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# Formation of Hydroxyapatite in the Presence of Phosphorylated Polyvinylalcohol as a Simplified Model Compound for Mineralization Regulator Phosphoproteins

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**Transformation of amorphous calcium phosphate (ACP) to hydroxyapatite (HAP) and subsequent crystal growth of HAP were retarded in the presence of phosphorylated polyvinylalcohol (Phos. PVA) and phosphoserine (PSer), while the mother compounds, polyvinylalcohol (PVA) and serine (Ser), showed no specific effect on these factors over the concentration range investigated. The retardation was caused through the competitive adsorption between inorganic phosphate ion (Pi, one of the lattice ions) and the phosphate group of the organic compounds (*i.e.*, Phos. PVA and PSer) for the active growth sites on the HAP crystal or nucleus. Phos. PVA was about 20 times stronger than PSer in its effect owing to the fact that the thick adsorption layer of the former repels Pi more efficiently than the thin adsorption layer of the latter. These results suggest that high molecular phosphorylated compounds such as phosphoproteins are more significant in regulation/retardation of biological mineralization/crystallization in mammalian body than low molecular phosphorylated compounds such as PSer.**

**Keywords** hydroxyapatite; crystal growth; induction time; phosphoprotein; polyvinylalcohol; phosphorylated polyvinylalcohol; serine; phosphoserine

Hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$; HAP) is known as the main inorganic component of hard tissues (*i.e.*, bones and teeth). It directly crystallizes and grows in the mother solution when the degree of supersaturation is low. On the other hand, it is formed *via* amorphous calcium phosphate (ACP), octacalcium phosphate (OCP), or dicalcium phosphate dihydrate (DCPD) when the degree of supersaturation in the mother solution is high.[1] Formation of hard tissues and/or crystallization of HAP in the human body are affected physicochemically by many kinds of proteins, which play an important role in the regulation of mineral deposition and HAP crystal growth on the matrix. These proteins are called regulator proteins. It is known, for example, that amelogenin, present in immature dental enamel tissue, regulates enamel mineralization through the adsorption on the HAP crystal seeds.[2] Kuboki *et al.*[3] suggested that calcium-induced precipitation of osteonectin (a bone protein) is a possible mechanism *via* which osteonectin might interact with $Ca^{2+}$ and participate in the initial immobilization of $Ca^{2+}$ to induce the nucleation of calcification in bone tissues. Doi *et al.*,[4] however, showed that precipitation formation of ACP, transformation of ACP to HAP, and crystal growth of HAP are retarded in the presence of osteonectin due to the adsorption and blocking effect on the active growth sites of these nucleated calcium phosphates. Thus, the function of osteronectin on calcification is not yet clear.

The function of phosphoproteins is also ambiguous. It has been suggested that both the matrix-bound and soluble phosphoproteins may serve dual roles: making the initial mineral deposition on the collagen matrix and inhibiting the calcification of soft tissues.[5,6] Moreno *et al.*[7] and Hay *et al.*,[8] however, recently showed that salivary phosphoprotein such as statherin could readily prevent accretion of minerals of ACP and/or HAP on the tooth surface by adsorption onto the enamel surface. They further pointed out the physiological significance of the salivary protein: it inhibits unwanted precipitation (*i.e.*, calculi) of calcium phosphates in the salivary glands, although human salivary secretions are supersaturated with respect to most calcium phosphate salts.

A strong retardation of HAP formation in an aqueous phase was noted in the presence of a small amount of milk protein casein.[9] The inhibitory action increased in the order $\kappa$-$<\beta$-$<\alpha_{s1}$-casein corresponding with the content of phsphoserine (PSer) residues in these caseins. Adsorption through phosphorylated serine groups of the casein molecules to the active growth sites of HAP resulted in retardation of the HAP growth. Retardation was also observed by the addition of a simple phosphorylated amino acid, PSer,[10] instead of phosphoproteins containing PSer residues such as that quoted above. Low molecular diphosphate[11] and diphosphonate[12] are also strong inhibitors of HAP crystallization and transformation of ACP to HAP. Ethane-1-hydroxy-1,1 diphosphonate (EHDP), for example, is known as an anticalcification agent for bioprosthetic heart valve,[12] whereas it depresses the dissolution rate of ripened HAP when it is added to the HAP suspending medium.[13] Both effects originate from the adsorption *via* terminal phosphonate groups to the surface of seed or crystals of HAP in a manner similar to that of ester phosphates. The effects of low and high molecular phosphorylated compounds, however, have not been compared to date.

In the present paper, phosphorylated polyvinylalcohol (Phos. PVA) was prepared as a polymeric model compound for phosphoproteins. PSer was assumed to be a monomeric model compound because phosphoproteins usually contain PSer residues. The effects of these model compounds on the formation of HAP and on the transformation of ACP to HAP will be discussed, taking into consideration the effects of unphosphorylated PVA and serine (Ser). This make it possible to examine the differences in the influence between the phosphorylated and unphosphorylated compounds of a given molecular size (*i.e.*, PSer *vs.* Ser, and Phos. PVA *vs.* PVA), and between the low and high molecular compounds at a given ester phosphate concentration (*i.e.*, PSer *vs.* Phos. PVA).

## Experimental

**Materials** All the reagents used in the present study were of analytical grade and were used without further purification. Polyvinylalcohol was

Gohsenol NL-05, kindly provided by Nippon Gohsei Kagaku Co., Ltd. Although the degree of saponification of the raw material was almost 100%, it was thoroughly saponified in the presence of an excess amount of NaOH, and dialyzed against water to remove low molecular contaminants. The viscosity average molecular weight, $M$, and the degree of polymerization were determined as $1.98\times10^4$ and $4.50\times10^2$ by the following equation,[14]

$$[\eta]=6.60\times10^{-4}M^{0.64} \quad (1)$$

where the intrinsic viscosity, $[\eta]$, at 30 °C of PVA was 0.375 dl/g.

Phosphorylation of PVA was done as follows according to the method of Kurose *et al.*[15] dicyanodiamide (10 g) and urea (15 g) were dissolved in 50 ml of *N,N'*-dimethylformamide (DMF). An additional 50 ml of DMF and 10 ml of 100% phosphoric acid, prepared from 100 g of 85% phosphoric acid and 39.4 g of $P_2O_5$, were poured into the above solution. Six grams of PVA was added after the temperature of the bath reached 140 °C, and allowed to react for 80 min. The raw reaction solution was dialyzed with Visking cellulose tubing for 24 h against cold water, and a further 7 d against cold water after the sample solution was made acidic with hydrochloric acid. The Phos. PVA thus obtained was freeze-dried and kept in acetone.

The phosphorylation of PVA was confirmed by phosphate-31 nuclear magnetic resonance ($^{31}$P-NMR) spectroscopy, elemental analysis, pH-titration, and viscosity measurement. According to the $^{31}$P-NMR spectroscopy with an external reference of 85% $H_3PO_4$ (200 MHz, JEOL JNM-FX200 FT-NMR), the peak corresponding to the ester phosphate was found around −11 ppm. Elemental analysis for phosphorus showed that hydroxyl groups of original PVA were phosphorylated by 10—20%. The reproducibility of the degree of phosphorylation was not good, and varied in every batch. The pH-jump was observed at the first equivalence point of the ester phosphoric acid, but it was weaker than that of inorganic phosphoric acid of the same phosphorus concentration. The content of the ester phosphoric acid, determined by pH-titration, was in fair agreement with that determined by elemental analysis. The jump at the second equivalence point was not found experimentally, but the solution pH gradually increased with the volume of titrant NaOH. The viscosity data is given in the next section (see Fig. 1).

**Methods** Total calcium concentration was determined by ethylenediamine tetraacetic acid (EDTA) chelatometry at pH 13 with 1-(2-hydroxy-4-sulfo-1-naphthylazo)-2-hydroxy-3-naphthoic acid (*i.e.*, NN indicator). Total inorganic phosphate concentration was determined by colorimetry at 720 nm according to the method of Gee *et al.*[16] Organic ester phosphate was determined by the residue on ignition.

The X-ray powder diffraction (Cu$K_\alpha$ radiation at 35 kV and 10 mA) was examined with a diffractometer (Toshiba ADO-301) at the diffraction angle $2\theta=31.8$ deg, which is specific for HAP, at room temperature. Samples for the diffraction measurement were obtained from time to time by filtration (0.2 $\mu$m Millipore filter) of a precipitate prepared by mixing 10 mM $K_2HPO_4$ with 5 mM $CaCl_2$ in the presence of 0.9% NaCl (= 154 mM) and a known concentration of additive at 25 °C. The jelly-like precipitate was thoroughly dehydrated and kept in acetone. The pH of the filtrate was 6.8—7.5, depending on the added species and on the elapsed time following the precipitate formation.

Mean diameter ($d_m$) of precipitate particles was measured by a Coulter counter (Coulter Electronics, Inc., type TA-II, aperture size 100 $\mu$m) at room temperature (*ca.* 25 °C) at 100 min after mixing 2.50 mM $K_2HPO_4$ with 1.25 mM $CaCl_2$ in the presence of 0.9% NaCl and a known concentration of various additives. The pH of the medium (7.6—7.9) after precipitate formation did not vary significantly with the concentration of additives because excess phosphate behaved as a buffering agent.

Calcium ion activity was determined at 35 °C using an Orion calcium-sensitive electrode (type 9320BN) connected to an Orion expandable ion analyzer (model EA940). Prior to the measurement on the sample solution, the calcium electrode was calibrated with an aqueous solution of $CaCl_2$ in the presence of 0.9% NaCl, taking the activity coefficients into consideration. The elecrode exhibited Nernstian response over the range of concentrations of the present work. If necessary, measured activity was converted to the concentration of free $Ca^{2+}$, $[Ca^{2+}]$. The suspension/solution pH was measured by a Hitachi-Horiba pH-meter (model M-1). The pH and $[Ca^{2+}]$ of the mother solution were traced for about 100 min from immediately before mixing 1.25 mM $CaCl_2$ with 2.5 mM $K_2HPO_4$ in the presence of 0.9% NaCl and a given amount of the additive in order to determine the induction time from ACP to HAP. The calcium phosphates (ACP and/or HAP), formed by mixing these chemicals, were colloidal white precipitate.

The measurement of solution viscosity was made in a Ubbelohde type viscometer at 30 °C.

The adsorption amounts of PSer and Ser were not determined in this study. However, it was reported elsewhere[7] that PSer is 8—10 times more highly adsorbed by HAP than Ser by virtue of the ester phosphate group, which shows higher affinity toward HAP than the OH-group of Ser. Although the adsorption amount of PVA NL-05 and Phos. PVA were not determined in the present paper, both compounds were assumed to be absorbed to some degree by HAP on the basis of the behavior shown in Figs. 2—6. The adsorption amount of Phos. PVA was considered to be higher than that of PVA NL-05 by the fact that Phos. PVA has the ester phosphate group and OH-group while PVA NL-05 has only OH-group in its molecules. The affinity of phosphate group to HAP must be higher than that of OH-group even in the macromolecules, in the same manner as mentioned above. In the present paper, discussion will be made on the assumption that Ser, PSer, PVA NL-05, and Phos. PVA are adsorbed by HAP.

## Results

**Viscosity of a Dilute Solution of Phos. PVA** Figure 1A shows the relationship between polymer concentration, $c_p$, and reduced viscosity, $\eta_{sp}/c_p$, for PVA NL-05 and Phos. PVA. Reduced viscosity of PVA NL-05 (○) decreased linearly with decrease in $c_p$. This is typical behavior of nonionic linear flexible polymer in an aqueous solution.[17] The reduced viscosity of Phos. PVA, on the other hand, increased when diluted with an aqueous solution of a given concentration of NaCl ([NaCl] = 0—20 mM), while it decreased with an increase in NaCl concentration at given $c_p$. Figure 1B shows the relationship between $\eta_{sp}/c_p$ and degree of neutralization of Phos. PVA, where $c_p$ was kept

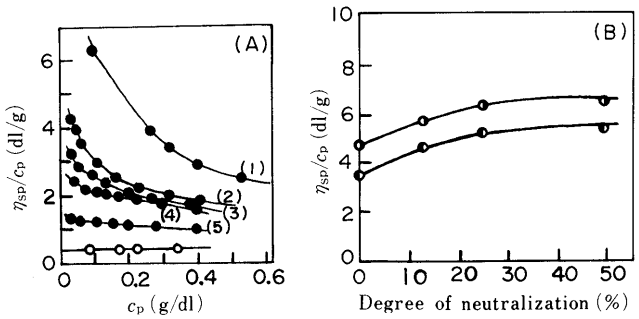


Fig. 1. Changes of Reduced Viscosity with Polymer Concentration (A), and Degree of Neutralization (B)

The degree of phosphorylation of the polymer was 15.2%. Viscosity was measured at 30 °C. (A) Open circles show reduced viscosity for PVA NL-05 in pure water. The closed circles show reduced viscosity for the acid type Phos. PVA in the presence of various concentrations of NaCl. [NaCl] (mM) = 0 (curve 1), 0.5 (2), 1.0 (3), 2.0 (4), and 20.0 (5). (B) Concentration of Phos. PVA as an acid type, $H_2$ Phos. PVA, $c_p$(g/dl) = 0.2 (◑) and 0.3 (◐).

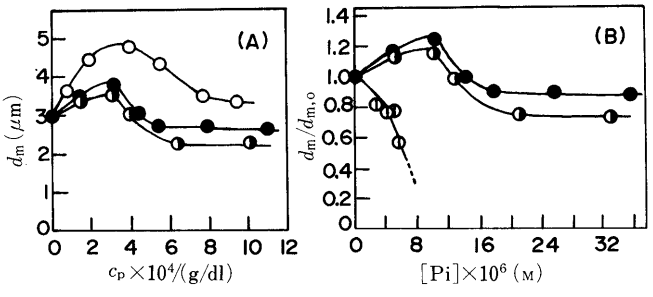


Fig. 2. Mean Diameter of Precipitate Particles

Additives: ○, PVA NL-05; ●, $H_2$Phos.PVA; ◑, $Na_2$Phos.PVA; ⊕, PSer. Abscissas in (A) and (B) show the polymer concentration converted to that of PVA NL-05, and the concentration of phosphorus of the esters, respectively, in order to compare the effects of additives on a common scale. The degree of phosphorylation of Phos.PVA was 19.0%.

constant at 0.2 or 0.3 g/dl. With increase in the degree of neutralization adjusted by NaOH, $\eta_{sp}/c_p$ increased monotonously. The results shown in Fig. 1A and B are typical viscometric behavior of linear flexible polyelectrolyte.[17] In other words, these facts confirmed the phosphorylation of PVA NL-05 (see Experimental).

**Mean Diameter of Secondary Particles** Figure 2A shows the relationship between mean diameter, $d_m$, of the secondary particles of the calcium phosphates and concentration, $c_p$, of added polymer. After attaining maximum, $d_m$ decreased with $c_p$. This tendency was found to be common with three polymers, PVA NL-05, acidic Phos. PVA ($H_2$Phos. PVA), and Phos. PVA neutralized with NaOH ($Na_2$Phos. PVA). The increase in $d_m$ (*i.e.*, aggregation) was due to the interparticle bridging effect of polymers adsorbed on the precipitate particles, while the decrease in $d_m$ (*i.e.*, dispersion) was caused by the interparticle repulsion effect through polymers adsorbed on the surface of each particle. The value of $d_m$ decreased in the order PVA NL-05 > $H_2$Phos. PVA > $Na_2$Phos. PVA, depending on the charge density of the polymer chain.

Mean diameter was barely affected by Ser up to $5\times10^{-2}$ mM (data not shown), while it monotonoulsy decreased with increase in the concentration of PSer. These effects of Ser and PSer were quite different from those of polymers, as mentioned above. Figure 2B shows the relationship between relative value of the mean diameter, $d_m/d_{m,0}$, and total phosphorus concentration, [Pi], of added Phos. PVA or PSer, where $d_{m,0}$ is the mean diameter at [Pi]=0, or at $c_p=0$ in Fig. 2A. In this way, it is easy to compare the effect of Phos. PVA phosphate group with those of PSer.

Mean diameter of the precipitate particles in the presence of PSer steeply decreased with the concentration, and became too small to be precisely determined by a Coulter counter when the concentration of PSer exceeded $8\times10^{-3}$ mM. The concentration of added PSer, [Pi]$=8\times10^{-3}$ mM, for example, is very small compared with the initial concentration of $Ca^{2+}$ (=1.25 mM). Therefore, it can be said that the decrease in $d_m$ does not originate from the consumption of $Ca^{2+}$ through ion binding with PSer; it must rather come from the specific adsorption of PSer to the surface of particles through the phosphate group, since, as stated, Ser did not affect $d_m$. It is interesting that phosphorylated compounds (*i.e.*, PSer and Phos. PVA) are more effective than unphosphorylated compounds (*i.e.*, Ser and PVA NL-05) in decreasing the mean diameter ($d_m$), owing mainly to the esterified phosphate group.

**X-Ray Powder Diffractometry of the Precipitate** No diffraction peaks were detected on a precipitate immediately after precipitation. However, a specific diffraction peak developed at diffraction angle $2\theta=31.8$ deg, and the diffraction strength increased with time. It levelled off at around 20 min after the precipitate formation. This result means the initial precipitate is ACP which subsequently crystallizes to HAP.

Figure 3A shows the diffraction strength as a function of elapsed time after the precipitation formation. The strength after the levelling-off depended on species and concentration of the added polymer. Figure 3B shows the relationship between peak height and concentration of the added polymer. The peak height was almost constant irrespective of the concentration of PVA NL-05, whereas it decreased with the concentration of $H_2$- and $Na_2$Phos. PVA. The effect of these polymers seemed almost the same, although, strictly speaking, the peak height in the presence of $Na_2$Phos. PVA was slightly lower than that in the presence of $H_2$Phos. PVA.

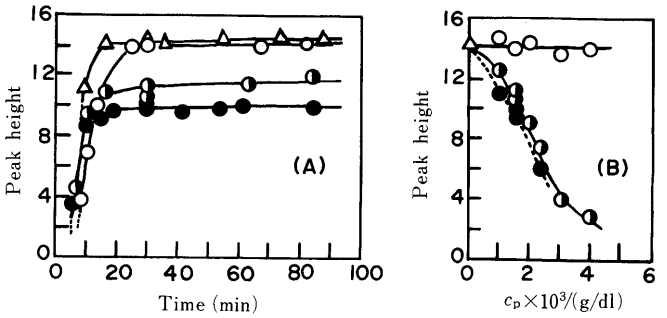


Fig. 3. Diffraction Strength as a Function of Time (A) and Polymer Concentration (B)

Additives: △, none; ○, PVA NL-05; ◑, $H_2$Phos.PVA; ●, $Na_2$Phos.PVA. The degree of phosphorylation of Phos.PVA was 8.17%. The diffraction was examined at $c_p=1.6\times10^{-3}$ g/dl (A), and at $t=30$ min after the precipitate formation (B). Polymer concentration, $c_p$, at the precipitate formation is shown as the concentration reduced to that of PVA NL-05 in order to compare the polymer effects on a common scale.

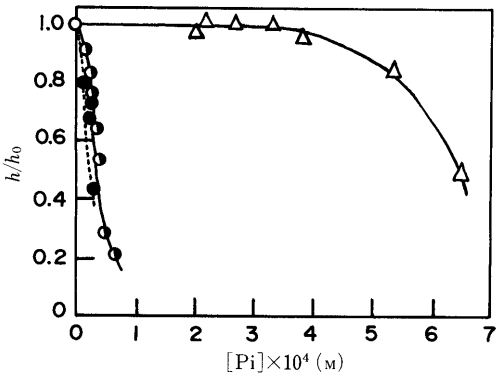


Fig. 4. Relative Diffraction Strength as a Function of the Phosphorus Concentration of the Added Ester Phosphate

Experimental conditions were the same as in Fig. 3. The experimental data for Phos.PVA are quoted from Fig. 3. Additive: ○, none; △, PSer; ◑, $H_2$Phos.PVA; ●, $Na_2$Phos. PVA.

Similar measurement was done in the presence of Ser and PSer to study their effects on the crystallinity of the precipitate. The diffraction strength at $2\theta=31.8$ deg decreased with increase in the concentration of PSer, while Ser showed no effect up to 1.2 mM. That is, unphosphorylated compounds such as Ser and PVA NL-05 hardly affect the peak height and/or degree of crystallization, but phosphorylated compounds such as Pser and Phos. PVA inhibit the crystallization to HAP. To compare the effect of Phos. PVA with that of PSer on a common scale, the relationship between [Pi] and relative peak height ($h/h_0$) is shown in Fig. 4, where [Pi] is a concentration of the added ester phosphorus in mole/liter units, and $h$ and $h_0$ are the peak height in the presence and absence of phosphorylated compounds at $t=30$ min after precipitate formation. According to Fig. 4, the concentration of PSer should be about 20 times higher than that of Phos. PVA to achieve the same effect on $h/h_0$.

**Transformation of Amorphous Calcium Phosphate to Hydroxyapatite** In a previous paper[18] and in Fig. 3A here, it was shown that ACP was transformed to crystalline HAP spontaneously after a certain induction period. The induction time was little affected in the presence of sodium chondroitin-6-sulfate,[18] while it increased with the concentration of added condensed phosphate such as diphosphate or triphosphate.[19] In the present paper, the effects of the phosphorylated and unphosphorylated organic compounds on induction time were studied using ion-selective electrodes.

Figure 5A shows the time courses of pH and concentration of free $Ca^{2+}$ ($[Ca^{2+}]$) in the presence and absence of $Na_2Phos$. PVA. The pH increased while $[Ca^{2+}]$ decreased steeply almost along the ordinate immediately after addition of $K_2HPO_4$ to an aqueous solution of $CaCl_2$. The decrease in $[Ca^{2+}]$ is attributable to the formation of

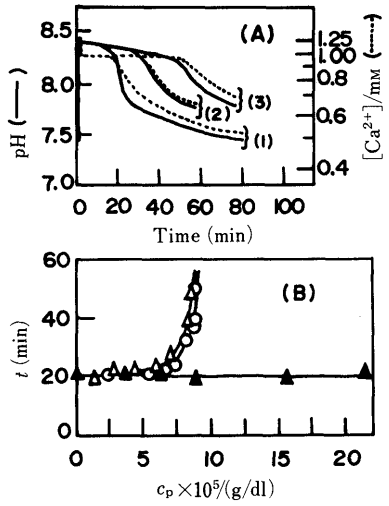


Fig. 5. Time Courses of pH and $[Ca^{2+}]$ (A), and Induction Time as a Function of Polymer Concentration (B)

(A) ——: time course of pH, ------: time course of $[Ca^{2+}]$ [$Na_2Phos.PVA$] $\times 10^5$ (g/dl) = 0 (curve 1), 9.75 (2), and 10.8 (3). Degree of phosphorylation of Phos.PVA was 8.17%. Measurements were done at 35 °C. (B) Polymer: $H_2Phos.PVA$ (△), $Na_2Phos.PVA$ (○), and PVA NL-05 (▲). Abscissa shows the polymer concentration converted to that of PVA NL-05 in order to compare the effect of Phos.PVA with that of PVA NL-05.

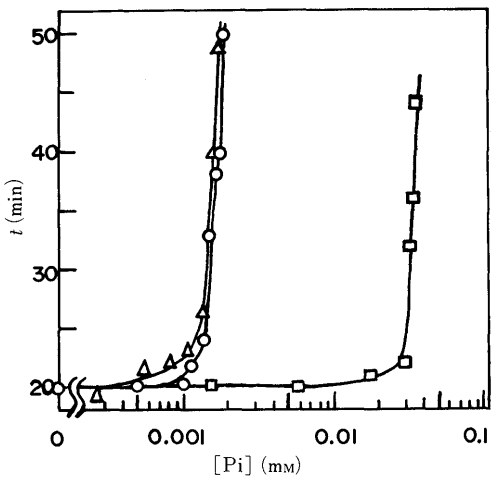


Fig. 6. Relationship between Induction Time and Phosphorus Concentration of Added Ester Phosphate

Data for $H_2$- and $Na_2Phos.PVA$ are quoted from Fig. 5 (B). Additive: PSer (□), $Na_2Phos.$ PVA (○), $H_2Phos.$ PVA (△).

ACP, and the increase in pH is due to the protonation of the excess Pi remaining in the mother solution. After an induction period, pH and $[Ca^{2+}]$ steeply decreased again; that is, $OH^-$ and $Ca^{2+}$ were simultaneously consumed from the mother solution. The decreases reflect the transformation of ACP to HAP, because the solubility of HAP is lower than that of ACP[19] and, therefore, HAP consumes $OH^-$, $Ca^{2+}$, and $PO_4^{3-}$ in the mother solution as the lattice ions when it crystallizes from ACP. The induction period, $t$, was determined from the intersection of the tangents drawn to the curves of pH and $[Ca^{2+}]$ just before and after the second steep decrease.[11,18] The induction periods obtained from these two curves agreed reasonably well.

The relationships between polymer concentration, $c_p$, and the induction time thus determined are shown in Fig. 5B. In $Na_2$- and $H_2Phos.$ PVA, the induction time began to increase at $c_p = ca.\ 7 \times 10^{-5}$ g/dl, while no significant increase in the induction time was observed up to $c_p = 2.2 \times 10^{-4}$ g/dl in PVA NL-05.

Similar measurements were done in the presence of bovine serum albumin (BSA), Ser, and PSer. Little specific effect of BSA (up to $2 \times 10^{-2}$ g/dl) or Ser (up to $7 \times 10^{-2}$ mM) was found, whereas PSer showed the retardation effect on the transformation of ACP to HAP in the concentration range higher than *ca.* $3 \times 10^{-2}$ mM.

Figure 6 shows the relationship between the induction time and phosphorus concentration of the ester phosphates, [Pi], in millimolar units. According to the figure, the concentration of PSer should be *ca.* 20 times higher than that of Phos. PVA to observe the same induction time. It is interesting that the value obtained here is quite the same as that obtained by X-ray powder diffractometry (see Fig. 4).

## Discussion

The transformation of ACP to HAP occurs through synchronous re-dissolution of ACP and recrystallization to HAP with consumption of $OH^-$, $Ca^{2+}$, and $PO_4^{3-}$ from a mother solution, but not through ion rearrangement in the solid state.[11,18] The binding of phosphorylated compound to the embryo and/or nucleus of HAP results in retardation of the initiation of HAP crystal growth (Figs. 5 and 6), while the competitive adsorption between the ester phosphate group and inorganic phosphate ion results in the reduced crystallinity of HAP (Figs. 3 and 4). This competition occurs easily on the active growth sites where inorganic phosphate ion should bind as the lattice ion, because the structure and size of the ester phosphate group are similar to those of inorganic phosphate ion. That is, adsorption and/or binding of ester phosphate, instead of inorganic phosphate ion, thwarts the crystal growth of HAP.

Although both phosphorylated (PSer and Phos. PVA) and unphosphorylated compounds (Ser and PVA NL-05) were assumed to be absorbed by HAP,[7] the former thwarted crystallization but the latter not. This is because the affinity of the ester phosphate group for the active growth site is higher than that of the hydroxyl group of Ser and PVA NL-05. Thus, the ester phosphate group of organic compounds as well as condensed phosphates and phosphonates[11-13] has a significant role in the regulation of the crystal growth of HAP. On the other hand, Dales *et al.*[20] recently reported that the rate of crystal growth of

HAP is reduced in the presence of glucose. This is probably due to the cooperative adsorption blocking effect of the OH groups of glucose on a portion of the active growth sites. This depressing effect of polyhydric glucose is in contrast to the sparse effects of monohydric Ser.

Some phosphate groups along the polymer chain of adsorbed Phos. PVA participate in the adsorption in contact with HAP, whereas others remain on the polymer loops or tails protruding from the HAP surface. Negative charges of the ester phosphate groups in the thick adsorption layer effectively repel the inorganic phosphate ions toward the growth sites of HAP, resulting in strong inhibition of the crystal growth. Therefore, the effect of Phos. PVA is more remarkable than that of PSer which is adsorbed separately on the surface of HAP (see Figs. 4 and 6). As for the formation of secondary particles of calcium phsophates (Fig. 2), adsorbed PSer simply inhibits the aggregation through electrostatic and steric repulsion. Phos. PVA, in contrast, showed both dispersing and flocculating (or bridging) effects, depending on the polymer concentration and/or the amount of adsorbed polymer. This is another specific character of the polymer.

In conclusion, inhibition of the crystal growth of HAP was observed with Phos. PVA and PSer but not with PVA NL-05 and Ser. The significance of the phosphate group in the ester was recognized. The effect of Phos. PVA was 20 times greater than that of PSer by virtue of its specific polymer chain effect. These facts suggest that polymeric phosphorylated compounds in the animal body (*i.e.*, phosphoproteins) are more important than monomeric or low-molecular phosphorylated compounds in the regulation of the crystal growth of HAP and the formation of hard tissues.

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