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Hydrolysis of Diphosphate Ion to Orthophosphate Ion in the Presence of Calcium Ion. Fundamental Study of the Development of the Mixed Crystal Deposition Disease

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The hydrolysis of diphosphate ion (pyrophosphate ion, PPi) to orthophosphate ion (Pi) in the presence of Ca^{2+} in aqueous systems were examined. The systems studied were (1) $\text{K}_4\text{P}_2\text{O}_7 + \text{CaCl}_2$ with/without KOH, (2) $\text{K}_4\text{P}_2\text{O}_7 + \text{Ca}(\text{OH})_2$, (3) aqueous suspension of the precipitate isolated from the mother solution immediately after mixing of $\text{K}_4\text{P}_2\text{O}_7$ and CaCl_2 , and (4) $\text{K}_4\text{P}_2\text{O}_7$ in an aqueous suspension of $\text{Ca}_2\text{P}_2\text{O}_7$. It was found that the gradual release of Ca^{2+} to the aqueous phase from the Ca^{2+} -reservoir (*i.e.*, $\text{Ca}(\text{OH})_2$ formed (system 1) or added (system 2)) is effective for the hydrolysis of PPi. When the temporary precipitate containing PPi was suspended in the aqueous phase (system 3), the hydrolysis of PPi was delayed because re-dissolution of the precipitate was necessary prior to the hydrolysis. The result in system 4 suggested that the hydrolysis reaction occurs on the surface of $\text{Ca}_2\text{P}_2\text{O}_7$. These results are discussed in connection with a possible physico-chemical mechanism of deposition of mixed crystals (calcium diphosphate and calcium orthophosphate) in the human body, causing mixed crystal deposition disease.

Keywords—hydroxyapatite; diphosphate hydrolysis; pyrophosphate hydrolysis; calcium diphosphate; calcium orthophosphate; crystal deposition disease; calcium pyrophosphate

Diphosphate ion (pyrophosphate ion, PPi) is present in body fluids such as blood, urine, and saliva. It has been suggested that PPi takes a role in preventing and/or regulating calcification of soft tissues and formation of undesirable concretion.²⁾ It is also known that the crystal growth and crystallite aggregation of hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$, HAP) is retarded or inhibited by the presence of PPi in water.³⁾

On the other hand, ectopic deposition of calcium salt of PPi ($\text{Ca}_2\text{P}_2\text{O}_7$, *i.e.*, Ca_2PPi) in arthrosis and yellow ligament causes pyrophosphate arthropathy (pseudo-gout syndrome)⁴⁾ and cervical radiculomyelopathy.⁵⁾ These diseases are associated with increased levels of PPi in the body fluids. Sometimes two kinds of calcium salts (*i.e.*, Ca_2PPi and HAP) are found in the affected part in crystal deposition diseases like these (mixed crystal deposition diseases).⁶⁾ Therefore, studies on the formation of these salts are physiologically and pathologically important.

In the previous papers,⁷⁾ it was shown that PPi is partially or totally hydrolyzed to orthophosphate ion (Pi) in the presence of Ca_2^{+} , forming amorphous calcium orthophos-

phate and/or HAP. The degree of crystallinity of HAP formed was higher in the presence^{7a)} than in the absence^{7b)} of alkaline substances (NH_4OH , for example). It was also found that hydrated calcium diphosphates prepared in an aqueous phase through the literature methods are contaminated with several kinds of by-products of orthophosphate salts.^{7c)} In the present paper, the transformation of the precipitates, which were formed temporarily immediately after mixing of $\text{K}_4\text{P}_2\text{O}_7$ with CaCl_2 (or $\text{Ca}(\text{OH})_2$) in an aqueous phase, was studied as a function of time by considering re-dissolution of the precipitate, release of Ca^{2+} , K^+ , and PPi from the precipitate, and/or the surface effect of the precipitate. A possible physico-chemical mechanism of deposition of mixed crystals (Ca_2PPi and HAP) in the human body is also discussed briefly.

Experimental

Materials—All chemicals used were of reagent grade from Nakarai Chemicals Ltd. or Wako Pure Chemical Industries Ltd. These were used without further purification. HAP was prepared as described elsewhere.⁸⁾ Crystalline calcium diphosphate ($\text{Ca}_2\text{P}_2\text{O}_7$, Ca_2PPi) was prepared by heating CaHPO_4 at 450°C for 6 h, according to the method of McIntosh and Jablonsky.⁹⁾

Preparative Methods— MeCl_2 ($\text{Me} = \text{Mg}^{2+}$, Ca^{2+} , or Sr^{2+}) was added to 112.5 mM $\text{K}_4\text{P}_2\text{O}_7$ (K_4PPi) in the presence or absence of 1 M KOH at 30°C . The solution containing the precipitate formed was filtered through a $0.22\text{ }\mu\text{m}$ Millipore filter after incubation for 2 h. The contents of total phosphorus (P_t) and PPi in the filtered precipitates were analyzed.

A known weight of $\text{Ca}(\text{OH})_2$ was added to an aqueous solution of 0.5 M K_4PPi (25 ml, pH 9.85) at 30°C , resulting in the formation of a suspension. The pH immediately after mixing was 13.5–13.9, depending on the amount of $\text{Ca}(\text{OH})_2$ added. The suspension was filtered through a $0.22\text{ }\mu\text{m}$ Millipore filter after various time intervals. The filtrate and the filtered precipitate were analyzed.

An aqueous solution of CaCl_2 (2M, 1.5 l) was added to 1.5 M K_4PPi (1 l), where the molar ratio of Ca^{2+} to PPi is 2.0, under vigorous stirring at 30°C to obtain a precipitate immediately after mixing. The precipitate obtained within 5 min after the reaction was washed with water and methanol, air-dried, and finally dried *in vacuo* at room temperature. It was a white fine powder and was amorphous as judged by X-ray powder diffractometry. Its composition was determined to be $\text{Ca}_{1.53}\text{K}_{1.06}(\text{P}_2\text{O}_7)_{0.94}(\text{PO}_4)_{0.12} \cdot 4.0\text{H}_2\text{O}$ (precipitate 1) by means of chemical analysis. The precipitate 1 was suspended in various kinds of media (water, 4 M KCl , and 2 M CaCl_2) and incubated at 30°C . The suspension thus obtained were filtered at various time intervals, and the filtrates and the remaining precipitates were analyzed.

Analysis—Immediately before the chemical analysis, the precipitates were dissolved quickly in an aqueous solution of ethylenediaminetetraacetate (EDTA). Calcium content was determined by measuring the excess EDTA remaining 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). The content of PPi was determined according to the method of Kato *et al.*¹⁰⁾ Zinc diphosphate (Zn_2PPi), precipitated from the sample solution by addition of an excess amount of zinc acetate at pH 3.8–3.9, was dissolved in NH_4Cl – NH_4OH buffer solution at pH 10. This solution, containing zinc ion ($[\text{Zn}^{2+}] = 2[\text{PPi}]$), was analyzed by chelatometry with EDTA standard solution and BT indicator at pH 10. It was confirmed that neither Ca^{2+} nor Pi , which is contained in the sample precipitate and/or filtrate, interferes with the titration, because these ions were excluded through the above procedure.

Content of P_t , which is present as Pi and PPi , was determined by colorimetry according to the method of Gee *et al.*¹¹⁾ after hydrolysis of PPi to Pi by boiling the sample in 0.5 M HCl for 30 min. 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 Pi was determined as the difference between the content of total phosphorus ($[\text{P}_t] = [\text{Pi}] + 2[\text{PPi}]$) and that of PPi ($2[\text{PPi}]$).

X-Ray Diffraction—The X-ray powder diffraction patterns (Co K_α radiation at 35 kV and 10 mA with an iron filter, wave length $1.789\text{ \AA}$) were obtained with a Norelco Geiger Counter diffractometer (Philips Electronics and Pharmaceutical Industry) at room temperature.

Henceforth, subscript “l” or “s” indicates liquid phase (filtrate) or solid phase (precipitate).

Results

Effects of Cationic Species and OH^- on Hydrolysis of PPi

The effects of species of divalent metal cations ($\text{Me} = \text{Mg}^{2+}$, Ca^{2+} , and Sr^{2+}) and of the

mixing ratio of Me to PPi on the hydrolysis of PPi were examined in the presence and absence of 1 M KOH.

Chemical analyses after incubation for 2 h showed that PPi was not hydrolyzed to Pi in neutral or alkaline solution in the absence of Me. In the presence of Me, PPi was hydrolyzed to Pi, and the content of Pi in the precipitate increased with the molar ratio of Me to PPi because Me catalyzes the hydrolysis of PPi to Pi. The extent of hydrolysis was larger in the presence of OH^- than in the absence of OH^- , and in the presence of OH^- and Ca^{2+} (or Sr^{2+}) than in the presence of OH^- and Mg^{2+} . The molar ratio of Pi to P_t (Pi/P_t), as a parameter of the extent of hydrolysis, in the precipitate became almost unity when OH^- (as KOH) was added and the initial molar ratio of Me/PPi ($\text{Me}=\text{Ca}^{2+}$ or Sr^{2+}) was higher than 3.0. However, the molar ratio of Pi/P_t was less than 0.4 even though $\text{Mg}^{2+}/\text{PPi}$ was higher than 4.0 in the presence of KOH. The presence of $\text{Ca}(\text{OH})_2$ was confirmed by X-ray powder diffractometry when KOH was added to the system containing Ca^{2+} . Crystalline $\text{Sr}(\text{OH})_2$ or $\text{Mg}(\text{OH})_2$ was not detected by X-ray powder diffractometry. However, amorphous $\text{Sr}(\text{OH})_2$ or $\text{Mg}(\text{OH})_2$ should have precipitated in the system containing Sr^{2+} or Mg^{2+} and OH^- . The reason why the extent of hydrolysis is smaller in the presence of $\text{Mg}(\text{OH})_2$ than in the presence of $\text{Ca}(\text{OH})_2$ or $\text{Sr}(\text{OH})_2$ may be lower solubility of $\text{Mg}(\text{OH})_2$ than of $\text{Ca}(\text{OH})_2$ or $\text{Sr}(\text{OH})_2$ (see Discussion also).

Hydrolysis of PPi in the Presence of $\text{Ca}(\text{OH})_2$

After the mixing of 0.5 M K_4PPi (25 ml) and $\text{Ca}(\text{OH})_2$ (1.25 g), the filtrate and the precipitate were analyzed chemically at various time intervals. It was found that the concentration of P_t in the filtrate decreased and the content of P_t in the precipitate increased with time. On the other hand, a small amount of Pi appeared in the filtrate, while the content of Pi in the precipitate increased gradually with time. These results show that PPi in the solution was hydrolyzed to Pi in the presence of Ca^{2+} ,⁷⁾ which was supplied gradually through the dissolution of $\text{Ca}(\text{OH})_2$ added and was consumed by the precipitate formation with PPi and Pi formed. Thus, the hydrolysis of PPi and precipitate formation continued for more than 100 h.

The above analytical values were converted to molar ratio of Pi to P_t . Figure 1 shows the time courses of the molar ratios of Pi to P_t , Pi/P_t , in the filtrate, in the precipitate, and in the

Fig. 1. Molar Ratio of Orthophosphate Ion to Total Phosphorus

Initially 1.25 g (A), 2.5 g (B), or 5.0 g (C) of $\text{Ca}(\text{OH})_2$ was added to 0.5 M K_4PPi (25 ml). The initial molar ratio of Ca^{2+} to total phosphorus, $\text{Ca}^{2+}/\text{P}_t$, was 0.675 (A), 1.35 (B), or 2.7 (C), respectively. The concentrations of Pi and P_t in the filtrate of system (B) were too low to determine accurately. Neither Pi nor PPi were detected in the filtrate of system (C).

Δ , in the filtrate; $\circ$, in the precipitate; $\bullet$, for the total system (the filtrate+the precipitate).

Fig. 2. Concentration of Potassium Ion in the Filtrate and Equivalent Fraction of Potassium Ion with Respect to Cations in the Precipitate

The initial molar ratio of mixing of Ca^{2+} to P_i , $\text{Ca}^{2+}/\text{P}_i$, was 0.675 ((A) and (a)), 1.35 ((B) and (b)), or 2.7 ((C) and (c)), as in Fig. 1. The equivalent fraction (not shown) of Ca^{2+} in the precipitate increased with time synchronously with the decrease in K^+ content.

Fig. 3. Time Courses of the Peak Heights in X-Ray Powder Diffraction

The initial molar ratio of Ca^{2+} to P_i , $\text{Ca}^{2+}/\text{P}_i$, was the same as in Fig. 1.
 —●—, $\text{Ca}(\text{OH})_2$ (spacing $d=1.93 \text{ \AA}$)¹³⁾; —○—, HAP ($d=2.81 \text{ \AA}$)⁸⁾;
 ---▲---, $\text{CaK}_2\text{P}_2\text{O}_7 \cdot 4\text{H}_2\text{O}$ ($d=2.89 \text{ \AA}$)¹²⁾; ---△---, $\text{CaK}_2\text{P}_2\text{O}_7$ ($d=2.18 \text{ \AA}$)¹²⁾

total system. The values of P_i/P_t in each phase increased with time. The initial slope in the figure became steeper with increase in the initial mixing ratio of $\text{Ca}^{2+}/\text{P}_i$. When the ratio, $\text{Ca}^{2+}/\text{P}_i$, was high enough (Fig. 1(C), for example), PPi was completely exhausted from the system and the ratio, P_i/P_t , became unity within a short period.

Figure 2 shows the time courses of the potassium concentration ($[\text{K}^+]_l$) in the filtrate ((A), (B), and (C)) and those of the equivalent fraction of potassium with respect to cations in the precipitate ($[\text{K}^+]_s / (2[\text{Ca}^{2+}]_s + [\text{K}^+]_s)$), (a), (b), and (c)). When the initial ratio, $\text{Ca}^{2+}/\text{P}_i$, was low ((A) and (a), for example), K^+ was captured in the precipitate and the level of K^+ in the filtrate decreased immediately after mixing of the reagents. However, when the ratio, $\text{Ca}^{2+}/\text{P}_i$, was high enough, K^+ was released from the temporary precipitate containing much K^+ . That is, $[\text{K}^+]_l$ initially decreased and then increased ((B) and (C)), while the equivalent fraction of K^+ in the precipitate initially increased and then decreased ((b) and (c)) with time. Especially in the cases of (C) and (c), the figures suggest that K^+ was excluded completely from the

precipitate within 75 h and that the only cationic species in the precipitate was Ca^{2+} . Acidic salt containing H^+ would not appear because the pH of the mother solution was high enough (13.5–13.9).

Figure 3 shows the time courses of the diffraction intensity determined by X-ray powder diffractometry of the crystalline substances found in the precipitate.^{12,13)} The intensity of the diffraction by $\text{Ca}(\text{OH})_2$ decreased gradually for about 50 h ((A) and (B)) or more than 50 h (C), depending on the amount of $\text{Ca}(\text{OH})_2$ added, after a steep decrease immediately after mixing of the reagents. These results show that the dissolution and/or consumption of $\text{Ca}(\text{OH})_2$ is gradual, as expected.

The species $\text{CaK}_2\text{P}_2\text{O}_7 \cdot 4\text{H}_2\text{O}$ was present for a while. However, $\text{CaK}_2\text{P}_2\text{O}_7$ (Fig. 3(A)) or HAP (Fig. 3(B) and (C)), both of which are more stable than $\text{CaK}_2\text{P}_2\text{O}_7 \cdot 4\text{H}_2\text{O}$,¹²⁾ appeared later synchronously with the disappearance of $\text{CaK}_2\text{P}_2\text{O}_7 \cdot 4\text{H}_2\text{O}$. The diffraction peak of HAP appeared earlier and the diffraction was stronger in (C) than in (B). These results may be due to higher contents of P_i , Ca^{2+} , and OH^- in the system shown in (C) than in the system shown in (B).

Transformation of the Precipitate Formed Temporarily

The amorphous precipitate **1** (5 g), prepared according to the method mentioned in Experimental, was suspended in 25 ml of water, 4 M KCl, or 2 M CaCl_2 to examine its transformation to a stable form in contact with an aqueous phase.

Partial dissolution and change of the chemical composition of the precipitate were suggested by the fact that the concentrations of P_i and K^+ in the supernatant increased and the total amount of P_i in the precipitate decreased with time when the precipitate **1** was soaked in water or in 4 M KCl. However, Ca^{2+} was not detected in the supernatant. On the other hand, when the precipitate **1** was suspended in 2 M CaCl_2 , the concentration of K^+ increased and that of Ca^{2+} decreased in the supernatant with time. This means that the K^+ content decreased and the Ca^{2+} content increased in the precipitate through the absorption of Ca^{2+} from the medium.

The ratios of P_i to P_t , P_i/P_t , in the supernatant, in the precipitate, and in the total system (solution phase + precipitate phase) are shown in Fig. 4 as functions of the incubation time. The molar ratio of P_i/P_t increased with time irrespective of the suspending medium. The ratio P_i/P_t in 2 M CaCl_2 (C) is higher than that in water (A) and in 4 M KCl (B). This result shows

Fig. 4. Molar Ratio of P_i to P_t as a Function of Time

The precipitate **1** (5 g) was suspended in 25 ml of water (A), 4 M KCl (B), or 2 M CaCl_2 (C). The initial pH was adjusted to 7.0. Neither P_i nor P_t was detected in the filtrate of system (C).

△, molar ratio in the filtrate; ○, molar ratio in the precipitate; ●, molar ratio with respect to the total system (the filtrate + the precipitate).

Fig. 5. Equivalent Fraction of Potassium Ion in the Precipitate and Peak Heights in X-Ray Powder Diffraction

The precipitate **1** (5 g) was suspended in 25 ml of water ((A) and (a)), 4 M KCl ((B) and (b)), or 2 M CaCl_2 ((C) and (c)).

Δ , equivalent fraction of K^+ in the precipitate; $\circ$, peak height in X-ray powder diffraction of crystallites of $\text{Ca}_5\text{K}_2(\text{P}_2\text{O}_7)_3 \cdot 6\text{H}_2\text{O}$ ($d = 2.82 \text{ \AA}$)¹²⁾; $\bullet$, peak height in diffraction of crystallites of $\text{Ca}_2\text{P}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$ (monoclinic, $d = 3.03 \text{ \AA}$)¹²⁾

Fig. 6. Hydrolysis of PPi in the Presence of Ca_2PPi

A known amount of Ca_2PPi was suspended in 20 mM K_4PPi , and the initial pH was adjusted to 7 (A), 9 (B), or 13 (C) by the addition of HCl or KOH. The filtrate was analyzed after incubation for 72 h at 30 °C. The horizontal dotted line shows 40 mM, which is the total concentration of phosphorus added as 20 mM K_4PPi .

$\circ$, [Pi]; $\bullet$, 2[PPi]; $\bullet$, [Pi] + 2[PPi].

that PPi is easily hydrolyzed to Pi in the presence of Ca^{2+} .⁷⁾ The data for the liquid phase are not shown in Fig. 4(C), because the concentrations of Pi and PPi in the solution were too low to be determined precisely due to excess Ca^{2+} in the solution.

Figures 5(A), (B), and (C) show the equivalent fractions of K^+ with respect to cations in the precipitate as functions of the incubation time. Simultaneously with the rearrangement of cations and anions in the precipitate, as mentioned above, crystallites of $\text{Ca}_5\text{K}_2(\text{P}_2\text{O}_7)_3 \cdot 6\text{H}_2\text{O}$ or $\text{Ca}_2\text{P}_2\text{O}_7 \cdot 2\text{H}_2\text{O}$ appeared and the diffraction intensity in X-ray powder diffractometry increased with time (Figs. 5(a), (b), and (c)). Molar ratios of K^+ to Ca^{2+} in these crystalline substances (0.40 for the former and 0.00 for the latter) became less than the initial molar ratio in the precipitate **1** (=0.69) owing to the decrease in the K^+ content in the precipitate.

Crystallites of $\text{Ca}_5\text{K}_2(\text{P}_2\text{O}_7)_3 \cdot 6\text{H}_2\text{O}$ were not found in the precipitate treated in 2M CaCl_2 , probably because almost all K^+ in the precipitate was extracted into the aqueous phase and Ca^{2+} was absorbed into the precipitate. In contrast to the result shown in Fig. 3, HAP crystallites were not detected in the precipitate by means of X-ray powder diffractometry, although the precipitate contains Pi and Ca^{2+} . This may be because of an insufficient quantity of OH^- in the mother solution (almost neutral) and because PPi, an inhibitor of HAP crystallization, is still present in the system.

The Effect of the Amount of Ca_2PPi Added and pH on the Extent of Hydrolysis of PPi

It was found that PPi in the aqueous phase was hydrolyzed to Pi to an extent depending on the added amount of Ca_2PPi (which is practically insoluble in water), and on the pH of the solution (Fig. 6). The concentration of Pi, formed by hydrolysis of PPi during the incubation for 72 h at 30 °C, increased with increase in the amount of Ca_2PPi added, $[\text{Ca}_2\text{P}_2\text{O}_7]$, and with decrease in the pH of the mother solution. Concomitantly, PPi remaining in the solution decreased with increase in $[\text{Ca}_2\text{P}_2\text{O}_7]$ and with decrease in pH. The closed circles, which show the concentration of total phosphorus ($[\text{Pi}] + 2[\text{PPi}]$) in the solution, should be on the dotted line if these anions were not adsorbed by added Ca_2PPi . The figure, however, shows that adsorption occurs, and the extent of adsorption decreases with increase in pH owing mainly to the competitive adsorption of OH^- with Pi and PPi.

Discussion

The mechanism of hydrolysis and precipitate formation is considered as follows, taking into account the experimental results. An amorphous precipitate is formed immediately after mixing of PPi and Ca^{2+} ; this precipitate contains Ca^{2+} , K^+ , PPi, and Pi, the last-mentioned of which is formed through the hydrolysis of PPi. This precipitate crystallizes to a stable form synchronously with release of K^+ and PPi. The released PPi is also hydrolyzed to Pi in the presence of the catalyst Ca^{2+} , which is supplied through the gradual dissolution of $\text{Ca}(\text{OH})_2$ as a Ca^{2+} -reservoir or which remains in the solution as excess CaCl_2 . Therefore, the Pi content in the precipitate increases with time through the re-precipitation of Pi with Ca^{2+} . When an excess amount of Ca^{2+} with respect to PPi is present in the system, the hydrolysis may continue until the complete exhaustion of PPi, forming a precipitate of calcium orthophosphate. On the other hand, when extra Ca^{2+} is not supplied to the system, the amount of Pi in the precipitate decreases because PPi released from the precipitate and/or Pi formed from PPi cannot re-precipitate without Ca^{2+} . HAP crystallites appear in the precipitate when enough OH^- is present (Fig. 3). However, HAP was not detected in the system shown in Fig. 5, where OH^- was not supplied. HAP crystallites may appear after long-term ageing and/or ripening because the most stable form of calcium orthophosphate is HAP when the pH of the mother solution is higher than ca. 4.5.¹⁴⁾

The hydrolysis proceeds through another mechanism, being accelerated by the adsorption of PPi on the surface of Ca_2PPi (Fig. 6).^{7a)} Therefore, it is reasonable to say that the dependence of the extent of hydrolysis on the OH^- concentration (or pH) is different under the conditions of Figs. 1—3 from that under the conditions of Fig. 6.

Calculus formation in the human body, as well as the hydrolysis and precipitate formation mentioned in the present paper, is the result of a rate process, not an equilibrium process. Therefore, the results mentioned above may be relevant to the mechanism of development of mixed crystal deposition disease,⁶⁾ although the experimental conditions here were not strictly physiological. The surface of the calculi, which are present in the affected part, and calcium ion, which is supplied continuously from the body fluids as a Ca^{2+} -reservoir (instead of $\text{Ca}(\text{OH})_2$ or CaCl_2 added), may take part in the hydrolysis of PPi in the tissues and

in the body fluids in a similar manner to that mentioned in the text. Of course, the hydrolysis of PPi is catalyzed mainly by the enzyme pyrophosphatase. The PPi and Pi formed, in addition to Pi contained originally in the body fluids, precipitate together with Ca^{2+} on the matrices, and the mixed mineral deposit grows as calculi with time.

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