Interaction of Hydroxyapatite with Sodium Chondroitin Sulfate and Calcium Chondroitin Sulfate in an Aqueous Phase

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The suspension pH at a given concentration of hydroxyapatite (HAP) decreased with the concentration of calcium chondroitin-6-sulfate (CaChs), whereas it increased with the concentration of sodium chondroitin-6-sulfate (Na_2Chs). The former effect is due to the increase in the concentration of H^+ by ion exchange between H^+ on the surface of HAP and Ca^{2+} of CaChs, and the latter is due to the protonation of phosphate ion (Pi) released from the surface of HAP. The adsorbed amount of chondroitin-6-sulfate anion (Chs) by HAP was higher with CaChs than with Na_2Chs over the concentration range examined. The equilibrium concentration of Pi decreased with increasing concentration of added CaChs because the concentration of free Ca^{2+}, which dissociates from CaChs, regulates the free concentration of Pi through the restriction of the solubility product of HAP (K_{sp}). In contrast, that in the presence of Na_2Chs increased with increasing concentration of added Na_2Chs owing to the anion exchange between Chs and Pi of the HAP surface. The total concentration of Ca^{2+}, which was released from HAP into the solution phase, increased after passing through a minimum with increasing concentration of added Na_2Chs. That is, the concentration of Ca^{2+} free from Chs decreased with an increase in the concentration of released Pi owing to the restriction of the solubility product, whereas that of Ca^{2+} bound by Chs increased with increasing concentration of added Na_2Chs through the ion exchange of Na^+ with Ca^{2+}. It was confirmed by the dialysis method that the value of K_{sp} was almost constant around 10^{-115}, although HAP dissolves incongruently in the presence of Na_2Chs.

Keywords hydroxyapatite; calcium chondroitin-6-sulfate; sodium chondroitin-6-sulfate; chondroitin-6-sulfate; solubility product; adsorption; ion exchange

Chondroitin sulfate (Chs) is one of the most abundant mucopolysaccharides in the human body, and is found in hard tissues (*i.e.*, bones and teeth) and soft connective tissues (cartilage and tendon, for example) as a form of protein complex.[1] It is reported that the weight-average molecular weight of Chs from normal articular cartilage is $1.9—2.1 \times 10^4$ in newborn and young individuals and *ca.* 1.6×10^4 in adults or the aged.[2] Chs also occurs in human urine, but the molecular weight ($0.8—1.3 \times 10^4$) of urinary Chs is lower than that reported for tissue Chs.[3] Chs in urine is one of the inhibitors of renal calculus formation.[4] The sulfated mucopolysaccharides, including Chs, from normal urine generally remain soluble in the presence of Ca^{2+}, whereas highly sulfated mucopolysaccharides from stone-forming urine appear to be a significant factor in calcium stone formation.[5]

Hydroxyapatite (HAP), $Ca_{10}(PO_4)_6(OH)_2$, is the main component of hard tissues and one of the constituents of urinary stones. Therefore, it is necessary to understand the interaction of HAP with Chs. Chen and Boskey[6] suggested that Chs plays an important role in the regulation of mineral deposition and HAP crystal growth through coating and/or adsorption effects. They emphasized the effect of sulfate groups of Chs by comparing the inhibitory effects before and after desulfation of Chs. On the other hand, Hunter *et al.*[7] showed that a high concentration of Chs inhibits the formation of HAP in collagen gels owing mainly to the binding of Ca^{2+} by Chs; that is, the initiation of the precipitation is delayed and the equilibrium amount of the precipitate decreases. They[8] also showed that Chs inhibits the formation of calcium pyrophosphate dihydrate. Chondroitin sulfate was a more potent inhibitor than keratan sulfate, in agreement with the greater affinity of Chs for Ca^{2+}.

In the previous papers, the dispersing action of sodium chondroitin sulfate (Na_2Chs) on HAP particles,[9,10] competitive adsorption of Na_2Chs with phosphate ion(Pi) on HAP,[9] concurrent adsorption of Na_2Chs with Ca^{2+} on HAP,[11] and precipitate formation of HAP in the presence of Na_2Chs[12] were discussed. In the present paper, the interaction of HAP with calcium chondroitin sulfate (CaChs) and with Na_2Chs will be discussed from the viewpoints of adsorption, ion exchange, and incongruent dissolution of HAP, by taking the counter ion effect of Chs into consideration. Significant differences were observed in the effects of Na_2Chs and CaChs. This is fundamental research to understand the interaction of biological Chs with normal hard tissues, pathological calculi, and artificial bones from the standpoint of physical chemistry.

Experimental

Materials Na_2Chs was of the C-type, *i.e.*, sodium chondroitin-6-sulfate (molecular weight $7—8 \times 10^4$; kindly provided by Kaken Yakukako Co., Ltd.), extracted from shark cartilage. It was the same sample as that used before.[12] CaChs was prepared by the use of cation exchange resin (Amberlite IR-120B) from Na_2Chs. The unit of concentration for Chs 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.

HAP used in the present study was commercial tricalcium phosphate (Nakarai Chemicals, Ltd.). Although it was labelled as tricalcium phosphate ($Ca_3(PO_4)_2$), the X-ray diffraction patterns and infrared (IR) spectrum were typical of HAP and chemical analysis showed the sample to be almost stoichiometric HAP (Ca/P = 1.66).

Other reagents used were of analytical grade. These were used without further purification.

Methods All the experiments were done at 30 °C. Adsorbate solution (100 ml) containing various concentrations of Na_2Chs or CaChs was prepared immediately before the addition of HAP powder (5 g). The HAP suspension was shaken vigorously from time to time.

After attaining equilibrium (at least 3 d after preparation), the filtrate (Millipore filter, pore size 0.22 μm) was analyzed for Chs, Ca^{2+}, and Pi. Immediately before the filtration, the suspension pH was measured by means of a pH meter (Yanaco model PH-8A).

The equilibrium concentrations of Chs and Pi were determined by colorimetry according to the methods of Disch, and Bitter and Muir[13] (at

530 nm), and Gee *et al.*[14] (at 720 nm), respectively. Total calcium (Ca^{2+} plus CaChs) free from HAP in the equilibrium solution 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). It was confirmed that the coexistence of Chs, Ca^{2+}, and Pi in the sample solution does not cause mutual interference in the determinations. It was also confirmed that the Millipore filter does not adsorb Chs, Ca^{2+}, or Pi. Therefore, the adsorbed amounts of Chs, Ca^{2+}, and Pi were calculated from the difference of adsorbate concentrations before and after the addition of HAP. In the present paper, the "desorbed amount" or "negative amount of adsorption" means the released amount of a certain ion from the HAP surface through dissolution and/or ion exchange (see Fig. 2).

Dialysis of the HAP suspension against the water phase was done as follows. The schema of the dialysis system is shown on the right-hand side of Fig. 5. Visking cellulose tubing containing distilled water (20 ml, compartment II) was sunk into an aqueous suspension of HAP (2 g) containing a given amount of Na_2Chs (50 ml, compartment I). This dialysis method is the reverse of the usual manner, because it was easier to add the HAP powder outside than inside of the dialysis bag. It was confirmed that equilibrium was attained within 3 d after the preparation with respect to the adsorption of Chs by HAP, the dissolution of HAP in an aqueous solution of Na_2Chs, the partition of the membrane-permeable species between compartments I and II, and the binding of the counter ions (Ca^{2+} and Na^+) with Chs. Through this dialysis procedure and the filtration on the Millipore filter, the concentration of Ca^{2+} free from both HAP and Chs (Ca^{2+} in compartment II), that free from HAP (free Ca^{2+} plus Ca^{2+} bound by Chs but free from HAP in compartment I), and the concentrations of Pi free from both HAP and Chs in compartments I and II can be determined directly at the same time.

Results

Adsorption of CaChs and Na_2Chs The adsorption isotherms of CaChs and Na_2Chs are shown in Fig. 1. Both isotherms increased steeply along the ordinate when the added concentration of Chs was low. The adsorbed amount of CaChs was higher than that of Na_2Chs when the added concentration of Chs was intermediate or high. The difference in the adsorbed amount between CaChs and Na_2Chs was caused by the following two factors: firstly, inter- and intra-molecular electrostatic repulsion of CaChs is lower than that of Na_2Chs because the degree of dissociation of CaChs is lower than that of Na_2Chs[15,16]; secondly, some Ca^{2+} of CaChs provides adsorption sites for Chs through direct adsorption on HAP (see the next section). It was shown elsewhere[9] that the negatively charged groups of Chs (*i.e.*, $-SO_4^-$ and $-COO^-$) bind to positively charged sites on the HAP surface, that is, to Ca^{2+} exposed on HAP and to defects and/or vacant Pi and OH^- sites on HAP.

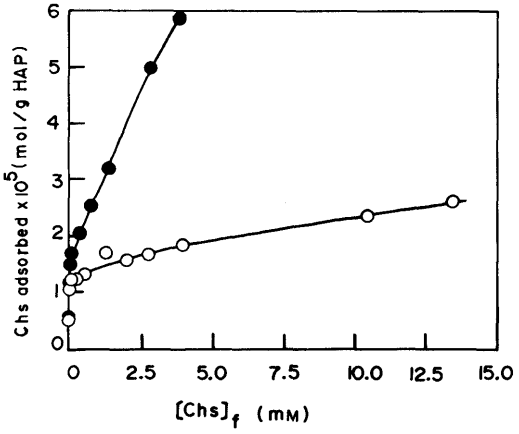

Fig. 1. Adsorption Isotherm of Chs as a Function of the Free Concentration of Chs

○, Na_2Chs; ●, CaChs.

Behavior of Ca^{2+} and Pi Figure 2 shows the amount of adsorption of Ca^{2+} as a function of the added concentration of CaChs or Na_2Chs. The adsorbed amount of Ca^{2+} from CaChs increased monotonously, starting from a negative value. That is, Ca^{2+} was released from HAP (*i.e.*, dissolution of HAP) when the CaChs concentration was very low, while positive adsorption of Ca^{2+} was observed as CaChs concentration was increased. The adsorbed amount of Ca^{2+} was almost the same as that of Chs when the concentration of CaChs was high enough. Adsorbed Ca^{2+} is located in part on the protruding segment (*i.e.*, the loop) of the adsorbed Chs as the counter ion, and the rest is attached directly to the surface of HAP with the Chs segments (*i.e.*, the bridging effect of Ca^{2+} between HAP and Chs).[11] On the other hand, Ca^{2+} was released from HAP over the whole range of the examined concentration of Na_2Chs. Strictly speaking, the amount of released Ca^{2+} increased with the concentration of added Na_2Chs after passing through a minimum. The significance of the minimum will be discussed later (see Fig. 5).

The amount of Pi released from HAP increased and decreased monotonously with the added concentration of Na_2Chs and CaChs, respectively, as shown in Fig. 3. The increase in Pi concentration with Na_2Chs was the result of anion exchange between Pi on the surface of HAP and the anionic groups of Chs when the latter was adsorbed by HAP. The decrease in Pi concentration with increasing

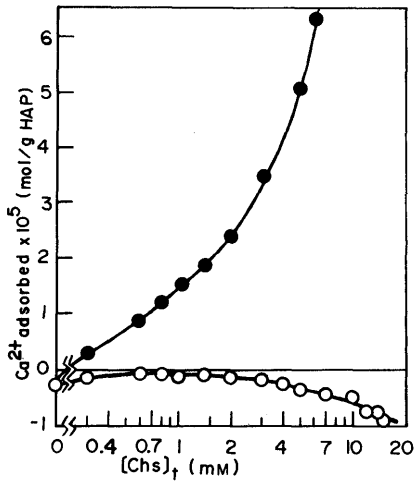

Fig. 2. Adsorbed Amount of Ca^{2+} as a Function of the Total Concentration of Chs

HAP concentration, 5 g/100 ml. ○, Na_2Chs; ●, CaChs. A negative value shows the amount of Ca^{2+} released from HAP. In this figure and the following Figs. 3—5, the data are shown as a function of the total concentration of Chs instead of the free concentration of Chs to make the effect of added amount of Chs clear.

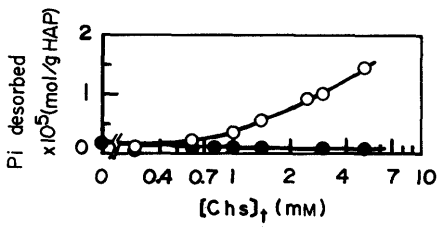

Fig. 3. Released Amount of Phosphate Ion from HAP as a Function of the Total Concentration of Chs

HAP concentration, 5 g/100 ml. ○, Na_2Chs; ●, CaChs.

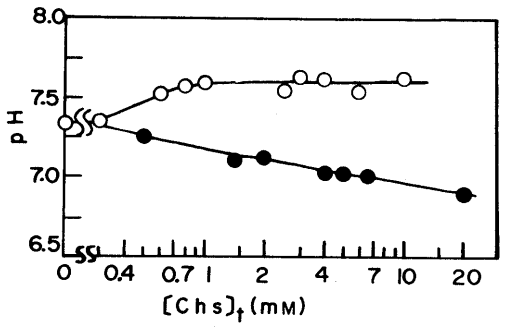

Fig. 4. Suspension pH as a Function of the Total Concentration of Chs
HAP concentration, 5 g/100 ml. ○, Na_2Chs; ●, CaChs.

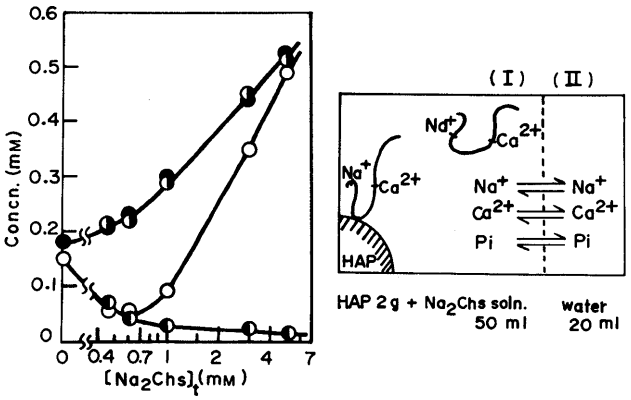

Fig. 5. Dialysis System of HAP–Na_2Chs–H_2O
The dialysis system is illustrated on the right-hand side, where the dotted line indicates the dialysis membrane. The equilibrium concentrations of each species are shown in the left-hand side as a function of the total concentration of Na_2Chs. ○: Ca^{2+} concentration in compartment I, $[Ca^{2+}]_I$. ◐: Ca^{2+} concentration in compartment II, $[Ca^{2+}]_{II}$. ●: phosphate ion concentration in compartment I, $[Pi]_I$. ◑: phosphate ion concentration in compartment II, $[Pi]_{II}$.

concentration of CaChs occurred to maintain the solubility product, K_{sp}, of HAP constant, because the free Ca^{2+} concentration (or Ca^{2+} activity) increases with the concentration of added CaChs.

The suspension pH also increased and decreased monotonously with increasing concentration of added Na_2Chs and CaChs, respectively, as shown in Fig. 4. The increase in pH with increasing concentration of Na_2Chs was attributed to the protonation of Pi released from HAP (see Fig. 3). The decrease in pH with increasing concentration of CaChs was caused mainly by the ion exchange between Ca^{2+} of CaChs and H^+ of the protonated Pi on the surface of HAP.[17-19]

Dialysis of HAP + Na_2Chs System Concentrations of Pi and Ca^{2+} in compartments I and II are shown in Fig. 5 as a function of the concentration of added Na_2Chs. The concentration of Pi in compartment I was almost the same as that in compartment II, because the dialyzing membrane is permeable to Pi which does not interact with Chs. The concentration of Pi increased with increasing concentration of added Na_2Chs or with the amount of adsorption of Na_2Chs owing to anion exchange in a similar manner to that mentioned before (see open circles in Fig. 3). The value on the ordinate (●) shows the solubility of HAP in distilled water through the equilibrium concentration of Pi. The concentration of Ca^{2+} in compartment I was almost the same as that in compartment II in the concentration range of less than 0.7 mM Na_2Chs, because almost all of the Chs was adsorbed by HAP and the effect of free Chs, which scarcely remains in the aqueous phase of compartment I, was negligibly small (see Fig. 1). In this case almost all of the calcium ion, determined by EDTA chelatometry, is free from Chs and can permeate through the dialyzing membrane. Therefore, Ca^{2+} is distributed almost equally in both compartments. When the concentration of added Na_2Chs becomes higher than *ca.* 0.7 mM, a significant amount of Chs free from HAP remains in the aqueous phase of compartment I. The Chs in the solution exchanges Na^+, the original counter ion, with Ca^{2+} released from HAP. As a result, it forms calcium sodium chondroitin-6-sulfate.[16] The Ca^{2+} bound by Chs does not permeate through the dialyzing membrane and does not contribute to the thermodynamic activity of Ca^{2+}, while the Ca^{2+} free from Chs is distributed almost uniformly between compartments I and II under the restriction of the concentration of Pi and/or the solubility product. Therefore, the difference in the total concentration of Ca^{2+} between compartments I (○) and II (◐) becomes large with an increase in the concentration of added Na_2Chs. The trend of the released Ca^{2+} shown in Fig. 2 (○) is quite similar to that in compartment I (○), because the concentrations of Ca^{2+} in both cases were determined as the total ones (*i.e.*, free Ca^{2+} plus Ca^{2+} bound by Chs) in the filtrate.

Chemical potential, μ, for each species in compartment I becomes equal to that in compartment II when the membrane and dissolution equilibria are attained. As for HAP, the following equations are obtained, where subscripts I and II indicate compartment I and II.

$$\mu_I = \mu_I^0 + RT \ln a_{HAP,I} \quad (1)$$

$$\mu_{II} = \mu_{II}^0 + RT \ln a_{HAP,II} \quad (2)$$

and

$$\mu_I = \mu_{II} \quad (3)$$

where μ^0, R, T, and a_{HAP} are standard chemical potential, gas constant, temperature in degrees Kelvin, and activity of HAP, respectively. Assuming that

$$\mu_I^0 = \mu_{II}^0 \quad (4)$$

the relationship

$$a_{HAP,I} = a_{HAP,II} \ (= K_{SP}) \quad (5)$$

is obtained, where

$$K_{SP} = (Ca^{2+})^{10}(PO_4^{3-})^6(OH^-)^2 \quad (6)$$

where parentheses ( ) mean the activity of the parenthesized ion. In other words, Donnan equilibrium is attained when Eq. 3 holds.

According to these equations, the ionic activity product or the solubility product of HAP, K_{SP}, was determined from the analytical concentrations in compartment II and the activity coefficients for each species, taking the solution pH into consideration, through the same procedure as that mentioned elsewhere.[20] The results are shown in Table I as pK_{sp} value ($= -\log K_{SP}$) at each concentration of added Na_2Chs. The numerical values of pK_{sp} are almost constant and in agreement with the literature values,[21] irrespective of the concentration of added Na_2Chs, although the concentrations of Pi and Ca^{2+} in compartment II were in-

TABLE I. pK_{sp} Value of HAP

Initial concentration of added Na_2Chs (mM)	pK_{sp}
0	114
0.45	116
0.60	115
1.00	116
3.00	116
5.00	114
Mean	115.2

congruent (*i.e.*, $Ca^{2+}/Pi \neq 10/6$) with respect to the stoichiometry of the HAP. Of course, electroneutrality in compartments I and II is attained by virtue of the other ions such as Na^+, H^+, and OH^-.

Discussion

The releasing tendencies of Pi and Ca^{2+} from HAP in the presence of Na_2Chs are quite similar to those in the presence of sodium dodecyl sulfate (SDS). The latter effect was reported elsewhere.[20] The equilibrium concentration of Pi increased monotonously with increasing concentration of added Chs or dodecyl sulfate ion (DS^-) through anion exchange between these anionic species and Pi on the surface of HAP. On the other hand, the concentration of Ca^{2+} in the aqueous phase decreased with increasing concentration of added Chs when almost all of the Chs was adsorbed, or with the concentration of free DS^- when it was less than the critical micelle concentration (cmc). In these cases, the concentration of total Ca^{2+} is regulated by the solubility product of HAP, K_{SP}, and the free concentration of Pi.[20] However, when a fair amount of Chs or DS^- remains in the solution as free Chs or DS^- micelles, these negatively charged polymers (*i.e.*, Chs and DS^- micelles) capture free Ca^{2+} as one of their counter ions (*i.e.*, Na^+ and Ca^{2+}). Therefore, the total concentration of Ca^{2+} liberated from HAP increases with increasing concentration of Chs free from HAP, or with the concentration of DS^- micelles. The bound Ca^{2+}, however, does not contribute to the thermodynamic activity of Ca^{2+}. In this sense, free Chs and DS^- micelles are regarded as a Ca^{2+} reservoir in the aqueous phase.

In biological systems, Ca^{2+} is stored in biopolymers such as Chs as the counter ion, and it is supplied from the reservoir when normal or pathological calcification begins. During and/or after the maturation of calcification, biopolymers in the connective tissues might interact electrostatically with the biological HAP, in the same manner as that mentioned above. The biopolymers might interact with and show affinity for implanted artificial bones and teeth, which are prepared from synthetic HAP, by the same mechanism as that mentioned above.

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