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Effect of Chondroitin Sulfate on the Precipitate Formation of Calcium Phosphate in Water

SABURO SHIMABAYASHI* and MASAYUKI NAKAGAKI

Faculty of Pharmaceutical Sciences, Kyoto University, Yoshida-Shimoadachi-cho, Sakyo-ku, Kyoto 606, Japan

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The properties of calcium phosphate (CaPi) formed in supersaturated solution (prepared by mixing $CaCl_2$ and K_2HPO_4 at neutral pH) were studied by means of a Coulter counter and a calcium ion-specific electrode. It was found that the mean particle diameter (ϕ) of CaPi formed initially increased and then levelled off with time. The rate of increase of ϕ, however, decreased with increase in the amount of sodium chondroitin-6-sulfate (Na_2Chs) added to the mother solution, presumably because of the dispersing effect of Na_2Chs. The number concentration of formed particles of CaPi increased at first but after a short period it decreased with time because of the formation and aggregation of secondary particles. Remarkable consumption of Ca^{2+} was observed at two points in the time course of the precipitation reaction: (1) immediately after mixing the reactants, and (2) after an induction period (t_{trans} required for the crystallization of amorphous CaPi as hydroxyapatite (HAP), as was confirmed by measurement of the X-ray powder diffraction pattern. The lag time, t_{trans}, was not affected by the amount of Na_2Chs added, but decreased with increase in the amount of CaPi precipitated or of seed HAP inoculated. The physiological significance of the present work is briefly discussed.

Keywords——calcium phosphate; hydroxyapatite; hard tissue; renal calculus; chondroitin sulfate; dispersion; suspension; Coulter counter; calcium ion-specific electrode

Hydroxyapatite (HAP; $Ca_{10}(PO_4)_6(OH)_2$) is the main component of biological hard tissues (bone and tooth) and human renal calculi. Chondroitin sulfate (Chs) is found in bone matrix, cartilage,[1,2] dentine,[3] and urine.[4] Therefore, it seems important to understand the interaction of Chs and HAP in an aqueous phase. In the previous papers,[5,6] it was shown that Chs stabilizes HAP suspension as a result of adsorption, but does not affect the formation of HAP crystallites if aging is sufficient. In the present paper, the properties of calcium phosphate formed in a supersaturated solution are discussed on the basis of experimental data obtained by means of a Coulter counter and a calcium ion-specific electrode.

Experimental

Materials——Sodium chondroitin sulfate (Na_2Chs) was of C-type, *i.e.*, sodium chondroitin-6-sulfate (molecular weight 7—8×10^4), kindly provided by Kaken Yakukako Co., Ltd.[5,6] Seed HAP was the same sample as that used in the previous papers.[5,6] All the reagents used were purchased from Nakarai Chemicals Ltd. or Wako Pure Chemical Industries Ltd.

The unit of concentration for Na_2Chs in the present paper is that of molarity (mM) of the repeating two sugar residues. Therefore, 1 g/dl of Na_2Chs is equivalent to 19.9 mM Na_2Chs.

Methods——The sample solutions were made as dust-free as possible by filtration through a 0.22 μm Millipore filter prior to being introduced into the reaction vessel, which was also made dust-free by rinsing with clean water. Particle formation of calcium phosphate (CaPi) was carried out in a supersaturated solution prepared by adding $CaCl_2$ (1 M, 0.25 ml) and K_2HPO_4 (1 M, 0.50 ml) to an aqueous solution of 0.9% NaCl (= 154 mM, 200 ml) containing a known concentration of Na_2Chs. The molar mixing ratio of phosphate (Pi) to calcium ion (Ca^{2+}) was chosen as 2 (=[Pi]/[Ca^{2+}]) in order to inhibit the formation of $CaCO_3$ through the absorption of atmospheric CO_2 by excess Ca^{2+}.

Ripened HAP was inoculated as crystal seeds only in the case of ion-activity measurement. The inoculation was

not done in the measurement of particle size development because large particles of HAP often clog the orifice of the aperture tube of a Coulter counter.

Mean diameter (ϕ) and number concentration (n) of precipitate particles were measured by means of a Coulter counter (Coulter Electronics, Inc., type TA-II; aperture size 100 μm and sample volume 2 ml) at room temperature under constant stirring. The reagents were mixed by pouring them into the vessel from a pipet along the vessel wall in a consistent manner throughout the present work. The stirring speed was kept constant ("medium") by means of a stirrer fixed on the Coulter counter. Stirring was continued during the experiment, *i.e.*, from the time immediately before the preparation of a supersaturated solution through to the termination of the measurements.

Calcium ion activity ($a_{Ca^{2+}}$) was measured at 35 °C by using an Orion calcium-sensitive electrode (type 93-20) connected to an Orion Microprocessor Ionalyzer (model 901). The electrode was calibrated with an aqueous solution of $CaCl_2$ containing 154 mM NaCl. The electrode exhibited a Nernstian response over the range of concentrations used in the present 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 of calcium ions was approximately constant due to the high ionic strength (154 mM NaCl). The pH was measured with a pH-meter (Toa HM-5ES) at 35 °C. Both pH and E were recorded concurrently on an electronic polyrecorder (Toa EPR-200A).

Each electrode (reference electrode, Ca^{2+}- and H^+-specific electrodes) was rinsed from time to time with a dilute aqueous solution of hydrochloric acid or sodium ethylenediaminetetraacetate (EDTA) to remove precipitates stuck to the surface, which may act as crystal seeds. This rinsing made the reproducibility of the data better than rinsing simply with distilled water.

The X-ray powder diffraction pattern (CuK_α radiation at 35 kV and 10 mA with a nickel filter) was obtained with a Norelco Geiger counter diffractometer (Philips Electronics and Pharmaceutical Industry) at room temperature.

Results and Discussion

Time Courses of Mean Diameter and Number Concentration

Figure 1 shows time courses of ϕ and n of the CaPi particles formed in a supersaturated solution containing a given concentration of Na_2Chs. The value of ϕ increases monotonously and then levels off. The value of n, however, decreases with time after attaining the maximum. Some information about the effect of addition of Na_2Chs is apparent from Fig. 1: (1) the appearance of particles detectable by a Coulter counter is retarded, (2) the initial slopes of ϕ

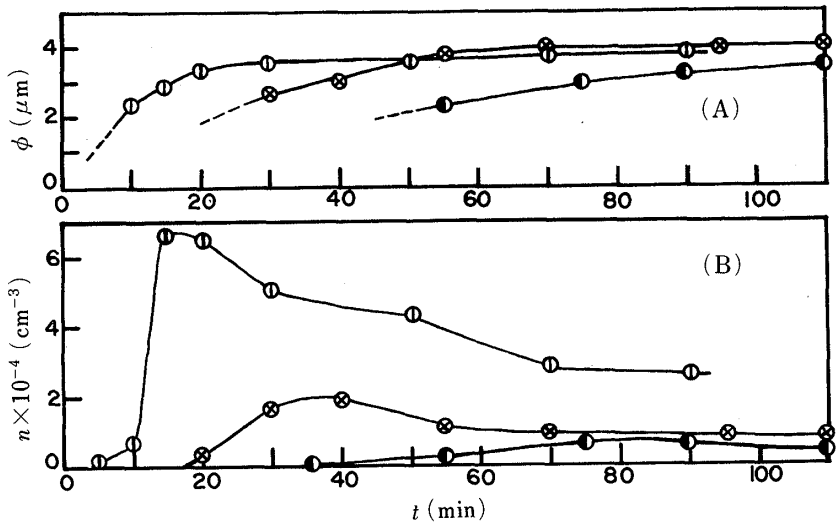

Fig. 1. Time Courses of Mean Particle Diameter and Number Concentration of Particles Formed at pH 7.4

(A) Mean particle diameter (ϕ) *vs.* reaction time (t).
(B) Number concentration of particles formed (n) *vs.* reaction time (t).
Initial concentration of each component: $[Na_2Chs]=0$ (◯), 0.018 (⊗), and 0.072 mM (◐). $[NaCl]=154$ mM, $[K_2HPO_4]=2.50$ mM, and $[CaCl_2]=1.25$ mM. K_2HPO_4 and subsequently $CaCl_2$ were added to an aqueous solution of NaCl containing Na_2Chs. When the particle size is small, a sample of 2 ml (see Experimental) is enough for particle counting but not for particle sizing. Therefore, initial data for n (B) of each run are shown but those for ϕ (A) are omitted because of their inaccuracy.

and n decrease, (3) the time at which n becomes maximum is delayed, and (4) the maximum value of n decreases with increase in the concentration of Na_2Chs added.

As ϕ is the mean diameter of particles and n is the number concentration of total particles irrespective of ϕ, $\pi\phi^3 n/6$ is the approximate volume concentration of particles detected by the Coulter counter. The time course of $\pi\phi^3 n/6$ was similar in pattern to that of n, having a lag in time and a maximum (not shown).

According to the previous paper,[5] the mean diameter of dried particles of CaPi was 0.15 μm. The value of ϕ shown in Fig. 1 (A) is far larger than the above. It can, therefore, be concluded that the particles observed by the Coulter counter are aggregated secondary particles of CaPi. Particles formed immediately after mixing the reactants might be too small to be detected by the Coulter counter. In a short time, however, the small primary particles grow and aggregate to form large secondary particles, resulting in the apparent time-lag and maximum observed in the time-course of n (Fig. 1 (B)), while ϕ increased monotonously (Fig. 1 (A)).

When Na_2Chs is added to a sample solution as dispersing agent,[5,6] ϕ should decrease and n should increase concomitantly with increasing concentration of Na_2Chs added, by virtue of its inhibiting effect on interparticle aggregation of CaPi particles formed. However, the counter cannot detect particles having a diameter smaller than the lower limit of the detection. Therefore, n apparently decreases with the amount of Na_2Chs added (Fig. 1 (B)) in spite of the decrease of ϕ (Fig. 1 (A)).

As the Coulter counter gives the relative volume (or weight) distribution of particles as a function of particle size on a logarithmic scale, the volume percentage (or volume concentration) of fine particles in the size distribution is relatively small as compared with their particle number concentration. Therefore, the change of ϕ (Fig. 1 (A)) with t or concentration of Na_2Chs was small as compared with the change of n (Fig. 1 (B)). The shape of the distribution curve scarcely changed, but moved along the abscissa of particle size as time passed and/or the concentration of Na_2Chs was increased (not shown).

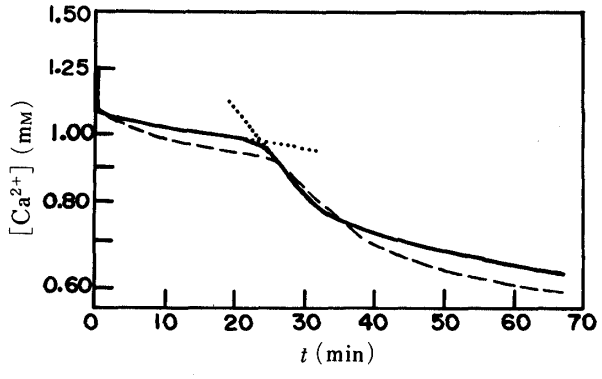

Fig. 2. Time Courses of Concentration of Free Calcium Ion at pH 7.4 and 35 °C

Initial concentration of each component: $[Na_2Chs]=0$ (——) and 0.0786 mM (------). $[NaCl]=154$ mM, $[K_2HPO_4]=2.50$ mM, and $[CaCl_2]=1.25$ mM. $CaCl_2$ and subsequently K_2HPO_4 were added to an aqueous solution of NaCl containing Na_2Chs. This order of addition is inverted from that of Fig. 1. The induction period for recrystallization (transition time, t_{trans}) is obtained from the intersection of the tangents (dotted lines) drawn to the curve, as illustrated on the full line.

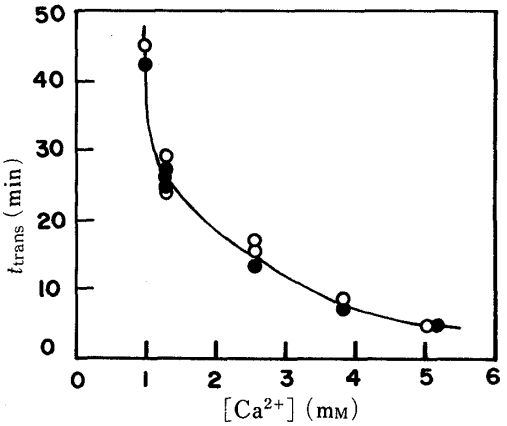

Fig. 3. Relationship between Concentration of Calcium Ion Added and Induction Period at 35 °C

Molar ratio of reactants: $[Ca^{2+}]/[Pi]=0.50$, and $[Chs]/[Ca^{2+}]=0$ (○) and 0.063 (●). The dispersing medium was 154 mM NaCl.

Time Course of Concentration of Free Calcium Ion

Figure 2 shows a time course of concentration of free calcium ion, $[Ca^{2+}]$. The concentration decreases sharply immediately after the addition of K_2HPO_4 to an aqueous solution of $CaCl_2$ (plus 154 mM NaCl) because of Ca^{2+} consumption owing to the formation of a precipitate of CaPi. A second steep decrease of $[Ca^{2+}]$ is found at around $t=25$ min, following a gradual lowering of $[Ca^{2+}]$, irrespective of the presence or absence of Na_2Chs.

The concentration of Na_2Chs shown in Fig. 2 is high enough to disperse CaPi particles according to the results shown in Fig. 1 (◐). The small difference between the full line and the broken line in Fig. 2 could be within the limits of experimental error. Therefore, it can be concluded that the time and the degree of the steep decrease of $[Ca^{2+}]$ are almost independent of the concentration of Na_2Chs added and of the degree of the dispersion of CaPi (*i.e.*, ϕ and n).

The relationship between initial concentration of Ca^{2+} and the time (t_{trans}) at which the second steep drop begins is shown in Fig. 3, where the ratios of the initial concentration, $[Ca^{2+}]/[Pi]$ and $[Chs]/[Ca^{2+}]$, are kept constant. The presence of Chs has almost no effect on t_{trans}, but t_{trans} decreases with increase in the concentration of Ca^{2+} added, that is, in the amount of CaPi precipitated. On the other hand, t_{trans} seems to increase to infinity when $[Ca^{2+}]$ decreases to about 1 mM (and $[Pi]=2$ mM), and the amount of the precipitate (CaPi) formed decreases concomitantly. This concentration is at the solubility limit of CaPi, and at lower concentrations than this, no precipitate of CaPi will appear.

Füredi-Milhofer *et al.*[7] showed that t_{trans} decreases slightly in the presence of gelation (0.04%). Their result is in contrast with that of the present work (Figs. 2 and 3). However, they did not discuss the reason why t_{trans} decreases.

X-Ray Powder Diffraction Pattern

Precipitates isolated from the reaction vessel at intervals were tested by an X-ray powder diffraction technique (Fig. 4(A)). The diffraction intensity at $2\theta=31.8°$ (a specific diffraction angle for HAP) is shown in Fig. 4(B) as a function of the reaction time. Figures 4(A) and (B) show that CaPi formed is amorphous initially, followed by a transformation to crystalline HAP. The abrupt increase of the diffraction intensity (Fig. 4(B)) indicates that the transition occurs after an induction period.

With reference to Figs. 2 and 3, therefore, it can be concluded that the steep decrease of

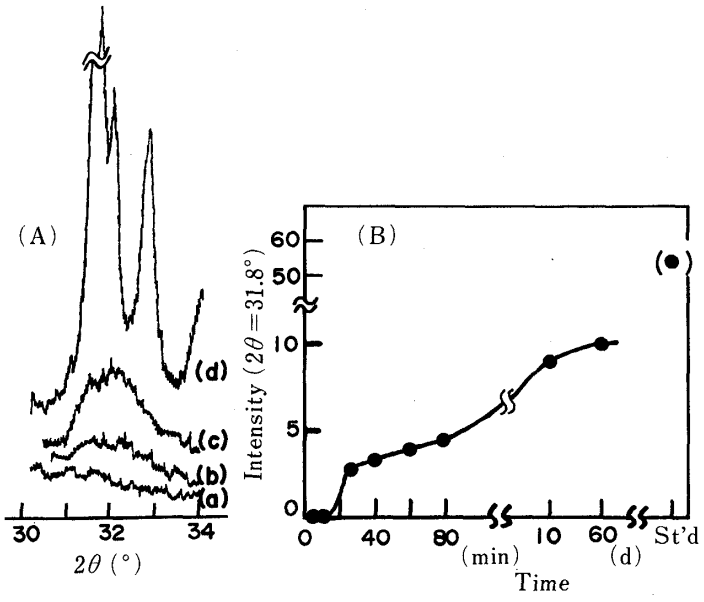

Fig. 4. X-Ray Powder Diffraction Patterns of the Precipitates

(A) X-Ray powder diffraction patterns of the precipitates. Precipitates were obtained at 5 min (a), 25 min (b), and 10 d (c) after mixing K_2HPO_4 (9.85 mM) and $CaCl_2$ (4.93 mM) in an aqueous solution of NaCl (154 mM) in that order. These concentrations of the reactants are higher than those shown in Figs. 1, 2, and 5 in order to obtain a large amount of precipitate. Sample (d) is a standard HAP.[5,6]

(B) Time course of the diffraction intensity at $2\theta=31.8°$. Abbreviation "St'd" on the abscissa indicates the standard HAP.

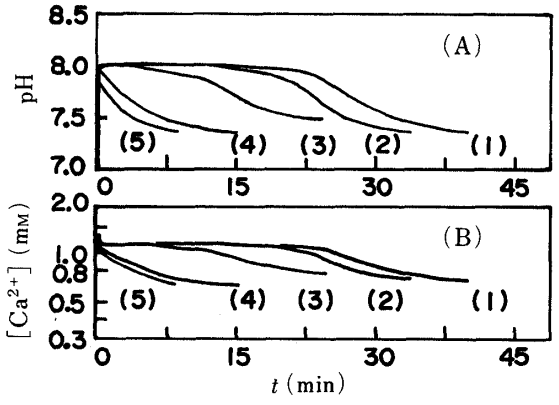

Fig. 5. Time Courses of pH and Concentration of Free Calcium Ion at 35 °C

(A) pH *vs.* time.
(B) $[Ca^{2+}]$ *vs.* time.
Concentration of HAP inoculated/(mg/l): (1) 0, (2) 2.5, (3) 24, (4) 250, (5) 500. The precipitate was formed by mixing $CaCl_2$ (1.25 mM), seed HAP, and K_2HPO_4 (2.50 mM) in an aqueous solution of NaCl (154 mM) in that order of mixing. The ordinate for $[Ca^{2+}]$ is graduated with a logarithmic scale.

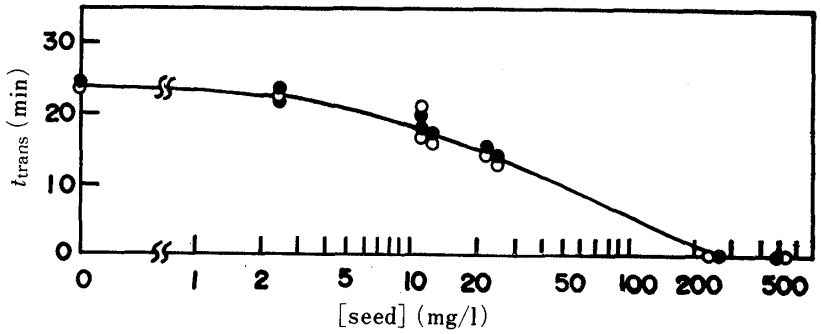

Fig. 6. Relationship between Concentration of HAP Inoculated and Induction Period

○: t_{trans} obtained from the pH data. ●: t_{trans} obtained from the Ca^{2+} concentration data.

$[Ca^{2+}]$ at t_{trans} is also due to the transformation of amorphous CaPi to crystalline HAP.

This conversion can be explained in terms of the so-called Ostwald law of stage.[8] That is, amorphous CaPi plays the roll of a precursor, which is not the thermodynamically stable phase but appears easily as a metastable phase. Therefore, HAP is preceded by an amorphous CaPi.[9,10]

Effect of HAP Added as a Seed

Figure 5 shows the consumptions of OH^- and Ca^{2+} in the presence of various amounts of seed HAP as a function of the reaction time. Decrease of pH (*i.e.*, consumption of OH^-) is synchronized with consumption of Ca^{2+}. Concurrent consumption of Ca^{2+} and OH^- supports the idea of crystallization of amorphous CaPi as HAP, in accordance with the results of the X-ray diffraction pattern (Fig. 4).

The induction period (t_{trans}), obtained in the same manner as that shown in Fig. 2, is shown in Fig. 6 as a function of the amount of seed added. The time, t_{trans}, decreases with an increase in the amount of seed added, falling to zero ultimately.

As the transition of amorphous CaPi to crystalline HAP occurs through Ostwald ripening, metastable CaPi dissolves prior to the crystallization as HAP. Therefore, the greater the total surface area of the seed HAP, the more easily the lattice ions (Ca^{2+}, OH^-, and PO_4^{3-}) can deposit on the surface of the seed HAP and the more rapidly the crystallization as HAP occurs. This results in the decreased of t_{trans} (Fig. 6).[11]

Similarly, the greater the amount of CaPi precipitation, the larger is the total surface area of the precipitate. Therefore, the dissolution rate of amorphous CaPi increases, and crystallization as HAP is accelerated with increasing amount of precipitate formed, as shown in Fig. 3.

Physiological Meaning of the Present Results

Chs in urine is known to be an inhibitor of urinary stone formation. This effect may arise for two reasons: (1) it blocks the formation of abnormally large aggregates of CaPi in a supersaturated solution (Fig. 1); and (2) it can, moreover, disperse HAP aggregates to small secondary particles, even though large aggregates are formed temporarily.[6]

On the other hand, it is known that glycosaminoglycans such as Chs are broken down just prior to the time when the matrix begins to calcify to form the hard tissues.[12] If this did not happen, calcified tissues (*i.e.*, physiologically systematized aggregates) might be partially destroyed because of the dispersing ability (Fig. 1) and Ca^{2+}-binding capacity of Chs.[13] However, crystallization of the precursor CaPi as biological HAP might be accelerated and proceed smoothly by virtue of the presence of the HAP crystallized in advance (Fig. 5) irrespective of the concentration of Chs (Fig. 2). Chs does not hinder the crystal growth and/or the adsorption of the lattice ions of HAP.[5,6,14]

In summary, Chs makes the particle size small but scarcely affects the induction time of the HAP crystallization. The induction period, however, decreases with increase in the concentration of CaPi formed or HAP seed crystals inoculated. The effect of Chs present in the human body requires further study, however.

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