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Competitive Adsorption of Phosphate Ion with Chondroitin Sulfate Ion and Its Effect on the Dispersion of Hydroxyapatite

SABURO SHIMABAYASHI,* SHIGEYUKI SUMIYA, TOSHITAKA AOYAMA, and MASAYUKI NAKAGAKI

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

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The amounts of concurrent adsorption of anionic species, *i.e.*, chondroitin sulfate (Chs) and phosphate (Pi), to synthesized hydroxyapatite (HAP) were determined. The amount of adsorption of Chs decreased with increasing concentration of Pi added. On the other hand, the adsorption of Pi was not inhibited in the presence of Chs. The affinity of HAP for Pi, one of the lattice ions, is significantly greater than that for Chs. Such a specific adsorption seems favorable for crystal growth in solutions containing various kinds of solutes and/or in the body fluids. The optical density (O.D.) of the HAP suspension, as an index of the suspension stability, was measured at 550 nm. The contour lines of the O.D. as a function of the concentrations of Pi and Chs showed that Chs, coexisting with Pi, exhibits a dispersing and protecting effect on the HAP suspension except at high concentrations of both Pi and Chs. It was concluded that Chs affects the formation of secondary particles of HAP (*i.e.*, aggregation and disaggregation), but does not affect the formation of the primary particles or the crystal growth of HAP through the selective adsorption of the lattice ions.

Keywords——hydroxyapatite; chondroitin sulfate; phosphate ion; competitive adsorption; crystal growth; suspension stability; aggregation; optical density; hard tissue; renal calculus

Chondroitin sulfate (Chs) is found in cartilage, bone, and so on, as a form of protein complex, which plays a role of paramount importance in the calcification of biological hard tissues.[1] Furthermore, Chs in urine is an inhibitor of renal calculi formation.[2] On the other hand, it is well-known that the major constituent of the biological hard tissues, urinary stones, dental calculus, and other pathological depositions of calcium salt in abnormal or unusual locations is hydroxyapatite (HAP), $Ca_{10}(PO_4)_6(OH)_2$. The formation and/or crystallization of the calcified tissues are achieved by capturing and adsorbing the lattice ions from the body fluids at the surface of the crystal seeds.

Adsorption isotherms of phosphate ion (Pi), one of the lattice ions, on HAP were studied in the previous paper.[3] It was shown that the adsorption is neither of simple Langmuir type nor completely reversible. Adsorption of the lattice ions should be regarded as an important elemental process in the crystal growth of HAP. In the present paper, concurrent adsorption of Chs and Pi to HAP is discussed. Competitive adsorption may occur because the negative charges of the adsorbates, Chs an Pi, would repel each other and the ions may compete for the adsorption sites on the surface of the adsorbent, HAP.

Secondary particles of aggregated HAP may be concomitantly disaggregated into small particles by virtue of the mutual repulsion between negative charges of Pi and Chs adsorbed on the surface of HAP. Aggregation and disaggregation of HAP particles might be related to the formation and destruction of clusters of calcified tissues, and, furthermore, to the structural strength and hardness of biological hard tissues.[4] It is, therefore, important to investigate the effects of Pi and Chs on the dispersion of HAP particles. The purpose of the present work was to study the adsorption and dispersion properties of HAP particles in the presence of Pi and Chs in water.

Experimental

Materials——Chondroitin sulfate (Chs) used was the same sample as described in the previous paper.[5] It is the C-type sodium salt (molecular weight 7×10^4—8×10^4), kindly provided by Kaken Yakukako Co., Ltd.

Hydroxyapatite (HAP) was prepared by mixing stoichiometric amounts of $Ca(OH)_2$ (230 g) and H_3PO_4 (3 M: 900 ml) in boiling water (6 l) in the same manner as described elsewhere.[6] The HAP formation reaction is shown by Eq. 1.

$$10\ Ca(OH)_2 + 6\ H_3PO_4 \longrightarrow Ca_{10}(PO_4)_6(OH)_2 + 18\ H_2O \quad (1)$$

The preparation of HAP in the presence of Chs was carried out in an aqueous solution of 0.03% NaChs instead of pure water, through the same procedure as described above. According to the stoichiometry of Eq. 1, the concentration of HAP which should be formed in the solution under these conditions is *ca.* 5 g/dl, which is the same concentration as that used in the dispersion experiment shown in Fig. 4. The concentration of Chs added (0.03%) is, therefore, enough to disperse the HAP particles, as mentioned later.

The precipitated HAP was filtered off, washed with water and acetone, and dried at 110 °C *in vacuo*. The pH of the filtrate was about 6.3. The X-ray diffraction patterns for both HAP samples were typical of HAP, and chemical analysis showed the samples to be almost stoichiometric (Ca/P = 10.3/6).

The other reagents used were of analytical grade.

Methods——X-Ray powder diffraction patterns (Cu K_α radiation at 35 kV and 10 mA with a nickel filter) were obtained with a Norelco Geiger counter diffractometer (Philips Electronics and Pharmaceutical Industry) at room temperature. The crystallite size of the (hkl) plane, D_{hkl}, was calculated from the half-width of the X-ray powder diffraction peak by means of Eq. 2,[6]

$$D_{hkl} = \frac{0.94\ \lambda}{\beta_{1/2} \cos\theta} \quad (2)$$

where λ is the wavelength (1.5405 Å), $\beta_{1/2}$ is the half-width of the diffraction peak (rad), and θ is the diffraction angle (rad) by the (hkl) plane.

Specific surface area, S, and/or mean particle diameter, d, of the dried HAP powder were measured by means of a Shimadzu powder surface area instrument (model SS-100).[7]

Adsorbate solutions containing various concentrations of both Chs and Pi were prepared by mixing aqueous solutions of NaChs and Na_2HPO_4 immediately before the addition of adsorbent HAP powder. HAP (1 g) was suspended in a test tube containing 20 ml of a given adsorbate solution of known concentrations, [Chs] and [Pi], at 25 °C, and shaken vigorously for about 5 min.

After 45 h at 25 °C, the optical density (O.D.) at 550 nm of the suspension in the upper part of the test tube was measured with a Shimadzu UV-180 spectrophotometer after decantation. A portion of the added HAP had already sedimented to the bottom and the rest remained suspended in the water phase. These was not a sharp interface between the sediment phase and the dispersion liquid phase. Therefore, the O.D. can be regarded as a convenient index of the stability of the HAP suspension. The higher the O.D., the more stable the suspension. It was confirmed that the O.D. is approximately proportional to the concentration of HAP remaining suspended in the medium. Therefore, the O.D. can be determined after dilution to a suitable concentration if the O.D. is too high to measure directly. It was also confirmed that there is no difference between HAP particles settled at the bottom and those remaining in the suspending medium with respect to X-ray powder diffractometry.

After standing for 5 d at 25 °C, the filtrate through a Millipore filter (0.22 μm pore size) was analyzed for Pi an Chs. The equilibrium concentrations of Pi and Chs were determined by colorimetry according to the methods of Gee *et al.* (at 720 nm),[3,8] and of Bitter *et al.* (at 530 nm),[5,9] respectively. It was confirmed that the coexistence of Pi and Chs in the sample solution does not cause mutual interference in the determinations. It was also found that the Millipore filter does not adsorb Pi or Chs. Therefore, the amounts of adsorbed Pi, x_{Pi}, and adsorbed Chs, x_{Chs}, were calculated from the difference of adsorbate concentrations before and after addition of HAP particles. The adsorption isotherm was determined as a function of free concentration of adsorbate ion.

It is known that the adsorptions of Chs and Pi are not exactly reversible with respect to dilution.[3] Therefore, the amounts adsorbed and the extent of HAP dispersion in the presence of the ions vary depending upon the order of mixing of water, adsorbate solution (Chs and Pi), and HAP powder. Accordingly, HAP powder was consistently added last to the pre-mixed adsorbate solution in the present work.

The pH of adsorbate solution before addition of HAP was 5.9 through 8.8, depending upon the concentrations of NaChs and Na_2HPO_4 added. The pH after attaining adsorption equilibrium became slightly higher (8.1—9.1). It is known that the equilibrium pH is increased by the adsorption of anionic species owing to the concomitant adsorption of H^+ and/or desorption of OH^-.[10]

Results

Adsorption Isotherm for Chs

The adsorption isotherms for Chs at various ratios of the concentrations of the added adsorbates, $[Pi]_t/[Chs]_t$, are shown in Fig. 1, where the subscripts "t" and "f" mean the total concentration (*i.e.*, added concentration) and the free concentration (*i.e.*, equilibrium concentration) of each species. The initial steep slope of the isotherm without Pi (◐) indicates high-affinity adsorption of Chs, but the amount of Chs adsorbed, x_{Chs}, decreases with increase in $[Pi]_t/[Chs]_t$.

The relationship between x_{Chs} and $[Pi]_t/[Chs]_t$ is shown in Fig. 2, where $[NaChs]_f$ is kept constant. The curves show that the amount of adsorption, x_{Chs}, decreases steeply with $[Pi]_t/[Chs]_t$. These results show that the presence of Pi inhibits the adsorption of Chs.

Adsorption Isotherm for Pi

The adsorption isotherms for Pi at various ratios of the concentrations, $[Chs]_t/[Pi]_t$, are shown in Fig. 3, where almost all of the data points appear to lie on the same curve. The data enclosed with the dotted line in Fig. 3(A) are shown on a large scale in Fig. 3(B), where all the experimental points also lie on almost one curve. This adsorption isotherm was not of the Langmuir type, as mentioned elsewhere.[3] The results show that the presence of Chs does not affect the amount of adsorption of Pi in contrast to the case of Chs adsorption in the presence of Pi.

Optical Density of HAP Suspension

The relationship between O.D. and $[NaChs]_t$ are shown in Fig. 4, where the ratio of the concentrations, $[Pi]_t/[Chs]_t$, is kept constant for each curve. The prominent feature of this figure is a maximum in the O.D., except in the case when Pi was not added (●, $[Pi]_t=0$). This maximum decreases with increasing concentration ratio, $[Pi]_t/[Chs]_t$, as shown by the dotted line, which is an envelope line for the curves.

The optical density is also shown as a function of $[Pi]_t$ (Fig. 5), where $[NaChs]_t/[Pi]_t$ is maintained constant. Again, the O.D. changes were bell-shaped, having a maximum. This maximum increases with $[NaChs]_t/[Pi]_t$. Figures 4 and 5 show that the dispersion stability of HAP is higher in the Chs solution without Pi (● in Fig. 4) than in the Pi solution without Chs (● in Fig. 5).

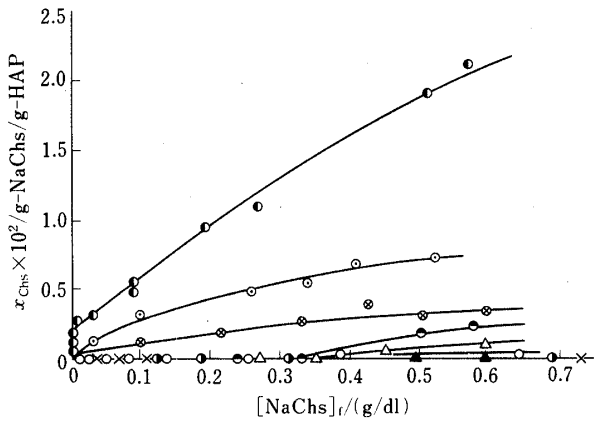

Fig. 1. Adsorption Isotherms for Chondroitin Sulfate at a Constant Concentration Ratio of $[Pi]_t/[Chs]_t$

$[Pi]_t/[Chs]_t$/(mol/base mol): ◐, 0; ⊙, 0.0395; ⊗, 0.170; ◓, 0.338; △, 0.847; ○, 1.70; ▲, 3.95; ◑, 8.48; ×, 23.7.

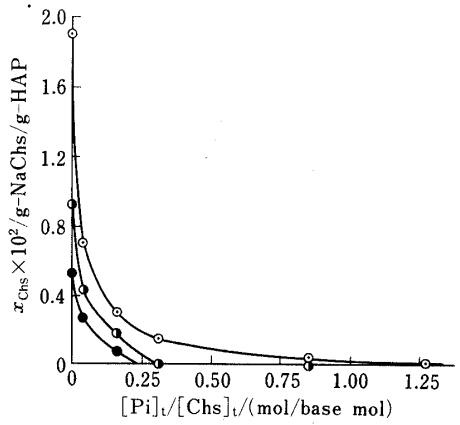

Fig. 2. Relationship between x_{Chs} and $[Pi]_t/[Chs]_t$ at a Constant Concentration of Free NaChs

$[NaChs]_f$/(g/dl): ⊙, 0.5; ◑, 0.2; ●, 0.1.

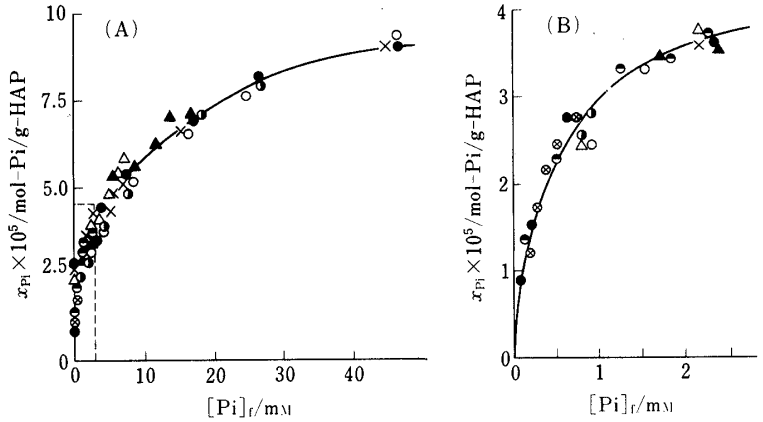

Fig. 3. Adsorption Isotherm for Phosphate Ion at a Constant Ratio of $[Chs]_t/[Pi]_t$

$[Chs]_t/[Pi]_t$/(base mol/mol): ●, 0; ×, 0.0422; ◑, 0.118; ○, 0.253; ▲, 0.590; △, 1.18; ◓, 2.95; ⊗, 5.90.

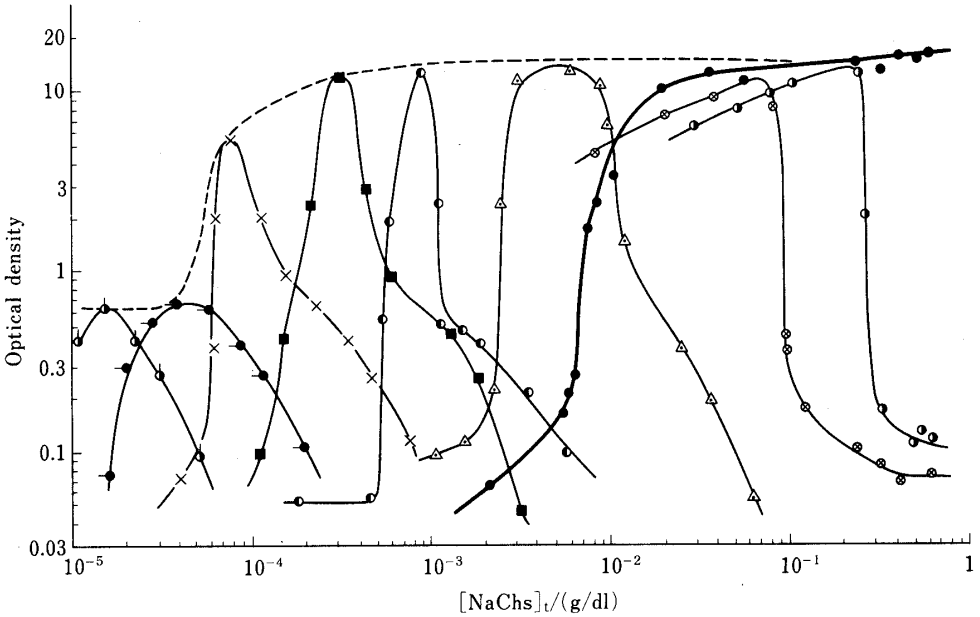

Fig. 4. Relationship between Optical Density and Concentration of NaChs Added

The concentration of HAP was kept constant at 5 g/dl. $[Pi]_t/[Chs]_t$/(mol/base mol): ◑, 1.06×10^4; -●, 2.66×10^3; ×, 664; ■, 177; ◐, 86.4; △, 8.47; ⊗, 0.847; ◑, 0.169; ●, 0.

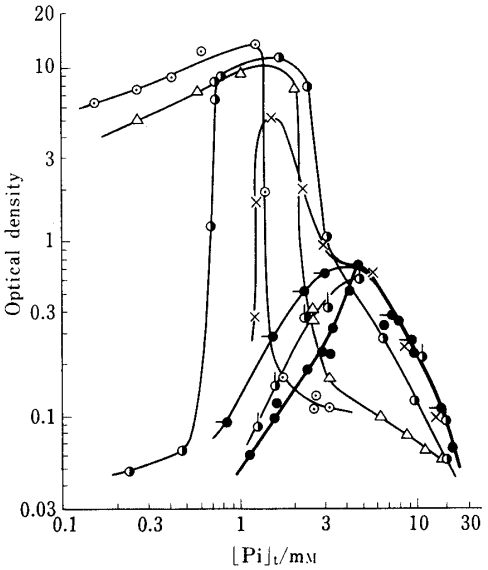

Fig. 5. Relationship between Optical Density and Concentration of Phosphate Added

The concentration of HAP was kept constant at 5 g/dl. $[NaChs]_t/[Pi]_t$/(base mol/mol): ●, 0; ◑, 9.40×10^{-5}; -●, 3.76×10^{-4}; ×, 1.51×10^{-3}; ◑, 0.253; △, 1.18; ⊙, 5.90.

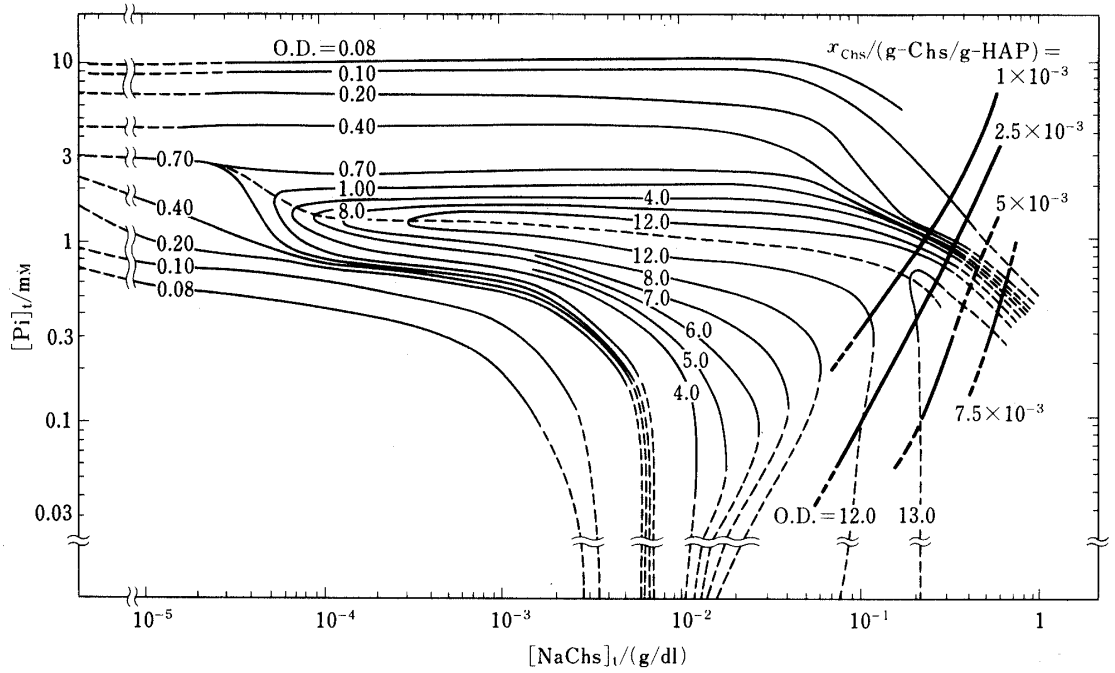

Fig. 6. Contour Lines for Optical Density and for the Amount of Adsorbed NaChs

The concurrent effects of $[Chs]_t$ and $[Pi]_t$ on the O.D. may become clearer when contour lines for the O.D. are drawn based on the results in Figs. 4 and 5. The diagram is shown in Fig. 6 with $[NaChs]_t$ as the abscissa and $[Pi]_t$ as the ordinate, where the HAP concentration is fixed at 5 g/dl. The contour lines for x_{Chs} are also shown on the right-hand side of the figure, but those for x_{Pi} are omitted because they become simply horizontal lines owing to the fact that x_{Pi} is independent of the concentration of added Chs, as shown in Fig. 3.

There is a "ridge" in Fig. 6 almost parallel to the abscissa, which corresponds to the envelope curve in Fig. 4. The O.D. (*i.e.*, the degree of suspension stability) decreases with an increase in $[Pi]_t$ when the concentration of Pi is higher than that at the ridge and the concentration of NaChs is lower than *ca.* 7×10^{-2} g/dl. The reason for the decrease of the O.D. may be the electrostatic shielding effect due to the increase of ionic strength in the solution caused by Na_2HPO_4 addition.

When $[NaChs]_t$ is larger than *ca.* 7×10^{-2} g/dl, the contour lines become crowded and the slope of the "descent" becomes steep. This steep slope corresponds to the steep decrease of the curves on the right-hand side of each maximum in Fig. 4 (◑ and ⊗, for example). This means that the HAP particles under these conditions aggregate more easily than under other conditions. This aggregation is found even though $[Pi]_t$ increases along the contour line of x_{Chs} (*i.e.*, at constant x_{Chs}), and even though $[NaChs]_t$ increases at constant $[Pi]_t$. The particle aggregation seems to be accelerated in the presence of a high concentration of Chs with coexistence of Pi.

Polymer chains of Chs adsorbed may bridge HAP particles to each other. The aggregation of HAP particles in this case may be attributed to both effects: the bridging effect of adsorbed Chs and the shielding effect of Na_2HPO_4 in the solution. However, the effect of polymer chains should be stressed because the suppression of the suspension stability becomes more marked with increase in the concentration of NaChs even though $[Pi]_t$ is kept constant. Polymer in solution, in general, behaves as a flocculating agent sometimes and as a dispersing agent at other times, depending on the experimental conditions.[11] NaChs also behaves as a dispersing agent, as mentioned later.

TABLE I. Mean Size of the HAP Particles Synthesized with or without Sodium Chondroitin Sulfate

	Specific surface area $S/(m^2/g)$	Particle mean diameter $d/\mu m$	Size of crystallite $D_{211}/Å$
HAP (A)	12.2	0.155	188
HAP (B)	12.6	0.152	195
$\frac{(B)-(A)}{(A)}$	+3.3%	−1.9%	+3.7%

HAP (A): synthesized without NaChs as a reference.
HAP (B): synthesized with 0.03% NaChs.

On the other hand, in the region where the Pi concentration is lower than that at the ridge and $[NaChs]_t$ is smaller than *ca.* 10^{-2} g/dl, the contour lines are also inclined to the right and/or downwards to the abscissa. This result means that Chs becomes a dispersing agent working together with Pi under these conditions. When $[NaChs]_t$ becomes high and $[Pi]_t$ remains low, the suspension stability increases with increase of the amount of Chs adsorption due to electrostatic repulsion and steric hindrance effects. This dispersing effect is prominent even though the amount of Chs adsorbed is still too low to be detected analytically, as can be seen from Fig. 6. These results are in contrast to the aggregation of HAP by Chs and Pi in the region where both $[NaChs]_t$ and $[Pi]_t$ are high, as mentioned previously.

The stability increases consistently with an increase in $[Pi]_t$ up to the ridge. This is caused by the effect of adsorption of Pi and Chs, *i.e.*, electrostatic repulsion and steric hindrance effects. These effects are greater than the shielding effect of the ionic "atmosphere" formed by the free Na_2HPO_4 in the bulk solution. The latter effect becomes dominant at higher concentrations than that at the ridge, as mentioned previously.

Synthesis of HAP in the Presence of NaChs

Specific surface area, S, particle mean diameter, d, and mean crystallite size for the (211) plane, D_{211}, of the HAP synthesized with or without NaChs (0.03%) are shown in Table I.

The bottom row in Table I shows the difference between the values for HAP (A) and HAP (B). The small difference between (A) and (B) may be within the limits of experimental error and it is clear that reproducibility of the particle formation of HAP is good irrespective of the coexistence of Chs. There was also no difference in D_{300} between HAP (A) and (B) (not shown). Therefore, it could be concluded that the presence of NaChs during the formation reaction of HAP affects neither the size of the primary particles nor the crystallite size present in the primary particles. It merely affects the size of secondary particles in the suspending medium.

Discussion

Phosphate ions become almost irreversibly attached to the surface of HAP by displacing coexisting Chs ions (Figs. 1—3). Similar inhibition of the adsorption of organic compounds by the lattice ions of HAP is also known: cetylpyridinium cation by Ca^{2+};[12] salivary proteins by Pi;[13] and human serum albumin by OH^-,[14] for example.

The adsorption sites for Chs and Pi seem to have positive charges generated by surface calcium ions and/or by defects of the lattice anions on the surface of HAP.[15] Therefore, it is reasonable to consider that the competitive adsorption of Chs and Pi takes place due to the mutual electric repulsions between their negative charges, followed by the desorption of Chs.

The reason for the preference for the lattice ion (Pi) may be that the size of the lattice ion is more appropriate for the adsorption site on the surface of HAP than that of the ionized group of Chs (*i.e.*, sulfate or carboxylate).[16] The adsorption of the lattice ion could be regarded as one of the microscopic processes of crystal growth. From this point of view, the preferential adsorption of the lattice ions is essential for the HAP crystals to grow in the mother solution or in body fluids containing various kinds of adsorbable compounds.

It is known that Chs ($\leqq$30 mg/l) affects the crystal growth of calcium carbonate (aragonite and calcite) by blocking some of the growth centers on the crystal surface due to its adsorption.[17] However, the results in Table I show that Chs does not interfere with the crystal growth of HAP. This is in accord with the fact that the amount of adsorption of Pi is independent of the concentration of Chs added, as shown in Fig. 3. The added Chs affects only the stability of the HAP suspension and the size of the secondary particles of the HAP aggregates, operating together with Pi. Therefore, the two processes, crystal growth and aggregation or disaggregation, seem to be governed by different factors.

Normal people can harmlessly excrete particles of calcium salt in their urine on occasions.[18] This fact implies that crystalluria alone may not be sufficient to cause urinary stones, although it may be a necessary contributory factor. The critical difference between stone-formers and normals may lie in the size, type and frequency with which calculi are passed by the two groups. Normal urines contain some inhibitors of crystal aggregation or growth (chondroitin sulfate, citrate and diphosphate, for example),[2] whereas the urines from stone-formers may contain less of the inhibitors. By blocking the formation of abnormally large aggregates and/or crystals, the inhibitors may play an important role in preventing crystalluria leading to stone formation.

Chs seems to inhibit the formation of undesirable urinary stones in at least two ways: its dispersing effect on the HAP particles, as mentioned in the present paper, and its strong calcium binding capacity.[5] The former may prevent the aggregation, sedimentation, and/or increase of the size of the secondary particles of HAP inside the kidney and/or the bladder in co-operation with other inhibitors. The latter may reduce the activity of calcium ion in the aqueous phase, leading to retardation of the crystal growth of HAP as calculi. However, the former effect may be predominant because of the low concentration of Chs in the urine.

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