even be detected below 70 °C after the addition of a high calcium concentration (Jacobson & Papahadjopoulos, 1975; Newton et al., 1978; Van Dijck et al., 1978). According to a recent study, the phase transition temperature of phosphatidylserine in the presence of Ca^{2+} is above 100 °C (Portis et al., 1979).

The increase in T_t induced by Ca^{2+} can be associated with a structural rearrangement of the phospholipid molecules. It was shown by X-ray diffraction that a low and a high Ca^{2+} concentration can induce two different lamellar structures in phosphatidylserine. On the addition of excess Ca^{2+}, the structure of phosphatidylserine is transformed into a crystalline-like packing of the hydrocarbon chains. This transformation was found to be exothermic (Papahadjopoulos et al., 1978; Portis et al., 1979).

Apart from inducing particular lamellar structures, Ca^{2+} is also known to induce structures with hexagonal symmetry. These so-called "hexagonal phases" have been reported in the case of cardiolipin after the addition of Ca^{2+} (Deamer et al., 1970; Rand & Sengupta, 1972; Cullis et al., 1978) and for phosphatidic acid after the addition of Mg^{2+} (Papahadjopoulos et al., 1976). At a very early stage specific models for phases with hexagonal symmetry had already been suggested (Marsden & McBain, 1948; Luzzati & Husson, 1962; Luzzati, 1968). 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.

In the present study the influence of a high Ca^{2+} concentration on the structure of DTPG was investigated by differential scanning calorimetry and by X-ray diffraction. The ether analogue of dimyristoylphosphatidylglycerol was chosen

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[1] Abbreviations used: T_t, phase transition temperature; DTPG, *rac*-1,2-ditetradecylglycerol-3-phosphoglycerol; T_1 and T_2, main transition temperatures of phase A and B, respectively; TLC, thin-layer chromatography.

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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 (Vaughan & Keough, 1974) but otherwise show the same titration characteristics (Blume, 1976; Eibl & Blume, 1979).

Materials and Methods

rac-1,2-Ditetradecylglycerol 1,2-Ditetradecylglycerol was purchased from Medmark (München-Grünwald, West Germany). It did not contain the 1,3 isomer, as was shown by TLC on boric acid plates using the solvent system chloroform–hexane–diisopropyl ether (5:4:1 v/v). Solvents of a purity >99% were used.

The crude products of the synthesis were purified by column chromatography on Kieselgel 60 (Merck, Darmstadt, West Germany). Mixtures of chloroform, methanol, and aqueous ammonia (25%) were used for elution. Starting with $CHCl_3$–CH_3OH–ammonia in a ratio of 200:15:1 (v/v), the polarity of the elution mixture was increased step by step up to 65:30:3 to elute DTPG.

Synthesis of DTPG. Freshly distilled phosphorus oxychloride (boiling range 105–107 °C), 1.8 g (0.012 mol), was cooled in an ice bath. A solution of *rac*-1,2-ditetradecylglycerol, 4.8 g (0.01 mol), in 60 mL of tetrahydrofuran and triethylamine, 1.5 g (0.015 mol), was added in drops to the phosphorus oxychloride while stirring continuously. After that the temperature was raised to 25 °C within 60 min. As shown by TLC, the original *rac*-1,2-ditetradecylglycerol was completely transformed into *rac*-1,2-ditetradecylglycerol-3-phosphoric acid dichloride. Stirring was continued, and 1,2-acetoneglycerol, 1.6 g (0.012 mol), in 40 mL of tetrahydrofuran and triethylamine, 3 g (0.03 mol), was added drop by drop. The temperature of the reaction mixture was raised to 45 °C. After 60 min *rac*-1,2-ditetradecylglycerol-3-phosphoric acid dichloride was no longer detected by TLC. The reaction mixture was filtered by suction to remove the precipitated triethylamine hydrochloride. The phosphoryl chlorides were hydrolyzed by the addition of 50 mL of 1 M sodium carbonate to the filtrate. Hydrolysis was completed after 6 h at 20 °C. The addition of 100 mL of acetone to the upper tetrahydrofuran phase led to precipitation of the contaminating phosphatidic acid, which was removed by filtration. The filtrate was evaporated to dryness, and the residue was dissolved in 100 mL of acetic acid (70% solution in water). After 60 min the protected phosphatidylglycerol was completely transformed into DTPG, which was extracted by using chloroform and purified by chromatography. The yield in the chromatographically pure product was 70% based on the original *rac*-1,2-ditetradecylglycerol. The purity of the compound was checked by TLC and elemental analysis. Anal. Calcd for $C_{34}H_{72}NaO_9P$ monohydrate (M_r 678.92): C, 60.15; H, 10.69; P, 4.56. Found: C, 60.01; H, 10.58; P, 4.46.

After the experiments the purity of the lipid was rechecked by TLC using the solvent system $CHCl_3$–CH_3OH–25% NH_3 (100:60:15 and 65:15:1 v/v). The plates were developed with "Dittmer" spray (Dittmer & Lester, 1964) followed by charring. Throughout the present study the acid form of phosphatidylglycerol was used.

Calorimetry. 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 (3–4 mg) were weighed into stainless steel pans ("large volume capsules"), and 50 μL of the desired $CaCl_2$ solution was added before the pans were sealed. The samples were equilibrated at $T > 100$ °C 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 a heating rate of 2.5 °C/min in the sensitivity range of 1 mcal/s (full scale). The enthalpies were calculated from the peak areas, which were determined by weight.

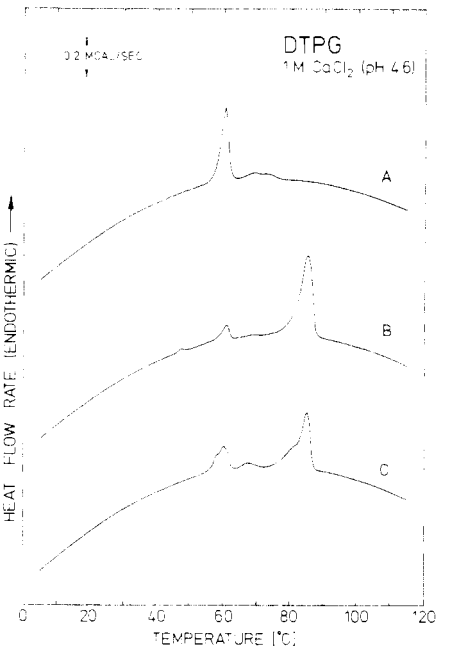

FIGURE 1: Differential scanning calorimeter traces of DTPG in 1 M $CaCl_2$ (pH 4.6) as a function of the pretreatment of the sample: (A) equilibration at $T > 100$ °C for 10 min and cooling to 1 °C; (B) equilibration at $T > 100$ °C for 10 min, cooling to 1 °C, and then storage at $T = 70$ °C for $t \geq 2$ h; (C) as in (B) except that storage at $T = 70$ °C was for only 1 h. The heating rate was 2.5 °C/min.

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, West Germany). The monochromator was set to isolate the Cu $K\alpha_1$ line ($\lambda = 1.5405$ Å). A camera with movable film was used in order to record a number of exposures on the same film, so that it was possible to compare the X-ray diffraction lines more easily. Further details of the experimental setup have been reported elsewhere (Harlos, 1978).

The lipid samples for the X-ray diffraction experiments were prepared by adding ~20 μL of the desired $CaCl_2$ solution to ~2 mg of dry lipid. The samples were then sealed with Teflon between two mica plates and equilibrated at $T > 100$ °C for at least 10 min. The specific temperature treatment after this equilibration varied and is stated under Results. Before starting the actual X-ray exposure, the sample was kept at the desired temperature for 5–10 min. The exposure times of the photographic films (Kodak, "Kodirex") varied between 15 min and 8 h. The density of the reflections was scanned with a Joyce-Loebl type 3CS microdensitometer.

Results

The purity of DTPG was checked after the X-ray measurements had been carried out. The possible decomposition products *rac*-1,2-ditetradecylglycerol and *rac*-1,2-ditetradecylglycerol-3-phosphoric acid were not detected by TLC.

Calorimetry. The calorimetric scans of DTPG in 1 M $CaCl_2$ (0.25 M sodium acetate, pH 4.6) are shown in Figure 1. The curves A–C show heating scans of the same sample after different pretreatments. In curve A the sample was kept at $T > 100$ °C for 10 min, cooled to 1 °C, and then scanned

in the calorimeter. In curve B the sample was equilibrated at $T > 100$ °C for 10 min and also cooled to 1 °C but then stored at 70 °C for $t \geq 2$ h. In scan C the sample was prepared in the same way as in scan B, except that the storage at 70 °C was for only 1 h.

The curves A and B show distinctly different calorimetric transitions. In curve A one transition at $T_1 \approx 57$ °C with a transition enthalpy of $\Delta H \approx 5.0$ kcal/mol is followed by a small broad transition. The heating curve B shows a main transition at $T_2 \approx 81$ °C ($\Delta H \approx 9.0$ kcal/mol) and two small transitions at 46 and 58 °C. (It should be mentioned that the transition temperature of DTPG in 0.25 M sodium acetate, pH 4.6, in the absence of $CaCl_2$ is 26 °C.) The small transition at 46 °C was not observed with all the samples. Moreover, after prolonged storage at 70 °C ($t \geq 10$ h), the transition at 58 °C was no longer present in some of the samples. As the calorimetric scans A and B in Figure 1 were found to be characteristic for two different lipid structures, the phases corresponding to scan A and scan B are defined in the present paper as phase A and phase B.

One can consider phase A as a metastable state, which is obtained only after the sample had been equilibrated at $T >$ 100 °C. To reach phase B, one has to anneal the sample at 70 °C. With the Ca^{2+} solution used in the present study (1 M $CaCl_2$ and 0.25 M sodium acetate, pH 4.6), the transformation from phase A to phase B at 70 °C takes a relatively long time. This is demonstrated in scan C, where the sample was annealed for only 1 h. It can be seen that the transition enthalpies at T_1 and T_2 lie between the transition enthalpies of scans A and B.

Slightly different results were obtained when a plain $CaCl_2$ solution (without buffer) was used. Although the same phases could be detected, the transformation from phase A to phase B at 70 °C took place at a much faster rate than when using the pH 4.6 buffer. Furthermore, the transition at T_1 was followed by an exothermic heat change. The transformation from phase A to phase B also occurred at low temperature, although at a much slower rate than at 70 °C. These results with a plain $CaCl_2$ solution are similar to the calorimetric results reported for didodecanoylphosphatidylglycerol in the presence of Mg^{2+} (Ververgaert et al., 1975) and for phosphatidylethanolamine at low water content (Chapman et al., 1966; Ladbrooke & Chapman, 1969). The present study was carried out with a 0.25 M sodium acetate buffer of pH 4.6, so that the bulk pH was accurately defined. Furthermore, the transformation from phase A to phase B was not found at low temperature when using the buffer. It was therefore possible to clearly distinguish the two phases by X-ray diffraction.

X-ray Diffraction. The X-ray diffraction patterns of DTPG in 1 M $CaCl_2$ (0.25 M sodium acetate, pH 4.6) at 20 °C are shown in Figures 2 and 4. The X-ray samples were prepared in the same way as the calorimetric ones. It can be seen that the diffraction lines for A and B differ completely. Therefore, the sample is present in two separate phases, as has already been indicated by the calorimetric results. Phases A and B both show a lamellar structure with a repeat distance of 59.4 and 48.4 Å, respectively. In A and B higher orders of the lamellar repeat can be seen. Like the lamellar reflections (long spacings), the wide angle reflections (short spacings) also show remarkable differences. In phase A a single sharp line at 4.13 Å is detected. A single wide angle diffraction line is characteristic for a hexagonal packing of the chains in the plane perpendicular to the chain axes. Phase B gives rise to a number of wide angle reflections, indicating a high degree of lateral order. The most intense lines are at 4.29, 4.20, and

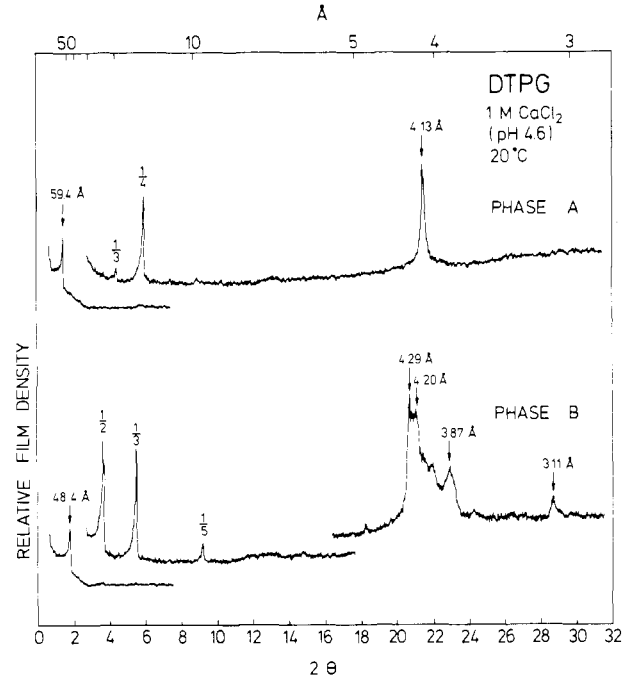

FIGURE 2: X-ray diffraction lines of DTPG in 1 M $CaCl_2$ (pH 4.6) at 20 °C. The pretreatment of the sample to obtain phases A and B was the same as that in parts A and B of Figure 1.

Table I: Observed X-ray Diffraction Spacings of DTPG in 1 M $CaCl_2$, pH 4.6

T (°C)		d (Å)	
20 °C, phase A	59.4, 19.8, 14.9, 11.9, 9.90	4.13	
20 °C, phase B	48.4, 24.2, 16.1, 9.68	4.84, 4.40, 4.29, 4.20, 4.03, 3.87	3.11
70 °C	48.6, 24.3, 16.2, 9.72	4.87, 4.42, 4.31, 4.19, 4.05, 3.91	3.12
90 °C	54.7, 31.6, 27.4	4.55 (broad line)	

3.87 Å. Furthermore, a faint reflection at 3.11 Å could be observed. This 3.11-Å line is probably a higher order lateral reflection. (The spacings of all the phases are listed in Table I.) Some of the wide angle reflections are probably higher orders of the lamellar repeat: 4.84 Å = d_{0010}; 4.40 Å = d_{0011}; 4.03 Å ≈ d_{0012}. Because of the number of wide angle diffraction lines, it is reasonable to assume that the packing of the chains in phase B is crystalline-like. In Figure 2 a longer exposure (8 h) was necessary to record the wide angle reflections of phase B, and therefore the intensities of the wide angle reflections of phases A and B cannot be compared directly in this figure.

Samples in phase A and B, respectively, were dried under high vacuum ($p < 1 \times 10^{-4}$ torr) for $t \geq 2$ h in order to obtain a rough estimate of the thickness of the water layer. In the case of phase B, it was found that drying did not change the position of the lamellar and the wide angle reflections (measured at 20 °C). Thus, DTPG is unhydrated in 1 M $CaCl_2$ (pH 4.6) in this phase. Drying the phase A sample caused a decrease in the lamellar repeat from 59.4 to 55.9 Å. In this phase the sample is therefore hydrated. Phase A in the dried state shows only a single sharp wide angle diffraction line, indicating that the lateral packing of the dried and the hydrated states is very similar. It is interesting to note that there are certain similarities between the diffraction lines of the hydrated state of phase A (with $CaCl_2$) and other hydrated phospholipids (without $CaCl_2$) [see, for example, phosphatidylethanolamine (Harlos, 1978)].

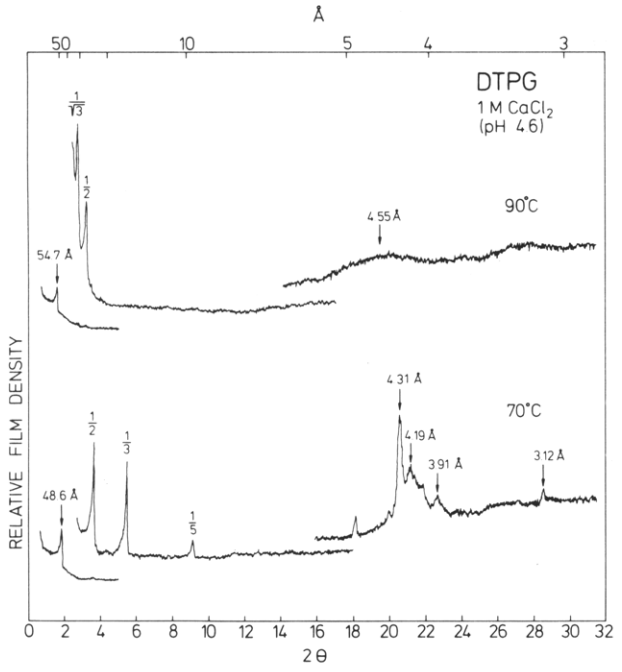

FIGURE 3: X-ray diffraction lines of DTPG in 1 M $CaCl_2$ (pH 4.6) at 70 and 90 °C.

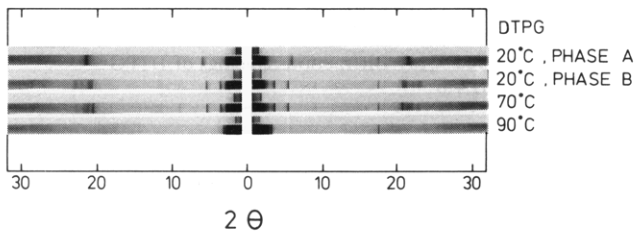

FIGURE 4: X-ray diffraction patterns of the different phases of DTPG (1 M $CaCl_2$, pH 4.6). For each phase the exposure time was 15 min for the upper trace and 4 h for the lower trace. The very sharp line at 17.5 (2θ), which is only present on the right side of the patterns, is due to a reflection of the mica windows. The same lipid sample has been used for all the patterns shown in this figure.

The X-ray diffraction lines of DTPG in 1 M $CaCl_2$ (0.25 M sodium acetate, pH 4.6) at 70 and 90 °C are shown in Figures 3 and 4. Irrespective of whether the samples were originally in phase A or phase B at 20 °C, heating the samples to 70 °C resulted in identical diffraction lines. This was also true when the samples were heated to 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 identical with the reflections observed for phase B at 20 °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 Figures 3 and 4, transformation from phase A to phase B must have taken place. This had already been concluded from the calorimetric measurements. The calorimetrically observed transition at T_1 could be the ordered–disordered phase transition of the phase A lipid. In this case there would be a disordered phase present at 70 °C, which is shown as A′ in Figure 5.

The calorimetric measurements indicated that the transformation from phase A (A′) to phase B at 70 °C took ~2 h when the 0.25 M sodium acetate buffer was used. Phase A (A′), however, could not be detected in the X-ray diffraction patterns taken at 70 °C. As the equilibration time prior to the X-ray exposure was 5–10 min, the transformation from phase A (A′) to B must have taken place within this time. The transformation therefore seems to occur faster in the X-ray

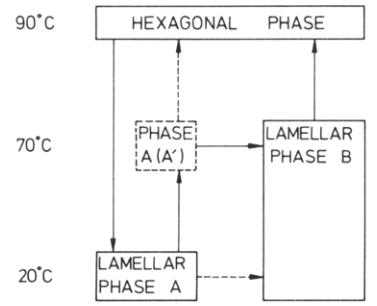

FIGURE 5: Schematic diagram showing the different phases of DTPG (1 M $CaCl_2$). Phase A is obtained by cooling the sample from 90 °C. Phase B is reached by storing the sample at 70 °C. When a plain 1 M $CaCl_2$ solution (without buffer) was used, the transformation of phase A to phase B was also observed at 20 °C, although at a much slower rate than that at 70 °C. The existence of phases A and B as well as the hexagonal phase is demonstrated in the present paper; the presence of phase A (A′) at 70 °C, however, is not yet certain.

samples than in the calorimetric ones.

One difficulty in detecting phase A (A′) at 70 °C by X-ray diffraction is due to the fact that the transformation continues during the actual measurement. A mixture of phases was obtained when the sample was kept at 70 °C for only 5 min and then cooled to 20 °C. The phases present in this mixture (at 20 °C) were closely related although not identical to phases A and B. The reason a mixture of phases could be observed was because the transformation did not take place any more after the sample had been cooled to 20 °C (when using the 0.25 M sodium acetate buffer).

At 90 °C the diffuse wide angle halo in the region of 4.55 Å indicates that the hydrocarbon chains are in the disordered state. In the small angle region the reflections are in the ratio $1:1/\sqrt{3}:1/2$. Assuming that all the reflections have to be attributed to one phase, the ratio $1:1/\sqrt{3}:1/2$ indicates the presence of a hexagonal lattice. The next higher order would be $(1/\sqrt{7}) \times 54.7$ Å = 20.7 Å, but this line could not be observed.

A summary of all the X-ray diffraction spacings observed is given in Table I, and a schematic diagram of the different phases of DTPG is shown in Figure 5. It would be interesting to find out how this transition to the hexagonal phase takes place and whether the small angle reflections are related to the repeat distances of the lamellar phases. As the lamellar phase A is only found after the sample has been equilibrated at temperatures where the hexagonal phase is present ($T > T_2$), phase A must be formed when the sample is cooled down from the hexagonal phase.

The transition of phase A at T_1 and of phase B at T_2 could be observed visually by using a common microscope with a heating stage. In both cases the sample changed from a white color to being partially transparent. Thus, when a sample in phase A was heated to 70 °C it became partially transparent at T_1. When this sample was stored at 70°C, its color slowly changed back to white. This was because the sample had changed from phase A to B but was still below T_2 at this temperature.

Discussion

As calcium can induce two different lamellar structures in DTPG, it is possible that both structures are present in the sample at the same time and that the phases then separate. In this case a calcium-induced phase separation would be present in a sample which contains only one type of phospholipid molecule. Clearly, the study of Ca^{2+}-induced phase separations in samples containing more than one kind of

phospholipid molecule then becomes rather complicated.

It has already been pointed out that the transformation of phase A to phase B can be exothermic when a plain $CaCl_2$ solution is used. Similar exothermic heat changes can be seen in the calorimetric scans of phosphatidylglycerols using excess Ca^{2+} in Figure 3, curve 5, of Papahadjopoulos (1977) and in Figure 2B, curve 7, of Van Dijck et al. (1978). An exothermic transition has also been reported for didodecanoylphosphatidylglycerol in the presence of Mg^{2+} (Ververgaert et al., 1975). These exothermic heat changes already reported in the literature are probably also due to the transformation from phase A to phase B. This assumption is supported by the observation that, on adding excess Ca^{2+}, the formation of the crystalline-like packing in phosphatidylserine is exothermic (Papahadjopoulos et al., 1978; Portis et al., 1979).

Because the X-ray diffraction patterns of phases A and B show higher orders of the lamellar reflections, both phases are present as multilamellar structures. However, it has not yet been shown whether any of the phases described in the present paper exhibit the cylindrical structure which has been reported for phosphatidylglycerol (Tocanne et al., 1974; Verkleij et al., 1974; Ververgaert et al., 1975) or the "cocleate" structure reported for phosphatidylserine (Papahadjopoulos et al., 1975). It would also be interesting to investigate the structure of DTPG at 90 °C by using freeze–fracture electron microscopy to determine whether this hexagonal phase shows similar fracture faces to other hexagonal phases (Deamer et al., 1970; Papahadjopoulos et al., 1976; Van Dijck et al., 1976; Cullis et al., 1978).

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