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EFFECTS OF CATIONS ON DIPALMITOYL PHOSPHATIDYLCHOLINE/CHOLESTEROL/WATER SYSTEMS

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SUMMARY

X-ray diffraction studies have been made on the effects of cations upon the dipalmitoyl phosphatidylcholine/water system, which originally consists of a lamellar phase with period of 64.5 Å and of excess water. Addition of 1 mM $CaCl_2$ destroys the lamellar structure and makes it swell into the excess water. The lamellar phase, however, reappears when the concentration of $CaCl_2$ increases: a partially disordered lamellar phase with the repeat distance of 150–200 Å comes out at the concentration of about 10 mM, the lamellar diffraction lines become sharp and the repeat distance decreases with increasing $CaCl_2$ concentration A small amount of uranyl acetate destroys the lamellar phase in pure water. $MgCl_2$ induces the lamellar phase of large repeat distance, whereas LiCl, NaCl, KCl, $SrCl_2$ and $BaCl_2$ exhibit practically no effect by themselves. Addition of cholesterol to the phosphatidylcholine bilayers tends to stabilize the lamellar phase.

The high-angle reflections indicate that molecular arrangements in phosphatidylcholine bilayers change at $CaCl_2$ concentrations around 0.5 M. The bilayers at high $CaCl_2$ concentration seem to consist of two phases of pure phosphatidylcholine and of equimolar mixture of phosphatidylcholine and cholesterol.

INTRODUCTION

The inter-membrane interactions which produce the lamellar phase in lipid/water systems seem to have close connection with inter-cell interactions and have been discussed by several workers. The van der Waals forces will be an origin of attractive forces and there must be an electrostatic repulsive force if the head groups have net electric charges, as discussed by Parsegian [1–3]. One experimental approach to this problem might be to investigate the effects of net charges on the membrane, and another to change the physical and chemical properties of water by introducing

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solutes such as electrolytes. Gulik-Krzywicki et al. [4] demonstrated that the repeat distance of the lamellar phase increases remarkably when lipids bearing net charges are added to egg lecithin. Palmer et al. [5] observed that the lamellar phase of lipids extracted from beef spinal cord disappears at the $CaCl_2$ concentration of 60 mM. Also several workers [4–7] reported that parameters which define lamellar structures are varied when electrolytes are added to water. More detailed studies, however, seem to be needed to clarify the roles of electrolytes in water. The present paper reports our results on the effects of electrolytes upon dipalmitoyl phosphatidylcholine/water and dipalmitoyl phosphatidylcholine/cholesterol/water systems, with special emphasis on $CaCl_2$.

MATERIALS AND METHODS

Material preparation

Synthetic β,γ-dipalmitoyl-D,L-(α)-phosphatidylcholine was purchased from Sigma Chemical Co., and cholesterol from Nakarai Chemicals Ltd. These chemicals were used without further purification. Phosphatidylcholine and cholesterol were dissolved in chloroform at a concentration of 2 mM and were stored at −20 °C. These solutions were mixed at the desired molar ratio of phosphatidylcholine and cholesterol. These mixtures were dried with rotary evaporator at about 40 °C, and then the remaining chloroform was further evaporated by keeping the specimen in an evacuated desiccator for at least 1 h. The dried lipids were dispersed in aqueous solutions of LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, $SrCl_2$, $BaCl_2$ or uranyl acetate. Ample solution was used, so that there always existed excess water phase together with the lamellar phase.

Experimental observations proved that the nature of the lamellar phase is determined by the concentration in the solution in cases of the above-mentioned inorganic salts, whereas it is determined by the total amount of dissolved salt in the case of dilute uranyl acetate solution. This fact suggests that almost all uranyl ions bind to the membranes in dilute solutions. Obtained X-ray patterns, however, indicated coexistence of two phases (assigned as I and II later) near the phase boundary, suggesting non-uniform distribution of bound uranyl ions. Therefore, a more adequate method was looked for in the case of uranyl acetate. Titration experiments (Yamaguchi, T., Furuya, K., Inoko, Y. and Mitsui, T., unpublished) indicated that phosphatidylcholine molecules can bind uranyl ions up to about equimolar ratio if enough uranyl acetate is present in the solution. Uranyl acetate is insoluble in chloroform but the uranyl-bound phosphatidylcholine molecules can be dissolved, possibly as a compound of phosphatidylcholine and uranyl acetate. Thus bilayers slightly decorated by uranyl ions were prepared by mixing chloroform solutions of pure phosphatidylcholine and uranyl-bound phosphatidylcholine. X-ray diffraction patterns did not indicate the coexistence of two phases in this case.

The dispersions were sealed in thin-walled glass capillaries having 1.0 mm internal diameter. Except for the case of uranyl acetate, the density of solution becomes comparable to, and then larger than, that of membrane with increasing concentration of the salt. Therefore, to produce a region which is rich in lamellar phase in the capillary, the dispersions were centrifuged at $30\,000 \times g$ for 30 min by holding the capillary in the hole of a modified centrifuge tube filled with 0.5 M sucrose solution.

X-ray diffraction experiments

The glass capillary was placed on the metallic sample holder maintained at about 5 °C. X-ray diffraction patterns were recorded on Fuji Medical KX X-ray film using Ni-filtered Cu Kα radiation (λ = 1 542 Å) from a Rigaku Denki rotaing anode microfocus generator. Exposures were made on Elliott toroidal and Franks point focusing cameras, operating in vacuum Sample-to-film distances were varied from 48 to 78 mm according to the desired scattering angle region. Exposure time was between 4 and 60 h. Diffraction spacings were calibrated with $Pb(NO_3)_2$ and sodium myristate powder patterns

RESULTS

Four states in $CaCl_2$ solutions

X-ray photographs of phosphatidylcholine/water systems were taken at various concentrations of $CaCl_2$ between 0 and 1 M. Results obtained revealed that there are basically four different states depending upon the $CaCl_2$ concentration, which will be called I, II, III and IV below Fig. 1 shows typical diffraction patterns for them. Characteristics of the four states are as follows.

State I appears in the $CaCl_2$ concentration range of 0–1 mM. It consists of the lamellar phase and excess water phase. Fig. 1a shows a diffraction pattern from the lamellar phase. The repeat distance of the phase is practically independent of $CaCl_2$ concentration and is about 64.5 Å. According to Tardieu et al [8], the lamellar phase contains one lipid bilayer per repeat unit. Patterson functions calculated with the lamellar diffraction lines supported this model.

State II appears in the concentration range of 1–10 mM. Here the lipid-rich phase swells into the excess water. Fig. 1b shows the diffraction pattern, which gives only diffuse peaks in low-angle region, whereas sharp high-angle ($\sim$ 4 Å) lines persist. The low-angle diffraction patterns are similar to that obtained from sonicated dispersions of egg lecithin in water reported by Wilkins et al. [9], suggesting that State II corresponds to dispersion of lipid bilayers. Autocorrelation functions and profiles of electron densities calculated with the diffuse peaks supported this model The high-angle lines suggest the crystalline packing of hydrocarbon chains in membranes.

State III appears in the concentration range of 10–200 mM. Here again the excess water phase coexists. Fig. 1c shows the diffraction pattern which gives two low-angle diffraction lines, though they are not very sharp, and additional diffuse peaks as well as the high-angle lines around 4 Å. The two low-angle lines can be indexed assuming lamellar structure with a large repeat distance. Therefore, a partially disordered lamellar phase seems to exist with thick water layers between lipid bilayers.

State IV appears at concentrations greater than 200 mM. Fig. 1d shows the diffraction pattern, which is quite similar to that in Fig. 1a, but the lines are sharper and background lower than those in Fig. 1a, suggesting that the lamellar structure has more perfect order Calculated Patterson functions were consistent with a bilayer structure of lipid membrane, similarly to State I.

Diffraction patterns from the same lipids in solutions of different $CaCl_2$ concentrations revealed that the transitions between the four states are reversible. In these experiments the lipids were washed by repeated replacements of solutions of new concentration. To reobtain samples free of Ca^{2+}, 10 mM EDTA was used.

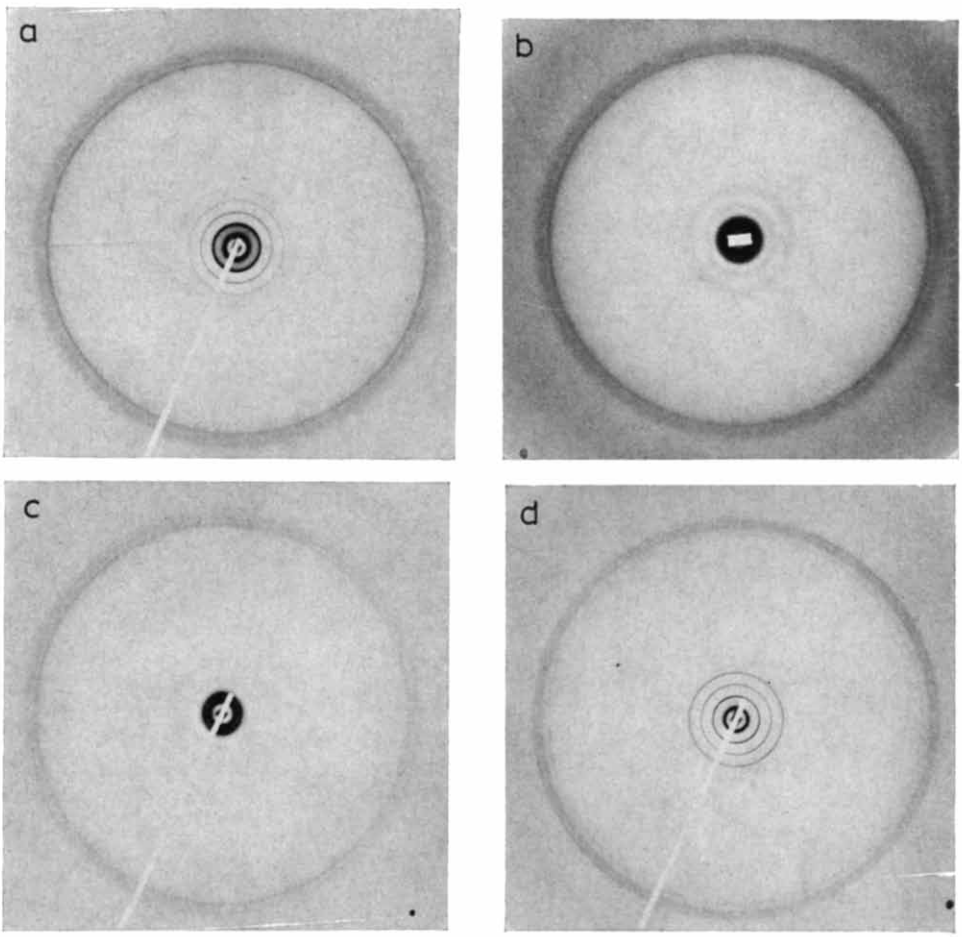

Fig. 1. X-ray diffraction photographs of phosphatidylcholine/water systems taken at 5 °C Large rings correspond to a spacing of about 4 Å A Franks point focusing camera was used for taking (a), (c) and (d), and an Elliott toroidal point focusing camera for (b). (a) Lamellar phase of State I in pure water, repeat distance $d = 64\ 5$ Å (b) State II, at 5 mM $CaCl_2$ (c) Lamellar phase in State III at 50 mM $CaCl_2$; $d = 120$ Å (d) Lamellar phase in State IV at 0 5 M $CaCl_2$; $d = 64\ 5$ Å. (For definition of these states, see the text)

Similarly, dispersions of mixtures of phosphatidylcholine and cholesterol were examined up to the molar ratio of cholesterol of 50 %, beyond which crystalline cholesterol precipitated. The effects of Ca^{2+} were similar those in the case of pure phosphatidylcholine on the whole. The $CaCl_2$ concentration at which the I-II transition takes place tends to increase with molar ratio of cholesterol/phosphatidylcholine: 1 mM for ratio 0/10, 1 mM for 2/8, 5 mM for 3/7, 50 mM for 4/6. When the ratio reaches 5/5, no transition was observed.

Fig. 2 gives repeat distances of the lamellar phases measured on X-ray photographs as functions of $CaCl_2$ concentration. The curves correspond to the molar ratio of cholesterol/phosphatidylcholine of 0/10, 2/8 and 5/5.

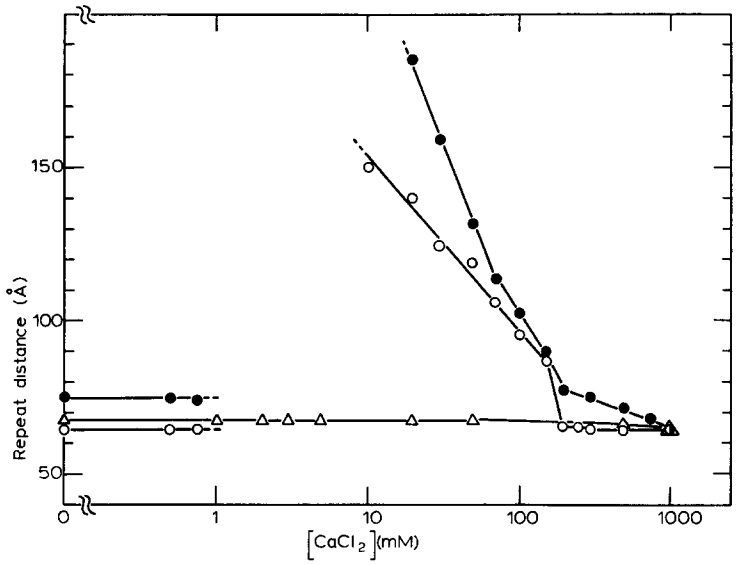

Fig. 2 Repeat distances of the lamellar phase as functions of $CaCl_2$ concentration for various molar ratios of cholesterol/phosphatidylcholine ○, 0/10; ●, 2/8; △, 5/5

Changes in high-angle reflections with $CaCl_2$ concentrations

The high-angle reflections corresponding to spacing of about 4 Å change their profile with $CaCl_2$ concentration and cholesterol content as shown in Fig 3 Observed results may be summarized as follows. (1) Addition of cholesterol makes the diffraction lines diffuse in pure water as seen in Curves b, c, d and e, as also reported by other authors [10]. (2) Three peaks appear as seen in Curves a′, b′, c′ and d′ at high $CaCl_2$ concentration in State IV. (3) Positions of these three peaks do not depend upon $CaCl_2$ concentration and cholesterol content. (4) The general feature of the Curves b′, c′ and d′ can be reproduced by a superposition of a′ and e′ at the ratio of $(c_p - c_c)/2c_c$, where c_p and c_c are molar concentrations of phosphatidylcholine and cholesterol, respectively. (5) For the equimolar mixture of cholesterol and phosphatidylcholine, a single diffuse peak was observed at all $CaCl_2$ concentrations examined, as seen in Curves e and e′.

Effects of cations other than Ca^{2+}

Effects of additions of uranyl acetate, LiCl, NaCl, KCl, $MgCl_2$, $SrCl_2$ and $BaCl_2$ on the phosphatidylcholine/water system were studied in the same manner as for $CaCl_2$

The lamellar phase persists when the molar ratio of uranyl acetate/phosphatidylcholine is less than 1/800, but adding more uranyl acetate causes diffuse X-ray patterns, as shown in Fig. 1b. The critical molar ratio of uranyl acetate/phosphatidylcholine to induce this State II is between 1/800 and 1/400. No further transition was observed up to the molar ratio of 1/1. Structural studies on the uranyl-decorated membranes by Yamaguchi et al. (Yamaguchi, T., Furuya, K., Inoko, Y. and Mitsui, T., unpublished) showed that the membrane has a bilayer structure. Well defined high-angle ($\approx$ 4 Å) reflections were observed at all the concentrations of uranyl

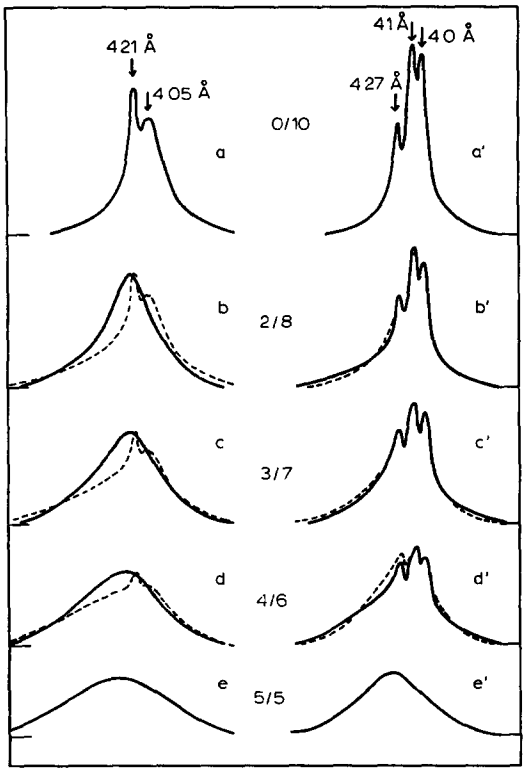

Fig 3. Profiles of high-angle reflections at 5 °C. a, b, c, d and e, in pure water, a', in 0 5 M $CaCl_2$ solution, b', c', d' and e', in 1 M $CaCl_2$ solutions. The fractions in the figure indicate the molar ratios of cholesterol/phosphatidylcholine Spacing of the peaks are also given The dotted lines show superpositions of a and e on the left-hand side, and of a' and e' on the right-hand side at the ratio of $(c_p - c_c)/2c_c$

acetate, indicating crystalline packing of hydrocarbon chains in membranes.

Pronounced changes in diffraction patterns were also observed for $MgCl_2$. In this case, however, State I is directly followed by State III, which is succeeded by State IV. High-angle reflections did not change appreciably, that is, the peaks such as shown on the right-hand side of Fig. 3 were not observed at all $MgCl_2$ concentrations up to 1 M. The observed repeat distance of the lamellar phase is given as a function of $MgCl_2$ concentration in Fig. 4.

As mentioned above, the lamellar phase disappears at a $CaCl_2$ concentration of 5 mM. All the above-mentioned inorganic salts induce the States III and IV from this disordered State II. Their effects are similar to that of Ca^{2+} in this respect. The concentrations where the transition III to IV occurs are 50 mM for $BaCl_2$, 100 mM for KCl and $SrCl_2$, 125 mM for NaCl and 200 mM for LiCl, $MgCl_2$ and $CaCl_2$. Also, it was observed that phosphatidylcholine bilayers bound by equimolar uranyl ions form a lamellar phase in solutions of 3 M NaCl.

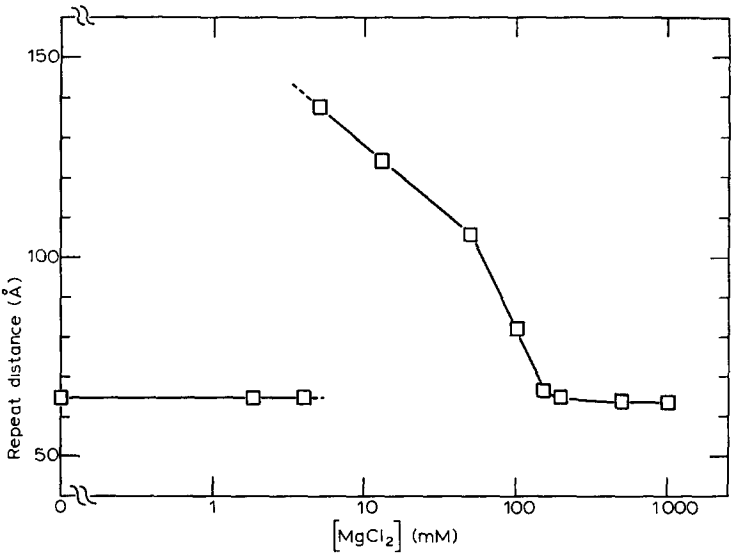

Fig 4 Repeat distance of the lamellar phase of pure phosphatidylcholine as a function of $MgCl_2$ concentration.

DISCUSSION

The results obtained may be summarized as follows.

(1) $CaCl_2$ induces the States II, III and IV, uranyl acetate State II, and $MgCl_2$ States III and IV.

(2) The salts LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, $SrCl_2$ and $BaCl_2$ produce the States III and IV when they are added to the State II induced by 5 mM $CaCl_2$.

(3) At high $CaCl_2$ concentrations, three peaks were observed in high angles when the molar ratio of cholesterol/phosphatidylcholine is less than 5/5, as shown in Fig. 3

(4) The Curves b', c' and d' in Fig 3 can be reproduced by superposition of a' and e' at the ratio of $(c_p - c_c)/2c_c$.

(5) Cholesterol in the bilayers tends to stabilize the lamellar phase against the effects of electrolytes.

Uranyl acetate induces only the State II in which the lipid-rich phase swells into the water phase. The effect of this salt is very strong: the critical molar ratio of uranyl acetate/phosphatidylcholine to produce the transition is between 1/800 and 1/400, that is, the transition occurs when about 2 per 10^3 phosphatidylcholine molecules bind the uranyl ions. Also, the affinity of uranyl ions to phosphatidylcholine is very large: titration measurement (Yamaguchi, T., Furuya, K., Inoko, Y. and Mitsui, T., unpublished) proved that the water phase is almost free of uranyl ions when the molar ratio of uranyl acetate/phosphatidylcholine is less than 1 in the whole system. Structural analysis (Yamaguchi, T., Furuya, K., Inoko, Y. and Mitsui, T., unpublished) of phosphatidylcholine bilayers decorated with equimolar uranyl ions has revealed that the uranyl ions sit near the head groups of phosphatidylcholine molecules. All these observations suggest that ions bound at membrane surfaces cause repulsive forces

between membranes and tend to induce State II. Studies on phosphatidylcholine monolayers indicated that dipalmitoyl phosphatidylcholine is able to bind more Ca^{2+} than egg lecithin or oleoyl phosphatidylcholine [11, 12] but its ability is less than that of phosphatidylethanolamine and far less than that of phosphatidylserine [13]. These imply that some ions are bound by bilayers and others are free in water in the case of $CaCl_2$ and the situation may be more or less similar for the above-mentioned inorganic salts. $CaCl_2$ and $MgCl_2$ induce States II and III from I, respectively. These transitions are associated with increase of inter-membrane distance. Therefore, the bound ions may be responsible for these transitions. In the case of $CaCl_2$, State III appears with further increase of concentration. Figs 2 and 4 indicate that in State III the inter-membrane distance decreases and therefore the attractive forces between membranes increase relatively to the repulsive forces with increasing $CaCl_2$ and $MgCl_2$ concentrations. It seems possible that these phenomena are related to the existence of free ions in water, since no such effect was observed in case of uranyl acetate. At present it is difficult to explain why the transition from I to II is so abrupt and why the disordered dispersion (State II) is more stable than the lamellar structures. Results (2) suggest that all inorganic salts examined tend to increase attractive force between membranes relatively to the repulsion from some concentrations.

The $L\beta'$ phase [8] in which the hydrocarbon chains are tilted to the membrane plane gives the high-angle peak profile similar to Curve a in Fig. 3 (cf. Fig. 11d of ref. 8). In the $L\beta$ phase the hydrocarbon chains are perpendicular to the membrane [8, 14]. If the chain can be regarded as a cylinder, close packing of them results in a two-dimensional hexagonal lattice in the membrane [8]. Now let us consider a general two-dimensional unit cell of which the edges are a and b and the angle is γ. The hexagonal lattice is characterized by a single parameter a, since $b = a$, $\gamma = 120°$, and thus there will appear a single sharp peak around the 4 Å region (cf. Fig. 11b of ref. 8). In actual phosphatidylcholine molecules, two hydrocarbon chains are paired by the head group. This implies that the hexagonal unit cell with $a \approx 4 \cdot (2/\sqrt{3})$ Å contains half a molecular unit of the head group. Such a situation can happen only when the arrangement of the head groups is disordered, keeping the hexagonal symmetry in an averaged structure. It is well known that crystals that have high symmetry due to partial disorder tend to transform into other crystal forms of lower symmetry with changes of external parameters such as temperature and pressure. By analogy, it seems possible that a phase having symmetry lower than hexagonal can appear in the membrane at some $CaCl_2$ concentration. It is easy to prove that a non-hexagonal lattice can bring about a group of three diffraction peaks around the 4 Å region corresponding to the three lattice parameters if deviations from the hexagonal lattice are appropriate, as actually observed and shown on the right-hand side of Fig. 3. Therefore, the results (3) imply that the structures of bilayers at high $CaCl_2$ concentrations are different from the usual model of $L\beta$.

The results (4) seem to be related to the phase separation. Generally, in studies of the phase diagram of materials A and B, the uniform mixed-crystal phase is recognized by a shift of diffraction peaks of A with increasing B concentration. On the other hand, coexistence of A and B phases (or of A phase and an equimolar mixture A-B phase, etc.) is characterized by a superposition of diffraction peaks originated from the two phases. The contribution of each phase to the diffraction pattern is proportional to the amount of material in it. If a pure phosphatidylcholine phase and

an equimolar phosphatidylcholine/cholesterol phase coexist in membranes, the ratio of materials in the two phases is approximately $(c_p - c_c)/2c_c$. Therefore, the results (4) suggest coexistence of the pure phosphatidylcholine phase which gives the diffraction pattern a′ in Fig. 3 and of the equimolar phosphatidylcholine/cholesterol phase of which the diffraction pattern is e′. No agreement between the solid and dotted lines was obtained on the left-hand side of Fig. 3, suggesting that a well defined phase separation does not occur in pure water at 5 °C. These results, however, do not deny the possibility of the local phase separation, which can not be detected with X-rays, and in this sense they do not necessarily contradict the reports on phase separations by several authors [15–18].

Concerning the results (5), the transition from I to II occurs at almost the same $CaCl_2$ concentration ($\approx$ 1 mM) for cholesterol/phosphatidylcholine mixtures of 0/10 and 2/8, as seen in Fig. 2. Beyond this molar ratio, the transition occurs at 5 mM for the ratio 3/7, 50 mM for 4/6, and no transition was observed for 5/5 These results suggest that affinity of Ca^{2+} to the membranes tends to decreases with increasing cholesterol content and also that cholesterol prevents the binding of Ca^{2+} tc phosphatidylcholine, as was discussed by Shah and Schulman [19] in case of egg lecithin monolayers.

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