Intercalation and Bilayer Formation of Phospholipids in γ-Type Layered Transition Metal Phosphates

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Intercalation of natural phospholipids, phosphatidylethanolamine (PEA) and phosphatidylcholine (PC), into inorganic layered compounds was examined in ethanol and chloroform solutions. γ-Type titanium(IV) phosphate and zirconium(IV) phosphate were selected as host layered compounds. They are strong solid acids and also known as inorganic ion exchangers. The intercalation of PEA was propagated in chloroform solution in the presence of *n*-alkylamine forming a bilayers of PEA–alkylamine mixture. The direct reaction of PEA with γ-phosphates was not successful. On the other hand PC reacted directly with the layered compounds either in the chloroform or in the ethanol solution of PC and successfully formed bilayers.

Biological cells are the minimum unit of our life system. Most of the cells are surrounded by a phospholipid bilayer and retain each fixed shape due to the van der Waals forces between long alkyl chains of the lipids. The bilayers of phospholipids also act as a selective membrane for various bio-functional compounds such as various proteins, inorganic ions, etc. The intercalation of *n*-alkylamine and formation of the bilayers were examined in detail in the preceding paper.[1] The bilayer formation of phospholipids in layered inorganic compounds such as clay minerals was planned in this study. Such hybrid compounds of phospholipid bilayer with inorganic materials may be utilized as various media for biological functional compounds.

γ-Type titanium(IV) phosphate γ-TiP[2,3,4,5] (typical composition: $Ti(HPO_4)_2 \cdot 2H_2O$) and γ-type zirconium-(IV) phosphate γ-ZrP[6,7] (typical composition: $Zr(HPO_4)_2 \cdot 2H_2O$) both prepared hydrothermally were selected as host materials. They are strong solid acids and the acidity seems convenient to distinguish whether the intercalation is based on the acid–base neutralization of the end group of lipids or on the van der Waals forces between alkyl chains. In addition, these γ-type phosphates have rather large interlayer spacings (about 1.2 nm) which may be favorable for the intercalation of large molecules. The scanning electron micrographs of the layered compounds used were shown in the previous paper.[1] Structural data and geometric size of these compounds are listed in Table 1.

Table 1. Structural Data for γ-Phosphates[7,9]

Phosphate	System	*d*-Spacing / nm	Free area around active site/nm²
γ-TiP	Monoclinic	1.16	0.166
γ-ZrP	Monoclinic	1.225	0.178

Experimental

Reagents. Three phospholipids were used for the intercalation: L-α-Cephalin (L-α-phosphatidylethanolamine: PEA) from egg yolk (Sigma Chemical Co.), Lecithin from egg yolk (phosphatidylcholine: PC-E, Wako Pure Chemical Ind.), and another lecithin from soybeans (phosphatidylcholine: PC-B, Wako Pure Chemical Ind.). Typical chemical formula are shown in Fig. 1.[8] (Exact chemical formula are not shown for the reagents used contained some components with different alkyl chain lengths.) Chloroform (reagent grade) was used to dissolve each phospholipid, and ethanol (reagent grade) to dissolve PC's.

Procedures. Three different procedures were adopted for the intercalation of phospholipids.

Procedure [1]: Prior to the intercalation of phospholipids, the interlayer spacing was expanded by intercalating *n*-alkylamines,[1,9] the length being smaller than those of phospholipids. The *n*-alkylamine intercalation compounds were then contacted with phospholipid solutions.

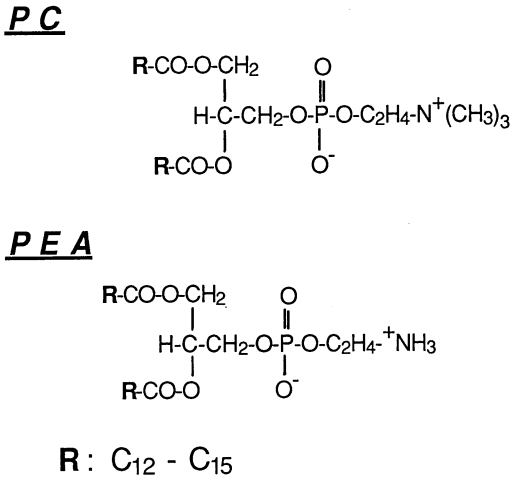

Fig. 1. Typical chemical formula of PC and PEA.[14]

Procedure [2]: Layered phosphates were contacted with phospholipid solutions in the presence of a small amount of *n*-alkylamines (cointercalation with *n*-alkylamines).

Procedure [3]: Layered phosphates were directly contacted with phospholipid solutions.

In procedures [2] and [3], intercalation was carried out as follows. Powdered γ-TiP (0.04 g: ca. 1.5×10^{-4} mol) or γ-ZrP (0.047 g) was contacted with 5 mL chloroform or ethanol solution containing about 60 mg phospholipids (ca. 8×10^{-5} mol) at 37 °C for one week unless otherwise stated. Didecylamine (DDA) and some *n*-alkylamines were used to assist the intercalation of phospholipids. Ratios of PEA to *n*-alkylamines were 17.4—0.44 for the intercalation reaction of PEA. Experimental conditions in procedure [3] were similar to procedure [2].

Characterization. The intercalation compounds were filtered with a qualitative filter paper and washed with pure solvent which was used for the intercalation. The amount of intercalated phospholipid was calculated from the residual phospholipid which was analyzed gravimetrically after vaporization of the solvent. The amount of amine cointercalated was analyzed by acid–base titration of the filtrate in organic media. Low-angle X-ray diffraction analysis for the products which developed on a glass plate was carried out with Fe-*Kα* radiation (λ=0.10007 nm) down to $2\theta=0.9°$, which corresponds to the interlayer spacing of 12 nm.

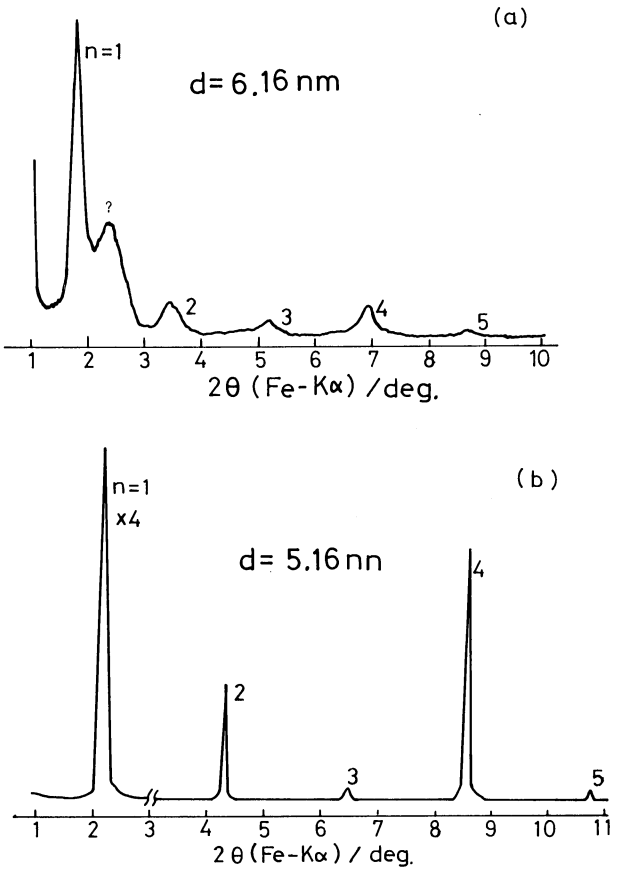

Fig. 2. X-Ray diffraction (Fe-*Kα*) patterns for bilayer of (a) Phosphatidylethanolamine (PEA) and (b) Phosphatidylcholine from egg yolk (PC-E) developed on a glass plate. Numerals in figure indicate *nλ* sequence (*n*=1, 2, ···).

Results and Discussion

The bilayers of PEA and PC-E were easily formed by developing chloroform or ethanol solutions of these phospholipids on a glass plate. The X-ray diffraction patterns of PEA and PC-E are shown in Fig. 2. PC-B is not shown because it was a viscous liquid at room temperature. Characteristic view of these X-ray patterns is that the intensity sequence of the basal plane reflection (*nλ*:*n*=1,2,3,···) is not continuous and the peak for *n*=4 was higher than that for *n*=3 because of some extinction rule, and differed definitely from those of intercalation compounds of phospholipids in which a regular decrease in intensity sequence was observed as shown below. Although the peak at $2\theta=2.3°$ for PEA was not identified, it suggests that some extent of molecular orientation is maintained along basal plane.[10] The *d*-spacings of these species are shown in the figures. Since geometric lengths of these phospholipids are estimated to be about 3 nm, the width of these *d*-spacings may imply the formation of "bilayers" of phospholipids similar to those in biological cells in which alkyl chains inclined to some extent with respect to the basal plane.[11]

Procedure [1]: The reaction of PEA with *n*-alkylamine intercalation compounds seemed to be unsuccessful. Only small increases in *d*-spacing were observed after the reaction and indicated the horizontal intercalation of PEA. Some of the results are shown in Table 2. The finding indicated that the chemical bonding of γ-phosphates with *n*-alkylamines was too tight to allow the insertion of PEA because these alkylamines are fairly strong bases and host layered phosphates are strong solid acids, while the end amino group in the phospholipids is a weak base.

Procedure [2]: Intercalation of phospholipids, especially PEA, was successful in the presence of *n*-alkylamines. Single phase intercalation compounds were obtained by this procedure, and an appreciable increase in *d*-spacing was observed.

(1) PEA: Didecylamine (DDA) was mainly used for the cointercalation because it has dual alkyl chains which have a similar length to hydrophobic tails (chains) of PEA. Figure 3 shows X-ray diffraction patterns of intercalation compounds at various concentrations of coexisted DDA under a constant γ-TiP:PEA ratio 1.0:0.6. Mixed X-ray diffraction patterns of PEA intercalation compound (closed circle) and DDA

Table 2. *d*-Spacings (in nm) of DDA Intercalation Compounds Before and After Contact with PEA

Phosphates	Before	After
γ-TiP	3.36	3.46
γ-ZrP	3.40	3.68

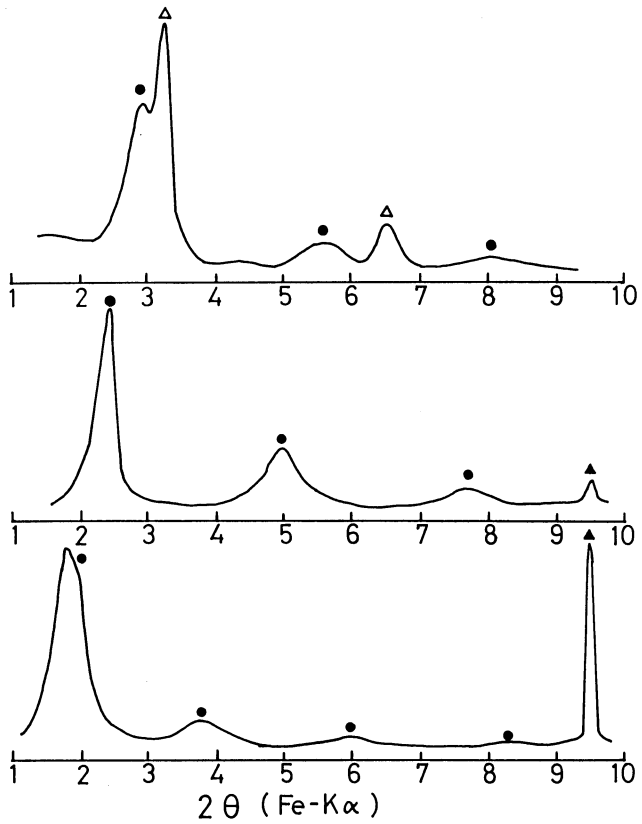

Fig. 3. X-Ray diffraction pattern of PEA–DDA co-intercalation compounds of γ-TiP. (●): Peaks for PEA–DDA cointercalation compound, (△): Peaks for DDA intercalation compound, and (▲): residual γ-TiP. γ-TiP:PEA:DDA: (a) 1.0:0.60:0.67, (b) 1.0:0.60:0.33, (c) 1.0:0.60:0.07.

Table 3. Time Dependence of *d*-Spacing (in nm) for PEA (+DDA) Intercalation at γ-Ti(Zr)P:PEA:DDA=1.0:0.6:0.33

	1 h	6 h	1.5 h	6 d
γ-TiP (PEA)	4.46 (s)	3.96 (s)	4.19 (s)	4.27 (s)
γ-TiP (DDA)	3.36 (s)	3.36 (s)	3.36 (w)	—
PEA	6.16 (s)	—	—	—
γ-ZrP (PEA)	4.62 (s)	4.62 (s)	4.62 (s)	4.62 (s)
γ-ZrP (DDA)	3.36 (w)	—	—	—
PEA	—	—	—	—

(PEA): Peak height for PEA intercalation compound, (DDA): Peak height for DDA intercalation compound, PEA: Peak height for PEA bilayer. s: Strong, w: Weak, —: Not detected.

Table 4. *d*-Spacings for PEA–Alkylamine Cointercalation Compound and for Corresponding *n*-Alkylamine Intercalation Compound (nm)

γ-TiP

C_n	4	6	10	14	18
Amine+PEA	4.16	4.04	4.04	4.44	5.04, 4.27
Amine only*	1.63	2.20	2.52	3.03	3.57

γ-ZrP

C_n	4	6	10	14	18
Amine+PEA	4.23	3.93	4.23	4.42	4.93
Amine only*	1.57	2.16	2.52	3.06	3.55

γ-Ti(Zr)P:PEA:Amine=1.0:0.6:0.33, γ-Ti(Zr)P:Amine=1.0:0.33 (*: monolayer compounds), C_n: Carbon number of *n*-alkyl chain.

intercalation compound (open triangle) were observed when the concentrations of PEA and DDA were comparable (Top). If PEA/DDA ratio exceeded 2, a single phase X-ray diffraction pattern was observed though a small peak for residual γ-TiP (2θ=9.7°: closed triangle) remained (Middle). The mixed X-ray diffraction patterns for the PEA intercalation compound and host γ-TiP were obtained at a lower concentration ratio (Bottom). The *d*-spacing for the PEA intercalation compound increased with increasing PEA–DDA ratio. The spacing almost corresponded to that of PEA-bilayer shown in Fig. 2a at the PEA–DDA ratio of about 10 (Bottom). Details of this result will be shown below.

The rate of intercalation was examined in the solution at the γ-phosphate:PEA:DDA ratio of 1.0:0.6:0.33. The *d*-spacings for the products are shown in Table 3. In the case of γ-TiP, the X-ray diffraction pattern showed the coexistence of PEA intercalation compound, DDA intercalation compound, and residual PEA at the initial stage of the reaction. A single phase X-ray diffraction pattern was obtained after 6 d. On the other hand, the intercalation into γ-ZrP was rather easy and a single phase X-ray diffraction pattern was obtained even at the reaction time of 6 h.

The effect of the length of *n*-alkyl chain was examined at γ-phosphate:PEA:amine=1.0:0.6:0.33. The *d*-spacings for the products are shown in Table 4. Single phase X-ray diffraction patterns were observed in any combination except for the octadecylamine cointercalation compound of γ-TiP. *d*-Spacings for cointercalation compounds were larger than those for the corresponding pure amine intercalation compounds. The values once decreased with increasing carbon number C_n of alkyl chain and showed a minimum at C_n=6 and increased again. The largest value was observed for the octadecylamine cointercalation compounds.

Table 5 shows concentration dependence of *d*-spacing and the appearance of single phase X-ray diffraction pattern at the constant γ-phosphate:PEA ratio of 1:0.6. The reproducibility of *d*-spacings was rather poor though the error was not so large. Single phase patterns were observed between PEA/DDA 1.16—1.74 and 0.87—3.5 for γ-TiP and γ-ZrP, respectively. Below the limits, mixed patterns were observed indicating the presence of DDA intercalation compound. Above the limit the residual X-ray diffraction based on the host γ-phos-

Table 5. Concentration Dependence of *d*-Spacings of PEA–DDA Cointercalation Compounds

γ-TiP							
R	0.44	0.87	1.16	1.74	3.5	8.7	17.4
d	4.10	4.10	4.29	4.42	4.77	5.55	—
*d***	4.44	3.90	4.38	4.62	5.04	6.06	(6.42)
— Mixed phase —|– Single phase ——|— Residual γ-TiP							

γ-ZrP							
R	0.44	0.87	1.16	1.74	3.5	8.7	17.4
d	4.14	4.04	4.27	4.53	5.00	5.41	5.51
– Mixed phase–|— Single phase ————|– Res. γ-ZrP							

γ-Ti(Zr)P:PEA=1.0:0.6, *R*: PEA/DDA, *d*: *d*-Spacing (nm) —: Could not be identified, **: Prepared under intermittent ultrasonic stirring.

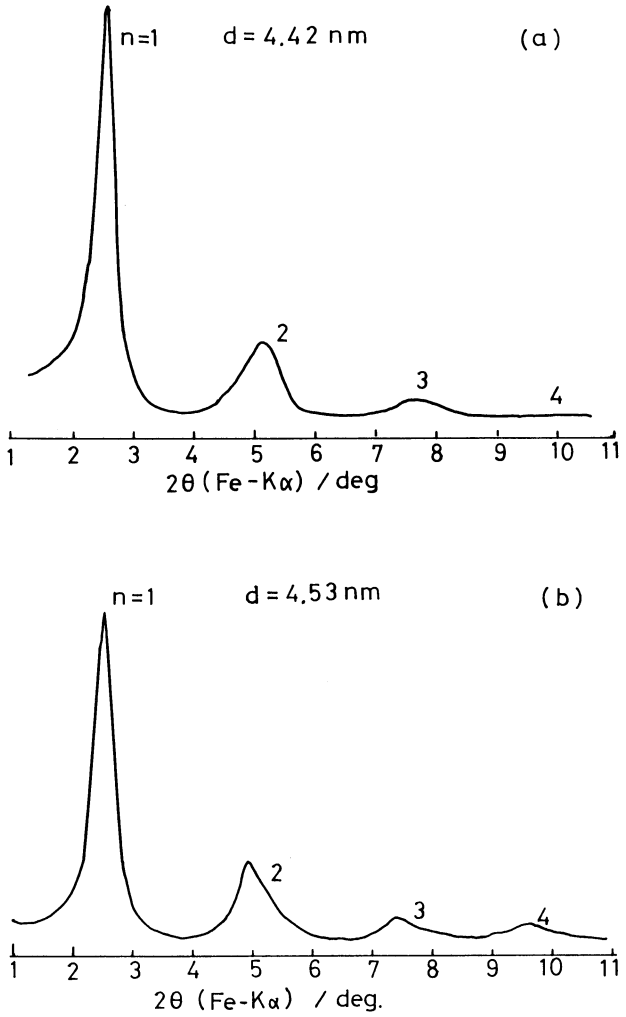

Fig. 4. Typical X-ray diffraction patterns for PEA–DDA cointercalation compound of (a) γ-TiP and (b) γ-ZrP. Numerals in figure indicate $n\lambda$ sequence (n=1, 2, ···). γ-Ti(Zr)P:PEA:DDA=1.0:0.60:0.33.

Table 6. Analytical Data of PEA and DDA (relative mole ratio)

	Phosphate	PEA	DDA
TiP	1.00 (1.00)	0.15 (0.60)	0.11 (0.33)
	1.00 (1.00)	0.20 (0.60)	0.04 (0.08)
ZrP	1.00 (1.00)	0.20 (0.60)	0.23 (0.33)
	1.00 (1.00)	0.21 (0.60)	0.05 (0.08)

( ): Added amounts.

phates became significant. A typical example for the X-ray diffraction pattern is shown in Fig. 4 for the PEA–DDA cointercalation compound of γ-Ti(Zr)P at the γ-Ti(Zr)P:PEA:DDA ratio 1.0:0.6:0.33. Effect of the ultrasonic wave (US) stirring is shown in Table 5.

The *d*-spacing of PEA intercalation compounds was found to increase with increasing PEA–DDA ratio. The maximum *d*-spacings observed were 6.1 nm and 5.5 nm for γ-TiP (US stirred sample) and γ-Zr, respectively. The *d*-spacings which were obtained by extrapolating PEA–DDA ratio to infinity may be those for pure PEA intercalation compounds because the *d*-spacing of the intercalation compound of PEA and DDA mixture must be smaller than that of the pure intercalation compound of PEA. (The length of DDA was estimated to be 1.2 nm, and the resulting *d*-spacing of DDA intercalate was about 3.4 nm as shown in Fig. 3 and Table 2.) However, the intercalation compounds which had *d*-spacings larger than the parent PEA bilayer (6.16 nm) was not obtained. The US dependence on *d*-spacing (Table 5) suggests that US was effective to arrange alkyl chains regularly.

The analytical data for the intercalation compound prepared are shown in Table 6. It was surprising that an appreciable amount of DDA remained in the supernatant solution. If the intercalation occurred through acid base reaction only, DDA was expected to be fully intercalated, for DDA is far more basic than PEA while γ-phosphates are strong acids.[12] Thus the significant amount of residual DDA indicated that van der Waals forces between PEA's, or PEA and DDA, took precedence over the acid base neutralization. Furthermore, it was found from this procedure that alkyl amines were effective in initiating the intercalation of PEA and then cointercalation compounds which were preference for PEA were obtained.

The possibility on the formation of bilayers was discussed as follows. Geometric cross section of an alkyl chain has been estimated to be 0.24 nm^2 for the tightly packed bilayers of *n*-alkylamine observed in α-phosphates or clay minerals.[13,14] The free area of the host γ-ZrP sheet has been estimated to be 2×0.178 nm^2 (2× means upside and reverse side).[9] On the other hand, *n*-alkylamines could form bilayers in γ-ZrP and γ-TiP, and were found to occupy about 0.35 (γ-ZrP)—0.58 (γ-TiP) nm^2 per alkyl chain[9] which was referred to

0.24 nm^2 for the closely packed bilayers. The apparent large cross section might be ascribable to the conformation[15] or the tilting[16] of alkyl chains. This fact means that loosely packed alkyl chains can form bilayers in the layered compounds.

The cross sections of PEA have been estimated to be 0.6 and 0.7 nm^2 for hydrophobic dual alkyl chains and a hydrophilic head, respectively,[17] and that of DDA about 2×0.3 nm^2. In PEA–DDA cointercalation compounds the sum of the cross sections of intercalated species corresponded to about 50% of the free areas of γ-phosphates assuming that they form bilayers within the crystal lattice. Compared this calculation with the above discussion on loosely packed bilayers, it can be concluded that intercalated PEA and DDA could form bilayers of PEA and DDA mixture if tilting and conformation of alkyl chain were taken into account.

(b) PC's: The intercalation of PC's was examined under similar conditions to those in PEA, e.g. γ-TiP:PC-E:DDA=1:0.6:0.17 in chloroform solution. The observed X-ray diffraction pattern showed the formation of single phase species and *d*-spacing of the intercalation compound was 5.39 nm as compared with 5.16 nm for the parent PC-E bilayers. In the case of γ-ZrP, *d*-spacing was 5.41 nm at γ-ZrP:PC-E:DDA=1:0.6:0.08 in chloroform solution. The intercalation compounds showed *d*-spacing larger than parent PC's. However, the products prepared must be cointercalation compounds of PC and DDA if the thickness of a sheet of γ-ZrP, 0.9 nm for dehydrated state, is taken into consideration.

The intercalation compounds for PC-B showed a similar behavior to PC-E(+DDA) intercalation compound.

Procedure [3]: This procedure was unsuccessful in the intercalation of PEA. On the other hand, the intercalation of PC's was found to occur even in the absence of alkylamines. Figure 5 shows the X-ray diffraction pattern of PC-E intercalation compound of γ-TiP (a) prepared in ethanol and γ-ZrP (b) prepared in chloroform. *d*-Spacings of these intercalation compounds are listed in Table 7. The *d*-spacings lay between 5.50—5.75 nm as compared with 5.16 nm for the bilayers of parent PC-E except for γ-TiP in chloroform solution.

PC-B was also intercalated directly and gave similar intercalation compounds to those in PC-E. Since X-ray diffraction for PC-B bilayers was difficult to observe as stated above, the intercalation compounds were not compared with the parent PC-B bilayers.

The thickness of the host γ-phosphate lamellae has been estimated to be 0.9 nm if lattice water molecules were removed.[3] Thus the increase in *d*-spacing from 5.16 nm to 5.75 nm must indicate the success of the formation of phospholipid bilayers. Chemical analysis indicated that the γ-phosphate:PC-E ratio was about 1:0.3 for both intercalation compounds. It means about

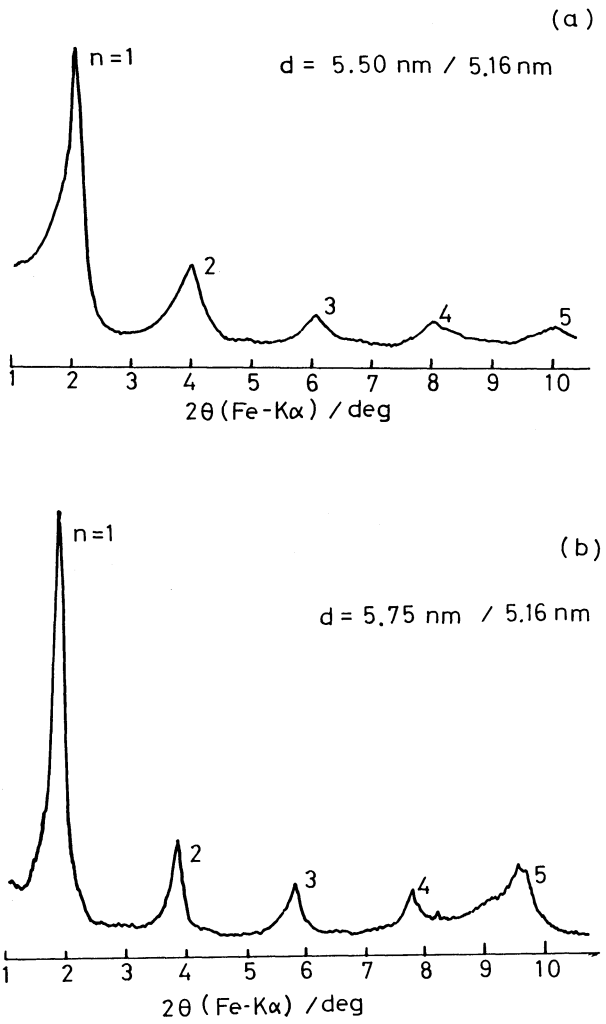

Fig. 5. X-Ray diffraction pattern of PC–E intercalation compounds prepared by direct reaction. (a) γ-TiP in ethanol, (b) γ-ZrP in chloroform. Numerals in figure indicate *d*-spacing.

Table 7. *d*-Spacings (nm) of PC-E Intercalation Compound Prepare by Direct Reaction

	In ethanol	In chloroform
γ-TiP	4.72	5.50
γ-ZrP	5.75	5.69

58% free area of γ-phosphate was occupied assuming that PC's formed bilayers within the lattice. Such packing density seems enough to form bilayers of PC's discussed above. The success of the formation of natural phospholipids' bilayers in the layered inorganic compounds has not been reported as far as we know.

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