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Concurrent Binding of Calcium Ion and Chondroitin Sulfate Ion to the Surface of Hydroxyapatite

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Concurrent adsorption of chondroitin sulfate ion (Chs) and calcium ion (Ca^{2+}) to hydroxyapatite (HAP) from an aqueous solution of Na_2Chs mixed with $CaCl_2$ was studied at 25 °C. The adsorbed amount of Chs increased with increasing concentration of Ca^{2+} for the following two reasons: (a) Ca^{2+} adsorbed on the surface of HAP offers an adsorption site for Chs owing to its positive charges, and (2) the binding of Ca^{2+} to anionic sites of Chs depresses the inter- and intramolecular electrostatic repulsion between Chs segments, resulting in the dense adsorption of Chs. The apparent amount of Ca^{2+} adsorbed by HAP is also increased in the presence of Chs, because Ca^{2+} is captured by negative charges of Chs adsorbed on the surface of HAP as well as by adsorption sites for Ca^{2+} on the surface of HAP itself. However, it was concluded that the amount of Ca^{2+} adsorbed directly on the surface of HAP in the presence of Chs is almost the same as that in the absence of Chs when the adsorbed amount of Ca^{2+} is shown as a function of the concentration of free Ca^{2+}.

Keywords——hydroxyapatite; calcium chondroitin sulfate; sodium chondroitin sulfate; hard tissue; renal calculus; concurrent binding; adsorption; ion binding; calcium binding; calcium phosphate

Chondroitin sulfate (Chs) is an acidic mucopolysaccharide, which has been found in bone and cartilage as the major constituent of connective tissue proteoglycans. The role of the proteoglycans may be one of calcium storage, so as to make calcium available to the matrix vesicles. Acid mucopolysaccharides such as Chs may serve to transport minerals from the cell to the matrix to be calcified.[1] Chs is also found in human urine, but its molecular weight is considerably lower than that reported for tissue chondroitin sulfate.[2] Hydroxyapatite (HAP, $Ca_{10}(PO_4)_6(OH)_2$) is the main component of biological hard tissues (bone and tooth) and human renal calculi. Therefore, it seems important to understand the interaction of Chs and HAP in the presence of calcium ion in the aqueous phase.

In the previous papers, the binding ratio of calcium and sodium ions to Chs in water was obtained as a function of equivalent fraction of calcium ion ($2[Ca^{2+}]/(2[Ca^{2+}]+[Na^+])$).[3] It was found that the amount of chondroitin sulfate ion adsorbed by HAP decreases with increasing concentration of phosphate ion owing to a competitive adsorption between these anions.[4] On the other hand, the apparent degree of dissociation of Ca^{2+} from calcium chondroitin sulfate (CaChs) was almost independent of the amount of HAP added.[5] It was concluded that some of the ionized groups of Chs adsorbed by HAP are not directly attached to the surface of HAP.[6] In the present paper, concurrent binding of Chs and Ca^{2+} to HAP is discussed on the basis of the previous results.

Experimental

Materials——Sodium chondroitin sulfate (Na_2Chs) was the C-type, *i.e.* sodium chondroitin-6-sulfate (molecular

weight 7—8 × 10^4), kindly provided by Kaken Yakukako Co., Ltd.[3-5] HAP was the same sample as that used in the previous papers.[4,5] All reagents were purchased from Nakarai Chemicals Ltd. or Wako Pure Chemical Industries Ltd.

Methods——Adsorbate solution containing various concentrations of both Chs and Ca^{2+} was prepared by mixing aqueous solutions of Na_2Chs and $CaCl_2$ immediately before the addition of adsorbent (*i.e.* HAP powder). HAP (1 g) was suspended in a test tube containing 20 ml of a given adsorbate solution of known concentrations, $[Chs]_t$ (=$[Na_2Chs]_t$) and $[Ca^{2+}]_t$ (=$[CaCl_2]_t$), at 25 °C and shaken vigorously. HAP powder was consistently added last to the pre-mixed adsorbate solution, because the adsorption of Chs is not exactly reversible with respect to dilution.

After at least 3 d at 25 °C, the filtrate (Millipore filter, pore size 0.22 μm) was analyzed for Chs and Ca^{2+}. The equilibrium concentration of Chs, $[Chs]_f$, was determined by colorimetry at 530 nm according to the method of Disch *et al.*[7] Total calcium (Ca^{2+} and CaChs) free from HAP in the equilibrium solution was determined by ethylenediaminetetraacetic 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 Na_2Chs and $CaCl_2$ in the sample solution does not cause mutual interference in the determinations. It was also confirmed that the Millipore filter adsorbs neither Ca^{2+} nor Chs. Therefore, the amounts of adsorbed Chs and adsorbed Ca^{2+} were calculated from the difference of adsorbate concentrations before and after addition of HAP particles. The adsorption isotherm was determined as a function of total concentration of added adsorbate, instead of the free concentration at the adsorption equilibrium, with a logarithmic scale on the abscissa in order to expand the region of low concentrations of the adsorbates.

The unit of concentration for Chs in the present paper is that of weight percent (*i.e.* g/dl) or molarity (base mM) of the repeating two sugar residues. As the formula weight of the repeating unit of Na_2Chs is 503, 1 g/dl of Na_2Chs is equivalent to 19.9 base mM of Chs. Henceforth, subscripts "t" and "f" mean total and free concentrations of the adsorbates, respectively.

Results

Adsorption Isotherm for Chondroitin Sulfate Ion

The amount of Chs adsorbed by HAP is shown in Fig. 1 as a function of the total concentration of Na_2Chs, $[Na_2Chs]_t$. The amount of adsorption of Chs increases with increase of molar ratio of Ca^{2+} to Chs, $[Ca^{2+}]_t/[Chs]_t$, as well as with increase of $[Na_2Chs]_t$.

The relationship between the amount of Chs adsorbed and the molar ratio of mixing is shown in Fig. 3(A), where $[Na_2Chs]_t$ is kept constant. The amount of adsorption seems to

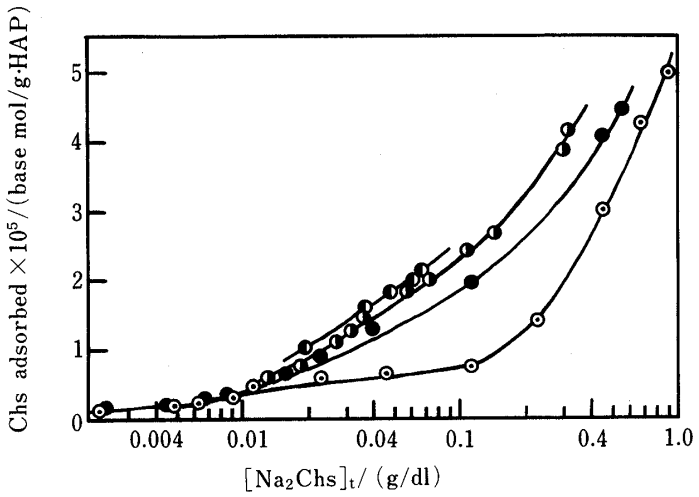

Fig. 1. Adsorption Isotherms for Chs to the Surface of HAP in the Presence of $CaCl_2$

Molar ratio, $R: \dfrac{[Ca^{2+}]_t}{[Chs]_t} \Big/ \left(\dfrac{\text{mM}}{\text{base mM}}\right)$ = 0.00 (⊙), 2.49 (●), 7.45 (◑), and 20.25 (◐).

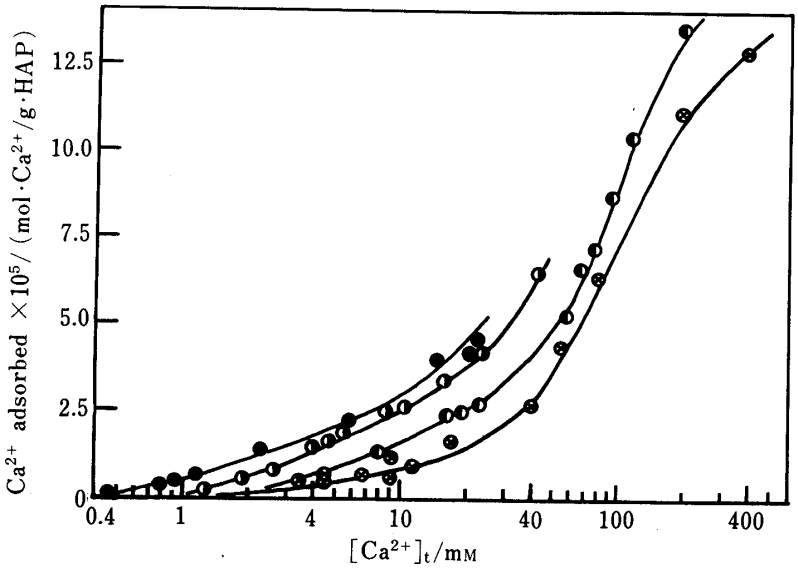

Fig. 2. Adsorption Isotherms for Ca^{2+} to the Surface of HAP in the Presence of Na_2Chs

Molar ratio, $\frac{1}{R}:\frac{[Chs]_t}{[Ca^{2+}]_t}\Big/\left(\frac{\text{base mM}}{\text{mM}}\right)$=0.000 (⊗), 0.049 (◐), 0.134 (◑), and 0.402 (●).

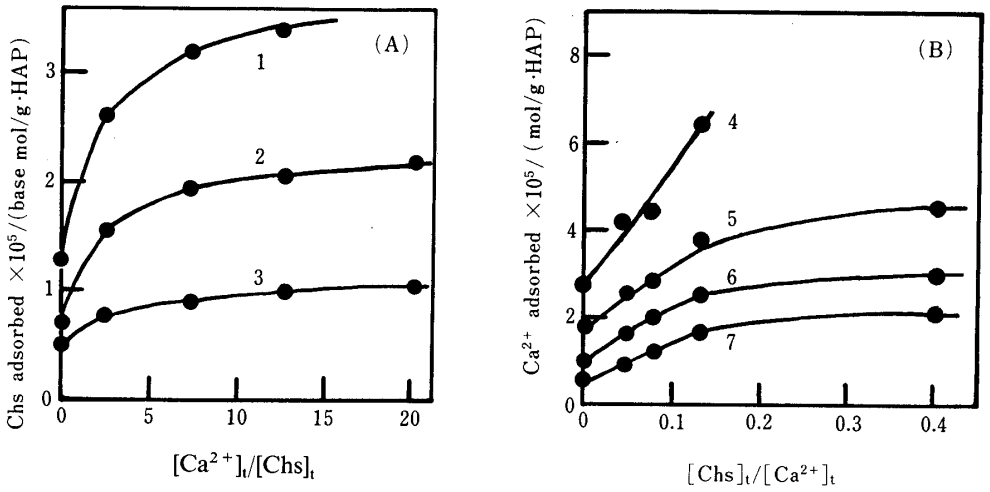

Fig. 3. Adsorbed Amounts of Chs (A) and Ca^{2+} (B) in the Presence of both $CaCl_2$ and Na_2Chs as a Function of Molar Ratio of Adsorbates

$[Na_2Chs]_t$/(g/dl)=0.20 (1), 0.07 (2), and 0.02 (3).
$[CaCl_2]_t$/mM=40 (4), 20 (5), 10 (6), and 5 (7).

level off when the ratio increases.

Adsorption Isotherm for Calcium Ion

The amount of Ca^{2+} adsorbed by HAP is shown in Fig. 2 as a function of total concentration of calcium ion, $[Ca^{2+}]_t$. The amount of adsorption of Ca^{2+} increases with increase of molar ratio of Chs to Ca^{2+}, $[Chs]_t/[Ca^{2+}]_t$, as well as with increase of $[Ca^{2+}]_t$.

The relationship between the amount of Ca^{2+} adsorbed and the molar ratio of mixing is shown in Fig. 3(B), where the total concentration of $CaCl_2$ added was kept constant.

Discussion

Amount of Calcium Ion Adsorbed Directly on the Surface of HAP

The total concentration of calcium ($[Ca^{2+}]_t$), prior to the addition of HAP, in the sample solution containing Ca^{2+} (from $CaCl_2$) and Chs (from Na_2Chs) consists of free Ca^{2+} ($[Ca^{2+}]_f$) and Ca^{2+} bound to Chs ($[Ca^{2+}\text{–Chs}]$). Therefore, Eq. 1 is obtained.

$$[Ca^{2+}]_t=[Ca^{2+}]_f+[Ca^{2+}\text{–Chs}] \tag{1}$$

The mixing molar ratio, R, of total Ca^{2+} to total Chs is shown by Eq. 2, the value of which is known from the experimental conditions (see Figs. 1 and 2).

$$R=\frac{[CaCl_2]_t}{[Na_2Chs]_t}=\frac{[Ca^{2+}]_t}{[Chs]_t} \tag{2}$$

The binding ratio, x, of Ca^{2+} to Chs is expressed by Eq. 3,

$$x=\frac{[Ca^{2+}\text{–Chs}]}{[Chs]_t} \tag{3}$$

where x has already been measured as a function of equivalent fraction of free Ca^{2+}, $2[Ca^{2+}]_f/([Na^+]_f+2[Ca^{2+}]_f)$, at the equilibrium of counter-ion binding to Chs.[3] Therefore, from Eqs. 1—3, $[Ca^{2+}]_f$ is expressed as follows:

$$[Ca^{2+}]_f=\left(\frac{R-x}{R}\right)[Ca^{2+}]_t \tag{4}$$

By means of Eq. 4, numerical calculation for $[Ca^{2+}]_f$ becomes possible at each mixing ratio.

On the other hand, there are two kinds of Ca^{2+} adsorption by HAP: the first is Ca^{2+} adsorbed directly on the surface of HAP, the amount of which is $[\![Ca^{2+}\text{–HAP}]\!]$; and the second is that captured by Chs adsorbed on the surface of HAP, the amount of which is $[\![Ca^{2+}\text{–Chs–HAP}]\!]$. The sum of these can apparently be assumed to be the amount of Ca^{2+} adsorbed by HAP since the result of chemical analysis gives the concentration of Ca^{2+} free from HAP in the filtrate (see Fig. 2). Therefore, the following equation is obtained, where the total amount of Ca^{2+} adsorbed is $[\![Ca^{2+}\text{–HAP}]\!]_t$.

$$[\![Ca^{2+}\text{–HAP}]\!]_t=[\![Ca^{2+}\text{–HAP}]\!]+[\![Ca^{2+}\text{–Chs–HAP}]\!] \tag{5}$$

The binding ratio (x_{HAP}) of Ca^{2+} to Chs adsorbed by the surface of HAP is expressed by Eq. 6 in the same manner as in Eq. 3,

$$x_{HAP}=\frac{[\![Ca^{2+}\text{–Chs–HAP}]\!]}{[\![Chs\text{–HAP}]\!]} \tag{6}$$

where $[\![Chs\text{–HAP}]\!]$ is the total amount of Chs adsorbed by HAP. Therefore, $[\![Ca^{2+}\text{–HAP}]\!]$ in Eq. 5 can be expressed as follows:

$$[\![Ca^{2+}\text{–HAP}]\!]=[\![Ca^{2+}\text{–HAP}]\!]_t-x_{HAP}[\![Chs\text{–HAP}]\!] \tag{7}$$

In the previous paper[5] it was shown that the degree of dissociation of counter-ions (Ca^{2+} and Na^+) from Chs adsorbed at the surface of HAP is almost the same as that from Chs free from HAP. Therefore, x_{HAP} in Eq. 6 or 7 can be replaced by x in Eq. 3 as follows:

$$[\![Ca^{2+}\text{–HAP}]\!]=[\![Ca^{2+}\text{–HAP}]\!]_t-x[\![Chs\text{–HAP}]\!] \tag{8}$$

Accordingly, $[\![Ca^{2+}\text{–HAP}]\!]$ can be obtained by means of Eq. 8, because both $[\![Ca^{2+}\text{–HAP}]\!]_t$ and $[\![Chs\text{–HAP}]\!]$ are determined experimentally and x is available from the literature,[3] as mentioned above.

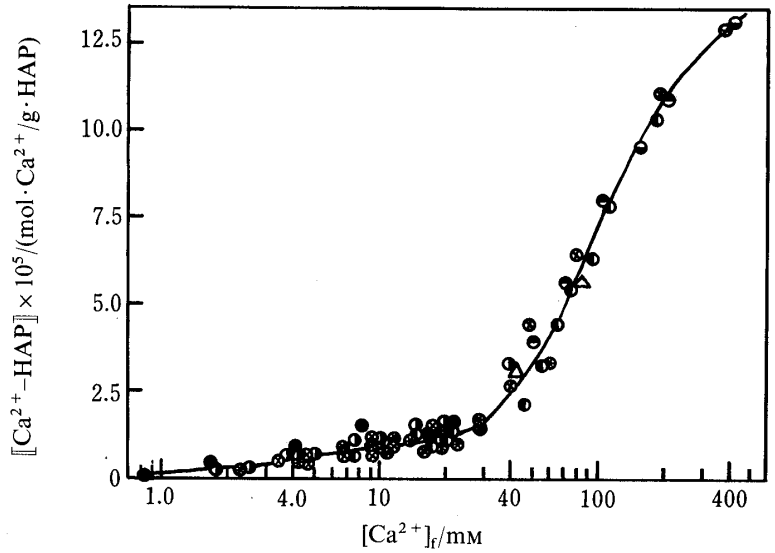

Fig. 4. Amounts of Ca^{2+} Adsorbed Directly on the Surface of HAP as a Function of Free Concentration of Ca^{2+}

Molar ratio, $\frac{1}{R}:\frac{[Chs]_t}{[Ca^{2+}]}\Big/\left(\frac{\text{base mM}}{\text{mM}}\right)$=0 (⊗), 0.000468 (◒), 0.00187 (◓), 0.00943 (△), 0.0494 (◐), 0.0785 (⊛), 0.134 (◑), and 0.402 (●).

The relationship between $[Ca^{2+}]_f$ in Eq. 4 and $[\![Ca^{2+}\text{–HAP}]\!]$ in Eq. 8 is shown in Fig. 4, where the experimental points re-calculated from Fig. 2 are almost on one curve irrespective of the value of R (or $[Chs]_t/[Ca^{2+}]_t$). This result shows that the adsorption isotherm for Ca^{2+} adsorbed directly at the surface of HAP in the presence of Chs is almost identical to that in the absence of Chs (⊗), at least as a first approximation, within the limits of experimental error.

Therefore, we can conclude that the amount of Ca^{2+} adsorbed directly at the surface of HAP is determined only by the concentration of free Ca^{2+} ($[Ca^{2+}]_f$) irrespective of the total concentration of Chs added and/or the amount of Chs adsorbed by HAP. This means that the effect of steric hindrance due to Chs adsorption on the amount of direct Ca^{2+}-adsorption ($[\![Ca^{2+}\text{–HAP}]\!]$) is negligible, probably because Chs adsorption is sustained only by a small number of ionized groups attached to the surface of HAP.

Adsorption Isotherms for Chs to the Surface of HAP Carrying a Constant Amount of Adsorbed Calcium Ions

Adsorption sites for Chs may be surface positive charges localized on the calcium ions and/or on defects of the lattice anions (phosphate and hydroxyl ions) of the HAP surface, where carboxyl and sulfate groups of Chs are anchored.[8-10] The amount of adsorption of Chs is enhanced by the presence of adsorbed Ca^{2+} (Figs. 1—3) in the same manner as found for the adsorption of salivary proteins with Ca^{2+}.[11] These results suggest that surface-adsorbed Ca^{2+} offers an adsorption site for Chs (*i.e.* simultaneous adsorption of cations and anions).[12]

The relationship between $[Chs]_t$ and the amount of Chs adsorbed at a constant value of $[\![Ca^{2+}\text{–HAP}]\!]$ is shown in Fig. 5. The values of $[Chs]_t$ in Fig. 5 were obtained from the concentration of free calcium ion ($[Ca^{2+}]_f$) at a given value of $[\![Ca^{2+}\text{–HAP}]\!]$ (Fig. 4) by means of Eqs. 2 and 4, with known values of R and x. The adsorbed amount of Chs, corresponding to $[Chs]_t$ (*i.e.* $[Na_2Chs]_t$) obtained by the procedure mentioned above, was determined from Fig. 1.

The relationship in Fig. 5 shows that the amount of adsorbed Chs increases with increase

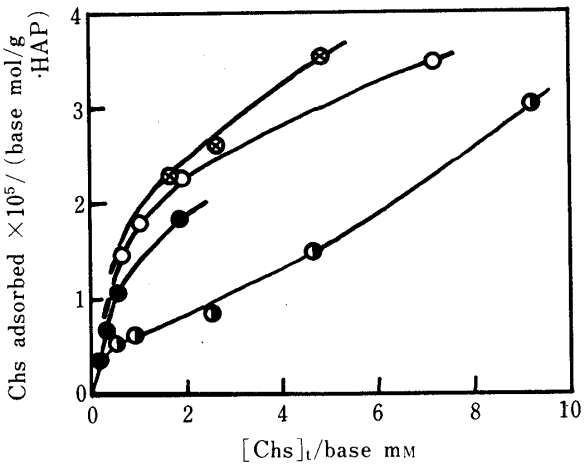

Fig. 5. Adsorption Isotherms for Chs on the Surface of HAP Treated with Various Amounts of Ca^{2+}

Amount of Ca^{2+} adsorbed directly on the surface of HAP: $[\![Ca^{2+}\text{–HAP}]\!]$/(mol-$Ca^{2+}$/g-HAP)=0 (◑), 0.5×10^{-5} (●), 1.0×10^{-5} (○), and 2.0×10^{-5} (⊗).

of $[\![Ca^{2+}\text{–HAP}]\!]$. This result means that the adsorbed amount of Chs at the surface of HAP increases with increase of surface positive charges due to Ca^{2+} adsorption (*i.e.* Ca^{2+} effect on the adsorbent), as would be expected. Needless to say, Ca^{2+}-binding to Chs certainly depresses the inter- and intramolecular electrostatic repulsions between charged groups of Chs chains. Therefore, the amount of Chs adsorbed by HAP also increases by virtue of the dense binding of Ca^{2+} to Chs with increase of $[Ca^{2+}]_t$ (*i.e.* Ca^{2+} effect on the adsorbate). Thus, Ca^{2+} affects Chs-adsorption by two means. It was confirmed that the effect of addition of $CaCl_2$ in increasing the adsorbed amount of Chs is distinctly larger than that of NaCl when the ionic strength is the same (not shown). Therefore, the effect of $CaCl_2$ discussed here is not due to an ionic strength effect, but is specific to the calcium ion itself, as mentioned above.

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