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Effect of Condensed Phosphate on the Precipitate Formation and Dispersion of Calcium Phosphate in Water

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Fine particles of calcium phosphate were obtained by mixing K_2HPO_4 and $CaCl_2$ in 154 mM NaCl in the presence of condensed phosphate. The effects of condensed phosphate on the particle formation of amorphous calcium phosphate (ACP), on the transformation of ACP to hydroxyapatite (HAP), and on the dispersibility of ripened HAP particles were studied by means of a Coulter counter, a calcium-ion specific electrode, X-ray powder diffraction analysis, and so on. The effective concentration of the condensed phosphate to retard the transformation of ACP to HAP was found to be between 10^{-4} and 10^{-3} mM; that to disperse the particles sufficiently was between 10^{-3} and 10^{-2} mM; and that to disaggregate ripened HAP particles was between 3 and 10^2 mM. The order of effectiveness of the condensed phosphate was common irrespective of the measuring method: hexametaphosphate > triphosphate > diphosphate. Adsorption and/or competitive adsorption of condensed phosphate and orthophosphate, which is the lattice ion of calcium phosphate, was taken into account to explain the above results. The physiological significance of the present work is briefly discussed.

Keywords——amorphous calcium phosphate; hydroxyapatite; condensed phosphate; triphosphate; hexametaphosphate; diphosphate; renal calculus; hard tissue; Coulter counter

Condensed phosphates, compounds characterized by one or more P–O–P bonds, are often used industrially as dispersing and/or peptizing agents. Physiologically, on the other hand, diphosphate (pyrophosphate; PPi), one of the condensed phosphates, has been found in blood plasma (1—6 μM), urine (10—100 μM), and other body fluids.[1] It is known to be a natural inhibitor which prevents the formation of undesirable concretion (such as renal stones) and the calcification of soft tissues (artery, cartilage, and skin).[2,3] In the field of dentistry, condensed phosphates exhibit anticaries activity through their adsorption on the surface of dental hydroxyapatite.[4]

However, clarification of these properties from the standpoint of physical chemistry is not yet complete. Therefore, precise studies on the interaction of PPi and other condensed phosphates with calcified tissues are necessary. In the present paper, the effect of condensed phosphates on the formation and dispersion of amorphous calcium phosphate (ACP) and hydroxyapatite (HAP; $Ca_{10}(PO_4)_6(OH)_2$) will be discussed. HAP is the main component of physiologically and/or pathologically calcified tissues of mammalian animals.

Experimental

Materials——HAP is the same sample as that used in the previous paper.[5] It is a white and fine powder. The mean diameter of HAP particles was 0.155 μm.[5]

Phosphate (orthophosphate; Pi), diphosphate (pyrophosphate; PPi), and triphosphate (tripolyphosphate; TPi) samples used were K_2HPO_4, $K_4P_2O_7$ (Nakarai Chemicals, Ltd.), and $Na_5P_3O_{10}$ (Wako Pure Chemical Industries, Ltd.), respectively. Sodium hexametaphosphate (NaHMPi) was of commercial origin (Wako Pure Chemical Industries, Ltd.). The mean degree of condensation of HMPi was estimated to be 8.0 by means of end-group assay through pH titration (*ca.* 4.5—9.5).[6] Therefore, HMPi in the present paper is regarded as an octamer of orthophosphate on average. The effect of different cationic species (Na^+ and K^+) was neglected in the present work.[7]

Other reagents used were purchased from Nakarai Chemicals, Ltd., or Wako Pure Chemical Industries, Ltd. All chemicals used were of analytical grade. These were used without further purification.

Methods——Mean particle diameter (ϕ) and number concentration of particles (n) were measured by means of a Coulter counter (type TA II; Coulter Electronics, Inc.) at room temperature. The medium (154 mM NaCl) for particle counting and sizing with the Coulter counter was saturated with respect to a ripened HAP and filtered through a Millipore filter (0.22 μm pore size) prior to the measurement.

Activity ($a_{Ca^{2+}}$) or free concentration ($[Ca^{2+}]$) of calcium ion and pH were determined in 154 mM NaCl at 35 °C by using an Orion calcium-sensitive electrode and a Toa glass electrode, respectively. In this work, the relationship between Ca^{2+}-electrode potential (E) and the logarithm of $[Ca^{2+}]$ (as well as $a_{Ca^{2+}}$) was linear because the activity coefficient for calcium ion was approximately constant due to the high ionic strength (154 mM NaCl).

X-Ray powder diffraction patterns were obtained with Cu K_α radiation. The methods are described elsewhere.[8]

Precipitate formation of calcium phosphate (CaPi) was performed as follows: 0.25 ml of 1 M $CaCl_2$, 1 ml of a given solution of a condensed phosphate, and 0.50 ml of 1 M K_2HPO_4 were added to 200 ml of 154 mM NaCl in that order of mixing at a constant stirring speed. Time-courses of ϕ and n of the CaPi particles formed were obtained by means of a Coulter counter, and those of pH and E (*i.e.*, $[Ca^{2+}]$) were followed simultaneously by using an Electronic Polyrecorder (Toa EPR-200A). The pHs of 154 mM NaCl, 1.25 mM $CaCl_2$, and condensed phosphate solutions of low concentration (less than 5×10^{-2} mM; see Figs. 1—5) were almost the same (pH 5.4—5.9). The pH of 2.5 mM K_2HPO_4 was 8.2.

Optical density (OD) was measured as follows:[5] ripened HAP (1 g) was suspended in a test tube containing 20 ml of a given solution and shaken vigorously for about 5 min. After 24 h at room temperature, the OD of the suspension in the upper part of the test tube was measured at 550 nm with a Shimadzu UV-180 spectrophotometer after decantation. A portion of the added HAP had already sedimented and the rest remained suspended in the water phase. There was no sharp interface between the sediment phase and the dispersion liquid phase. Therefore, the OD may be regarded as a convenient index of the stability of the HAP suspension. The higher the OD, the more stable the suspension is.

The degree of peptization of the HAP suspension was measured as follows: HAP (2 g) was suspended in a given solution (20 ml) and shaken vigorously for about 5 min. After standing for 24 h, the suspension was filtered through a Toyo filter paper (qualitative No. 2, 7 cm diameter; pore size $<$ *ca.* 5 μm). The filtrate was turbid, however, because a portion of small-sized HAP particles had passed through the filter paper due to the peptizing and/or dispersing ability of the condensed phosphate added. The concentration of HAP in the filtrate was determined by ethylene diaminetetraacetic acid (EDTA) chelatometry for calcium ion after dissolving the HAP in dilute hydrochloric acid. The ratio of the HAP concentration in the filtrate to the initial value (2 g/20 ml) was regarded as a convenient index of the degree of peptization in the present paper.

Results

Transformation of Amorphous Calcium Phosphate to Crystalline Hydroxyapatite

In the previous paper[8] it was shown that ACP, formed immediately after mixing orthophosphate (Pi) with Ca^{2+}, was transformed to crystalline HAP spontaneously after a certain induction period (t_{trans}). The time, t_{trans}, was not affected by the presence of sodium chondroitin-6-sulfate (Na_2Chs), but the mean particle diamerer of CaPi particles formed decreased with increase in the concentration of Na_2Chs added.

On the other hand, addition of condensed phosphate affected the time, t_{trans}, and made the transformation slower. Figure 1 shows time-courses of the X-ray powder diffraction intensity for CaPi precipitates formed in the presence of condensed phosphate. The period during which the precipitate remains amorphous (*i.e.*, specific X-ray diffraction is hardly found) was longer in the presence than in the absence of condensed phosphate (see ● and ◑,

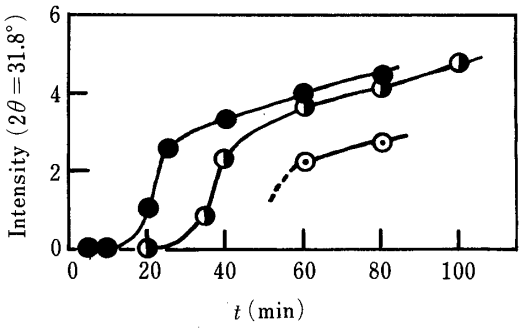

Fig. 1. Time-Course of the X-Ray Powder Diffraction Intensity at $2\theta=31.8°$

Condensed phosphate added: ●, none; ◑, 0.5×10^{-3} mM $K_4P_2O_7$; and ⊙, 0.5×10^{-3} mM $Na_5P_3O_{10}$. The precipitated samples were obtained by filtration at a given time after mixing the reagents: 9.85 mM K_2HPO_4, a given solution of condensed phosphate, 4.93 mM $CaCl_2$, and 154 mM NaCl. The diffraction angle cited ($2\theta=31.8°$) is specific for HAP crystal.

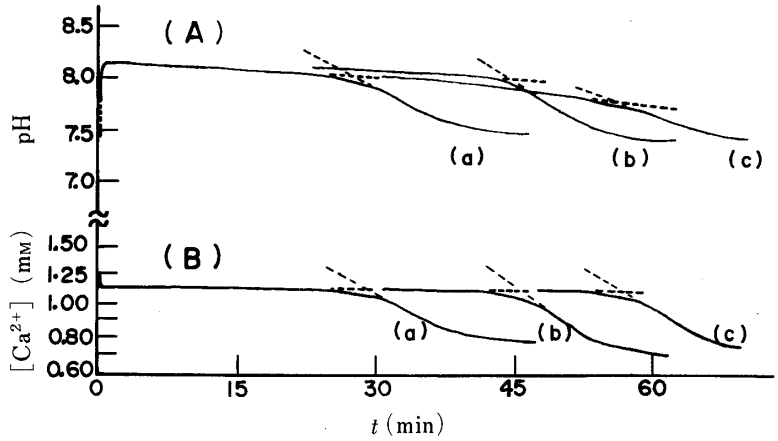

Fig. 2. Time-Courses of pH and $[Ca^{2+}]$ in the Presence of Added PPi

(A) pH *vs.* time, (B) $[Ca^{2+}]$ *vs.* time.
Concentration of PPi added/mM: 0 (a), 0.25×10^{-3} (b), 0.75×10^{-3} (c). Molar ratio of PPi to Pi added: 0 (a), 10^{-4} (b), 3×10^{-4} (c). The precipitate of CaPi was formed by mixing 154 mM NaCl, 1.25 mM $CaCl_2$, a given solution of $K_4P_2O_7$, and 2.50 mM K_2HPO_4 in that order.

for example). The diffraction intensity was correspondingly weaker (◑ and ⊙). The time-course immediately after the precipitate formation in the presence of TPi or HMPi was not obtained because the particle size of the precipitate was too small to allow rapid filtration (see Figs. 4 and 5).

Figure 2 shows the time-courses of pH and $[Ca^{2+}]$ after mixing K_2HPO_4 and $CaCl_2$ in the presence of PPi. The pH increased and $[Ca^{2+}]$ decreased steeply almost along the ordinate immediately after the mixing. The $[Ca^{2+}]$ decreased by virtue of ACP formation, and the pH increased due to the free Pi remaining in the mother solution. After an induction period, ACP was transformed to HAP (see Fig. 1), and OH^- and Ca^{2+} were consumed simultaneously again, resulting in the decrease of both pH and $[Ca^{2+}]$ of the mother solution. The induction periods (t_{trans}), obtained from the intersection of the tangents drawn to the curves of pH and $[Ca^{2+}]$ (dotted lines in Fig. 2) agreed reasonably well. Similar tendencies were observed in the presence of added TPi or HMPi (not shown).

Variation of t_{trans} is shown in Fig. 3 as a function of concentration of condensed phosphates added. The order of the effectiveness on t_{trans} with respect to concentration of phosphorus atom (Fig. 3(A)) was TPi $\simeq$ PPi $>$ HMPi; and that with respect to molar concentration (Fig. 3(B)) was HMPi $>$ TPi $>$ PPi, although the situation was complicated in the region of low concentration. Therefore, the following conclusion may be drawn: the longer the chain of the condensed phosphate, the more effective it is with respect to the molar concentration but the less effective it is with respect to each phosphate residue of the condensed phosphate.

Number Concentration and Mean Diameter of CaPi Particles Formed

Number concentration, n, and mean diameter, ϕ, of CaPi particles formed in the presence of TPi were measured by means of a Coulter counter. Time-courses of these parameters are shown in Fig. 4.

The ϕ increased monotonously with time, while it decreased with increase in concentration of TPi. The n value increased with time at first and after a short period it decreased when little or no TPi was added (○ and ⊗). However, it increased monotonously with time in the presence of 1.66×10^{-2} mM TPi (◑). These differences in the time-courses, depending on concentration of added TPi, may be explained in terms of the dispersing effect of TPi. That is, the higher the concentration of TPi, the more the aggregation among CaPi particles formed is inhibited.[8] Similar tendencies of the changes in ϕ and n were found when PPi or HMPi was added instead of TPi (not shown).

No particles were detected by the Coulter counter when $CaCl_2$ was mixed with a condensed phosphate at the concentration used in the present work in the absence of

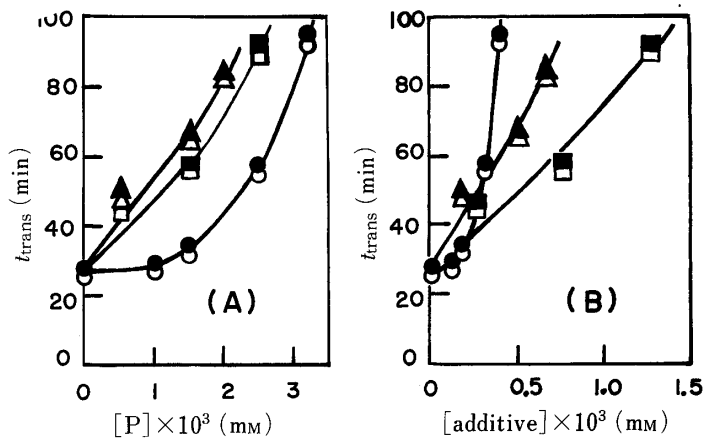

Fig. 3. Relationship between Concentration of Condensed Phosphate and Induction Period for the Crystallization of ACP to HAP

(A) Concentration of phosphorus atom of the condensed phosphate added, [P], *vs.* induction period, t_{trans}.
(B) Molar concentration of condensed phosphate added, [additive], *vs.* t_{trans}.
PPi: □ and ■, TPi: △ and ▲, HMPi: ○ and ●.
Open symobls show the data obtained from the time-courses of pH, and closed symbols from those of $[Ca^{2+}]$.

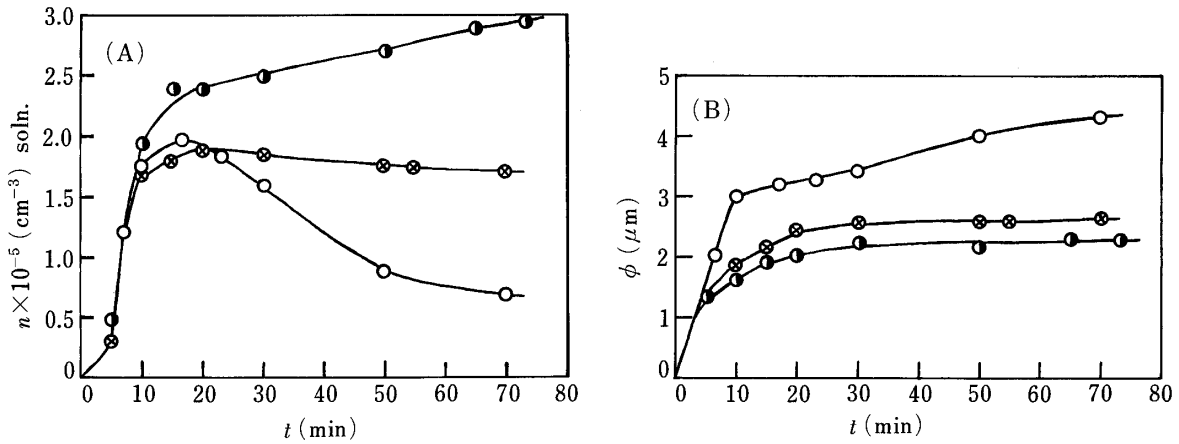

Fig. 4. Time-Course of Particle Formation in the Presence of TPi

(A) Number concentration of precipitated particles, n, *vs.* time, t.
(B) Mean diameter of precipitated particles, ϕ, *vs.* t.
Concentration of TPi added/mM: ○, 0; ⊗, 0.21×10^{-2}; ◑, 1.66×10^{-2}.

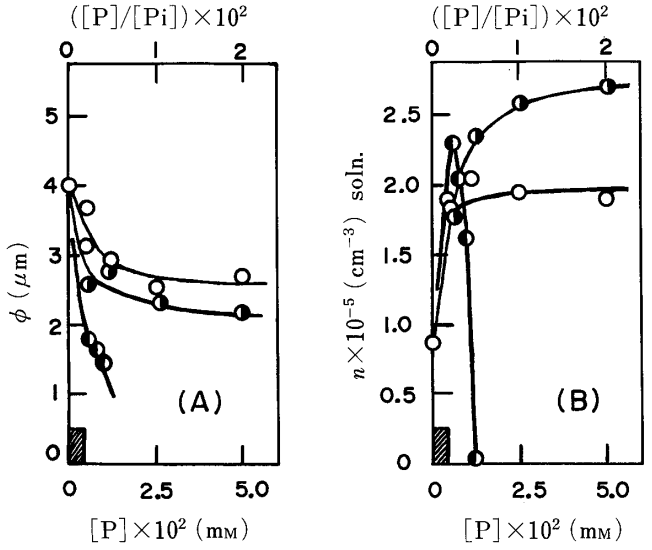

Fig. 5. Dispersing Effect of Condensed Phosphate on the Precipitated Particles

(A) Mean particle size, ϕ, *vs.* concentration of phosphorus atom of the condensed phosphate added, [P].

(B) Number concentration of the particles formed, *n*, *vs.* [P].

○: PPi, ◑, TPi, ◐: HMPi.

The data shown here were obtained from the time-courses for ϕ and *n* at 50 min after precipitate formation. The concentration range hatched on the bottom is that shown in Fig. 3. This shows that the effective concentration to disperse particles is considerably higher than that to inhibit the transformation.

When these figures are re-drawn as a function of the molar concentration of the condensed phosphate, the order of effectiveness of the condensed phosphates on ϕ and *n* is the same as that shown here, but the scale of the abscissa is different. Therefore, these relationships are omitted.

K_2HPO_4. Therefore, the particles counted by the Coulter counter were those of calcium orthophosphate (ACP and/or HAP), not calcium condensed phosphate. No remarkable changes, corresponding to t_{trans} in Figs. 1—3 and/or to the transformation of ACP to HAP were found in the time-courses of ϕ and *n* in Fig. 4.

Figure 5 shows the relationship between ϕ or *n* *versus* concentration of phosphorus atom, [P], of the condensed phosphate added. The ϕ and *n* data were obtained from the time courses at 50 min after precipitate formation (from Fig. 4 for TPi, for example). The abscissa on the top is graduated for molar ratio of phosphorus atom of the condensed phosphate added to orthophosphate added as a reactant, [P]/[Pi], where $[Pi]=[K_2HPO_4]=2.50$ mM. The figure shows that the remarkable dispersing effect was already seen even at a ratio of less than 1/100. The ϕ decreased and *n* increased monotonously with increasing concentration of condensed phosphate (except *n* for HMPi (◐)).

The order of effectiveness of the dispersing ability was: HMPi > TPi > PPi, where the sequence was the same as that found in Fig. 3(B). The marked decrease of *n* for HMPi (◐; $[P]>0.5\times10^{-2}$ mM) was due to the fact that fine particles, having a diameter smaller than that of the lower limit of detection of the Coulter counter (1 μm in the present work), were extensively formed by virtue of the strong dispersing effect of HMPi added. The possibility that the precipitate might not be formed (that is, the solution is supersaturated) was ruled out by the fact that $[Ca^{2+}]$ decreased immediately after mixing $CaCl_2$ with K_2HPO_4 even in the presence of HMPi (see Fig. 2, for example).

In order to compare the effect of the amount of Pi added with that of condensed phosphate added, the precipitate was formed in a 5% excess of K_2HPO_4 (*i.e.*, 2.63 mM instead of 2.50 mM K_2HPO_4) in the absence of condensed phosphate. The ϕ and *n* data were obtained at 50 min after the precipitate formation. The ϕ increased by 12% and *n* decreased by 7% as compared with those for 2.50 mM K_2HPO_4 (see the values at $[P]=0$ in Figs. 5(A) and (B)). On the other hand, when 2.37 mM Pi was added instead of 2.50 mM Pi, ϕ decreased by 12% and *n* increased by 23% at 50 min. Therefore, excess Pi in this case acted as an aggregating agent rather than a dispersing agent, in contrast to the effect of condensed phosphates.

Dispersing Effect of Condensed Phosphates on Ripened HAP Particles

The degree of peptization (▽), the optical density (●) and the mean particle diameter (○) of ripened HAP are shown in Fig. 6 as a function of concentration of added PPi. The effective concentration ranges for the dispersion were broad and obscure but roughly coincide

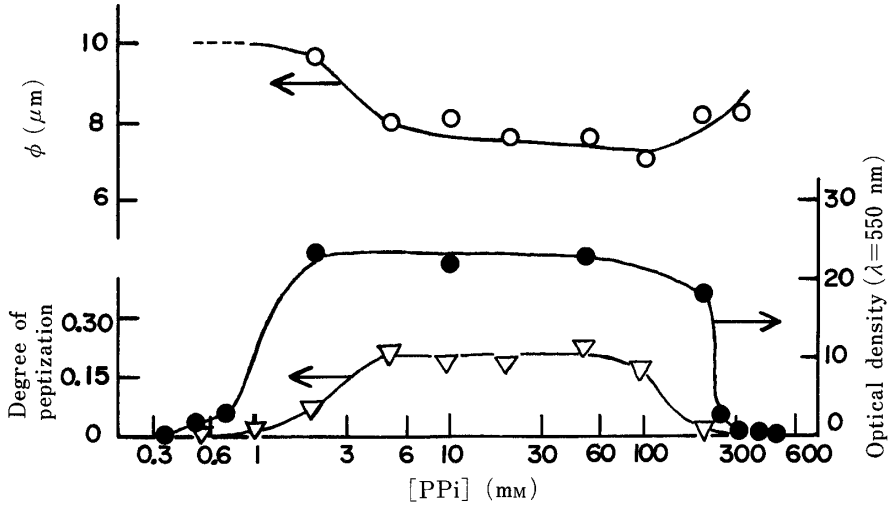

Fig. 6. Dispersion of Ripened HAP as a Function of Concentration of Diphosphate Added

○, particle mean diameter; [HAP]=0.02 g/dl; ●, optical density; [HAP]=5 g/dl; ▽, degree of peptization; [HAP]=10 g/dl. 154 mM NaCl is used as a dispersing medium.

TABLE I. The Effect of Condensed Phosphates on the Dispersion of HAP Particles

Condensed phosphate	Conc. for the max. degree of peptization (mM)[f]	Max. degree of peptization	Conc. for the min. value of particle mean diameter (mM)[g]	Min. value of particle mean diameter (μm)[h]
154 mM NaCl[a]	—	*ca.* 0	—	(10.0)
Pi[b]	10—20	0.02	*ca.* 20	7.8
PPi[c]	5—80	0.20	10—100	7.5
TPi[d]	3—20	0.27	7—30	6.2
HMPi[e]	10—40	0.29	5—40	5.9

a) As a reference. *b*) K_2HPO_4/154 mM NaCl. *c*) $K_4P_2O_7$/154 mM NaCl. *d*) $Na_5P_3O_{10}$/154 mM NaCl. *e*) Sodium hexametaphosphate (NaHMPi)/154 mM NaCl. *f*) [HAP]=10 g/dl. *g*) [HAP]=0.02 g/dl. *h*) Mean particle diameter of dried HAP powder is *ca.* 0.155 μm.[5]

among the three measurements. A similar trend was also observed in the presence of TPi or HMPi (not shown). The degree of dispersion increased and leveled off with increase in the concentration of the condensed phosphate. When the concentration was further increased, the degree of dispersion decreased again owing to the high ionic strength of the condensed phosphate added.

The effects of these condensed phosphates are summarized in Table I, from which the following conclusions may be drawn: the effective concentration ranges (the 2nd and 4th columns) for the maximum degree of dispersion (the 3rd and 5th columns) were roughly the same among these condensed phosphates; and the order of effectiveness in terms of the degree of dispersion was HMPi > TPi > PPi > Pi > 154 mM NaCl. This sequence is the same as that obtained from Figs. 3(B) and 5 although the effective concentrations (and/or concentration ranges) are different from those in Figs. 3(B) and 5.

Discussion

The effective concentrations of the condensed phosphates were 10^{-4}—10^{-3} mM for

retarding the transformation (Fig. 3), 10^{-3}—10^{-2} mM for inhibiting the aggregation (Fig. 5), and 3—10^{2} mM for disaggregating the ripened HAP particles (Table I). Although these numerical values are different, these effects are all caused by the adsorption of the condensed phosphates on the surface of the particles. As the transformation of ACP to HAP (the Ostwald ripening) is delayed by the adsorption of condensed phosphate on the active sites for the crystal growth on HAP,[8] the effective concentration is the lowest (see the molar ratio in Figs. 2 and 3; [PPi]/[Pi] $\sim 10^{-4}$). On the other hand, as the disaggregation and/or inhibition of aggregation are caused through electrostatic repulsion and steric hindrance by the condensed phosphate adsorbed on the surface of the particles, a higher concentration is needed than that for the inhibition of crystal growth. The difference in the concentrations effective for the dispersion of precipitated CaPi particles and of synthesized HAP particles might be due to the difference in the particle properties (ripened or not, for example) or due to the fact that the precipitate particles are formed in the presence of excess orthophosphate, while the ripened HAP is not contaminated by any excess electrolytes by virtue of the process of water-rinsing during the preparation.[5]

In the previous paper it was shown that Na_2Chs disperses the HAP particles efficiently,[5,9] though the induction period, t_{trans}, for the transformation of ACP to HAP was not affected in the presence of Na_2Chs.[8] On the other hand, as mentioned above, condensed phosphate retards the transformation (see Figs. 1—3). The difference in the effects of Na_2Chs and condensed phosphate may be due to the difference in their affinity for CaPi (ACP and HAP): condensed phosphate may be more tightly adsorbed on the surface of CaPi than Na_2Chs by virtue of the terminal phosphate group, which is similar in structure to the orthophosphate ion, *i.e.*, the lattice ion of CaPi. It is already known that the amount of adsorption of Pi on the surface of HAP is scarcely affected in the presence of Na_2Chs because Pi is selectively adsorbed by virtue of being the lattice ion.[5] The condensed phosphate ion may compete for the adsorption site with Pi and interrupt the crystal growth of HAP by replacing Pi on the surface of HAP and by blocking the site of crystal growth.[10,11]

The inhibiting effect of PPi against pathological and undesirable concretion, such as urolithiasis,[1-3] may be explained by the following mechanism on the basis of the results mentioned above: (1) PPi prevents the aggregation of small calcified particles even though they have been formed from supersaturated body fluids; (2) particles adsorbing PPi may not deposit easily on the surface of the soft tissues due to the effect of electrostatic repulsion between them; (3) small particles thus remaining in the body fluids may be excreted more easily than agglomerated ones; and (4) the fact that PPi retards the transformation of ACP to HAP is favorable for preventing the formation of calculi because ACP is more soluble than HAP (see Fig. 2, for example). Thus, PPi is useful in small amounts to prevent concretion, although a portion of PPi may be hydrolyzed by enzymes in the human body or on the surface of CaPi formed.[12]

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