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COMPARATIVE STUDIES ON THE EFFECTS OF pH AND Ca^{2+} ON BILAYERS OF VARIOUS NEGATIVELY CHARGED PHOSPHOLIPIDS AND THEIR MIXTURES WITH PHOSPHATIDYLCHOLINE

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Summary

(1) The thermotropic behaviour of dimyristoyl phosphatidylglycerol, phosphatidylserine, phosphatidic acid and phosphatidylcholine was investigated by differential scanning calorimetry and freeze-fracture electron microscopy as a function of pH and of Ca^{2+} concentration.

(2) From the thermotropic behaviour as a function of pH, profiles could be constructed from which apparent pK values of the charged groups of the lipids could be determined.

(3) Excess Ca^{2+} induced a shift of the total phase transition in 14 : 0/14 : 0-glycerophosphocholine and 14 : 0/14 : 0-glycerophosphoglycerol mixtures. In 14 : 0/14 : 0-glycerophosphocholine bilayers containing 16 : 0/16 : 0-glycerophosphoglycerol lateral phase separation was induced by Ca^{2+}.

(4) Up to molar ratios of 1 : 2 of 14 : 0/14 : 0-glycerophosphoserine to 14 : 0/14 : 0-glycerophosphocholine, excess Ca^{2+} induced lateral phase separation. Addition to mixtures of higher molar ratios caused segregation into different structures: the liposome organization and the stacked lamellae/cylindrical organization.

(5) Addition of excess Ca^{2+} to mixtures of 14 : 0/14 : 0-glycerophosphocholine and 14 : 0/14 : 0-phosphatidic acid caused, independent of the molar ratio, separation into two structural different organizations.

(6) The nature of Ca^{2+}-induced changes in bilayers containing negatively charged phospholipids is strongly dependent on the character of the polar headgroup of the negatively charged phospholipid involved.

Abbreviations: PIPES, piperazine-*N*,*N*'-bis-(2-ethanesulfonic acid).

Introduction

Negatively charged phospholipids may play an important role in biomembrane functioning as the properties of these negatively charged phospholipids are largely dependent on environmental conditions. This, for instance, is apparent from the large effects of pH and Ca^{2+} or Mg^{2+} concentration on the thermotropic properties of negatively charged phospholipids. With respect to the effects of divalent ions like Ca^{2+} and Mg^{2+} there appear to be differences between the various types of negatively charged phospholipids. On one hand, in phosphatidylcholine bilayers containing either phosphatidylserine or phosphatidic acid, Ca^{2+} induced a lateral phase separation [1—5]. In contrast, in either equimolar dilauroylglycerophosphoglycerol-dilauroyl-glycerophosphocholine or dimyristoylglycerophosphoglycerol-dimyristoyl-glycerophosphocholine bilayers, Ca^{2+} and Mg^{2+} only shifted the total transition peak to higher temperatures but no phase separation occurred [6,7]. As in most studies with phosphatidylserine and phosphatidic acid where natural phospholipids are used it is difficult to decide whether the discrepancies are caused by the differences in the polar headgroup or by differences in miscibility properties of the acyl chains of the mixtures concerned. Furthermore, as the ionization of the polar headgroup is strongly pH dependent the pH at which the studies have been performed may be an important factor.

In this paper we describe the influence of pH on the thermotropic properties of negatively charged synthetic phospholipids and the effects of Ca^{2+} on the phase behaviour of phospholipid mixtures. The phospholipids which were used were dimyristoylglycerophosphocholine, dimyristoylphosphatidic acid, dimyristoylglycerophosphoserine and dimyristoylglycerophosphoglycerol. The thermotropic properties were studied by differential scanning calorimetry and structural information on these mixtures was obtained by freeze-fracture electron microscopy.

Materials and Methods

1,2-Dimyristoyl-*sn*-glycero-3-phosphorylcholine (14 : 0/14 : 0-glycerophosphocholine) was synthesized according to the method of van Deenen and de Haas [8]. 1,2-Dimyristoyl-*sn*-glycero-3-phosphatidylglycerol (14 : 0/14 : 0-glycerophosphoglycerol) and 1,2-dipalmitoyl-*sn*-glycero-3-phosphatidylglycerol (16 : 0/16 : 0-glycerophosphoglycerol) were obtained by phospholipase D-catalyzed base exchange and subsequently purified as described by van Dijck et al. [7]. 1,2-Dilauroyl-*sn*-glycero-3-phosphate (12 : 0/12 : 0-phosphatidic acid) and 1,2-dimyristoyl-*sn*-glycero-3-phosphate (14 : 0/14 : 0-phosphatidic acid) and 1,2-dimyristoyl-*sn*-glycero-phosphatidylserine (14 : 0/14 : 0-glycerophosphoserine) were converted from 12 : 0/12 : 0-glycerophosphocholine and 14 : 0/14 : 0-glycerophosphocholine, respectively, and purified on carboxymethyl-cellulose as described by Comfurius and Zwaal [9]. As the products were slightly contaminated by neutral compounds they were subjected to a purification procedure developed for egg phosphatidic acid but applicable for all acidic phospholipids. A concentrated lipid solution was slowly added to a stirred methanol/water (10 : 1, v/v) mixture (100 ml/g lipid) which contains a

5-fold excess of bariumacetate. The Ba-salt of the negatively charged phospholipid precipitated in this medium and was collected by centrifugation. The neutral contaminants remained in solution [10]. These Ba-salts of the lipids proved to be chromatographically pure. The negatively charged lipids were converted to the (di)sodium salt in a Bligh and Dyer extraction [11] which contained sodium sulfate [12], EDTA and NaCl (final concentration in the one phase system: lipid 5 mM, $NaSO_4$ 100 mM, EDTA 10 mM, NaCl 100 mM). The formed precipitate of barium sulfate was separated by filtration of the one phase system. After the phase separation was accomplished by addition of water and chloroform the sodium salts of the lipids were isolated from the lower chloroform layer.

When negatively charged lipids are dispersed in a small volume of buffer (3 μmol in 50 μl) the pH of the medium is affected due to insufficient buffering capacity. This explains why in a previous study [7] hardly any influence on the transition temperature was found when the pH of the dispersion buffer was decreased from pH 7 to 3. Therefore, in this study for calorimetric analysis the lipid was dispersed in excess buffer. 3 μmol of phospholipid were dispersed at 60°C in 1.5 ml of 25 mM piperazine-*N*,*N*'-(2-ethanesulfonic acid) (PIPES), 100 mM NaCl, pH 6.0, unless stated otherwise. Mixtures to which Ca^{2+} was added were dispersed in 1.0 ml of the above buffer, subsequently 0.5 ml of 25 mM PIPES, 100 mM $CaCl_2$, pH 6.0, was added. After 10 min of equilibration at 60°C the lipid dispersions were collected by centrifugation (20 min, 37 500 × *g*, 4°C). Samples of the wet pellet were analyzed by differential scanning calorimetry. The instrument was operated and calibrated as described before [13]. Samples of the wet pellet were quenched from 4°C, fractured and analyzed by electron microscopy as described previously [14]. In order to be able to interpret the pH profile of 14 : 0/ 14 : 0-glycerophosphoserine correctly, ^{31}P NMR spectroscopy experiments were carried out on a Bruker WH-90 machine operated as described elsewhere [15].

Results

Effect of pH on the thermotropic properties of negatively charged phospholipids

The packing properties and, consequently, the thermotropic behaviour of negatively charged phospholipids will be largely dependent on the degree of ionization of the charged polar headgroups. Therefore, the miscibility properties of negatively charged lipids with phosphatidylcholines will be influenced by pH. To choose a suitable constant pH for all lipid mixtures we first compared the relationships between the transition temperatures of the various lipids and the pH. These profiles (Fig. 1) show that the transition temperature of a given phospholipid is sensitive to small variations in pH in those region where the ionization of the polar headgroup is affected. All phospholipid species tested showed changes in the transition temperature in the region of pH 3—5. For phosphatidylglycerol or phosphatidic acid species these changes can only be attributed to the phosphate ionization. The apparent p*K* values of these phosphate ionization were estimated as 3.1—3.5 for phosphatidylglycerol and 4.0 for phosphatidic acid. The possibility in phosphatidic acid to have the

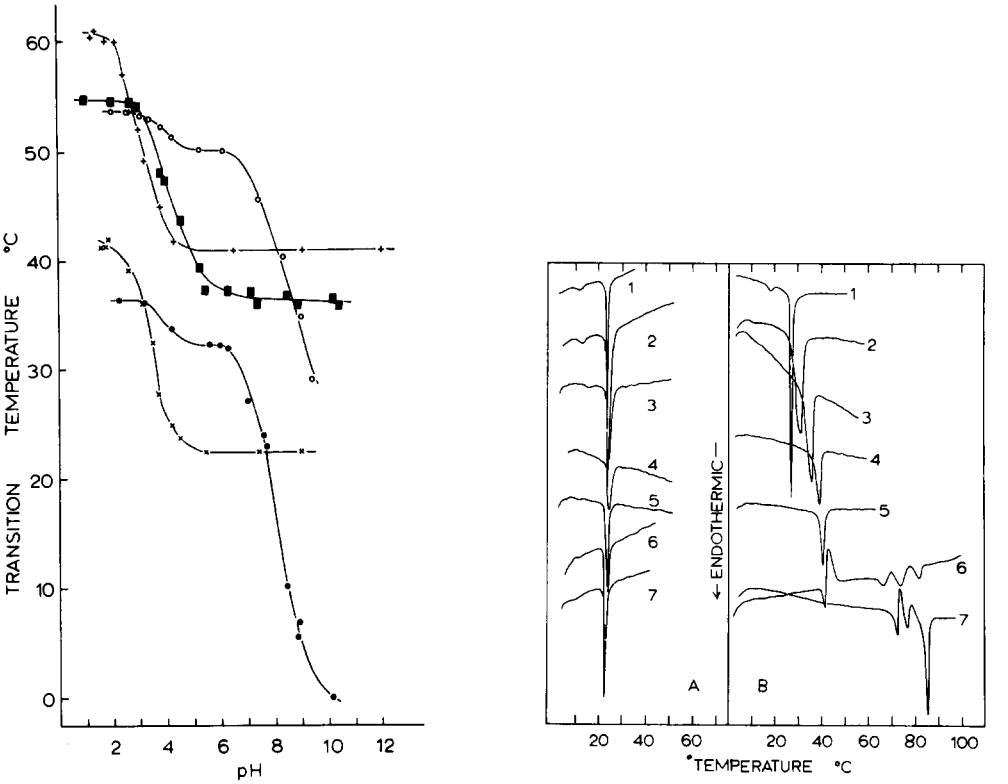

Fig. 1. Relationship between the transition temperature and pH of various charged phospholipids. ●———●, 12 : 0/12 : 0-phosphatidic acid; ○———○, 14 : 0/14 : 0-phosphatidic acid; X———X, 14 : 0/14 : 0-glycerophosphoglycerol; +———+, 16 : 0/16 : 0-glycerophosphoglycerol; ■———■, 14 : 0/14 : 0-glycerophosphoserine. The phospholipid (3 μmol) was dispersed in 1,5 ml of 25 mM buffers at the pH desired, all containing 100 mM NaCl, at 70°C and incubated for 10 min. The pH was checked and adjusted if necessary. Finally the lipid dispersion was collected by centrifugation. Samples of the wet pellet were analyzed by differential scanning calorimetry. The intercept of the slope of the peak with the baseline in the heating curve was plotted versus the pH as measured in the buffer. The pH was measured at various temperatures between 4 and 70°C. The pH at the transition temperature was determined by interpolation.

Fig. 2. Differential scanning calorimetric tracings of mixtures of 14 : 0/14 : 0-glycerophosphoglycerol and 14 : 0/14 : 0-glycerophosphocholine both in the absence (A) and presence (B) of excess Ca^{2+} at pH 6.0. (1), 0%; (2), 25%; (3), 50%; (4), 66%; (5), 80%; (6), 90%; (7), 100% of phosphatidylglycerol.

phosphate group doubly ionized is reflected in a dramatic change in transition temperature in the region of pH 6.5—10. An estimation of the apparent pK value for this second phosphate ionization gave a value of 8.1—8.5. The transition curves of phosphatidic acid are in agreement with those obtained by Träuble and Eibl [16].

The apparent pK value calculated from our transition temperature profile of 14 : 0/14 : 0-glycerophosphoserine is in good agreement with the value which was obtained by de Haas et al. [17] from a titration curve of a synthetic 18 : 1_c/18 : 0-glycerophosphoserine in a tetrahydrofuran/water (1 : 1, v/v) system. Phosphatidylserine loses one proton going from pH 3.8 to 6.8 from a group with an apparent pK of 4.4. The additional deprotonation of the amino group with a pK of 9.8 did not give any transition temperature change in our

calorimetric study. The change in the transition temperature around pH 4.4 for phosphatidylserine can be caused by a change in molecular packing brought about either by the protonation of the phosphate or by the carboxylic group. In order to be able to discriminate between these two possibilities we studied the ^{31}P NMR signal of phosphatidylserine in a tetrahydrofuran/water (1 : 1, v/v) system containing 100 mM NaCl in which the phosphate diester can no longer experience influences of the other charged groups. The position of the ^{31}P signal will be dependent on the ionic condition of the phosphate group only. As gradual pH changes from 7 to 4 appeared to have no influence on the position of the phosphate diester signal it can be concluded that the protonation of the carboxylic function is indeed responsible for the observed phenomena.

Effects of excess Ca^{2+} on the thermotropic properties of synthetic negatively charged phospholipids mixed with phosphatidylcholine

At pH 6.0 the transition temperatures of the various charged phospholipids remain constant and all the phospholipids bear at this pH one net negative charge. As we wanted to compare the miscibility properties of the charged phospholipids with phosphatidylcholine and the effects of Ca^{2+} on these miscibility properties we decided to carry out all experiments at pH 6.0 to avoid artificial interferences of small pH variations.

Phosphatidylglycerol. 14 : 0/14 : 0-Glycerophosphocholine and 14 : 0/14 : 0-glycerophosphoglycerol display comparable temperatures and enthalpy changes of the phase transition. It was, therefore, not remarkable to find that mixtures of these lipids in various ratios, in a medium containing only monovalent cations, showed one homogeneous transition peak (Fig. 2A). The ΔH values, calculated from these curves, were constant and independent on the molar ratios (Fig. 3b). In agreement with previous reports [6,7,18] excess Ca^{2+} was not able to induce a phase separation in mixtures up to 80 mol% of phos-

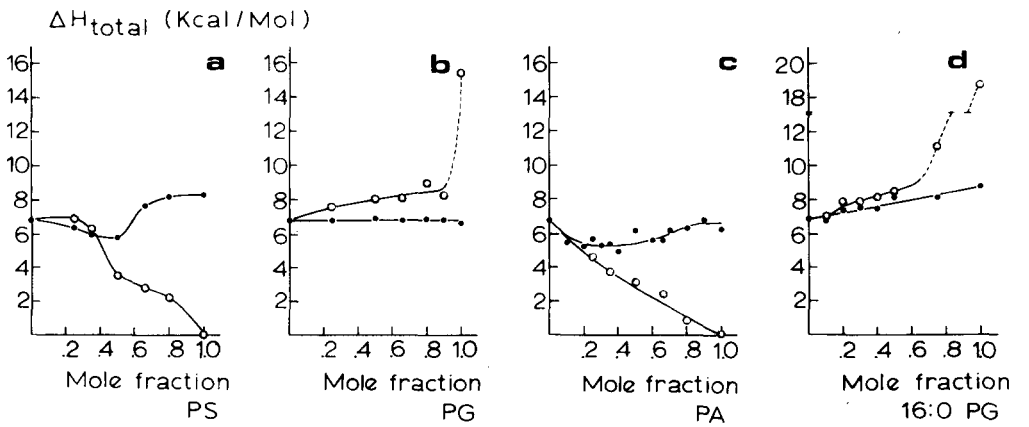

Fig. 3. Plots of the total enthalpy changes observable between 0 and 100°C both in the absence (•) and presence (○) of excess Ca^{2+}. Total observable ΔH values are plotted versus the mol fraction of the charged phospholipid. Data are obtained from the analyses of samples from Figs. 2, 4—6. For PA some additional data are incorporated. a (PS), 14 : 0/14 : 0-glycerophosphoserine; b (PG), 14 : 0/14 : 0-glycerophosphoglycerol; c (PA), 14 : 0/14 : 0-phosphatidic acid; d (16 PG), 16 : 0/16 : 0-glycerophosphoglycerol.

phatidylglycerol. In these mixtures Ca^{2+} addition led to a shift of the total transition peak to higher temperatures (Fig. 2B) coupled to an increase in the ΔH value (Fig. 3b), dependent on the mol fraction of the charged phospholipid. The liposomal conformation of these mixtures was confirmed by freeze-fracture electron microscopic analysis [6,7,18].

Addition of excess Ca^{2+} to pure phosphatidylglycerol liposomes results in a disorganization of the liposomal bilayer and a conversion into highly packed bilayers of Ca^{2+} complexes which precipitate in water. As seen by freeze-fracture electron microscopy [7] these precipitates have the appearance of stacked lamellae or cylindrical structures. Upon calorimetric analysis they display a phase transition at a very high temperature and with a ΔH value which is about double that of the lipid organized in liposomal bilayers (refs. 6 and 7, Figs. 2B (7) and 3b). A mixture consisting of 90 mol% of phosphatidylglycerol showed a thermotropic behaviour (Fig. 2B (6)), which was quite complex. The combination of an endo- and exothermic transition at 40° C followed by a series of endothermic peaks in the high temperature region is reminiscent of the behaviour observed with excess Mg^{2+} on pure phosphatidylglycerol bilayers and has been interpreted as metastable behaviour of the liposomal bilayers [7,19]. At 40°C the liposomal gel phase transforms to the liquid crystalline state but the liposome structure is not stable at this temperature. It is converted by an exothermic process to a gel-phase structure of highly packed lamellae. At very high temperatures these highly packed lamellae undergo a reconversion to stable liposomes which are in the liquid crystalline state [7,19]. In the presence of Ca^{2+} the gel-phase liposome of pure phosphatidylglycerol does not exist. Here the complex is either in the form of gel-phase lamellae or of liquid crystalline liposomes [6,7].

Ca^{2+} effects were also studied on mixtures of 14 : 0/14 : 0-glycerophosphocholine and 16 : 0/16 : 0-glycerophosphoglycerol. Mixtures of 16 : 0/16 : 0-glycerophosphoglycerol and 14 : 0/14 : 0-glycerophosphocholine showed at all ratios in the presence of only monovalent cations one homogeneous transition peak (Fig. 4A). Excess Ca^{2+} induced a shift in the total transition peak accompanied by a partial phase separation (Fig. 4B). This was accomplished without any loss in total peak intensity. The ΔH values of the mixtures in the presence of Ca^{2+} were consistently higher than the ones without Ca^{2+} (Fig. 3d) in agreement with the data for the mixtures of 14 : 0/14 : 0-glycerophosphocholine and 14 : 0/14 : 0-glycerophosphoglycerol. No other structures besides liposomes were observed after Ca^{2+} addition in these mixtures up to 1 : 1 molar ratios upon electron microscopic examination. The liposomes display smooth and banded domains, indicative of lateral phase separation. Only at very high concentrations of phosphatidylglycerol could part of the molecules be converted to high melting structures. This destabilization of the liposomal structure took place at a lower percentage of phosphatidylglycerol than in case of mixtures with the 14 : 0/14 : 0 compound.

Phosphatidylserine. Differential scanning calorimetry scans of mixtures of 14 : 0/14 : 0-glycerophosphocholine and 14 : 0/14 : 0-glycerophosphoserine, in the presence of only monovalent cations, are shown in Fig. 5A. Mixtures of 66 or 80 mol% of phosphatidylserine show two separated peaks indicating gel phase immiscibility. The phosphatidylserine/phosphatidylcholine mixtures

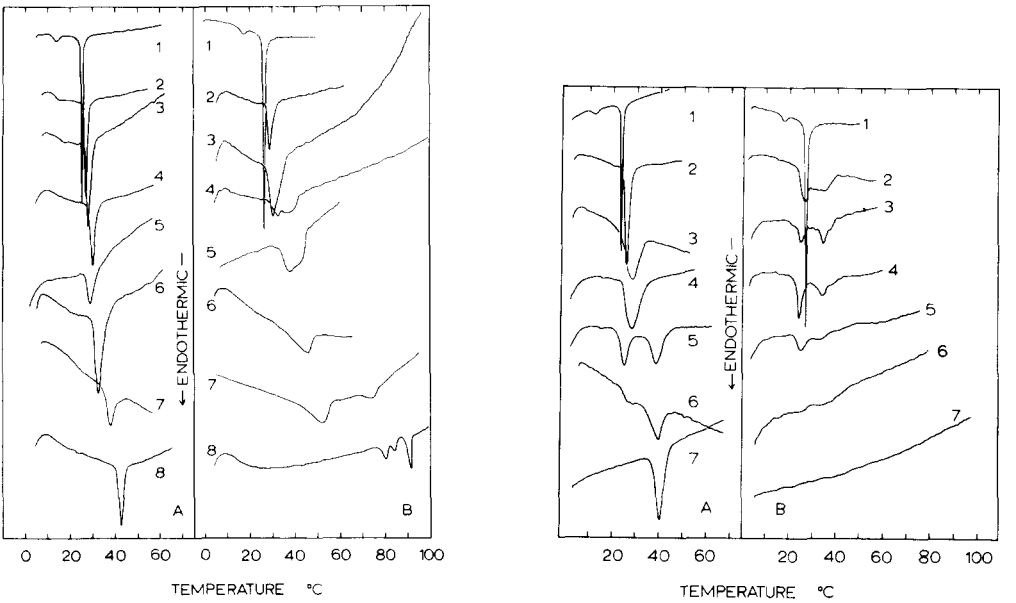

Fig. 4. The influence of excess Ca^{2+} on the thermotropic behaviour of mixtures of 14 : 0/14 : 0-glycerophosphocholine and 16 : 0/16 : 0-glycerophosphoglycerol at pH 6.0. Mixtures in the absence (A) and in the presence (B) of excess Ca^{2+}. (1), 0%; (2), 10%; (3), 20%; (4), 30%; (5), 40%; (6), 50%; (7), 75%; (8), 100% of phosphatidylglycerol.

Fig. 5. The influence of excess Ca^{2+} on the calorimetric behaviour of 14 : 0/14 : 0-glycerophosphocholine and 14 : 0/14 : 0-glycerophosphoserine mixtures at pH 6.0. Mixtures in the absence (A) and in the presence (B) of excess Ca^{2+}. (1), 0%; (2), 25%; (3), 35%; (4), 50%; (5), 66%; (6), 80%; (7), 100% of phosphatidylserine.

all show, upon analysis by freeze-fracture electron microscopy, liposomal structures (Fig. 6A).

The effects of Ca^{2+} on phosphatidylserine/phosphatidylcholine mixtures was totally different from that of mixtures containing phosphatidylglycerol. Excess Ca^{2+} addition to mixtures up to 35 mol% of phosphatidylserine, which show in the presence of only monovalent ions one homogeneous peak, resulted in an increase in the total $\triangle H$ value of the transition which was observed (Fig. 3a). In this way the mixtures resembled the phosphatidylglycerol-containing mixtures. However, there is one important difference. Upon addition of Ca^{2+} two separated transitions could be observed instead of one homogeneous upward shifted peak (Fig. 5B). Freeze-fracture electron microscopy of these samples showed the presence of only liposomal structures. The banded and smooth domains represent the phosphatidylcholine- and phosphatidylserine-rich areas in the liposomal bilayers, respectively (Fig. 6B). Thus, the effect of Ca^{2+} can be appreciated as the induction of an isothermic phase separation in the plane of the membrane.

In mixtures containing more than 50 mol% of phosphatidylserine, in the presence of only monovalent cations, two peaks were observed by differential scanning calorimetry (Fig. 5A), indicating separation in the gel phase into phosphatidylcholine- and phosphatidylserine-rich domains in the membrane. When excess Ca^{2+} was added to these mixtures, analysis of the calorimetric scans

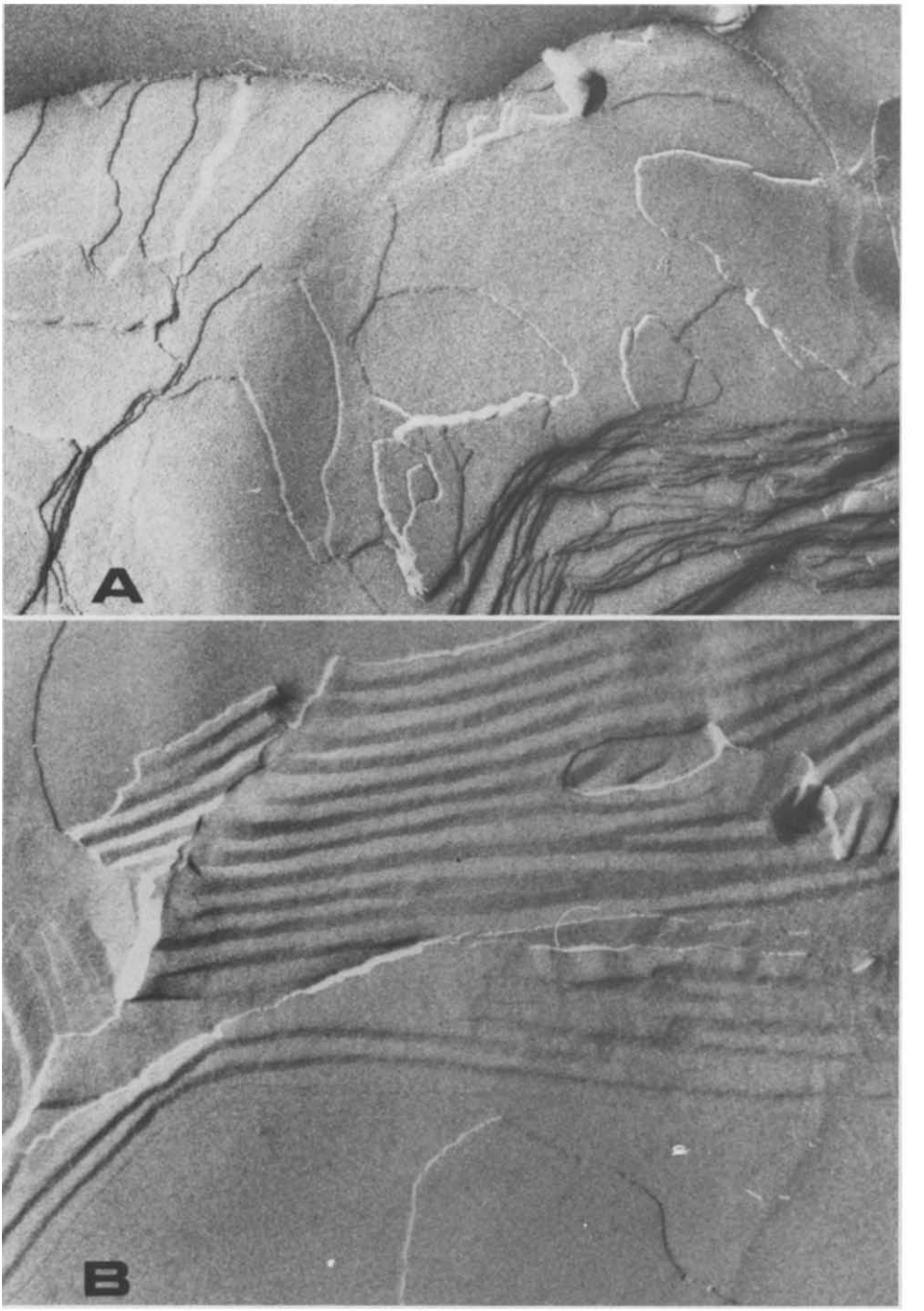

Fig. 6. Morphological effects of excess Ca^{2+} on mixtures of 14 : 0/14 : 0-glycerophosphocholine/14 : 0/14 : 0-glycerophosphoserine up to 35 mol% of phosphatidylserine. A. Glycerophosphoserine/glycerophosphocholine (1 : 2, molar ratio) in the presence of only monovalent cations. Magnification 80 000X. B. A similar mixture in the presence of excess Ca^{2+}. Magnification 80 000X. The samples were prepared as described in Materials and Methods. They were quenched from 4°C.

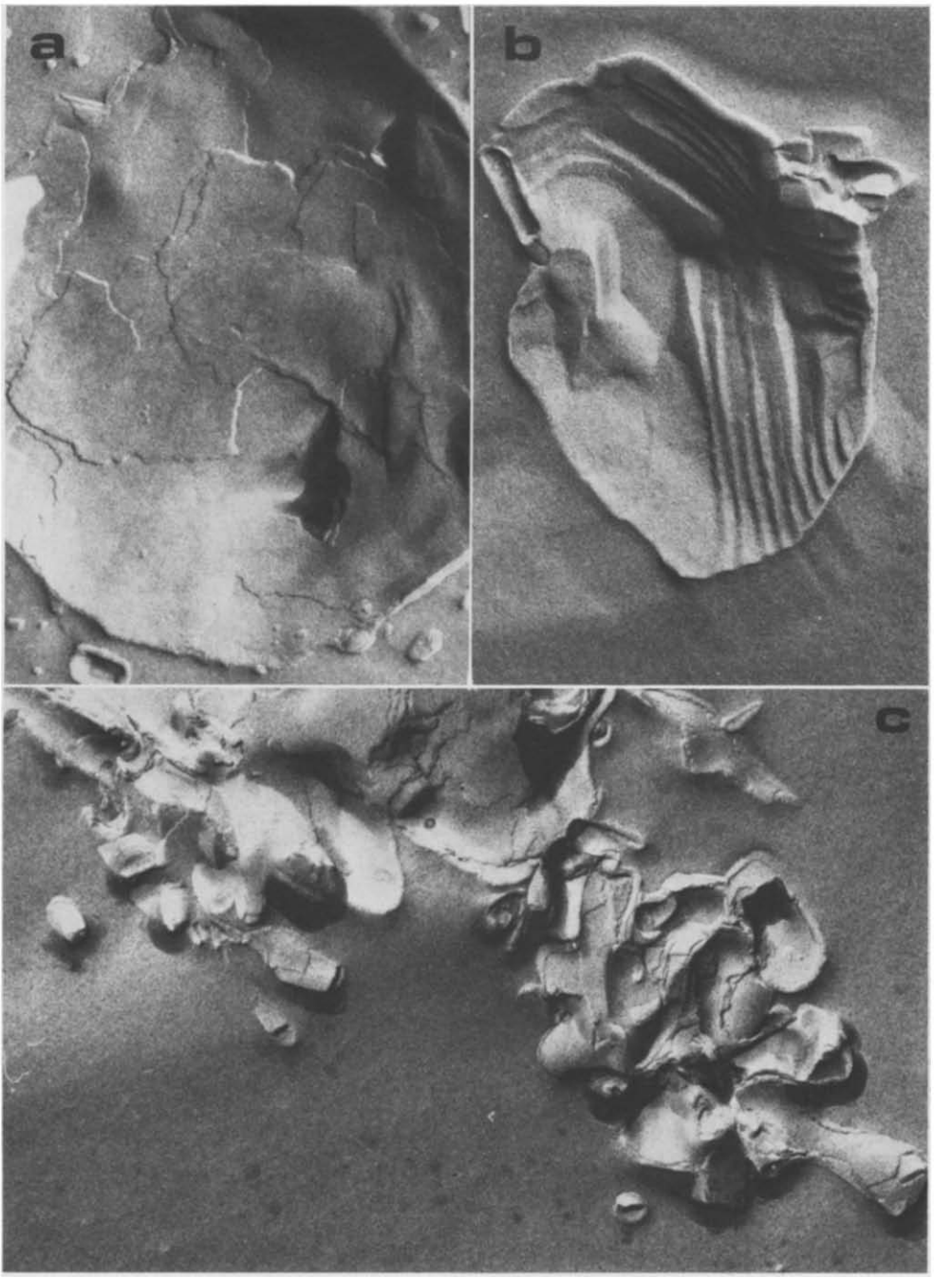

Fig. 7. Morphological effects of excess Ca^{2+} on an equimolar mixture of 14 : 0/14 : 0-glycerophosphoserine/14 : 0/14 : 0-glycerophosphocholine. The fracture plane consisted of a complex mixture of smooth (a) and banded liposomes (b) as well as cylindrical bilayers (c). The sample was prepared as described in Materials and Methods and quenched from 4°C. Magnification 80 000×.

revealed that an increasing part of the detectable transition intensities was lost with increasing phosphatidylserine concentration (Fig. 3a). When excess Ca^{2+} is added to pure phosphatidylserine bilayers a precipitate is formed which is highly packed and does not show a transition below 100°C (Fig. 5B (7)). Therefore, it is reasonable to conclude that excess Ca^{2+} in these 14 : 0/14 : 0-glycerophosphocholine and 14 : 0/14 : 0-glycerophosphoserine mixtures leads to a separation in different structures; liposomal phosphatidylcholine-rich bilayers and the highly packed Ca^{2+} phosphatidylserine-rich lamellae with a thermal transition which is not observable below 100°C. In agreement with this view, these samples show upon freeze-fracture electron microscopic analysis a complex mixture of bilayers of both the liposomal and the stacked lamellae/cylinder type. Part of the liposomal bilayers even exhibit banded regions indicative for 14 : 0/14 : 0-glycerophosphocholine-rich gel-phase domains (Fig. 7).

Phosphatidic acid. 14 : 0/14 : 0-Glycerophosphocholine and 14 : 0/14 : 0-phosphatidic acid undergo phase transitions at 23 and 50°C at pH 6.0, respectively, so it can be expected that these two components, in the absence of Ca^{2+}, will mix less ideally than 14 : 0/14 : 0-glycerophosphocholine and 14 : 0/14 : 0-glycerophosphoglycerol or 14 : 0/14 : 0-glycerophosphoserine. The calorimetric scans of the several mixtures displayed a broad peak, whereas in the extreme mol fraction regions a more narrow but tailing transition was observed (Fig. 8A). Excess Ca^{2+} addition to these mixtures (Fig. 8B) led, at all molar ratios, to a loss in total observable enthalpy-change intensity which was nearly linear to the mol fraction of phosphatidic acid present in the mixture (Fig. 3c). The Ca^{2+} · phosphatidic acid complex formed with the pure phosphatidic acid underwent no observable transitions between 0 and 100°C.

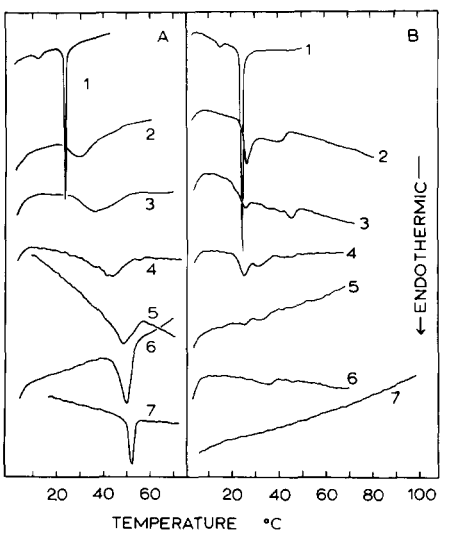

Fig. 8. The influence of excess Ca^{2+} on the thermotropic properties of mixtures of 14 : 0/14 : 0-glycerophosphocholine and 14 : 0/14 : 0-phosphatidic acid mixtures at pH 6.0. A, Mixtures in the absence and (B) in the presence of excess Ca^{2+}. (1), 0%; (2), 25%; (3), 35%; (4), 50%; (6), 80%; (7), 100% of phosphatidic acid.

Freeze-fracture analysis showed for all mixtures the coexistence of liposomal bilayers and closely packed lamellae and cylinders analogous to the structures shown for phosphatidylserine/phosphatidylcholine in Fig. 7.

As Galla and Sackmann [4] observed the largest effects of Ca^{2+} on mixed phosphatidylcholine/phosphatidic acid bilayers at pH 9.0 we also studied the effects of excess Ca^{2+} on 14 : 0/14 : 0-phosphatidic acid/14 : 0/14 : 0-glycerophosphocholine mixtures at pH 9.0 (data not shown). Our results indicated that the effects of Ca^{2+} are comparable to those obtained at pH 6.0. However, the scans obtained were not reproducible, perhaps as a consequence of chemical hydrolysis at this basic pH.

Discussion

Our results indicate that differential scanning calorimetry can be used to determine p*K* values of negatively charged phospholipids in the bilayer configuration. All p*K* values obtained from our profiles of the transition temperature as a function of the pH are apparent p*K* values as the pH values were measured in the bulk solution. Since the transition temperatures were obtained from heating curves the values obtained represent the apparent p*K* values for the gel state of the lipids. The value of 4.4 for the phosphatidylserine is in agreement with that of MacDonald et al. [20] who reported 4.6 at 25°C for 16 : 0/16 : 0-glycerophosphoserine. Titration curves for several phosphatidylserines have yielded p*K* values between 3.2 and 4.6 [20—25]. The physical state of the phosphatidylserine bilayers was shown to affect the apparent p*K* value [28]. Due to a decrease in surface charge density upon going from the gel to the liquid-crystalline state the apparent p*K* value will decrease. This may account for the variation in the different reported values.

Another factor which will interfere with the found p*K* value is the concentration of monovalent ions in the buffer. Sacré and Tocanne [26] have found in monolayer studies that, in agreement with the Gouy-Chapman theory of the diffuse electrical double layer [27], an increase in concentration of monovalent cations will decrease the apparent p*K* value. This effect was maximum at 100 mM NaCl. For 12 : 0/12 : 0-glycerophosphoglycerol a value of 3.1 was found [34], which is in excellent agreement with the values of 3.1 and 3.5 found in this study. This similarity in results indicates that concentration of lipid by centrifugation, necessary for the calorimetric analysis, has not affected the determination of the p*K* values.

From studies concerned with the miscibility of binary phosphatidylcholine systems [28—34] it is known that a minimal difference of 30°C between the transition temperatures of two phosphatidylcholines is necessary to create a gel phase miscibility gap. The difference in transition temperature is only 15°C in the binary system 14 : 0/14 : 0-glycerophosphocholine/14 : 0/14 : 0-glycerophosphoserine. We have previously noted a miscibility gap in the gel state in a system of 16 : 0/16 : 0-glycerophosphocholine and hydrogenated bovine brain phosphatidylserine which shows a transition temperature difference of 20°C (van Dijck, P.W.M., unpublished data).

Our results clearly show that one can differentiate between the effects of Ca^{2+} on the various types of negatively charged lipids. In mixed phosphatidyl-

glycerol/phosphatidylcholine bilayers with identical acyl chains Ca^{2+} induced only a shift of the co-crystallizing system. At high molar ratios of phosphatidylglycerol the spherical liposomal bilayers were destabilized and organized in highly packed (cylindrical) lamellae. Ca^{2+}-induced phase separation in the bilayer could be achieved by variation of the fatty acid composition of the mixed phosphatidylglycerol/phosphatidylcholine bilayers. Ca^{2+} effects in mixed phosphatidylserine/phosphatidylcholine or phosphatidic acid/phosphatidylcholine bilayers with identical acyl chains appeared to be dependent on the miscibility properties of the mixtures. In bilayers of co-crystallizing phosphatidylcholine/phosphatidylserine mixtures, up to 35 mol% of the latter component, Ca^{2+} induced a phase separation in the plane of the membrane. Mixtures containing higher molar ratios of phosphatidylserine already displayed partial gel-phase immiscibility; Ca^{2+} introduction in these mixtures yielded a destabilition of the phosphatidylserine-rich domains in the membrane and, consequently, a conversion into lamellae of cylindrical structures. Mixed phosphatidic acid/phosphatidylcholine bilayers showed gel-phase immiscibility at all ratios and Ca^{2+} introduction always yielded a mixture of bilayers of the spherical liposomal and of the stacked lamellae/cylindrical type. The extrusion of non-liposomal highly packed structures which occurred only in dispersions with phosphatidylserine and phosphatidic acid and not in those with phosphatidylglycerol was not a consequence of the difference in transition temperature. This can be deduced from the differential scanning calorimetry results with mixtures of 14 : 0/14 : 0-glycerophosphocholine and 16 : 0/16 : 0-glycerophosphoglycerol. It is apparent that the divalent ion-charged phospholipid interaction is strongly dependent on the nature of the charged phospholipid. The relative tendency of Ca^{2+} to break up the liposomal bilayer is phosphatidic acid > phosphatidylserine > phosphatidylglycerol.

The capability of Ca^{2+} to induce lateral phase separation in lipid bilayers was first demonstrated by electron spin resonance spectroscopy [1—4]. However, in view of the present study these ESR data may also be explained by structural segregation of the lipids as the ESR measurements were performed on samples with high mol fractions of charged lipid (phosphatidic acid or phosphatidylserine). Although the Ca^{2+} concentrations in the ESR studies (up to 1 Ca^{2+} per charged lipid molecule) were much less than we used, it must be realized that one Ca^{2+} per two charged lipid molecules is enough to convert the lipid into the highly packed salt [6,7].

Differential scanning calorimetry has been used previously to study Ca^{2+} effects on mixtures of defined phosphatidylcholines and phosphatidylserines obtained from natural sources (refs. 5 and 18 and van Dijck, P.W.M., unpublished). In equimolar mixtures of these lipids only an upward shift of the total (broad) transition was observed (ref. 5 and van Dijck, P.W.M., unpublished). Freeze-fracture electron microscopy of similar lipid mixtures revealed the coexistence of both smooth and banded domains in the liposomal structure, indicative of a lateral phase separation [18] in mixtures containing more phosphatidylserine Ca^{2+} induced the appearance of a somewhat changed phosphatidylcholine transition and a progressive decrease of the mixed lipid transition [5]. Although that was interpreted as a Ca^{2+}-induced segregation of individual lipids in separate domains within the vesicle membrane [5] our results suggest

that segregation into different structures had occurred.

The subtle differences in interaction between the various charged lipids and Ca^{2+} can only be explained in terms of differences in the polar headgroup as the acyl moieties were kept identical. Both the size and charge of this headgroup may be of importance. As both phosphatidylglycerol and phosphatidic acid bear only one negative charge at pH 6.0 and yet experience extreme differences in Ca^{2+} effects the size of the polar headgroup and its consequences for the geometry of the lipid molecule must be the predominant factor.

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