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Hydrolysis of Diphosphate Ion to Orthophosphate Ion and Formation of Hydroxyapatite in the Aqueous Phase

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The formation of mixed precipitates of calcium diphosphate (Ca_2PPi) and calcium orthophosphate (hydroxyapatite, HAP, mainly) from alkaline solutions of $CaCl_2$, K_2HPO_4, $K_4P_2O_7$ and NH_4OH was studied. The chemical composition of the precipitate determined by direct chemical analysis of the precipitate was different from that obtained by calculation from the filtrate composition after incubation (72 h, 37°C). It was concluded that, although diphosphate (PPi) is usually hydrolyzed only in acidic solution, PPi was hydrolyzed to orthophosphate (Pi) on the surface of the precipitate in alkaline solution during the incubation, followed by further precipitate formation (CaPi) with excess Ca^{2+} in the solution. The extent of hydrolysis of PPi depended on the amounts of Ca^{2+} added and precipitate formed. It was confirmed that PPi is hydrolyzed in an alkaline solution of $K_4P_2O_7$ depending on the amount of synthesized HAP or Ca_2PPi, but is not hydrolyzed in the absence of any solid. HAP seems to catalyze the hydrolysis reaction of PPi more effectively than Ca_2PPi. When the amount of Ca^{2+} was less than that of total phosphate, another counter cation in the precipitate was K^+. X-Ray powder diffractometry of the precipitates revealed diffraction peaks of HAP, but those of Ca_2PPi were not found. The precipitates formed in solutions containing enough Ca^{2+} were crystalline HAP, although the initial solution did not contain Pi but just PPi. The significance of this hydrolysis is discussed in relation to the formation of biological hard tissues.

Keywords——hydroxyapatite; calcium phosphate; calcium diphosphate; hydrolysis of diphosphate; hard tissue formation

Body fluids of animals contain various inhibitors of the formation of calcium phosphate, which precipitates and aggregates as hard tissues in the animal body. Soft tissues are protected from becoming calcified by these inhibitors. Diphosphate (*i.e.* pyrophosphate, PPi) is known to be one of the inhibitors, and is found in normal plasma, urine, saliva, synovial fluids, and so on. On the other hand, PPi does not prevent calcification of bone in living animals. This might be due to local destruction of the diphosphate to phosphate (*i.e.* orthophosphate, Pi) by the enzyme alkaline phosphatase, which possesses pyrophosphatase activity. Thus the action of the alkaline phosphatase would allow the tissues to calcify to hard tissues, of which the main constituent is basic calcium phosphate, *i.e.* hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$).[1]

Francis[2] studied the effect of PPi on the formation of HAP from aqueous solutions of $CaCl_2$ and NaH_2PO_4 *in vitro*. According to electron diffractometry and electron micrography, the precipitates of calcium phosphate from these solutions were found to be gel-like agglomerates and amorphous, or pronouncedly rectangular crystallites of HAP even when sufficient PPi was added. The characteristic feature of the precipitate seemed to depend on the reaction conditions. It was concluded that PPi inhibits HAP crystal growth as long as enough PPi is present in the system, and that activity of PPi as an inhibitor decreases at higher temperature and/or lower pH because of hydrolytic instability of PPi. However, the chemical composition of these precipitates has not been analyzed precisely yet.

The formation and inhibition of formation of HAP are fundamentally important to man because calcium phosphate in the form of HAP is the main constituent of the skeletal system and of ectopic calcification. In the present paper, the crystallinity and chemical components of the precipitates formed from alkaline solutions of Ca^{2+}, Pi and PPi are discussed as a function of the initial constituents of the mother solution. It is shown that PPi in the solution is hydrolyzed on the surface of the precipitate by a mechanism distinct from that of enzyme- or acid-catalyzed hydrolysis.

Experimental

Materials——All chemicals used were of reagent grade from Nakarai Chemical Ltd. or Wako Pure Chemical Industries Ltd. These were used without further purification. Water was doubly distilled. HAP was prepared as described elsewhere.[3] Crystalline calcium diphosphate (Ca_2PPi) was prepared by heating $CaHPO_4$ at 450°C for 6 h according to the method of McIntosh *et al.*[4]

Methods——Aqueous NH_4OH (4 M, 35 ml) was added to an aqueous solution (20 ml) of K_2HPO_4 mixed with $K_4P_2O_7$ at various mixing ratios; the total concentration was, however, kept constant at 0.6 M. Aqueous $CaCl_2$ (15 ml) of various concentrations was added to the above alkaline solution to produce an immediate precipitate of calcium salt. Solutions were always brought to the appropriate temperature (37°C) before mixing. The total volume of the mother solution was 70 ml, and therefore the initial concentrations of NH_4OH and total phosphate ([Pi]+[PPi]) became 2 M and 171.4 mM, respectively, in the mother solution. Calcium hydroxide may or may not precipitate, but ultimately the stable precipitate phase is calcium phosphate and diphosphate in this experimental system.

After incubation of the sample solution at 37°C for 72 h with vigorous stirring, the solution was filtered through a 0.22 μm Millipore filter. Both the precipitate and the filtrate were obtained and characterized chemically and by X-ray powder diffraction. The pH of the filtrate remained between pH 9.5 and 11.0 throughout the reaction, depending on the initial concentration of orthophosphate and/or diphosphate. The precipitate was washed with water and acetone, and then dried at 110°C *in vacuo*.

Immediately prior to chemical analysis, the precipitate was dissolved quickly in an aqueous solution of ethylenediaminetetraacetic acid (EDTA). Calcium content was determined by determining the excess EDTA in the solution by chelatometry with a standard solution of $MgCl_2$ at pH 10 with BT indicator (1-(1-hydroxy-2-naphthylazo)-6-nitro-2-naphthol-4-sulfonic acid). Potassium content was determined by flame photometry (Hitachi Model 205) with "Lithium Stock Standard for Dilution" (Daiichi Pure Chemicals Co., Ltd).

Diphosphate content was determined according to the method of Kato *et al.*[5] Zinc diphosphate ($Zn_2P_2O_7$), precipitated from the sample solution by addition of excess zinc acetate at pH 3.8—3.9, was dissolved as $[Zn(NH_3)_4]^{2+}$ in NH_4Cl-NH_4OH buffer solution at pH 10. This solution, containing zinc ion ($[Zn^{2+}]=2[PPi]$), was analyzed by chelatometry with EDTA standard solution and BT indicator at pH 10. It was confirmed that neither calcium nor orthophosphate ion, which are contained in the precipitate, interfere with the titration, because these ions were excluded through the above procedure.

Total content of phosphorus, which is present as Pi and PPi, was determined by colorimetry according to the method of Gee *et al.*[6,7] after hydrolysis of PPi to Pi by boiling the sample in 0.5 M HCl for 30 min.[8] The phosphate ammonium molybdate complex formed was reduced with stannous chloride. The absorbance of the resulting color after 15 min was determined at 720 nm in a Shimadzu model UV-180 spectrophotometer.

The concentration of orthophosphate ion was obtained from the difference between the content of total phosphorus ([Pi]+2[PPi]) and that of diphosphate (2[PPi]).

X-Ray powder diffraction patterns (Cu $K\alpha$ radiation at 35 kV and 10 mA with a nickel filter) were obtained with a Norelco Geiger counter diffractometer (Philips Electronics and Pharmaceutical Industry) at room temperature and at scanning speed of 1 degree/min.

Henceforth, the molar fraction of PPi, x, in this paper is defined as the molar ratio of PPi content to the sum of the contents of PPi and Pi, [PPi]/([PPi]+[Pi]), without the numerical coefficient of 2 for PPi, either in solution or in the precipitate. Subscript "i" or "f" means initial condition before incubation or final condition after incubation. Superscript "s" or "l" indicates solid phase (precipitate) or liquid phase (filtrate).

Results

Chemical Analysis of the Filtrate

The filtrate after the precipitation reaction was analyzed chemically. The concentration of Pi and PPi in the filtrate after incubation both decreased with initial concentration of calcium, because calcium consumes them to form insoluble precipitates, CaPi and/or Ca_2PPi. The concentration of Pi and PPi became practically zero when the initial concentration of Ca^{2+} was high enough. The Pi and PPi concentrations in the present paper are converted to molar fractions as defined in the previous section. The molar fraction of PPi in the filtrate (x_f^l) also decreases with the initial concentration of Ca^{2+}, as shown in Fig. 1-a as a function of initial molar fraction of PPi (x_i^1). It is interesting that x_f^1 becomes less than one even though x_i^1 is equal to one, depending on the initial Ca^{2+} concentration. This apparent discrepancy will be discussed later.

The apparent molar fraction of PPi in the precipitate, $(x_f^s)'$, can be calculated from the composition of the filtrate by means of Eq. (1),

$$(x_i^s)' = \frac{\Delta[\mathrm{PPi}]}{\Delta[\mathrm{PPi}] + \Delta[\mathrm{Pi}]}, \qquad (1)$$

where $\Delta[\ ]$ is the difference of concentration of PPi or Pi between that in the initial solution and that in the filtrate. The results are shown as a function of x_i^1 in Fig. 1-b, where the dotted line shows the relation $(x_f^s)'=x_i^1$. Consumption of PPi and Pi from the mother solution increases with Ca^{2+} added, and therefore, the numerical values of $\Delta[\mathrm{PPi}]$ and $\Delta[\mathrm{Pi}]$ approach the initial concentrations of PPi and Pi, respectively. Consequently, $(x_f^s)'$ approaches x_i^1 (*i.e.* to the dotted line) with increasing concentration of Ca^{2+} added, as shown in Fig. 1-b.

Some of the values of $(x_f^s)'$ curiously became larger than one (● and ■, for example). The experimental reason is that the Pi concentration in the filtrate became larger than that in the initial solution (and therefore, $\Delta[\mathrm{Pi}]<0$) during the incubation. This problem will be discussed later.

Chemical Analysis of the Precipitate

To obtain the composition accurately, the precipitate should be analyzed directly. The molar fraction of PPi in the precipitate obtained by direct analysis, x_f^s, is shown in Fig. 2 as a function of x_i^1. If the composition in the precipitate is the same as that in the initial solution, the experimental points should be on the dotted line ($x_i^1=x_i^s$). However, the experimental curves form an S-shaped curve. In the case of low concentration of added Ca^{2+}, x_f^s is smaller than x_i^1 when x_i^1 is small, and x_f^s is larger than x_i^1 when x_i^1 is large (■ and ●, for example). In the case of high concentration of Ca^{2+}, x_f^s is smaller than x_i^1 everywhere (▲, for example).

The molar fraction of PPi in the precipitate, x_f^s, decreases with initial concentration of Ca^{2+} added, that is, with the amount of precipitate fromed. The experimental values of x_f^s do not become unity, although $x_i^1=1$ except in the case of low concentration of Ca^{2+} (■ and ●).

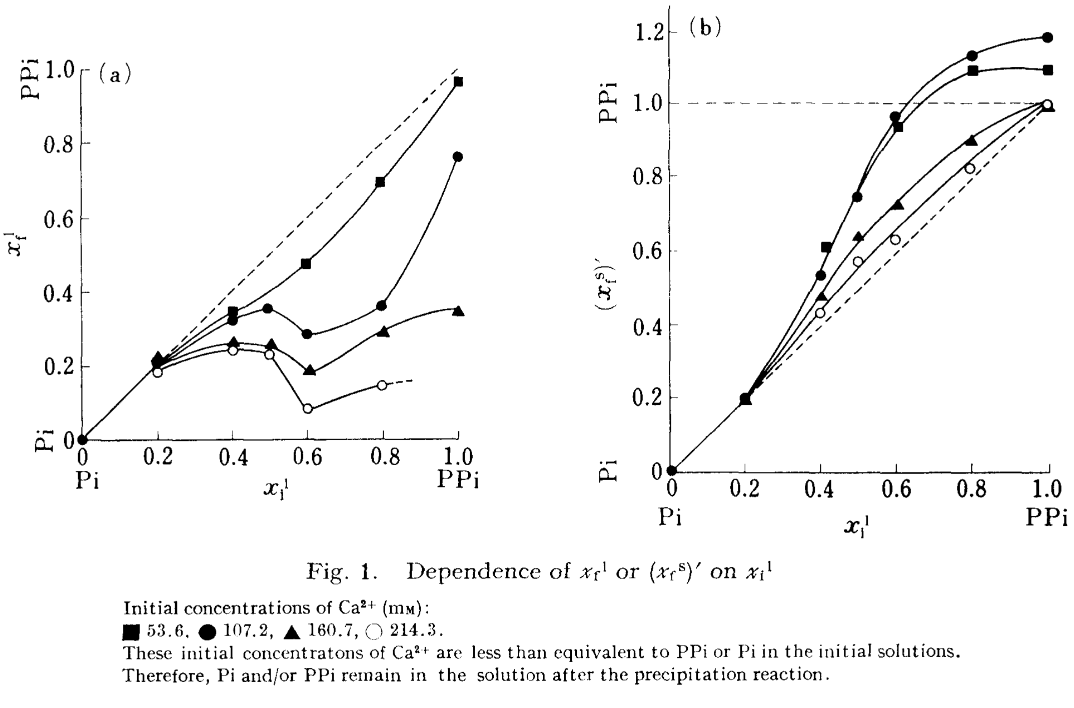

Fig. 1. Dependence of x_f^1 or $(x_f^s)'$ on x_i^1

Initial concentrations of Ca^{2+} (mM):
■ 53.6, ● 107.2, ▲ 160.7, ○ 214.3.
These initial concentratons of Ca^{2+} are less than equivalent to PPi or Pi in the initial solutions. Therefore, Pi and/or PPi remain in the solution after the precipitation reaction.

In the case of the highest concentration of Ca^{2+} (▲), all the experimental points are on the abscissa ($x_f{}^s=0$), and the concentrations of Pi and PPi remaining in the filtrate were almost zero. In this case, PPi disappears from both the liquid and solid phase. The findings that $x_f{}^s$ (Fig. 2) and $x_f{}^l$ (Fig. 1-a) decrease with the amount of Ca^{2+} added even though $x_l{}^l=1$, and that some values of $(x_f{}^s)'$ (Fig. 1-b) are larger than unity suggest that PPi may be hydrolyzed to Pi during the incubation for 72 h. The extent of the hydrolysis seems to increase with the amount of calcium added in the initial solution or the amount of the precipitates formed.

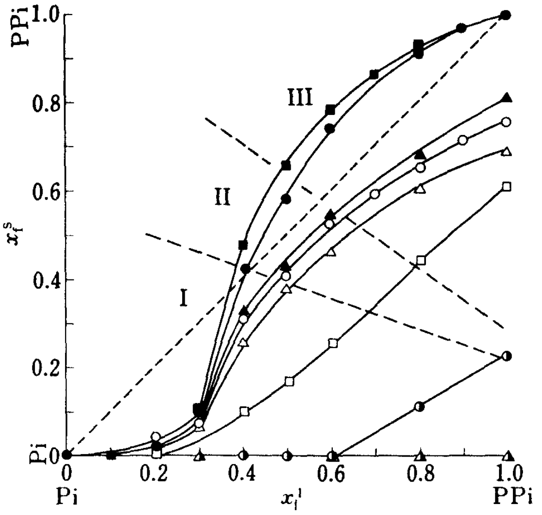

Fig. 2. Releationship between $x_l{}^l$ and $x_f{}^s$

Initial concentrations of Ca^{2+} (mM):
■ 53.6, ● 107.2, ▲ 160.7, ○ 214.3, △ 267.8,
□ 321.3, ◑ 428.4, ▲ 535.5.
Full lines show the experimental curves. Broken lines show the boundaries of regions I, II, and III.

Cations in the Precipitate

The equivalent ratio, E_1, of Ca^{2+} to Pi and PPi in the precipitate was calculated by means of Eq. (2),

$$E_1=\frac{2[Ca^{2+}]_f{}^s}{3[Pi]_f{}^s+4[PPi]_f{}^s}, \tag{2}$$

where $[Ca^{2+}]_f{}^s$, $[Pi]_f{}^s$, and $[PPi]_f{}^s$ are the concentrations of Ca^{2+}, Pi and PPi in the precipitate, and the numerical factors of 2, 3 and 4 are the valences of the ions. When Ca^{2+} in the precipitate is less than equivalent to Pi and PPi in the precipitate, E_1 becomes less than one. When the amount of Pi and PPi is less than equivalent to Ca^{2+}, E_1 becomes larger than one. In the case of pure HAP or Ca_2PPi, E_1 becomes 1.11 or 1.00 from the definition, Eq. (2). The relationship between E_1 and $x_f{}^s$ is shown in Fig. 3-a, where E_1 decreases with $x_f{}^s$ from a value larger than one to one smaller than one, depending on the concentration of Ca^{2+} added.

Although there are small variations due to the experimental inaccuracy, E_1 is larger than 1 at low $x_f{}^s$. This means that the precipitates have OH^- and/or Cl^- as other anions. Chloride anion may not be contained in the precipitates because insoluble chloride salts, such as chlorapatite, are not formed under these experimental conditions. Therefore, the precipitates contain OH^-, which forms HAP. Furthermore, the fact E_1 becomes less than 1 with increasing $x_f{}^s$ means that the amount of available Ca^{2+} as a counter ion for Pi and PPi is not sufficient at high $x_f{}^s$. The precipitate, therefore, may be assumed to be a mixture of HAP and Ca_iM_jPPi, where the subscripts i and j are numerical parameters and M is monovalent cations to be determined. M might be H^+, K^+ and/or $NH_4{}^+$ in this system.

Dotted lines in Fig. 3-a show the calculated values of E_1 given by Eq. (2), assuming that the precipitate consists of HAP and Ca_iM_jPPi. The calculated values of E_1 become smaller with increasing $x_f{}^s$, and with decrease of calcium content in Ca_iM_jPPi. The experimental values of E_1 also decrease with $x_f{}^s$ and with decrease of initial concentration of Ca^{2+} added.

To determine the chemical species of M, flame photometry for K^+ was carried out. Potassium content in the solid, $[K^+]_f{}^s$, increased with $x_f{}^s$, as shown in Fig. 3-b, in contrast with decrease of the calcium content with increasing $x_f{}^s$, as shown in Fig. 3-a.

The equivalent ratio, E_2, of Ca^{2+} and K^+ to Pi and PPi was calculated by means of Eq. (3) in the same manner as with Eq. (2),

$$E_2=\frac{2[Ca^{2+}]_f{}^s+[K^+]_f{}^s}{3[Pi]_f{}^s+4[PPi]_f{}^s}. \tag{3}$$

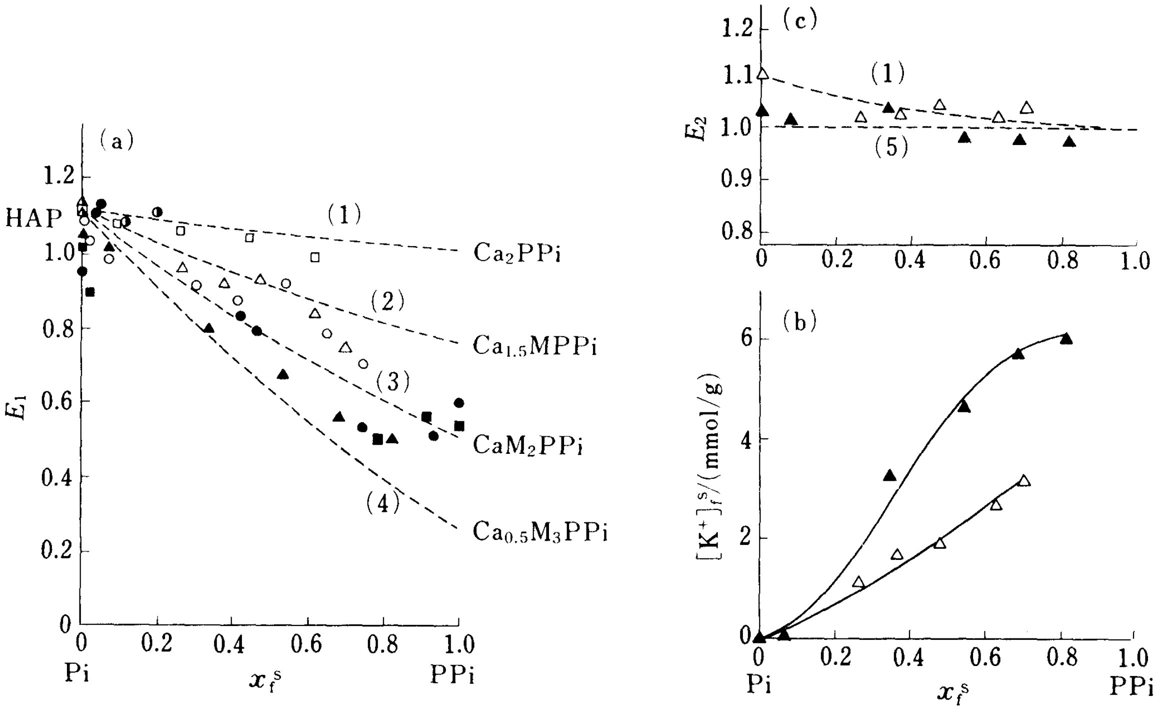

Fig. 3. Dependence of E_1, $[K^+]_f^s$ or E_2 on x_f^s

All symbols in the figure are the same as in Fig. 2. Dotted lines are theoretical curves for the mixture of HAP and Ca_2PPi (1), HAP and $Ca_{1.5}MPPi$ (2), HAP and CaM_2PPi (3), HAP and $Ca_{0.5}M_3PPi$ (4), and $Ca_3(PO_4)_2$ and Ca_2PPi (5).

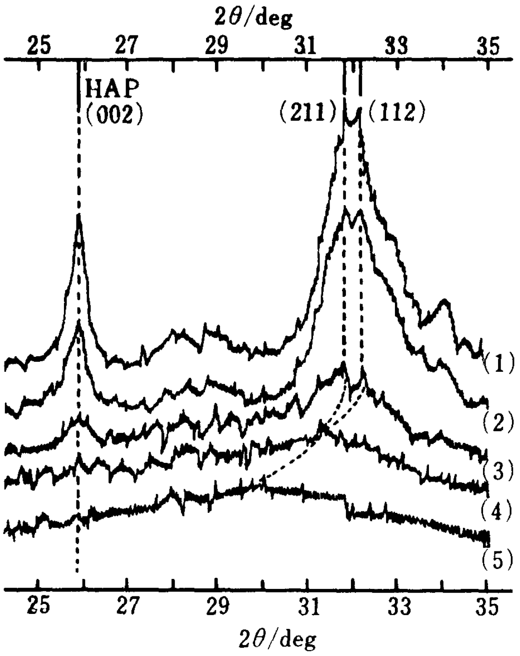

Fig. 4. X-Ray Powder Diffraction Patterns for the Precipitate

Initial concentration of Ca^{2+} was 214.3 mM for all samples in the figure, and the total molar concentration of phosphate, $[Pi]_i^1+[PPi]_i^1$, was 171.4 mM.
Anionic compositions (x_i^1 and x_f^s):
(1) 0, 0, (2) 0.30, 0.070, (3) 0.60, 0.537, (4) 0.90, 0.710, (5) 1.00, 0.750.

The results are shown in Fig. 3-c as the relationship between E_2 and x_f^s. The dotted line (1) in Fig. 3-c is the same as the dotted line (1) in Fig. 3-a. Dotted line (5) shows $E_2=1.0$ from $x_f^s=0$ through $x_f^s=1.0$. All the experimental points are in the neighborhood of the two curves. This means that almost all of the calcium deficit relative to PPi and Pi is compensated for by K^+, and that other ions such as H^+, NH_4^+ and Cl^- are not included in the precipitate.

X-Ray Diffraction Patterns

X-Ray powder diffraction patterns for the precipitates are shown in Fig. 4, where curve (1) for $x_f^s=0$ ($x_i^1=0$) is typical of HAP. Specific diffraction peaks at $2\theta=25.9$, 31.8 and 32.2° are due to the diffractions by the crystal planes (002), (211) and (112) of HAP, respectively. These diffraction peaks decrease and eventually disappear with increasing x_f^s and/or x_i^1. The precipitates with high contents of PPi (curve(5), for example) do not show distinct diffraction peaks for calcium and/or potassium diphosphate, nor HAP, but appear to be almost amorphous.

Therefore, it may be concluded that calcium orthophosphate in the precipitate is crystalline HAP even though it contains small amounts of diphosphates ion (*i.e.* at small values of x_f^s), and that crystalline diphosphate salt does not appear in the precipitate even when the diphosphate ion is the dominant anionic species of the precipitate (*i.e.* at large values of x_f^s).

According to the relative intensity of specific diffraction peaks of HAP, the precipitates can be classified into three regions, I, II and III, as shown in Fig. 2 by broken lines. In region I, the precipitates show sharp and strong diffraction peaks, such as shown by curves (1) and (2) in Fig. 4. The precipitates which belong to region II have weak and obscure diffraction peaks, such as shown by curve (3) in Fig. 4. In region III, the precipitates do not show any specific diffraction peaks, such as shown by curves (4) and (5) in Fig. 4, where diffraction peaks due to the (211) and (112) planes of HAP have already moved to lower diffraction angles.

Hydrolysis of Diphosphate with Formation of Precipitate

The relationship between x_f^s and the amount of added Ca^{2+}, read out from Fig. 2 at $x_i^1=1$, is shown in Fig. 5-a. The abscissa on the top is graduated for initial concentration of Ca^{2+}, $[Ca^{2+}]_i^1$, and that on the bottom is graduated for the equivalent ratio of added Ca^{2+} to added PPi, $([Ca^{2+}]/2[PPi])_i^1$.

The results in Fig. 5-a show that Ca^{2+} enhances the Pi content in the precipitate because Pi, produced by the hydrolysis of PPi, forms a precipitate with Ca^{2+} in the solution. One mole of PPi is decomposed to two moles of Pi, and the amount of Ca^{2+} required to form the precipitate with the latter is thus more than that with the former. Consequently, Ca^{2+} added in amounts more than equivalent to initial PPi enhances the precipitate formation.

Therefore, the Pi content in the precipitate $(1-x_f^s)$ increases, and PPi content (x_f^s) simultaneously decreases with $[Ca^{2+}]_i^1$ or with the amount of the precipitate formed, although x_f^s is one when $[Ca^{2+}]_i^1$ is small, as shown in Fig. 5-a. Other data for $x_i^1<1$, not shown here, exhibited the same tendency.

The vertical distance from the horizontal dotted line $(x_f^s=1)$ to an experimental point in Fig. 5-a, such as that indicated by the dotted arrow, shows the apparent extent of hydrolysis of PPi during incubation for 72 h. The time course of x_f^s, due to hydrolysis and precipitation, is shown in Fig. 5-b; x_f^s decreases steeply at the early stage of the incubation and subsequently levels off. The data of x_f^s given in Fig. 2 are those at the peroid of leveling off.

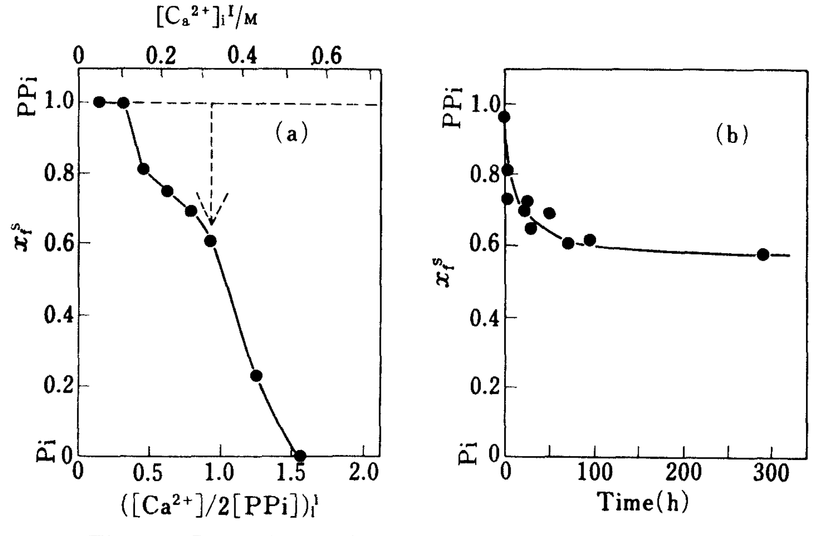

Fig. 5. Dependence of x_f^s on the Initial Concentration of Calcium or Incubation Time

Initial concentration of diphosphate was 171.4 mM and x_i^1 was 1.0 for both (a) and (b). The incubation time for (a) was 72 h. The initial concentration of calcium ion for (b) was 321.3 mM.

Hydrolysis of the Diphosphate at the Surface of Synthesized HAP or Calcium Diphosphate

The results in Figs. 1, 2 and 5 show that the amount of diphosphate ion hydrolyzed increased with increasing amount of the precipitate, which is assumed to be a mixture of HAP and calcium diphosphate (Ca$_2$PPi) with some K$^+$ to compensate for deficiency of Ca^{2+}. Therefore, the hydrolysis of PPi by synthesized HAP or Ca$_2$PPi was investigated.

The concentrations of PPi ([PPi])$_f{}^1$), Pi([Pi]$_f{}^1$), and total phosphorus ([Pi]$_f{}^1$+2[PPi]$_f{}^1$) in the filtrate after incubation for 72 h at 37°C are showni n Fig. 6. as a function of amount of HAP or Ca$_2$PPi (Ca$_2$P$_2$O$_7$) added. Correction of suspension concentrations for dissolution was not done, because they are practically insoluble in water, especially in alkaline solution. The concentration of PPi is shown as twice the analytical value, 2[PPi]$_f{}^1$, in Fig. 6, because the mass balance of the total phosphorus, [Pi]$_f{}^1$ +2[PPi]$_f{}^1$, in the solution after incubation will be checked later. PPi concentration in the solution without any solid phase was exactly the same as that initially (=20 mM). This result means that PPi in the alkaline solution (pH 10) is not hydrolyzed during this incubation period. On the other hand, the concentration of PPi decreases, and that Pi increases with increasing amount of added HAP or Ca$_2$PPi. The concentration of total phosphorus, [Pi]$_f{}^1$+2[PPi]$_f{}^1$, also decreases with increasing amount of added solid.

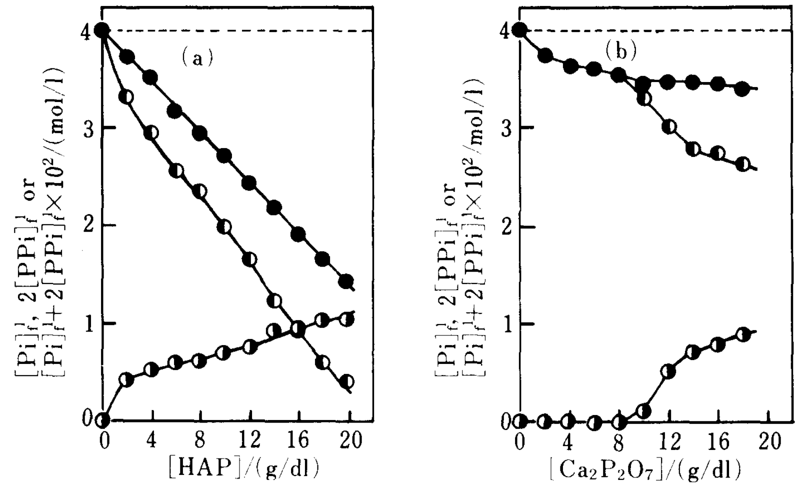

Fig. 6. Concentration Changes of Pi, PPi and Total Phosphorus after Hydrolysis and Adsorption

The initial concentration of K$_4$P$_2$O$_7$ was 20 mM. Incubation temperature and time were 37°C and 72 h.

Symbols: ◑ [Pi]$_f{}^1$, ◐ 2[PPi]$_f{}^1$, ● [Pi]$_f{}^1$+2[PPi]$_f{}^1$.

(a) concentration *vs*. the amount of HAP added (pH 9.8—10.0).

(b) concentration *vs*. the amount of Ca$_2$P$_2$O$_7$ added (pH 8.8—10.0).

Added HAP and added Ca$_2$P$_2$O$_7$ were synthesized according to the literature methods.[3,4)]

One of the reasons why the concentration of Pi increases with the amount of added HAP might be ion-exchange between PPi in the solution and Pi on the surface of added HAP, but this explanation is not valid for the increase of Pi concentration in the system of Ca$_2$PPi plus K$_4$P$_2$O$_7$ in water, because the chemical species present do not include Pi. Consequently, it may be concluded that PPi in the solution is hydrolyzed to two moles of Pi on the surface of the solid, HAP or Ca$_2$PPi.

The decrease of the total phosphorus in the filtrate with increasing amount of solid added, as shown by closed circles (●), may be due to the adsorption of Pi and PPi on the solid surface, as described elsewhere.[7-9)] This would be compatible with the speculation that PPi is hydrolyzed *on the solid surface* and that some of the Pi formed as a reaction product may remain on the surface.

The change of PPi hydrolysis, or that of Pi formation, with solid concentration is different for the HAP system and for the Ca_2PPi system. In the concentration range of Ca_2PPi below 8 g/dl, Pi (◑ of Fig. 6-b) does not appear in the solution, while the concentration of total phosphorus (● in Fig. 6-b) decreases owing to adsorption. As PPi is the lattice ion for Ca_2PPi but not Pi, it might be difficult to consider that Pi, formed by hydrolysis, is selectively adsorbed by Ca_2PPi in preference to PPi in the solution. Therefore, the catalytic activity of Ca_2PPi seems to be smaller than that of HAP. The reason might be differences of their surface properties, such as specific surface area, surface density of adsorption sites for PPi, and stability of PPi on the surface. Adsorbed PPi might be more stable on the surface of Ca_2PPi than on the surface of HAP because PPi is the lattice anion of Ca_2PPi. For the same reason, Pi, formed by hydrolysis from PPi, may be better adsorbed by HAP than by Ca_2PPi. This adsorption of Pi as the lattice ion is similar to crystal growth of HAP on a seed of HAP crystallite. Therefore, consumption of PPi by hydrolysis and consumption of Pi by adsorption from the mother solution seem to be much greater in the HAP system than in the Ca_2PPi system. However, neither weight changes nor composition changes in the added solid were checked after the hydrolysis reaction shown in Fig. 6.

Discussion

Mechanism of Precipitate Formation

As the fourth dissociation constant, K_4, for PPi is 3.6×10^{-9},[10] the dominant ionic species of PPi at pH 9.5—11.0 (*i.e.* under the present experimental conditions) is $P_2O_7^{4-}$. On the other hand, the dissociation constants for Pi ($K_2=6.23\times10^{-8}$ and $K_3=3.0\times10^{-13}$)[11] imply that the dominant ionic species of Pi at pH 9.5—11.0 are HPO_4^{2-} and PO_4^{3-}. As HAP is known to be the most stable calcium orthophosphate at around these pH values,[12] the pH of the solution may decrease with the formation of HAP owing to the consumption of OH^- or liberation of H^+. However, NH_4OH in the solution buffers the pH change in this experimental system. Therefore, the pH of the mother solution was kept alkaline, and the precipitate formed in the solution was composed of $P_2O_7^{4-}$, PO_4^{3-}, OH^-, Ca^{2+}, and K^+, but not H^+, as mentioned before.

When the initial concentration of Pi and/or PPi is more than equivalent to Ca^{2+} added to the solution, almost all calcium (except for some reflecting the solubility of the precipitate) and some of the potassium in the solution are consumed to form precipitates with these anions, and some of the PPi and/or Pi (excess amount) remains in the solution after the precipitation reaction. It may apparently be assumed from Fig. 2 that the precipitates with high contents of Pi or PPi selectively capture Pi or PPi remaining in the solution, respectively, because the composition curves for low concentrations of Ca^{2+} were S-shaped, crossing the diagonal (dotted line) which shows the relation of $x_i^1=x_f^s$. However, attention should be paid to the fact that some of the PPi is hydrolyzed to Pi. The reason why x_f^s is smaller than x_i^1 at low x_i^1 (Fig. 2) and why x_f^1 is smaller than unity at $x_i^1=1$ (Fig. 1-a) may be hydrolysis of PPi.

Diphosphate (PPi), present as the lattice ion or as the adsorbate, is hydrolyzed to Pi on the surface by a surface-catalyzed reaction, as mentioned before (Figs. 5 and 6). The resulting Pi may remain on the precipitate surface, or may desorb and enter the solution. Consequently, the Pi concentration in the mother solution increases with increasing extent of hydrolysis and desorption, leading to the precipitation of CaPi with excess Ca^{2+} in the solution. Therefore, the molar fraction of PPi in the filtrate and in the precipitate decreases with increasing extent of hydrolysis and with increasing initial concentration of Ca^{2+}, as shown in Figs. 1-a and 2. When the Ca^{2+} concentration is not equivalent to Pi in the solution, the excess Pi remains in the solution. As a result, the solution concentration of Pi after incubation becomes lager than the initial concentration of Pi under some conditions (low $[Ca^{2+}]_i^1$ and high x_i^1). Accordingly, Δ[Pi] of Eq. (1) becomes negative (<0), as mentioned in the previous section, and $(x_f^s)'$ in

Fig. 1-b becomes larger than unity.

When the initial concentration of Ca^{2+} is high enough, almost all Pi and/or PPi in the solution is consumed to form the precipitate, in which the K^+ content is very low due to the Ca^{2+}–selectivity of Pi and PPi. Some of the Ca^{2+}, which is in excess over Pi and /or PPi, stays in the solution. The hydrolyzed Pi remaining on the surface may capture Ca^{2+} remaining in the solution, and the precipitates may grow as a mixture of Ca^{2+} salts of PPi and Pi. The Pi desorbed into the solution also forms a precipitate of HAP with Ca^{2+}. The precipitate, obtained for chemical analysis after incubation, was found to be a mixture of the former and the latter. Therefore, the greater the initial concentration of Ca^{2+}, the more the content of Pi or HAP (*i.e.* $1-x_f{}^s$) increases even though $x_f{}^1$ is constant, and the more the content of K^+ in the precipitate decreases, as shown in Figs. 2 and 3. Ultimately, at extremely high concentration of Ca^{2+}, pure crystalline HAP can be obtained from Ca^{2+} and PPi without Pi after the formation and decomposition of Ca_2PPi to calcium orthophosphate, as shown in Fig. 2 ($x_f{}^1=1.0$ and $x_f{}^s=0.0$, ▲)

Mechanism of Hydrolysis of Diphosphate

It is known that the hydrolysis of PPi in water is catalyzed by strong acids, such as H_2SO_4, HCl and $HClO_4$, and that the rate of the reaction increases with acid concentration and temperature.[13] However, the hydrolysis of PPi in the alkaline solution was negligible even at 70°C for 41 d.[14] It is interest that chromate, in contrast, condenses to dichromate in acidic solution. This reaction is applied to qualitative analysis to detect the chromium ion. On the other hand, diphosphate ion, a condensed oxoacid ion like dichromate, decomposes to orthophosphate in acidic solution. Diphosphate is also hydrolyzed by pyrophosphatase with a free energy change of -7.3 kcal/mol.[15]

Electric charges of PPi on the surface of the precipitate may be lower than in the mother solution, because immobile Ca^{2+} as the lattice ion on the surface and mobile Ca^{2+} as the counter ion in the electric double layer may bind rather strongly to PPi on the surface and may partially neutralize the electric charges. Therefore, the valence of PPi ion on the surface becomes similar to that at low pH rather than that in the mother solution, owing to the neutralization of electric charges by Ca^{2+} instead of by H^+ in acidic solution. As the rate constant of the hydrolysis increases with decreasing pH, that is, with decrease of the electric valence of PPi, PPi is assumed to be more easily hydrolyzed to Pi on the surface. The fact that the electron distribution in diphosphate exposed or adsorbed on the surface might be distorted by the asymmetric field at the surface might be also one of the reasons for rapid hydrolysis on the surface.

On the other hand, the possibility of hydrolysis of PPi in the bulk solution might also have to be considered. However, this effect is assumed to be minor, because the extent of hydrolysis increases with the surface area, that is, with the amount of precipitate formed (Fig. 5) and of solid added (Fig. 6). Therefore, the surface effect of the solid, as mentioned above, was considered here to be most important for the hydrolysis of PPi.

Significance of PPi Hydrolysis

Although PPi is usually assumed to be stable in alkaline solution, PPi with some kind of solid is easily hydrolyzed, as shown here. Therefore, attention should be paid to this when the effect or properties of PPi in solution are going to be investigated strictly, and when pure Ca_2PPi is to be synthesized with Ca^{2+} and PPi in the aqueous phase. On the other hand, Nelson *et al.*[16] made use of this property of easy hydrolysis to synthesize long-chain monoalkyl phosphates from alcohols and diphosphoric acid, which is hydrolyzed to orthophosphate during the reaction process.

In biological systems, the reason why soft tissues are protected from calcification is the presence of calcification inhibitors, one of which is known to be PPi.[17] PPi is, however, hydrolyzed by alkaline phosphatase or pyrophosphatase, which is present wherever calcification occurs in biological tissues. Therefore, the concentration of PPi decreases and that of Pi increases locally, and precipitation for the formation of hard tissues (HAP) takes place with Ca^{2+} in the body fluids.1) The biological hydrolysis of PPi and formation of hard tissue is, thus, mainly regulated by the enzyme, but some of the PPi in the body fluids might be hydrolyzed on the surface of biological HAP in hard tissues by the mechanism that we have discussed here.

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